Supporting Information

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SI Materials and Methods

Primary Human Adult Hepatocytes. Primary human hepatocytes were purchased from vendors permitted to sell products derived from human organs procured in the United States by federally designated Organ Procurement Organizations. Vendors included: Celsis In Vitro Technologies (Celsis), Triangle Research Labs (TRL), and VWR through Xenotech (VWR) (See Table S1). Human hepatocytes were pelleted by centrifugation at 50–100 × *g* for 5–10 min at 4 °C, resuspended in hepatocyte culture medium, and assessed for viability using trypan blue exclusion (typically 70–90%).

Inducible Pluripotent Stem Cell Culture and Induced Hepatocyte-Like Cell Generation. In brief, inducible pluripotent stem cells (iPSCs) were cultured in monolayers on matrigel (Becton Dickinson), and directed differentiation was achieved by sequential exposure to activin A (R&D Systems), bone morphogenic protein 4 (R&D Systems), basic fibroblast growth factor (Invitrogen), hepatocyte growth factor (R&D Systems), and oncostatin M (R&D Systems) (1).

Micropatterned Cocultures. Off-the-shelf tissue-culture polystyrene (24-) or glass-bottom (24-) multiwell plates, coated homogenously with rat tail type I collagen (50 μg/mL), were subjected to soft-lithographic techniques to pattern the collagen into microdomains (islands of 500 μm in diameter with 900-μm center-to-center spacing). To create micropatterned cocultures (MPCCs), cryopreserved adult human hepatocytes were seeded on collagen-patterned plates that mediate selective cell adhesion. The cells were washed with medium 2–3 h later (~4×10^4 adherent hepatocytes in 96 collagen-coated islands in a 24-well plate) and incubated in hepatocyte medium overnight. Hepatocyte culture medium was DMEM with high glucose, 10% (vol/vol) FBS, 1% (vol/vol) ITS premix (BD Biosciences, cat No 354352), 7 ng/mL glucagon, 40 ng/mL dexamethasone, and 1% penicillin-streptomycin. 3T3-J2 murine embryonic fibroblasts were seeded (9×10^4 cells in each well of a 24-well plate) 24-h later. Hepatocyte culture medium was replaced 24 h after fibroblast seeding and subsequently replaced every other day. Randomly cultured cocultures (RCCs) of hepatocytes and 3T3-J2 fibroblasts were created as described previously (2). Briefly, RCCs were generated by seeding 2×10^5 hepatocytes per well of a collagen-coated 24-well plate, followed by addition of 9×10^4 J2-3T3 cells the next day, all in the same hepatocyte culture medium as used in MPCCs. There is a fivefold increase in hepatocytes per well in RCCs compared with MPCCs, but these numbers were chosen because hepatocyte survival is improved in denser culture.

Hepatitis B Virus Infection of MPCCs and Induced Hepatocyte-Like Cells. The concentration of entecavir was chosen as 30× the EC_{50} according to the literature (3). De-identified plasma positive for hepatitis B virus (HBV) but negative for HCV and HIV was obtained from the Red Cross. For all of the experiments presented in this study, three stocks of plasma derived from three different donors were used. Two stocks were genotype D the other genotype A. Genotypes were determined using DNA extracted from plasma by PCR using primers (F) 5′-CTCCACCAATCGGCAGTC-3′ and (R) 5′-AGTCCAAGAGTCCTCTTATGTAAGACCTT-3′. PCR products were sequenced using the following primer: 5′-CCTCTGCCGATCCATACTGCGGAAC-3′ and genotypes were determined using the National Center for Biotechnology Information genotyping online tool (www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi). For infection, calcium chloride was added to the plasma at a final concentration of 1.25 mM and incubated for 30 min at 37 °C. The gelled plasma was then spun at 14,000 × *g* for 5 min, and the prior two steps repeated until no gelled clots remained. The supernatant remaining (serum) was then used to inoculate MPCC or induced hepatocyte-like cells (iHeps) at a 1:10–1:20 dilution in standard culture medium for 24 h. The calculated multiplicity of infection, based on initial viral (DNA) titer and cell number, was between 300 and 350 HBV genomes per cell. Cells were washed five times with DMEM, then new hepatocyte culture media or iHep culture media was added. Every 48 h, medium was collected and stored at −80 °C for subsequent analyses and replaced with fresh medium.

Quantification of Intracellular or Secreted HBV DNA in iPSC-Derived and Primary Hepatocytes. Cell pellets or media were collected and DNA was extracted using the QIAamp DNA blood mini kit (QIAGEN, cat No 51104) or QIAamp Minielute Virus spin kit (QIAGEN, cat No 57704), respectively. DNA was extracted according to the manufacturer's protocol, and the final product was eluted in 60 μL of water. Five microliters was taken for a quantitative PCR (qPCR).

