2 Unmet Needs in Basic Research of Hepatitis B Virus Infection: In Vitro and In Vivo Models

C. Nelson Hayes and Kazuaki Chayama

Abstract

Chronic infection with hepatitis B virus (HBV) is a preventable but incurable disease that affects more than 250 million people. Current therapies are effective in controlling infection, but complete elimination of the virus will require targeting covalently closed circular DNA (cccDNA). HBV relies on numerous host factors, some of which are promising drug targets. However, HBV replicates efficiently only within differentiated human hepatocytes. This specificity has complicated the development of in vivo and in vitro experimental models and hindered drug discovery. The identification of NTCP as the HBV receptor explained the poor infectivity in hepatoma cell lines and small animal models, but a number of approaches not directly tied to NTCP have also been established. The pressing need for more effective HBV therapies coupled with the unique challenges in the development of HBV models has propelled advances in transgenic and chimeric

C. N. Hayes
Department of Gastroenterology and Metabolism, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan

Research Center for Hepatology and Gastroenterology, Hiroshima University, Hiroshima, Japan
e-mail: nelsonhayes@hiroshima-u.ac.jp

K. Chayama (✉)
Department of Gastroenterology and Metabolism, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan

Research Center for Hepatology and Gastroenterology, Hiroshima University, Hiroshima, Japan

Institute of Physical and Chemical Research (RIKEN) Center for Integrative Medical Sciences, Yokohama, Japan
e-mail: chayama@hiroshima-u.ac.jp

J.-H. Kao (ed.), *Hepatitis B Virus and Liver Disease*,
https://doi.org/10.1007/978-981-16-3615-8_2

mouse models, small primate models such as macaque and tree shrew, adenoviral and hydrodynamic delivery of infectious particles, stably transfected cell lines, induced pluripotent stem cells, and 2D and 3D microarchitecture models that blur the line between in vitro and in vivo models. An array of impressive tools is now available in the search for a cure for chronic HBV infection.

Keywords

Adeno-associated virus · Covalently closed circular DNA · Hepatitis B virus HepaRG · HepG2 · NTCP · Human hepatocyte chimeric mice · Induced pluripotent stem cells · Primary human hepatocytes

Abbreviations

AAV	Adeno-associated virus
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
cccDNA	Covalently closed circular DNA
CPD	Carboxypeptidase D
DMSO	Dimethyl sulfoxide
HBsAg	HBV surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
iPS	Induced pluripotent stem
LHBsAg	Large S antigen
NK cells	Natural killer
NOD	Nonobese diabetic
NTCP	Sodium taurocholate cotransporting polypeptide
uPA/SCID	Urokinase-type plasminogen activator/severe combined immunodeficiency

1 Introduction

Nearly 250 million people throughout the world currently suffer from chronic hepatitis B virus (HBV) infection. The introduction of an effective HBV vaccine in 1986 has greatly reduced the incidence of new infections, and the disease is now largely preventable due to improvements in public health and awareness. Even when exposed to the virus, most adults are able to successfully clear acute infection. Despite this progress, chronic infection is still difficult to treat, and long-term management of the infection rather than outright cure is usually the primary goal of treatment. Current therapies can successfully but temporarily suppress viral

replication, slowing progression of liver disease and reducing the risk of complications such as cirrhosis and hepatocellular carcinoma (Kwon and Lok 2011). Long-term or even life-long treatment with interferon or nucleoside analogues such as tenofovir disoproxil fumarate, tenofovir alafenamide, or entecavir is necessary to suppress HBV replication. Long-term therapy is expensive and inconvenient and poses a small risk of adverse events and eventual development of drug resistance. HBV reactivation may also occur if therapy is discontinued, especially in immunosuppressed individuals.

The main reason for this intractability is that current therapies target essential but late-acting stages in viral replication but fail to target the virus's failsafe in the form of covalently closed circular DNA (cccDNA) mini-chromosomes that are able to persist long-term within the nucleus.

