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REVIEW

In vivo and *in vitro* Models of Hepatitis B Virus Infection

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Abstract—Hepatitis B virus (*Orthohepadnavirus hominoidei*, HBV) is a hepatotropic virus from the *Hepadnaviridae* family and the causative agent of both acute and chronic hepatitis B (CHB). The possible outcomes of CHB include liver cirrhosis and hepatocellular carcinoma (HCC) that pose a significant burden on the healthcare systems worldwide. In the nuclei of infected hepatocytes of patients with CHB, the HBV genome persists as a pool of covalently closed circular DNA (cccDNA) molecules. Current therapeutic strategies cannot directly target cccDNA. Instead, the available treatments focus on long-term suppression of viral replication and require lifelong administration. Development and evaluation of novel antiviral agents capable of achieving complete HBV eradication require relevant *in vivo* and *in vitro* models of HBV infection. Among the available animal models, the following categories are distinguished: (i) animals naturally susceptible to HBV; (ii) surrogate models using animal species susceptible to the related hepadnaviruses; (iii) non-susceptible animals receiving HBV genome via recombinant viral vectors; (iv) models utilizing human hepatocyte xenografts. Among the available *in vitro* models, primary human and northern treeshrew (*Tupaia belangeri*) hepatocytes fully support the HBV replication cycle, but they rapidly lose susceptibility to the virus in cell culture. In turn, unmodified human hepatoma cell lines are not susceptible to HBV but can support viral replication after transfection with the viral genome. This review discusses key characteristics, advantages, limitations, and areas of application of the currently available *in vivo* and *in vitro* models of HBV infection.

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INTRODUCTION

According to the WHO estimates, approximately 1.2 million new cases of hepatitis B virus (HBV) infection are recorded annually and more than 250 million people are living with chronic hepatitis B (CHB) (https://www.who.int/news-room/fact-sheets/detail/hepatitis-b, accessed 10.07.25). The HBV genome is represented by relaxed circular partially double-stranded DNA (rcDNA) (~3200 base pairs) that contains four overlapping open reading frames (ORF): S, pre-C/C, P and X. ORF S includes pre-S1, pre-S2, and S regions, which encode three isoforms of the surface protein (HBsAg): small (SHBsAg), middle (MHBsAg)

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and large (LHBsAg), all sharing a common C-terminus but differing in N-terminal extensions and different glycosylation levels. ORF pre-C/C encodes precore protein (HBeAg) and core protein (HBcAg). ORF P encodes polymerase with reverse transcriptase and RNase H activities. ORF X encodes a key transcription activation factor (HBxAg) [1]. HBV hepatotropism is determined by three factors: (i) the unique receptor expressed on hepatocyte surface, (ii) HBV capsid nuclear import, and (iii) hepatocyte-specific factors for viral transcription. HBV infection begins with low-affinity binding of the virus to heparan sulfates proteoglycans on the cell surface, followed by specific interaction between the pre-S1 domain of LHBsAg and its receptor – sodium-taurocholate cotransporting polypeptide (NTCP). Next, the HBV/NTCP complex is internalized into cells by interacting with the epidermal growth factor receptor (EGFR) via clathrin-dependent endocytosis. HBV enters the endolysosomal compartment, where the rcDNA-containing capsid is released into the cytosol. Import of the viral genome into the nucleus is ensured by the interaction of the nuclear localization signal in the terminal region of HBcAg on the surface of the viral capsid with the importin α1 [2]. In the nucleus, the HBV genome is released from a capsid followed by conversion of rcDNA into covalently closed circular DNA (cccDNA) in a multistep process mediated by cellular enzymes. The host RNA polymerase II mediates cccDNA transcription into pregenomic RNA (pgRNA), which serves as a template for reverse transcription of the (−) strand of rcDNA [3]. The hepatocyte-specific factors such as hepatocyte nuclear factors (HNF) HNF1α, HNF3β, HNF4α, HNF6, and factor C/EBP, play a crucial role in the HBV replication cycle [4]. The rcDNA is released from cells within *de novo* virions or re-imported into the nucleus, thereby ensuring maintenance of the intranuclear pool of cccDNA [5].

HBV causes both acute and chronic infection, transmitted from mother to child or via bloodborne transmission. Incubation period of the infection varies from 30 to 180 days. In most cases, acute infection in adults is self-limiting and results in virus elimination. However, in rare cases, fulminant hepatitis develops followed by liver failure and patient death. CHB is characterized by a long-term inflammatory process in the liver eventually leading to the development of liver cirrhosis and hepatocellular carcinoma (HCC). The risk of developing CHB in adults is low (~5%), whereas in children under one year it reaches 95%. In 2022, 1.1 million deaths related to CHB outcomes, such as liver cirrhosis and HCC, had been recorded worldwide [6].

Serological markers related to HBV infection include viral antigens HBsAg and HBeAg and antibodies – anti-HBs, anti-HBe, and anti-HBc (IgM and IgG classes). These markers allow for the identification of patients with HBV infection as well as the diagnosis of CHB and the differentiation of the clinical phases of the disease, being also important for monitoring of the effectiveness of antiviral therapy [7].

HBsAg can be detected in the blood of the patients during both acute infection and CHB. HBsAg is located at the surface of the virions and circulates in the blood as a part of subviral particles of various shapes. Quantitative assessment of HBsAg levels as a marker of viremia is important for monitoring of the response to antiviral therapy. Anti-HBsAg antibodies exert virus-neutralizing activity and provide protection against HBV infection [8]. HBsAg seroconversion, defined as the lack of detectable HBsAg (<0.05 IU/ml) and emergence of anti-HBs antibodies (>10 IU/ml) is a crucial endpoint of CHB functional cure, implying (in rare cases) a long-term decline in viral replication and immunologically controlled infection. However, in some cases occult HBV infection has also been described, when patients with undetectable HBsAg in the blood serum still have viral replication and inflammation in the liver, which represents an increased risk of developing HCC and infection reactivation [9].