Quantification of Total HBV DNA. qPCR for HBV DNA was performed using the TaqMan Universal PCR Master Mix (Applied Bio systems, cat No 4304437) and the following primers and probe: 5′-CCGTCTGTGCCTTCTCATCTG-3′ (sense), 5′-AGTCCAAGAGTCCTCTTATGTAAGACCTT-3′ (antisense), 5- /56-FAM/CCG TGT GCA /ZEN/CTT CGCTTC ACCTCT GC/ 3IABkFQ/ -3 (probe). PCR was performed using the Roche LightCycler 480 and the following conditions: (*i*) denaturation at 50 °C for 5 min followed by 95 °C for 10 min (one cycle); (*ii*) qPCR at 95 °C for 15 s, 56 °C for 40 s, and 72 °C for 20 s (40 cycles); (*iii*) melting at 65 °C for 10 s, followed by 95 °C (continuous).

Quantification was done by using a standard curve composed from 2× HBV plasmid over a range of 10^9–10^1 copies.

HBV Covalently Closed Circular DNA Quantification. DNA extracted from cells was subjected to overnight digestion with a plasmid-safe DNase (Epicentre), as previously described (4). Following enzyme inactivation at 70 °C for 30 min, DNA was subjected to real-time PCR using SYBR Premix Ex Taq (TaKaRa) following a protocol previously described (4) and using the covalently closed circular DNA (cccDNA) -specific primers described by Glebe et al. (5).

The primers used for cccDNA amplification were 5′-TGCACTTCGCTTCACCTF-3′ (sense), 5′-AGGGGCATTTGGTGGTC-3′ (antisense). For quantification, a standard curve derived from decreasing concentrations of 2× HBV plasmid was used.

PCR was performed using the Roche LightCycler 480 and the following conditions: (*i*) denaturation at 95 °C for 2 min (one cycle); (*ii*) qPCR at 95 °C for 10 s, 63 °C for 20 s, and 72 °C for 45 s (40 cycles); (*iii*) melting at 95 °C for 10 s, 65 °C for 10 s, and 95 °C (continuous).

Analysis of HBV DNA Forms. Total DNA was extracted using the QIAamp DNA blood mini kit (QIAGEN, cat No 51104) in a procedure involving cell lysis and proteinase K treatment (without prior DNaseI treatment). Total DNA was later run on 0.8% agarose-TAE gel, followed by denaturation and Southern blotting to a Hybond N nylon membrane (Amersham). Viral DNA was detected by hybridization with a ^{32}P random primed HBV probe, using the Prime-It II Random Primer Labeling Kit (Agilent

Technologies, Cat No 300385). Following incubation and washing, hybridized species were visualized by phosphorimaging and film exposure.

Analysis of Viral and Cellular mRNAs in MPCC and iPSC-Derived iHeps. Total RNA was isolated with the RNeasy Plus Mini Kit (Qiagen) or via TRIZOL RNA/DNA extraction. RNA was quantified using a NanoDrop, and first-strand cDNA was synthesized using Moloney murine leukemia virus RT (Bio-Rad) or SuperScript III RT kit (Invitrogen). qPCR for various genes/mRNAs including HBV 3.5 kb and total transcripts, IFN-stimulated genes, bile acid pump sodium taurocholate cotransporting polypeptide (NTCP), and differentiation factors was carried out with Taq polymerase and SYBR Green in the supplier's reaction buffer containing 1.5 mM $MgCl_2$ (Bio-Rad). For amplification of HBV 3.5-kb mRNA or total HBV mRNAs, we used primers spanning the 5′ end common only to the HBV long transcript or the region upstream to the poly(A) signal spanning the 3′ of all mRNAs, respectively, as previously described (4). To rule out HBV DNA contamination, total RNA was pretreated with DNaseI before first-strand synthesis and for every qRT-PCR analysis, a negative control (without reverse transcriptase) was included. qRT-PCR results for HBV transcripts were normalized to the human RPS11 housekeeping gene; other qRT-PCR results were normalized to β-actin (and verified with GAPDH). Oligonucleotide primer sequences are available by request. Amplicons were analyzed by 2% (wt/vol) agarose gel electrophoresis (Bio-Rad).

Detection of Secreted Hepatitis B Surface Antigen. One-hundred microliters of medium was loaded on ELISA plates coated with mouse monoclonal anti-hepatitis B surface antigen (HBsAg) antibodies (Bio-Rad, GS HBsAg EIA 3.0, Cat. No. 32591). ELISA was carried out according to the manufacturer's instructions. Plates were read using the FLUOstar Omega luminometer (BMG LABTECH). HBsAg positivity (cut-off) was calculated as an average of three negative controls + 0.07 (this value was optimized to avoid false–positive identification of HBsAg).

Detection of Secreted Hepatitis B e-Antigen. Fifty microliters of medium was loaded on ELISA plates coated with mouse monoclonal anti-hepatitis B e-antigen (HBeAg) antibodies (AbNova KA3288). ELISA was performed according to the manufacturer's instructions using HRP detection with 3,3′,5,5′-tetramethylbenzidine (Thermo Scientific) substrate.

