New insights into hepatitis B virus biology and implications for novel antiviral strategies

Jieliang Chen1,2, Min Wu2, Kuancheng Liu1,3, Wen Zhang1,2, Yaming Li1, Xiaohui Zhou2, Lu Bai1 and Zhenghong Yuan1,3,*

ABSTRACT

Hepatitis B virus (HBV), a small DNA virus with a unique replication mode, can cause chronic hepatitis (CHB), which is characterized by the persistence of the viral covalently closed circular DNA that serves as the template for HBV replication and the production of large amounts of secreted HBV surface antigen (HBsAg) that is present in excess of the levels of infectious virus. Despite the success of currently approved antiviral treatments for CHB patients, including interferon and nucleotide analogs, which suppress HBV replication and reduce the risk of CHB-related liver diseases, these therapies fail to eradicate the virus in most of the patients. With the development of the cell and animal models for HBV study, a better understanding of the HBV life cycle has been achieved and a series of novel antiviral strategies that target different stages of HBV replication have been designed to overcome the viral factors that contribute to HBV persistence. Such basic HBV research advancements and therapeutic developments are the subject of this review.

Keywords: HBV, cccDNA, HBsAg, persistence, antiviral targets

INTRODUCTION

Although hepatitis B virus (HBV) vaccines have been used widely for prevention, HBV infection remains a major health problem worldwide, with an estimated 350 million chronically infected individuals, many of whom will eventually develop severe liver diseases, including liver cirrhosis, and hepatocellular carcinoma (HCC). Currently, two approaches have been approved for antiviral treatment of chronically infected HBV (CHB) patients, including nucleot(s)ide analogs (NAs) and interferon alpha (IFN-α). NAs effectively suppress HBV replication to undetectable levels by inhibiting the viral polymerase (Pol), but rarely induce seroconversion of the HBV surface antigen (HBsAg), and viremia rebound always occurs after termination of drug treatments. Therefore, lifelong therapy is required for the majority of chronically infected persons. Treatment with IFN-α or pegylated (PEG)-IFN-α results in prolonged clinical remission with an increased rate of HBsAg seroconversion and improved liver histology; however, this treatment is only effective for a minority of CHB patients and is limited by intolerance and adverse reactions [1]. More importantly, currently available antiviral therapies do not target defective responses of the immune system or the persistence of covalently closed circular DNA (cccDNA) in the infected hepatocytes. Thus, developing different approaches to achieve sustained cure or elimination of HBV is urgently needed.

In recent years, the development of new cell- and animal-model systems combined with novel technologies has allowed the HBV replication cycle and its interaction with the host antiviral immune system to be better characterized. Advancements in the understanding of the details of viral replication and the mechanisms involved in an inadequate induction of anti-HBV immune responses have guided the development of strategies aimed at achieving a functional cure for HBV infection. Several comprehensive reviews have summarized the progress regarding the molecular biology of HBV infection [2], how HBV
deals with host immunity, and how the immune response can be harnessed to potentially achieve infection control [3,4,5]. Hence, in this review, we will focus primarily on the new findings in key steps of HBV biology that are essential for HBV replication and hotspots for novel drug development. We also review the progress in regarding the development of cell and animal models that drive the HBV research and advance the search for novel strategies to treat chronic HBV infection.

**KEY STEPS AND FACTORS IN THE HBV REPLICATION CYCLE**

HBV is an enveloped DNA virus comprising a partially double-stranded relaxed circular DNA (rcDNA) genome with tropism to infect hepatocytes. The replicative cycle of HBV progresses through several well-defined steps including the binding and entry of the virus, cytosolic transport and uncoating of the nucleocapsid, formation of cccDNA in the nucleus, the transcription and translation of virus-specific genes, assembly of capsids and initiation of reverse transcription, followed by budding and secretion of virions and subviral particles (SVPs). This cycle is a sophisticated process that is tightly regulated by both host and viral factors.

**Viral receptor, entry and cytosolic transport**

The preS1 domain of the large envelope protein of HBV has been well recognized as the key factor that mediates viral attachment to cell surface heparin sulfate proteoglycans (HSPGs) [6] and binding to the specific receptor(s) on hepatocytes. The critical role of the preS1 domain in the initial steps of viral interaction with hepatocytes and cell entry has been confirmed using synthetic peptides derived from the preS domain that inhibited virus infection [7–9]. Moreover, using an N-terminal myristoylated preS1 peptide as a bait in conjunction with a novel method of near-zero distance photo-cross-linking, Li et al. identified Na-taurocholate cotransporting polypeptide (NTCP) from primary tupaia hepatocytes as the receptor supportive of HBV and its satellite hepatitis D virus (HDV) infection [10]. An independent work by Urban et al. also determined NTCP as a receptor used by HBV to obtain access to hepatocytes [11]. However, notably, the experiments that led to the identification of NTCP as a receptor used by HBV were performed in the presence of polyethylene glycol and used extremely high virus titers prepared from HBV-producing cells. These conditions differ from natural infection where an extremely small HBV inoculum is sufficient to induce infection [12,13]. Moreover, S267F (rs2296651) variant of the NTCP was shown to be supportive of HBV infection in vitro cells, and recent clinical data pointed out that although the distribution of the genotype and allele frequency of rs2296651 polymorphism was significantly different among the HBV patients, HBV infection resolvers, and healthy controls, the genotype and allele frequency between infection resolvers and healthy controls and between HBV patients with different clinical diseases had no significant difference [14,15]. Thus, it remains to be determined whether other potential coreceptor(s) participate in mediating a more efficient HBV infection.