HBV DNA detection in the blood is the most relevant and accurate marker of viral replication, which is also widely used in HBV infection diagnostics. Serum DNA level in CHB patients correlates with the risk of liver damage progression. Assessment of serum DNA levels is used to determine the need for antiviral therapy and for monitoring of treatment effectiveness [7].

cccDNA is a stable form of HBV DNA that persists as a viral mini-chromosome capable of binding histones to form a nucleosome [10]. Up to 50 copies of cccDNA are detected in the nuclei of HBV-infected human hepatocytes. Due to the presence of the nuclear pool of cccDNA and the lack of approved drugs that can directly block or destroy cccDNA, viral replication resumes following therapy cessation [11].

Development of new approaches to the treatment of CHB requires adequate *in vitro* and *in vivo* models reproducing all of the described features of HBV infection, which, however, is challenging. For example, HBV exhibits strict species tropism partially determined by the species specificity of the receptor NTCP [12]. Furthermore, development of productive HBV infection requires several host intracellular factors, additionally limiting the range of biological species suitable for infection modeling [13]. Apart from searching for the species susceptible and permissive to HBV, studies with other *Hepadnaviridae*, such as duck hepatitis B virus (DHBV), woodchuck hepatitis virus (WHV), and woolly monkey hepatitis B virus (WMHBV), are underway. This review is aimed to describe the current *in vivo* and *in vitro* models used to study HBV and related viruses as well

as for testing of CHB-specific antiviral therapies. In addition, we paid close attention to the stages and features of HBV infection in humans that can be reproduced by each model, which, we believe, could help to develop next-generation drugs aiming at a functional cure of CHB.

In vivo HBV INFECTION MODELS

Naturally susceptible animals. Chimpanzees (*Pan troglodytes*) are the only non-human primates fully susceptible to HBV [14]. They reproduce acute HBV infection and can develop its chronic and persistent forms, resulting in less pronounced pathological changes in the liver as compared to humans [15]. Since 1972, when hepatitis B development in chimpanzees following inoculation of viral material obtained from humans was first reported [16], this species has been used as an animal model to investigate both HBV pathogenesis and immune response against HBV. For instance, cross-reactive immune response to HBsAg derived from the diverse viral subtypes has been proven using chimpanzees [17]. Delayed-type hypersensitivity was demonstrated in chimpanzees following subcutaneous HBsAg inoculation, thereby proving HBsAg immunogenicity [18]. Hence, these studies laid the foundation for the development of the first HBV vaccines, efficacy and safety of which were also tested in chimpanzees. First-generation vaccines were made by purifying HBsAg from the serum of asymptomatic HBV carriers [19]. The resulting preparation became the first human serum-derived vaccine licensed in the United States [20]. However, due to the concerns about the residual presence of HBV and other viruses in the biomaterial, alternative vaccine types have been later developed and tested on chimpanzees, including the pre-S2 region-encoded synthetic peptide [21], human hepatoma cell line PLC/PRF/5-derived HBsAg [22], DNA vaccines [23], etc. Furthermore, the currently used vaccine consisting of recombinant HBsAg produced in yeast (*Saccharomyces cerevisiae*), has been tested in the chimpanzee model [24]. Studies using chimpanzees revealed the crucial role of proinflammatory cytokines in the clearance of HBV during acute infection, achieved without extensive destruction of hepatocytes [25].

Due to the ethical concerns and high cost, laboratory use of chimpanzees is limited, which has led to the search for new models of HBV infection primarily among other primates. However, the species-specific differences in the amino acid sequence of the NTCP orthologs make Old World primates insusceptible to HBV. To skip the viral entry stage, a plasmid encoding HBV genome dimer was directly injected into the liver of Barbary macaques (*Macaca sylvanus*). Two days after the injection and several weeks onward, the presence of HBsAg and HBV DNA was detected in the serum and liver tissue of the animals. Subsequently, the analysis of animal serum samples revealed the HBV-like viral particles along with pathological liver changes resembling those observed during hepatitis B infection in humans. Hence, the obtained data demonstrate that the HBV replication could occur in the Barbary macaque hepatocytes [26].

In 2013, HBV DNA as well as HBsAg and HBcAg were detected in the blood serum and liver tissue, respectively, of cynomolgus macaques (*Macaca fascicularis*) from the island of Mauritius, whereas no pathological changes in the liver tissue were observed [27]. However, it was later reported that the virus isolated during this study was unable to induce HBV infection in the same macaque species [28].

The northern treeshrew (*Tupaia belangeri*) is an HBV-susceptible small rodent-like mammal that is phylogenetically more closely related to primates [29]. Adult animals directly infected with HBV exhibited a self-limiting acute infection with increased HBsAg levels in the serum 2-3 weeks post-infection along with the rapidly produced anti-HBeAg antibodies [30]. Injection of the virus into neonatal animals caused chronic infection lasting over 48-weeks. cccDNA has been detected in the liver of northern treeshrews with CHB along with pathological changes in the liver morphology, which developed at a slower rate than in humans [31]. Nonetheless, chronic HBV infection in treeshrews could result in HCC development [32], which is not typical for some other animal models of HBV infection. Additionally, immunohistochemical staining revealed the presence of HBcAg in hepatocytes during both acute and chronic infection, confirming viral replication process [31]. Studying of HBV using treeshrews is hampered by the difficulty in selecting adequate species-specific reagents and protocols, low viral loads, and the need to infect neonatal animals, while the incidence of CHB development is low (13%) [30].