HBV is most vulnerable to elimination during the early stages of infection. Although the virus is thought to largely avoid immune surveillance during initial establishment, most individuals eventually mount an effective immune response that clears the virus during the acute phase of the infection and does not progress to chronic HBV. However, if the initial response falls short, HBV becomes highly entrenched in the liver and infects nearly all hepatocytes. RNA viruses such as hepatitis C virus that must replicate continuously can be effectively targeted using direct acting antiviral (DAA) agents. Conversely, while peg-interferon and nucleos(t)ide analogs are able to suppress HBV replication, the presence of cccDNA in the nucleus allows HBV to persist even when active replication is suppressed and allows it to quickly reactivate if viral suppression is relieved. Therefore, a successful cure for chronic HBV must contend with this contingency and will likely involve direct elimination or silencing of cccDNA or host factors involved in its maintenance (Allweiss and Strick-Marchand 2020).

Although HBV replication can be suppressed, patients with chronic HBV infection have a much greater risk of developing hepatocellular carcinoma, and it is important to continue to strive for a true cure that completely eliminates the virus. Development of such a cure is daunting due to the complexity of the HBV life cycle and the virus's high specificity to human hepatocytes, which has long hindered the search for suitable in vitro and in vivo models. Although imperfect, several models have recently been developed that facilitate analysis of HBV replication and evaluation of potential drug candidates. However, antiviral therapy is likely to be only one aspect of a successful cure, and immunocompetent models are also needed to evaluate immunomodulation strategies to restore exhausted adaptive immune responses (Maini and Burton 2019). The models reviewed below have greatly expanded our knowledge of HBV.

1.1 Hepatitis B Virus

Hepatitis B virus is a member of the *Hepadnaviridae* family in the genus *Orthohepadnavirus*. The woodchuck hepatitis virus, the ground squirrel hepatitis virus, and the woolly monkey hepatitis B virus also belong to this genus. Other

viruses within the *Hepadnaviridae* infect birds (Avihepadnaviruses) and rodents (Orthohepadnaviruses). The virus that infects humans is thought to be specific to humans and chimpanzees and does not fully infect other animals (Wieland et al. 2004). This diverse host range suggests an ancient origin for HBV followed by a long period of adaptation to each host. Therefore, while these animals can provide insight into the HBV life cycle, key differences in required host factors and other species-specific adaptations must be considered.

1.2 Woodchuck Hepatitis Virus

Given the dearth of experimental models, woodchuck hepatitis virus (WHV) has often served as a useful system to investigate the HBV life cycle and interactions with immune effectors. As another member of the *Hepadnaviridae*, WHV shares similar morphology and genome organization with HBV but differs with respect to transcriptional regulation and pathogenesis. Laboratory maintained woodchuck colonies yield high rates of chronic infection and may develop hepatocellular carcinoma (HCC) (Tennant and Gerin 2001). The woodchuck (*Marmota monax*) is also not an ideal experimental organism due to a lack of information about its genome and immune system and practical experimental difficulties such as hibernation and lack of available reagents. Nonetheless, the *Marmota monax* genome sequence was recently published (Alioto et al. 2019), and the WHV model has been used to test antivirals and immunomodulatory drugs such as the TRL7 agonist GS-9620 (Menne et al. 2015) and has served as an important model for drug toxicity (Allweiss and Dandri 2016).

1.3 Duck Hepatitis B Virus

Duck hepatitis B virus is another useful infection model. The virus is a distant relative of human HBV and supports the full viral life cycle in duck hepatocyte tissue culture, including formation of cccDNA and production of DNA replication intermediates and HBc and HBs antigens (Tuttleman et al. 1986). However, key species-specific differences must be considered, including a critical role of carboxypeptidase D (CPD) for binding in duck but not in human (Spangenberg et al. 2001).

2 HBV In Vivo Experimental Models

Chronic HBV infection often requires life-long treatment with only a small likelihood of cure and a continuing risk of progressive liver disease. Although the need for novel therapies is clear, it is challenging to find animal models that adequately mimic the biochemical details of HBV infection in humans. HBV is indigenous to chimpanzees, gorillas, orangutans, gibbons, and other large primates, but these animals are impractical as experimental systems on ethical and logistical grounds.

Conversely, the lack of NTCP receptor prevents HBV infection in smaller primates. Nonetheless, despite the well-known species- and tissue-specificity of HBV, transduction of hNTCP in macaques, baboons, and pig hepatocytes confers support for HBV infection in vitro, suggesting that NTCP is largely responsible for this species barrier (Lempp et al. 2017). The advantages and disadvantages of several in vivo models are shown in Table 2.1.

