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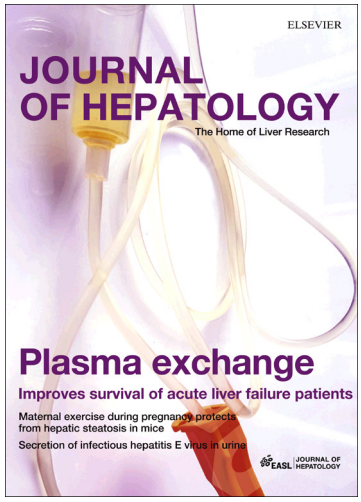

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Human stem cell-derived hepatocytes as a model for hepatitis B virus infection, spreading and virus-host interactions

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Abbreviations: HBV (hepatitis B virus), HCC (hepatocellular carcinoma), NUC (nucleos(t)ide analogs), IFN-α (interferon-α), NTCP (sodium-taurocholate cotransporting polypeptide), rcDNA (relaxed circular DNA), cccDNA (covalently closed circular DNA), pgRNA (pregenomic RNA), HLCs (hepatocyte-like cells), PHHs (primary human hepatocytes), HBVcc, (cell culture derived HBV), MOI

(multiplicity of infection), hESCs (human embryonic stem cells), iPSCs (induced pluripotent stem cells), AFP (α-fetoprotein), HBsAg (hepatitis B virus surface antigen), HBeAg (hepatitis B virus e antigen), ETV (entecavir), RT (reverse-transcription), DHBV (duck hepatitis B virus)

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Abstract:

Background and Aims: One major obstacle of hepatitis B virus (HBV) research is the lack of efficient cell culture system permissive for viral infection and replication. The aim of our study was to establish a robust HBV infection model by using hepatocyte-like cells (HLCs) derived from human pluripotent stem cells.

Methods: HLCs were differentiated from human embryonic stem cells and induced pluripotent stem cells. Maturation of hepatocyte functions was determined. After HBV infection, viral total DNA, cccDNA, total RNA, pgRNA, HBeAg, HBsAg were measured.

Results: More than 90% of the HLCs expressed strong signals of human hepatocyte markers like albumin as well as known host factors required for HBV infection, suggesting that these cells present key features of mature hepatocytes. Notably, HLCs expressed the viral receptor sodium-taurocholate cotransporting polypeptide more stably than primary human hepatocytes (PHHs). HLCs supported robust infection and some spreading of HBV. Finally, by using this model, we identified two host-targeting agents, Genistin and PA452, as novel antivirals.

Conclusions: Stem cells-derived HLCs fully support HBV infection. This novel HBV infection HLCs model offers a unique opportunity to advance our understanding of the molecular details of the HBV life cycle, to further characterize virus-host interactions and to define new targets for HBV curative treatment.

Lay summary

Our study used human pluripotent stem cells to develop hepatocyte-like cells (HLCs) capable of expressing hepatocyte markers and host factors important for HBV infection. These cells fully support HBV infection and virus-host interactions, allowing for the identification of two novel antiviral agents. Thus, stem cell-derived HLCs provide a highly physiologically relevant system to advance our understanding of viral life cycle and provide a new tool for antiviral drug screening and development.

Introduction

Hepatitis B virus (HBV) remains a major public health threat with more than 240 million individuals chronically infected worldwide and at high risk of developing liver cirrhosis and hepatocellular carcinoma (HCC) [1, 2]. Currently, there are two classes of approved treatments: nucleos(t)ide analogs (NUC) and interferon-α (IFN-α). NUC targets viral polymerase, which limits virus replication, but cannot clear virus infection. Thus, patients need to take life-long treatment with the risk of developing drug resistance. Although IFN-α can be effective in certain situations, the response rate remains low and side effects are often difficult to tolerate. Thus there is an urgent need to develop novel antivirals to cure HBV infection [2]

HBV is a small, enveloped DNA virus that targets hepatocytes. The virus infects host cells through binding to the specific receptor sodium-taurocholate

cotransporting polypeptide (NTCP) [3]. After entry, viral capsid is transported to the nucleus, where the relaxed circular DNA (rcDNA) is released and converted into a covalently closed circular DNA (cccDNA) by host enzymes [4]. This cccDNA persists as a minichromosome and serves as a template for transcription of the different viral RNA transcripts. The 3.5 kb pregenomic RNA (pgRNA) is encapsidated and reverse transcribed into new rcDNA. rcDNA-containing capsids are then either enveloped and released as progeny virions or recycled back to the nucleus for cccDNA pool amplification.

Although HBV was identified more than 50 years ago [5], many fundamental aspects of the virus remain poorly understood. This includes the molecular mechanisms of HBV entry, spreading, innate immune sensing of HBV, and signaling pathways involved in HBV-induced HCC. These gaps in knowledge are partly attributable to the lack of a cell culture system that resembles the physiological status of hepatocytes and permits efficient HBV infection, replication and dissemination. Primary human hepatocytes (PHHs) have been used for many years as a gold standard for HBV *in vitro* studies, but their utility is restricted by availability, donor variability, and limitation for long-term culture. Alternatively, HepaRG, a liver progenitor cell line, can be used after one month differentiation, but the low HBV infection efficiency, as well as a mixed cell phenotype (half biliary-like cells and half hepatocyte-like cells), hampers clear interpretation of hepatocyte-specific interaction with HBV [6]. Other models, such as NTCP-overexpressing hepatoma cells, are not optimal due to their

transformed nature. Most importantly, virus spreading, which occurs efficiently *in vivo*, could not be observed with the aforementioned *in vitro* models.

Here, we describe a novel *in vitro* HBV infection model based on stem cell-derived hepatocyte-like cells (HLCs), which fully supports HBV infection for at least one month. HLCs closely resemble PHHs and highly express factors important for HBV infection and replication, making them suitable for many applications, including virus-host interaction studies and drug development in patient-derived hepatocytes. Importantly, HLCs can be maintained with a long-lasting hepatocyte-specific phenotype as compared to PHHs, which easily lose their differentiation status *in vitro*. Furthermore, it supports HBV spreading.

Materials and Methods

Stem cell culture

Induced pluripotent stem cells (iPSCs) SC3D cell line was generated from foreskin fibroblast (CRL-2097, ATCC, Manassas, VA, USA), and reprogrammed using STEMCCA Lentivirus Reprogramming Kits (Millipore Corp., Bedford, MA, USA). Non-colony monolayer type culture human embryonic stem cells (hESCs) H9 and iPSCs SC3D cell line have been described previously [7, 8] and were maintained on growth factor-reduced (GF(-)) Matrigel (0.4 mg/ml)(Corning, NY, USA) in mTeSR1 (StemCell Technologies, Vancouver, Canada). Cells were routinely passed 1 in 8 every 5-6 days with Accutase (Life Technologies,

Frederick, USA) and reseeded with mTeSR1 medium in presence of 10 µM of Rock Inhibitor Y-27632 (Millipore, Billerica, USA) as described before [7, 8].

HLCs differentiation

Definitive endoderm was induced using the STEMdiff™ Definitive Endoderm Kit (StemCell Technologies, Vancouver, Canada), following manufacturer's instructions. Briefly, monolayer type culture of hESCs or iPSCs were resuspended using Accutase and 2 million cells were seeded per well of 6 well plates previously coated with GF(-) Matrigel (0.125 mg/mL). Cell monolayer was then cultured in STEMdiff Definitive Endoderm Basal medium with Supplements A and B for 1 day followed by STEMdiff Definitive Endoderm Basal medium with only Supplement B for 3 days with daily medium change. For hepatic specification, DE cells were resuspended using accutase, and seeded at a concentration of 79000 cells / cm^2 in differentiation medium (High glucose DMEM, F12, 10% KOSR, 1% Glutamine, 1% NEAA, 1% Penicillin/Streptomycin)(Life technologies, Frederick, USA) containing 100ng/ml of HGF (Peprotech, Rocky Hill, USA), 1% DMSO (Sigma Aldrich, St. Louis, USA) and 10 µM Rock Inhibitor Y-27632. After one day, the cells are cultured for 7 more days in Differentiation Medium containing 100 ng/mL of HGF and 1% DMSO, with daily medium change. Hepatoblasts are then maturated for 3 days by culturing the cells in differentiation medium containing 10^{-7}M of dexamethasone (Sigma Aldrich, St. Louis, USA). Differentiated HLCs can then be maintained for one month in the WEM medium based on William's E Medium

containing 10% FBS, 1% Penicillin/Streptomycin, 0.17 µM of human Insulin (Sigma Aldrich, St. Louis, USA), 10 µM of hydrocortisone 21-hemisuccinate (Sigma Aldrich, St. Louis, USA), and 1.8% DMSO, with medium changed twice per week.

