A novel primary human hepatocyte co-culture system for the investigation of hepatitis B viral persistence and evaluation of novel drugs.

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ABSTRACT:

Approximately 400 million individuals worldwide are chronically infected with Hepatitis B virus (HBV). These individuals can develop fibrosis, cirrhosis, and liver disease. Current antiviral treatments for HBV are effective in reducing the viral load, but they are rarely capable of curing the disease. HBV has a limited tropism only infecting human hepatocytes, and is able to persist, through the formation of covalently closed circular DNA (cccDNA), which is an extra-chromosomal viral episome that serves as the template for transcription of all viral mRNAs[1]. To develop a drug testing platform, a reliable *in vitro* system must be established that is capable of supporting persistent HBV infection. The discovery of the human bile acid transporter NTCP has paved the path for creating an infectious cell culture system for HBV. There is now ample evidence that hepatoma cell lines expressing human NTCP can be infected with HBV[2]. However, due to their transformed nature, human hepatoma cells do not adequately mimic hepatocytes, leading to aberrant host responses. To analyze hepatitis virus infection in a more physiologically relevant context, cultures of primary human hepatocytes (PHHs)–including adult and stem-cell derived hepatocyte like cells (HLCs)–have been developed that support HBV infections, albeit transiently and only at low levels[3,4,5]. Previously published PHH models for HBV infection have deteriorated within 2-3 weeks after initiating the culture and require blunting of antiviral innate immunity to establish a persistent infection[6]. To address these limitations, we have created a more robust, self-assembling co-culture method for PHHs. In this culture format, PHHs maintain major hepatic functions, including phase I cytochrome P450 mediated metabolism, canalicular formation and albumin secretion for weeks[7]. We demonstrated that exposure of these cultures to cell culture derived HBV results in persistent infection for up to 40 days, mimicking chronic infection as evidenced by HBsAg secretion, detection of HBV DNA in supernatant and cells and the presence of cccDNA. Pre-selection of specific hepatocytes lots and pre-conditioning of the cultures with inhibitors of antiviral immunity were not necessary to establish infection. The platform is scalable to microtiter well formats, enabling high throughput screens of anti-HBV drug candidates in a physiologically relevant cell. The system has further utility for combined efficacy, toxicity and drug metabolism. Our current efforts focus on applying the model to investigate the role of host factors that have been implicated in playing a role in establishing HBV persistence.

INTRODUCTION:

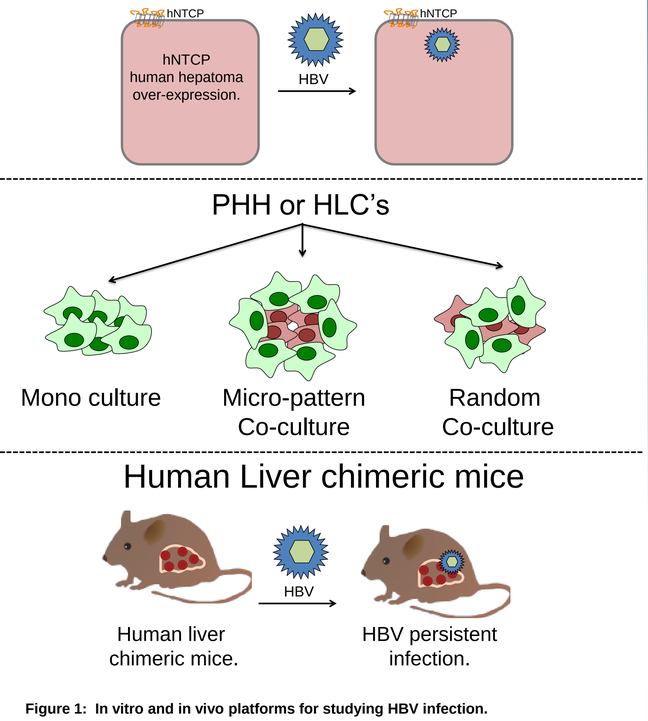


Figure 1: In vitro and in vivo platforms for studying HBV infection.

