Forthcoming Developments in Models to Study the Hepatitis B Virus Replication Cycle, Pathogenesis, and Pharmacological Advancements

Sajad Ahmad Bhat, Ishfaq Ahmad Ahanger, and Syed Naqui Kazim*

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ABSTRACT: Hepatitis, liver cirrhosis, and hepatocellular carcinoma are all manifestations of chronic hepatitis B. Its pathogenesis and molecular mechanism remain mysterious. As medical science progresses, different models are being used to study the disease from the physiological and molecular levels. Animal models have played an unprecedented role in achieving in-depth knowledge of the disease while posing no risk of harming humans throughout the study. The scarcity of acceptable animal models has slowed progress in hepatitis B virus (HBV) research and preclinical testing of antiviral medicines since HBV has a narrow species tropism and exclusively infects humans and higher primates. The development of human chimeric mice was supported by a better understanding of the obstacles to interspecies transmission, which has substantially opened the way for HBV research *in vivo* and the evaluation of possible chronic hepatitis B therapeutics. Animal models are cumbersome to handle, not accessible, and expensive. Hence, it is herculean to investigate the HBV replication cycle in animal models. Therefore, it becomes essential to build a splendid *in vitro* cell culture system to demonstrate the mechanisms attained by the HBV for its multiplication and sustenance. We also addressed the advantages and caveats associated with different models in examining HBV.
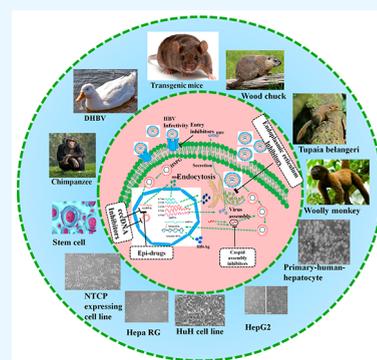
INTRODUCTION

The hepatitis B virus (HBV) is a member of the hepadnaviridae family, which has a short genome (size 3.2 kilobases) of partially double-stranded DNA. In an extremely species-specific manner, it enters human hepatocytes through a coupling between a viral envelope protein and human sodium taurocholate co-transporting polypeptide (NTCP).[1−5] After viral entry, the HBV core particle is released into the cytoplasm and transported to the nucleoplasm. The DNA is released and repaired by cellular factors to the covalently closed circular DNA (cccDNA).[6] The cccDNA serves as a template for pregenomic RNA (pg RNA) transcription and other mRNAs that encode at least seven different proteins and then uses a transcription−reverse transcription mechanism to replicate its genome. The cccDNA persistence is responsible for the failure of viral clearance, the absence of a functional cure, and in particular relapses after treatment termination.[7] The pregenomic RNA for the viral core and polymerase serves not only as a messenger but also as a reverse transcription template which involves the use of polymerase to produce core particles (Figure 1).

The hepatitis B virus is a hepatotropic virus that affects the liver with specific host and tissue tropism.[10] HBV is considered to be a noncytopathic virus whose pathogenesis is mostly attributed to liver injury caused by the host immunological response. Virus components such as the HBV e antigen (HBeAg), HBx, and the three HBV surface antigens (HBsAg) suppress the host immune response, leading to HBV chronic infection.[11] Though HBV vaccination is available as a preventative measure, hundreds of millions of people globally are chronically infected with HBV, and due to the refractory nature of the HBV intracellular replication intermediate cccDNA, amalgamation therapies using interferon in blending with nucleos(t)ide analogs hardly achieve infection elimination or a functional cure, i.e., the permanent inactivation of the virus in HBV infected cells.[12]

Furthermore, there is proof of virological relapse in patients who have stopped using antiviral drugs for treatment[13] and hepatitis flares during and following termination of antiviral therapies[14] including lamivudine resistance.[15] This indicates that chronic hepatitis B (CHB) patients may require longer treatment because of the refractory HBV nature, and extremely effective antiviral therapies against HBV may need to be adopted in amalgamation with current treatments to support and restore the immune system, like checkpoint inhibitors or
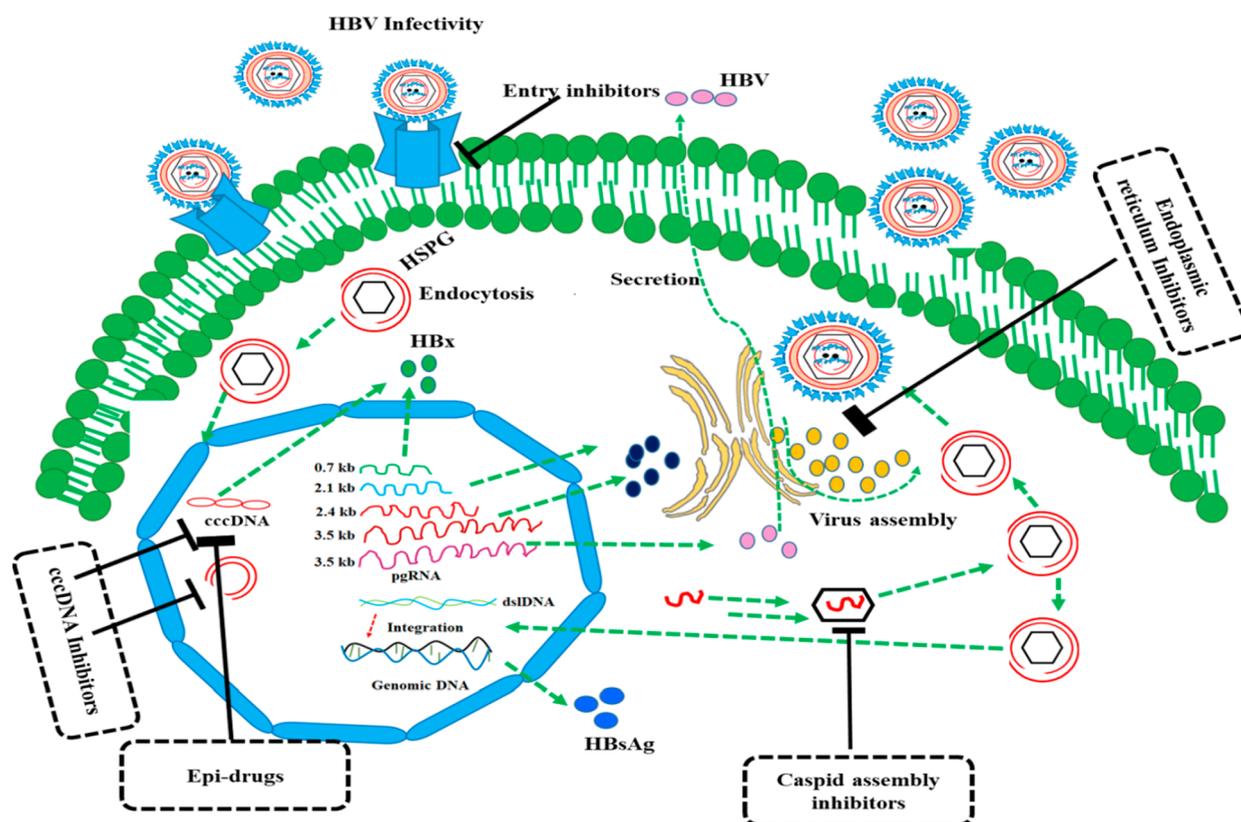
Figure 1. HBV replication cycle and potential targets. HBV replication cycle: Binding and attachment, penetration or ingress, biosynthesis, nucleocapsid assembly, and secretion. Different treatments targets: inhibitors of entry point (virus binding receptors NTCP and HSPG), cccDNA inhibitors (impeding cccDNA genesis), epi-drugs (suppressing the synthesis of viral RNA), inhibitors of the endoplasmic reticulum (blocking viral capsid assembly), and secretory protein inhibitors (impeding secretion of HBV virions).[8,9] Adapted and modified with permission from Bhat et al., 2022.

