

Human stem cell-derived hepatocytes as a model for hepatitis

B virus infection, spreading and virus-host interactions

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Supplementary methods

HBV infection

Cell culture derived HBV (HBVcc) was concentrated from the supernatant of HepG2.2.15 cells using centrifugal filter devices (Centricon Plus-70, Biomax 100.000, Millipore Corp., Bedford, MA) and titered by HBV-DNA qPCR. Immediately after collection, the virus stock was divided in aliquots and stored at -80°C until use. Sera from two treatment naïve hepatitis B patients were directly used for infection. Viral genotype and titer was determined by PCR. For infection, inoculation of cells was performed with multiplicity of infection (MOI) 300 in WEM medium containing 5% PEG 8000 (Sigma Aldrich, St. Louis, USA) for 16 h. At the end of incubation period, cells were washed three times with PBS and cultured in WEM medium.

Detection of HBV antigens by ELISA

Secreted HBeAg and HBsAg were determined using Human HBeAg Elisa Kit (CD BioScience, Shirley, USA) and Human HBsAg Elisa Kit (CD BioScience, Shirley, USA) respectively. Signal-to-noise ratio (S/N) is presented in the figures.

Quantitative real time PCR

For intracellular HBV total DNA and cccDNA quantification, total cellular DNA was purified from infected cells using NucleoSpin Tissue Tissue kit (Macherey-Nagel, Düren, Germany). HBV total DNA qPCR was performed using rcDNA1745 fw / rcDNA1844 rev primer pair. DNA samples for cccDNA qPCR were treated with 500 U/ml T5 exonuclease (NEB, Ipswich, USA) at 37°C for 30 min. cccDNA specific primer pair cccDNA 92 fw and cccDNA 2251 rev were used for qPCR as described [1]. Extracellular HBV DNA quantification was performed with DNA extraction using DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). rcDNA1745 fw / rcDNA1844 rev primer pair were used for qPCR. For qRT-PCR, RNA was extracted using RNeasy-kit (Qiagen, Hilden, Germany) and transcribed into cDNA with Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham,

USA) according to the manufacturer's instructions. qPCR were performed using the LightCycler™ 480 system with SYBR Green Master (Roche, Mannheim, Germany) or Probe Master (Roche, Mannheim, Germany). All primers and probes are listed in supplementary table 1. A relative unit, defined as the expression ratio of target gene against the reference housekeeping gene (ACTB for RNA, PRNP for DNA), is presented.

Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde in PBS pH 7.4 for 10 min at room temperature and permeabilized with 0.5% saponin. Slides were blocked in PBS containing 0.5% saponin and 10% serum from the species that the secondary antibody was raised in at room temperature for two hours. After blocking, primary antibodies diluted in PBS with 0.1% saponin and 10% blocking serum were added overnight at 4°C. After extensive washing, cells were incubated with the secondary antibody in PBS with 0.1% saponin and 2% blocking serum for 2 hours at room temperature in the dark. Samples were mounted with Dapi Fluoromount-G

(SouthernBiotech, Birmingham, Alabama, USA) and images were captured with EvosFL (Thermo Scientific, Waltham, USA) or LSM 700 confocal microscope (Zeiss, Oberkochen, Germany). All antibodies used are listed in supplementary table 2.

Southern blot analysis.

For the detection of cccDNA, a modified Hirt method was used to extract protein-free viral DNAs as described [2]. Briefly, protein-free viral DNAs were isolated from a full 6 well plate HBV infected HLCs cells. The extracted DNA was treated with EcoR I overnight. The viral DNAs were separated on agarose gel, transferred to an Amersham Hybound-N+ membrane (GE Healthcare, UK). HBV-specific digoxigenin-labeled probe was generated and detected with DIG DNA labelling and detection Kit (Roche, Mannheim, Germany).

siRNA knock down

siRNAs were obtained from Thermo Fisher Scientific Dharmacon® (Lafayette, USA).

HLCs cells were transfected with 25 nM siRNA using different transfection reagents:

2.5 μ L TransIT-TKO (Mirus, Houston, USA), 2.5 μ L TransIT-siQUEST (Mirus, Houston, USA), 2 μ L TransIT-X2 (Mirus, Houston, USA), 2 μ L RNAiMAX (Invitrogen, Carlsbad, USA), 2 μ L lipofectamin 2000 (Invitrogen, Carlsbad, USA), 2 μ L Oligofectamin (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions.