HBV Transcription During iHep Differentiation. For HBV transcription during iHep differentiation, 1.3 × HBV-Luc, in which a luciferase cassette is cloned downstream of the EnhII and pre-C/C promoter, was a kind gift from Y. Shaul (The Weizmann Institute, Rehovot, Israel) (6). Ten micrograms of HBV-Luc plasmid DNA was transfected using TransIT-2020 reagent (Mirus) to 1.0×10^6 iPSC/iHeps at varying stages of differentiation. Cells were analyzed 72–96 h posttransfection for luciferase expression. Briefly, cells were incubated with D-luciferin (Invitrogen) for 10 min and then imaged using an IVIS Spectrum optical imaging system. Bioluminescent images were acquired using the autoexposure function. Data analyses for signal intensities and image comparisons were performed using Living Image software (Caliper Life Sciences). To calculate radiance for each well, the well size was delinated and each signal was expressed as radiance (photons per s/cm^2 per steradian). To rule out variations in transfection efficiency, cells were cotransfected with a GFP-expressing plasmid and GFP$^+$ cells quantified by fluorescence microscopy 72 h posttransfection. In addition, intracellular DNA was quantified by amplification of the luciferase fragment and normalization to β-globin DNA.

Immunofluorescence Analyses. Cells were fixed in 4% (wt/vol) paraformaldehyde (Electron Microscopy Services) or −20 °C methanol. After washing and blocking in 0.1% donkey serum/0.1% Triton X- 100 in PBS, cells were incubated in primary antibodies overnight at 4 °C (mouse or rabbit anti-human albumin (Sigma Aldrich); goat anti-human α-1-antitrypsin (Bethyl Laboratories); mouse or rabbit anti-human cytokeratin 18 (Sigma Aldrich); rabbit anti-human α-fetoprotein (Santa Cruz); mouse anti-human SOX17 (R&D Systems); goat anti-human HNF4α (Santa Cruz); polyclonal rabbit anti-HBV Core (generously provided by Y. Shaul, The Weizmann Institute, Rehovot, Israel) (7). Secondary antibodies were donkey anti-mouse DyLight 594, donkey anti-rabbit DyLight 488, donkey anti-mouse DyLight 488, or donkey anti-rabbit DyLight 594 conjugates (Jackson Immunoresearch). Cells were counterstained with Hoechst dye (Invitrogen).

Western Blot Analysis of NTCP. Total protein was extracted with radioimmunoprecipitation assay lysis buffer, and samples were separated by electrophoresis on 12% (wt/vol) polyacrylamide gels and electrophoretically transferred to a PVDF membrane (Bio-Rad Laboratories). Blots were probed with NTCP antibody (Aviva Biosystems) followed by HRP-conjugated anti-rabbit secondary antibodies (Amersham), and developed using Super-Signal West Pico substrate (Thermo Scientific).

Albumin and Transferrin ELISA. Media samples were stored at −20 °C. Transferrin and albumin concentrations were measured by sandwich ELISA using HRP detection (Bethyl Laboratories) and 3,3′,5,5′-tetramethylbenzidine (Thermo Scientific) substrate.

Generation of an NTCP-Expressing HepG2 Cell Line. HepG2 cells (p25) in collagen-coated six-well plates were transduced with VSV-G pseudotyped TRIP-based lentiviral pseudoparticles expressing FLAG-hNTCP1-GFP. Transduced cells were expanded to a P100 plate then scaled to a T175 flask. Transduction efficiency was confirmed by flow-cytometry. GFP$^+$ cells were sorted by FACS to intermediate and high GFP$^+$ populations. Intermediate and high GFP-expressing cells were singly sorted in 3× collagen-coated 96-well flat-bottom plates and monitored for growth under the microscope. Multiple clones were tested for HBV infection permissiveness, with clone 3E8 demonstrating the highest infectivity.

1. Schwartz RE, Fleming HE, Khetani SR, Bhatia SN (2014) Pluripotent stem cell-derived hepatocyte-like cells. *Biotechnol Adv* 32(2):504–513.
2. Khetani SR, Bhatia SN (2008) Microscale culture of human liver cells for drug development. *Nat Biotechnol* 26(1):120–126.
3. Langley DR, et al. (2007) Inhibition of hepatitis B virus polymerase by entecavir. *J Virol* 81(8):3992–4001.
4. Yan H, et al. (2012) Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *eLife* 1:e00049.
5. Glebe D, et al. (2003) Pre-s1 antigen-dependent infection of Tupaia hepatocyte cultures with human hepatitis B virus. *J Virol* 77(17):9511–9521.
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7. Cooper A, Shaul Y (2005) Recombinant viral capsids as an efficient vehicle of oligonucleotide delivery into cells. *Biochem Biophys Res Commun* 327(4):1094–1099.