Table 1. Various *in Vitro* Experimental Models of Cell Culture Frequently Used for Studying Persistent HBV Infection

Cell Biology	HepG2 cell line	HuH-7 cell line	HepaRG line	Primary human hepatocytes (PHHs)	Hepatocyte derived from stem cells
Functional Innate immunity	Minimal Slight	Average	Entirely functional	Entirely functional	Entirely functional
Length of propagation	Indefinite	Indefinite	Indefinite	Less than 30 days	Less than 30 days
Polarized property	No	Yes	Yes	Yes	Yes
Transformed	Yes	Yes	No	No	No
Availability	Extreme	Extreme	Average	Less	Less
Lot variability*	Negligible	Negligible	Slight	Slight	High
Favors chronic disease HBV	No, needs NTCP receptor	No, needs NTCP receptor	Yes needs differentiation	Yes	Yes

adoptively transferred chimeric antigen receptor T cells, in the future.[7,16]

Chronic HBV infection can lead to many liver diseases, including fibrosis, cirrhosis, and hepatocellular carcinoma. Despite significant advancements in the prevention and therapeutic approaches to HBV disease, over 0.24 billion individuals worldwide are infected with the virus, and the global mortality rate is 650,000 annually.[8,17] To further minimize these figures, novel ways of therapy that can cure chronic infection are required, in addition to expanding HBV vaccination coverage.

The constant development of cell culture and various animal models for CHB diseases has been critical in gaining a better understanding of a virus' replication cycle and the generation of anti-HBV drugs. Since the discovery of the "Australian antigen" (Au), a number of models have been established.[18]

Their pros and cons are discussed. There are also suggestions for future improvements to meet the basic requirements of an HBV treatment.

Cell Culture Models. Tumor-derived cell lines, like those used for other viruses, have helped us learn more about viral hepatitis.[19] General molecular biology approaches have been used to overexpress viral proteins and establish cell lines that persistently express the hepatitis viral genome. HBV DNA genomes and viral protein-coding plasmids have been transported intracellularly using different methods of transfection and systems for viral transduction. Besides, screenings of small molecules have also been performed using cell lines that carry viral genomes in order to discover compounds that could inhibit viral replication.[20] Broadly, the usage of cell lines that have been transformed has relied on the fact that a few of them have typically failed in natural innate immune antiviral

pathways in cells that would usually inhibit viral genomes and proteins from being expressed.[21] Although dysfunctional, these cell lines allowed researchers to study these viruses without being hampered by host defense signaling pathways. A successful strategy for HBV was determined by using the wide range of cell lines that stably integrated into the genome of the HBV DNA in several loci of the chromosome.[22] A number of cell culture systems have been used to study HBV replication. What are the advantages and disadvantages of each of these systems? No system is ideal for all studies, but findings from different systems can be complementary.

Table 1 and Figure 3 show a list of experimentally transformed liver cell line models used to investigate HBV disease.

HuH-7 Cells. The cell line HuH-7 was developed in 1982 from a hepatocellular carcinoma tumor, extracted from a 57-year-old Japanese man who underwent a liver resection for the tumor.[23] This cell line was proved to be beneficial in HBV research[24] though it has been more frequently used as *in vitro* experimental cell culture models to investigate the hepatitis C virus (HCV). This cell line was primarily doing well in multiplying luciferase-harboring subgenomic mutants that exclusively encoded HCV nonstructural proteins.[25] As a result, this cell line was utilized as the major substrate for developing a replicon that would constantly express and replicate the HCVRNA genome, without the integration of DNA intermediate into the cellular genome.

The HuH-7 cell line has been proved effective in HBV-related research, but it has played a less important role than it has in HCV research. This cell line like HCV was developed to overexpress the viral proteins from the HBV genome.[24] The linear, full-length HBV cDNA transfection into HuH-7 cells was one of the most important uses of this cell line for studying HBV.[26] During the HBV replication cycle, this model allowed for the formation of cccDNA. It was used to investigate more difficult facets of HBV biology, such as HBV episomal DNA and epigenetic alteration.[27] In 2012, with the discovery of the putative entry receptor, HBV NTCP, encoding a sodium/bile acid cotransporter also called SLC10A1 (the solute carrier family 10 member 1), stably expressing this gene in a HuH-7 cell line was used as an experimental *in vitro* model to favor chronic HBV infection.[1]

HepG2 Cells. A different cell line that has been developed to explore viral hepatitis agents is the HepG2 cell line. The pathophysiology of HBV infection has been studied using human hepatoma cell lines (HepG2 cell line). This cell line was established from the hepatic tissue of a 15-year-old Caucasian white male patient who had a well-differentiated hepatoblastoma. As a hepatocyte surrogate model, the cell line is commonly used. These hepatoma cells are transiently transfected with replication-competent HBV DNA to commence the viral replication cycle within the host and generate the HBV particles secretion.[28−30]

In contrast to the HuH-7 cell line, the HepG2 cell line is polarized and has proved to be extremely beneficial for studying HBV compared to HCV. The HBV genome could be continuously expressed in HepG2 cells, which led to early advancements in HBV research. In selection media, this feat was achieved by growing HepG2 cells stably transfected with the HBV genome.[29,31] Although these transfected cells were capable of the HBV replication and the cccDNA formation, albeit at a low level production, they were incapable of being infected by HBV virions, which is now understood to be due to the lack of NTCP gene expression. (HepG2-NTCPsec+) cells, a novel HepG2 cell clone, successfully favor the entire replication cycle, prolonged viral transmission, and HBV amplification obtained from patients or cell culture, reflecting the important properties of HBV-infected individuals.[32]

Similar approaches have been used to generate HepG2 H1.3 and HepAD38 cell lines, which were selected for harboring the HBV genome.[33,34] These cell lines are persistently used to examine aspects of the HBV replication cycle, the primary steps of viral entry, and hepatocyte trafficking, as well as later steps of the HBV replication and cccDNA clearance by host defense pathways.[35] Besides, HepAD38[34] and HepG2.2.15[31] being a stable replicating system, these cells are fascinating models for evaluating the antiviral compounds.[36] These cells were most frequently used as a source of infectious HBV virions for performing infection assays, as these were produced by a stable transfection incorporated with the HBV genome.[28,37,38]

HepG2.2.15 cells favor not only HBV DNA replication but also packaging and Dane particle release. This cell line can be passaged for a prolonged period because it was developed from liver cells. Since this cell line has been developed as a useful *in vitro* culture model for investigating the structure, activity, and gene expression, it regulates HBV DNA replication as well as the *in vitro* assessment of antiviral drugs against HBV. This cell line also has several drawbacks, which are as follows. (1) It does not mimic a natural infection since the HBV DNA is integrated into the host cell genome, which allows it to reproduce the viral replication but not the process of virus invasion into cells. (2) Because of the absence of NTCP, a bonafide receptor responsible for HBV infection, this cell line is insensitive to direct infection with serum containing HBV.[39] (3) HBV adsorption, cellular entrance, and viral uncoating are impossible to examine using HepG2.2.15 cells. (4) HepG2.2.15 cells are descendants of HepG2 cells and are unable to be used to investigate the HBV carcinogenicity.

Another cell line called the HepAD38 cell line generates larger quantities of HBV than HepG2.2.15 cells and may precisely control the onset of viral replication. The HepAD38 cell line, unlike HepG2.2.15 cells, has a disadvantage; i.e., it is not appropriate for examining the interaction between the virus and host cells in the initial stage of HBV disease.[34] The HepAD38 cell harbors 1.1 copies of the HBV genome, and its expression is regulated by the inducible cytomegalovirus immediate-early (CMV-IE promoter). The HepAD38 cell line generates roughly 11-fold more HBV DNA than HepG2.2.15 cells. Tetracycline can be used to regulate the HBV replication in the HepAD38 cell line.[34] HBV cannot be developed when tetracycline is present in the medium, as the synthesis of pgRNA is suppressed. The cells instantly express pgRNA, cccDNA, and HBV the moment tetracycline is removed. This HBV cell culture system is ideal for understanding the HBV replication and testing anti-HBV drugs.[34]

Baculovirus and adenovirus constructs were used to transduce HepG2 cells and used as a vehicle for delivery to efficiently express the HBV genome in these cells, according to a new model established afterward.[40,41] These transduction models outperformed transiently transfected or stable cell lines, and they are extremely reproducible compared to transfected cells because the replication and HBV genes expression highly predominated those of HepG2.2.15. The adenovirus system is without species restriction and has the potential to replicate HBV in the liver cells of any nonspecific

host. Unfortunately, due to its substantial cytotoxicity, this approach limits its further use. It is not appropriate to evaluate the anti-HBV effects of drugs, for example, because of the inadequacy of the enhanced vector dosage to boost the antigen development.[39]

The HBV baculovirus system when compared with HepG2.2.15 cells has about a 100-fold larger rate of HBV replication. The potential of the recombinant HBV baculovirus/HepG2 approach to monitor the replicative intermediates such as rcDNA and cccDNA is a distinctive feature; as a result, the system may be used to assess the impact of anti-HBV drugs on HBV DNA. It also has the potential to study the HBV resistance to nucleoside analogs (NAs) and interferons.[39] The HBV baculovirus has its limitations which restrict its wide use. (1) Instead of using a receptor-mediated approach, baculovirus reaches mammalian cells by a nonspecific process of the endosomes uptake.[42] (2) Gene transfer through baculovirus is limited to a few species. (3) Baculovirus vectors are not appropriate models to exploit them in animal research because the complementary system quickly deactivates them.[43]

NTCP-expressing HepG2 cells are now used to study HBV disease with infectious virions, due to the discovery of the putative HBV receptor (NTCP).[44] The systematic and efficient investigation of the full replication cycle in a sustainable in vitro cell culture system has been made possible by this approach.

