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Research Article

Establishment and characterization of a new cell culture system for hepatitis B virus replication and infection

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ABSTRACT

Hepatitis B virus (HBV) is a primary cause of chronic liver diseases in humans. HBV infection exhibits strict host and tissue tropism. HBV core promoter (Cp) drives transcription of pregenomic RNA (pgRNA) and plays a key role in the viral life cycle. Hepatocyte nuclear factor 4α (HNF4α) acts as a major transcriptional factor that stimulates Cp. In this work, we reported that BEL7404 cell line displayed a high efficiency of DNA transfection and high levels of HBV antigen expression after transfection of HBV replicons without prominent viral replication. The introduction of exogenous HNF4α and human sodium taurocholate cotransporting polypeptide (hNTCP) expression into BEL7404 made it permissive for HBV replication and susceptible to HBV infection. BEL7404-derived cell lines with induced HBV permissiveness and susceptibility were constructed by stable co-transfection of hNTCP and Tet-inducible HNF4α followed by limiting dilution cloning. HBV replication in such cells was sensitive to inhibition by nucleotide analog tenofovir, while the infection was inhibited by HBV entry inhibitors. This cell culture system provides a new and additional tool for the study of HBV replication and infection as well as the characterization of antiviral agents.

1. Introduction

At least 257 million people worldwide are chronically infected with hepatitis B virus (HBV), which is associated with hepatitis and higher risks for liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (Polaris Observatory, 2018; Jia et al., 2020; Tan et al., 2021). Unfortunately, current antiviral therapies based on interferon-alpha (IFN-α) and polymerase-targeting nucleos(t)ide analogs (NAs) such as entecavir (ETV) and tenofovir (TDF) have limited effects on HBV clearance (Konig et al., 2019).

HBV is an enveloped DNA virus replicating via an RNA intermediate (Trepo et al., 2014). The virion contains a 3.2 kb partially double-stranded relaxed circular DNA (rcDNA). HBV enters hepatocytes through receptor-mediated endocytosis. In the nucleus of infected hepatocyte, rcDNA is converted to covalently closed circular DNA (cccDNA), which serves as the template for viral transcription (Gao and Hu, 2007; Guo et al., 2007, 2010). Four promoters (Cp, Sp1, Sp2, Xp) and two enhancers overlapping Xp and Cp (EnI and EnII respectively) drive viral transcription (Chang et al., 1989; Seeger and Mason, 2000). The core promoter (Cp) plays a key role in the viral life cycle: it is responsible for the synthesis of 3.5 kb pregenomic RNA (pgRNA), which is both the template for reverse transcription and for core protein and polymerase translation (Lopez-Cabrera et al., 1990). Multiple liver-enriched transcription factors (LETFs), including hepatocyte nuclear factor 1α (HNF1α), 3 (HNF3), 4α (HNF4α) and CCAAT/enhancer-binding protein (C/EBP), regulate viral gene expression and replication by binding to HBV promoters and enhancers (Raney et al., 2001; Tang et al., 2001; Tang and McLachlan, 2001, 2002; Schrem et al., 2002, 2004). HNF4α in particular has been demonstrated to be a major transcriptional factor that stimulates Cp to promote pgRNA synthesis (Garcia et al., 1993; Yu and Mertz, 2003).

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HBV exhibits highly restricted host and tissue tropism. Several cell culture models have been established for HBV infection and replication. Primary human hepatocytes (PHHs) reproduce HBV natural infection *in vitro*. However, high cost, complex procedure, donor variability, and rapid loss in susceptibility limit the application of PHHs (Konig et al., 2014). The HepaRG cell line requires a lengthy differentiation process to allow moderate HBV infection (Gripon et al., 2002). Human HCC cell lines HepG2 and Huh7 support HBV replication but not infection. Since its establishment as the functional receptor for HBV infection (Yan et al., 2012), human sodium taurocholate cotransporter polypeptide (hNTCP) has been overexpressed in HepG2, Huh7, and mouse hepatocyte line AML12 cells to make them susceptible to HBV infection (Iwamoto et al., 2014; Zhou et al., 2017; Qiao et al., 2018; Konig et al., 2019). The DNA transfection efficiency of these cell models ranges from extremely low (PHH) to moderate (Huh7), which makes the manipulation of cellular gene expression rely on stable transfection or viral vector-mediated transduction.

BEL7404 is a hepatoma cell line originating from a 69-year-old Chinese male in 1974 (Chen et al., 1980). The cell line is easy to culture and has a relatively high DNA transfection efficiency compared to other commonly used human liver cell lines. In the present study, we report that although BEL7404 cells do not support HBV replication, overexpression of HNF4α and hNTCP in BEL7404 resulted in efficient HBV replication and verifiable infection. By establishing BEL7404-derived cell lines that express hNTCP constitutively and HNF4α inducibly, HBV replication post-infection could be modulated. This new BEL7404-based system provides an additional tool for the study of HBV replication and infection as well as the evaluation of antiviral drugs.

2. Materials and methods

2.1. Cell culture and transfection

Huh7, BEL7404, HeLa and HEK293T cells were obtained from Stem Cell Bank, Chinese Academy of Sciences, HepAD38 was provided by Prof. Shuping Tong of Fudan University. Stable NTCP-transfected HepG2 (HepG2-NTCP) was provided by Dr. Yongxiang Wang of Fudan University. The cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, USA) supplemented with 10% fetal bovine serum (Gibco), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco) at 37 °C with 5% CO_2. HepG2-NTCP was additionally supplemented with 2 μg/mL puromycin. For induction of Tet-on system, doxycycline (MedChemExpress, USA) was supplemented at 1 μg/mL or as indicated. DNA transfections were performed at cell confluence of 80%–90% with Turbofect transfection reagent (Thermo Fisher Scientific, Waltham, USA), according to the manufacturer's instructions. Fam-labeled control siRNA (sense, 5′-UUCUCCGA ACGUGUCACGUTT-3′; antisense, 5′-ACGUGACACGUUCGGAGAATT-3′) was chemically synthesized by GenePharma (Shanghai, China) and transfected using GP-transfect-Mate (GenePharma) according to manufacturer's instructions. Tenofovir disoproxil fumarate (TDF) was purchased from Selleck Chemicals (USA).

2.2. Plasmids and lentiviruses

HBV replicon plasmid pCMV-1.1-B56 carries 1.1-fold genome of strain B56 (genotype B, GenBank Acc. No. AF100309.1), with pgRNA transcription controlled by cytomegalovirus (CMV) promoter (Nassal and Rieger, 1996). HBV replicon plasmids p1.3 × B6, p1.3 × B56, and p1.3 × B200 contain 1.3-fold genome of genotype B strains B6 (GenBank Acc. Nr. KR152339), B56 and B200 (GenBank Acc. Nr. KR232337) respectively (Shen et al., 2017), with pgRNA transcription driven by the core promoter of HBV. Firefly luciferase reporters for each of HBV promoters (pGL3-Cp, pGL3-Sp1, pGL3-Sp2, and pGL3-Xp) have been described previously (Qin et al., 2009). Renilla luciferase expression plasmid pRL-TK and pGL3-basic vector were from Promega (Madison, USA). pCMV-EGFP, pTRE-Luc, pCMV-HNF1α, and pCMV-HNF4α were constructed by inserting the corresponding cDNA into pcDNA3 vector. pCDH-NTCP-Blast was constructed by inserting hNTCP cDNA into pCDH-CMV-MCS vector (System Biosciences, USA). The Tet-On inducible system was from Clontech (Mountain View, USA).