Hepatocytes cultures

Cryopreserved primary human hepatocytes (PHH) from different donors (Hu1420, Hu1457, Hu8209, Hu8210) were purchased from Life Technologies Corporation (New York, USA). Cells were recovered in Hepatocyte Thaw Medium (Thermo Scientific, Waltham, USA) and plated in William's E Medium + Plating Supplements (Thermo Scientific, Waltham, USA). One day after plating, PHHs were maintained in WEM medium. Huh7-NTCP and HepG2-NTCP were maintained in DMEM plus 10% FBS, 1% NEAA, 2mM L-gutamine, 1% penicillin/streptomycin, 30 µg/mL blasticidin S HCl (Thermo Scientific, Waltham, USA). HepaRG cell culture and differentiation were performed as described [9].

Statistical analysis

Student's unpaired two-tailed t test were performed with GraphPad Prism 5.0a (GraphPad Software, La jolla, CA, USA). Data are means ± s.d. Two-sided P values < .05 are considered significant. *P< .05, **P< .01, ***P< .001.

Results

Differentiated HLCs express hepatocyte markers and HBV proviral host factors

Different protocols have been described for differentiating hESCs and patient-derived iPSCs into hepatocyte-like cells [7, 10]. The main challenges are to obtain HLCs exhibiting a mature and homogeneous level of differentiation and maintain their differentiation status *in vitro*. Here, we optimized our previous method to differentiate stem cells more efficiently into a homogeneous population of mature HLCs capable of retaining their phenotype for a prolonged period of time [8]. HLCs were obtained from hESCs or iPSCs (non-colony type monolayer-adapted) through a three-step differentiation protocol (**Fig. 1A**) [8]. After maturation, the cells were maintained in the WEM medium. Secreted human albumin was detected after hepatic specification and dramatically increased during the hepatic maturation stage (**Supplementary Fig. 1A**). Expression of hepatocyte markers were determined by qPCR (**Supplementary Fig. 1B**) and further confirmed by immunostaining (**Supplementary Fig. 1C**). Differentiated HLCs presented key features of human hepatocytes, but still expressed α-fetoprotein (AFP). Compared to HLCs, the differentiation of HepaRG revealed a slower process and lower expression level of these proviral factors (**Supplementary Fig. 2A**).

To determine the HLCs' utility as an infection model for HBV, we first evaluated the expression of known host factors essential for HBV infection and replication in HLCs. mRNA levels of viral transcription factors RXR, HNF4α, as well as viral

receptor NTCP, increased continuously in both hESCs and iPSCs derived HLCs during the differentiation process (**Fig. 1B**). At the end of differentiation, immunostaining revealed that nearly 100% of the cells presented nuclear expression of RXR, HNF4α, NTCP (**Fig. 1C**), while only around 50% of the differentiated HepaRG (dHepaRG) expressed albumin, NTCP and HNF4α (**Supplementary Fig. 2B**). Notably, the expression of NTCP in PHH declined following isolation and subsequent culture [3, 11]. NTCP staining of HLCs showed a ubiquitous membrane expression, though at varying levels (**Fig. 1C**). HLCs differentiated from either hESCs (HLC-1 and HLC-2) or iPSCs (HLC-3, HLC-4) expressed similar levels of RXR and HNF4α, as well as higher levels of NTCP in comparison to PHHs from different donors or dHepaRG (**Fig. 1D**). Immunostaining uncovered that only around 50% of dHepaRG expressed albumin, NTCP and HNF4α (**Supplementary Fig. 2B**).

Stem cell-derived HLCs support productive HBV infection

We infected hESCs-derived HLCs with HBV at a multiplicity of infection (MOI) of 300 (genome equivalents per cell), and monitored kinetics of different HBV infection markers. There was a gradual increase of cccDNA from day 0 to day 14 (**Fig. 2A**) followed by an increase of total viral RNA (**Fig. 2B**). The establishment of cccDNA was confirmed by Southern blot (**Supplementary Fig. 3**). Secreted hepatitis B virus surface antigen (HBsAg) and hepatitis B virus e antigen (HBeAg) showed a similar pattern (**Fig. 2C**). Extracellular HBV-DNA representing newly produced virions peaked at day 11 and thereafter remained stable (**Fig.**

2d). To determine the efficiency of infection, HBsAg staining was performed. The signal was weak on day 3 post-infection but became strong on day 7. On day 11, more than 30% of the cells were strongly positive for this viral protein (**Fig. 2E**). To determine the efficiency of viral infection, a highly sensitive RNAscope assay was also used to detect HBV nucleic acid 10 days post infection. As shown in **Fig. 2F**, a MOI 50 gave 30% HBV-positive cells while MOI 200 resulted in 60% infected cells. When MOI was increased to 1000, nearly all cells were infected. No signal was detected from uninfected cells, demonstrating the high specificity of this assay. Similar infection efficiency was achieved with iPSCs-derived HLCs (**Supplementary Fig. 4A, B**).

To confirm viral infection, HBV-infected HLCs were treated with three known antivirals targeting different steps of viral infection. As expected, Myrcludex B, an entry inhibitor [12], blocked all HBV infection markers, while entecavir (ETV), a reverse-transcription (RT) inhibitor, inhibited mainly HBV-DNA [13]. IFN-α affected all HBV markers due to its multi-functional antiviral properties (**Supplementary Fig. 4C**).

To test the involvement of known HBV host factors in our model, we used siRNA to knock down NTCP and APOBEC3A. Different transfection reagents were tested on HLCs, and RNAiMax showed the best effect with around 60% of knock-down efficiency (**Supplementary Fig. 4D**). As expected, siRNA-mediated knock-down of viral receptor NTCP prevented efficient HBV infection, while knock-down

of the antiviral factor APOBEC3A [14, 15] led to increased viral replication (**Supplementary Fig. 4E, Fig. 2G)**. These findings indicate that HLCs is a suitable model to study HBV-cell interactions.

Next, we evaluated the infectivity of viral particles produced from HBV-infected HLCs. We concentrated supernatant from infected HLCs by CsCl ultracentrifugation and observed distinct peaks of HBsAg and HBV-DNA in the gradient, which is consistent with the gradient profile of HBV serum samples (**Supplementary Fig. 4F**). Electron microscopy revealed the presence of 42-nm Dane particles, 22-nm spherical and filamentous subviral particles produced by the infected cells (**Fig. 2H**). Infection of fresh HLCs with concentrated supernatant led to production and secretion of extracellular HBV-DNA and viral antigens (**Supplementary Fig. 4G**). Establishment of infection was also confirmed by HBsAg staining (**Supplementary Fig. 4H**). Taken together, these data indicate that HLCs support productive HBV infection.

HLCs support long-term HBV infection

Previous studies show that PHHs can maintain HBV infection and replication for only 5 to 10 days [16]. This short window hinders longer-term studies. Their loss of hepatocyte-specific functionality, after isolation from their *in vivo* microenvironment, including a rapid decrease of NTCP expression, probably contributes to the loss of HBV susceptibility over time [17].