Genetically modified animals. Susceptibility to HBV depends on the species-specific host factors, primarily the sequence of NTCP orthologs. Human *NTCP* gene (*hNTCP*) transduction into mouse, rat, and dog hepatocytes followed by HBV infection ensures virus entry into the cells but does not result in effective viral replication, suggesting that species-specific intracellular factors affect the HBV replication cycle. Hepatocytes derived from cynomolgus macaques, rhesus macaques, and pigs, after transduction of *hNTCP*, supported complete HBV replication cycle comparable to that in human hepatocytes, thereby opening the way to use immunocompetent macaques in studies of HBV infection, immune response, and viral pathogenesis [33].

Due to the general similarity between the human and macaque immune systems, rhesus macaques (*Macaca mulatta*) have several advantages over other HBV infection models [34]. Inoculation of rhesus macaques with the *hNTCP*-encoding adenoviral vector followed by infection with HBV [28] led to moderate viremia. However, no chronic infection developed in this model. Immunohistochemical staining of the liver sections revealed the presence of HBcAg solely inside the nuclei of 0.5-1.0% hepatocytes [28]. In order to achieve chronic infection, immunosuppression was additionally induced in infected rhesus macaques, which resulted in persistent 20-week long HBV replication identified by the presence of HBsAg and HBeAg in the blood as well as HBV DNA in liver biopsies. However, after cessation of immunosuppression, immune-mediated virus elimination occurred in most animals with emergence of serum anti-HBs and anti-HBc IgG antibodies, as well as $CD8^+$ and $CD4^+$ T cell activation [35]. Thus, the genetically modified *hNTCP*-expressing rhesus macaques could maintain prolonged HBV infection subsequently resulting in specific immune-related virus elimination.

Surrogate models. *Duck hepatitis B virus.* The studies with other members of *Hepadnaviridae* family were conducted in parallel with the search for HBV-susceptible animal species. In 1980, a virus later named duck hepatitis B virus (DHBV) was discovered in the blood serum obtained from domestic ducks (*Anas domesticus*). Viral DNA was detected mainly in the liver, suggesting hepatospecificity of the virus. Electron microscopy analysis of the purified virus revealed particles (40 nm in diameter) similar to those of other members of the *Hepadnaviridae* family [36]. Similar to HBV, the DHBV genome is represented by rcDNA [37]. The DHBV-infected ducks were used as a model for testing of antiviral drugs such as polymerase inhibitors [38] and nucleocapsid assembly inhibitors [39]. Although ducks offer a relatively convenient model for laboratory use, extrapolation of the research data to humans is complicated because of the differences in the genome sequence and pathogenesis between DHBV and HBV. In particular, nucleotide sequence of the DHBV genome is only 40% homologous to that of HBV [40]. Moreover, specific virus receptors also are different: the entry receptor for DHBV is carboxypeptidase D [41], which does not allow to study the drugs targeting the interaction between HBV and its receptor. In addition, no correlation was found between chronic hepatitis and the development of HCC in various duck species infected with DHBV [42].

Woolly monkey hepatitis B virus. Besides HBV, *Orthohepdnavirus* genus also includes woolly monkey hepatitis B virus (WMHBV), identified in the blood of common woolly monkeys (*Lagothrix lagotricha*). The WMHBV amino acid sequence is 44% homologous to that of HBV, however the HBcAg gene which is presumably conserved among the *Hepadnaviridae* family, has 85-86% and 75-77% homology to the HBV HBcAg in amino acid and nucleotide sequence, respectively. The WMHBV causes either acute (fulminant) or chronic infection in common woolly monkeys [43]. Other primates were also experimentally infected with WMHBV. HBsAg and WMHBV DNA were detected in black-handed spider monkey (*Ateles geoffroyi*) blood serum for 4-6 weeks after infection, while no pathological changes were detected in liver biopsies [43]. In order to establish chronic WMHBV infection, neonatal black-handed spider monkeys were infected with a WMHBV clone, which did not lead to the development of a chronic disease [44]. As common woolly monkeys and black-handed spider monkeys are endangered species, ethical reasons hamper their large-scale research use, which motivated researchers to use a related primate species, squirrel monkeys (*Saimiri sciureus*), as an alternative. Infection of squirrel monkeys with WMHBV-containing biomaterial lead to acute infection with viremia that lasted for 4 weeks. To prolong the infection, the animals were inoculated with the adeno-associated virus (AAV8) encoding infectious WMHBV genome, which resulted in viremia lasting 32 weeks. In all neonatal squirrel monkeys infected with WMHBV viremia lasted for 3 months, followed by virus elimination [45]. Thus, despite the absence of chronic infection, squirrel monkeys infected with WMHBV can be used as a surrogate model of HBV infection.

Woodchuck hepatitis virus (WHV). WHV also belongs to the genus *Orthohepdnavirus*. Along with general similarity in the genome and virion structure (WHV genome has 62-70% nucleotide sequence homology to HBV), WHV and HBV exhibit antigenic cross-reactivity. Also, these viruses have the same timeline of viral antigens appearance in the liver and blood after infection, which indicates the similarity of the stages of infection course [46]. In adult woodchucks, acute WHV infection may result either in complete virus elimination or progression into chronic form. In neonatal woodchucks, the infection mainly becomes chronic [47]. Chronic WHV-infection leads to exhaustion of the WHV-specific T-cell response as well as to low interferon production by hepatocytes [48].

WHV was first detected in the tissues of dead woodchucks (*Marmota monax*), which showed signs of HCC development [47]. Similar to HBV, WHV is an oncogenic virus [49], with HCC developing in 90% of cases of verified chronic WHV infection [49]. Therefore, high HCC incidence allows to use woodchucks as a convenient model in studies of viral oncogenesis. The ability of the WHV genome to integrate into the cell genome and cause increased proliferation of hepatocytes was studied using the marmot model [50].

