

# Modeling host interactions with hepatitis B virus using primary and induced pluripotent stem cell-derived hepatocellular systems

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Hepatitis B virus (HBV) chronically infects 400 million people worldwide and is a leading driver of end-stage liver disease and liver cancer. Research into the biology and treatment of HBV requires an *in vitro* cell-culture system that supports the infection of human hepatocytes, and accurately recapitulates virus–host interactions. Here, we report that micropatterned cocultures of primary human hepatocytes with stromal cells (MPCCs) reliably support productive HBV infection, and infection can be enhanced by blocking elements of the hepatocyte innate immune response associated with the induction of IFN-stimulated genes. MPCCs maintain prolonged, productive infection and represent a facile platform for studying virus–host interactions and for developing antiviral interventions. Hepatocytes obtained from different human donors vary dramatically in their permissiveness to HBV infection, suggesting that factors—such as divergence in genetic susceptibility to infection—may influence infection *in vitro*. To establish a complementary, renewable system on an isogenic background in which candidate genetics can be interrogated, we show that inducible pluripotent stem cells differentiated into hepatocyte-like cells (iHeps) support HBV infection that can also be enhanced by blocking interferon-stimulated gene induction. Notably, the emergence of the capacity to support HBV transcriptional activity and initial permissiveness for infection are marked by distinct stages of iHep differentiation, suggesting that infection of iHeps can be used both to study HBV, and conversely to assess the degree of iHep differentiation. Our work demonstrates the utility of these infectious systems for studying HBV biology and the virus' interactions with host hepatocyte genetics and physiology.

HBV persistence | innate immunity | viral hepatitis

Hepatitis B virus (HBV) is a small 3.2-kb DNA virus that selectively infects hepatocytes in the human liver (1). The global disease burden is large, with ~400 million people chronically infected worldwide, of whom about one-third will develop severe HBV-related complications, such as cirrhosis and liver cancer. Lifelong treatment is often required because of the stable nature of viral episomal DNA, known as covalently closed circular DNA (cccDNA), which maintains basal levels in infected cell nuclei even upon nucleos(t)ide inhibitor treatment. To date, HBV research has been hampered by a distinct lack of robust infectious model systems that both support productive HBV infection and accurately mimic virus–host interactions. Recently, the bile acid pump sodium taurocholate cotransporting polypeptide (NTCP) has been identified as a receptor for both HBV and hepatitis D virus (2), and overexpression of NTCP in hepatoma cell lines renders them susceptible to HBV infection. However, hepatoma cells are known to be defective in many cellular pathways implicated in the innate immune response (3, 4), metabolism (5), and cell proliferation (6), which may

contribute to published contradictory evidence regarding the extent to which HBV activates the innate immune response, and the importance of this response in curtailing infection (for a review, see ref. 7).

As the sole host cell infected by HBV *in vivo*, primary adult human hepatocytes represent the gold-standard for studying HBV interactions with the host. Prior studies have shown that primary human hepatocytes support HBV infection, although infection is usually not robust even upon supplementation of cell-culture medium with dimethyl sulfoxide (8) or polyethylene glycol (9). Moreover, primary human hepatocytes rapidly lose their hepatic phenotype shortly after isolation from the *in vivo* microenvironment (10, 11). We have previously developed a miniaturized system in which primary hepatocytes are organized in micropatterned colonies and surrounded by supportive stromal cells, providing hepatocytes with the necessary homotypic and heterotypic cell–cell interactions to promote long-term maintenance of their hepatic function (12). This micropatterned coculture (MPCC) system maintains hepatocyte phenotype and function over several weeks and has been shown to support robust infection with hepatitis C virus (HCV) and *Plasmodium*

## Significance

Major obstacles for using human hepatocytes to study hepatitis B virus (HBV) pathobiology are rapid loss of hepatocyte function after plating and the variability between hepatocyte donors. We show that micropatterning and coculturing of primary human hepatocytes with fibroblasts (MPCC format) maintains prolonged infection that is restricted by the innate immune response, and can be further boosted by suppression of this response. To address the problem of donor variability, we show that induced pluripotent stem cells (iPSC) differentiated into hepatocyte-like cells support HBV infection in a differentiation-dependent manner. Our study opens an avenue for using these systems to study virus–host interactions and test antiviral drugs, and suggests HBV permissiveness as a surrogate reporter to assess the degree of differentiation of candidate iPSC-derived hepatocyte-like cells.