To evaluate whether HLCs can maintain their differentiation state longer, we compared gene expression of HLCs and PHHs side-by-side after maturation or plating, respectively. Consistent with previous studies [18], PHHs lost differentiated gene expression within 10 days after *in vitro* culture (**Fig. 3A**). In contrast, HLCs maintained high levels of proviral factors NTCP, RXR and HNF4α for a longer period of time, which only slightly decreased after three weeks following maturation (**Fig. 3A**). Immunostaining revealed that although 90% of HLCs lost albumin expression after 30 days, only 50% of HLCs reduced their NTCP expression (**Fig. 3B**). To assess how long we could maintain HBV infection in our model, we monitored the infected HLCs cultures for 48 days. There was a gradual decrease of albumin production (**Fig. 3C**) and HBV replication (**Fig. 3D**) after 20 to 24 days. Interestingly, intracellular HBV-RNA showed a similar decreasing pattern but the cccDNA remained relatively stable, suggesting that the attenuation of HBV infection might be due to the loss of viral transcription factors (**Fig. 3E**). Overall, HLCs can support efficient HBV infection and replication for at least four weeks with levels slowly decreasing after day 24. To evaluate whether HLCs are still suitable for HBV infection 30 days after maturation, HLCs were infected with cell culture derived HBV (HBVcc) at a MOI 300. Even though viral replication markers were lower than infection directly after maturation, the increasing viral antigens and HBV-DNA indicated a successful infection (**Fig. 3F**). Together, our results showed that the HLCs can maintain their hepatic differentiation state longer than the PHHs, which makes it a useful tool for long-term HBV infection.

HLCs support HBV spreading

We next compared HBV replication in HLCs with three other models: HepG2-NTCP, dHepaRG and PHHs. With a MOI 300, HepG2-NTCP, PHHs and HLCs showed around 50-70 % infection efficiency, while less than 20% dHepaRG were HBV-positive as revealed by RNAscope (**Fig. 4A**). To probe the details of viral replication, ETV was used to block the reverse -transcription step. ETV strongly inhibited both intracellular and extracellular HBV-DNA in all four models (**Fig. 4B, Supplementary Fig. 5A**). Interestingly, viral antigens, RNA and cccDNA were also reduced by ETV in HBV-infected HLCs, while only little or no effect was seen in PHHs, HepG2-NTCP or dHepaRG cells (**Fig. 4C-E, Supplementary Fig. 5B,**). Initially after infection, the cccDNA pool is derived from the rcDNA of input virus, but additional cccDNA can be generated from either newly synthesized rcDNA via capsid recycling or virus spreading to un-infected cells [19]. The relatively unchanged cccDNA levels under ETV treatment in HepG2-NTCP and dHepaRG cells suggest that their cccDNA pool was established predominantly from the input virus. Furthermore, these data suggest that viral spreading or recycling of mature capsids may be occurring in our HLC infection model.

To investigate this possibility, we used virus entry inhibitor Myrcludex B alone or together with ETV one day after HBV infection. Interestingly, Myrcludex B treatment reduced both viral antigen and cccDNA in HBV-infected HLCs and PHHs, but not HepG2-NTCP or dHepaRG cells (**Fig. 4F**). When we blocked viral

reinfection by Myrcludex B, ETV treatment did not further inhibit viral antigens and cccDNA levels, indicating that the expansion of cccDNA pool in this model resulted from viral spreading and not capsid recycling (**Fig. 4F**). To validate this hypothesis, Myrcludex B was added at different time points of HBV-infected HLCs. As expected, Myrcludex B completely blocked HBV replication when applied together with the HBV inoculum (0-14) (**Fig. 5A**). In accordance with Fig 4F, viral replication was also reduced when Myrcludex B was added on 1 day (1-14) or day 4 (4-14) after infection, indicating that Myrcludex B inhibited HBV spread (**Fig. 5A**). Suppressed viral replication correlated with a reduction of the cccDNA level (**Fig. 5A**).

Spreading of HBV infection has been reported in humanized mice and chimpanzees, but not in previously described *in vitro* models [20, 21]. In HLCs, we frequently observed clusters of HBV infected cells (**Fig. 5B upper picture**). These clustered structures were reduced significantly when HBV infected HLCs were treated with viral entry inhibitor Myrcludex B one day after infection (**Fig. 5B lower picture, Supplementary Fig. 6A**), indicating that NTCP-mediated viral spreading occurs in HLCs. To investigate the net amplification of HBV, we used different MOI's to infect HLCs. As expected, a high MOI (200 or 1000) resulted in a plateauing of virus replication after day 17 (**Fig. 5C**), whereas a low MOI (8 or 40) led to a gradual and continuous increase of virus infection over time (**Fig. 5C**).

To obtain direct evidence of viral spreading, HBV infected HLCs (donor cells) and CMFDA-labeled HLCs (acceptor cells) were co-cultured. CMFDA is a stable green dye that is retained by the cells for an extended period of time. After 3 days, HBsAg was observed within acceptor cells suggesting viral spreading (**Supplementary Fig. 6B**). To exclude the possibility that CMFDA dye contaminating the donor cells and further validate the finding, GFP-lentiviral transduction was used to label the acceptor cells. Similarly, acceptor cells became HBV-positive after 7 days of co-culture as indicated by either RNAscope or HBsAg staining (**Fig. 5D, Supplementary Fig. 6C**). The presence of Myrcludex B in the co-culture significantly blocked infection of the acceptor cells, supporting an NTCP-mediated viral spreading mechanism. Together, our results indicate that the HLCs support NTCP-mediated HBV spreading to some extent.

Evaluation of HLCs as a model for antiviral study

There is an urgent need for development of antivirals with novel mode-of-action to improve the treatment for chronic HBV infection. In this light, our HLCs model provides a suitable model for novel antiviral discovery as it supports the full viral life cycle and responds to known antivirals. Here we further evaluated the anti-viral activity of two compounds, Genistein and PA452, in our HLC infection model.

As productive HBV infection depends extensively on host factors, host-targeting agents would provide a novel antiviral approach to treat HBV infection.

Considering the potential role of estrogen receptors in the transcription of HBV genes [22], Genistein, a plant-derived phytoestrogen that activates estrogen receptor [23], may be a viable antiviral agent against HBV. Unexpectedly, Genistein did not show antiviral effect on HBV-infected Huh7-NTCP or HepG2-NTCP cells (**Fig. 6A**). However, when tested in HLCs (**Fig. 6B**) or PHHs (**Fig. 6C**), Genistein significantly reduced different HBV replication markers without cytotoxicity (**Supplementary Fig. 7A**). This finding indicates that intracellular signaling like estrogen receptor pathway may be impaired in hepatoma cell lines, thus making HLCs a better model for this purpose.

RXR is an important hepatocyte factor that regulates HBV transcription by activating the HBV enhancer 1[24]. PA452 is a RXR antagonist, which triggers dissociation of RXR tetramers [25]. In HBV-infected HLCs, PA452 treatment reduced all HBV infection markers (**Fig. 6D**) in a dose and time dependent manner (**Fig. 6E**) without cytotoxicity (**Supplementary Fig. 7B**). As expected, viral RNAs were preferentially affected, supporting the concept that PA452 blocks viral transcription by antagonizing RXR. Similar results were obtained in HBV-infected PHHs (**Fig. 6F**) and HepG2-NTCP cells (**Fig. 6G**).

Together, our results showed that the anti-viral potential of two compounds with a novel mode-of-action against HBV highlights the utility of the HLCs infection model for drug discovery targeting the full HBV life cycle and its host

dependency. This model offers a unique opportunity to discover novel antivirals that can be developed for curative treatment of HBV infection.

Discussion

HBV basic research and the development of novel antivirals have been hampered by the lack of suitable infection models. The first cell-culture model system used HBV-expressing hepatoma cell lines generated by stably transfecting HBV-DNA genomic construct [26]. Other models have been developed through delivery of viral genome by baculovirus or adenovirus vectors, resulting in productive HBV replication and formation of infected viruses [27, 28]. Although these models have been used for drug testing and resistance studies, there are significant limitations, such as the absence of viral replication from cccDNA and other crucial steps of HBV life cycle. The discovery of HepaRG cells, a liver progenitor hepatoma cell line, which supports the entire HBV life cycle, provides an important tool for HBV *in vitro* studies [9]. However, both hepatocyte and biliary lineages are obtained from HepaRG cell cultures after one-month of differentiation. Moreover, HBV infection rates in this model are relatively low. PHHs served as the gold standard for HBV infection experiments for many years, and yet are not used widely because of their limited availability and large donor-to-donor variations. Additionally, PHHs provide a narrow time window for experimentation and rapidly lose permissiveness for HBV infection [3, 11].