Woodchuck models have been useful for preclinical testing of antiviral drugs, including nucleoside analogues [51]. In 2011, Himalayan marmots (*Marmota himalayana*) were also shown to be susceptible to WHV, expanding the panel of available surrogate models of HBV infection [52]. However, susceptibility to WHV varied between different animals [53]. Applicability of woodchucks as an HBV infection model is limited due to the difficulties in laboratory housing and care of these animals and limited availability of reagents for the immune response studies.

Mouse models. For biomedical research, mice are the most common and easy-to-use model, with standardized methods and reagents available for a wide range of experiments. Because mice are normally neither susceptible nor permissive to HBV, gene modification and human tissue transplantation are used to generate mouse models of HBV infection.

Transgenic mice. Due to the development of the embryonic microinjection technology, transgenic mouse models have been created, which carry in their genome either individual HBV genome regions encoding HBsAg [54], HBeAg [55], and HBxAg [56] or full-length HBV genome [57]. The hepatocyte-specific promoters such as the mouse metallothionein or albumin promoters were used to control HBV gene expression in the mouse liver [58].

HBsAg-producing transgenic mice were used for the modeling of the asymptomatic HBV carriage [54] as well as for the validation of the indirect oncogenic effect of HBsAg [59]. Moreover, the oncogenic effect of HBxAg was demonstrated in transgenic mice, as production of HBxAg was directly associated with histopathological changes in the liver [56]. HBV vertical transmission was shown in transgenic female mice bearing complete HBV genome. In addition, it has been shown that HBeAg could induce the production of the regulatory peptide PD-L1 by liver macrophages. Production of PD-L1 leads to the decrease in $CD8^+$ T cell proliferation [60], resulting in HBV persistence in the transgenic mouse offspring [61]. A disadvantage of transgenic mouse models is that the proteins produced from the transferred genes are not recognized as foreign by the immune system of the animal, which hinders the assessment of the immune response. Furthermore, there is no recirculation of the assembled viral particles into hepatocytes of transgenic mice bearing complete HBV DNA sequence, as the mouse NTCP ortholog is unable to facilitate HBV entry into cells. The transgenic mouse model is not relevant for testing of therapeutic drugs aimed at blocking or destroying cccDNA, because this form of HBV genome is not formed in mice. The virions assemble due to the expression of linear genomic sequences of the virus integrated into the mouse genome, which makes their complete elimination impossible [62].

Mouse models with in vivo transfection. To avoid genetic aberrations caused by the HBV genome integration into the mouse genome, models of episomal HBV assembly have been developed. Currently, two methods are used to deliver HBV genome into the mouse hepatocytes either as HBV-encoding plasmids or as recombinant cccDNA (rcccDNA): high-pressure hydrodynamic injection-based HBV transfection (HDI-HBV) and transduction using adeno-associated virus (AAV) or adenovirus (Adv) vectors, bearing HBV genome. The HDI-HBV results in transfection of 10% hepatocytes, whereas adenoviruses provide transduction of 90% hepatocytes. The drawbacks of such approaches are as follows: (i) lack of the infection stage and full viral replication cycle, as the “surrogate” HBV cccDNA is formed in the transduced mouse cells, from which viral transcripts are produced; (ii) non-physiological viremia levels – tens of times higher than in natural infection; (iii) potential for development of the robust immune response both against viral components due to the high level of virion production and against adenovirus components; (iv) tissue non-specificity of HDI-HBV, as along with hepatocytes the viral genome may potentially enter other cell types [63]. Since hepatotoxic effects are observed upon using the plasmids/rcccDNA at high doses in HDI-HBV or when inoculating $>10^9$ adenovirus particles encoding the HBV genome (AdHBV), 10 μg DNA in HDI-HBV and up to 10^8 AdHBV should be used in *in vivo* experiments [64, 65].

Genetic background of the mice used in the experiments also affects the course of HBV infection. In particular, BALB/c mice after HDI-HBV (10 μg of plasmid in a volume equivalent to 8% of mouse body weight) are predisposed to HBV elimination with production of anti-HBsAg antibodies within 14 days post-injection, whereas in C57BL/6 mice HBsAg was detected for 35 days after plasmid injection [66]. The main drawback of the described mouse models is the absence of cccDNA. To overcome this obstacle, a precursor recombinant cccDNA plasmid (prcccDNA) was synthesized and transduced using Adv into the C57BL/6 and Alb-Cre Tg mice (C57BL/6-Tg [Alb-cre] 21Mgn/J). Recombination of prcccDNA occurred in the nuclei of the transduced hepatocytes resulting in the production of rcccDNA. Viremia in C57BL/6 mice was not observed following HDI-HBV, whereas HBcAg was detected at low levels and located primarily in the hepatocyte nuclei. At the same time, in Alb-Cre Tg mice rcccDNA was detectable for 62 weeks accompanied by the pronounced viremia as evidenced by the detection of HBcAg and HBsAg in the blood. Increased PD-L1 production in the liver also resulted in the decrease in specific T-cell response against the rcccDNA-positive hepatocytes, which contributed to the rcccDNA persistence [67].