RNAscope assay

In situ detection of HBV nucleic acid was performed using the RNAscope assay (Advanced Cell Diagnostics, Newak, CA, USA)[3]. HBV-specific probe sets targeting nt141-1402 of genome type D viral genome were provided by the manufacturer. Hybridizations were performed for 2 h with target probes at 40°C. Following signal amplification, probes conjugated to Atto 550 were imaged using Zeiss LSM 700 confocal microscope. HBV non-infected cells were used as negative control.

Electron microscopy

Supernatant of HBV infected HLCs was first concentrated by Amicon Ultra centrifugal filters (Millipore, Darmstadt, Germany), and then applied on CsCl gradient at 35,000 rpm for 16 hours at 4°C using SW60 rotor (Beckman Coulter, Brea, USA). HBV-DNA and HBsAg from different fractions were determined by qPCR and ELISA respectively. Fraction 15 and 16 (**Supplementary Fig. 4F**) were then fixed in 4% paraformaldehyde and loaded on FF400-Cu grid (Electron Microscopy Sciences, Hatfield, USA). Negative staining was performed with 1% uranyl acetate solution and then observed by JEOL JEM 1200EXII transmission electron microscope (JEOL, Waterford, USA).

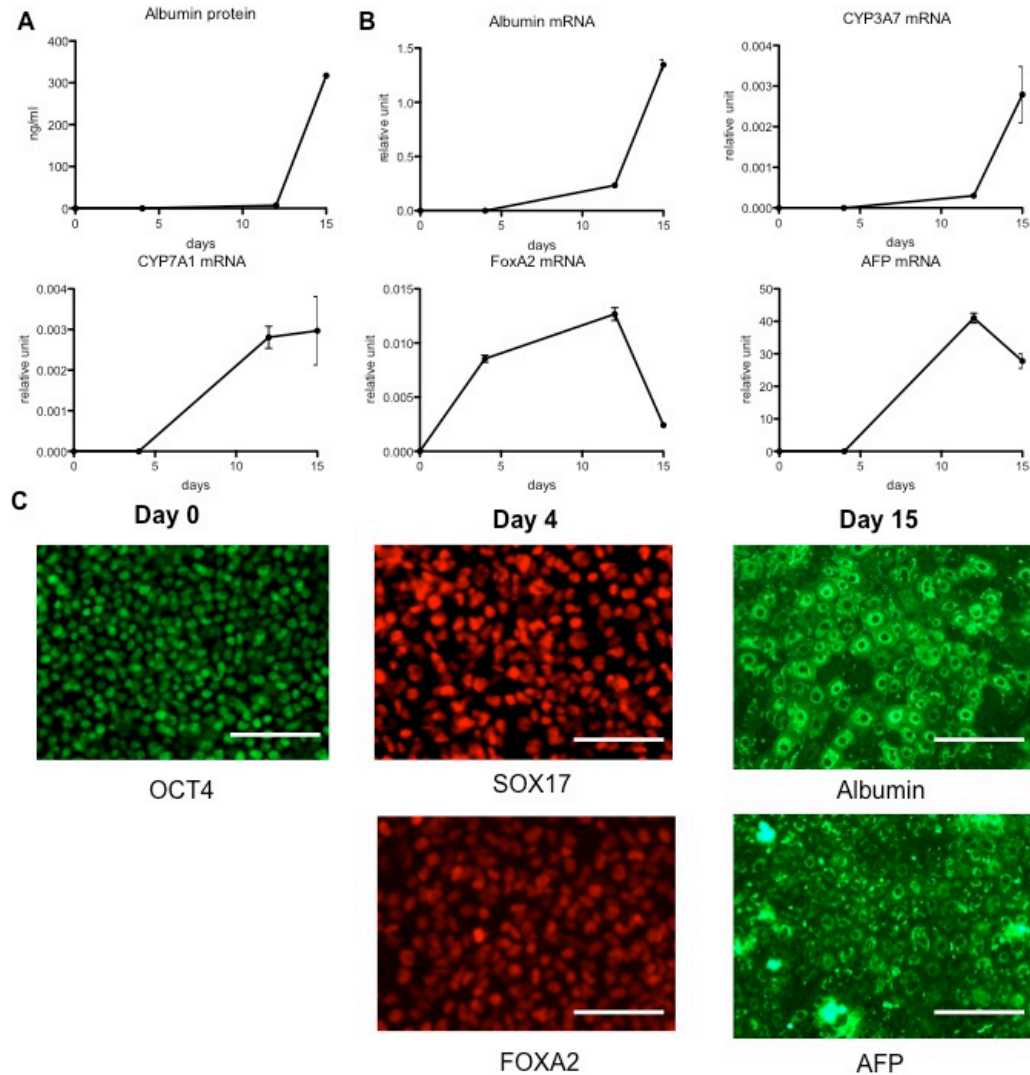
Supplementary table1. Primers and probes for qPCR