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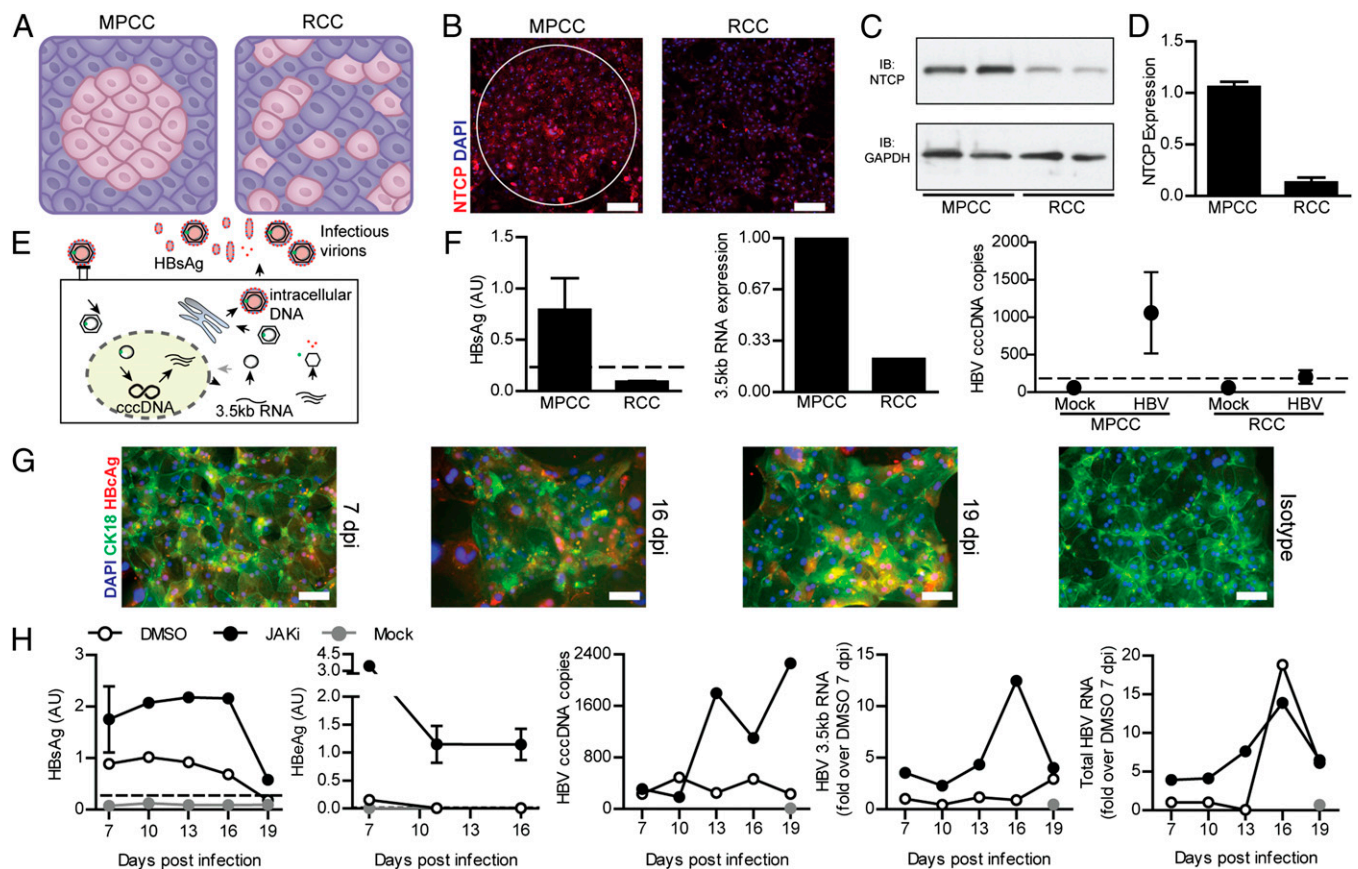
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*falciparum* and *vivax* malaria (13, 14). We hypothesized that this system would be ideal for modeling HBV infection in vitro.

Beyond its potential utility for assessing virus–host interactions, studying the role of host factors in the MPCC system is complicated by limited availability and variability between donor hepatocytes. As a complementary approach that enables more facile genetic manipulation on an untransformed and isogenic hepatocyte background, we also sought to establish robust HBV infection in induced pluripotent stem cell (iPSC)-derived hepatocyte-like cells (iHeps) (15, 16). These cells have demonstrated their utility for modeling inherited metabolic disorders (17), incorporating genetic manipulations (18), and supporting infection with HCV (19–21). During iHep generation, the progression of differentiation is characterized by the sequential emergence of various hepatocyte-specific host factors known to play a role in the HBV life cycle, such as the transcription factor HNF4 $\alpha$  and the nuclear receptor RXR (22). We show that permissiveness to HBV infection likewise progresses in a differentiation stage-specific manner. Thus, in this paper we use a system of stabilized primary hepatocytes for disease modeling to establish HBV infection in vitro and explore the use of directed differentiation of iPSCs to demonstrate that they serve as a suitable host population for the study of HBV and host–virus interactions.

## Results

**Micropatterned Human Hepatocytes Stably Express the HBV Receptor for Weeks in Culture.** It has been hypothesized that primary human hepatocytes lose their permissiveness to HBV infection because of down-regulation of NTCP receptor expression upon isolation and subsequent culture (2). Our MPCCs of primary human hepatocytes and stromal fibroblasts (J2-3T3 fibroblasts, or J2s) maintain hepatocyte functions as well as polarity and promote the accurate localization of membrane proteins to hepatocytes' basolateral and apical domains (12). Although many distinct hepatocyte culture models have been explored in the literature, a telling control to probe the importance of tissue microarchitecture is seeding similar cellular constituents in a random configuration (random coculture, RCC) (Fig. 1A). NTCP was readily detected on the plasma membrane by immunostaining of hepatocytes in MPCCs (Fig. 1B), whereas the NTCP level at 18 d postplating was drastically reduced in RCCs (Fig. 1B–D). Because viral spread is dependent upon consistent expression of the entry receptor, we analyzed NTCP levels over a course of 14 d, and found that NTCP protein and mRNA levels remained stable over time (Fig. S1A). These results establish the capacity of the MPCC format to maintain expression of the HBV receptor, NTCP, in vitro.