The binding of HBV to the receptor and attachment to the cell surface permit the virions to fuse with either the plasma or an endosomal membrane. Recent studies have suggested that cholesterol has an important role in hepadnaviral infection and that cholesterol depletion impaired HBV endosomal escape during the virus entry step [16]. In HBV infection cell models, caveolin-1- and clathrin-mediated endocytosis pathways were shown to be required for HBV infection [17,18]. Following the fusion of viral envelope proteins with the membrane, nucleocapsids are actively transported via microtubules to nuclear pore complexes (NPCs). The understanding of the HBV viral capsid transport and HBV genome release into the cell nucleus remains limited. Current results are primarily based on methods of HBV capsid transfection, microscopic injection and the translocation motif (TLM) fusion of capsids models. HBV capsid transfection is used to replace the viral surface proteins with a lipid shell; this method was used to detect hepadnaviral capsids that were to be readily transported toward the nucleus. Additionally, capsids were microscopic-injected into the cytosol of Xenopus laevis oocytes, which confirmed that HBV uses microtubules to transport capsids to the nuclear membrane [19]. TLM-fused HBV capsids are able to package polymerase and the pregenome, allowing for a clear observation of uptake of HBV capsid into nucleus [20]. Upon reaching the NPC, HBV capsids then interact directly with nucleoporin 153 (Nup153), an essential protein involved in nuclear transport via importin α. Binding importin α with HBV capsids is regulated by the genome maturation and/or phosphorylation status of the capsid protein. Only the mature form of the capsid protein, which exposes its nuclear localization signal in its C-terminal, could bind to importin α. Then, while the HBV capsid disintegrates, the genome is released into the nucleus, and the capsid protein enters the nucleus as protein dimers or irregular polymers [21,22].
cccDNA formation and regulation

Once within the nucleus, the HBV rcDNA is converted into a cccDNA molecule. cccDNA formation is a multistep process that is composed of the removal of all the redundant elements including the covalently linked polymerase protein, the RNA primer and the short terminal redundancy of the minus-strand DNA. The completion of the plus strand and the ligation of the ends of both strands finalize this process. Currently, very little is known regarding the exact mechanism and factors that may be involved in cccDNA formation. The fact that the conversion of HBV rcDNA to cccDNA is less efficient in hepatoma cell lines than in infected livers [23] and the observation that the duck hepatitis B virus (DHBV) rcDNA converted more efficiently into cccDNA than HBV rcDNA [24] suggest that both distinct cellular and viral factors are involved in this process. Recently, Nassal et al. have identified tyrosyl-DNA-phosphodiesterase (TDP) 2 as one of the host DNA-repair factors involved in HBV cccDNA formation. TDP2 was shown to specifically cleave the Tyr-DNA bond and release polymerase protein from rcDNA in vitro. Interfering RNA (RNAi)-mediated TDP2 depletion in human cells could significantly slow the conversion of rcDNA to cccDNA, while ectopic TDP2 expression in the same cells restored faster conversion kinetics [25]. Further studies are required to examine the in vivo role of TDP2 in cccDNA formation.

Similar to other DNA virus, such as polyoma viruses and simian virus 40 (SV40) [26], HBV cccDNA molecules are organized into a host chromatin-like structure called a ‘minichromosome’ [26]. However, HBV cccDNA is not replicated by the host’s semiconservative DNA synthesis machinery. In contrast, the amplification and maintenance of the cccDNA pool is achieved by intracellular nuclear reimporting of the newly synthesized nucleocapsids which contain mature rcDNA generated by reverse transcription [27]. Thus, only one round of infection and a single copy of viral cccDNA are theoretically sufficient for HBV to establish a cccDNA pool. The unique characteristics of hepatocytes and virus make the HBV cccDNA pool stable in infected cells [28]. The long half-life of quiescent hepatocytes ensures that the nuclear cccDNA pool is not diluted by cell proliferation and death while the viral minichromosome structure allows cccDNA to persist in the nucleus without being detected by the host innate immune responses.

The size of the cccDNA pool is not constant among infected cells and varies during the course of infection. Evidence from woodchuck HBV (WHV) and DHBV models indicated that the number of cccDNA molecules per cell ranges from several to dozens and may fluctuate at different periods post-infection [29,30]. Results obtained from CHB patients showed that intrahepatic cccDNA levels decrease as the disease progresses from a HBeAg-positive to an anti-HBeAg-positive phase [31]. So far, the viral and host factors that regulated cccDNA pool remain poorly defined. Data from DHBV models have shown that the viral large surface protein (L-HBs) is an important viral factor that regulates cccDNA amplification through a negative-feedback mechanism [32,33]. However, whether this mechanism works in HBV remains further validation. Zhen et al. have shown that HBc protein preferentially binds to cccDNA at CpG island II in CHB patients. The abundances of HBc interactions with cccDNA were correlated with increased binding of the transcription factor CBP and with reduced methylation of DNA and binding of histone deacetylase HDAC1 at CpG island II, thus promoting an epigenetic permissive state [34]. HBx was also reported to modify the epigenetic regulation of cccDNA function [35]. HBx could decrease the methylation status of cccDNA-bound histones by competitively binding Tudor domain protein Spindlin1 [36] or the methyltransferase PRMT1 [37].

Similar to host chromosomes, HBV cccDNA is complexed with both histone and non-histone proteins, including transcription factors, coactivators, corepressors, chromatin-modifying enzymes and viral proteins (e.g. HBc and HBx) [26]. Therefore, the cccDNA transcriptional activity is highly regulated by epigenetic factors, such as cccDNA modification by methylation and/or by post-translational acetylation, methylation, ubiquitylation and SUMOylation [38]. Recently, a work by Zhang et al. has shown that the transcription of HBV cccDNA is regulated by CpG methylation in CHB patients. CpG island II methylation correlated with the low level of HBV DNA in patient serum. Moreover, in vitro methylation studies further confirmed that the CpG island II methylation markedly reduces cccDNA transcription and subsequent viral core DNA replication [39]. In occult hepatitis B infection, which is characterized by the persistence of HBV genomes in the hepatocytes of individuals testing negative for serum HBV HBsAg and HBV DNA, the CpG island II was also found to be more densely methylated than in nonoccult HBV infection [40]. Recently, using the chromatin immunoprecipitation assays combined with a sensitive and specific real-time PCR for cccDNA quantification [41], HBV replication was shown to parallel the acetylation and methylation status of HBV cccDNA-bound-H3 and -H4 histones in HBV-replicating cells and in patients [41,42].
Nucleocapsid assembly

The HBV nucleocapsid plays a central role in the viral life cycle. The icosahedral HBV nucleocapsid is formed by multiple copies (240 or 180) of the viral core protein \([43,44]\), which in turn encloses the viral pgRNA together with the Pol protein. After the pgRNA is converted to the characteristic rcDNA in the nucleocapsid, which is defined as nucleocapsid maturation, the mature rcDNA-containing nucleocapsid is encapsulated by the viral envelope proteins and secreted extracellularly as a virion or recycled to the nucleus to replenish the cccDNA pool.