HepG2 cells have become increasingly useful for HCV research due to advancements in the past decade. HepG2 cells had been observed to lack miRNA 122 (miR-122), which is important to support the HCV replication cycle. Specifically, miR-122 might support the HCV RNA stability, replication, and protein synthesis via several distinct mechanisms, by directly binding homologous regions in the virus genome's untranslated region.[41,45] Furthermore, HepG2 cells have a relatively poor CD81 expression (a known entry factor for HCV).[40,46] Therefore, it was shown that HepG2 cells are selected to express that both the CD81 receptor and miR-122 can be infected by HCV and support viral replication.[47] This model has proven to be very beneficial in the research of host defense mechanisms and to validate findings derived in primary human hepatocytes (PHH), based on disparities in antiviral innate immunity in this cell line when compared with HuH-7 cells. In response to HCV infection, the genetically modified cell line was capable of producing large amounts of type III IFNλ which has also been observed in primary human hepatocytes.[48]

HepaRG Cell Line. For studies of both HBV (Gripon, Rumin et al. 2002) and HCV,[49] an additional cell line has proven to be useful to the HepaRG cell line. This cell line was developed from cells obtained from a resection of tumor from an HCV-infected individual with liver cancer, although the infection was not sustained in vitro after isolation of these cells. HepaRG cells serve as hepatic progenitor cells that are bipotent and can proliferate indefinitely. These cells develop into both biliary and hepatocyte-like cells.[50] In order to support complete HBV infection, these cells must also be exposed to dimethyl sulfoxide to foster further development into more specialized cells like hepatocytes. According to head-to-head comparisons, HepaRG cells are similar to PHHs, which are regarded as the gold standard for hepatocyte studies in vitro studies in terms of drug metabolism and the ability to support infection by HBV.[51,52] Intially, it was shown that this cell line could favor HBV infection and replication.[50]

Regrettably, polyethylene glycol (PEG) is required to promote viral entry by supporting HBV binding to HepaRG. It develops the interaction with heparan sulfate proteoglycans, resulting in augmented infection efficiency.[53] Generally, infection rates are modest, and the virus transmission from cell to cell is minimal.[53] Thereafter, researchers were competent enough to use this cell line as a substrate for HCV infection.[49] In this study,[49] the investigators were able to utilize serum-derived HCV genotype 3 to validate susceptibility to HCV infection.[54]

HepaRG cells, in comparison to other cells originating from hepatic cancer, retain many physiological properties associated with the liver and more accurately show a similar transcriptomic profile close to that of hepatocytes. Surprisingly, cytochrome P450 expression level and innate element expression displayed that of PHH.[55,56] Particularly, they are widely employed for drug metabolism and toxicology studies.[52] It is the first cell line that is suitable for a cell culture system for infection studies.[50] This is the only available cell line infected so far. It is evident that HepaRG cells favor both viral entry and cccDNA formation[57] and are therefore a relevant model for investigating the HBV replication cycle.

A study was conducted by Schulze and colleagues in 2007[53] that explained the significance of HSPGs in the HepaRG cell line which triggers HBV entry. It was evident that the heparin sulfate binding region of the HBV envelope protein in the antigenic loop plays a vital role in HBV and HDV infection.[58] Despite being useful in many ways as summarized above, the use of the HepaRG model remained limited, especially in cases when the cell needs a complete differentiation procedure which is cumbersome and may influence the repeatability of experiments. This disadvantage associated with this in vitro culture model further limits its use for high-throughput studies.

NTCP-Overexpressing Hepatoma Cell. This is another in vitro cell culture model to study HBV infection. This cell line was developed after the discovery of an NTCP receptor in 2012. NTCP is a carrier of bile acid expressed primarily at the basolateral surface of the hepatocyte membrane.[59] Notably, both the HuH7 and HepG2 cells fail to express NTCP molecules,[60] illustrating their incompetence to mediate viral infection. Human NTCP receptors in hepatoma cells were exogenously expressed to make them prone to HBV and HDV infection,[37,60] forming the first vigorous in vitro model to study the complete viral replication cycles and to explore the underlying mechanism of the virus−host interaction. Interestingly, HepG2-NTCP cells seem to be more highly prone to HBV infection than HuH7-NTCP cells, indicating that many more studies are needed to obtain a full viral replication cycle.[37] The hepatoma cell lines overexpressing NTCP have also been used to check cell entry inhibitors (Verrier et al., 2016). These robust cell culture systems provide useful information for a better understanding of HBV pathogenesis. The disadvantage related to this cell line is that these are well-defined from hepatocytes with respect to chromosomal abnormalities, mutations, fluctuating gene expression, and metabolic characteristics.[61]

Primary Human Hepatocytes (PHHs). Primary human hepatocytes (PHHs) are an extremely crucial model for the study of the hepatitis B virus disease. These normal cells are extracted from the exterior margin of the tumor in a patient that underwent liver resection (generally for a tumor of the liver). PHHs can also be reliably formed from aborted fetal liver embryos and used as a substrate for infection.[62]
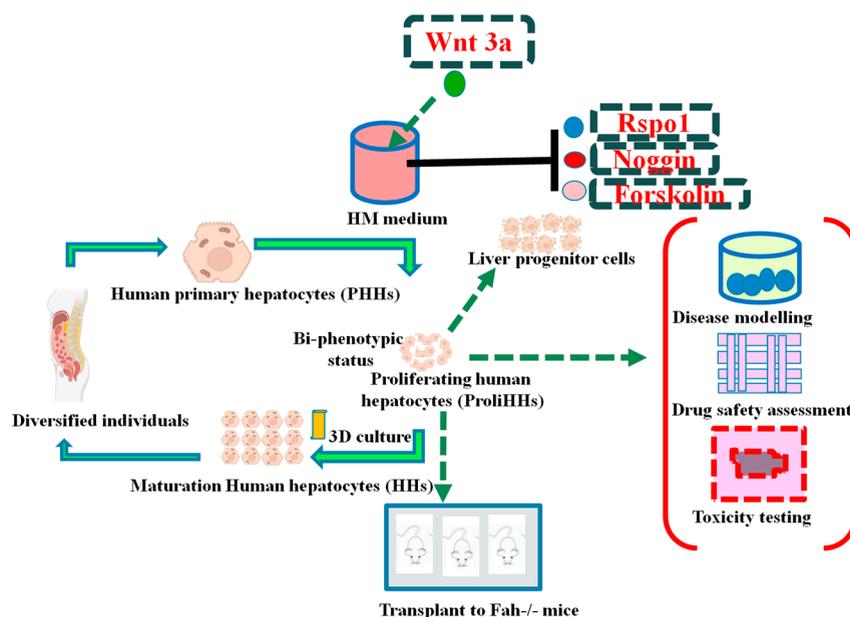
Figure 2. Schematic representation of the *in vitro* culture of PHHs under standard conditions.[72] Adapted and modified with permission from Feng et al., 2016.

PHHs, unlike HepaRG cells, do not multiply once plated, and their tissue culture survival is limited, usually between 1 and 2 weeks; however, fetal hepatocytes can last for several weeks.[63] *In vitro*, these cells quickly dedifferentiate, down regulating biological characteristics related with mature hepatocytes at the same time.[64] According to various studies, PHHs have also been successfully infected with serum-derived antigens and HBV developed through a cell culture.[65,66] From other species, such as chimpanzees and Tupaia, primary hepatocytes have also been demonstrated to support HBV disease and associated hepadnaviruses that infect ducks and woodchucks (Dandri, Burda et al. 2001, Nair, Arathy et al. 2011).