**Fig. 1.** HBV infection in micropatterned primary human hepatocytes is augmented by innate immune inhibition. (A) MPCC vs. RCC schematic. Hepatocytes in pink, fibroblasts in purple. (B) NTCP immunostaining; white circle marks hepatocyte island boundary. (Scale bar, 100  $\mu$ m.) (C) NTCP Western blot. (D) NTCP quantitative RT-PCR (qRT-PCR; mean  $\pm$  SEM,  $n = 3$ ). (E) Schematic of viral life-cycle readouts used. (F, Left) ELISA for HBsAg, expressed as a mean  $\pm$  SEM ( $n = 3$ ), secreted into supernatant between 14 and 16 dpi; (Center) HBV 3.5-kb mRNA expression (one cell pellet per condition) at 16 dpi; (Right) Copies of cccDNA at 16 dpi, expressed as an average of biological duplicates  $\pm$  range. (G) Hbc immunostaining of MPCCs at the indicated days postinfection. Isotype-matched negative control shown. (Scale bars, 50  $\mu$ m.) (H) Time course of HBV infection in MPCCs. (Left panels) HBsAg and HBeAg levels (average of triplicates) in supernatant; (Center) cccDNA levels; (Right panels) qRT-PCR for HBV 3.5-kb mRNA and total mRNA. Expression relative to DMSO-treated samples 7 dpi, one pellet per condition per experiment, verified across independent experiments. Dotted lines: limit of quantification (qPCR), cut-off (ELISA).

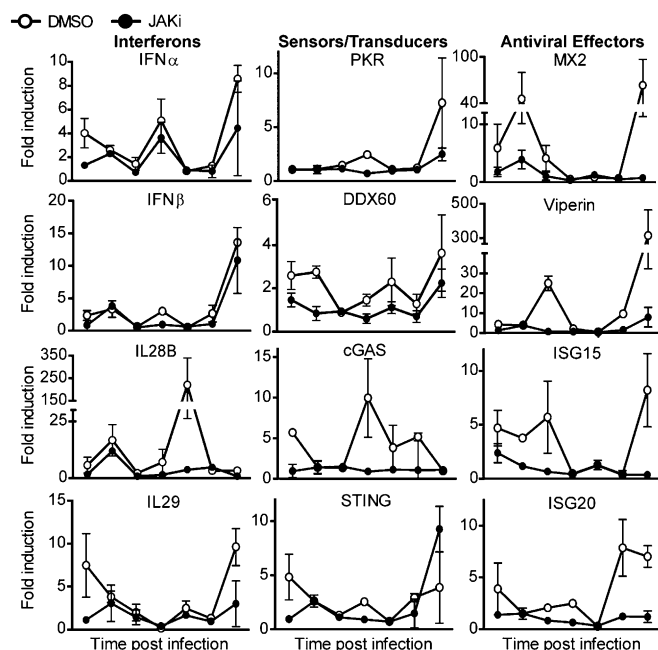
**MPCCs Support Productive HBV Infection.** Given the importance of the MPCC format for maintaining expression of the HBV receptor as well as other hepatocyte functions (Fig. S1 *B* and *C*), we next investigated whether MPCCs support HBV infection. We assayed various viral life-cycle stages, including viral gene expression, reflected by HBV surface antigen (HBsAg) secretion and 3.5-kb mRNA (the main HBV transcript) production, and the presence of the viral transcription template cccDNA, considered a hallmark of productive infection (Fig. 1*E*). We found that HBV derived from human infectious serum infects human hepatocytes more efficiently in MPCCs than RCCs (Fig. 1*F*). Furthermore, MPCCs support productive infection throughout the culture period of nearly 3 wk, based on immunostaining for HBV core (HBc) protein (Fig. 1*G*).

Based on previous data that the innate immune response can restrict HCV infection in hepatocytes (23), we explored pretreatment of MPCCs or RCCs with a broad-spectrum Janus kinase (JAK) inhibitor (JAKi), known to interfere with a major pathway of the innate immune axis by dampening expression of IFN-stimulated genes (ISGs) (24), in an attempt to elicit enhanced HBV replication efficiency. With the addition of JAKi, we observed more robust HBV infection in MPCCs (Fig. 1*H*) and detected cccDNA almost exclusively in this format (Fig. 1*F*, *Right*). Augmentation of HBV infection was also observed upon introduction of an inhibitor of TANK-binding kinase 1 (TBK1), an upstream activator of the IFN response pathway, although JAKi was more efficient in maintaining cccDNA following infection (Fig. S2). Collectively, these results suggest that the MPCC format supports the maintenance of productive infection over time in primary human hepatocytes, and that inhibition of major pathways of the hepatocyte innate immune response enhances infection in this system.

**Temporal Expression of ISGs Following HBV Infection.** In response to intracellular pathogen sensors, an innate immune pathway typically activates a set of ISGs, leading to autocrine/paracrine

signaling by interferons. Based on our observation that addition of a JAK pathway inhibitor improved HBV infection in MPCCs, we hypothesized that the innate immune response may induce antiviral ISGs, at least in culture. To assay for this response, we incubated MPCCs with HBV infectious serum and analyzed the relative expression of type I IFNs, IFN- $\alpha$ , and IFN- $\beta$ , as well as two genes implicated in type III IFN response, over the next 16 d (Fig. 2, *Left*). Both IFN- $\alpha$  and IFN- $\beta$  were induced mainly late during the course of infection, although a modest induction (>two-fold) was detected as early as 12 h postinfection. The expression of a variety of ISGs implicated in antiviral responses were also detected following HBV infection, with a range of kinetic patterns, including several that function as sensors and transducers of these pathways (Fig. 2, *Center*), and a selection of antiviral effectors (Fig. 2, *Right*). Consistent with our hypothesis, we found that blunting the innate immune response with JAK inhibition blocked the expression of many downstream ISGs but had no significant effect on the expression of either IFN- $\alpha$  or IFN- $\beta$ , which are regulated upstream of JAK-STAT signaling. We note that some exhibit a biphasic elevation over time, possibly representing reinfection events or expression in response to the emergence of later stage viral components. Importantly, ISG induction was dependent on productive HBV infection, because pretreatment of MPCCs with the HBV inhibitor entecavir largely abolished the induction of most early- and late-stage interferons and downstream ISGs (Fig. S3*A*).