2.1 Chimpanzee Model

Recently chimpanzees (*Pan troglodytes*) have received endangered species protection status, and their use in medical research has been expressly banned in the United States and other countries. However, chimpanzees have long played an important role in HBV research as the only primate that can fully support HBV infection (reviewed in (Wieland 2015)). After HBV was first characterized, HBsAg and anti-HBsAg antibodies were detected in blood drawn from chimpanzees (Hirschman et al. 1969; Lichter 1969; Maynard et al. 1971). It was shown that chimpanzees can become infected with as little as one to three genome equivalents of

Table 2.1 Advantages and disadvantages of in vivo HBV experimental models

Model	Advantages	Disadvantages
Chimpanzee	Fully infectious, immunocompetent, most similar to human infection	Banned in several countries, ethical and practical limitations, potential differences from human in innate immune response
Macaque	Smaller, readily available, fewer restrictions; naturally occurring HBV infection; transferable; recently updated reference genome	Not as well characterized as chimpanzee
Tree shrew	Can be infected with patient sera	Transient, self-limited infection; animal handling difficulties
Transgenic mice	Consistent and well-characterized inbred lines; can achieve high replication rates	Does not support full HBV life cycle and not useful for analysis of drug resistance
Human hepatocyte chimeric mice	Supports full HBV life cycle, can be used to compare different host and viral genotypes, avoids confounding effect of adaptive immune response	Does not reflect adaptive immune response; animals are delicate and expensive
BRGS-uPA mice	Immunocompetent; can be used to analyze NK cell, T cell, and antibody responses	Defects in NK cell maturation cause differences with respect to human response
Hydrodynamic injection	Immunocompetent; can be used to analyze viral mutants	Transient expression; technically difficult
Adenovirus-mediated delivery	Persistent viremia	Low-level viremia; vector induces immune response and inflammation
Adeno-associated virus delivery	Persistent viremia; useful for development of immune therapies	Suppresses immune response but can be overcome with agonist

HBV DNA isolated from human plasma (Barker et al. 1973; Komiya et al. 2008) and can develop chronic HBV infection similar to that of humans. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels are elevated (Tabor et al. 1983), but symptoms are less severe than in humans (Barker et al. 1973). Chimpanzees also played an important role in the development of the first vaccines because the immune response is similar to that of humans (McAuliffe et al. 1980). A large overlap was observed in T-cell peptide-binding specificity (McKinney et al. 2000), but gene expression analysis revealed lower than expected induction of interferon-stimulated genes (ISGs), suggesting that HBV is able to avoid detection by innate immune defenses during early infection (Wieland et al. 2004; Wieland and Chisari 2005). However, later in vivo and in vitro studies appear to show that HBV plays a more active role in suppressing the innate immune response in humans (Shlomai et al. 2014; Luangsay et al. 2015; Yoneda et al. 2016) perhaps revealing an important distinction between the human and chimpanzee immune responses.

2.2 Macaque Model

Other primate models either present the same problems as chimpanzees or fail to support HBV infection or serial passage. One exception is macaques (*Macaca fascicularis*), small Old World monkeys that have been shown to harbor HBV DNA, HBsAg, and HBcAg from a strain of HBV genotype D that probably originated in humans (Bukh et al. 2013; Dupinay et al. 2013). Transduction of hNTCP allows macaques to support in vitro and in vivo infection for six or more weeks and demonstrate cellular and humoral immune responses that make them suitable for testing antiviral and immunomodulatory drugs (Burwitz et al. 2017). Several problems, possibly related to inadequate hNTCP delivery, may limit the usefulness of this model, including poor infection rates and undetectable levels of cccDNA. Nonetheless, macaques are the most widely used nonhuman primate model in biomedical research. A greatly improved *Macaca mulatta* reference genome was recently published in which nearly 100 million genetic variants were characterized (https://science.sciencemag.org/content/370/6523/eabc6617), providing an impetus to further develop this primate model of HBV infection.