They are widely accepted as the "gold standard" of liver functions because they possess all the vital elements required for the studies of hepatotoxicity, drug discovery, development, cellular metabolism, drug-induced liver injury (DILI), and hepatitis B virus infection.[67] There are limitations of PHH which restrict its use in clinical research for understanding HBV biology: donor-to-donor diversity, little availability of the best quality donors, expensive to isolate and purchase, and extremely tough to grow its culture for a long time (lack of continuous passages) and grow it *in vitro*.[68] Additionally, the study of potential genetic variants in a functionally suitable model has hampered the result that there is always failure to get best the PHH from patients with liver disease. For both HBV and HDV, they are complete hosts. The PHHs are the only relevant *in vitro* experimental cell culture model for the HBV investigation.[66] Besides, HBV's infectivity for PHH after their plating declines immediately, with the result that depletion of hepatocyte polarization occurs under standard culture conditions.[66] In addition to their shortcomings to date, the primary culture of hepatocytes is a highly suitable *in vitro* model for HBV and HDV infection, displaying exemplary features of the natural host of these viruses, like polarization of hepatocytes and the complete support of host factors of liver. They show a complete functional innate immune system that enables the antiviral response to HBV infection to be

studied.[55,69] They are extensively used to support host HBV/HDV-associated factors and to validate the efficacy of antiviral agents.[28,60,70]

Hui's study was published in *Cell Stem Cell* conducted by ref 71 and was considered a milestone in primary human hepatocyte *in vitro* cultivation. They documented that the most difficult problem has been sorted out for PHH *in vitro* culture by developing a novel culture system for primary human hepatocytes. PHHs are cultivated in an *in vitro* culture when grown in human hepatocyte (HM) media. Wnt3a is believed to be critical in triggering the HH maturation. Wnt3a (a signaling protein also known as a Wnt/β-catenin canonical pathway important for normal embryonic development, cell fate) has been supplemented with HM media and elimination of Rspo1 (a secreted protein promoter of the canonical Wnt signaling pathway by functioning as a ligand for receptors of LGR-6). Noggin (NOG) is important in embryonic cell development, and forskolin is a widely used as a laboratory substance to improve the concentration of cyclic Amp, in which immortalizing human hepatocytes (HHs) can be split to obtain a 10,000-times cultivation. They have further observed that proliferating human hepatocytes (ProliHHs) grown in this system retain a biphenotypic central tendency between mature hepatocytes and liver progenitor cells (LPCs) (Figure 2). This biphenotypic feature facilitates ProliHHs to work more similar to PHHs both *in vitro* and *in vivo* than liver progenitor cells. After the 3D culture, ProliHHs mature and display relatively uniform gene expression and cell activity compared to primary hepatocytes. When grafted into the liver of immune-deficient Fah knockout mice, they also are proliferated (Figure 2). Surely, this will impart a suitable model system for studying disease modeling like hepatotropism, along with drug safety assessment, analysis, and evaluation of cytotoxicity *in vitro*. This will open the door for patients of liver disease via hepatocyte transplantation therapy in the forthcoming days.[72] The dearth and herculean to culture PHH have motivated researchers to investigate other cell sources, for example, liver

cell lines HepG2, HUH-7, etc. and stem cells that are pluriopotent.

Primary human hepatocytes generated from chimeric mice offer a reliable *in vitro* infection model that can fully sustain the HBV replication cycle.[73]

Stem Cells. Progress in the propagation of stem cells in cell culture has resulted in some promising models for studying viral hepatitis. Methods are now available to differentiate stem cells toward the endodermal lineage and then forward to foster development into mature hepatocytes. Stems cells of embryonic origin are more controversial source because of the need to derive these cells from discarded embryos and because of the possibility of long-term biomedical applications (including cloning).[74] Induced pluripotent stem cells (iPSCs) are a newly developed source of hepatocyte that can be used for studies on viral hepatitis. Once developed, these cells can be transformed into fully mature hepatocytes. The potential to receive an abundant amount of primary hepatocytes and the notion that these cells are less fluctuating than PHHs derived from two donors, who can differ by sex, age, contact to medications and/or chemicals, genetic polymorphisms, and the occurrence or lack of a primary liver ailment, are all major benefits of hepatocytes derived from stem cells over PHHs.[75] HBV infection has been effectively supported using a peculiar strategy.[76] Regrettably, hepatocytes produced from stem cells do not develop into fully functional adult hepatocytes, although different methods have been used to counteract functional obstacles and other limitations of this model, such as the applications of tiny molecules that encourage differentiation and exploitation of the microenvironment used in *in vitro* culturing settings.[76,77]

The application of stem cells to generate vascularized and functional human liver *in vitro* has also provided an interesting promise for this field of research.[77] This model might offer oppurtunties to study the hepatitis virus in a three-dimensional system with a functional vasculature.

Other Cell Lines. PH5CH8 and Hep3B cells are two more cell lines that have been used in the studies of viral hepatitis. A mutation in the P53 gene in Hep3B cells makes the protein inactive. Therefore, it is used to investigate how p53 interacts with HBV in the setting of a nonfunctional p53 protein.[78,79] Additional research has been conducted using PH5CH8 cell lines, which contain active Toll-like receptor 3 systems (required for identifying double-stranded RNA involved in various viral infections) that exist in PHHs but are missing in HepG2 and HuH-7 cells.[79,80]

In nutshell, all the above cell culture models have broadened our understanding of virus−host interaction. It is noteworthy that all the models favor the important stages of viral infection from the viral entry to cccDNA formation, replication, and finally virions assembly. Moreover, viral transmission was cumbersome to monitor in these models.[81] Similarly, the cccDNA concentration formed in these cells is remarkably in a reduced amount.[7] This is a disadvantage of all the infectious model systems, i.e., a small quantity of cccDNA formation.[81] Since cccDNA has a predominant use in the HBV replication and the development of chronic viral liver infection. CccDNA, a replicative intermediate, becomes an important therapeutic for the establishment of novel anti-HBV drugs.[7] The existing literature on cccDNA biology is limited and not clearly understood, and much comprehensive knowledge of cccDNA production and stubborness will be essential to assess its potential therapeutic target for HBV treatment. Surprisingly,

the establishment of *in vitro* cell culture models producing significant levels of cccDNA as a result of chronic disease becomes obligatory.

It is believed that progress in the structure of these models, for instance by enhancing their robustness, increasing the cccDNA formation, and facilitating viral transmission, will eventually support the exploration of innovative therapeutic approaches expected to treat chronic viral infection. Additionally, cutting edge technologies may provide suitable culture systems. The hepatocyte-like cells obtained from stem cells are the prominent examples which favor chronic HBV infection,[82] or liver organoid cultures[83] may serve as a few fascinating and powerful tools for the ample understanding of chronic HBV disease. All the limitations of HBV models become mandatory to take into consideration for future perspectives. The above cell culture models have a predominant use in elucidating the mechanisms responsible for the HBV replication cycle as well as HBV pathogenesis (Figure 3).
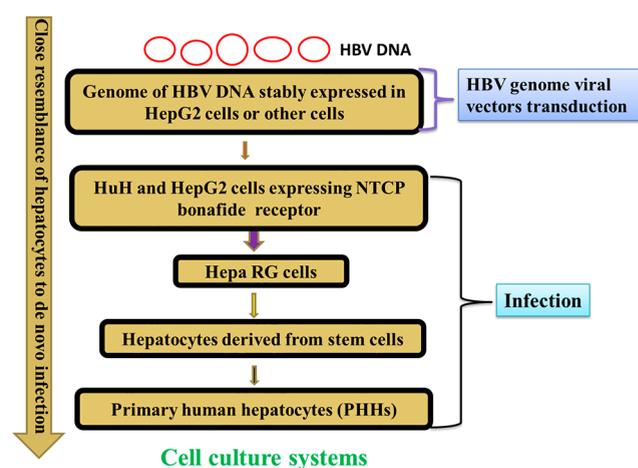
Figure 3. Evolving cell culture models for understanding the HBV replication cycle and pathogenesis. Infectious systems such as primary human hepatocytes (PHHs) and hepatocyte cells derived from stem cells with efficient innate antiviral responses in cells have replaced cell systems that largely depended on cell lines developed from hepatoma and DNA genomes that have been transfected or virally transduced. NTCP, sodium/bile acid cotransporter.[84] NTCP, sodium taurocholate cotransporting polypeptide or sodium/cotransporter of bile acid (also called SLC10A1). *Lot variability is vulnerability headed for fluctuation in investigational findings collected among various laboratories.[84] Adapted and modified with permission from Thomas et al., 2016.