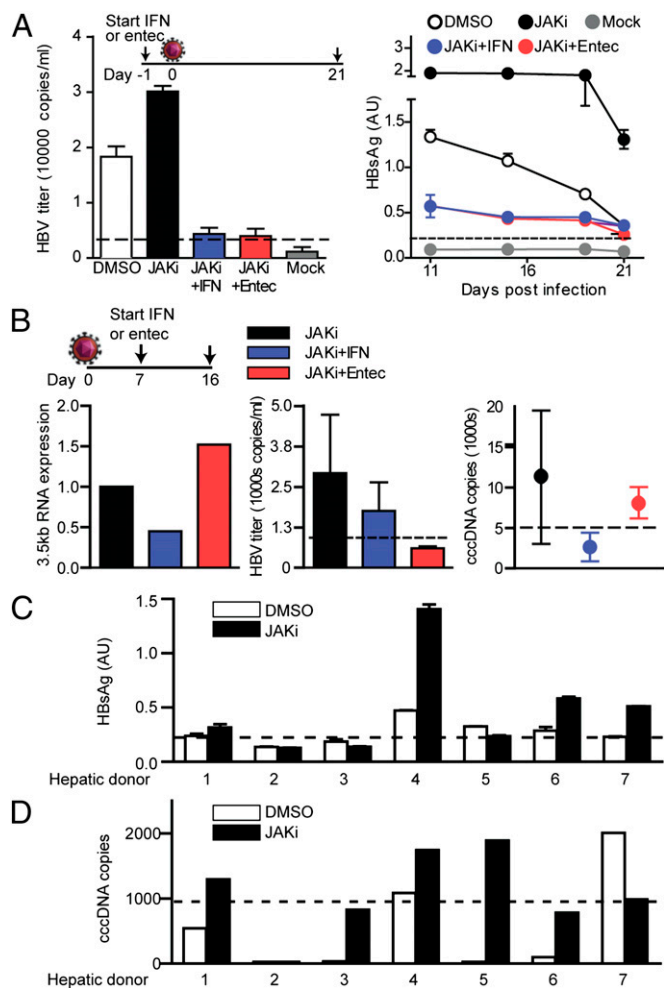
Interestingly, although productive infection could be clearly detected in NTCP-expressing HepG2 hepatoma cells, as evidenced by production of HBV 3.5-kb mRNA (Fig. S3*B*, *Left*), no significant ISG induction was observed in these cells compared with HBV nonpermissive HepG2 cells (Fig. S3*B*, *Right*). These data suggest that either HBV sensors or key transducers in this sensing pathway are defective in hepatoma cells, highlighting a distinct opportunity of the MPCC system in terms of its potential for studying virus-host interactions in HBV infection.



**Fig. 2.** Temporal induction of ISGs in HBV infected MPCCs. Primary human hepatocyte MPCCs were either mock- or HBV-infected with concomitant JAKi or DMSO (vehicle control) treatment. RNA expression was analyzed for the indicated ISGs at 12 h, 24 h, 48 h, 72 h, 7 d, 11 d, and 16 d postinfection and reported relative to the mock-infected cells, expressed as a mean  $\pm$  SEM ( $n = 3$ ).

**MPCCs Offer the Potential to Study Antiviral Candidates.** Following the demonstration that the MPCC format can support HBV infection, we asked whether the platform could be applied for use as an anti-HBV drug-testing tool. As proof-of-principle, we incubated MPCCs with HBV-infected serum with or without concomitant treatment with the HBV reverse-transcriptase inhibitor entecavir, or an alternate antiviral, IFN- $\beta$ . The addition of prophylactic entecavir or IFN- $\beta$  to MPCCs abrogated HBV infection, as indicated by a sharp decrease in the secretion of both HBV DNA and HBsAg into the medium over time (Fig. 3*A*). Consistent with these findings, levels of cellular 3.5-kb mRNA and cccDNA were also dramatically reduced in infected cells pretreated with IFN- $\beta$  or with entecavir, as long as 21 d postinfection (dpi) (Fig. S4).

Having established that MPCCs can successfully model prophylactic drug protection against HBV, we assayed their potential utility to model a more clinically relevant regimen by starting treatment with IFN- $\beta$  or entecavir 7 d after establishing HBV infection. Both treatments significantly reduced HBV DNA secretion into the medium by preinfected MPCCs, indicating an efficient inhibition of HBV replication (Fig. 3*B*). In contrast, only IFN- $\beta$ , but not entecavir, abolished the levels of 3.5-kb mRNA and cccDNA observed at 16 dpi (9 d after initiating drug treatment), consistent with published differences in the capacity of reverse-transcriptase inhibitors versus interferons to promote cccDNA elimination (25). Collectively, these results demonstrate that the MPCC system can serve as a platform for studying the efficacy and mechanism of action of diverse antiviral agents, and has the potential to be expanded to a medium-to-high throughput drug-discovery tool (13, 26).