Species	Route of infection	Receptor-mediated virus entry into hepatocytes	Formation of cccDNA	Chronic HBV infection	HCC development
(P.t.) 	HBV	+ [15]	+	+ [15]	+ [15]
(T.b.) 	HBV	+ [30]	+ [31]	+ [31]	+ [31,32]
(M.m.) 	Hydrodynamic injection with plasmid encoding HBV genome	–	–	+ [63,65]	–
	Transduction with viral vectors encoding HBV genome	–	–	+ [66]	–
(M.f.) 	Transduction with AAV encoding *hNTCP*, infection with HBV	+ [33]	–	–	–
(M.m-a.) 		+ [28]	–	–	–
(A.d.) 	DHBV	+ [41]	+ [38]	+ [42]	–
(M.m-x.) 	WHV	+ [47]	+ [46]	+ [47]	+ [49]
(M.h.) 		+ [51]	+	+	+
(L.l.) 	WMHBV	+ [43]	+ [43]	+ [43]	+ [43]
(A.g.) 		+ [43]	–	–	–
(S.s.) 		+ [45]	–	–	–

Fig. 1. Stages of HBV infection and related pathogenesis reproduced in the *in vivo* models. The animal species and relevant viruses as well as recombinant vectors used for each model are presented. Designations: DHBV, Duck Hepatitis B Virus; WHV, woodchuck hepatitis virus; WMHBV, woolly monkey hepatitis B virus; AAV, adeno-associated virus. Species-specific descriptions: P.t., *Pan troglodytes*; T.b., *Tupaia belangeri*; M.f., *Macaca fascicularis*; M.m-a, *Macaca mulatta*; M.m., *Mus musculus*; A.d., *Anas domesticus*; M.m-x, *Marmota monax*; M.h., *Marmota himalayana*; L.l., *Lagothrix lagotricha*; A.g., *Ateles geoffroyi*; S.s., *Saimiri sciureus*). cccDNA, covalently closed circular DNA; HCC, hepatocellular carcinoma.

To assess the efficacy of novel HBV treatments *in vivo*, an episomal vector based on the adeno-associated virus AAV9-HBsAg was developed. AAV9-HBsAg serves as a surrogate for cccDNA and contains a partial HBV sequence under the control of the hepatospecific promoter. Inoculation of AAV9-HBsAg into NOD-SCID IL2rg$^{-/-}$ mice resulted in HBsAg secretion lasting for 7 weeks. Subsequently, the AAV8-HBsAg was also used in the cynomolgus macaque (*Macaca fascicularis*) model to test the HBV genome-specific engineered nuclease (ARCUS-POL) [68].

Thus, a wide range of *in vivo* HBV infection models designed in diverse animal species is currently available, each of which (except for chimpanzee model) only partially reproduces the course of human HBV infection. The advantages and drawbacks of such models are shown in Fig. 1.

Xenograft-based mouse models. Transgenic and transduced mice models do not fully reproduce the natural course of HBV infection *in vivo*; moreover, many antiviral drugs cannot be tested using such models. To reproduce the complete HBV replication cycle in mice, the Trimera model was developed. For this, the immunocompetent CB6F1 mice were exposed to lethal gamma irradiation followed by inoculation with bone marrow cells derived from SCID/NOD mice. Fragments of human HBV-infected liver tissue were then transplanted under the subrenal capsule or into the auricle of the animals. HBV DNA was detected in the serum of the CB6F1 mice bearing human liver

xenografts from day 8 to day 25 post-transplantation and declined afterwards. A similar infection profile was achieved using the Trimera model in the immunodeficient BNX mice. cccDNA was detected in the liver grafts indicating viral replication inside the human hepatocytes. The Trimera model was used to evaluate the efficacy of antiviral drugs including polyclonal anti-HBsAg antibodies, reverse transcriptase inhibitors, and nucleoside analogues [69]. Another model was also created using *nude (nu/nu)* mice, which were subcutaneously injected with the HepAD38 cells bearing complete HBV genome under the control of tetracycline repressor. Viral replication was regulated by the presence of tetracycline (tet) in drinking water. In the absence of tet, the mice developed viremia lasting 35 days after infection, and cccDNA was detected in the liver. Viral load decreased after adding tet to drinking water and relapsed after tet removal [70]. The developed model was used to test combination therapy against HBV *in vivo* [71]. Long-term HBV viremia was achieved using a transplantation model based on mice with combined RAG-2 deficiency (RAG-2M). For this, a primary human hepatocyte culture was immortalized by transduction with the SV40 T-antigen followed by transfection with the full-length HBV genome and obtaining monoclonal cell line (IHBV6.7). The latter was transplanted into the mice via intrasplenic injection. Although the transplanted IHB6.7 cells comprised as few as 1% of the mouse hepatocyte mass, viremia lasted for at least 5 months post-transplantation [72]. The ability to not only integrate into, but also partially repopulate the mouse liver with human hepatocytes (up to 15% mouse liver) was first demonstrated in the uPA/RAG-2 model, using immunodeficient *RAG-2* knockout mice crossed with the uPA transgenic mice. Overexpression of the urokinase-type plasminogen activator (uPA) gene in mouse hepatocytes resulted in neonatal hemorrhage and liver necrosis. Following human hepatocyte xenotransplantation, mouse hepatocytes were replaced in the liver by human hepatocytes. Infection of the uPA/RAG-2 mice with serum samples derived from HBV carriers resulted in viremia and detection of HBsAg for 8 weeks following infection [73]. Another model was developed based on SCID mice homozygous for the Alb-uPA transgene [74]. Infection of the xenotransplanted SCID Alb-uPA mice with serum samples derived from HBV carriers resulted in viremia that was detected at week 2 after infection and persisted for 12 weeks. It was also shown that HBV particles derived from HepG2 cells bearing the full-length viral genome were able to infect SCID Alb-uPA mice. However, the viral load detected from week 4 was significantly lower compared to infection with blood serum samples. The drawback of the Alb-uPA SCID model was the lack of the control over uPA expression, the need for transplantation into neonatal mice, and increased risk of hemorrhage [75]. Therefore, an alternative *Fah*$^{-/-}$*Rag-2*$^{-/-}$*Il2rg*$^{-/-}$ mouse model was created using immunodeficient animals with deleted gene encoding fumarylacetoacetate hydrolase (Fah). Loss of the latter results in accumulation of toxic tyrosine metabolites in mouse hepatocytes causing their death, which promotes liver repopulation with the xenograft hepatocytes. Furthermore, the toxicity level could be controlled by the orally administered 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione (NTBC). Following human hepatocyte transplantation, the mouse liver comprised as many as 20% of the xenograft hepatocytes, and repopulation success rate ranged from 45 to 95% [76]. Mice with high proportion of liver repopulation were infected with HBV. From the 2nd to the 7th week of follow-up, cccDNA was detected in liver samples and viremia was confirmed with the peak of the viral load detected at the 6th week. Immunocytochemical staining for HBcAg demonstrated that the infection proceeded exclusively in the transplanted human hepatocytes, with HBcAg accumulation observed in both the nuclei and cytoplasm [77]. The model was used to evaluate the efficacy of novel antiviral drugs [78].