Animal Models. Study of HBV biology has been extremely difficult due to a dearth of animal models that favor hepatitis B virus replication. This is possibly due to the absence of receptor molecules required for viral replication in animal hepatic cells. The discovery of an SLC10A1 or NTCP receptor molecule[60] on human hepatocytes was a major breakthrough in HBV science, and its identification in the cell culture systems enables us to study various mechanisms like hepatotropism, species tropism, binding, and cccDNA formation. Multidisciplinary approaches are used to gain more insights in HBV infection by using different animal models.[85] The HBV research is stucked because of its highly narrow host range, high cost, unavailability, ethical issues, large size, and incompatibility of proper experimental models prone to HBV infection.[86] This restriction has greatly hampered

Table 2. Animal Models Used for Understanding the HBV Pathogenesis

Animal models	Hepadnavirus	Viral entry	Genesis of cccDNA	Status of immune response	Inbred/ outbred	AntiHBV investigation	Vaccine testing	Availability	ref.
Chimpanzee	HBV	Yes	Yes	Immunocompetent	No	Yes	Yes	Very low	146
Tupia	HBV	Yes	Yes	Immunocompetent	No	Yes	No	Low	147
Woodchuck	WHBV	Yes	Yes	Immunocompetent	No	Yes	Yes	Low	148
Duck	DHBV	Yes	Yes	Immunocompetent	No	Yes	Yes	Readily	149
Woolly monkey	WMHBV	Yes	Yes	Immunocompetent	No	Yes	Yes	Low	5
HBV transgenic mouse model	HBV	No	No	Immunotolerant	Yes	Yes	No	Moderate	150
Human liver chimeric mouse model	HBV	Yes	Yes	Immunodeficient	Yes	Yes	Yes	Moderate	151

functional studies and established the importance of suitable animal models in HBV study.[87]

In the present review, different categories of animal models, used for examining hepatitis B virus infection, are reviewed. Both pros and cons associated with animal models in studying hepatitis B virus infection are also addressed in this review (Table 2) (Figure 4).[88]
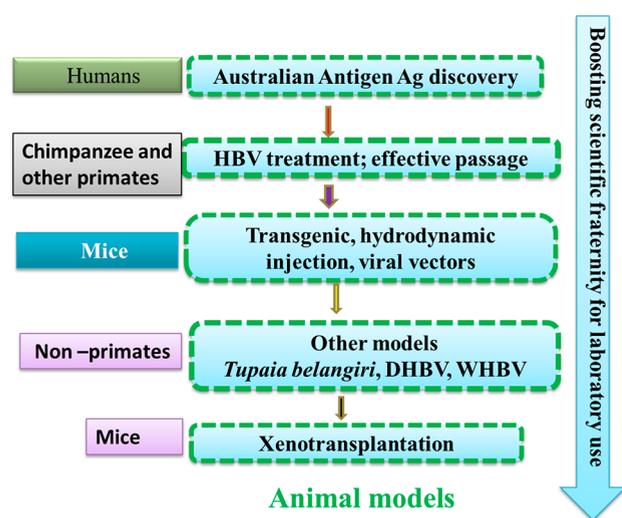
Figure 4. Evolving animal models for understanding the HBV replication cycle and pathogenesis. The use of animal models has shifted from mostly humans and primates to new organisms such as Tupaia, woodchucks (WHBV), and duck (DHBV) hepatitis viruses. Xenotransplantation in mouse models has more recently been developed with animals that are susecptible to HBV infection.[84] Adapted and modified with permission from Thomas et al., 2016.

Woodchuck Hepatitis B Virus. Woodchuck hepatitis virus (WHBV), a hepatitis B-like virus, was discovered that affected Eastern woodchucks (*Marmota monax*) in the Philadelphia Zoo.[89] It paves the path for new ways to investigate *in vivo* host responses to hepadnaviruses. WHBV is similar to HBV not just in terms of virological properties such as genome size, organization, nucleotide sequence (60−70% similarity), and protein expression but also in terms of the immunological responses, both innate and adaptive, that develop in the host during viral infection.[90] Because wood-chucks are extremely susceptible to WHBV, two types of woodchucks with known liver and spleen transcriptomes have been established and used as animal models for HBV-related research. The American woodchuck (*Marmota monax*), for example, has a high risk of chronic hepatitis and HCC after contracting WHBV,[91] while another is the Chinese woodchuck

(*Marmota himalayana*), for which breeding colonies have been well-known.[92] In comparison to HBV infections in humans, woodchucks infected with WHBV have a common natural history of infection, pathogenesis, and liver disease progression from chronic hepatitis to HCC. As a result, the woodchuck model has become frequently used in preclinical drug testing and HBV-related HCC studies. Woodchucks have been used as preclinical models for antiviral medications like nucleos(t)-ide analogs but not limited to lamivudine,[93] entecavir,[94] and tenofovir.[95,95] Moreover, experimental infection of newborn woodchucks frequently results in chronic illness, whereas adult woodchucks usually suffer from acute hepatitis, indicating that the immunological response to viral infection is comparable to that of humans. This discovery increased the use of this animal model for testing prophylactic vaccinations and immunological therapeutic strategies against HBV infection, like programmed death ligand-1 (PD-L1) antagonists and toll-like receptor (TLR)7 agonists.[96,97]

New WHBV-targeting approaches are being studied further. For instance, it has been shown that after injection into WHBV-infected woodchucks a cationic liposome carrying JVRS-100 (complexes of cationic liposomes and noncoding DNA) prevented new tumor growth in the liver.[95] Another group recently administered nanoparticles into HCC-bearing woodchucks as a potential way to deliver antiviral medications to the diseased liver, finding that the nanoparticles aggregated in macrophages and localized in the liver and spleen.[98] However, it must be highlighted that there are still a number of obstacles to using the woodchuck model in HBV studies. They are tough to work with, and there are not many reagents for studying the immunological response to viral infection. Furthermore, the HBV carcinogenesis and WHBV may differ because WHBV DNA quite often integrates into the Myc proto-oncogene, resulting in approximately all neonatally infected woodchucks developing HCC, whereas HBV DNA prefers to integrate into the TERT, MLL4, and CTNNB1 genes.[97] As a result, both viral and host-related variances represent significant obstacles that must be considered when evaluating the efficacy of HBV treatment and vaccine candidates.

The woodchuck is a member of the hepadnaviridae family and is closely linked with HBV in terms of genome organization and morphology. Despite that this model has been intensively studied for evaluating the antiviral potential of different compounds, its limitations need to be taken into account: distinction between the virus and host species; maintenance is cumbersome (hibernation); insufficient molec-ular reagents and assays; elementary understanding of woodchuck genetics; and molecular biology and immunology. Recently, the genome of woodchuck has been sequenced.[92,99]

Duck Hepatitis B Virus. DHBV was first discovered in the serum of domestic ducks in 1980, and it has since become an important model for studying the hepadnavirus replication cycle.[100] This model was crucial in studying the mechanisms of viral replication, including viral capsid assembly, initiation of reverse transcription, and finally rcDNA synthesis, due to the availability of duck hepatocyte cultures and ducklings.[96,101,102] The steps for cccDNA production were also examined using this model, increasing our actual understanding of the generation of cccDNA pools and their method of refilling within the nucleus.[102,103] Duck models with chronic DHBV infection have been utilized to test antiretroviral medicines, nucleocapsid assembly inhibitors, and combination therapy regimens.[104] DHBV and duck models, however, differ significantly from HBV and humans in the following areas: only 40% homology exists between DHBV and human HBV,[105] the carboxypeptidase D entry receptor is used by DHBV,[106] and drug toxicity may have considerable consequences on ducks.[106,107] As a result of the viral and host discrepancies, drug screening and mechanistic research from this system may require additional validation.