The first step of nucleocapsid assembly is the formation of capsid homodimers linked by a disulfide bridge between the cysteine residue 61 \([45–47]\). Once translated, the dimerization of core proteins occurs rapidly \([45]\). After the dimers reach a certain threshold concentration, they begin to aggregate and form capsid particles \([45,48]\). However, whether the higher order oligomer intermediates exist is not clear at the present time, although some reports suggested that dimers trimereize when they assemble into capsid particles \([49]\) and form higher oligomers with chaperones \([50]\).

Nucleocapsid assembly is followed via the formation of a specific pgRNA-Pol ribonucleoprotein (RNP) complex. A short-structured RNA signal, called \(\varepsilon\), locates at the S’ end of pgRNA, which harbors an internal bulge and an apical loop, separated by two short stems, was demonstrated to be essential for both RNP formation and pgRNA packaging into capsid particles \([51]\) and to be specifically recognized by the Pol protein \([52,53]\). The terminal protein (TP) domain and reverse transcriptase (RT) domains of the Pol protein are also required for \(\varepsilon\)-Pol binding \([54–56]\). Besides, some host cell factors are found to be involved in RNP complex formation and pgRNA packaging. Early studies showed that heat shock protein 90 (Hsp90) interacts with the viral RT as a chaperone protein to facilitate RNP complex formation \([57]\). Further studies indicated that in addition to Hsp90, Hsp70 and the other co-chaperones are also required to establish and maintain the Pol conformation which permits \(\varepsilon\) binding \([58–60]\).

At present, how the RNP complex is recognized by the assembled core protein dimers is not clearly understood. The RNP complex may bind to the first core protein dimer, and this interaction may mediate the nucleation of nucleocapsid assembly. This notion has been supported by a recent study showing that the localization of Pol in the nucleocapsids is unique \([61]\).

Reverse transcription

Once the RNP complex is formed and packaged into the viral nucleocapsids, the viral genomic DNA, rcDNA, will be synthesized from the pgRNA, via reverse transcription, which is triggered by the interaction of Pol and pgRNA. But the initiation of HBV minus-strand DNA occurs via a unique mechanism, defined as protein priming, during which the Pol protein is used as a specific protein primer, while the internal bulge in the \(\varepsilon\) RNA is used as the template for the protein priming \([62–64]\).

Several critical elements of the Pol protein and \(\varepsilon\) RNA that are required for protein priming have been identified. The S’ cap structure near the \(\varepsilon\) RNA signal, the apical loop in the \(\varepsilon\) RNA and the structural conformation change of \(\varepsilon\) RNA are important for protein priming \([62,65]\). Some studies reported that the \(\varepsilon\) RNA could also activate the enzymatic activity of the Pol protein during protein priming \([66]\). As the specific primer, the Pol protein plays a central role in the protein priming. The Tyr residue in TP domain of Pol is reported as the binding site of the first dGMP to prime minus-strand DNA synthesis \([62,67,68]\), and the other sequences in this domain are also essential for protein priming \([56,62,69]\). The RT domain harbors the polymerase catalytic center and thus is needed in the formation of the initial phosphorysyl linkage between the S’ dGMP residue and the priming Tyr site in the TP domain and in the DNA polymerization that follows \([62,70,71]\). The other sequences in the RT domain are also required for \(\varepsilon\) RNA binding and DNA synthesis during protein priming \([56,69]\).

Following protein priming, minus-strand DNA synthesis starts after the translocation of the short DNA oligomer from the S’ end of pgRNA to an acceptor site in the 3’ end \([63,72,73]\). At the same time, the RNase H activity of Pol degrades pgRNA but leaves the extreme S’ end undegraded \([74]\), which is subsequently used as primer to initiate plus-strand DNA synthesis. After the plus-strand DNA synthesis reaches the S’ end of the template minus-strand DNA, the elongating 3’ end of the plus-strand DNA translocates from the S’ end to the 3’ end of the minus-strand DNA and leads to the circularization of the DNA product. Then, the synthesis of plus-strand DNA continues and accesses to the rest of the minus-strand DNA but does not proceed to completion, leading to the formation of characteristic rcDNA. In end, the maturing nucleocapsid containing rcDNA is enveloped and secreted extracellularly as virions, or it recycles the rcDNA to the nucleus to amplify more cccDNA.
Maturation and secretion of infectious virions and SVPs

Following their formation, nucleocapsids bud through the endoplasmic reticulum (ER) membrane [75] and either are recycled to the nucleus or become associated with HBs envelope proteins already localized at a post-ER, pre-Golgi compartment, followed by their release from the cells as mature enveloped virions through the multivesicular body pathway [76], referred as endosomal sorting complex required for transport-dependent machinery [77]. HBsAg plays a critical role in this process. HBsAg itself can also be exported as empty SVPs through the ER/Golgi secretory pathway. Secreted SVPs consist of 20 nm spheres and filaments of the same diameter but with a variable length. Thus far, relatively few data have described a detailed mechanism of HBV release and the effect of viral secretion on the secretion of cellular proteins and vesicles.

MODEL SYSTEMS FOR HBV STUDY

The understanding of HBV biology has been hampered for many years by the lack of an in vitro HBV infection system and small animal models for infection. Thus, tremendous efforts have been undertaken to develop cell and animal models that can mimic the in vivo human hepatocyte and HBV replication cycle. HCC cell lines, primary hepatocytes and newly developed cells expressing HBV receptor have contributed to the understanding of viral replication. HBV genomes can be delivered to the livers of mice using hydrodynamic injection (HI) or an adenoviral vector. Human-induced pluripotent stem cells (iPSCs) can provide cells with defined features of human hepatocytes and contributed to the development of a personalized humanized mouse model for HBV.

Cell models

Since the discovery of HBV, various HBV cell models have been developed to meet the needs of HBV studies (Table 1). From 1990s, most studies have primarily used HBV transient expression or stable cell lines, such as HepG2.2.15 [78] and HepAD38 [79]. These cell models represent valuable tools to study HBV DNA synthesis, viral assembly and virion secretion, but have obvious limitations that do not exactly mimic the natural HBV life cycle. For long, primary human hepatocytes (PHHs) [80] and a differentiated human hepatoma cell line HepaRG cells [81,82] have been the main source for studying the entire life cycle of HBV. However, while unpre-dictable variability between hepatocyte donors and rapid loss of hepatocyte function after plating hampers the use of PHHs for HBV infection models, long time of differentiation and low efficiency of infection restrict the wide use of the HepaRG cell line. So, much effort has been put into the development of new HBV cell infection systems.