Assessment of the immune response in xenograft mouse models is complicated, as it requires the use of immunocompromised animals. To overcome this limitation, additionally to human hepatocytes, mice were transplanted with human immune system (HIS) elements. In particular, the neonatal A2/NSG mice expressing human *HLA-A2* gene were inoculated with donor-derived CD34$^+$ hematopoietic stem cells (HSC) and human hepatocyte progenitor cells (Hep). This resulted in the development of a functional human immune system along with liver repopulation. In this process, *HLA-A2* expression increased the selection of T cells producing only human MHC. To promote liver repopulation with human cells, mouse hepatocyte death was selectively induced by anti-murine Fas antibodies. It was found that the A2/NSG-hu HSC/Hep mice developed low viremia over 12 weeks after HBV infection. The immune response was characterized by the increased production of IFN-γ, IP10, and IL-6 along with the low levels of IL-10 and IFN-α, as well as the presence of anti-HBsAg antibodies and HBV-specific CD8$^+$ T cells. While examining the liver tissue in HBV-infected mice, increased collagen deposition was described, which is typical for HBV-induced tissue fibrosis during CHB [79]. For dual humanization, the BALB/c *Rag-2*$^{-/-}$*Il2rg*$^{-/-}$*Sirpa*^{NOD} *Alb-uPA*$^{tg/tg}$ mice were also used for transplantation of human hematopoietic stem cells. The resulting model (HIS-HUHEP) demonstrated high human hepatocyte engraftment rate (about 20-50% of mouse liver was replaced with human cells) [80]. Infection of HIS-HUHEP mice with

HBV showed that the viral load and accumulation of T cells, Kupffer cells and natural killer cells (NK cells) in the liver correlated with the administered HBV dose. However, the disease progressed to chronic course without the development of liver fibrosis. It was also shown that the liver PD-L1 protein expression in infected animals was elevated in comparison with intact mice. It has been shown previously that PD-L1 expression leads to T-cell exhaustion, which is one of the causes of CHB [81]. Another alternative model is based on Fah$^{-/-}$Rag-2$^{-/-}$IL-2Rγc$^{-/-}$ SCID (FRG) mice transplanted with human bone marrow-derived stem cells (hBMSCs). After hBMSC-FRG animals were infected with HBV, viral DNA and antigens were detected in serum at relatively high levels for 56 weeks. Immunohistochemical staining of the liver sections for human albumin, HBsAg, and HBcAg revealed that only human hepatocytes were infected with HBV. The percentage of infected hepatocytes increased up to 80% over time. Analysis of mouse liver sections also showed increased levels of proinflammatory cytokines, human chemokines, as well as of hCD3, hCD86, and NK cells, suggesting an active immune response. Moreover, the humoral response was also characterized by the presence of specific IgG against HBsAg and HBcAg, which, however, declined sharply at the week 32. Pathological liver changes typical to chronically infected patients were observed throughout the experiment, which included lobular inflammation, lymphoid aggregates, and duct damage, as well as lymphocyte infiltration from the portal vein. At the later stages, signs specific to the developing cirrhosis were observed: collagen accumulation, fibrous tissue hyperplasia, and abnormal liver lobe structure [82].

Xenograft mouse models played an important role in investigating HBV pathogenesis *in vivo* as well as efficacy assessment of antiviral therapy. The double-humanized mice also allowed to assess the impact of chronic HBV infection on the development of immune response. However, use of such animal models is complicated by the labor-intensive process of their obtaining, high cost, and risk of sudden death. The advantages and drawbacks of the described models are summarized in Table S1 (Online Resource 1).

In vitro HBV INFECTION MODELS

Primary human hepatocytes. Primary human hepatocytes are derived from the livers of organ donors, tissues after liver surgery including resections, cadaveric material, and liver biopsies [83]. Due to the natural susceptibility to HBV infection, human hepatocyte culture serves a reference model for HBV research. Hepatocytes fully support HBV replication cycle and, moreover, possess a functional intracellular innate immunity allowing for studies of the innate antiviral immune response against HBV [84]. However, primary hepatocytes undergo rapid phenotypic changes during *in vitro* culturing [85]. Initially, primary hepatocytes could be maintained *in vitro* for up to one month. Modifications in culturing conditions allowed to extend hepatocyte viability up to 2 months without affecting their metabolism [86], which is still insufficient for CHB modeling. Culture media supplementation with dimethyl sulfoxide (DMSO) has been shown to promote hepatocyte differentiation, leading to a significant increase in HBV replication level [87]. HBV viral particle and protein production levels vary between the primary hepatocyte cultures obtained from different donors [88]. Thus, although primary human hepatocytes support full HBV replication cycle, use of this model is limited due to the low accessibility and heterogeneity of the biomaterial, which profoundly complicates their large-scale application.