Name	Sequence 5'-3'
cccDNA 92 fw	GCCTATTGATTGGAAAGTATGT
cccDNA 2251 rev	AGCTGAGGCGGTATCTA
PRNP fw	GACCAATTTATGCCTACAGC
PRNP rev	TTTATGCCTACAGCCTCCTA
GAPDH fw	ACCAACTGCTTAGCCC
GAPDH rev	CCACGACGGACACATT
APOBEC3A fw	AAGGGACAAGCACATGGAAG
APOBEC3A rev	TGTGTGGATCCATCAAGTGTC
pgRNA 383 fw	CTCCTCCAGCTTATAGACC

pgRNA 705 rev	GTGAGTGGGCCTACAAA
rcDNA1745 fw	GGAGGGATACATAGAGGTTTCCTTGA
rcDNA1844 rev	GTTGCCCGTTTGTCTCTAATTC
HNF4A fw	GATGTAGTCCTCCAAGCTCAC
HNF4A rev	GCCATCATCTTCTTTGACCCA
HNF4A probe	FAM/AAGATCAAG/ZEN/CGGCTGCGTTCC/3IABkFQ
RXRA fw	GGAGGTGAGGGAGGAGTT
RXRA rev	GCATGAGTTAGTCGCAGACAT
RXRA probe	FAM/TGGAGAAAT/ZEN/CGAGCGGCAGGAAAT/3IABkFQ
PPARA fw	TTCTGTTCTTTTTCTGGATCTTGC
PPARA rev	CAGGCTATCATTACGGAGTCC
PPARA probe	FAM/AGAAGCCCT/ZEN/TGCAGCCTTCACA/3IABkFQ
NTCP fw	TGCTCTTCCCCACATTGATG
NTCP rev	TCCTGGTTCTCATTCTTGC
NTCP probe	FAM/TCC AAA CGG /ZEN/CCACAATACATGCG/3IABkFQ