A Japanese group described a high-efficiency HBV infection system that achieves approximately 80% infection rates, using fresh human hepatocytes isolated from humanized livers of chimeric mice [83]. By fusing PHHs with special HepG2 cells, HepCHLine-4/-7, an immortal cell line was established and was shown to be susceptible to HBV and support its replication [84,172]. Inspiringly, micropatterning and coculturing of PHHs with fibroblasts (MPCC format) was reported to promote long-term maintenance of PHH functions, thus maintaining prolonged HBV infection [85]. Recently, a new hepatoma cell line, HLCZ01, isolated from a liver tumor of a male patient suffering from chronic HCV infection, was shown to support the entire life cycles of both HBV and HCV. Importantly, the virus particles from HBV-infected HLCZ01 cells could be transferred to naive HLCZ01 cells confirming the production of infectious HBV virions [86]. After NTCP was identified as a HBV/HDV functional receptor, a stable HepG2-hNTCP cell line [11] overexpressing human NTCP was established. HepG2-hNTCP cells can be infected with high concentrations of HBV genome equivalents. More importantly, iPSC-derived hepatocyte-like cells (iHeps) have been reported to support HBV infection [85], which will open new avenue for studying host interactions with HBV and for developing antiviral indentations.

As HBV cccDNA is a vital replicative intermediate and crucial for HBV persistence, lots of attention has been paid to explore cell model in which HBV cccDNA can be studied. In addition to infected cell models, monomeric linear HBV genome-transfected [87] and loxP chimeric monomeric HBV DNA plasmid-transfected [88] hepatoma cells were developed to investigate the formation and regulation of cccDNA. Specifically, in cells transfected with the monomeric linear HBV genome, circularization of linear genome leads to the formation of cccDNA, which then, following association with epigenetic factors, organizes itself into minichromosome [89]. In cells expressing the monomeric linear HBV genome under the control of the loxP-Cre expression system, recombinant cccDNA (rccccDNA) accumulates in the nucleus [88]. Therefore, these cell models can help to investigate host factors regulating viral cccDNA or evaluate novel antiviral drugs targeting HBV cccDNA.
<table>
<thead>
<tr>
<th>Cell models</th>
<th>Sources of viral genome</th>
<th>Applications</th>
<th>Limitations</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2.2.15</td>
<td>Four tandem copies of HBV genome integrated into HepG2 genome</td>
<td>Testing the effect of drugs that could interfere with viral replication; analyzing the involvement of immune system in disease process</td>
<td>Life cycle of HBV is not the same to natural one. Unsuitable for studying HBV entry and uncoating.</td>
<td>[78]</td>
</tr>
<tr>
<td>HepAD38</td>
<td>Plasmid pTetHBV under the control of a tetracycline-responsive promoter in HepG2 cells</td>
<td>High-throughput, cell-based screening for inhibitors of HBV replication</td>
<td></td>
<td>[79,168]</td>
</tr>
<tr>
<td>HepG2.117</td>
<td>Plasmid pTA2 and pTRE-HBV</td>
<td>Higher levels of HBV expression, and particles from supernatant is infectious for primary tupaia hepatocytes; responsive to HBV inhibitors</td>
<td></td>
<td>[169]</td>
</tr>
<tr>
<td>HBV baculovirus infection of HepG2 cells</td>
<td>pBB45HBV1.3 (recombinant baculovirus)</td>
<td>A highly flexible system for studying HBV replication and pathogenesis at the molecular level</td>
<td></td>
<td>[170]</td>
</tr>
<tr>
<td>HBV adenovirus infection of HepG2 or PHH</td>
<td>pAdEasy1-HBV1.3 (AdHBV)</td>
<td>Understanding virus–host interaction of HBV infection; determining pathogenicity of mutant viruses; studying the role of viral protein in regulating the virus life cycle and in transferring hepadnavirus genomes across the species barrier</td>
<td></td>
<td>[171]</td>
</tr>
<tr>
<td>Monomeric linear HBV genome-tranfected cells</td>
<td>Monomeric linear HBV genome (self-circularization)</td>
<td>Study for regulation of HBV minichromosome; sensitive amplification and simplified functional analysis of full-length HBV genomes</td>
<td></td>
<td>[41,87,89]</td>
</tr>
<tr>
<td>loxP chimeric monomeric HBV genome plasmid-transfected cells</td>
<td>loxP chimeric monomeric HBV genome plasmid, Cre/loxP-mediated 3.3kb rcccDNA</td>
<td>A model system in vitro and in vivo for evaluating antiviral treatments against HBV cccDNA</td>
<td></td>
<td>[88]</td>
</tr>
<tr>
<td>Primary human hepatocyte</td>
<td>HBV infection (average 50% infection efficiency with HBV in the presence of 5% PEG)</td>
<td>A tool for investigation of HBV infection</td>
<td>Limited availability and unpredictable variability of human liver</td>
<td>[80]</td>
</tr>
<tr>
<td>Fresh human hepatocytes isolated from humanized mice</td>
<td>HBV infection (The maximum infection rate is approximately 80%)</td>
<td>A tool for studying the entire HBV life cycle</td>
<td>Same as above</td>
<td>[83]</td>
</tr>
<tr>
<td>HepCHLine-4/-7</td>
<td>HBV infection (serum)</td>
<td>A tool for studying the complete process of HBV infection and testing the efficacy of antiviral drugs</td>
<td>Uncertain genetical stability during maintenance</td>
<td>[84,172]</td>
</tr>
<tr>
<td>HepaRG</td>
<td>HBV infection (around 10% infection efficiency with HBV in the presence of PEG, maximum rate is 20%)</td>
<td>A tool for deciphering the mechanism of HBV entry and they are suitable for many applications including drug metabolism studies</td>
<td>Differentiation and infectability are maintained only when these cells are cultured in the presence of DMSO; at least 2 weeks for differentiation before HBV infection</td>
<td>[81,82]</td>
</tr>
<tr>
<td>HLCZ01</td>
<td>HBV infection. (around 30% cells are infected at 65 dpi)</td>
<td>A tool for addressing multiple aspects of the HBV life cycles, and for studying HBV/HCV coinfection</td>
<td>Isolated from tumor but not normal liver tissue</td>
<td>[86]</td>
</tr>
<tr>
<td>HepG2-NTCP/Huh7-NTCP</td>
<td>HBV infection (approximately 70% infection efficiency at 4% PEG and 2.5% DMSO)</td>
<td>High-throughput analyzing to identify novel drugs and elucidate host–virus interaction</td>
<td>Using high concentrations of HBV genome equivalents to infect the cells</td>
<td>[10,11,173]</td>
</tr>
<tr>
<td>iPSC-derived iHeps</td>
<td>HBV infection (serum, late stage of hepatic differentiation)</td>
<td>Studying HBV biology and the virus’ interaction with host hepatocyte genetics and physiology</td>
<td>The capacity to support HBV transcription and initial permissive for infection strictly depending on iHep differentiation state</td>
<td>[85]</td>
</tr>
</tbody>
</table>
### Table 2. Current animal models for HBV study.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Characteristics</th>
<th>Advantage</th>
<th>Disadvantage</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chimpanzees</td>
<td>Susceptible to HBV naturally</td>
<td>Genetic proximity to human; without special treatment; similar to human infection</td>
<td>Ethical restriction, high cost, large size</td>
<td>[174]</td>
</tr>
<tr>
<td>Tree shrew (tupaia)</td>
<td>Close phylogenetic relationship with primate</td>
<td>Relatively small animal; infected HBV directly</td>
<td>Poorly efficient infection; mild, transient infection; low viral titer</td>
<td>[175]</td>
</tr>
<tr>
<td>Ducks, herons, woodchucks, squirrels, woolly monkeys, gibbons</td>
<td>HBV-related hepadnaviridae host</td>
<td>Can be infected with hepadnaviridae; accessible primary hepatocytes</td>
<td>Relatively large size; difficult to handle; immune systems are not characterized; the hepadnaviridae viruses are different from HBV which might limit the value of data on the viral lifecycle and the results of antiviral drug screening</td>
<td>[176]</td>
</tr>
<tr>
<td>Transgenic mice</td>
<td>Small, low cost, easy husbandry, well-characterized, clear genetic and immune background, easy-to-handle, short breeding period, techniques for genetic modification, abundance of immunological reagents</td>
<td>Have either complete or single HBV genome; can investigate specific aspects of HBV; replicate at high levels in vivo</td>
<td>Cannot apply to study HBV entry and spreading; no cccDNA; HBV tolerance</td>
<td>[177]</td>
</tr>
<tr>
<td>Hydrodynamically injected mice</td>
<td>Same as above</td>
<td>Break species-specific barriers; have high levels of HBV replication; different mutant viruses can be injected</td>
<td>Strong damage to liver; may alter cell or other part function; influence signaling analyses</td>
<td>[96,101]</td>
</tr>
<tr>
<td>Transfection mice</td>
<td>Same as above</td>
<td>Transfer the HBV genome efficiently</td>
<td>The vector induces immune response</td>
<td>[92,171]</td>
</tr>
<tr>
<td>Chimeric mouse models</td>
<td>Same as above</td>
<td>Have HBV-natural host hepatocytes; support HBV infection</td>
<td>Implanted hepatocytes remain functional for a limited time; the human hepatocytes are unavailable; genetically immune deficient; do not have a viral-induced immunopathogenesis</td>
<td>[103,104]</td>
</tr>
<tr>
<td>Humanized mouse models</td>
<td>Same as above</td>
<td>Human immune system and human liver tissues, more closer to human infection and response</td>
<td>The human hepatocytes are unavailable, need high technology and condition</td>
<td>[107,108]</td>
</tr>
</tbody>
</table>