Human hepatoma cultures. HepG2 and Huh-7 are the most widely used human hepatoma cell lines due to their *in vitro* stability and ease of cultivation. However, due to the lack of surface hNTCP, they are not susceptible to HBV infection. The hepatoma cells can only be used as HBV infection models after their modification by HBV genome transfer or by *hNTCP* expression followed by HBV infection.

Huh-7 cells, isolated from hepatocellular carcinoma in 1982 [89], were shown to be a useful *in vitro* model for studying of HBV replication cycle. After Huh-7 cells were transfected with a plasmid encoding HBV DNA genome dimer, HBsAg and HBeAg were detected in the culture medium for 25 days. Moreover, the presence of viral particles was confirmed in culture medium at day 20 after transfection [90]. The Huh-7 cell line was used as a model to investigate the molecular mechanisms underlying HBV replication [91, 92] including virion assembly and maturation [93], impact of the immune factors on viral replication [94] and efficacy assessment of antiviral agents [95, 96] including novel therapeutic approaches based on TALENs- and CRISPR/Cas9 cccDNA cleavage systems [97].

HepG2 cells were isolated from the hepatocellular carcinoma of a 15-year-old patient [98]. After transfection of HepG2 cells with a plasmid encoding an HBV DNA genome dimer, the HepG2.2.15 monoclonal cell line with genome-integrated HBV DNA was obtained [99]. Infectivity of the viral particles isolated from HepG2.2.15 cells was demonstrated in the chimpanzee model [100]. Additionally, HepG2.2.15 cells allowed to identify the potential molecular targets for CHB therapy [101]. In 1997, the HepAD38 cell line was derived from HepG2 cells after transfection with the plasmid encoding HBV genome under the control of tetracycline-responsive promoter [102]. Later, the modified

cell lines, HepDE19 and HepDES19, were obtained, in which viral particle production was tetracycline-depended [103]. These cell lines were used to screen potential inhibitors of cccDNA formation [104].

HepaRG cells were derived from the hepatocellular carcinoma cells of a hepatitis C-infected patient. After differentiation by adding DMSO and corticosteroids to the culture medium HepaRG cells morphologically and functionally resembled primary hepatocytes and were successfully infected with HBV. HBsAg, HBcAg, and HBV DNA were detected in the culture medium after infection [105]. The presence of cccDNA in the infected cells was later confirmed in a 50-day experiment. However, susceptibility of the HepaRG cells is strictly dependent on cell differentiation, which requires extended period of time to achieve (2 weeks for proliferation and 2 weeks for differentiation in the culture medium supplemented with DMSO). Moreover, the HepaRG cell line consists of differentiated and polarized hepatocyte-like cells and biliary-like epithelial cells, which results development of the infection in islands of differentiated cells [106]. Additionally, prolonged exposure to DMSO induces cell death, which also affects the HBV infection characteristics [107].

Upon discovering hNTCP as a functional receptor of HBV [12], the research focus was directed towards the generation of HBV-susceptible cell lines by *hNTCP* overexpression in cells. It was experimentally confirmed that *hNTCP*-expressing HepG2 and Huh-7 cell lines (HepG2-NTCP and Huh7-NTCP, respectively) are susceptible to HBV purified from serum samples of infected patients, as well as the virus isolated from culture medium. The HepG2-NTCP and Huh7-NTCP cells support complete HBV replication cycle, from viral entry to cccDNA formation. Due to the ease of their production, these cell lines have been widely used for testing and development of antiviral drugs [108, 109]. However, HBV infection efficiency in HepG2-NTCP and Huh7-NTCP cell lines varies. Although *hNTCP* expression allows HBV to enter Huh7-NTCP cells, further viral replication occurs in a smaller proportion of cells in comparison with the HepG2-NTCP cell line [110]. The current HepG2-hNTCP models allow for infection of up to 50-60% of cells (https://www.abmgood.com/ntcp-stable-hepg2-cell-line.html, accessed 18.04.25). Despite this, virus particle production by the HepG2-NTCP cell line is low. In order to create a cell culture with high virus yield, the monoclonal HepG2-NTCP-sec$^+$ culture with low proliferative activity was selected, in which more than 90% of the cells remained infected for 4 weeks after HBV infection without a decrease in the levels of detectable HBV DNA [111].

Overall, human hepatoma cells represent a useful and convenient tool for researchers, but their widespread use is profoundly limited by the fact that HBV infection is transient, without significant spread among new cells. Moreover, the hepatoma cells differ from the primary hepatocytes in multiple physiological functions and mechanisms, that complicates the data interpretation. Oncogenic origin of such cell lines complicates their use in investigating the mechanisms behind the HBV-induced carcinogenesis.

Animal cell lines. Being susceptible to HBV, the northern treeshrew played a crucial role not only in the *in vivo* but also in *in vitro* studies of HBV infection. The infection of primary treeshrew hepatocytes resulted in HBV DNA and RNA production lasting for 12 days as well as in the detection of HBsAg and HBeAg in the culture medium for up to 5-6 days [30]. Later, accumulation of cccDNA was also demonstrated. Furthermore, treeshrew hepatocytes were shown to be susceptible to WMHBV, with higher efficiency of viral replication compared to HBV [112]. The HBV-infected treeshrew hepatocytes were used as an *in vitro* model for efficacy assessment of antiviral agents [113] and in studies of virus-cell interactions [114]. This model was used to prove that the HBV pre-S1 domain enables HBV entry, which occurs in a species-specific manner [115]. Treeshrew hepatocytes were used to assess the infectivity of viral particles produced by human hepatoma cell lines [116]. Treeshrew primary hepatocytes are also a more readily available alternative to human primary hepatocytes. This allowed their use in the development of a xenograft mouse model, which was subsequently successfully infected with HBV [117] and used to test a potential inhibitor of HBV entry [118]. In addition, the treeshrew xenograft mouse model helped to describe the emerging hepatocyte proliferation, which resulted in the decreased population of the cccDNA-containing cells [119].