**Fig. 3.** MPCCs as a platform for anti-HBV drug studies. (A) MPCCs treated with DMSO or JAKi, with or without entecavir or IFN- $\beta$ , were incubated with HBV infectious serum for 24 h, followed by continued drug treatment every 2 d. Collected supernatants were analyzed for HBV DNA after 3 wk (Left), and for secreted HBsAg at the indicated time points (Right); results are expressed as a mean  $\pm$  SEM,  $n = 3$ . (B) HBV-infected MPCCs treated with JAKi were dosed with either IFN- $\beta$  or entecavir from 7 to 16 dpi, when cell pellets were analyzed for 3.5-kb mRNA expression relative to nonantiviral treated cells (one cell pellet per condition, verified across multiple experiments; Left) and for cccDNA, expressed as an average (per cell pellet) of duplicates  $\pm$  range (Right). Also at 16 dpi, medium (last changed at 14 dpi) was analyzed for secreted HBV DNA, expressed as a mean  $\pm$  SEM ( $n = 3$ ) (Center). (C and D) JAKi or DMSO-treated MPCCs bearing primary human hepatocytes from different donors were incubated with HBV infectious serum and assayed at 16 dpi for HBsAg, expressed as average of duplicates, and cccDNA quantification (qPCR) or cut-off (ELISA). Dotted lines indicate limit of quantification (qPCR) or cut-off (ELISA).

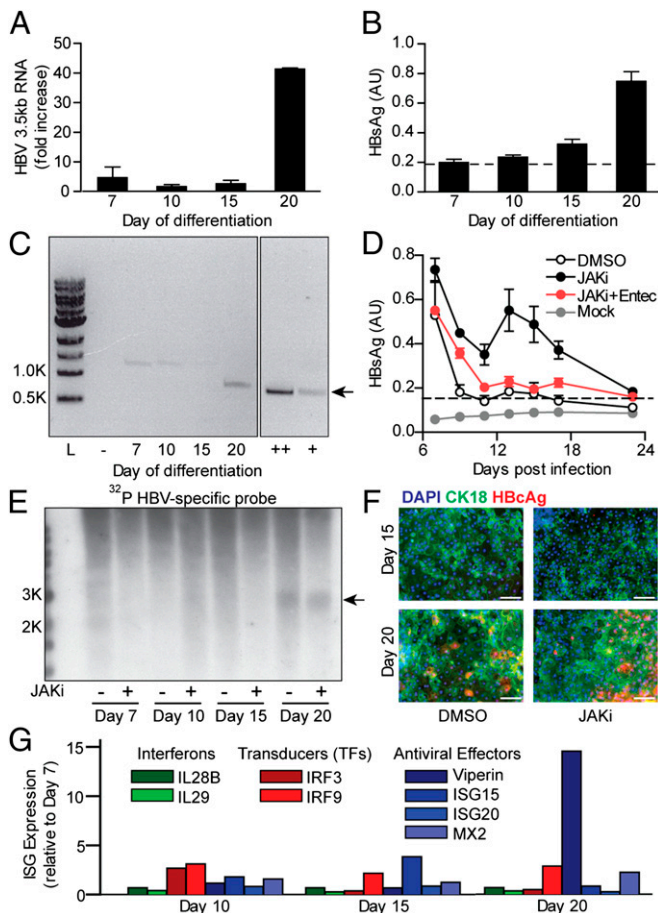
**Hepatic Donors That Share a Differentiated Phenotype *In Vitro* Exhibit Variable HBV Permissiveness.** To establish a viable drug testing platform, it is essential to identify a source of reproducibly infectible hepatocytes. The advent of cryopreserved human hepatocytes partially achieves this goal, in terms of offering a uniform donor source. To examine the importance of host variability in HBV infection of MPCCs, we used MPCCs seeded with hepatocytes derived from different hepatic donors (HD) (Table S1) and incubated them with patient-derived HBV infectious serum. Analyzing markers of productive infection revealed a wide variation between donors, with hepatic donor 4 (HD4) showing the most robust HBsAg production (Fig. 3C) and cccDNA formation (Fig. 3D). Notably, blocking the type I IFN

response by treating the cells with JAK inhibitor generally resulted in increased HBsAg secretion, as well as cccDNA production, although in some donors (such as HD2), even JAK inhibition was unable to promote the production of detectable levels of cccDNA. Furthermore, the interdonor variation in infection levels did not correlate with other established biomarkers of hepatocyte function (Fig. S5). Collectively, these results suggest that MPCCs support HBV infection in a hepatocyte donor-dependent manner. Although technical factors implicated in hepatocyte isolation and cryopreservation may play a role, it is also possible that a divergence in genetically determined host factors may underlie this variation.

**iPSC-Derived iHeps as a Candidate HBV Host *In Vitro*.** To overcome the variability in HBV permissiveness observed among donors of primary human hepatocytes, we considered options for generating a physiologically relevant *in vitro* system on an isogenic background. iPSCs are renewable, can be derived from a single donor, and repeatedly differentiated into iHeps that share features of human hepatocytes (15, 16, 26) (Fig. S6A). To ascertain whether iHeps might be permissive to HBV infection, we first investigated the expression kinetics of NTCP during the course of iHep differentiation. Using immunostaining, we observed that although NTCP was barely detectable on day 15, it was readily detectable 3 d later and increased throughout the remainder of the differentiation protocol (Fig. S6B, Left). Consistent with the protein-expression findings, quantitative analysis of NTCP mRNA levels also showed a gradual increase throughout differentiation, although the level achieved in day 20 differentiated iHeps remained less than that observed in cryopreserved primary adult hepatocytes (Fig. S6B, Right).