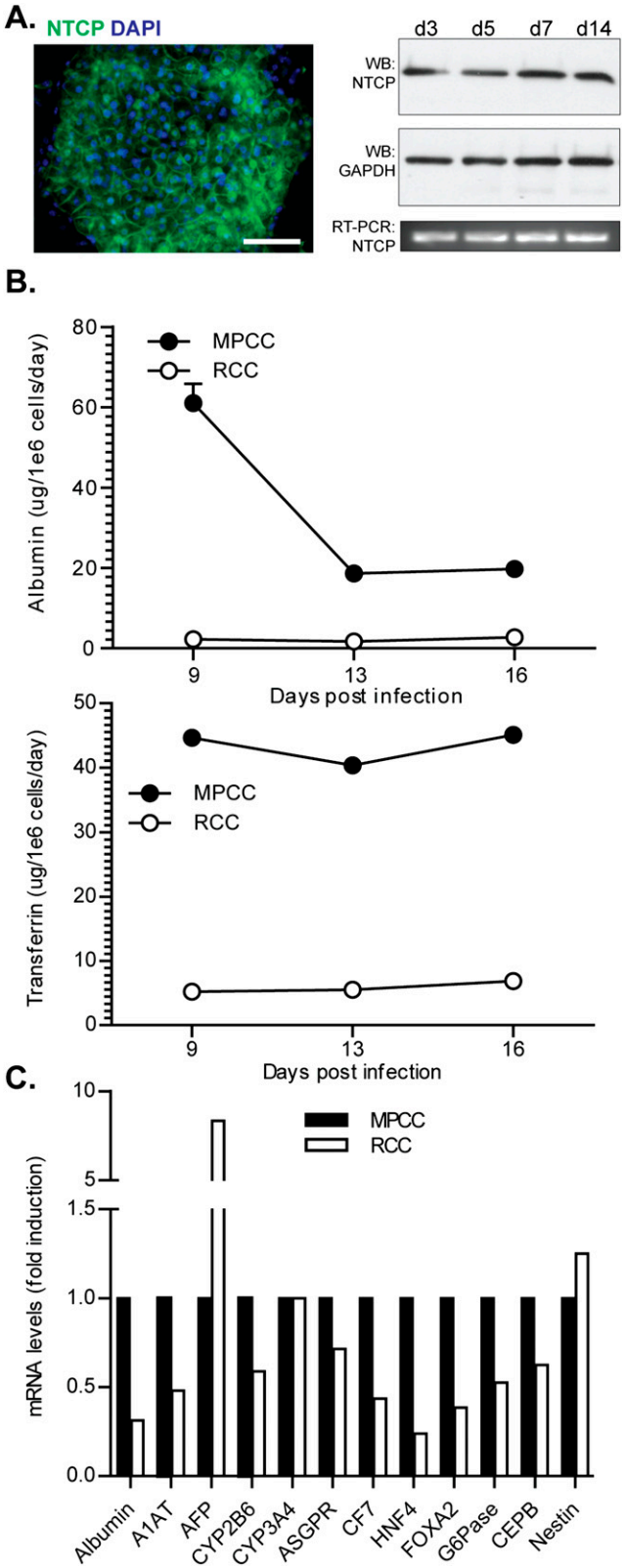

Fig. S1. MPCCs maintain hepatocyte-specific function compared with RCCs. (*A*, *Left*) Immunofluorescence (IF) staining of NTCP shows receptor expression only in circular hepatocyte islands. (Scale bar, 100 μm.) (*Right*) Western blotting and RT-PCR for NTCP protein and RNA, respectively, show receptor maintenance over at least 2 wk. (*B*) MPCCs and RCC hepatocytes (HD4) were analyzed for albumin (*Upper*) and transferrin (*Lower*) levels at the indicated time points after infection with HBV (infection was done 2 d after plating the cells); data expressed as mean ± SEM with $n = 3$. (*C*) MPCCs and RCCs were collected and analyzed for the expression of major genes implicated in normal hepatocyte function, with Janus kinase inhibitor (JAKi) treatment administered to mimic infection conditions.