Woolly Monkey Hepatitis B Virus. Another HBV-like virus that infects its native host is the woolly monkey hepatitis B virus (WMHBV) (*Lagothrix lagotricha*).[108] This is an exciting discovery since woolly monkeys are a host intermediate between humans and rodents that can be used to test antiviral treatments for hepadnavirus infection. Woolly monkeys are endangered, despite the fact that they give an additional arm for investigating HBV-like viruses in animal models;[101] as a result, there has been little research on the animals themselves. WMHBV, on the other hand, has been successfully used in a number of spider monkey studies (*Ateles geoffroyi*) and squirrel monkeys (*Saimiri sciureus*). In order to study WMHBV in spider monkey models, a WMHBV infectious clone was established,[101] leading to viremia of moderate severity (10E4−10E6 GE/mL), and immune-mediated clearance occurs 8 weeks after inoculation. However, spider monkeys are also endangered.[109] As a result, they are not widely available for HBV-related studies. Immunocompromised liver damage mice engrafted with primary hepatocytes from squirrel monkeys were specifically vulnerable to WMHBV rather than HBV.[64] Nevertheless, this nonhuman primate model could be useful; squirrel monkeys infected with WMHBV developed acute and, in some cases, persistent infections after receiving WMHBV by adeno-associated virus (AAV) delivery.[64]

Hepadnavirus Infections in Tupaias. Tupaias, sometimes known as tree shrews, are small nonprimate animals with a genetic relationship to primates rather than rodents.[110] Tupaias have been shown to be experimentally sensitive to HBV and HCV, making them a well-developed nonprimate animal model in recent decades.[111] Tree shrews, like other species, have a higher tendency for chronicity when infected as neonates, and their liver histological lesions are analogous to those seen in HBV-infected humans.[112] Tupaia primary hepatocytes are frequently used, to study HBV and WMHBV infections due to their widespread availability.[60,113] Tupaias' applicability as an animal model is currently limited by a number of factors, along with their genetic heterogeneity as an outbred species (which may be useful for representing real-world situations but is not ideal for large-scale animal experiments), overall low viral titers *in vivo*, and a limited supply of research tools and materials for this species. Tupaia

hepatocytes are still useful, as evidenced by their implantation into chimeric mice which resulted in effective HBV infection.[114]

■ HBV-SUSCEPTIBLE PRIMATE MODELS

Chimpanzees. The best model for HBV infection is the chimpanzee. The chimpanzee is the only immunocompetent nonhuman primate model known to be entirely susceptible to human HBV (Table 2), and it can effectively imitate the pathophysiology and disease development caused by HBV in individuals.[115] Even one genome equivalent (GE) of HBV DNA was considered adequate to productively infect chimpanzees.[116] Chimpanzees can get acute and chronic HBV infection after being inoculated with HBV obtained from chronic hepatitis B (CHB) patients, with immune response profiles and inflammation analogous to those seen in HBV-infected individuals.[117] The chimpanzee animal model was used extensively in the HBV research to evaluate the safety and efficiency of HBV vaccine candidates. The first-generation (plasma-derived) HBV vaccine and the later-developed vaccine incorporating HBsAg from yeast were both tested in chimpanzees.[118] In recent years, the chimpanzee model has also been used to assess the efficacy of modified recombinant vaccinations, vaccines against antiviral drug-resistant HBV mutations, and some therapeutic vaccination investigations.[119] Although chimpanzees were formerly the most important animal model for HBV research, their use as an experimental model has been limited due to a lack of availability, high related expenses, and considerable ethical issues. They are susceptible to both acute and chronic HBV infection and immune response that correspond to those in humans.[120] Their use is highly limited, expensive, large, and not easily accessible for HBV research as mentioned above, making them irrelevant models. Research on chimpanzees is not permissible in the United States and many other countries. Therefore, there are few efforts to adopt primate models for anti-HBV research.

HBV Transgenic Mice. Two different research groups produced the first HBV transgenic mice by microinjecting the entire or overextended genome of HBV into zygotes.[117,121] Furthermore, viral replication and protein expression were found exclusively in a few organs of a couple of transgenic mice, and only some developed particles of the virion were isolated from the serum. Remarkably, a rat hepatoma cell line was transfected with a tandem dimer HBV genome, and infectious HBV virions could be developed.[122] Guidotti and colleagues in 1995 developed transgenic mice with competent HBV replications using a genome vector construct that was terminally repetitive (1.3 X the HBV genome length).[123] With no signs of cytopathology, the transgenic mice lines of HBV (lineages 1.3.32 and 1.3.46) developed all of the HBV secretory proteins and mRNA transcripts at levels analogous to those found in a normally human infected liver. HBV replication was accomplished in the mice, as well as production of rcDNA, RNA synthesis, virions assembly, and finally discharge of the mature virus. This particular model has been used as the cornerstone for HBV research in small animals. The model was used to evaluate a number of anti-HBV drugs and provided substantial understanding of the HBV host−virus interaction.[105,124] Furthermore, in the HBV transgenic animals, the integrated HBV genome maintains viral replication, gene expression, and untimely onset of viral infection phases such as viral entrance as well as viral

dissemination and reinfection is absent. Interestingly, cccDNA, the template of virus transcription throughout spontaneous HBV infection, is not found in these mice for unknown reasons.[125] As HBV transgenic mice are inherently immuno-tolerant to HBV, immunological pathology studies in these models are not possible.[126]

HBV Plasmid DNA Hydrodynamic Injection Models. Yang et al. established the hydrodynamic injection of the HBV mouse model by hydrodynamically applying a high dose of salt water (equal to 8−10% of the body weight of the mouse (v/w)) with a plasmid harboring a 1.3 X genome of HBV DNA into the tail vein of the mouse for a small duration (5−8 s).[127] HBV DNA is mechanically squeezed into hepatocytes by transient hydrodynamic force, resulting in transient replication of HBV and translation. By day 7, immunologically competent B10.D2 mice's blood contains antiviral antibodies and antiviral CD8+ T cells, whereas liver transcripts of HBV and replicative intermediates are eliminated by day 15 after transfection, corresponding with the advent of antiviral antibodies and CD8+ T cells. In NOD/SCID mice devoid of active T, B, or natural killer (NK) cells, HBV gene expression and replication can continue for greater than 81 days, suggesting that an immune response of the host, especially a CTL response that is virus specific, may contribute to the elimination of HBV in the animal. The result produced by hydrodynamic injection is largely determined by the plasmid backbone and the genetic background of mice.[90,128,129] The hydrodynamic injection model is simple to implement and can be used to investigate various HBV genotypes, variations, or mutations. In this model, the viral genome does not become part of the host genome which provides chances to study the HBV stubbornness, immunological responses, and disease pathogenesis. The hydrodynamic process causes considerable liver damage, making it difficult to interpret research findings, particularly in the areas of immunological responses and pathogenesis.[130] Furthermore, the model's utility is limited by its poor rate of transfection (5−10%) and small period.[131]

HBV cccDNA Surrogate Mice Models. To imitate the functions of cccDNA, different approaches have been used in hepatocytes to incorporate molecules similar to HBV cccDNA. Site-specific recombination from a precursor plasmid facilitated by Cre/loxP harboring a HBV genome that is monomeric can be used to make recombinant cccDNA (rcccDNA) in vitro or in immunocompetent mice.[132] In immunocompetent mice, rcccDNA administered hydrodynamically could cause a slight (approximately 5−7 weeks) HBV antigenemia, analogous to severe resolving HBV disease. A recombinant adenovirus with a replication fault was recently employed to transport rcccDNA into the livers of Cre transgenic mice, and the rcccDNA resulted in significant stubbornness of HBV for over 62 weeks.[133,134] In the investigation, the mice liver showed a prolonged necroinflammatory response, fibrosis, and dysplastic changes, which are identical with the pathogenesis of symptomatic chronic HBV. One more investigation found that injecting immunocompetent C3H mice with a recombinant minicircle cccDNA hydrodynamically resulted in sustained and significant HBV concentrations in the serum (>1 year). The surrogate models of cccDNA can be applied to analyze the response of the immune system and pathophysiology of CHB disease as well as to evaluate antiviral treatments that target cccDNA.[135]

Human Liver Chimeric Mice. To studying the natural HBV disease, mice with PHH are excellent models. The production of humanized mice through xenotransplantation of PHHs is one strategy that has proven effective in conquering this species-specific obstacle. PHHs can be delivered into a mouse using this procedure, and the mouse liver can then be repopulated. To make this strategy effective, the mouse must be immunodeficient so that the transplanted human hepatocytes do not reject them, and the hepatocytes of normal mice must be substituted. Hepatocytes from mice can be used to substitute human hepatocytes, performed particularly by genetically causing a disease of the liver in the mouse, enabling the engrafted human hepatocytes a growth advantage. The SCID (severe combined immunodeficiency) mice harboring a urokinase plasminogen activator transgene triggered by a promoter of liver-specific albumin were the first to successfully use this method (Alb-uPA).[136] Following transgenic expression, acute hepatotoxicity develops, which can be alleviated by engraftment of PHHs delivered, which subsequently serve as an HCV disease substrate. Regrettably, due to the fragility of these mice, their utility has been restricted.