Among other studied animals, the common marmoset (*Callithrix jacchus*) is a promising animal model of HBV infection. Although common marmoset hepatocytes are not directly susceptible to HBV infection, it has been shown that the Adv-mediated delivery of the full-length HBV genome to a primary hepatocyte culture resulted in viral replication with detection of cccDNA and its derivatives, which was followed by HBV particle formation, confirming permissiveness of the common marmoset cells to HBV [120]. Recently it has been found that common marmoset hepatocyte resistance to HBV is associated with amino acid differences between common marmoset NTCP and hNTCP at two positions directly involved in HBV binding. In order to overcome this obstacle, a chimeric HBV/WMHBV pre-S1 virus was constructed and isolated. This virus successfully entered and replicated in common marmoset hepatocytes, as evidenced by the detection of HBeAg in the culture medium and the presence of HBV cccDNA in cells [121]. Taken together,

these results suggest high potential for further use of common marmoset hepatocytes in HBV research. However, currently common marmoset hepatocyte cell lines are not yet available.

Primary hepatocytes from woodchucks and ducks have also been used for *in vitro* studies. When primary woodchuck hepatocytes were infected with WHV, cccDNA formation was detected on day 2 post-infection followed by the detection of DNA fragments for 7-10 days, indicating active WHV replication [122]. HBV-infected woodchuck hepatocytes served as a model for efficacy assessment of antiviral drugs, such as DNA synthesis inhibitors [123]. Due to the relative ease of culturing, primary duck hepatocytes were more widely used [124]. The DHBV-infected duck hepatocyte culture allowed to explore reverse transcription activation [125] and cccDNA formation from rcDNA [126], as well as nucleocapsid assembly [127]. However, translational value of the obtained data is hampered by the above-mentioned genomic differences between HBV and DHBV.

Hence, hepatocyte cell cultures of various origins are widely used in the studies of various stages of HBV replication cycle, as well as in testing of potential antiviral drugs. However, similar to the *in vivo* models, each type of cell cultures has certain limitations that must be taken into consideration while making the decisions regarding their use in experiments. The hepatocyte culture-based models described here are summarized in Table S2 (Online Resource 1).

CONCLUSION

Undoubtedly, HBV infection poses a serious global health threat. Relevant and accessible models of viral infection are required to investigate the mechanisms underlying HBV pathogenesis, identify new antiviral targets, as well as to test novel antiviral vaccines and therapeutics. Here, we review most of the currently available models used to study HBV infection.

Among the *in vivo* models, the chimpanzee has historically served as a reference research model due to the natural susceptibility to HBV and phylogenetic proximity to humans. However, high cost and difficulty in maintenance in laboratory conditions limit the widespread use of chimpanzees. Among the *in vitro* models, primary hepatocyte cultures serve as a standard reference model. Both models are virtually inapplicable for large-scale studies due to ethical concerns and limited availability.

Ideally, a universal HBV model should meet the following parameters: (i) viral replication level similar to the one observed in natural infection, with maximum infection efficiency; (ii) recapitulate all stages of infection development, replication cycle and immune response; (iii) be widely accessible with regard to cost-effectiveness and availability of reagents; (iv) ensure data reproducibility. Owing to decades of research, a wide range of diverse HBV infection models have been developed, but none of them fully meet these criteria.

In this regard, modifications of the existing *in vitro* models such as HepG2-NTCP-sec$^+$ cell line and the search for new susceptible animals could provide most promising approaches to development of a universal model. It is worth noting that, despite the mentioned drawbacks, genetically modified and humanized mice are most widely used as *in vivo* models, in part due to accessibility and availability of the necessary reagents, particularly specific antibodies. In this regard, the model using common marmoset hepatocytes and chimeric HBV/WMHBV pre-S1 virus undoubtedly has an advantage due to the wide panel of available methods and reagents.

In conclusion, no universal model of HBV infection able to recapitulate all the stages of viral replication cycle and host immune responses is currently available. Its development might be a crucial step toward creating therapies for CHB and HBV-induced pathologies.

Abbreviations

AAV	adeno-associated virus
Adv	adenovirus vector
cccDNA	covalently closed circular DNA
CHB	chronic hepatitis B
DHBV	duck hepatitis B virus
HBV	hepatitis B virus
HBeAg	hepatitis B virus E antigen
HBcAg	hepatitis B virus core antigen
HBsAg	hepatitis B virus surface antigen isoforms
HCC	hepatocellular carcinoma
HDI-HBV	high-pressure hydrodynamic injection-based HBV transfection
NTCP	Na^+-taurocholate cotransporting polypeptide
rcDNA	relaxed circular DNA
rcccDNA	recombinant cccDNA
tet	tetracycline
uPA	urokinase-type plasminogen activator
WHV	woodchuck hepatitis virus
WMHBV	woolly monkey hepatitis B virus

Supplementary information

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Contributions

Y. V. Kolyako – wrote the initial version of the manuscript, edited the text; A. S. Zhitkevich and

D. V. Avdoshina – edited the text, created the illustrations; D. Y. Tanygina and V. D. Apolokhov – created the illustrations; T. V. Gorodnicheva – edited the text; E. O. Bayurova – edited the text, conceptualized the study; D. S. Kostyushev and I. V. Gordeychuk – edited the text, conceptualized the study, funding acquisition.

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Conflict of interest
The authors of this work declare that they have no conflicts of interest.

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