2.3 Tupaia Model

Although no longer classified as a primate, the tree shrew (*Tupaia belangeri*) provides another potential animal model for HBV infection. These small squirrel-like mammals are closely related to primates and can support HBV infection from patient sera in vivo (Walter et al. 1996). Isolated *Tupaia* hepatocytes support infection with HBV or woolly monkey hepatitis B virus and produce HBsAg and HBeAg (Walter et al. 1996; Kock et al. 2001). This model is notable for its critical role in the identification of NTCP as the primary HBV receptor (Yan et al. 2012). Tree shrews also support infection with hepatitis C virus and herpes simplex virus 1 and

2 (Walter et al. 1996; Tsukiyama-Kohara and Kohara 2014). Although promising, problems with handling of the animals have limited wide adoption of tree shrew as an animal model (Tsukiyama-Kohara and Kohara 2014).

2.4 Transgenic Mice

Attempts to improve infection efficiency using NTCP transgenic animal models have been disappointing, underscoring the need for greater insight into host factors mediating HBV host specificity (Li and Urban 2016). Transgenic mouse models have been developed that can partially support HBV replication (HBVtg), but they cannot be used to investigate critical early steps in viral entry or cccDNA formation (Ortega-Prieto et al. 2019). Although the mice are immunocompetent, they are nonetheless immune-tolerant to viral proteins, limiting their usefulness in investigating the adaptive immune response (Allweiss and Strick-Marchand 2020).

Development of animal models based on outbreeding animals such as duck and woodchuck is hindered by the problem of genetic variability that makes it more difficult to unravel the underlying immunobiology. Inbred transgenic mice have long provided a controlled genetic background for investigating HBV proteins. Lineages have been developed that express HBV surface, core, precore, and X proteins either individually or together, but replication efficiency is low (Araki et al. 1989; Farza et al. 1988). Mice harboring 1.3X-genome length HBV sequences have been developed that support higher replication efficiency (10^7–10^8 copies per mL) without inducing cellular damage. However, while models such as HBVtg can be used to investigate parts of the HBV life cycle, they do not undergo early stages such as viral entry and formation of cccDNA that are of great interest for development of antiviral therapies (Ortega-Prieto et al. 2019). Even though the mice have a functional adaptive immune system, they do not mount an immune response to the transgenic products and so are also unsuitable for analysis of the immune response (Allweiss and Strick-Marchand 2020). Similarly, it is difficult to evaluate viral clearance due to the presence of integrated HBV DNA (Yang et al. 2014).

2.5 Human Hepatocyte Chimeric Mice

Humanized mouse models in which human hepatocytes are transplanted into immunodeficient mice have successfully been used to investigate the early stages of HBV infection and to evaluate antiviral drugs. In the early trimera mouse model, human hepatocytes were transplanted into mouse kidneys (Ilan et al. 1999), Even though most mice became infected, viremia was poor (10^5 IU/mL), in part because key liver architectural features were lacking. Replication rates were improved in other mouse models by changing the genetic background of the mice to induce liver damage and promote establishment of human hepatocytes.

Transgenic mice were established in which the urokinase gene is regulated by the human albumin promoter. These urokinase-type plasminogen activator (uPA) mice

were then mated with severe combined immunodeficiency (SCID) mice (Heckel et al. 1990). Hepatocyte death in the uPA/SCID offspring causes subacute liver failure that is compensated via transplantation of human hepatocytes (Rhim et al. 1995). This mouse model supports infection with both HBV (Dandri et al. 2001) and HCV (Mercer et al. 2001). A small number of native mouse hepatocytes remain in the liver, and a herpes simplex virus type-1 thymidine kinase (HSVtk)/ganciclovir (GCV) system was developed to remove residual mouse hepatocytes, but the approach was unsuccessful (Douglas et al. 2010). However, measuring the level of human albumin provides an estimate of the repopulation rate and can be used to monitor graft failure. HBV has been found to remain infective after passage in mice (Tsuge et al. 2005; Meuleman et al. 2005; Sugiyama et al. 2006), and the model has made it possible to examine early host and innate immune responses and to evaluate therapeutic agents over the full viral life cycle.

Nonetheless, the model is limited due to the lack of key components of the innate immune response and the complete lack of an adaptive immune response that is required to avoid rejection (Li and Di Santo 2019). Chimeric mice also require a source of donor hepatocytes, although this may also be seen as an advantage because the effects of donors with different genotypes can be compared. Explanted hepatocytes can also be used as a source of human hepatocytes for in vitro experiments (Michailidis et al. 2020). An alternative approach is to generate chimeric mice using hepatocytes derived from iPS or dHepaRG cells (Yuan et al. 2018a, b), and mouse models with inducible liver failure have been developed using fumaryl acetoacetate (FAH)−/− mice in which accumulation of a toxic metabolite kills hepatocytes unless the mouse is supplied with 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cycloh exanedione (NTBC). Notably, male FAH−/− mice have higher mortality (Michailidis et al. 2020).