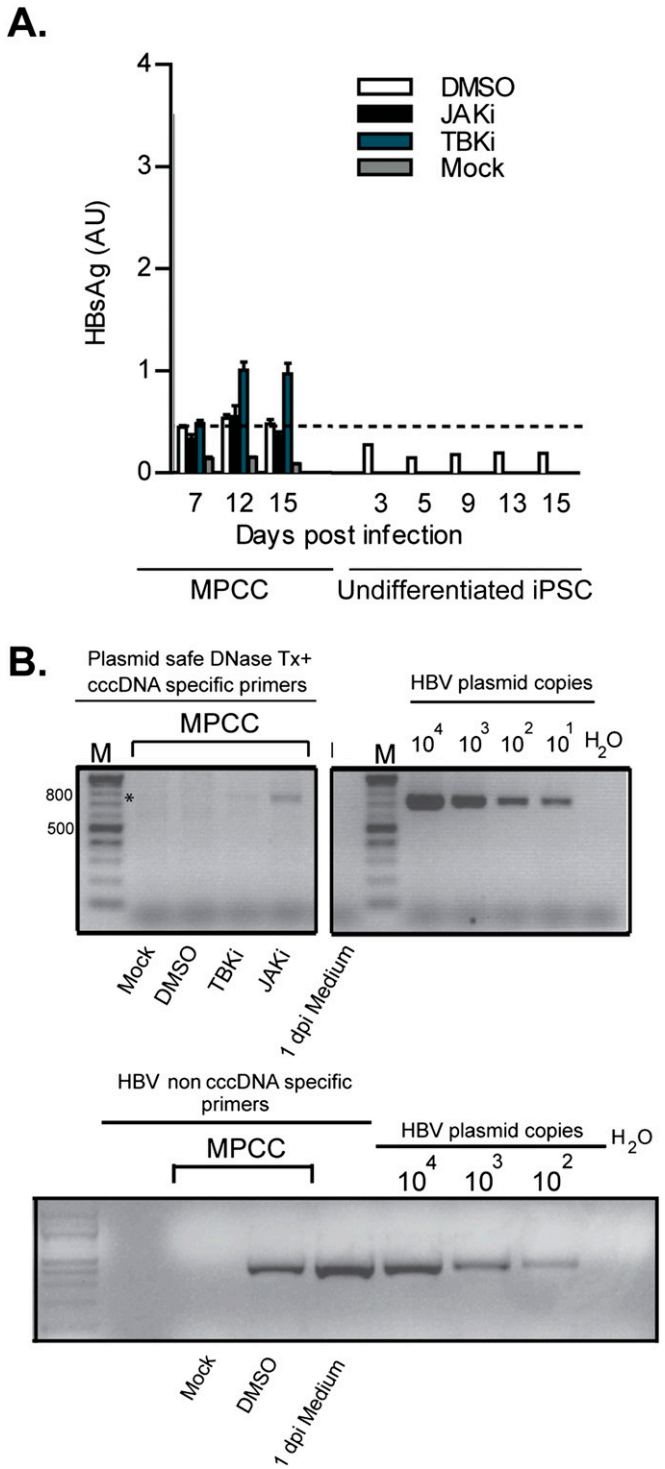

Fig. S2. JAK or TANK binding kinase 1 (TBK1) inhibition enhances HBV infection. (*A*) Primary human hepatocytes (MPCCs) were pretreated with DMSO, JAK, or TBK1 inhibitors 24 h before infection and every other day thereafter. Following incubation with HBV^+ serum for 24 h, medium was collected at the indicated time points for HBsAg analysis (data expressed as mean ± SEM, $n = 3$). iPSCs (nonpermissive for HBV) were used as negative controls. Dotted line indicates the cut-off. (*B*) DNA extracted from HBV-infected cells was amplified using cccDNA-specific primers or primers capable of amplifying all HBV DNA forms following treatment with plasmid-safe DNase, as detailed in *SI Materials and Methods*. PCR products were separated by agarose gel electrophoresis. Two-times HBV plasmid DNA, at the indicated genome equivalents, was used as a positive control. Medium from infected cells 1 day postinfection (dpi) was used as a negative control to show the specificity for cccDNA amplification.