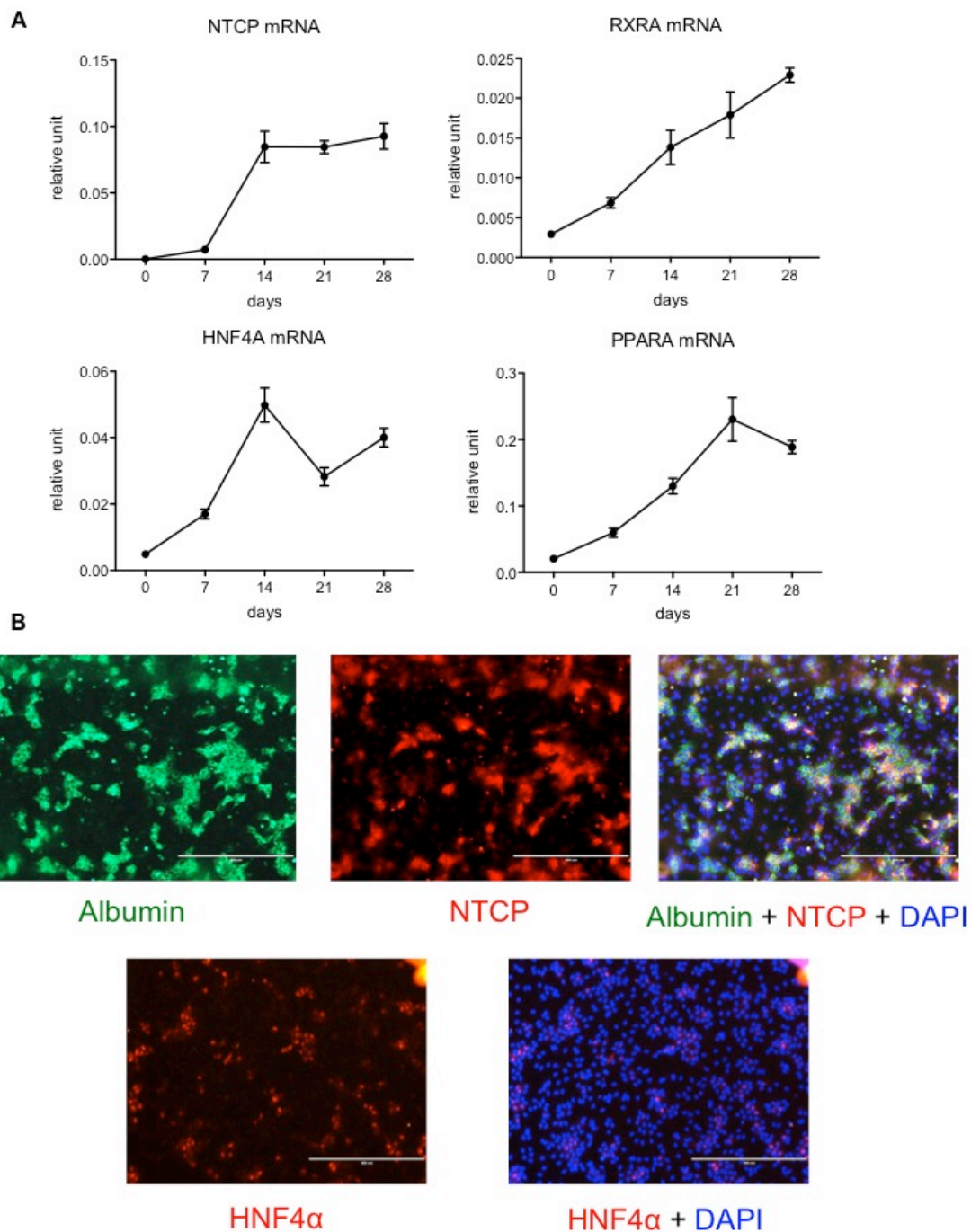
Supplementary table2. Antibodies used in immunostaining

Antigen	Source	Cat#	Host	Dilution
ALB	Cedarlane	CL2513A	Mouse	1:330
AFP	Sigma Aldrich	A8452	Mouse	1:330
HNF4A	Santa Cruz Biotechnology	sc-6556	Goat	1:100
OCT4	Santa Cruz Biotechnology	sc-9081	Rabbit	1:400
SOX17	R&D Systems	AF1924	Goat	1:100
FOXA2	Santa Cruz Biotechnology	sc-271103	Mouse	1:100
NTCP	Bruno Stieger		Rabbit	1:100
RXR	Santa Cruz Biotechnology	sc-553	Rabbit	1:100
PPAR	Santa Cruz Biotechnology	sc-9000	Rabbit	1:50
HBsAg	Invitrogen	18-0023	Mouse	1:50



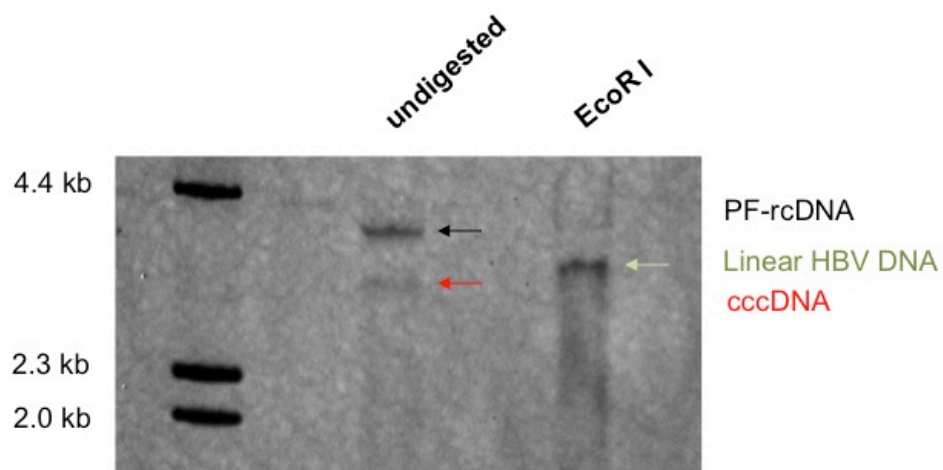
Supplementary figure 1. Differentiated HLCs express hepatocyte markers.