Lentiviruses were harvested 72 h after co-transfection of HEK293T cells with the packaging vector psPAX2 (Addgene, Watertown, USA), the envelope expression plasmid pMD2.G (Addgene) and pCDH-hNTCP, pLVX-Tet-on 3G, or pLVX-TRE-Flag-HNF4α-Puro at a ratio of 3:1:4. The virus-containing supernatant was passed through a 0.45-μm filter and used for transduction.

2.3. Transduction and establishment of stable cell lines

Stable cell lines were established by lentiviral transduction of BEL7404 cells. Transductants were selected and maintained in medium supplemented with 2 μg/mL puromycin, 5 μg/mL blasticidin or 400 μg/mL neomycin (G418) depending on the selection gene used. Western blot or quantitative real-time PCR (qPCR) was performed to confirm the expression of cargo gene.

2.4. HBsAg and HBeAg measurement

HBsAg and HBeAg in culture supernatants were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (Kehua, Shanghai, China). Absorbance at 450 nm was read using a microplate reader (Bio-Rad, Hercules, USA). Quantitative measurements of HBsAg and HBeAg were performed using commercial assays by Adicon (China).

2.5. Isolation of HBV DNA and Southern blot

Extraction of intracellular HBV capsid-associated DNA and analysis by Southern blot were performed as previously described (Bai et al., 2016). Hirt extraction was used for nuclear DNA isolation (Hirt, 1967; Gao and Hu, 2007) and after digestion with Plasmid-Safe ATP-Dependent DNase (Epicentre Technologies), cccDNA was quantified using qPCR (Yan et al., 2012; Zhang et al., 2017). The primers used are listed in Supplementary Table S1.

2.6. Dual luciferase assay

The Dual-Luciferase Reporter System Detection kit (Promega) was used for the promoter reporter assays. Cells were cultured in 24-well plates and transfected with 0.4 μg of one of the promoter reporter plasmids (Cp, Sp1, Sp2 and Xp) and 0.1 μg pRL-TK per well. At 48 h post-transfection, cells were washed with PBS and lysed in 100 μl lysis buffer. Cell lysates were centrifuged to remove cellular debris. Firefly and Renilla luciferase activities in cell lysate were detected with a Luminometer (Promega), according to the manufacturer's instructions.

2.7. HBV virion preparation and infection

HBV derived from HepAD38 (Ladner et al., 1997) cell culture supernatant was concentrated by precipitation with 8% polyethylene glycol 8000 (PEG 8000) (Sigma-Aldrich, USA) (Schulze et al., 2010) and centrifugation at 12,000 rpm for one hour at 4 °C. The pellet was resuspended in DMEM and filtered through a 0.45-μm filter. Aliquots were stored at −80 °C. The HBV DNA genome copy number was quantified by qPCR and presented as genome equivalent per mL (GE/mL). Cells were infected with HBV (MOI = 1000) in the presence of 2.5% DMSO and 4% PEG8000 for 12 h, washed with PBS for five times, and cultured in DMEM supplemented with 10% FBS and 2% DMSO. For inhibition of HBV binding to NTCP receptor, preS1 mimic Myrcludex-B (Peptidego Biotech, China) (Lutgehetmann et al., 2012; Zhao et al., 2018) and anti-HBsAg neutralization monoclonal antibody G12 (Wang et al., 2016) were used as indicated.

2.8. RNA extraction and qPCR analysis

Total cellular RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, USA). After phenol/chloroform extraction and isopropanol precipitation, mRNA was reverse transcribed using the Transcript one-step gDNA removal and cDNA synthesis supermix kit (TakaRa, Kusatsu, Japan) following the manufacturer's instructions. cDNA mixture was subjected to qPCR using the PowerUp SYBR Green Master Mix (Thermo Fisher) in LightCycler 480 system (Roche, Switzerland). Beta-actin mRNA was quantified in parallel and used as the normalization control. The primers used are listed in Supplementary Table S1.

2.9. Northern blot

Intracellular HBV RNA transcripts were detected using Northern blot hybridization as described previously (Lin et al., 2017). Each experiment was carried out three times.

2.10. Western blot

Cells were lysed in SDS buffer and incubated at 100 °C for 5 min. Proteins were separated in 10% SDS-PAGE and electro-transferred onto nitrocellulose membranes (Millipore, Billerica, USA). The membranes were blocked with 5% nonfat milk and incubated with specified primary antibodies [anti-β-actin (A3854, Sigma-Aldrich), anti-HNF4α (hepatocyte nuclear factor 4α) (C11F12, Cell Signaling Technology, Beverly, USA) and anti-SLC10A1 (solute carrier family 10 member 1/Na^+/taurocholate cotransporting polypeptide/NTCP) (HPA042727, Sigma-Aldrich), anti-ALPL (alkaline phosphatase, liver/bone/kidney) (11187-1-AP, Proteintech, Rosemont, IL, USA), anti-GSTM1 (glutathione S-transferase mu 1) (12412-1-AP, Proteintech), anti-KYNU (kynureninase/L-kynurenine hydrolase) (11796-1-AP, Proteintech), anti-HKDC1 (hexokinase domain containing 1) (25874-1-AP, Proteintech)] overnight at 4 °C, followed by incubation with the corresponding secondary antibody. Signal detection was performed using ECL Blotting Substrate (Millipore). Each assay was repeated at least three times.

2.11. Statistical analysis

Statistical analyses and graphical representation were performed using Prism 8 (GraphPad Software, USA). All experiments were repeated for at least three times, and data are presented as means or means ± standard deviations (SD). Statistical significance was determined using the Student's *t*-test or Mann Whitney *U* test. A P value < 0.05 was considered statistically significant and shown as * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$).

3. Results

3.1. BEL7404 cells are not permissive for HBV replication

The human hepatoma cell line BEL7404 routinely displayed higher transfection efficiency compared to Huh7 cells (Fig. 1A). To test its support for HBV replication, BEL7404 and Huh7 cells were transfected with various HBV replicon constructs, and viral antigen secretion, as well as genome replication were analyzed. As shown in Fig. 1B, secreted HBV surface and e antigens (HBsAg and HBeAg) were readily detectable in supernatants of both transfected Huh7 and BEL7404 cells. The only exception was HBeAg produced by pCMV-1.1-B56, which was not detected at prominent levels in either Huh7 or BEL7404. Despite its higher transfection efficiency, HBV replicon transfected BEL7404 secreted relatively lower levels of viral antigens compared to Huh7. When intracellular viral replication was measured, capsid-associated HBV DNA replication intermediates could be detected in Huh7 cells transfected with pCMV-1.1-B56, and at comparatively lower levels in Huh7 cells transfected with 1.3 × over-length genome replicons (Fig. 1C). In BEL7404 cells, however, only transfection of pCMV-1.1-B56, but none of the 1.3 × over-length genome replicons, resulted in marked HBV replication (Fig. 1C). Since the only difference between pCMV-1.1-B56 and p1.3-B56 is the promoter driving pgRNA transcription (CMV promoter in the former and HBV Cp in the latter), these results suggested that HBV replication in BEL7404 is likely blocked at the step of pgRNA synthesis.

3.2. Exogenous HNF4α rescues canonical HBV replication in BEL7404 cells

To investigate why BEL7404 cells do not support the replication of 1.3 × replicons, we compared the activities of HBV promoters in Huh7 and BEL7404 cells using reporter assay. As shown in Fig. 2A, activities of Cp and Xp were significantly lower in BEL7404 than Huh7 cells, whereas the activity of Sp2 was markedly higher in BEL7404 cells. Sp1 activity was low in both cells but slightly higher in Huh7 cells (Fig. 2A). As surface antigens are not required for HBV genome replication, low Cp (and possibly also Xp) activity is consistent with the inability of 1.3 × replicons to replicate in BEL7404 cells (Fig. 1C).