METHODS AND RESULTS:

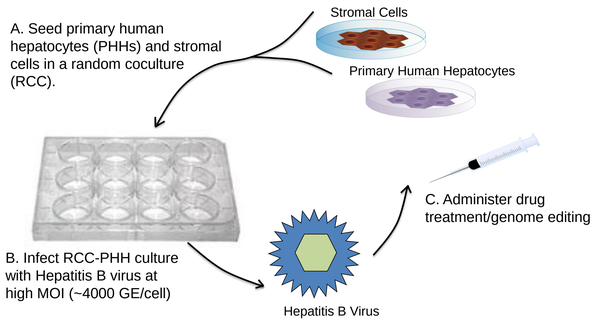


Figure 3. Creation, infection, and treatment of primary human hepatocytes (PHHs) in a random coculture[3]. Assays performed include albumin and HBsAg ELISAs as well as total HBV DNA and cccDNA quantification.

Donor Demographics	
Gender	Males (4), Female (1)
Age (yrs)	7-69
Race	Caucasian (3), Asian (2)
Cause of Death	Annoxia (2), Head Trauma (1), Cerebrovascular accident (2)

Serology (PCR detection)	
Cytomegalovirus (CMV)	Positive (3), Negative (2)
Hepatitis B Surface Antigen (HbsAg)	Negative (5)
Antibody to Hepatitis C Virus (HCV)	Negative (5)
Human Immunodeficiency Virus (HIV)	Negative (5)

Figure 4. Information about donor demographics and serological data.

Metabolic Activity (nmoles/hr/10^6 cells)

Substrate	Enzyme	Concentration (μM)	Day 1	Day 7	Day 14
Midazolam	CYP3A4	5	2.263	1.687	1.053
Dextromethorphan	CYP2D6	20	0.759	0.848	0.454
Tolbutamide	CYP2C9	20	0.556	0.443	0.320
Urea Synthesis (μg/24 hr/10^6 cells)			90.97	82.48	68.51

Figure 5. Metabolic activity data for RC-PHH cells.

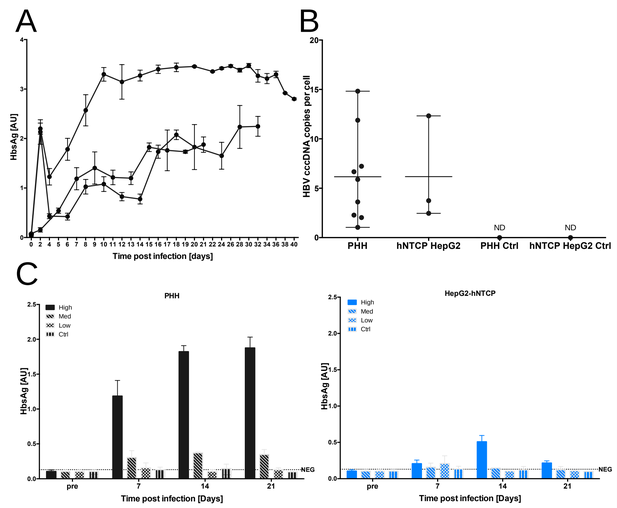
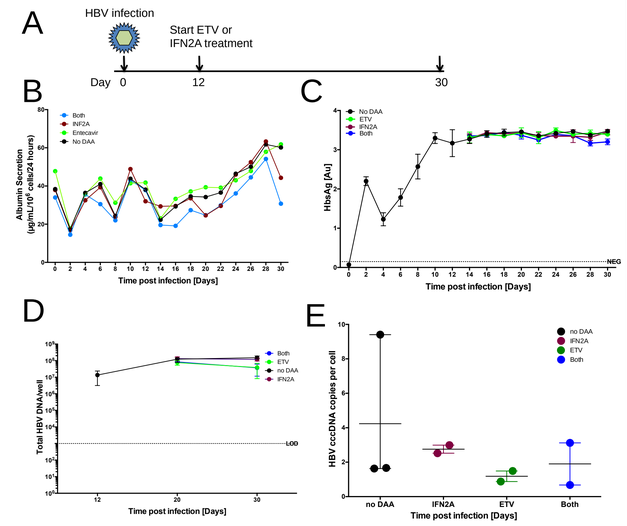