In addition to the dependence on an entry receptor, iHeps must also exhibit the capacity to support HBV transcription to support replication. Thus, we assessed the earliest stage at which differentiating iHeps achieved this milestone by transfecting them with an HBV luciferase reporter construct (Fig. S6C). Although the liver-enriched transcription factor HNF-4 $\alpha$ , central to the activation of the HBV transcriptional program (27), is expressed early during iHep differentiation (22, 28) (Fig. S6A), HBV transcriptional activity was not detected until day 18, after which it continued to rise (Fig. S6C).

**iHeps Support Productive HBV Infection During Late Stages of Differentiation.** To investigate whether iPSC-derived iHeps are permissive to productive HBV infection, we incubated differentiating iPSCs with HBV infectious serum and assayed for markers of the viral life cycle. Analysis performed at 16 dpi revealed signs of productive infection in fully differentiated iHeps (day 20 of differentiation), but not in cells infected at earlier stages of differentiation, as evidenced by 3.5-kb mRNA expression, HBsAg secretion, and cccDNA accumulation (Fig. 4A–C). To examine both the specificity and kinetics of HBV infection of day 20 iHeps, we incubated cells with HBV infectious serum with or without JAKi and the antiviral drug entecavir. When analyzed over a 3-wk period, only JAKi-treated iHeps maintained significant secretion of HBsAg over time, in contrast to a rapid loss of HBsAg produced by vehicle-treated or entecavir-treated cells (Fig. 4D). However, HBV DNA, both quantified (Fig. S7) and also analyzed by Southern blot (showing mainly relaxed circular replicative forms migrating at around 3.2 kb) (Fig. 4E), as well as HBV core protein (Fig. 4F) were detected in day 20 iHeps, largely independent of JAK inhibition. Given this discrepancy, we analyzed the effect of HBV infection on innate immune activation in iHeps, where we detected induction of many of the same ISGs that were observed in HBV-infected MPCCs (Fig. 4G). However, most of the ISG transcripts produced by infected iHeps were rarely elevated to the same magnitude, with the exception of viperin, which was induced over 15-fold in day 20 iHeps, relative to day 7 iHeps, which were



**Fig. 4.** HBV infection of iPSCs is drug-sensitive and differentiation-dependent. (A–C) iPSCs were differentiated in a stepwise fashion, treated with JAKi and incubated with HBV infectious serum upon treatment at the indicated days of the differentiation protocol. (A) At 16 dpi, HBV 3.5-kb mRNA was quantified by qRT-PCR, shown relative to DMSO-treated cells infected at day 10 of differentiation, and expressed as the mean  $\pm$  SEM across two separate experiments. (B) Also at 16 dpi, medium (last changed at 14 dpi) was analyzed for secreted HBsAg (mean  $\pm$  SEM,  $n = 3$ ). (C) Agarose gel separation of amplified cccDNA products (16 dpi) on a single gel with high- (++) and low- (+) expression positive controls (size mismatch because of slight curvature between distant lanes). (D) Differentiated iPSCs (day 20 of differentiation) treated as indicated were incubated with HBV infectious serum, followed by HBsAg measurements in media, expressed as a mean  $\pm$  SEM ( $n = 3$ ). (E and F) iPSCs were incubated with HBV infectious serum at the indicated days of differentiation with DMSO or JAKi. (E) Southern blot of cellular DNA extracted at 16 dpi (SI Materials and Methods) using an HBV-specific probe; arrow indicates bands corresponding to HBV DNA (predicted to be relaxed, circular DNA at this size). (F) HBcAg immunofluorescent staining of DMSO- or JAKi-treated 16-dpi iPSCs infected at day 15 and 20 of differentiation; (Scale bar, 50  $\mu$ m). (G) ISG mRNA expression by qRT-PCR of HBV-infected iPSCs at 16 dpi, normalized to the expression of HBV infected cells at day 7 of differentiation.

essentially uninfected by HBV. Collectively, these results suggest that iPSCs at advanced stages of the hepatic differentiation program support productive HBV infection, which can be maintained over a period of weeks with the addition of an innate immune response inhibitor.

## Discussion

In this study, we report HBV infection in two complementary patient-derived hepatocyte systems in which infection is limited by an innate immune response, and demonstrate the utility of these systems for studying virus–host interactions. The establishment of two complementary HBV model systems represents an opportunity to

tackle questions regarding major components of the viral entry process and viral life cycle, including host factors underlying establishment and persistence of a nuclear cccDNA pool, the key viral reservoir that leaves patients susceptible to viral reactivation (29).

The MPCC system uses micropatterned primary human hepatocytes cocultured with stromal fibroblasts, a format that ensures the prolonged viability and functionality of these delicate cells (12). By comparing the HBV permissiveness of MPCCs to that of cocultured cells seeded in a random format, we show that the patterning provided by the MPCC format is essential to maintaining productive infection for up to 3 wk. An advantage of the MPCC system is its facility for interrogating the identity of host factors responsible for the observed permissiveness, in that cryopreserved donor cells that exhibit comparable hepatocyte functions in culture but disparate permissiveness to HBV infection can be subjected to systematic molecular analysis. In this manner, candidate host-factor pathways and drug-targeting strategies may be explored *in vitro*.

We can also leverage inherent strengths of the iPSC system to identify candidate essential host factors that confer HBV sensitivity. In the differentiating populations, we observe that HBV transcriptional activity increases over time, and can be overlaid with the stepwise process of hepatocyte-specific factor acquisition. Thus far, our differentiation time-course data tracking both NTCP expression and HBV promoter/enhancer element-driven luciferase expression demonstrate that the up-regulation of both entry factors and other transcription-related host factors are essential for successful infection by HBV. We observe a tipping point for permissiveness at around days 18–20 of the iPSC differentiation process, corresponding to a switch from a hepatoblast-like phenotype to one resembling fetal hepatocytes. A comparison of populations on either side of this time point may identify candidate pathways, to be filtered based on findings from the MPCC system, and interrogated via directed mutagenesis or selective generation of iPSC lines from desired genotypes. In addition, the stage-specific acquisition of permissiveness to hepatotropic pathogens may offer a novel method for assessing the relative success of candidate *in vitro* differentiation protocols.