Human hESC H9 derived HLCs were obtained through a three-step differentiation protocol. Time line is indicated in Fig 1A. **(A)** Secreted albumin in the cell culture supernatant was measured by ELISA. **(B)** mRNA kinetics of differentiation markers were evaluated by qRT-PCR. The relative unit is defined as an expression ratio of target against reference ACTB. **(C)** Immunostaining of differentiation markers at different stages. Scale bar= 200 μ M.



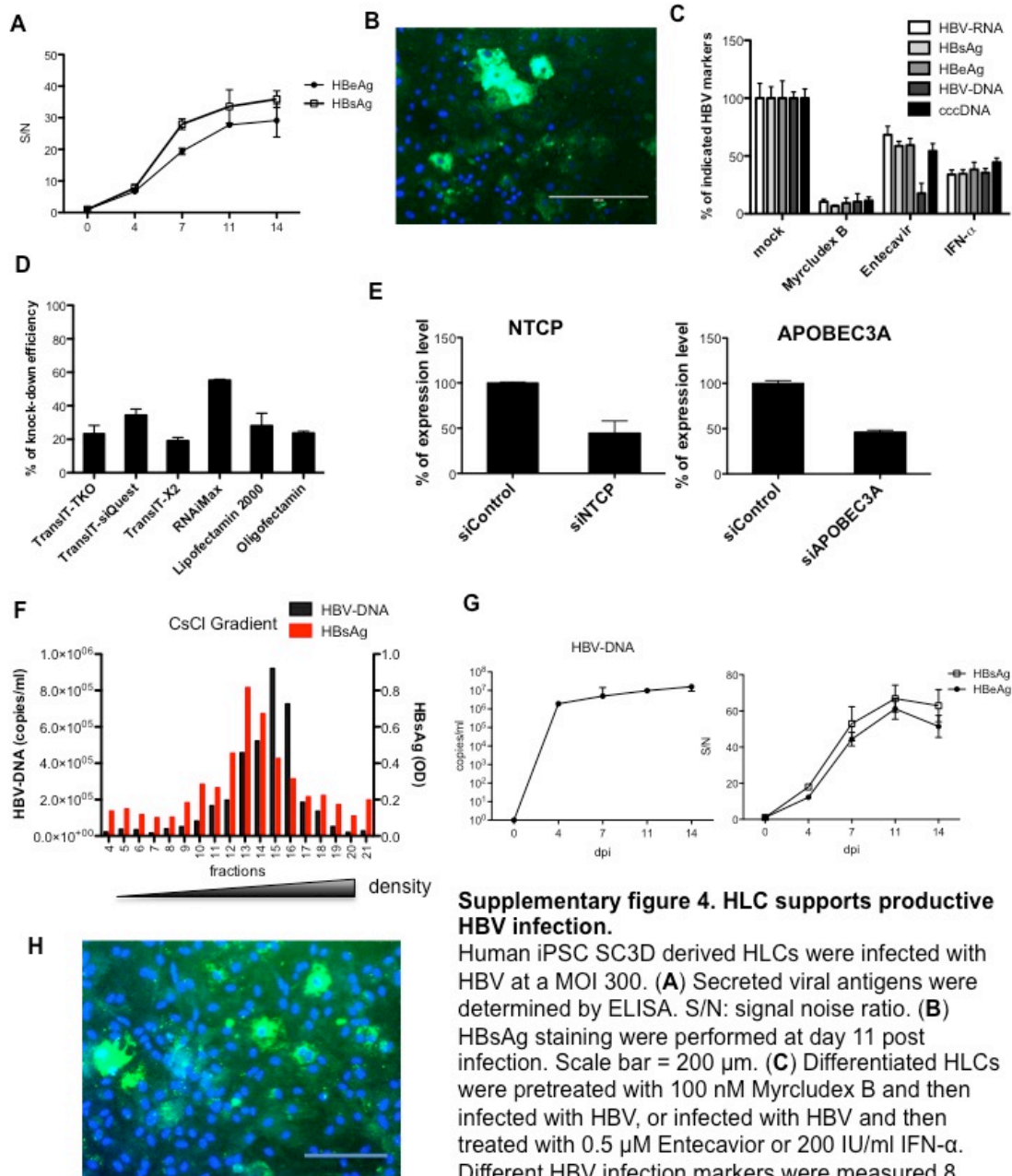
Supplementary figure 2. Differentiation of HepaRG cells.