Identification of the NTCP as a functional receptor for HBV provided opportunities for developing new model system like NTCP over-expressing hepatoma cell line [3, 29]. However, the transformed nature of hepatoma cells has altered physiological response, intracellular signaling and metabolic activities, thus making them less suitable for many applications. Recently, iPSC-derived HLCs have been described to support HBV infection [10]. However their differentiation takes 20 days and infection efficiency was very low. In the present study, we took advantage of our recently published optimized protocol [8] to differentiate non-colony type, monolayer-adapted culture of hESCs and iPSCs to HLCs in 15 days. After differentiation, the cells maintained their differentiated state and allowed HBV infection for more than 4 weeks.

Despite attempts to optimize protocols for maintaining PHHs isolated from fresh human liver tissue, PHHs can rapidly dedifferentiate in culture [18, 30, 31]. This limitation compromises their hepatocyte functionality, as well as their susceptibility to HBV infection. Previous reports suggest that dedifferentiation is caused by pro-inflammatory or proliferative response induced during PHHs isolation or subsequent *in vitro* culture [32, 33]. Recently, a global proteomic analysis revealed that many metabolic-associated factors are down-regulated in PHHs during culture [18]. Consistent with these studies, our results demonstrated the loss of the same metabolic factors in cultured PHHs like RXR, HNF4α and NTCP, which are important for HBV infection. Notably, the dedifferentiation process occurs at a slower rate in HLCs, as high expression

levels of proviral factors can be maintained for more than 3 weeks, making them a suitable model for long-term HBV infection study.

It has been demonstrated that a single HBV particle is sufficient to establish an infection and spread to all hepatocytes in the liver [21]. Due to the lack of experimental models, it is unknown whether HBV is transmitted directly from cell to cell or via infection of uninfected cells by extracellular progeny virus released from already infected cells. Similar to reports on duck hepatitis B virus (DHBV) [34], our findings indicate that human HBV mainly spreads by newly produced and secreted viruses that favored transmission to adjacent cells in the HLCs. Why the neighboring cells rather than random cells are preferred remains unknown. One explanation is that hepatocyte polarization may be required for HBV infection [35]. Newly produced virus may be released or infect preferentially within certain regions of the hepatocyte. However, the spreading kinetics of DHBV in primary duck hepatocytes are much faster than HBV in HLCs [34]. This difference can be attributable to key biological differences between the two viruses. Although DHBV is a close relative to human HBV, it has a unique gene structure, cccDNA formation mechanism and entry receptor [4, 36]. Data from HBV infected chimeric mouse model shows that HBV spreading is slow and takes more than 6 weeks *in vivo* [37]. Optimizations like maturation of HLCs using 3-dimensional collagen matrices, have been shown to improve HLCs functional longevity to over 75 days [38]. This approach will allow us to monitor HBV spreading even longer in HLCs.

This HLCs model system is highly suitable for antiviral discovery and testing. In the current study, we focused on inhibitors of key host factors required for viral replication, and identified Genistein and PA452 as two potential antivirals. Future studies could exploit this model for other antiviral development and combination therapy. For example, targeting viral receptors can inhibit viral spreading or targeting tyrosyl-DNA-phosphodiesterase 2 can block cccDNA formation [39]. While single treatment may not achieve HBV eradication, combination of antivirals targeting multiple steps of viral life cycle may lead to a functional cure.

This HBV infection HLCs model offers a highly physiological model system to advance our understanding of virus-host interaction including HBV pathogenesis and immune response, as well as provide new opportunities for antiviral drug screening and development. Producing iPSCs from different individuals will allow us to further characterize virus-host interactions and disease mechanisms in the context of diverse host genetics [40].

References

Author names in bold designate shared co-first authorship.

[1] Trepo C, Chan HL, Lok A. Hepatitis B virus infection. Lancet 2014;384:2053-2063.

[2] Liang TJ, Block TM, McMahon BJ, Ghany MG, Urban S, Guo JT, et al. Present and future therapies of hepatitis B: From discovery to cure. Hepatology 2015;62:1893-1908.

[3] **Yan H, Zhong G**, Xu G, He W, Jing Z, Gao Z, et al. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. eLife 2012;1:e00049.

[4] Kock J, Rosler C, Zhang JJ, Blum HE, Nassal M, Thoma C. Generation of covalently closed circular DNA of hepatitis B viruses via intracellular recycling is regulated in a virus specific manner. PLoS pathogens 2010;6:e1001082.

[5] Blumberg BS, Alter HJ, Visnich S. A "New" Antigen in Leukemia Sera. Jama 1965;191:541-546.

[6] Hantz O, Parent R, Durantel D, Gripon P, Guguen-Guillouzo C, Zoulim F. Persistence of the hepatitis B virus covalently closed circular DNA in HepaRG human hepatocyte-like cells. The Journal of general virology 2009;90:127-135.

[7] Carpentier A, Tesfaye A, Chu V, Nimgaonkar I, Zhang F, Lee SB, et al. Engrafted human stem cell-derived hepatocytes establish an infectious HCV murine model. The Journal of clinical investigation 2014;124:4953-4964.

[8] Carpentier A, Nimgaonkar I, Chu V, Xia Y, Hu Z, Liang TJ. Hepatic differentiation of human pluripotent stem cells in miniaturized format suitable for high-throughput screen. Stem Cell Res 2016;16:640-650.

[9] Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, et al. Infection of a human hepatoma cell line by hepatitis B virus. Proceedings of the

National Academy of Sciences of the United States of America 2002;99:15655-15660.

[10] **Shlomai A, Schwartz RE**, Ramanan V, Bhatta A, de Jong YP, Bhatia SN, et al. Modeling host interactions with hepatitis B virus using primary and induced pluripotent stem cell-derived hepatocellular systems. Proceedings of the National Academy of Sciences of the United States of America 2014;111:12193-12198.

[11] **Konig A, Doring B**, Mohr C, Geipel A, Geyer J, Glebe D. Kinetics of the bile acid transporter and hepatitis B virus receptor Na+/taurocholate cotransporting polypeptide (NTCP) in hepatocytes. Journal of hepatology 2014;61:867-875.

[12] Gripon P, Cannie I, Urban S. Efficient inhibition of hepatitis B virus infection by acylated peptides derived from the large viral surface protein. Journal of virology 2005;79:1613-1622.

[13] Langley DR, Walsh AW, Baldick CJ, Eggers BJ, Rose RE, Levine SM, et al. Inhibition of hepatitis B virus polymerase by entecavir. Journal of virology 2007;81:3992-4001.

[14] **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.

[15] **Xia Y, Stadler D**, Lucifora J, Reisinger F, Webb D, Hosel M, et al. Interferon-gamma and Tumor Necrosis Factor-alpha Produced by T Cells Reduce the HBV Persistence Form, cccDNA, Without Cytolysis. Gastroenterology 2016;150:194-205.

[16] Gripon P, Diot C, Theze N, Fourel I, Loreal O, Brechot C, et al. Hepatitis B virus infection of adult human hepatocytes cultured in the presence of dimethyl sulfoxide. Journal of virology 1988;62:4136-4143.

[17] Allweiss L, Dandri M. Experimental in vitro and in vivo models for the study of human hepatitis B virus infection. Journal of hepatology 2016;64:S17-31.

[18] Heslop JA, Rowe C, Walsh J, Sison-Young R, Jenkins R, Kamalian L, et al. Mechanistic evaluation of primary human hepatocyte culture using global proteomic analysis reveals a selective dedifferentiation profile. Arch Toxicol 2016.

[19] Tuttleman JS, Pourcel C, Summers J. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. Cell 1986;47:451-460.

[20] Petersen J, Dandri M, Mier W, Lutgehetmann M, Volz T, von Weizsacker F, et al. Prevention of hepatitis B virus infection in vivo by entry inhibitors derived from the large envelope protein. Nature biotechnology 2008;26:335-341.

[21] Asabe S, Wieland SF, Chattopadhyay PK, Roederer M, Engle RE, Purcell RH, et al. The size of the viral inoculum contributes to the outcome of hepatitis B virus infection. Journal of virology 2009;83:9652-9662.