**Animal models**

HBV has a narrow host range and can only naturally infect humans, chimpanzees and, to a certain extent, tree shrews and recently discovered *Macaca fascicularis* [90]. Alternatively, several HBV-related hepadnaviridae and their natural hosts were identified, such as DHBV and WHV. However, simultaneously, there are limitations associated with the application of these animal systems to HBV research (Table 2). In comparison, the mouse model is believed to be the most suitable laboratory animal for HBV infections; therefore, efforts are being made to develop an HBV infection mouse model.

In the ideal scenario, the experimental mouse model should express human receptors, which would allow HBV attachment and entry into hepatocytes, followed by DNA replication and cccDNA formation in the nuclei of infected cells. However, for a long time, the receptors mediating HBV entry were not known. Only recently, NTCP was identified as one of functional receptor(s) for human HBV; however, mouse NTCP does not contain molecular determinants required for viral entry [91]. Furthermore, investigations are underway to identify potential coreceptor(s) that are thought to mediate the high efficiency of HBV infection. Therefore, the success of HBV receptor expressing transgenic mice could take time to be realized. Despite the inability of HBV to naturally infect and propagate in mice, different surrogate murine models have been generated.

The transduction of the HBV genome into mice using viral vectors such as adenovirus (Ad) [92] or adeno-associated virus (AAV) [93] leads to efficient viral gene expression in the liver and activation of host immune response against HBV.
This transduction model has been used to study the mechanism of HBV immunotolerance and evaluation of novel immunotherapy, such as the TLR9 agonist CpG [94]. Moreover, an AAV/HBV-transduced murine HCC model was established for studying the pathogenesis and therapeutic interventions [95] of HBV-associated HCC.

Following the generation of an acute HBV transfection model by the HI of an engineered and replication-competent HBV DNA into mouse [96], Chen et al. established a chronic transfection mouse model by HI of HBV1.2 genomic DNA into C57BL/6 mice, which has accelerated further mechanistic and antiviral studies of HBV [97–99]. However, in this widely employed HI model, only less than 20–30% of injected mice express HBV for more than 24 weeks [99]. Recently, Chen et al. applied multiple inbred mouse strains (BALB/c, C57BL/6, C3H/HeN, DBA/2J, C57BL/6J, C3H/HeN, DBA/2J, CBA/cj) and showed that HBV sustained rates among these strains are different, which indicates that the HBV clearance after HI actually depended upon the mouse strains, and genetic constitution of the host influences the outcome of HBV infection [100]. Similar to the observation of Chen et al., Zhou et al. also succeeded in delaying the mouse immune clearance of HBV by hydrodynamically injecting the HBV plasmid into C3H/HeN mice. Approximately 90% of the injected C3H/HeN mice were shown to maintain HBV for up to 46 weeks post HI. More importantly, the administration of IFN-α or entecavir led to a decrease in HBV DNA in vivo [101]. Thus, these models based on HI of HBV may provide a more suitable platform for studying HBV persistence.