HepaRG cells were differentiated for 4 weeks. **(A)** mRNA kinetics of different HBV NTCP, RXR, HNF4α and PPAR were evaluated by qRT-PCR. The relative unit is defined as an expression ratio of target against reference ACTB. **(B)** Expression of albumin, NTCP and HNF4α by the end of differentiation were determined by immunostaining. Scale bar = 400 μm.



Supplementary figure 3. Southern blot analysis of HBV cccDNA.

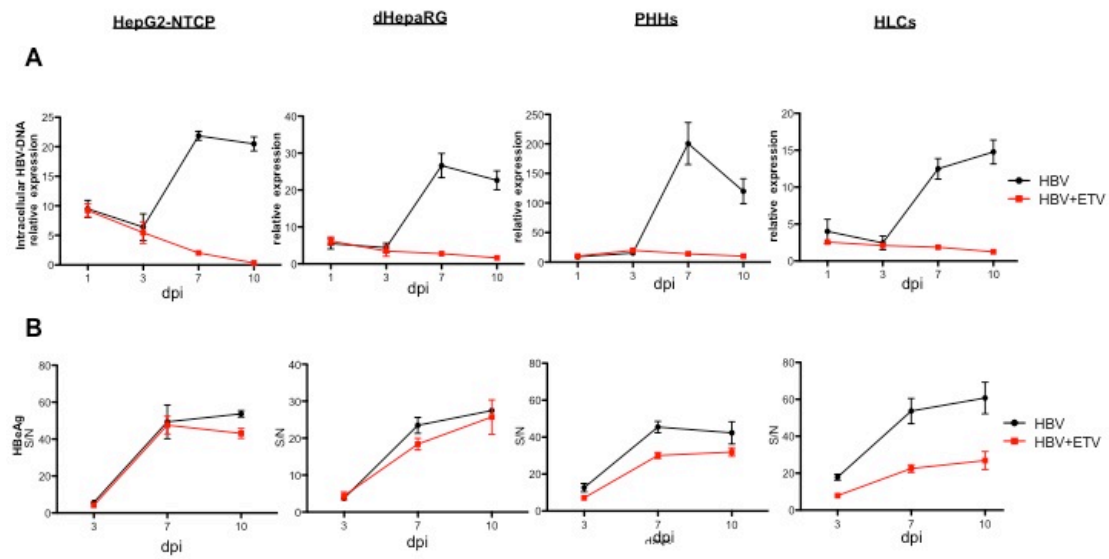
Human iPSC SC3D derived HLCs were infected with HBV at a MOI 300. Protein-free Hirt DNA was extracted 10 days after infection, treated with EcoR I and subjected to gel electrophoresis. Southern blot analysis was performed to detect viral DNA using a digoxigenin-labeled HBV-specific probe.



Supplementary figure 4. HLC supports productive HBV infection.