One important suite of host factors consists of the proteins involved in the hepatocyte innate immune response, the role of which in restricting HBV infection has been difficult to pin down (7). Much of this debate has resulted from deficiencies in the model systems used for HBV studies, because commonly used hepatoma cell lines possess well-documented defects in innate immune sensing and signal transduction (3, 4). In our systems, the establishment of productive and long-lasting infection was aided by inhibiting the innate immune response with inhibitors of the JAK family or the signaling intermediate TBK1. However, our data show that JAK inhibition boosted infection much more in MPCCs than in day 20 differentiated iPSCs, as evidenced by the similar levels of viral replication achieved with and without JAK inhibition in differentiated iPSCs (Fig. 4). This difference may be caused by the weaker induction of ISGs observed in HBV-infected iPSCs compared with MPCCs, reflecting differences in host innate immune response between fully differentiated primary human hepatocytes and iPSC-induced hepatocyte-like cells (30, 31). Still, the differential effect of JAK inhibition on different markers of viral replication (e.g., large effects on HBsAg secretion but minor effects on replicative intermediate formation) observed in iPSCs is not entirely clear and requires further study.

Our observation that innate immune activation restricts HBV infection is consistent with studies showing that HBV can be cleared from the liver in a cytokine-mediated, noncytotoxic manner (25, 32), and that HBV replication is significantly reduced in chimpanzees chronically infected with HCV because of the induction of the type I IFN response (33). In contrast to the robust induction of ISGs in HBV-infected MPCCs, and to a lesser extent in iPSCs, no ISG response was observed in HBV-permissive

HepG2 cells overexpressing NTCP. This finding emphasizes the advantage of both MPCCs and iHeps over hepatoma cell lines and traditional hepatocyte culture systems in the analysis of virus–host interactions, in particular innate immune responses. A similar induction of the type I IFN response accompanied by up-regulation of ISGs was previously observed in HBV-permissive HepaRG cells, but not in HepG2 cells upon induction of a baculovirus expressing HBV (34). However, even in HepaRG cells, the bipotential nature of the cells suggests an immature state in which the innate immune axis may not be fully developed. Similarly, our observation that iHeps display more modest, differentiation stage-dependent ISG induction relative to MPCCs, may be explained by the finding that the innate immune axis matures in concert with the hepatic phenotype of differentiating iHeps (35). Thus, our results support the model that the innate immune system plays a role in HBV infection and suggests that HBV may be less of a “stealth virus” than previously thought (36).

Notably, in the MPCC platform, we observed that a constant fraction of around 25% of the cells were HBe-positive between 7 and 19 d postinfection. This absence of apparent viral spread raises the possibility that viral production is not robust enough to support reinfection, or that the rate of new infection is offset by viral clearance from other cells. In addition, given the prolonged maintenance of normal hepatocyte function in the MPCC system, it is not clear why levels of HBV surface antigen and DNA secretion drop precipitously by 21 dpi. One possible explanation is that a gradual reduction in host factors essential to the viral transcription/replication machinery induces an eventual block at the level of gene expression or replication. The observation that the cccDNA level remains relatively stable even after the sharp decline in the viral gene expression and replication raises the intriguing possibility that these kinetics represent a switch from a more acute stage of infection to one that is more chronic and low-level.

In summary, we show that MPCCs and pluripotent stem cell-derived iHeps are both permissive to and support productive HBV infection. We envision these platforms to be complementary, each with their own advantages, and also each with the capacity to inform further optimization of the other. Thus, the combination of our HBV infectious systems will open new avenues to more fully characterize the HBV life cycle and its interaction with the host, thereby promoting the identification of potential drug targets for a disease infecting 400 million people globally.

## Materials and Methods

The experimental conditions used to generate MPCCs, maintain iPSCs, and differentiate iHeps have all been previously described (12, 15, 16, 19). For HBV infection of MPCCs and iHeps, cultures were pretreated for 24 h with dimethyl sulfoxide [0.01% (vol/vol)], JAKi (1  $\mu$ M; EMD Millipore), TBK1 inhibitor (1  $\mu$ M; EMD Millipore), IFN- $\beta$  (1,000 U/mL; R&D Systems), or entecavir (120 nM; Cayman Chemicals), as indicated, followed by infection with HBV<sup>+</sup> patient plasma. Additional details are described in *SI Materials and Methods*, including techniques used to assess HBV infection, such as total DNA, cccDNA, RNA analysis, immunofluorescence, and ELISA for HBsAg and HBeAg.