[22] Wang SH, Yeh SH, Lin WH, Yeh KH, Yuan Q, Xia NS, et al. Estrogen receptor alpha represses transcription of HBV genes via interaction with hepatocyte nuclear factor 4alpha. Gastroenterology 2012;142:989-998 e984.

[23] Martin PM, Horwitz KB, Ryan DS, McGuire WL. Phytoestrogen interaction with estrogen receptors in human breast cancer cells. Endocrinology 1978;103:1860-1867.

[24] Huan B, Kosovsky MJ, Siddiqui A. Retinoid X receptor alpha transactivates the hepatitis B virus enhancer 1 element by forming a heterodimeric complex with the peroxisome proliferator-activated receptor. Journal of virology 1995;69:547-551.

[25] Liu Y, Kagechika H, Ishikawa J, Hirano H, Matsukuma S, Tanaka K, et al. Effects of retinoic acids on the dendritic morphology of cultured hippocampal neurons. Journal of neurochemistry 2008;106:1104-1116.

[26] Sells MA, Chen ML, Acs G. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. Proceedings of the National Academy of Sciences of the United States of America 1987;84:1005-1009.

[27] Delaney WEt, Isom HC. Hepatitis B virus replication in human HepG2 cells mediated by hepatitis B virus recombinant baculovirus. Hepatology 1998;28:1134-1146.

[28] Sprinzl MF, Oberwinkler H, Schaller H, Protzer U. Transfer of hepatitis B virus genome by adenovirus vectors into cultured cells and mice: crossing the species barrier. Journal of virology 2001;75:5108-5118.

[29] Iwamoto M, Watashi K, Tsukuda S, Aly HH, Fukasawa M, Fujimoto A, et al. Evaluation and identification of hepatitis B virus entry inhibitors using HepG2 cells overexpressing a membrane transporter NTCP. Biochemical and biophysical research communications 2014;443:808-813.

[30] Fraczek J, Bolleyn J, Vanhaecke T, Rogiers V, Vinken M. Primary hepatocyte cultures for pharmaco-toxicological studies: at the busy crossroad of various anti-dedifferentiation strategies. Arch Toxicol 2013;87:577-610.

[31] Zeisberg M, Kramer K, Sindhi N, Sarkar P, Upton M, Kalluri R. De-differentiation of primary human hepatocytes depends on the composition of specialized liver basement membrane. Mol Cell Biochem 2006;283:181-189.

[32] Elaut G, Henkens T, Papeleu P, Snykers S, Vinken M, Vanhaecke T, et al. Molecular mechanisms underlying the dedifferentiation process of isolated hepatocytes and their cultures. Curr Drug Metab 2006;7:629-660.

[33] Godoy P, Hengstler JG, Ilkavets I, Meyer C, Bachmann A, Muller A, et al. Extracellular matrix modulates sensitivity of hepatocytes to fibroblastoid dedifferentiation and transforming growth factor beta-induced apoptosis. Hepatology 2009;49:2031-2043.

[34] Funk A, Hohenberg H, Mhamdi M, Will H, Sirma H. Spread of hepatitis B viruses in vitro requires extracellular progeny and may be codetermined by polarized egress. Journal of virology 2004;78:3977-3983.

[35] Schulze A, Mills K, Weiss TS, Urban S. Hepatocyte polarization is essential for the productive entry of the hepatitis B virus. Hepatology 2012;55:373-383.

[36] Tong S, Li J, Wands JR. Carboxypeptidase D is an avian hepatitis B virus receptor. Journal of virology 1999;73:8696-8702.

[37] Dandri M, Petersen J. Chimeric mouse model of hepatitis B virus infection. Journal of hepatology 2012;56:493-495.

[38] Gieseck RL, 3rd, Hannan NR, Bort R, Hanley NA, Drake RA, Cameron GW, et al. Maturation of induced pluripotent stem cell derived hepatocytes by 3D-culture. PLoS One 2014;9:e86372.

[39] Koniger C, Wingert I, Marsmann M, Rosler C, Beck J, Nassal M. Involvement of the host DNA-repair enzyme TDP2 in formation of the covalently closed circular DNA persistence reservoir of hepatitis B viruses. Proceedings of the National Academy of Sciences of the United States of America 2014;111:E4244-4253.

[40] **Peng L, Zhao Q, Li Q**, Li M, Li C, Xu T, et al. The p.Ser267Phe variant in SLC10A1 is associated with resistance to chronic hepatitis B. Hepatology 2015;61:1251-1260.

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Figure legend

Figure 1. Expression of proviral host factors in HLCs.

(**A**) Schematic of the differentiation process and phase contrast pictures of hESCs H9 derived HLCs during differentiation. Scale bar = 200 µm. (**B**) mRNA

kinetics of RXR, HNF4α and NTCP during differentiation were evaluated by qRT-PCR. (**C**) Expression of NTCP, RXR and HNF4α at the end of differentiation were determined by immunostaining. Scale bar = 400 µm. (**D**) mRNA levels of RXR, HNF4α and NTCP from different PHHs donors, dHepaRG or different batches of HLCs were compared. Mean value of PHHs was normalized as 1.

Figure 2. HBV infection of HLCs.

hESCs H9 derived HLCs were infected with HBVcc at MOI 300. Intracellular HBV infection markers (**A**) cccDNA (**B**) HBV-RNA were determined by qPCR or qRT-PCR respectively. (**C**) Secreted HBsAg and HBeAg were evaluated by ELISA. S/N: signal noise ratio. (**D**) HBV-DNA presented in cell culture supernatant was measured by qPCR. (**E**) HBsAg staining was performed on day 3, 7 and 11 post-infection (dpi). Goat anti-mouse secondary antibody was used (Red). Scale bar = 200 µm. (**F**) HLCs were infected with HBVcc at a MOI of 50, 200 or 1000. Ten days after infection, RNAscope staining was performed to detect HBV RNA replicates in the cells. Scale bar = 50 µm. (**G)** HLCs were transfected with siRNA against NTCP or APOBEC3A and then infected with HBV 48 h post-transfection. Eight days after infection, HBV-RNA, cccDNA and HBeAg were measured by qRT-PCR and ELISA respectively. (**H**) Electron microscopy was performed to detect Dane particles (upper panel, red arrow), spherical subviral particles (upper panel, black arrow) and filamentous subviral particles (lower panel) from supernatant of HBV infected HLCs. Scale bar = 100 nm. Data are means ± s.d. **p<0.01, *** p<0.001 by unpaired two-tailed *t* test.

Figure 3. Long-term maintenance of HLCs culture.

HBV infected hESCs H9 derived HLCs were maintained after differentiation. (**A**) Kinetics of NTCP, RXR and HNF4α were evaluated by qRT-PCR. (**B**) Expression of albumin and NTCP, RXR and HNF4α right after maturation and 30 days after maturation were determined by immunostaining. Scale bar = 400 µm. (**C**) Albumin secretion was evaluated by ELISA. (**D**) Extracellular HBV-DNA and secreted viral antigens as well as (**E**) intracellular HBV RNA and cccDNA were monitored. (**F**) hESCs H9 derived HLCs were infected with HBVcc 30 days after differentiation (day 45) at MOI 300. Different HBV infection markers were evaluated.

Figure 4. Comparison of HBV replication in different *in vitro* models.

HepG2-NTCP, dHepaRG, PHHs and hESCs H9 derived HLCs were infected with HBVcc at MOI 300 and treated with or without ETV. (**A**) Infection efficiency was determined by RNAScope. (**B**) Extracellular HBV-DNA, (**C**) HBsAg, (**D**) HBV-RNA and (**E**) cccDNA at different time points were determined. (**F**) HBV infected HepG2-NTCP, dHepaRG, PHHs or HLCs were treated with 100 nM Myrcludex B only or 100 nM Myrcludex B plus 0.5 µM ETV one day after infection. 14 days after infection, secreted HBeAg and cccDNA were determined by ELISA or qPCR respectively. Data are means ± s.d. ns: not significant.

Figure 5. HBV spreading in HLCs.