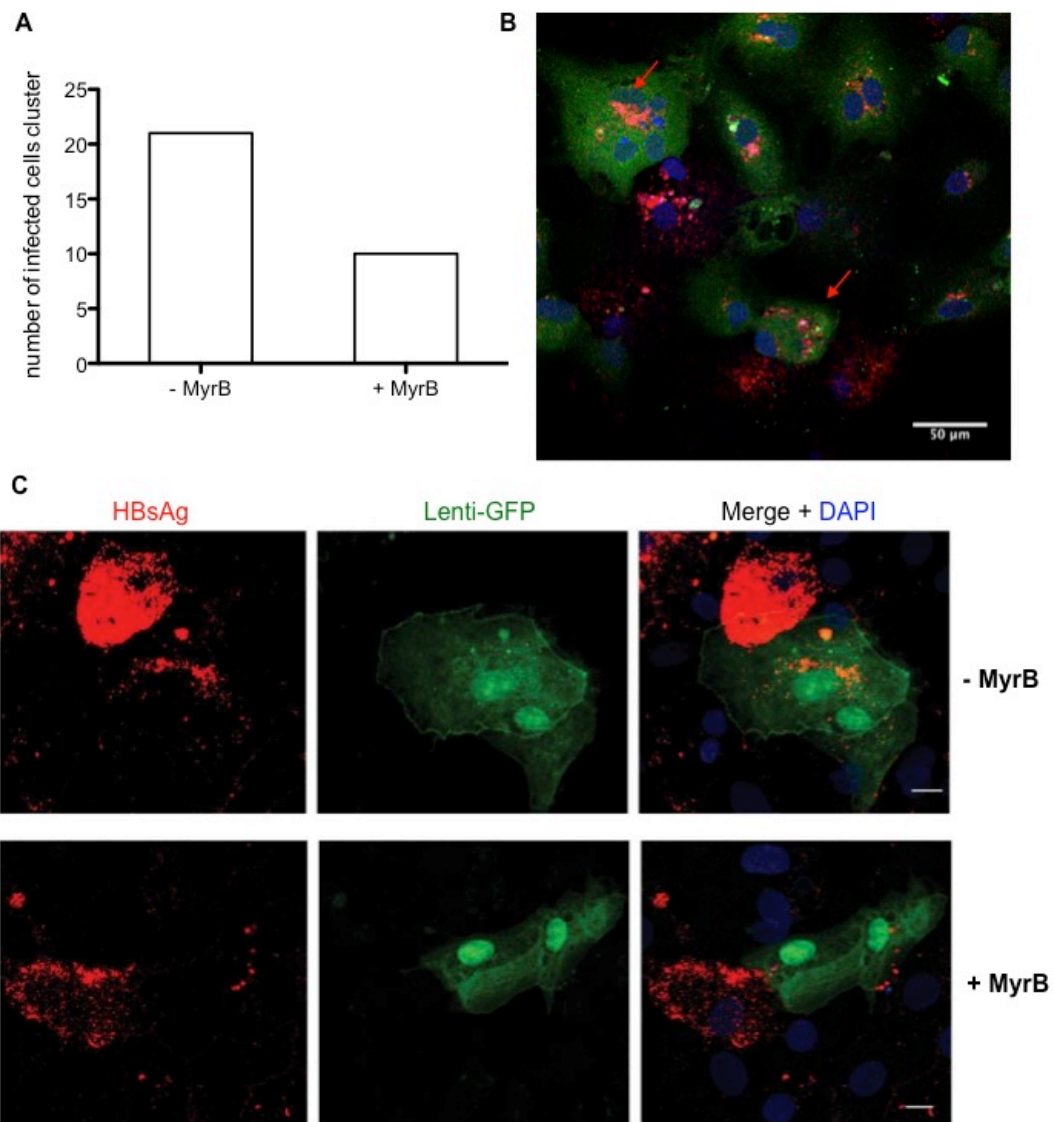
Human iPSC SC3D derived HLCs were infected with HBV at a MOI 300. **(A)** Secreted viral antigens were determined by ELISA. S/N: signal noise ratio. **(B)** HBsAg staining were performed at day 11 post infection. Scale bar = 200 μ m. **(C)** Differentiated HLCs were pretreated with 100 nM Myrcludex B and then infected with HBV, or infected with HBV and then treated with 0.5 μ M Entecavir or 200 IU/ml IFN- α . Different HBV infection markers were measured 8 days after infection.