Figure 4. Comparison of HBV infection between RC-PHH and HepG2-hNTCP expressing cells. A) PHHs in three separate experiments when challenged with tissue culture derived HBV from HepG2.2.15 cells demonstrate a high secreted HBsAg level in collected media, (minimum of three biological replicates per experiment and time point). RC-PHH's are persistently infected for up to 40 days post infection. B) 1-15 copies of HBV cccDNA per cell were observed for both RC-PHH's and HepG2-hNTCP expressing cells, measured in triplicate, challenged at a high MOI (4,000 HBV GE/cell). C) RC-PHH's and HepG2-hNTCP expressing cells when challenged with HBV exhibit different infection profiles with RC-PHH's showing persistence for three weeks when infected at High (4,000 HBV GE/cell) or Medium (400 HBV GE/cell) while only high MOI's of 4,000 HBV GE/mL resulted in persistent infection in HepG2-hNTCP expressing cells. Neither RC-PHH's or HepG2-hNTCP expressing cells showed any level of infection with low MOI's of infection (40 HBV GE/cell). In addition HBsAg secretion by persistently infected HepG2-hNTCP expressing cells was lower than RC-PHH's at the challenged at the same High MOI (4,000 HBV GE/cell) and showed a different profile with the highest levels of HbsAg secretion occuring at day 14 p.i. for HepG2-hNTCP expressing cells followed a decrease at day 21 while RC-PHH HbsAg secreted levels continued to increase until day 21 when cells were harvested.



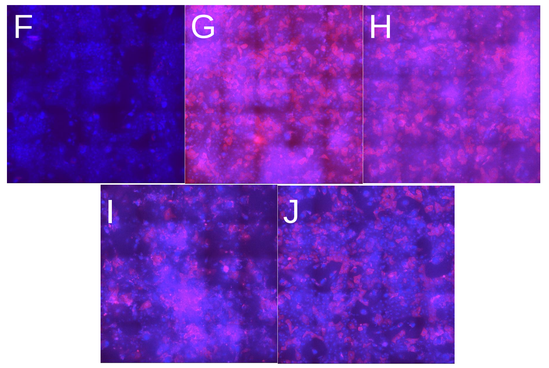
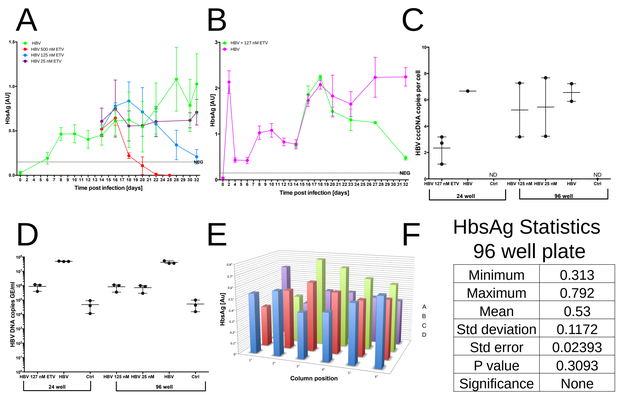


Figure 5. A) Experimental time course**.** B) Levels of albumin in PHHs. C) HBsAg levels for each treatment condition, measured in at least duplicate. HBV persistence was observed for 30 days, 2 weeks longer than any other PHH or iHep culturing system. No response to drug treatment was observed. D) Total HBV DNA per well, measured in minimum duplicate. E) HBV cccDNA copies per cell for each condition. Levels indicate that treatment does not seem to affect cccDNA levels. PHH were all infected with an MOI= ~4,000 HBV virions followed by treatment. After 40 days in cultures wells were fixed with 4% PFA and stained for HbcAg. The following images are of representative wells: **F.** No primary control. **G.** No DAA treated. **H.** INF2A treatment. **I.** Entecavir (127 nm). **J.** Both treatment with entecavir and INF2A.



HbsAg Statistics 96 well plate

Minimum	0.313
Maximum	0.792
Mean	0.53
Std deviation	0.1172
Std error	0.02393
P value	0.3093
Significance	None

Figure 6: RCC-PHH is scalable and can be used as a platform for drug testing. A) 96 well plate and B) 24 well plate HbsAg ELISA data for RCC-PHH infected cells treated with various dosages of entecavir. Both 127 nM (24 well) and 125 nM ETV treatment resulted in significant reduction of HbsAg. Treatment with 500 nM ETV resulted in significant cell death and loss of culture. C) HBV cccDNA is detected in persistently infected RCC-PHH's regardless of format and treatment with entecavir has no affect. D) A ~1.5 log drop in HBV DNA in supernatants corroborates that entecavir treatment worked in both 24 and 96 well formats. E) HbsAg values at day 14 p.i. across 96 well plate. F). No significant difference due to well position were observed across 96 well plate.