(**A**) HBV infected hESCs H9 derived HLCs were treated with Myrcludex B at the time of infection (0-14), one day after infection (1-14) or 4 days after infection (4-14). HBeAg and extracellular HBV-DNA were determined by ELISA or qPCR respectively. cccDNA level on day 14 was evaluated by qPCR. (**B**) 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. Clustered infected cells are highlighted with arrows. (**C**) HLCs were infected with HBVcc at different MOI. Kinetics of viral antigens and extracellular HBV-DNA were determined by ELISA or qPCR respectively. cccDNA level on day 21 was evaluated by qPCR. (**D**) 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, RNAscope was used to evaluate HBV replication. Scale bar = 50 µm. Data are means ± s.d. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by unpaired two-tailed *t* test.

Figure 6. Evaluation of novel antivirals by using HLCs.

(**A**) Huh7-NTCP, HepG2-NTCP, (**B**) HLCs and (**C**) PHHs were infected with HBVcc at a MOI 300 and treated with 10 µM Genistein. Eight days after infection, HBeAg or HBV-RNA was evaluated by ELISA or qRT-PCR respectively. (**D**) iPSCs SC3D derived HLCs were infected with HBVcc at a MOI 300 and treated with indicated dose of PA452 at the same time. Ten days after infection, different

HBV infection markers were determined. (**E**) HLCs were infected with HBVcc at a MOI 300. Eight days after infection, cells were treated with indicated dose of PA452. Kinetics of HBeAg was determined by ELISA. (**F**) PHHs and (**G**) HepG2-NTCP were infected with HBVcc at a MOI 300 and treated with 2 μM PA452 at the same time. Eight days after infection, HBeAg or HBV-RNA was evaluated by ELISA or qRT-PCR respectively. Data are means ± s.d. *** $p<0.001$ by unpaired two-tailed *t* test. ns: not significant.