HNF1α and HNF4α have been reported to be critical for Cp activity (Raney et al., 1991; Yu and Mertz, 2003). Western blot analysis readily detected endogenous HNF1α and HNF4α expression in Huh7 cells, whereas BEL7404 cells only endogenously express HNF1α and no HNF4α proteins (Fig. 2B). Cp reporter assay performed in the presence of co-transfected HNF4α in BEL7404 cells showed that exogenous HNF4α expression dose-dependently upregulated Cp activity (Fig. 2C and D), but not Sp1 or Sp2 activities (Fig. 2D). These data indicated that the lack of endogenous HNF4α protein expression could be a limiting factor of pgRNA synthesis and HBV replication in BEL7404 cells.

To test this possibility, the effects of exogenous HNF4α expression on HBV antigen expression and replication were examined in p1.3 × B200-transfected BEL7404 and Huh7 cells. As shown in Fig. 2E, co-transfection of HNF4α expression plasmid had no effect on levels of HBV antigens secreted by p1.3 × B200-transfected Huh7 cells, but markedly increased antigen secretion by similarly transfected BEL7404 cells. More importantly, although exogenous HNF4α did not affect HBV replication in Huh7 cells, supplementation with exogenous HNF4α alone was sufficient to rescue canonical HBV replication in p1.3 × B200-transfected BEL7404 cells (Fig. 2F). In contrast, HeLa cells, which have been suggested to contaminate BEL7404 cell stocks (Rebouissou et al., 2017), did not support HBV replication after p1.3 × B200 replicon transfection, with or without exogenous HNF4α (Fig. 2F).

3.3. BEL7404 cells with stable hNTCP and inducible HNF4α expression support HBV infection

In addition to HNF4α, BEL7404 also lacks endogenous expression of the HBV receptor hNTCP (Fig. 3A). To construct BEL7404-derived cell lines that support HBV infection, we first transduced BEL7404 cells with hNTCP-expressing lentivirus and selected transductants using blasticidin. The resultant cells, designated BEL7404-hNTCP, displayed significant hNTCP expression (Fig. 3A). We then transduced BEL7404-hNTCP cells with one lentivirus expressing the Tet-on regulator and another carrying Tet-responsive element controlled HNF4α expression cassette (Fig. 3B). After selection with puromycin, neomycin and blasticidin, triple transductants, designated BEL7404-NTCP-Tet-on-HNF4α (abbreviated as 7404NT-HNF4α) displayed typical dose-responsive DOX-induced expression from transfected TRE-controlled luciferase reporter (Supplementary Fig. S1) with maximum induction at ~1 μg/mL DOX and higher, and expressed HNF4α when and only when cultured in media containing 1 μg/mL DOX (Fig. 3B).

To test its support for HBV replication, 7404NT-HNF4α cells were transfected with p1.3 × B200. Northern blot analysis of intracellular viral RNA showed that in DOX-free media, only very low amounts of Sp1/Sp2-driven 2.4/2.1 kb transcripts were detectable (Fig. 3C). In the presence of DOX, however, prominent production of both Cp-driven pgRNA

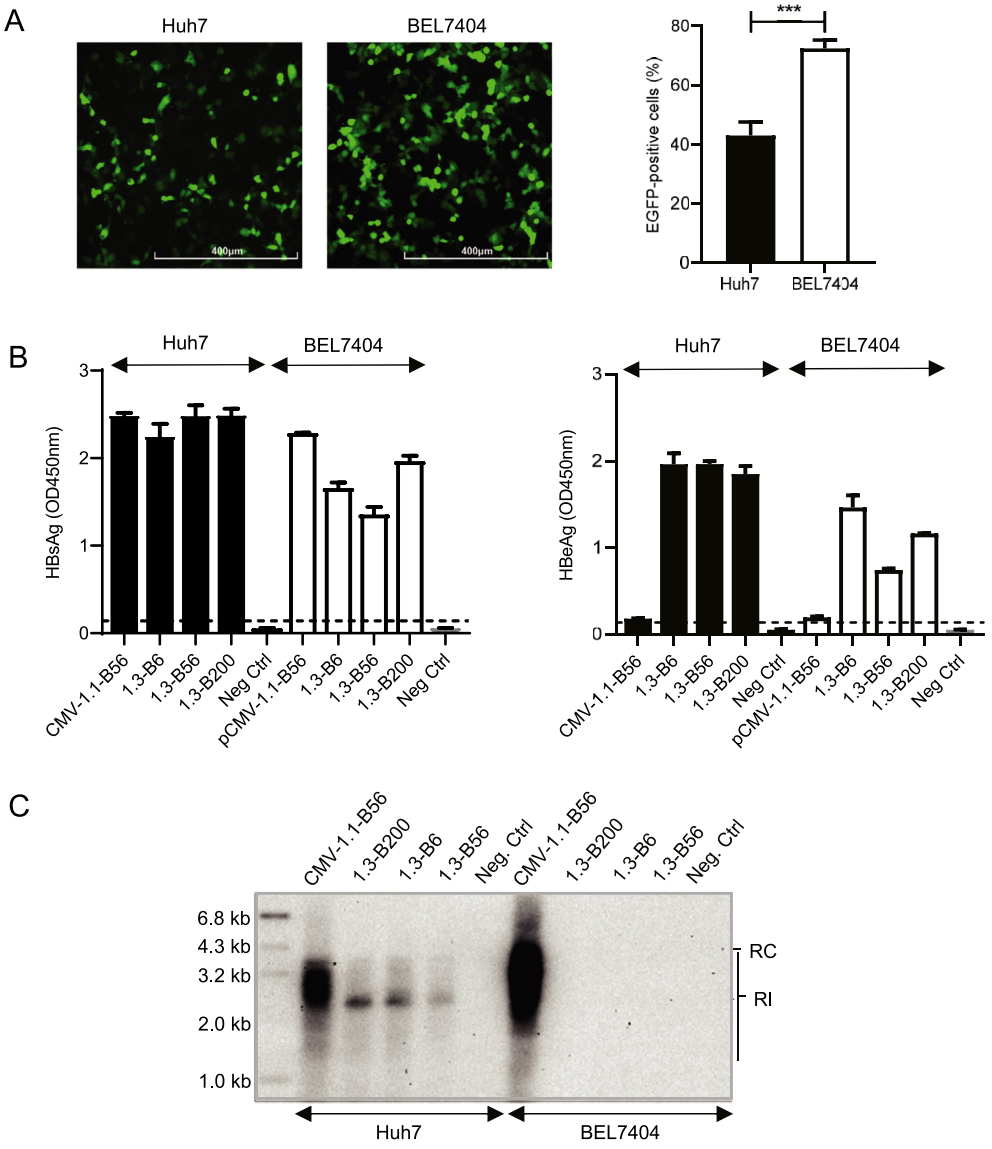

Fig. 1. BEL7404 cells are not permissive for canonical HBV replication. (**A**) Huh7 and BEL7404 cells in 6-well plate were transfected with 2 μg pCMV-EGFP plasmid and EGFP expression at 48 h was visualized using fluorescent microscopy (left). Percentage of EGFP-positive cells was analyzed using imageJ software. Data are presented as means ± the SD (n = 10). **B, C** Huh7 and BEL7404 cells in 6-cm dishes were transfected with 4 μg HBV replicon constructs as indicated. At 96 h post-transfection, HBsAg and HBeAg in the supernatants were determined using ELISA. Data are presented as means ± the SD (n = 3). Dotted lines represent cut-off thresholds (**B**). Intracellular capsid-associated HBV DNA was analyzed using Southern blot (**C**). Positions of relaxed circular (RC) and replication intermediates (RI) are indicated. Transfections were performed in triplicates and repeated twice independently.

transcripts and Sp1/Sp2-driven 2.4/2.1 kb transcripts were detected (Fig. 3C), consistence with a marked increase in HBsAg and HBeAg secretion induced by exogenous HNF4α in replicon-transfected BEL7404 cells (Fig. 2E). Correspondingly, marked production of capsid-associated replication products was only detectable in p1.3 × B200-transfected 7404NT-HNF4α cells with DOX induction (Fig. 3D). DOX treatment also resulted in higher levels of HBsAg and HBeAg being secreted into culture supernatants (Fig. 3E). These results were consistent with promoter activity reporter assay results (Fig. 2D), and demonstrated that 7404NT-HNF4α cells support canonical HBV replication in a DOX-dependent manner.