Despite the fact that this method has been effective in the investigations of the metabolism of drugs and hepatitis caused by viruses, their utility for many applications is limited due to high mortality, a restricted transplantation window, low reproductive efficacy, and kidney problems in repopulated mice. To conquer the limitations of the original Alb-uPA model, researchers developed a better and extremely strong model of animals (MUP-uPA) by using a promoter that is triggered later in mouse maturation like the major urinary protein (MUP) promoter.[137]

The knockout mice fumaryl acetoacetate hydrolase (Fah), NOD-FRG, and NRG-FAH are the second most extensively utilized liver humanized mouse models.[133,138] FAH is the final enzyme in the catabolism of tyrosine, and its loss causes a deposition of harmful metabolic intermediates of tyrosine in the liver, which kills mouse hepatocytes and provides transplanted human cells a selective growth advantage.[139] The chemical 2-(2-nitro-4-trifluoromethylbenzyl)-1,3-cyclo-hexanedione (NTBC) can inhibit the buildup of hepatotoxic metabolites by blocking the upstream enzyme of FAH. NTBC administration and cessation cycles could be regulated to ensure a significant regeneration of human hepatocytes transplanted in the liver of the recipient.[139]

Grompe and colleagues first effectively transplanted human hepatocytes into triple mutant mice (FRG) to circumvent the majority of the problems of the uPA mouse model. These mice were produced by breeding fumarylacetoacetate hydroxalse (FAH)$^{-/-}$ mice with mice containing a recombinant activation gene 2 (RAG2$^{-/-}$) and interleukin 2 receptor gamma (IL2Rγ)$^{-/-}$ immunodeficient environment.[140]

In some aspects, the humanized mouse model developed by FRG outperforms the uPA model. For instance, mice are usually stronger and quicker to treat; transplantation can be performed at several stages, and continuous transplantation is a viable option.[141] The delivery and removal of NTBC can be used to manage the severity of the liver injury and selective pressure.[133,142] On the other hand, FRG mice have some drawbacks: to promote human hepatocyte transplantation, further preconditioning of Fah mice with an adenovirus-expressing uPA may be needed.

Fah mice generate liver carcinomas frequently as a result of their type I tyrosinemia and need recurrent rounds of NTBC treatment to stop liver cancer from growing from the hepatocytes of the remnant mouse.

Another human liver injury model that can be developed for human hepatocyte transplantation is the TK-NOG mouse model, which was established by an albumin promoter stimulating liver-specific expression of a herpes simplex virus type 1 thymidine kinase (HSVtk) in NOG mice with severe immunodeficiency.[143] The fact is that the sterility of male TK-NOG mice is a limitation to the model.[144]

Humanized Mouse NTCP or Mouse Expressing the NTCP of Humans. Because NTCP was discovered to be the cellular receptor for HBV, researchers hope that exogenous production of NTCP of humans in mice would make them prone to HBV disease. Meanwhile, attempts to infect these models with HBV have so far failed.

Transgenic and Knock-in Mice Expressing Human NTCP. The first hNTCP-Tg (human NTCP transgenic C57BL/6 mice) animal model was developed by He et al.[2] Although the model does not favor HBV disease, it does bolster acute infection of HDV, with approximately 3% of infected hepatocytes.

New HBV Animal Models. Undoubtedly, new animal models are urgently needed for the establishment of therapeutic rationale in chronic hepatitis B virus disease.[86] The HBV receptor's high-sequence homology, the Na+/taurocholate cotransporting peptide (NTCP), between humans and squirrel monkeys is used by researchers to assess HBV infection in squirrel monkeys. The ability of the HBV PreS1 peptide to interact with human and squirrel monkey NTCP was investigated. HBV or woolly monkey HBV (WMHBV) was tested in immune-deficient Fah/NOD and Rag1/Il2Rgnull (FNRG) mice engrafted with human or squirrel monkey hepatocytes. Adult squirrel monkeys were also infected with HBV, WMHBV, AAV-HBV, AAV-WMHBV, and an adeno-associated virus harboring an infectious HBV genome (AAV-HBV). Furthermore, neonatal squirrel monkeys were examined for the possibility of persistent WMHBV infection. The PreS1 peptide was linked to NTCP in humans and squirrel monkeys but not in mice or capuchins. WMHBV infection was found in FNRG mice engrafted with squirrel monkey hepatocytes but not in human HBV-infected mice. Adult squirrel monkeys were prone to WMHBV but not to human HBV; however, chimeric mice engrafted with human hepatocytes were vulnerable to HBV but not to WMHBV. Squirrel monkeys infected with AAV-WMHBV developed viremia of 10^8 genomes/mL, with a quantifiable virus for up to 8 months. Surprisingly, the livers of these animals were reported to have covalently closed circular DNA. WMHBV viremia lasted 6−8 months in both adult and newborn squirrel monkeys. This is more than twice as long as other nonhuman primates' viremia lasts, implying that squirrel monkeys could be a good model for testing chronic HBV treatments.[5]

Another new model is macaque primary hepatocytes.[145] With the help of these new models, curative treatment is possible. The attempts are hampered, with the dearth of a physiologically appropriate, animal model of chronic HBV disease. It has been demonstrated that entry receptor expression of the HBV, the human sodium-taurocholate cotransporting polypeptide (hNTCP), on macaque primary hepatocytes promotes *in vitro* chronic HBV infection, where all replicative intermediates as well as cccDNA are present. Additionally, in vivo expression of hNTCP on liver cells by viral vectors makes rhesus macaques susceptible to chronic HBV infection. Longitudinal HBV DNA in serum and measurement of HBV DNA, RNA, and HBcAg in hepatocytes

define these in vivo macaque chronic HBV infections. Collectively, these findings demonstrate that expression of hNTCP on hepatocytes of macaque makes them vulnerable to chronic HBV disease, thus developing a physiologically suitable model of chronic HBV infection to examine immune-mediated clearance, test therapeutic, and preventive strategies.[4]

In addition, the above models are not inbred and not readily available. These animals are big and cumbersome to handle and thus not very accessible, and distinct animal models like mice are compulsory. It is believed that all animal models have their own limitations.[152,153] Hence, it is herculean to investigate the HBV replication cycle in animal models. Therefore, it is critically important to develop an *in vitro* culture system to demonstrate the mechanisms attained by the HBV for its replication.

NTCP and Its Expression Are Important for HBV Infection in Most Cell Culture and Animal Models. NTCP is a functional receptor molecule for the HBV virus and is located on the sinusoidal/basolateral side of the parenchymal cells of the hepatocytes.[154] It is a membrane-localized protein synthesized in the liver, and its principal role is characterized by its peculiar expression.[155] In liver cells, NTCP serves as a transporter. It is highly expressed on the sinusoidal/basolateral membrane and is important for the sodium-dependent import of bile acids into liver cells via the sinusoidal/basolateral cell membrane.[155] The spectrum of bile acid molecules carried by NTCP is extensive, and it contains cholic acid, chenodeoxycholic acid, deoxycholic acid, sarcosine cholic acid, ursodeoxycholic acid, glycocholic acid, glycochenodeoxycholic acid, glycodeoxycholic acid, etc.[156] All essential physiologically pertinent glycine- and taurine-conjugated bile acids are believed to be transported via NTCP.