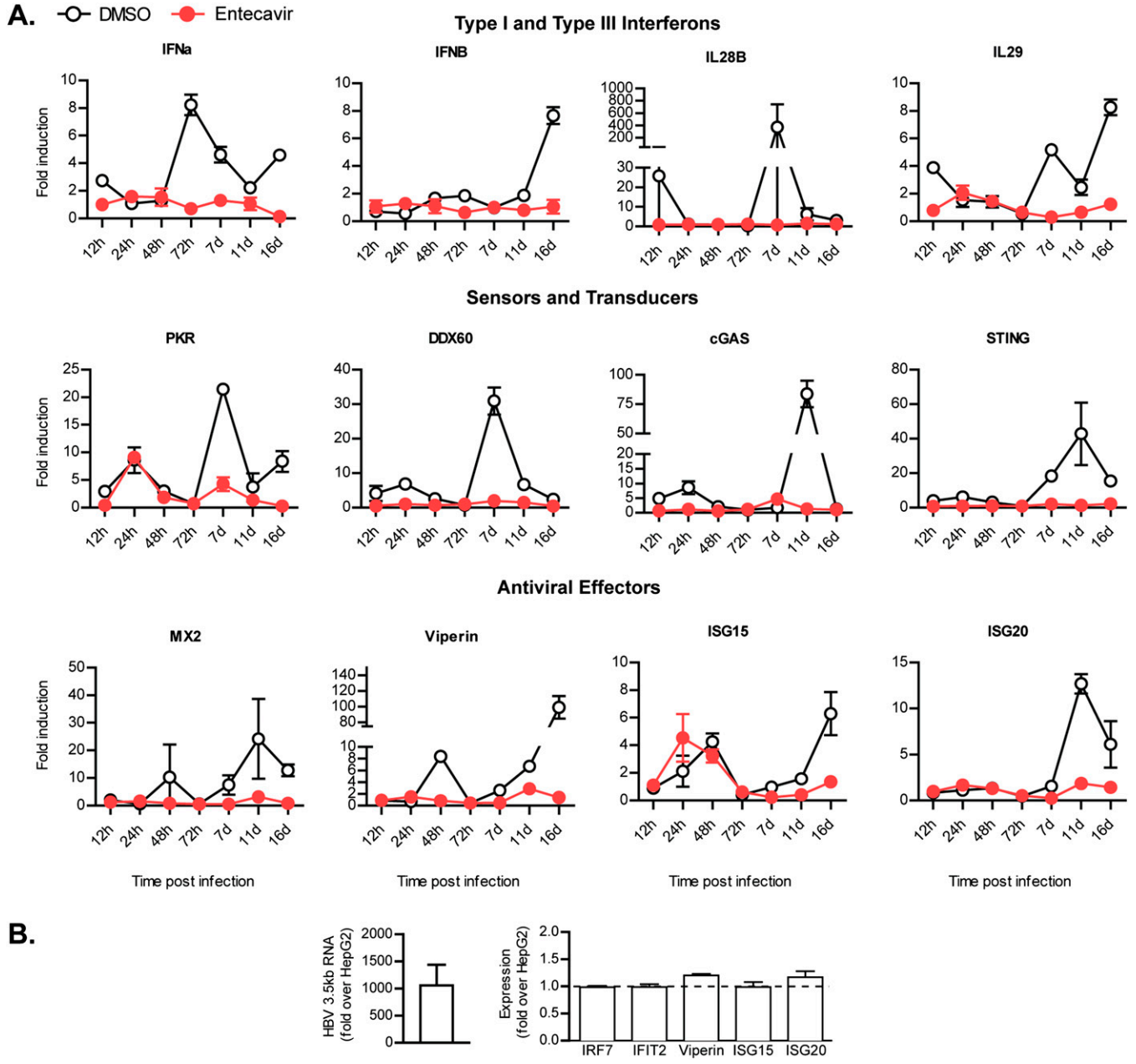

Fig. S3. HBV infection-dependent induction of interferon-stimulated genes (ISGs). (*A*) HD4-derived MPCCs were either mock- or HBV-infected and treated with the reverse-transcriptase inhibitor entecavir or DMSO (vehicle control). Cells were harvested at the indicated times postinfection and RNA was analyzed for the indicated ISGs. Results are normalized to β-actin (and verified with GAPDH) and reported as expression levels relative to the mock-infected cells for each time point. The experiment was done in triplicate and the numbers reflect the mean ± SEM. (*B*) HBV-infected naïve or human NTCP-expressing HepG2 cells were analyzed for 3.5-kb mRNA (*Left*) and ISG RNA expression levels at 9 dpi (*Right*). Results are reported relative to nonpermissive, naïve HBV-infected HepG2 cells ($n = 2$).

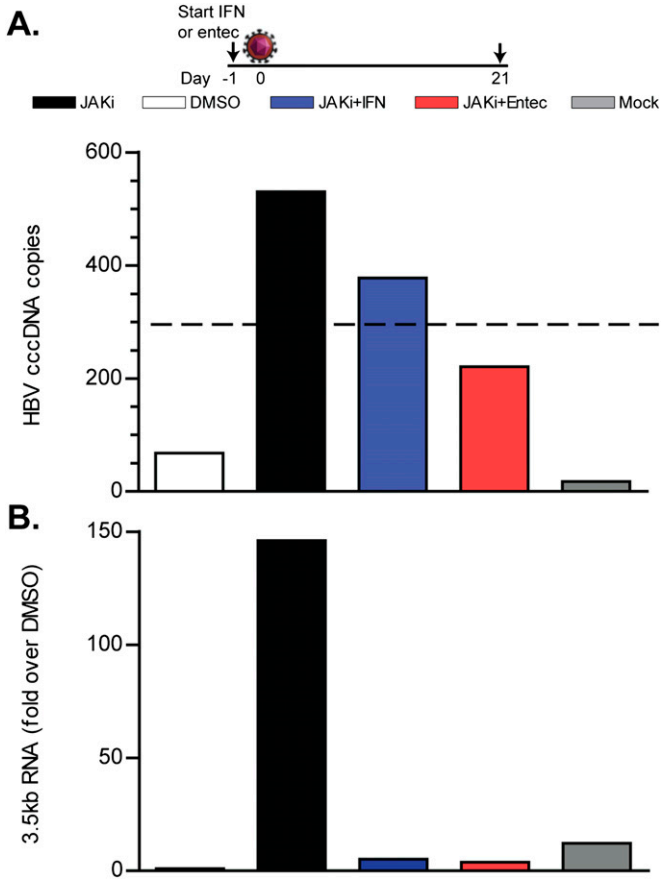

Fig. S4. Prophylactic drug treatment of MPCCs suppresses HBV infection. MPCCs were treated with DMSO (vehicle), JAKi, JAKi and entecavir, or JAKi and IFN-β starting at 1 d before infection. Cultures were subsequently incubated with infectious HBV serum for 24 h. (*A*) At 21 dpi, cell pellets were analyzed for HBV cccDNA (total copies per pellet). Limit of quantification (dotted line). (*B*) Cell pellets analyzed for HBV 3.5-kb mRNA (expression levels relative to HBV infected DMSO treated cells, one pellet per condition per experiment; convergent results were obtained in two independent experiments).