Finally, 7404NT-HNF4α cells were infected with HepAD38-derived HBV virions in the absence or presence of DOX, and e antigen production by infected cells was followed for 15 days after infection. As shown in Fig. 3E, no detectable HBeAg was secreted by infected 7404NT-HNF4α cells cultured without DOX, while HBV-infected 7404NT-HNF4α cells cultured in DOX-containing media started secreting detectable levels of HBeAg at 5 days post-infection (d.p.i.), which gradually rose to peak at 9 d.p.i. and remained detectable by 15 d.p.i. (Fig. 3F). Clearly, stable hNTCP expression enables HBV infection, while inducible HNF4α expression allows controllable HBV replication post-infection in 7404NT-HNF4α cells.

3.4. Characterization of 7404NT-HNF4α-derived clonal cell lines

The fact that 7404NT-HNF4α is a heterogeneous mixture of triple transductants might be problematic for future applications. We, therefore, performed cloning by limiting dilution using 7404NT-HNF4α and

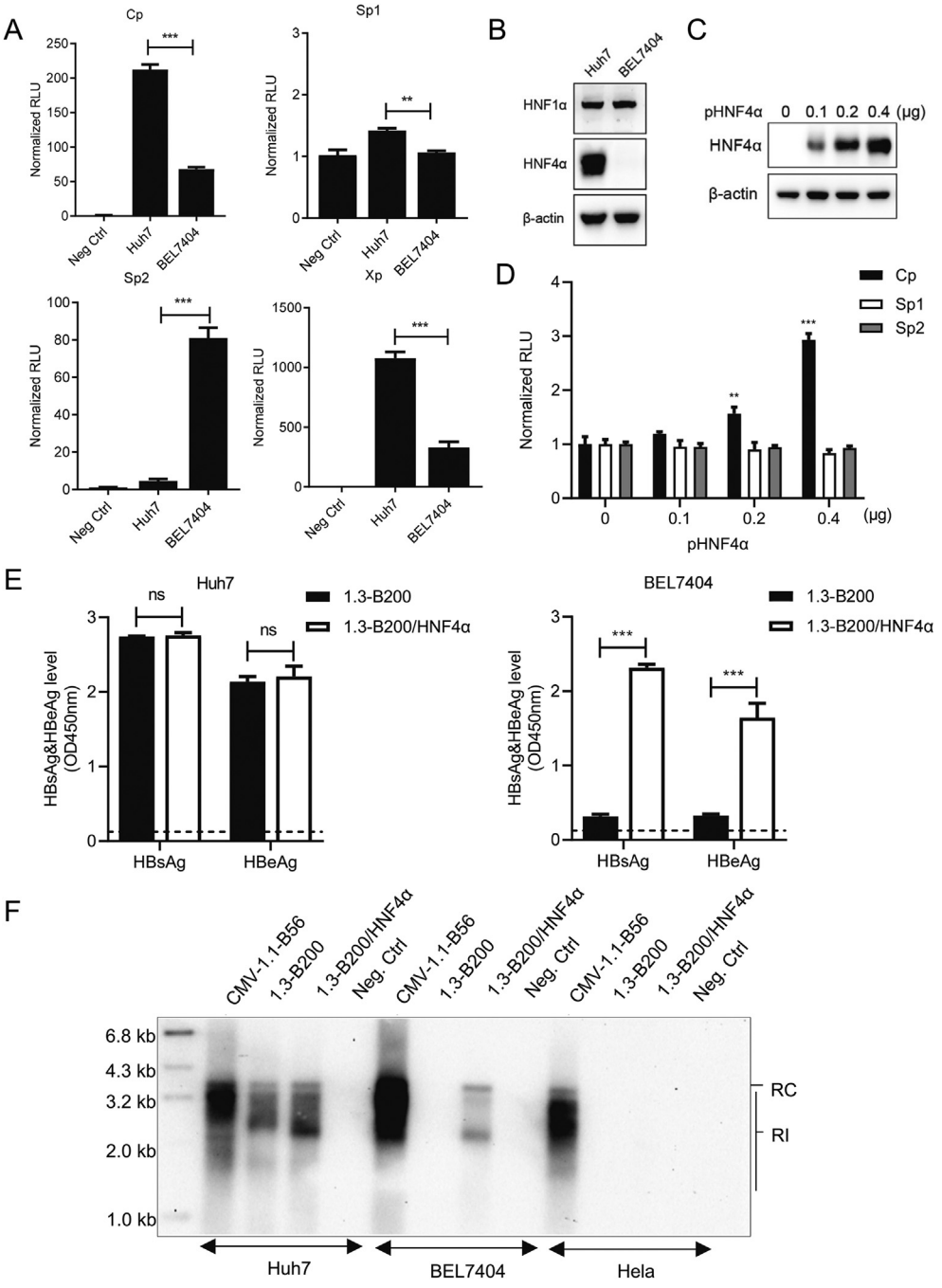

Fig. 2. Exogenous HNF4α rescues canonical HBV replication in BEL7404 cells. **A** Huh7 and BEL7404 cells in 24-well plates were transfected with indicated HBV promoter Firefly luciferase reporter plasmid and Renilla luciferase plasmid control. At 48 h post-transfection, cells were lysed and lysates were analyzed using dual-luciferase reporter assay. Means and SD of relative luciferase activities normalized against promoter-less pGL3-basic vector were plotted (n = 3). **B** Expression of endogenous HNF1α and HNF4α in Huh7 and BEL7404 cells was analyzed using Western blot. **C, D** BEL7404 cells in 24-well plate were co-transfected with Cp, Sp1, and Sp2 Firefly luciferase reporter, increasing amounts of pCMV-HNF4α plasmid, and Renilla luciferase plasmid control. Promoter activities were analyzed as described above. Means and SD of relative luciferase activities normalized against empty expression vector are plotted (n = 3). **E, F** Huh7, BEL7404 and HeLa cells in 6-cm dishes were transfected with 4 μg HBV replicon plasmid as indicated, with or without co-transfection of 2 μg pCMV-HNF4α. At 96 h post-transfection, HBsAg and HBeAg in the supernatants were determined using ELISA (**E**). Dotted lines represent cut-off thresholds. Data are presented as means ± the SD (n = 3). Intracellular capsid-associated HBV DNA was analyzed using Southern blot (**F**). Positions of relaxed circular (RC) and replication intermediates (RI) are indicated. Transfections were performed in triplicates and repeated twice independently.