The most recent studied animal models for studying HBV replication has been the use of the immunodeficient mice carrying human hepatocytes engrafted in their livers. Two previously established models relied on the use of mice that were immunodeficient (SCID, or DKO deficient in Rag2 and IL2rg genes), that were transgenic for the urokinase plasminogen activator (uPA-SCID) or that had the fumaryl acetoacetate hydrolase (Fah) gene knocked out to induce hepatocytes damage [102]. Both of these two murine models can support efficient HBV infection [103,104], but are also associated with drawbacks, including difficulties in breeding and the need for overly elaborated techniques. For example, the high neonatal lethality of uPA-SCID mice provides a narrow window usually 14–21 days post-natally for xenogen hepatocytes transplantation; thus, the handling and operation of this model is difficult. To overcome the problems associated with the uPA-SCID mouse model, Deng et al. in Beijing first generated a transgenic mouse in which the transcription of the reverse tetracycline transactivator (rTA) is driven by the mouse albumin promoter. Then, the rTA mice were crossed with SCID/bg mice to generate immunodeficient rTA/SCID mice. Next, the rTA/SCID mice were transduced with the recombinant adenoviral vectors Ad. TRE-uPA, which encodes the urokinase gene downstream of the tetracycline response element (TRE). The efficacy of this model system was demonstrated by the initiation of inducible liver injury caused by Tet-On-regulated urokinase [105]. The TK-NOG mouse model is another inducible liver injury model for human hepatocyte transplantation. In this model system, the herpes simplex virus type-1 thymidine kinase is expressed in mouse hepatocytes. A brief exposure to ganciclovir resulted in the ablation of transgenic mouse hepatocytes, followed by transplantation with human hepatocytes that stably repopulated the mouse liver. Similarly to the uPA/SCID mouse model, the efficacy of this system was demonstrated by the ability of HBV to infect the human hepatocyte-transplanted TK-NOG mice [106].

Notably, the above-mentioned human mouse liver chimeras do not have a functional human immune system and cannot mimic human responses to HBV infection. Therefore, generating a murine model with a human immune system and liver tissues is necessary. To reach this goal, Su et al. developed two novel humanized mouse models that meet these requirements. The AFC8 mouse model employs the BALB/c-Rag2−/−γc−/− mouse carrying a liver-specific inducible suicidal transgene. The transplantation of CD34+ human hematopoietic stem cells (HSCs) to AFC8 mice supports the development of a functional human immune system [107]. Very recently, the development of a second model was reported. This novel model, named A2/NSG-hu HSC/Hep, supported HBV infection, with approximately 75% of HBV-infected mice presenting an established persistent infection for at least 4 months [108].

It should be noted that the generation of a mouse model with detectable cccDNA is urgently needed for studying the critical steps in the HBV replication life cycle. Deng et al. developed a model based on the HI of a precursor plasmid (prcccDNA) that encodes a loxP–chimeric intron engineered into the monomeric HBV genome. During the course of transcription, the chimeric intron is excised from RNA transcripts by the Cre/loxP system without interfering with the HBV life cycle [88]. This model could be used for developing cccDNA-based antiviral strategies.
POTENTIAL TARGETS FOR THE DEVELOPMENT OF NEW ANTI-HBV AGENTS

As discussed above, the model systems for HBV study have been greatly improved in the recent years, thus promoted a deeper understanding of the molecular biology of HBV. Based on this, a number of new viral and host-associated antiviral targets have been revealed, and increasing numbers of new anti-HBV strategies have been reported. While many of these strategies have been developed to repair or restore the host immune responses to eliminate or clear the viral infection, different approaches have been designed to target viral factors including cccDNA, HBsAg, core and polymerase, as well as the host factors involved in different stages of the HBV replication cycle, of which some are currently under experimental development, and some have already reached clinical validation.

cccDNA

Although HBV DNA can integrate into the genome of the host cells, this integration involves only segments of the viral genome and is not essential for viral replication. cccDNA, the viral episomal minichromosome, has been shown to be a virological indicator for treatment response [109] and has long been considered the primary factor in HBV persistence. However, the current treatments with NAs or IFN-α, individually or in combination with each other, may efficiently inhibit the production of viral RNA and new viral DNA, but cannot eradicate the intrahepatic cccDNA [110–112]. Therefore, the identification of drugs that can eliminate cccDNA or silence the transcriptional activity of cccDNA is thought to be a direction for successful treatment of HBV infection (Fig. 1).

Cytokines including IFN-α and TNF-α were reported to reduce cccDNA accumulation by interfering with the maturation of nucleocapsids.
[113,114]. IFN-α was also found to suppress HBV replication by the epigenetic repression of cccDNA transcriptional activity [115,116], although the host and viral proteins involved in this process still need to be clarified. A recent study demonstrated that IFN-α and lymphotoxin-β receptor activation have a direct effect on cccDNA stability in HBV-infected, differentiated HepaRG cells, PHH cultures and chimpanzees. IFN-α activation was shown to upregulate APOBEC3A, resulting in cytidine-deamination, apurinic/apyrimidinic site formation, and finally cccDNA degradation [117]. In addition to the direct inhibition of HBV infection in hepatocytes, IFN-α was also shown to induce the transfer of antiviral molecules from liver non-parenchymal cells to hepatocytes via exosomes [118]. These work support the view that HBV could be eliminated through intrinsic non-cytopathic mechanisms [2,119]. A deeper understanding of the maintenance of cccDNA and the factors that lead to its active decay might eventually be exploited as strategies for functional cure of chronic HBV infection.