Figure 1

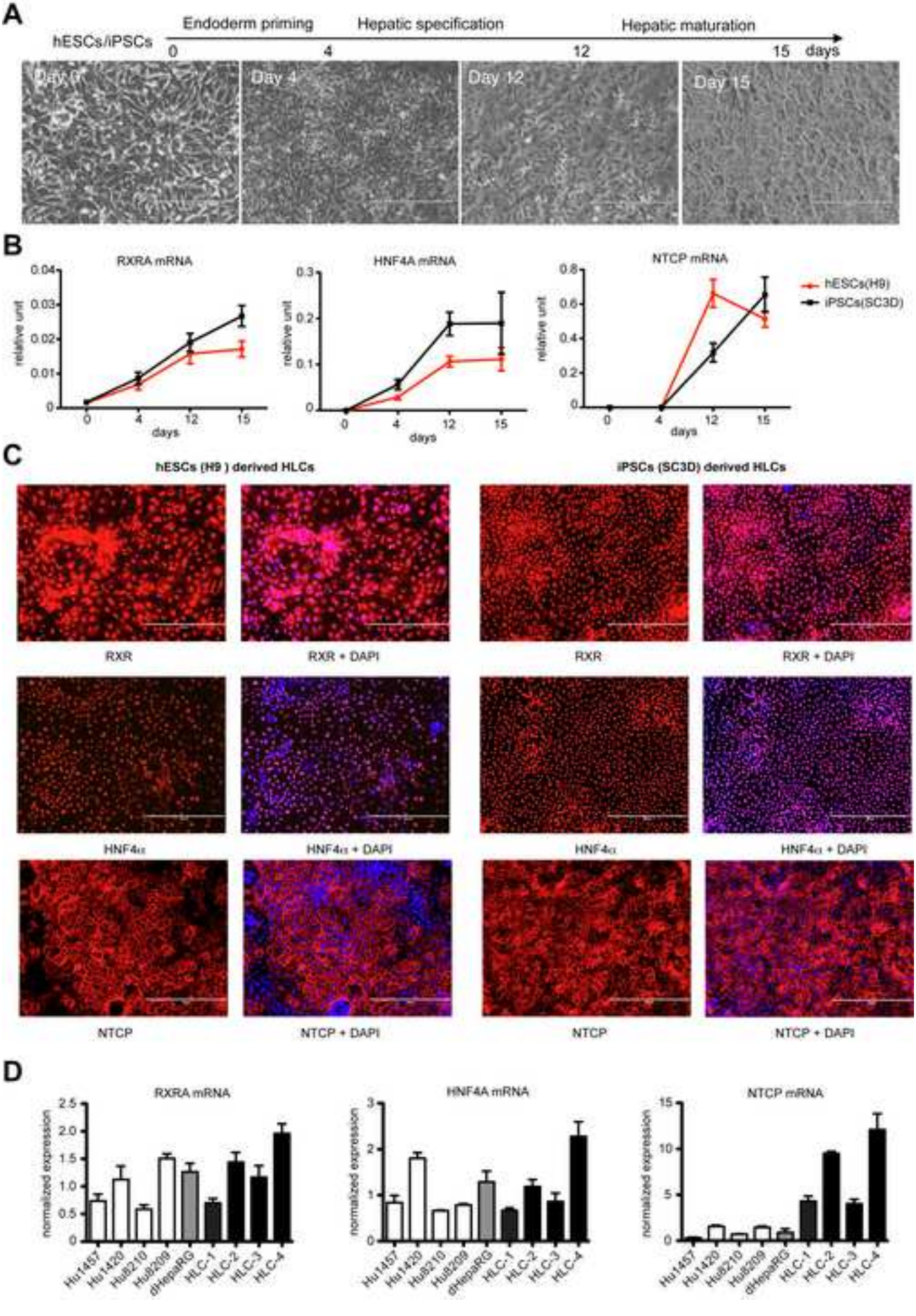


Figure 2

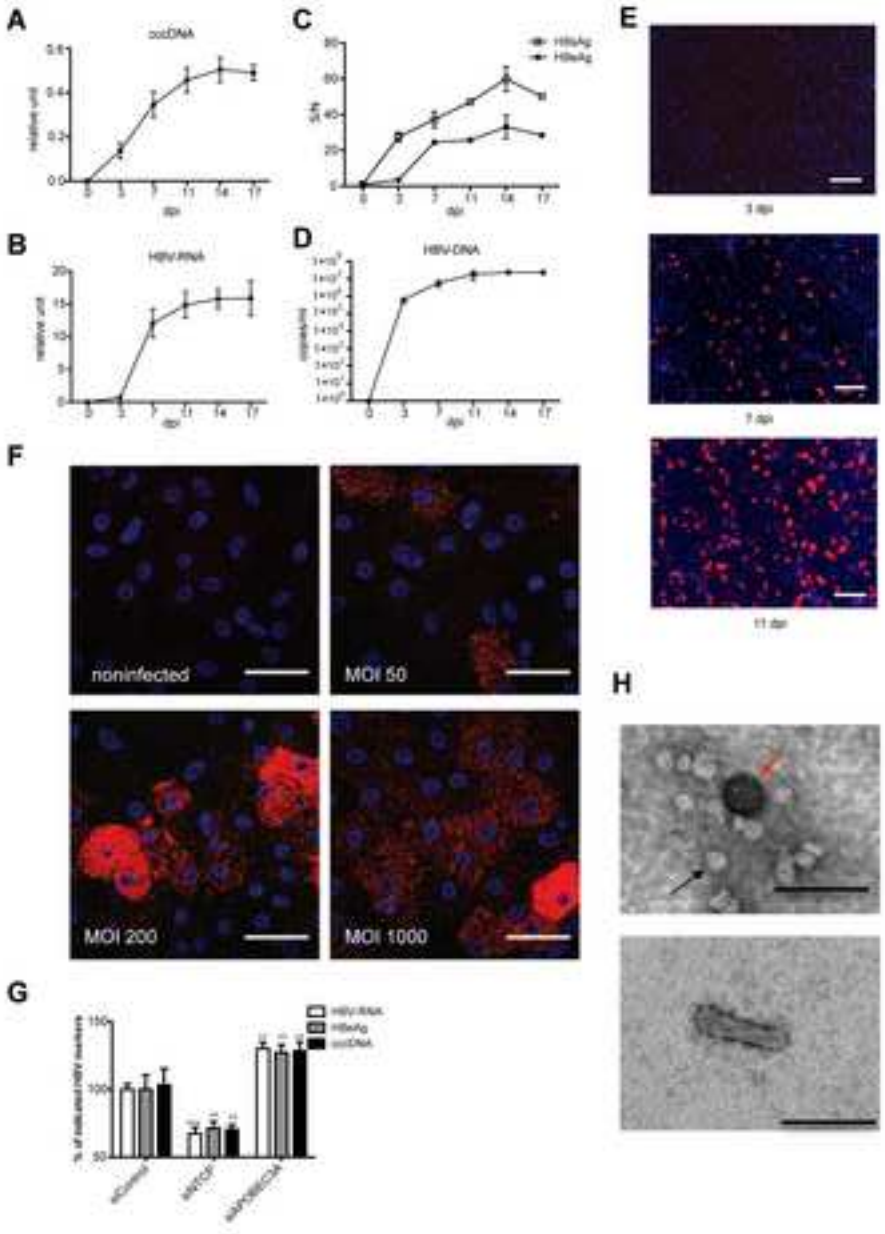


Figure 3

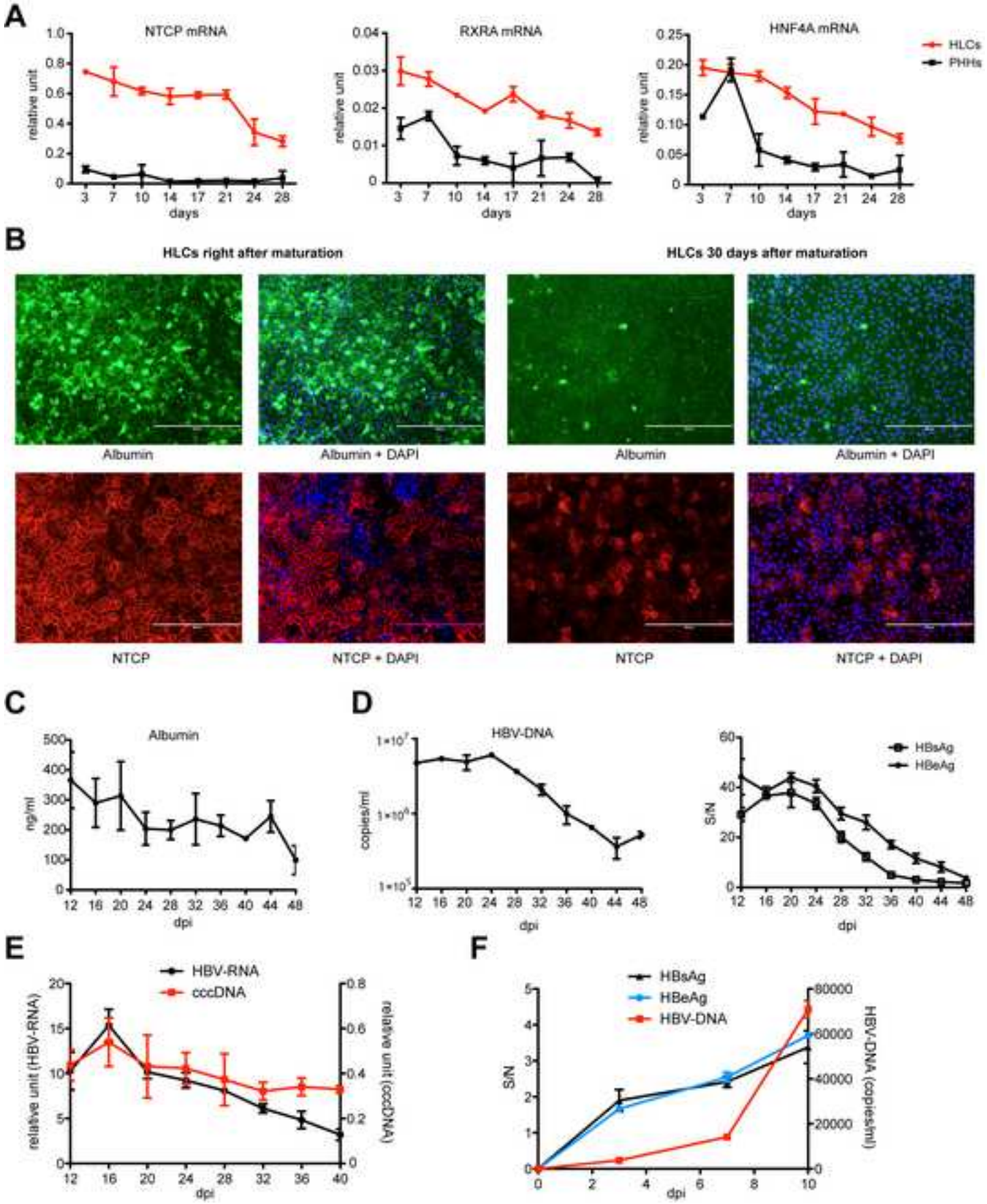


Figure 4