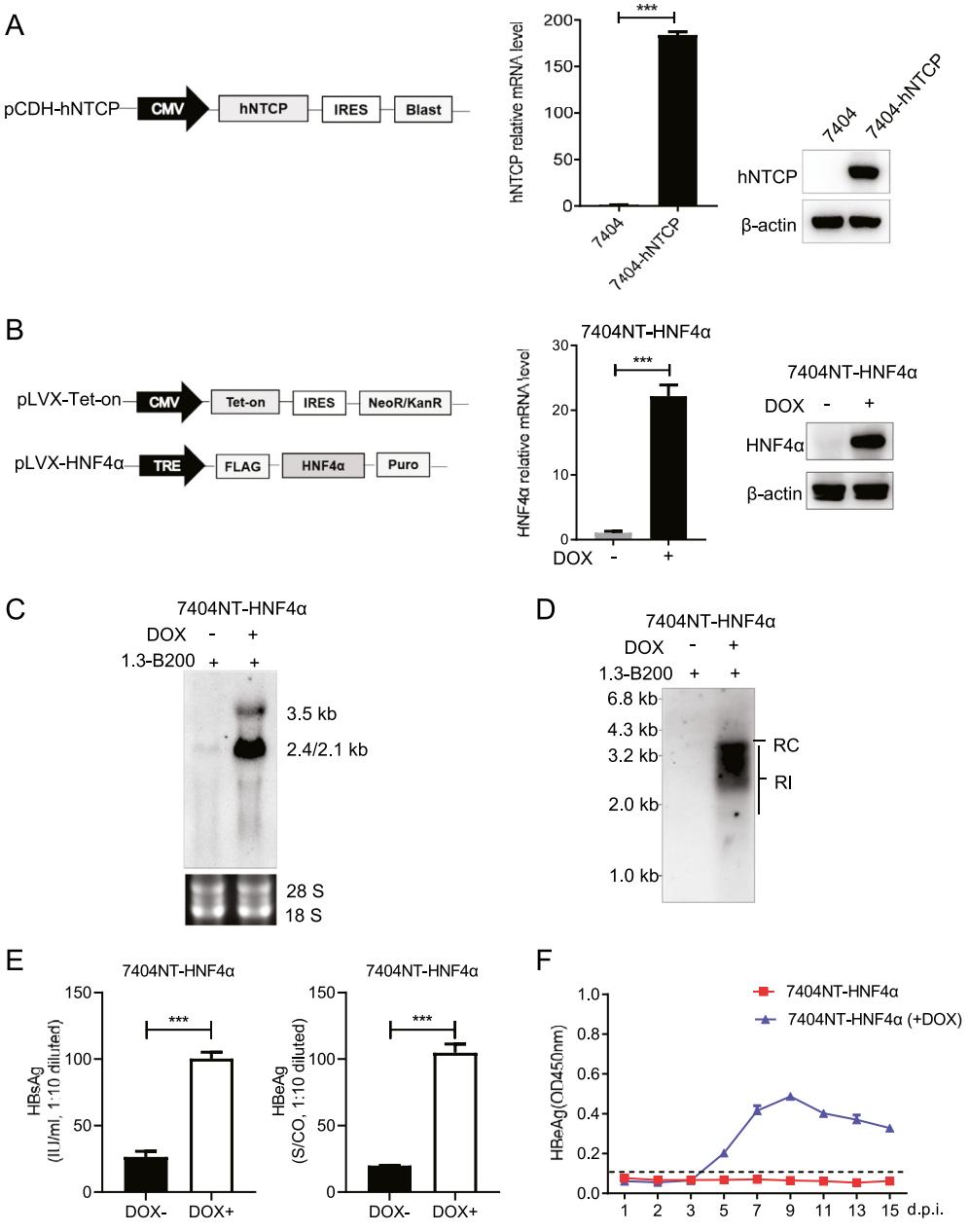

Fig. 3. BEL7404-derived cells with stable hNTCP and inducible HNF4α expression support HBV replication and infection. **A** Recombinant lentiviral virus expressing hNTCP (left) was used to transduce BEL7404 cells and transductants (BEL7404-hNTCP) were selected and maintained in blasticidin-containing media. Relative levels of hNTCP mRNA and protein in parental and transduced cells were analyzed using RT-qPCR (middle) and Western blot (right). Data are presented as means ± the SD (n = 3). **B** Recombinant lentiviral viruses expressing Tet-on regulator and Tet-responsive element (TRE) controlled HNF4α were used to transduce BEL7404-hNTCP cells and triple transductants (7404NT-HNF4α) were selected and maintained in media containing blasticidin, puromycin and G418. Relative levels of HNF4α mRNA and protein in un-induced and doxycycline (DOX)-induced 7404NT-HNF4α cells were analyzed using RT-qPCR (middle) and Western blot (right). **C-E** 7404NT-HNF4α cells in 6-cm dishes were transfected with p1.3 × B200 and cultured with or without DOX. At 96 h post-transfection, intracellular HBV RNA (**C**), capsid-associated DNA (**D**) and secreted HBsAg and HBeAg (**E**) were analyzed using Northern blot, Southern blot and commercial quantitative assay, respectively. **F** 7404NT-HNF4α cells were infected by HBV (MOI = 1000) in the presence of 2.5% DMSO and 4% PEG8000 for 12 h and cultured with or without DOX. Culture media collected at the indicated days post-infection (dpi) were analyzed for HBeAg using ELISA. Dotted lines represent cut-off thresholds. Data are presented as means ± the SD (n = 3). Transfections were performed in triplicates and repeated twice independently.

screened clones grown in 96-well plates for ones that retain triple antibiotic resistance, stable hNTCP and DOX-inducible HNF4α expression. Among a total of 10 selected and expanded clones, B7 and G8 were subjected to further propagation and characterization due to their apparent better support for HBV antigen expression (data not shown). Transfection efficiency analysis showed that both B7, G8, and the parental 7404NT-HNF4α retained the high transfection competence of BEL7404, which was remarkably higher than HepG2-NTCP (Fig. 4A). Transfection of chemically synthesized siRNA was also markedly more efficient in B7 and G8 cells (Supplementary Fig. S2). Similarly, both B7 and G8 stably expressed hNTCP at levels comparable to parental 7404NT-HNF4α, 7404-NTCP as well as HepG2-NTCP, while both B7, G8, and parental 7404NT-HNF4α expressed comparable HNF4α when and only when induced with DOX (Fig. 4B). Western blot analysis of endogenous expression of four cellular markers, selected from screening a panel of known genes expressed in liver cells (data not shown), showed that B7, G8, and parental BEL7404 shared the same pattern, which is different from both HeLa and Huh7/HepG2 (Fig. 4C). After transfection with p1.3 × B200 replicon plasmid, both B7 and G8 cells displayed DOX-dependent pgRNA transcription, genome replication and increased antigen secretion (Fig. 5A–D) that were nearly identical to parental 7404NT-HNF4α (Fig. 3C–E).