Another recently developed anti-HBV approach, which directly targets the viral cccDNA within cells, makes use of the sequence-specific genome-editing DNA nucleases including zinc finger nucleases (ZFN), transcription-activator like endonucleases (TALENS) and CRISPR/Cas9 [120–123]. Studies showed that these proteins could be used to block the viral transcription and DNA production in cell and mouse models. CRISPR/Cas9 combinations that are specific for multisites of the HBV sequence were shown to reduce total viral DNA by a factor of ~1000-fold and HBV cccDNA levels by up to 10-fold; they also mutagenically inactivated the majority of the residual viral DNA [123]. These data have provided proof of principle for the hypothesis that engineered nucleases have the potential to serve as effective tools for depleting the cccDNA pool. However, the safe and efficient delivery of these large systems to reach cccDNA in infected hepatocytes of CHB patients remains challenging.

In addition, a small-molecule library screen was recently conducted in cell models to uncover compounds inhibiting cccDNA formation. These efforts led to the discovery of disubstituted sulfonamide (DSS). Although DSS did not directly promote the degradation of rcDNA or cccDNA, it inhibited de novo cccDNA formation by interfering with rcDNA conversion into cccDNA [124].

HBsAg (viral entry and release)

HBsAg, which was first described as the Australian antigen by Dr Blumberg in 1967, persist in the circulation of most CHB patients. HBs antigens are composed of three glycoproteins, termed S-HBs, M-HBs and L-HBs, and include preS1, preS2 and S domains as indicated in Fig. 2. HBs proteins are the primary component of the envelope of the infectious viral particles and mediate HBV entry primarily via the preS1 domain of L-HBs protein. Agents targeting HBsAg may inhibit the entry and spread of the virus, including the drug-resistant variants, thus protecting the uninfected cells and the HBV-cured cells. Moreover, HBs proteins, which are secreted as the non-infectious SVPs of HBV, are produced in vast excess over intact virions and circulate in the blood at concentrations that may exceed 100 μg mL⁻¹. Mounting evidence suggests that the HBsAg proteins play a role in the persistence of HBV infection by effectively suppressing both innate and adaptive immune responses to HBV infection [125–127]. Therefore, agents targeting HBsAg may not only inhibit the dissemination of HBV but also relieve the given immune suppression to help the patients fight infection with their own immune system.

As the preS1 domain of the L-HBs protein plays a pivotal role in HBV entry, several preS1-derived peptides that compete with virus binding to hepatocytes have been developed to block viral entry. Particularly, Myrcludex-B, a synthetic lipopeptide consisting of the myristoylated 2–48 amino acids region of preS1, has been shown to efficiently block HBV infection in vitro [128,129] and in HBV-infected uPA/SCID mice [130,131], and is presently in clinical development as a potent HBV entry inhibitor. Interestingly, the entry receptor of HBV NTCP was recently identified by a pull-down assay using Myrcludex-B as a bait. With the discovery of NTCP, a series of small-molecule compounds that inhibit HBV entry by targeting NTCP have been reported. Among these, cyclosporine A, a calcineurin/Ca-pathway licensed for clinical suppression of the immunological failure of xenografts, has been reported to inhibit HBV replication by interfering with the interaction of NTCP and L-HBs [132]. Another example is ezetimibe, an anticholesteremic drug known to inhibit the NTCP transporter, that has been shown to block HBV entry [133]. NTCP is a host protein that has a fundamental physiological function including sodium-dependent transport of bile acids. Therefore, further use of these inhibitor compounds requires careful evaluation of their safety in vivo. To avoid the potential side effects that may accompany the long-term use of NTCP inhibitors or preS1-derived peptides, preS1-binding peptides that could interfere with HBV attachment by ‘wrapping’ the HBV virions have been identified by screening of a random peptide phage display library with HBV preS1 [134].
Figure 2. Key steps of HBV entry and release and targeting strategies. The entry of HBV follows a multiple step process: (1) HBV reversibly attaches to HSPOs with a low affinity, (2) the virion binds to specific receptor(s) with high affinity and (3) endocytosis-mediated internalization. Several PreS1-derived peptides, NTCP inhibitors and PreS1-binding peptides can inhibit the viral entry process. Mature nucleocapsids interact with the HBV envelope proteins at a post-ER, pre-Golgi compartment before being released from the cells as enveloped virions through the multivesicular body pathway (4), whereas the empty HBV SVPs are exported through the cell ER/Golgi secretory pathway (5). Several inhibitors of virions and SVPs secretion have been described as indicated.

The inhibition of HBV virions and/or SVP secretion into the circulation represents yet another target for the development of novel anti-HBV drugs and several agents targeting the HBV secretory pathway have been reported as novel antivirals, including α-glucosidase inhibitors, triazolopyrimidine derivatives, nitazoxanide and amphipathic oligonucleotides. Glucosidase inhibitors were reported to interfere with the morphogenesis and infectivity of the virus by inhibiting the glycosylation of the envelope protein in the ER, resulting in the development of a defective HBV envelope incapable of binding to host cells and establishing infection [135–137]. Triazolo-pyrimidine derivatives have been shown to inhibit the secretion of HBsAg produced by HBV variants that are resistant to current NAs [138,139]. The non-specific antimicrobial nitazoxanide and its active metabolite, tizoxanide, were also suggested to reduce the levels of extracellular HBsAg and HBeAg and to decrease the levels of intracellular HBeAg in a dose-dependent manner in vitro [140]. Amphipathic oligonucleotides, also known as amphipathic DNA polymers or nucleic acid-based polymers, belong to a class of compounds displaying an extremely broad spectrum of antiviral activity against enveloped viruses. These compounds can bind with high affinity to amphipathic protein structures commonly observed in the viral envelope proteins but very rarely observed in normal human proteins. One of such molecules, Rep 9AC, was reported to suppress the HBsAg release from infected hepatocytes. Early clinical trials involving HBV-infected animals and CHB patients have demonstrated that the administration of Rep 9AC results in a rapid reduction and elimination of HBsAg from the blood without affecting the production of HBV virions. More importantly, a decrease in the circulating levels of HBsAg was associated with a restoration of the immune function. Considering that the blockage of virions and SVPs secretion could lead to the accumulation of HBsAg and newly synthesized viral DNA in the cells, further studies are needed to examine the safety and off-target effects of this type of anti-HBV approach.