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1. Ganem D, Varmus HE (1987) The molecular biology of the hepatitis B viruses. *Annu Rev Biochem* 56:651–693.
2. Yan H, et al. (2012) Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *eLife* 1:e00049.
3. Li K, Chen Z, Kato N, Gale M, Jr, Lemon SM (2005) Distinct poly(I:C) and virus-activated signaling pathways leading to interferon- $\beta$  production in hepatocytes. *J Biol Chem* 280(17):16739–16747.
4. Tnani M, Bayard BA (1999) Evidence for IRF-1-dependent gene expression deficiency in interferon unresponsive HepG2 cells. *Biochimica et Biophysica Acta* 1451(1):59–72.
5. Lu H, Forbes RA, Verma A (2002) Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. *J Biol Chem* 277(26):23111–23115.
6. Tsukada Y, Miyazawa K, Kitamura N (2001) High intensity ERK signal mediates hepatocyte growth factor-induced proliferation inhibition of the human hepatocellular carcinoma cell line HepG2. *J Biol Chem* 276(44):40968–40976.
7. Bertoletti A, Ferrari C (2012) Innate and adaptive immune responses in chronic hepatitis B virus infections: Towards restoration of immune control of viral infection. *Gut* 61(12):1754–1764.
8. Gripon P, et al. (1988) Hepatitis B virus infection of adult human hepatocytes cultured in the presence of dimethyl sulfoxide. *J Virol* 62(11):4136–4143.
9. Gripon P, Diot C, Guguen-Guillouzo C (1993) Reproducible high level infection of cultured adult human hepatocytes by hepatitis B virus: Effect of polyethylene glycol on adsorption and penetration. *Virology* 192(2):534–540.
10. Guillouzo A (1998) Liver cell models in in vitro toxicology. *Environ Health Perspect* 106(Suppl 2):511–532.
11. Hewitt NJ, et al. (2007) Primary hepatocytes: Current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev* 39(1):159–234.
12. Khetani SR, Bhatia SN (2008) Microscale culture of human liver cells for drug development. *Nat Biotechnol* 26(1):120–126.
13. March S, et al. (2013) A microscale human liver platform that supports the hepatic stages of *Plasmodium falciparum* and *vivax*. *Cell Host Microbe* 14(1):104–115.
14. Ploss A, et al. (2010) Persistent hepatitis C virus infection in microscale primary human hepatocyte cultures. *Proc Natl Acad Sci USA* 107(7):3141–3145.
15. Schwartz RE, Fleming HE, Khetani SR, Bhatia SN (2014) Pluripotent stem cell-derived hepatocyte-like cells. *Biotechnol Adv* 32(2):504–513.
16. Si-Tayeb K, et al. (2010) Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. *Hepatology* 51(1):297–305.
17. Rashid ST, et al. (2010) Modeling inherited metabolic disorders of the liver using human induced pluripotent stem cells. *J Clin Invest* 120(9):3127–3136.
18. Yusa K, et al. (2011) Targeted gene correction of  $\alpha$ 1-antitrypsin deficiency in induced pluripotent stem cells. *Nature* 478(7369):391–394.
19. Schwartz RE, et al. (2012) Modeling hepatitis C virus infection using human induced pluripotent stem cells. *Proc Natl Acad Sci USA* 109(7):2544–2548.
20. Wu X, et al. (2012) Productive hepatitis C virus infection of stem cell-derived hepatocytes reveals a critical transition to viral permissiveness during differentiation. *PLoS Pathog* 8(4):e1002617.
21. Roelandt P, et al. (2012) Human pluripotent stem cell-derived hepatocytes support complete replication of hepatitis C virus. *J Hepatol* 57(2):246–251.
22. DeLaForest A, et al. (2011) HNF4A is essential for specification of hepatic progenitors from human pluripotent stem cells. *Development* 138(19):4143–4153.
23. Andrus L, et al. (2011) Expression of paramyxovirus V proteins promotes replication and spread of hepatitis C virus in cultures of primary human fetal liver cells. *Hepatology* 54(6):1901–1912.
24. Schneider WM, Chevillotte MD, Rice CM (2014) Interferon-stimulated genes: A complex web of host defenses. *Annu Rev Immunol* 32(1):513–545.
25. Lucifora J, et al. (2014) Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. *Science* 343(6176):1221–1228.
26. Shan J, et al. (2013) Identification of small molecules for human hepatocyte expansion and iPSC differentiation. *Nat Chem Biol* 9(8):514–520.
27. Li L, et al. (2009) Developmental regulation of hepatitis B virus biosynthesis by hepatocyte nuclear factor 4 $\alpha$ . *PLoS ONE* 4(5):e5489.
28. Li J, Ning G, Duncan SA (2000) Mammalian hepatocyte differentiation requires the transcription factor HNF-4 $\alpha$ . *Genes Dev* 14(4):464–474.
29. Dandri M, Locarnini S (2012) New insight in the pathobiology of hepatitis B virus infection. *Gut* 61(Suppl 1):i6–i17.
30. Burke DC, Graham CF, Lehman JM (1978) Appearance of interferon inducibility and sensitivity during differentiation of murine teratocarcinoma cells in vitro. *Cell* 13(2):243–248.
31. Pare JM, Sullivan CS (2014) Distinct antiviral responses in pluripotent versus differentiated cells. *PLoS Pathog* 10(2):e1003865.
32. Guidotti LG, et al. (1999) Viral clearance without destruction of infected cells during acute HBV infection. *Science* 284(5415):825–829.
33. Wieland SF, Asabe S, Engle RE, Purcell RH, Chisari FV (2014) Limited hepatitis B virus replication space in the chronically hepatitis C virus-infected liver. *J Virol* 88(9):5184–5188.
34. Lucifora J, et al. (2010) Control of hepatitis B virus replication by innate response of HepaRG cells. *Hepatology* 51(1):63–72.
35. Zhu S, et al. (2014) Mouse liver repopulation with hepatocytes generated from human fibroblasts. *Nature* 508(7494):93–97.
36. Durantel D, Zoulim F (2009) Innate response to hepatitis B virus infection: Observations challenging the concept of a stealth virus. *Hepatology* 50(6):1692–1695.