B7 cells were then subjected to further characterization. HBV-infected B7 cells displayed strict DOX-dependent HBeAg secretion (Fig. 6A), intracellular pgRNA synthesis (Fig. 6B), genome replication (Fig. 6C), and accumulation of cccDNA (Fig. 6D). Optimal infection of B7 cells by HBV required 2.5% DMSO and 4% PEG, similar to HepG2-NTCP cells (Fig. 6E). Under such optimal conditions, the level of secreted HBeAg produced by infected B7 cells with DOX was lower compared to infected HepG2-NTCP cells (Fig. 6E), yet the cccDNA level in the former as measured by qPCR was only slightly lower than the latter (Fig. 6F). This is similar to lower HBeAg production observed in

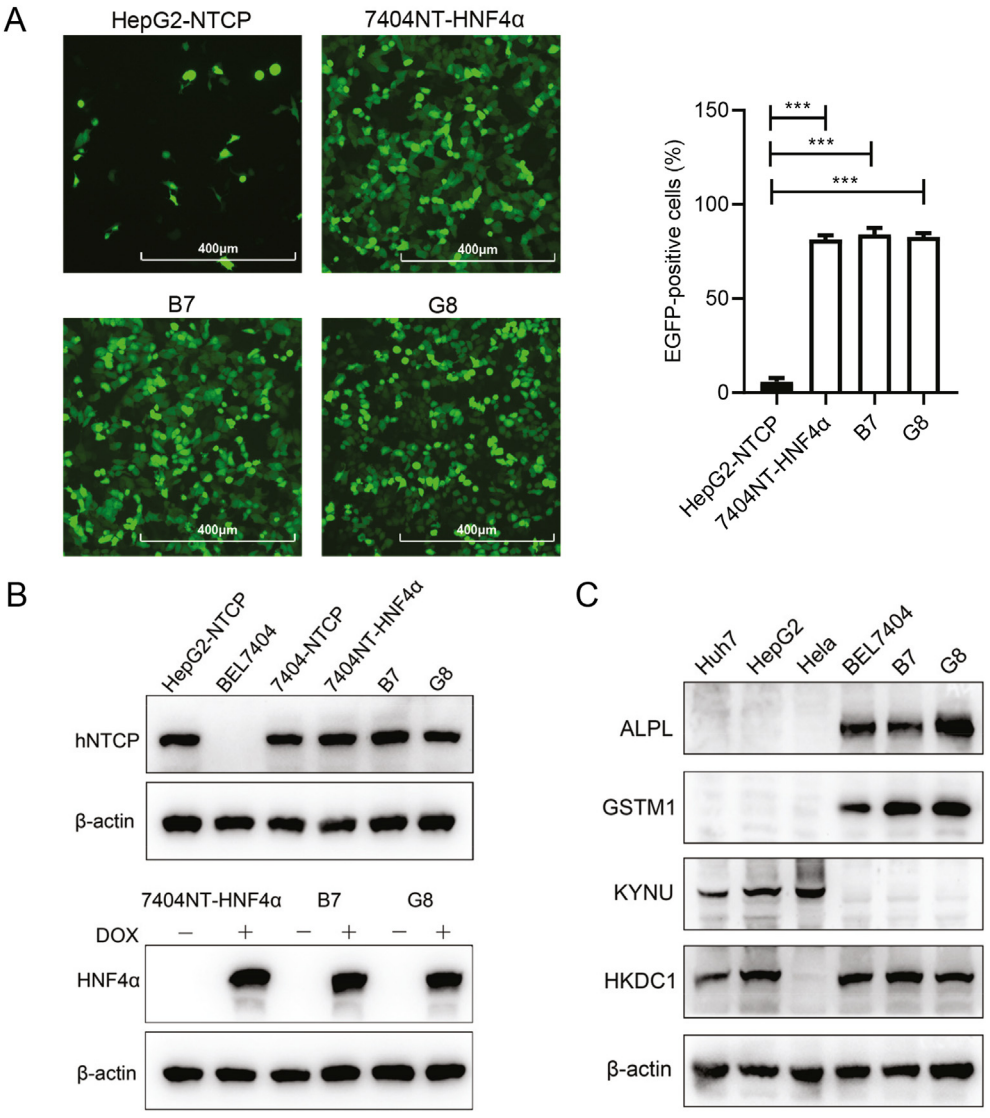

Fig. 4. Characterization of 7404NT-HNF4α-derived clonal cell lines established through limiting dilution. **A** HepG2-NTCP, 7404NT-HNF4α and 7404NT-HNF4α-derived clonal B7 and G8 cells cultured in 6-well plate were transfected with 2 μg pCMV-EGFP plasmid and EGFP expression at 48 h was visualized using fluorescent microscopy (left). Percentage of EGFP-positive cells was analyzed using imageJ software. Transfections were performed in triplicates and repeated twice independently. Data are presented as means ± the SD (n = 10). **B** Expression of hNTCP in HepG2-NTCP, BEL7404, 7404-NTCP, 7404NT-HNF4α, B7, G8 cells and the expression of HNF4α in 7404NT-HNF4α, B7 and G8 cells cultured with and without DOX were analyzed using Western blot. **C** Expression of ALPL, GSTM1, KYNU, HKDC1 and β-actin in Huh7, HepG2, HeLa, BEL7404, B7 and G8 cells was analyzed using Western blot.

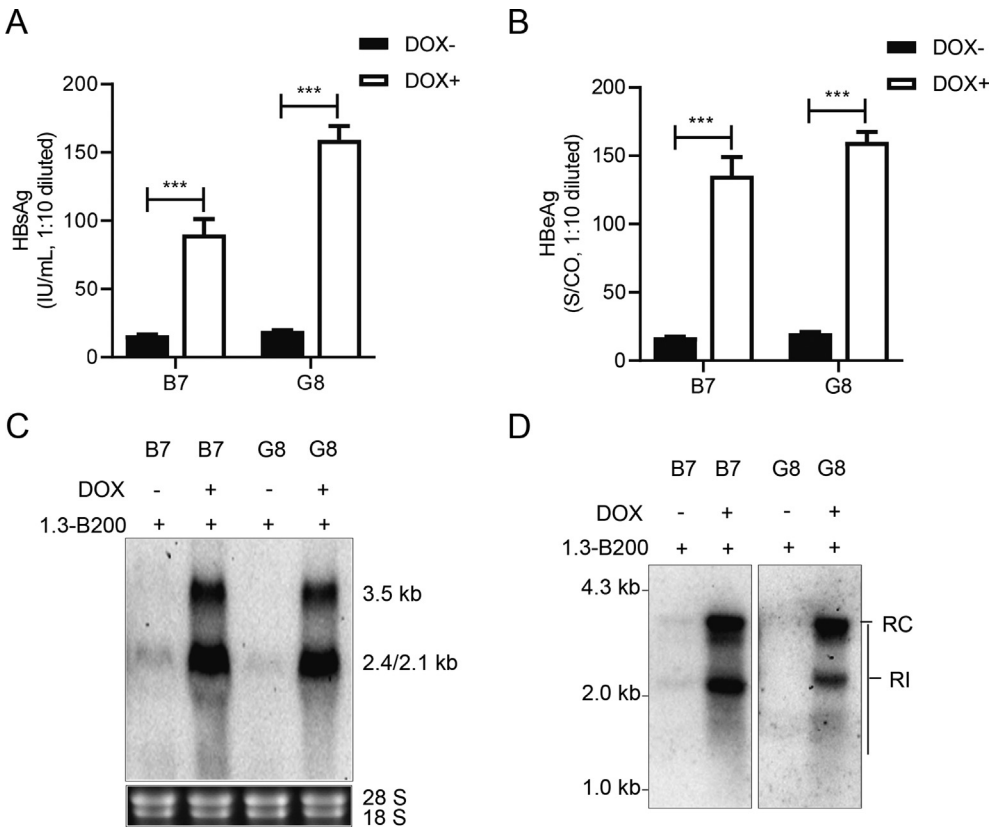

Fig. 5. DOX-dependent HBV transcription and replication in B7 and G8 cells. **A, B** B7 and G8 cells in 6-well plates were transfected with p1.3 × B200 and cultured with or without DOX. At 96 h post-transfection, secreted HBsAg **A** and HBeAg **B**, intracellular HBV RNA **C**, capsid-associated DNA **D** were analyzed using commercial quantitative assay, Northern blot and Southern blot, respectively. Data are presented as means ± the SD (n = 3). Transfections were performed in triplicates and repeated twice independently.

BEL7404 cells transfected with 1.3 × replicon plasmid despite its higher transfection efficiency compared to Huh7 cells (Fig. 1B, right), and apparently reiterates the effects of differences in the cellular environment on aspects of HBV life cycle.