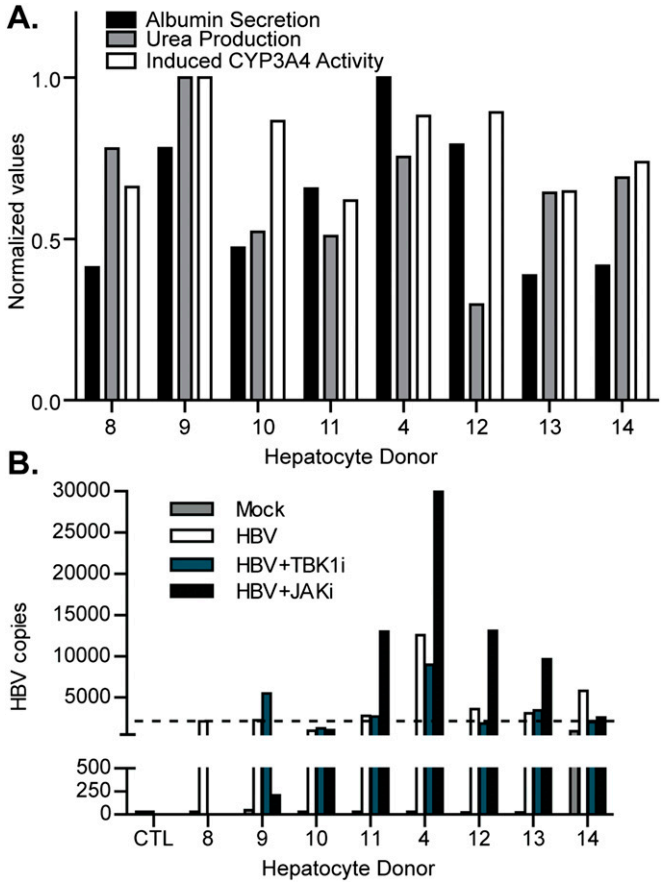

Fig. S5. Common markers of hepatocyte function do not predict donor permissiveness. (*A*) Hepatocytes screened for their HBV permissiveness were also tested for major hepatocyte functions (i.e., albumin secretion, urea production and induced CYP3A4 activity). The set of hepatocyte donors in this experiment is nonoverlapping with the set from Fig. 3, except for HD4, which was used for the majority of experiments in this study. A comparison is presented with the value of 1 representing the most robust activity observed for each of the analyzed parameters. (*B*) MPCCs created with hepatocytes from the donors in *A* were pretreated with DMSO, JAKi, or TBK1i, and infected with HBV. At 16 dpi, cell pellets were harvested and total HBV DNA was extracted and quantified. Limit of quantification (dotted line).