Core

As a viral structure protein, the core protein is the major component of the viral nucleocapsids that protects the viral genome. In addition to its role in capsid assembly, recent reports have suggested that the core protein is also involved in several processes that may contribute to the virus persistence: (1) the formation and maintenance of the mini chromosome or cccDNA copy number in the nuclei of
the assembly of aberrant capsid particles \[149\]. Similar to phenylpropenamide derivatives and SBAs, HAPs can efficiently inhibit HBV variants that are resistant to the currently used polymerase inhibitors \[150\]. Another core inhibitor candidate currently undergoing a Phase I clinical trial is NVR 3–778 (Novira Therapeutics), which is believed to bind all oligomeric forms of HBV core including the core dimer and the assembled viral capsid. Using cell culture models and a humanized liver mouse model, NVR 3–778 was shown to disrupt the HBV lifecycle by inducing the assembly of defective capsids and to function as a potent inhibitor of HBV replication.

### Polymerase
The HBV polymerase is a multifunctional protein that plays a key role in HBV replication. Pol contains 832 amino acid residues and consists of four separate domains, including the TP domain in the N-terminal, the spacer, the RT domain and the RNase H domain. The TP domain is required for \(\varepsilon\) binding, RNA packaging and protein priming. The RT domain mediates the initiation of reverse transcription and nucleocapsid assembly, which is the primary target of the NAs. The RNase H domain functions to degrade the pgRNA template during minus-strand DNA synthesis and is required for the pgRNA encapsidation \[151\]. Long-term NA treatment may lead to the emergence of drug-resistant HBV variants and thus a risk of rendering the NAs ineffective. Therefore, exploring novel drugs that can target HBV polymerase is necessary.

Because Pol-\(\varepsilon\) binding is a key step for both pgRNA encapsidation and protein priming, it represents an ideal anti-Pol target. Hu et al. found that iron protoporphyrin IX (heme), an endogenous component of hemoglobin and a number of other cellular enzymes, as well as several related porphyrins, block protein priming in both HBV and DHBV cell models \[152\]. Furthermore, a carbonyl J acid derivative was suggested to disrupt the Pol-\(\varepsilon\) interaction \[153\]. Recently, a series of compounds that inhibit the RNase H activity of Pol have been identified by screening of a group of known or predicted inhibitors of HIV RH domains and integrase enzymes \[154\], although the mechanism of action of these compounds remains unclear. Because distinct domains of Pol have been shown to interfere with the signaling pathways activated by the engagement of either pattern recognition receptors or IFN receptors in hepatocytes \[155–158\], the development of Pol inhibitors may also be beneficial for restoring the anti-HBV host innate immune responses.

Taken together, these findings indicate that Pol is a

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**Figure 3.** Assembly of Pol and core proteins with pgRNA into nucleocapsids and targeting strategies. Nucleocapsid assembly is triggered by the formation of pgRNA-polymerase RNP complex, which is facilitated by some host chaperon proteins like Hsp90. (1) Pol-\(\varepsilon\) binding and RNP formation; (2) HBc dimerization; (3) initiation of nucleocapsid assembly; (4) and (5) nucleocapsid assembling, and protein priming reverse transcription; (6) nucleocapsid maturation. Several compounds have been found to disturb Pol-\(\varepsilon\) binding, DNA elongation activity of Pol and viral nucleocapsid assembly and thus inhibit viral replication. Heteroaryldihydropyrimidines (HAPs), Sulfaomylbenzamide (SBA).
promising target for the development of new anti-HBV therapeutics (Fig. 3), although further studies regarding the structure and function of Pol are required.

Viral RNAs

A number of strategies targeting viral RNAs have been attempted. In vitro data have demonstrated that HBV transcription levels could be reduced by antisense oligonucleotides [159], hairpin ribozymes [160] or hammerhead ribozymes using lentiviral vectors for delivery [161]. McCaffrey et al. reported that RNAi could be applied to inhibit the production of HBV replicative intermediates in cell culture as well as in immunocompetent and immunodeficient mice transfected with a HBV plasmid [162]. Two independent groups showed that S′-triposphorylated HBV-specific siRNAs could directly silence the viral RNA in addition to their effect on activating RIG-I-like receptors-mediated innate antiviral immune responses [163,164]. Nevertheless, a number of challenges such as efficient in vivo siRNA delivery, RNA instability and potential off target effects need to be overcome before these molecules can be exploited in the treatment of CHB patients. Notably, the dynamic polyconjugate (DPC) technology provides a platform for targeted delivery of siRNA to liver hepatocytes. ARC-S20, which consists of a mixture of two siRNAs directed against conserved HBV RNA sequence conjugated with cholesterol and a hepatocyte-targeted membrane-lytic-peptide (NAG-MLP), is a DPC therapeutic for treatment of HBV infection developed by Arrowhead Research Corporation [165] and has been demonstrated to be safe and well tolerated and could efficiently reduce HBsAg in HBV patients in a dose-dependent manner in a preliminary phase II study. The recently reported recombinant HBV vectors developed from the naturally occurring, highly replicative HBV mutant bearing a 207-bp deletion in the preS1/polymerase spacer region have also been suggested as an effective tool for hepatocyte-targeted delivery of siRNAs or genes with anti-HBV activities [166].

CONCLUSIONS AND PERSPECTIVES

HBV is a highly efficient virus that makes the cure rates of currently available therapies for CHB patients quite low. The recent experience in developing new antiviral agents and therapy regimes for HCV was a great encouragement to researchers working on the development of anti-HBV agents. Similar to the process implemented for the development of HCV-targeting drugs, improved model systems and increasing understanding of HBV replication in the past decade have revealed many potential anti-HBV targets, which have guided the development of a large number of candidates targeting key factors of HBV infection such as cccDNA, HBsAg, core and the viral polymerase. Because these new strategies function through distinct mechanisms and affect broad aspects of viral replication, they will undoubtedly contribute to a cure of chronic HBV infection and to the prevention of CHB-related liver diseases in a significant manner. Moreover, in light of a growing body of evidence demonstrating that HBV has evolved active strategies to evade the immune surveillance and HBV infection can be characterized as a chronic immunological disorder as well as a viral infection [167], the development of immunotherapeutics and combination regimens composed of novel antiviral agents and immunomodulatory approaches could prove advantageous to maximize the antiviral efficacy. The challenge is on how to optimally combine the available antiviral agents together. Overall, the ultimate goal of HBV treatment is virus elimination. With greater understanding of the ins and outs of HBV infection, the chances of having efficient anti-HBV agents and concerted strategies toward a functional cure are foreseeable in the near future.

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