(D) siRNA transfections were tested on HLCs with indicated transfection reagents. Knock-down efficiency was determined by qPCR. **(E)** HLCs were transfected with siRNA against NTCP or APOBEC3A using RNAiMax, and then infected with HBV 48 hours post transfection. Efficiency of siRNA knock-down was determined by qPCR. **(F)** Supernatant of HBV infected HLCs were concentrated by CsCl ultracentrifugation, HBV-DNA and HBsAg from collected fractions were evaluated by qPCR and ELISA respectively. HLCs were inoculated with concentrated supernatant from HBV-infected HLCs (from day 7 to day 17) at a MOI 300. **(G)** Kinetics of viral DNA and antigens were determined by qPCR or ELISA respectively. **(H)** HBsAg staining was performed on day 14 after inoculation. Goat anti-mouse secondary antibody was used (Green). Scale bar = 200 μ m.



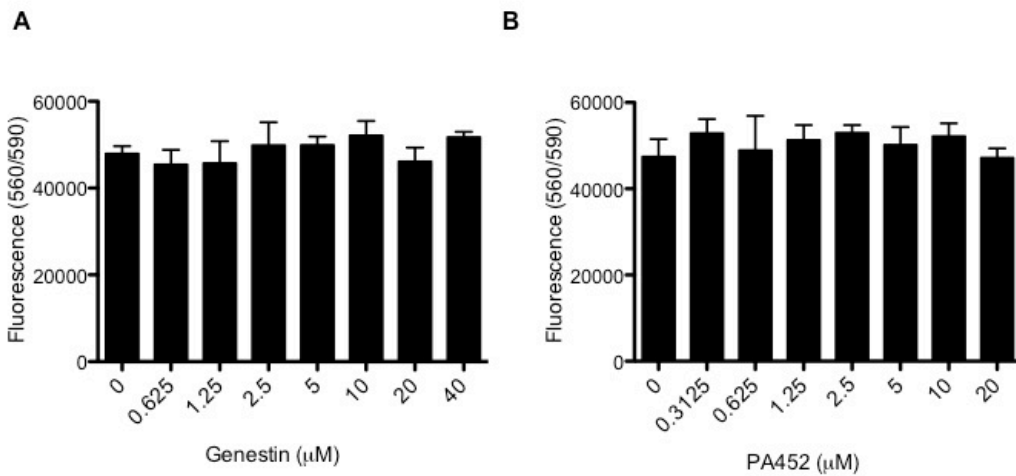
Supplementary figure 5. Comparison of HBV replication in different *in vitro* models.

HepG2-NTCP, dHepaRG, PHHs and HLCs were infected with HBVcc at MOI 300 and treated with or without ETV. **(A)** Intracellular HBV-DNA and **(B)** HBeAg at different time points were determined.



Supplementary figure 6. HBV spreading in HLC cultures.

(A) HLCs were infected with HBVcc at a MOI 300. One day after infection, cells remain untreated or treated with Myrcludex B for 10 days. HBsAg staining was performed. infected cells clusters (more than 2 connected HBV positive cells) from 10 random views were calculated. (B) HLCs were infected with HBV for 7 days, and then co-cultured with CMFDA (green) labeled HLC cells. Three days after co-culture, cells were fixed and stained for HBsAg (red). (C) HLCs were infected with HBV for 7 days, and then trypsinized and co-cultured with GFP-lentivirus transduced HLCs with or without Myrcludex B. Eight days after co-culture, cells were fixed and stained for HBsAg (red). Scale bar = 10 μ m.



Supplementary figure 7. Cytotoxicity assay

iPSCs SC3D derived HLCs were infected with HBVcc at a MOI 300 and treated with indicated dose of (A) Genestin or (B) PA452. Ten days after infection, viability of the cells were determined by CellTiter-Blue® Cell Viability Assay.

References

Author names in bold designate shared co-first authorship.

[1] Xia Y, Stadler D, Ko C, Protzer U. Analyses of HBV cccDNA quantification and modification. *Methods in Molecular Biology: Hepatitis B Virus* 2016. In press.

[2] **Lucifora J, Xia Y**, Reisinger F, Zhang K, Stadler D, Cheng X, et al. Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. *Science* 2014;343:1221-1228.

[3] Wang F, Flanagan J, Su N, Wang LC, Bui S, Nielson A, et al. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J Mol Diagn* 2012;14:22-29.