A Comparison of different in vitro systems for studying HBV infection

Cell type	hNTCP-expressing human hepatoma cell lines	Primary human hepatocytes	Fetal human hepatocytes	Hepatocyte like cells (HLC's)
Platform	Mono-culture	Mono-culture (Mono), Micro-patterned co-culture (MPCC), **Random co-culture (RCC-PHH)**	Mono-culture	Mono-culture (Mono), Micro-patterned co-culture (MPCC), Random co-culture (RCC-PHH)
HBV infection [Days]	Duration of infection is short [Days]	Variable depending on platform. Mono [Days], MPCC [2-2.5 weeks], **RCC-PHH [4-5 weeks]**	Duration of infection is short [Days]	Variable depending on platform. Mono [Days], MPCC [2-2.5 weeks]
Format	6, 12, 24 well plate. Can also be seeded in 96 well format.	6, 12, 24 well plate (Mono +MPCC). **Can also be seeded in 96 well format for RCC-PHH platform only.**	6,12, 24 well plate.	6, 12, 24 well plate.
Comments	1. Multiplicity of infection is very high. 2. PEG and DMSO must be used. 3. no viral spread. 4. Duration of infection is short. 5. Little cccDNA observed. 6. High variability between clones.	Mono 1. phenotype is unstable in vitro 2. large donor variability MPCC large donor variability. 2. Low infection (~30%) no viral spread. 3. Innate immune system must be supressed. **RCC-PHH** **1. Preserves hepatocyte function (weeks).** **2. Persistant prolonged infection (32-40 days).** **3. No need for blunting of innate immunity.** **4. Scalable**	Similar but not identical phenotype to adult hepatocytes	1. Similar but not identical phenotype to adult hepatocytes. 2. Technically difficult to work with. 3. Innate immune system must be supressed. 4. Not scalable.
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SUMMARY:

1. Persistent HBV infection up to 40 days has been established in a novel *in vitro* system using primary human hepatocytes in a random coculture without the use of JAK/STAT inhibitors.
2. Drug treatment with entecavir and IFN-2A has demonstrated this system's ability to be used as a drug screening platform.
3. Between 50-90% of PHH's in the RCC-PHH platform are infected. Of interest is that high levels of HbsAg are observed as well as total HBV DNA, pgRNA, and HBV cccDNA.
4. Scalable format 96 well microtiter plat which can be used as a high throughput platform for testing of novel drugs, genetic engineering, and metabolic studies.

DISCUSSION AND OUTLOOK:

1. Our results show that the RCC-PHH platform is a robust system that can be used to establish a persistent hepatitis B virus infection without the use of immune repressing molecules.
2. Furthermore, the RCC-PHH culture system described is scalable, as PHH's are susceptible to HBV infection in a 96 well format. Regardless of format HBV infection can persist for up to 40 days in challenged PHH's allowing for the investigation of viral persistence and kinetics of prolonged infection.
3. Both 24 or 96 well formats respond to the HBV antivirals entecavir but not IFN-2A. This supports this models viability as a drug testing platform for HBV.
4. Of note is that tissue culture produced HBV from HepG2.2.15 cells was used in all experiments as opposed to patient serum from infected individuals again showing how robust and versatile this platform is.
5. Another point of interest is that hNTCP-eGFP expressing HepG2 cells have significantly lower levels of HBsAg secretion when infected with HBV than RCC-PHH's.
6. In the future, direct acting antivirals and genetic manipulation using lentiCRISPR constructs to knock out host genes of interest that have been suggested to be important in the HBV life cycle

Future directions:

1. Testing whether secreted virus from persistently infected RCC-PHH's can be used to infect naïve cells.
2. Testing of novel drug candidates for inhibition of host factors thought to be involved in HBV replication.
3. Manipulation of RCC-PHH using CRISPR-Cas9 to probe host-viral interactions.

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