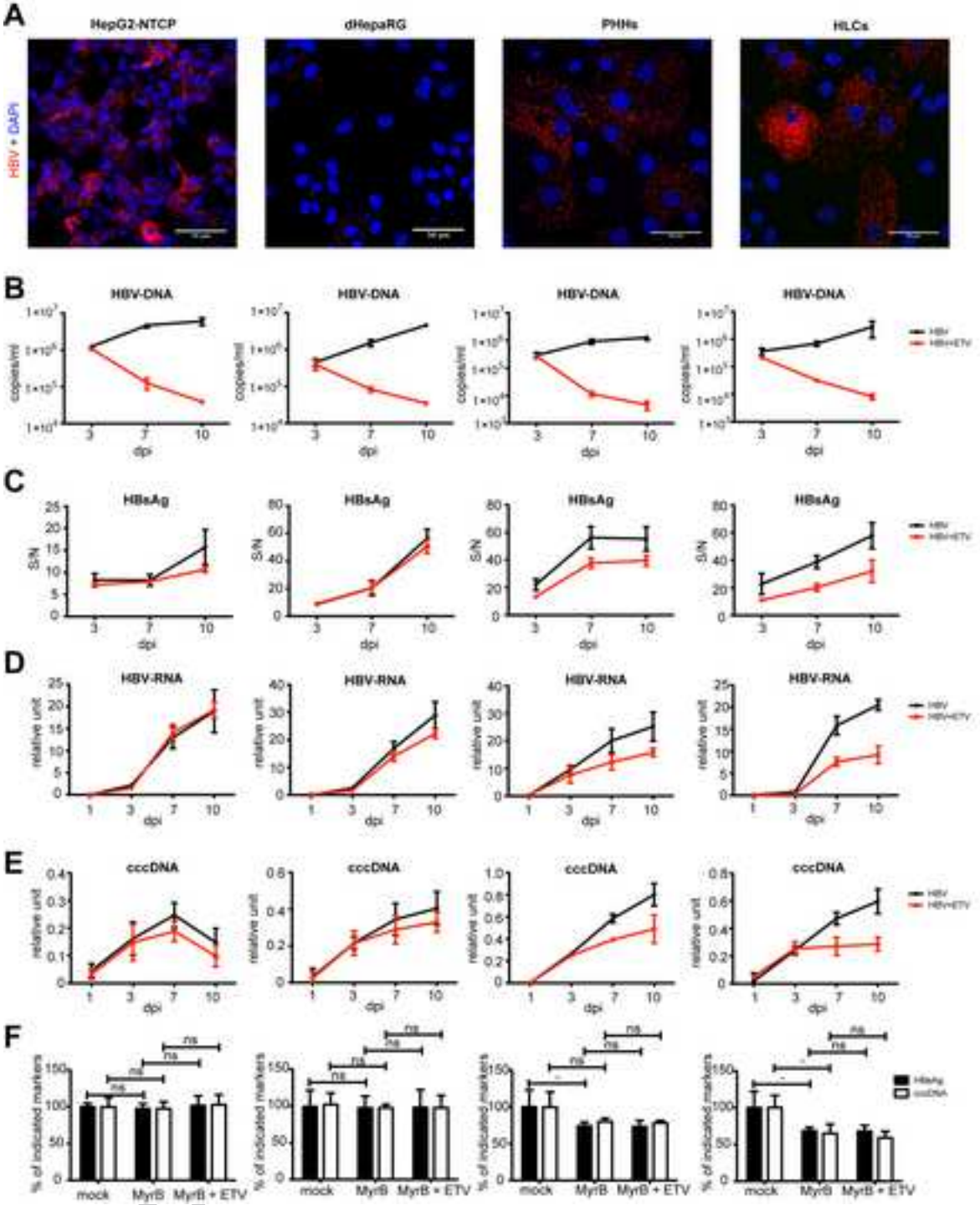


Figure 5

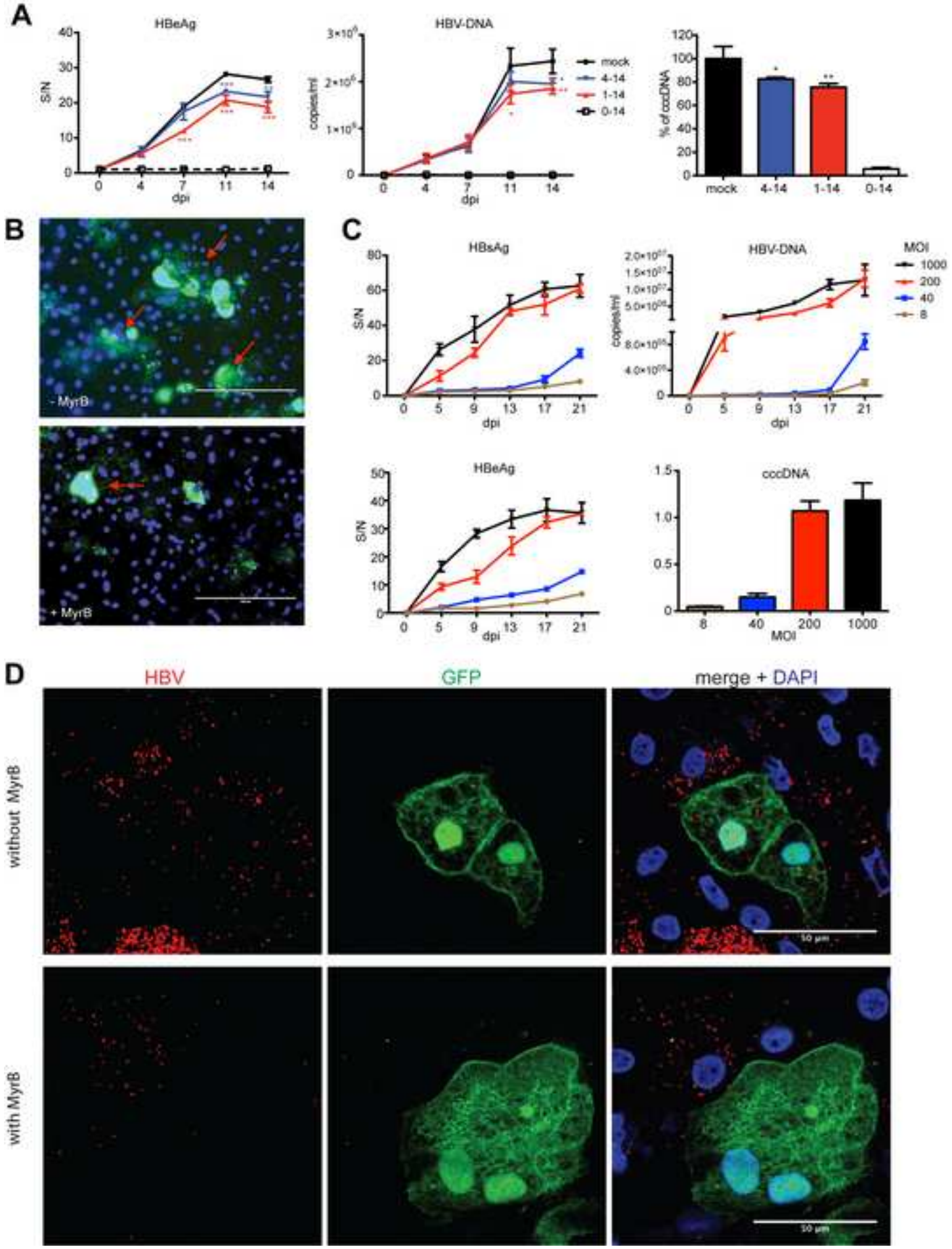
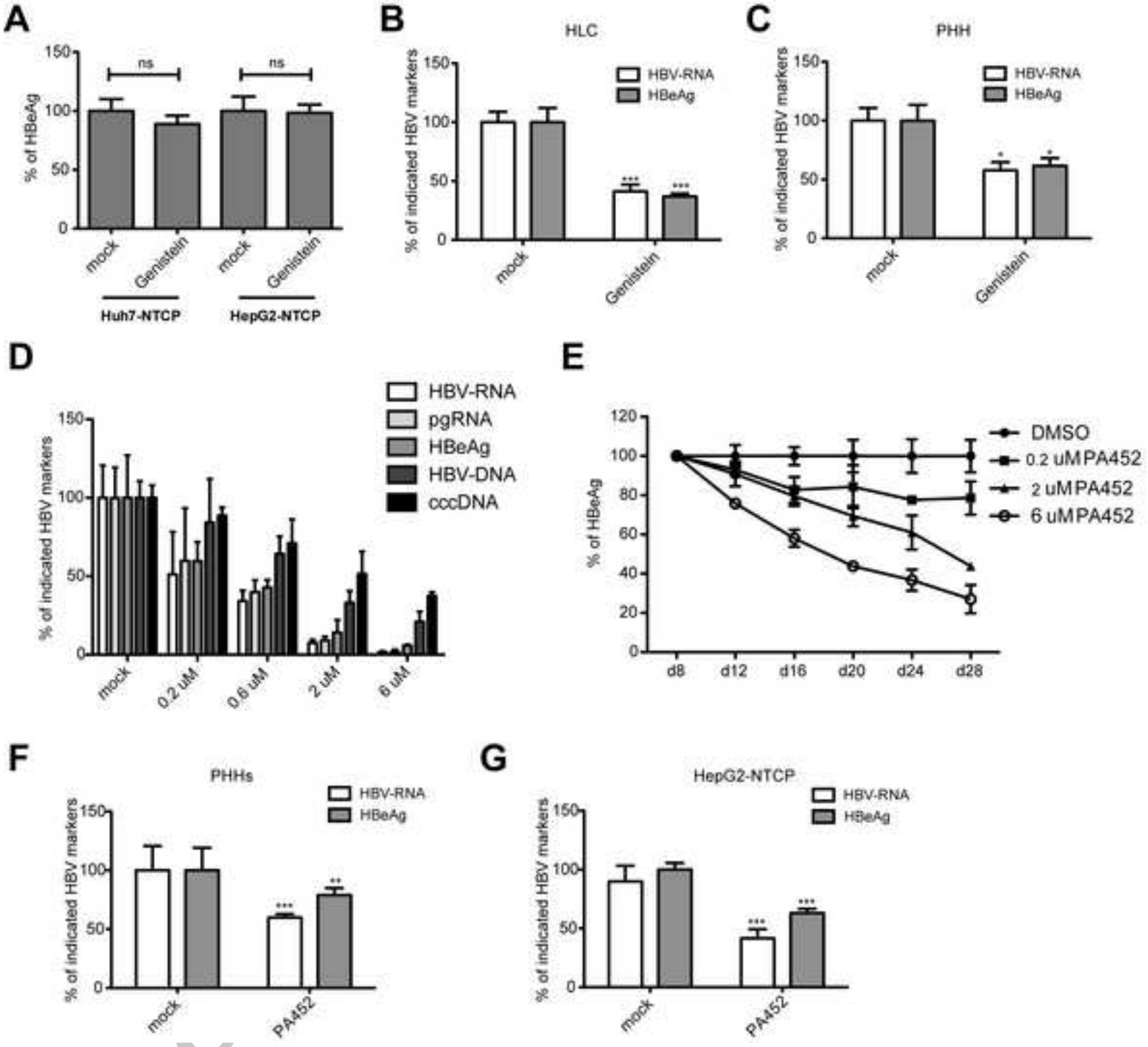


Figure 6



*Graphical Abstract