To demonstrate its suitability for HBV-targeted research applications, B7 cells were used to test selected polymerase and entry inhibitors of HBV. In DOX-treated p1.3 × B200-transfected B7 cells, TDF treatment reduced genome replication to a level close to DOX-untreated cells (Fig. 7A). Correspondingly, TDF treatment of HBV-infected B7 cells cultured in DOX-containing media resulted in nearly total inhibition of replication (Fig. 7B). In the infection-blocking assay, both preS1-mimick peptide (Myrcludex-B) and neutralizing monoclonal antibody against S (G12) markedly reduced levels of HBeAg secretion, pgRNA synthesis, and genome replication by infected cells (Fig. 7C–E). These data showed that 7404NT-HNF4α-derived cell lines like B7 are adequate for routine HBV-related applications.

4. Discussion

Although HBV infection *in vivo* is strictly hepatotropic, susceptibility and permissiveness of commonly available human hepatic cell lines vary considerably: a few of them, including Huh7, HepG2, *etc.*, support Cp-driven pgRNA synthesis, genome replication and virion production, while no such cell line is known to support infection without exogenous hNTCP expression. BEL7404 and its derivatives are characterized by consistently high transfection competence compared to both Huh7 (Fig. 1A) and HepG2 (Fig. 4A, Supplementary Fig. S2). In this work, we showed that low Cp activity (Fig. 2A), and consequently low pgRNA synthesis (Fig. 3C), is the main blocked step of canonical HBV replication in BEL7404, and identified insufficient endogenous HNF4α expression as a key factor of the block (Fig. 2B–F). Interestingly, HBeAg production, which also uses Cp-driven transcripts as mRNA, is readily detectable in 1.3 × HBV genome replicon transfected BEL7404 cells, albeit at lower levels compared to Huh7 (Fig. 1B), yet these cells required exogenous HNF4α expression to produce detectable pgRNA and viral replication (Figs. 1C and 2C). Whether this apparent inconsistency reflects different efficiencies of HBeAg translation versus reverse transcription warrants further studying. Alternatively, it has been suggested that Cp might actually consist of two distinct promoters that separately control pgRNA and preC mRNA transcription (Yu and Mertz, 1996), and if this is the case, our results would indicate that HNF4α is an essential positive regulator of pgRNA promoter, but less important for preC promoter. Regardless, BEL7404 and its derived cell lines constitute ideal tools to study both of these hypotheses.

It is also worth noting that in BEL7404 cells, although exogenous HNF4α had no marked effects on the activity of Sp1 or Sp2 in reporter assay (Fig. 2D), HNF4α prominently boosted the production of both Sp1/Sp2-driven 2.4/2.1 kb transcripts (Fig. 3C) and secreted HBsAg (Fig. 2D) in 1.3-fold replicon transfected cells. This could be attributed to activation of enhancers I and II by HNF4α (Yu and Mertz, 2003), but might also involve as yet unknown mechanisms that are specific to BEL7404.

In addition to HNF4α, BEL7404 also lacks sufficient endogenous hNTCP expression (Fig. 3A). Similar to HepG2 and Huh7 cells, supplementation of exogenous hNTCP resulted in susceptibility to HBV infection, while exogenously expressed HNF4α enabled genome replication, leading to the establishment of productive infection (Fig. 3F). Full HBV life cycle is thus reproduced in BEL7404. To facilitate further applications

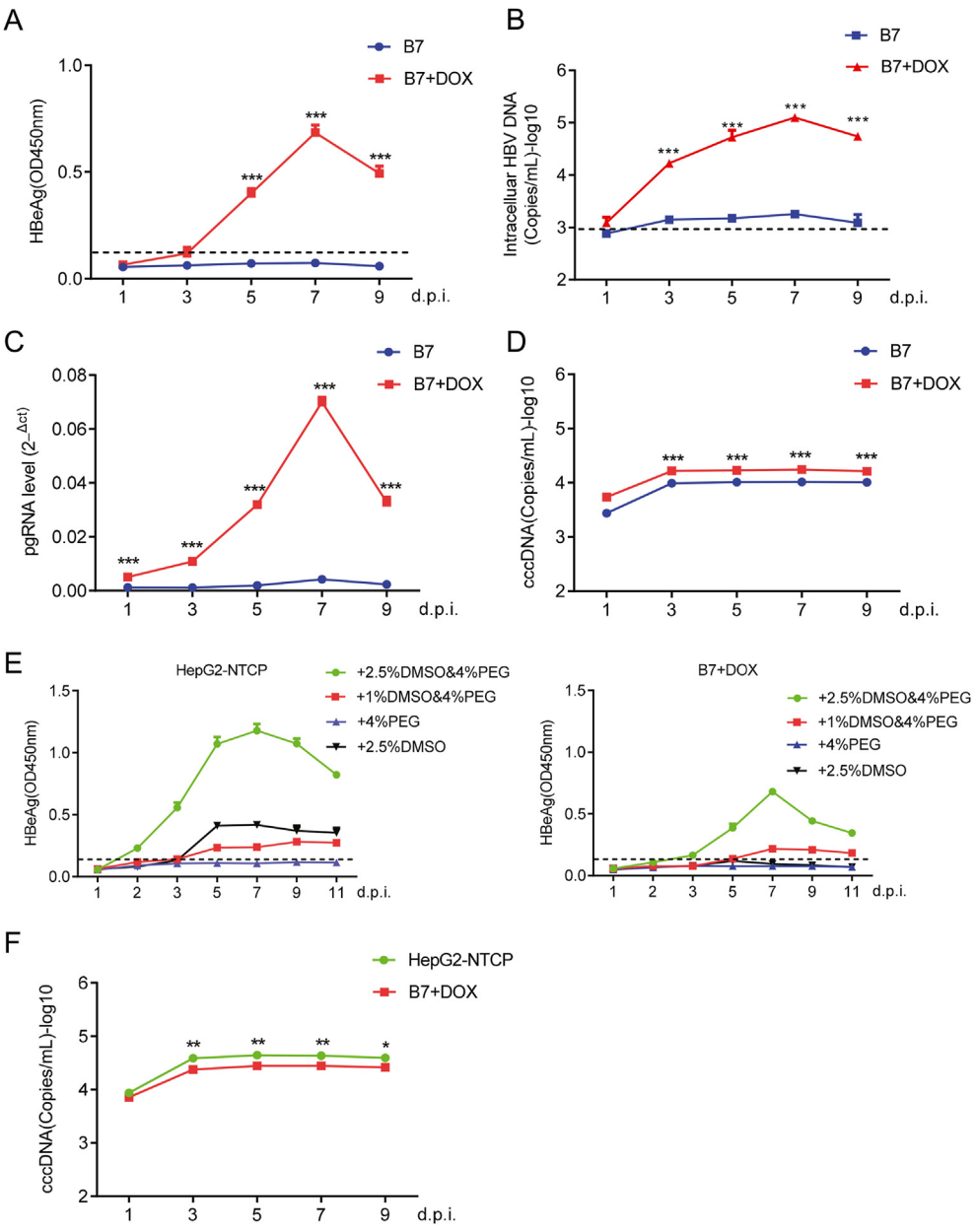

Fig. 6. DOX-dependent HBV transcription and replication in HBV-infected B7 cells. **A-D** B7 cells were infected with HBV (MOI = 1000) and cultured with or without DOX. Secreted HBeAg was analyzed using ELISA. Intracellular capsid-associated HBV DNA, pgRNA and nuclear cccDNA were analyzed using by qPCR or RT-qPCR. **E** DMSO and PEG requirement for HBV infection of B7 cells. HepG2-NTCP and B7 cells were infected with HBV (MOI = 1000) in the presence of indicated DMSO and/or PEG8000 for 12 h. Secreted HBeAg was analyzed using ELISA. **F** HepG2-NTCP and B7 cells were infected with HBV (MOI = 1000) in the presence of indicated DMSO and PEG8000 for 12 h. Nuclear cccDNA were analyzed using qPCR. Dotted lines represent cut-off thresholds. Data are presented as means or means ± the SD (n = 3).