Post-translational modifications (PTMs) regulate NTCP's membrane expression and localization. Because of the significant level of expression of NTCP in hepatocytes' sinusoidal membranes and its strong affinity for conjugated bile acids, NTCP effectively removes them from the portal blood and in this manner regulates enterohepatic circulation (EHC) of bile acids.[157] NTCP-mediated bile acid transport across the hepatic cell membrane can be impeded or severely affected by a number of inhibitors already in clinical use. Cyclosporine A, ketoconazole, furosemide, propranolol, glibenclamide, ritonavir, bosentan, efavirenz, rifamycin, gemfibrozil, and saquinavir are the important inhibitors reported so far.[158]

The identification of hNTCP as a limiting host factor for HBV and HDV infection in human hepatoma cells sparked hopes of establishing hNTCP transgenic animals that are totally prone to HBV. Such mice feebly favor HDV infection;[2,159] however, they are still at risk of contracting HBV. The unique species specificity of HBV and HDV is linked to hNTCP-dependent and hNTCP-independent stages in both viruses' early infection events, according to a growing body of evidence. HBV-transgenic mice are completely capable of generating infectious virions.[123] Myrcludex B (MyrB), the HBV/HDV entry inhibitor,[160] is a synthetic L-protein-derived lipopeptide that binds with hNTCP and thus hampers the development of a functional receptor complex.[161] We have demonstrated earlier that HBV/HDV can interact with hepatic hNTCP from some nonsusceptible species (such as the mouse, rat, and dog). Interestingly, MyrB and therefore HBV/HDV are incapable to bind with some types of NTCP, for example. that from cynomolgus macaques, rhesus macaques, and pigs.[162]

These unique binding features could be mapped to different sequence elements within the NTCP homologues in humans, mice, and macaques; NTCP from mice but not from cynomolgus macaques interacts with MyrB due to an important sequence element localized at positions 157−165.[37,60] Nevertheless, mouse NTCP cannot arbitrate effective virus entry because of sequence fluctuation within amino acids 84−87 (Ni et al. 2014; Yan et al. 2013). Only the human homologue, hNTCP, is capable of both binding and infection. When hNTCP is expressed in murine cell lines, the cells become HDV prone to a small extent but are largely HBV resistant.[163] These limitations can be conquered through amalgamation of mouse cells expressing hNTCP with nonsusceptible human HepG2 hepatoma cells, representing a missing host factor in murine cells (Lempp et al. 2016). To study hNTCP-mediated species constraints in laboratory animals that could be used as candidates for an immuno-competent HBV/HDV model, the researchers tested the potential of hNTCP to sustain HBV replication in primary hepatocytes from mice, rats, dogs, cynomolgus macaques, rhesus macaques, and pigs. They demonstrated that hepatocytes from macaques and pigs are fully prone to both HDV and HBV, implying that endogenous NTCP homologues are the sole element preventing HBV replication. Expression of NTCP is highly dependent on the differentiation and thus rapid disappears in explanted hepatocytes and absent in hepatoma cells.

Viruses use several host components to mediate the stepwise entry process and obtain efficient entry into host cells. Hepatitis B virus (HBV) internalization requires more than the sodium taurocholate cotransporting polypeptide (NTCP) expression. The epidermal growth factor receptor (EGFR) is recognized as a host factor that binds with NTCP to facilitate HBV internalization. The viral internalization and infection were reduced when the NTCP−EGFR relationship was disrupted or when EGFR was functionally depleted. The findings indicate that HBV uses an endogenous NTCP−EGFR combination as a driving mechanism to penetrate human hepatocytes. Consequently, EGFR serves as a missing link between the cell-surface HBV−NTCP attachment and viral invasion. As a result, EGFR is a necessary cofactor for HBV infection in the human hepatocytes.[164]

■ FUTURE DIRECTIONS

There is no ideal blueprint for the study of HBV or drug formulations. More work is required to establish suitable models. Cell culture methods that directly match natural humans hepatocytes but are extremely efficient, less costly, have a limitless supply or are more easily available, enable more efficient infection and spread amplification would be extremely useful. The optimization of iHep cells and parameters for primary human hepatocyte culture should be upgraded which is of great significance. Our knowledge of the antiviral immunological response and the progression of different liver diseases has increased through animal models of HBV infection. Moreover, mice that are completely susceptible to HBV disease, as well as the formation and cccDNA amplification, are desperately required, potentially beginning with mice to facilitate the NTCP transgene expressed in the liver. Such a model could emerge when more essential elements for virus entrance, replication, and spread are discovered. This information may be used in macaque or Tupaia animal models to produce alternate nonprimate or

primate drug development models. These methods can be used for drug development as well as studying the persistent HBV infection. Reporter systems that can rapidly detect modifications in particular aspects of the replication cycle of HBV could help in the design of novel antiviral agents. Building systems for finding therapeutic molecules that target cccDNA can aid in the discovery of a novel treatment for CHB disease.[165]

■ CONCLUSIONS

The surrogate, nonhuman primate, and mouse models described here have provided a wealth of information about the HBV replication cycle, pathogenesis, and host immune responses of virus. Considering the decline in the use of chimpanzees and the challenges of dealing with woodchuck, duck, tree shrew, and woolly monkey models, a mouse model for HBV is required for future prospective in HBV studies. In acute infections, we still do not know how the immune system attacks the virus, and in chronic infections, we do not know how HBV may circumvent the immune system. Our understanding of which immunological components are involved in acute and chronic HBV infections has considerably benefited from the use of mouse noninfection models and humanized xenotransplantation models. Humanized mouse models can be accurately generated for future HBV investigations in pharmacological treatments and for producing a functional cure with persistent attempts to replicate an immunological response analogous to that of humans. There are various drawbacks in animal models that hamper their applicability. It is important to emphasize that all HBV animal models have contributed a better knowledge in the existing literature of the disease and have suggested potential pharmaceutical targets for cure.

The *in vitro* cell culture model of HBV is useful for testing antiviral drugs against HBV, examining HBV's biological features, and studying virus−host relationships. We reviewed and analyzed the benefits and drawbacks of each cell culture model (displayed in Table 1). The accessibility to a robust and efficient *in vitro* cell culture model for HBV investigations is a major component influencing the understanding of the HBV action mechanism due to the host specificity and HBV organ specificity.

The established cell culture systems of HBV have significantly assisted in studies on the biological features, process of infection, and HBV etiology, including the development of antiviral drugs against HBV, vaccines, etc. Typically the *in vitro* cell culture system of HBV failed to infect with an HBV-positive serum because inhibitory factors are present in the blood serum of humans. Because the genome of HBV has been integrated into the HepG2.2.15 and HepAD38 cell lines, they can release viral particles indefinitely. HepAD38 cells release 11-fold greater viruses than HepG2.2.15 cells, are frequently utilized as a reservoir of virus for HBV disease in *in vitro* cell culture models, and are broadly used in similar investigations. Various laboratories have employed HepG2.2.15 cells to evaluate antiviral medicines against HBV. The discovery of the HBV NTCP receptor has accelerated work into the action mechanism of HBV disease. Certain HBV-infected liver carcinoma cell lines turned vulnerable to HBV after NTCP overexpression, while cell lines that could be infected with HBV, like the HepaRG cell line, developed greater vulnerability to HBV. On the other hand, cell culture model organisms that demonstrated

overexpression of NTCP still do not produce a significant level of cell-to-cell transmission and are unable to mimic the spontaneous processes of HBV disease. This finding also implies that NTCP is not the sole element regulating the host's HBV infection, and carcinoma cell lines may not display the factors involved in HBV disease and replication. Primary human hepatocytes (PHHs) are the most suitable *in vitro* cell culture experimental model for understanding the underlying HBV infection mechanism. Furthermore, because of the shortage of the source and the difficulties to culture them *in vitro* for extended time durations, their application is hampered. A 3D culture technique is being widely employed to improve the application of primary human hepatocytes (PHHs). It has its own merits and limitations. It is expected that in the forthcoming days the optimization of the *in vitro* cell culture system will take it to new heights.

■ AUTHOR INFORMATION

Corresponding Author

Syed Naqui Kazim − *Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi, India;* orcid.org/0000-0002-4248-0511; Phone: +91 9953621758; Email: skazim@jmi.ac.in

Authors

Sajad Ahmad Bhat − *Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi, India*

Ishfaq Ahmad Ahanger − *Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi, India; Clinical Biochemistry University of Kashmir, Srinagar, India*

Complete contact information is available at:
https://pubs.acs.org/10.1021/acsomega.2c07154

Author Contributions

S.N.K. and S.A.B. designed the study; data were collected by S.A.B.; and the literature was searched by S.A.B. I.A.A. helped in the preparation of some figures, and S.A.B. penned the manuscript. All authors approved the article for publication.

Notes

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