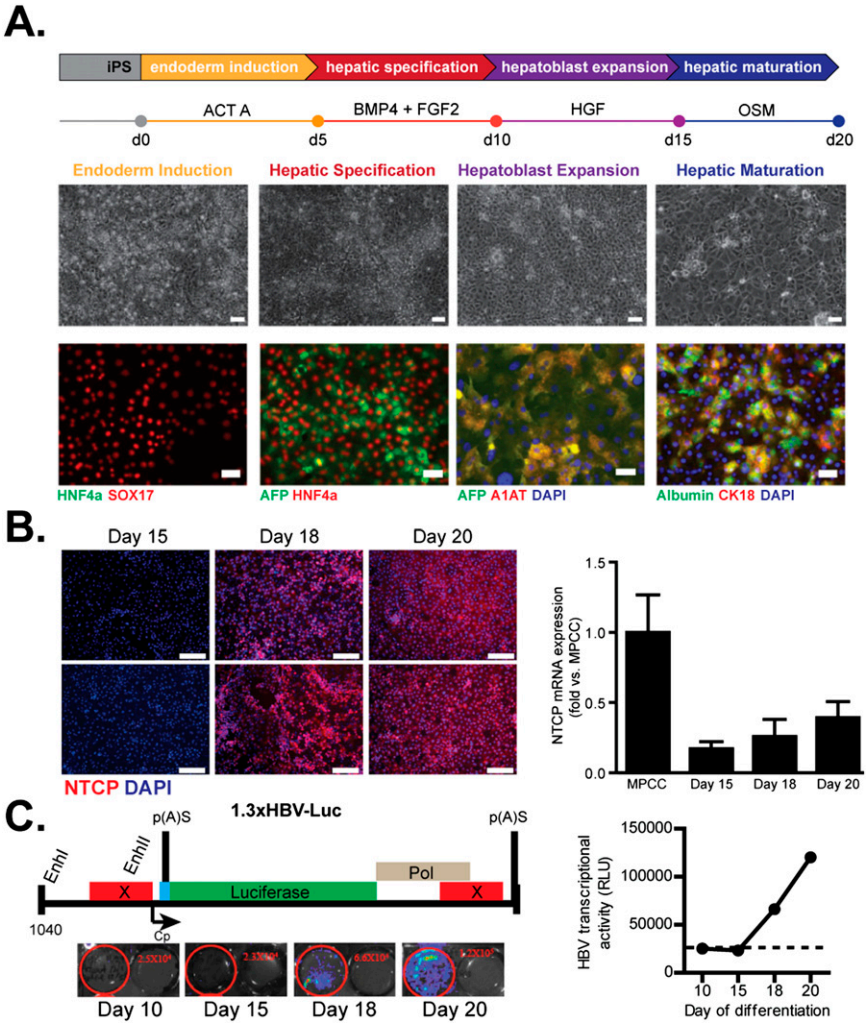

Fig. S6. Expression of NTCP and activation of HBV transcription during iPSC differentiation to iHeps. (*A*, *Upper*) Schematic representation of the stages of differentiation, including cell-culture medium additives used to stimulate the progression to each new stage. (*Lower*) Bright-field microscopy of cellular morphology (*Upper Row*) and immunofluorescence microscopy (*Lower Row*) of stage-specific markers expressed by iPSC-derived cells during differentiation steps. Albumin and CK18 double-positive cells are the most functional iHeps, corresponding to a fetal-like hepatocyte phenotype. (Scale bar, 50 μm.) (*B*) iPSCs were differentiated in a stepwise fashion and immunofluorescent staining for NTCP (two representative examples of each time point are pictured) (*Left*), as well as qRT-PCR for NTCP mRNA (*Right*; $n = 3$ expressed as mean ± SEM) were performed at day 15 (hepatoblast), day 18 (early hepatocyte-like cells), and day 20 (hepatocyte-like cells). (Scale bars, 100 μm.) (*C*) iPSCs were differentiated in a stepwise fashion and transfected with an HBV-luciferase reporter construct (*Upper Left*). Cells were visualized in six-well plates by IVIS imaging (*Lower Left*) and luminescent intensity was measured 72–96 h posttransfection (*Right*). Luminescence intensity is reported as radiance (photons per s/cm^2 per steradian). Dotted line, background luminescence. Data are shown from one representative experiment of three independent replicates yielding similar results.

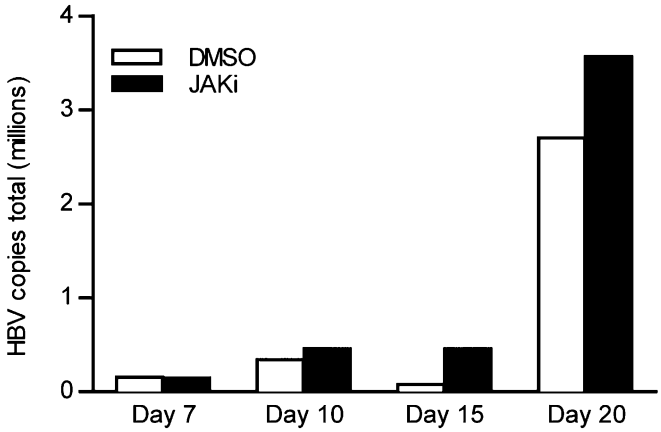

Fig. S7. Analysis of intracellular HBV DNA in iHeps. iPSCs were differentiated and incubated with HBV infectious serum at day 7, day 10, day 15, or day 20 of the differentiation protocol concomitant with treatment with either DMSO (vehicle control) or JAKi. DNA was extracted at day 16 postinfection, and quantified by qPCR (one pellet per condition per experiment, confirmed in two independent experiments).

Table S1. Basic identifying parameters of the hepatocyte donors screened for HBV permissiveness in this study

Lot number	Vendor	Lot	Race	Sex	Age
Donor information for Fig. 3					
HD1	Celsis	OFA		Female	78
HD2	Celsis	TSM		Female	49
HD3	TRL	HUM4012	Caucasian	Male	54
HD5	TRL	HUM4037	Caucasian	Male	8
HD6	TRL	HUM4038	Caucasian	Female	33
HD7	TRL	HUM4040	Caucasian	Female	23
HD4	Celsis	NON		Female	35
Donor information for Fig. S5					
HD8	VWR	8148	Caucasian	Female	55
HD9	VWR	4244	Caucasian	Male	3
HD10	Celsis	BHL		Male	28
HD11	Celsis	IZT		Female	44
HD4	Celsis	NON		Female	35
HD12	Celsis	TRZ		Female	35
HD13	Celsis	YEM		Female	46
HD14	Celsis	YJM		Female	47