of this new cell model, we established BEL7404-derived clonal cell lines that stably express hNTCP and inducibly express HNF4α (Figs. 4 and 5). The ability to switch on and off the expression of the key pgRNA transcription regulator in these cells allows for the probing of some interesting and difficult issues in HBV virology. For example, after HBV productive infection of HNF4α-expressing B7 cells, an intranuclear cccDNA pool is established (Fig. 6D). DOX can be withdrawn at this point to stop further genome replication and replenishment of cccDNA pool. Such cells would allow studying maintenance and decay mechanisms of cccDNA, as well as testing cccDNA-targeting therapeutic agents. Cell lines like B7 and G8 are also adequate for more common applications of HBV infection and replication models (Fig. 7).

Recently, STR (short tandem repeat) profile analysis results suggested that a number of previously presumed hepatic cell lines, including BEL7404, might have been contaminated by HeLa (Huang et al., 2017). Nevertheless, BEL7404 at our lab, as well as its derived clonal cell lines, displayed a distinct cellular protein expression profile that was different from both HeLa and Huh7/HepG2 (Fig. 4C). With regard to support for HBV replication, HeLa cells did not support replication of Cp-driven replicons, even when supplemented with exogenous HNF4α, which was

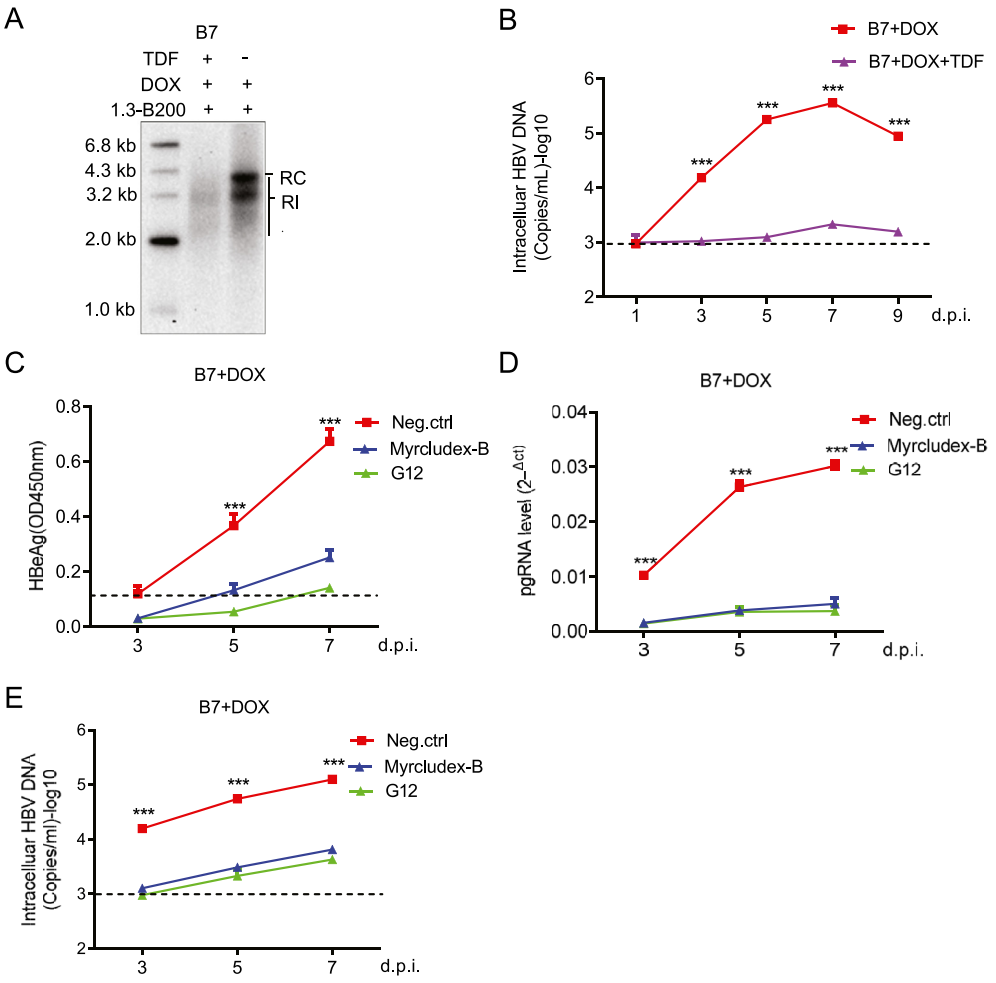

Fig. 7. Evaluation of HBV-targeting agents using B7 cells. **A** B7 cells were transfected with p1.3 × B200 and cultured in DOX-containing media with or without 2 μmol/L Tenofovir disoproxil fumarate (TDF). Intracellular capsid-associated HBV DNA was analyzed using Southern blot. Transfections were performed in triplicates and repeated twice independently. **B** B7 cells were infected with HBV and cultured in DOX-containing media with or without 2 μmol/L TDF. Intracellular capsid-associated HBV DNA in cells harvested at indicated time points was analyzed using qPCR. **C-E** B7 cells were infected with HBV in the presence of 2 μmol/L Myrcludex-B or 2 μg/mL anti-HBs monoclonal antibody G12 (simplified as G12). Secreted HBeAg was analyzed using ELISA. Intracellular pgRNA and capsid-associated HBV DNA were analyzed using by qPCR. Data are presented as means or means ± the SD (n = 3). Dotted lines represent cut-off thresholds.

also clearly different from BEL7404 and its derived cell lines (Fig. 2F). Such functional dissimilarities between BEL7404 and HeLa support the former to be considered as a different cell line, in spite of any possible historical connection between the two.

5. Conclusions

In summary, we have established a new cell culture model that constitutively supports HBV infection, and inducibly supports HBV replication. Availability of additional replication and infection cell models for HBV not only means more tools with different qualities such as higher transfection competence, but also allows comparison between different models for identification and characterization of regulators of various stages of HBV life cycle. For example, HBV infection of B7 cells was apparently less efficient compared to HepG2/NTCP cells (Fig. 6E), although both cells express comparable levels of hNTCP proteins (Fig. 4B). By comparing the two cell lines using transcriptomic and/or proteomic methods, additional factors affecting HBV productive infection might be revealed.

Data availability

The datasets generated and analyzed in the current study are available from the corresponding author on reasonable request.

Ethics statement

This article does not contain any studies with human or animal subjects performed by any of the authors.

Author contributions

Yingying Song: conceptualization, methodology, software, resources, formal analysis, investigation, data curation, validation, writing - original draft, writing - review & editing, supervision. Shuyu Shou: software, formal analysis, writing - review & editing. Huimin Guo: methodology, resources, investigation, data curation, validation. Zixiang Gao: methodology, data curation. Nannan Liu: methodology, data curation. Yang Yang: software, formal analysis. Feifei Wang: visualization, supervision.

Qiang Deng: resources, supervision. Jing Liu: conceptualization, resources, writing - review & editing, supervision, funding acquisition. Youhua Xie: conceptualization, resources, project administration, validation, writing - original draft, writing - review & editing, supervision, funding acquisition.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2022.05.002.

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