2.6 Immune-Competent Mouse Models

A major shortcoming of chimeric mouse models is the lack of an adaptive immune response, a critical factor in determining whether or not HBV is able to establish chronic infection. One approach to establishing an immune-competent HBV infection animal model is to transplant both human hepatocytes and human immune cells (Tzeng et al. 2013). Transplantation of fetal hematopoietic stem cells and hepatoblasts resulted in low chimerism and modest replication (Kremsdorf and Strick-Marchand 2017; Douam and Ploss 2018), although the use of oncostatin M has been shown to improve chimerism (Billerbeck et al. 2016). Formed by transplanting both human hepatocytes and hepatic stellate cells, BALB/cRag2$^{-/-}$IL2rg$^{-/-}$SIRPaNOD-uPA (BRGS-uPA) mice demonstrate a number of attractive features, including good infectivity over several months, a high repopulation rate, presence of NK cells, Kupffer cells, PD-1Hi effector memory T cells, and development of IgG antibodies against the HBV surface and core proteins (Allweiss and Dandri 2016; Kremsdorf and Strick-Marchand 2017; Dusseaux et al. 2017; Lopez-Lastra and Di Santo 2017). Although the presence of NK cells is a chief advantage of this model, deficient cytokine production in BRGS-uPA mice due to the lack of human MHC results in

defects in NK cell functionality relative to human NK cells (Lopez-Lastra and Di Santo 2017).

2.7 Hydrodynamic Injection HBV Mouse Model

Efficient delivery of HBV into mice also poses a challenge with respect to immune competence. Hydrodynamic injection is a technically challenging method in which a large volume of DNA is injected rapidly into the mouse tail vein. This hydrodynamic effect increases the pressure in the inferior vena cava, causing the viral DNA to pass through the hepatic portal vein and through the liver fenestrae where it comes into direct contact with hepatocytes (Tzeng et al. 2013). Yang et al. injected greater than full-length HBV genomic DNA (pT-MCS-HBV1.3) into the tail vein of both immune-competent mice and nonobese diabetic (NOD)/SCID mice lacking T, B, and natural killer (NK) cells (Yang et al. 2002). While HBV gene expression and viremia were observed in both mice, the virus disappeared rapidly in immunocompetent mice following CD8+ T cell proliferation, whereas the virus remained detectable for several months in the immunocompromised mice.

2.8 Adenovirus-Mediated Delivery

Another way to deliver viral DNA to the hepatocytes is via delivery with another virus. Infection with HBV DNA cloned into adenovirus or adeno-associated virus (AAV) yielded low-level per persistent viremia (Tzeng et al. 2013; Bramson et al. 1995; Huang et al. 2006). However, AAV both elicits an immune response, including the release of cytokines and chemokines, while also suppressing immune responses (Tzeng et al. 2013). Recent innovations, such as the less strongly immunogenic AAV2/8, have been used to establish persistent infections that last several months (Paulk et al. 2018). AAV can also be used to compare virus–host interactions and response to treatment with different HBV genotypes (Liu and Kao 2013), including genotypes A, B, and C (Huang et al. 2006; Li et al. 2013, 2016). While an advantage of AAV mouse models is that immunocompetent mice can be used, the full viral life cycle is not represented, as the virus is not able to reinfect hepatocytes, and it is not a suitable model to evaluate cccDNA (Lucifora et al. 2017). Furthermore, the murine immune response may not adequately reflect the human response.

3 HBV In Vitro Experimental Systems

Animal models are indispensable for elucidating complex host–virus interactions and evaluating antiviral therapies, but identification and testing of drug targets requires an efficient and reproducible in vitro model. Nonetheless, the development of a suitable in vitro model that supports viral entry and the complete viral life cycle has proven problematic. Each in vitro model has specific use cases as well as limitations, and no single model has so far proven superior for all applications (Table 2.2).

Table 2.2 Advantages and disadvantages of in vitro HBV experimental models

Model	Advantages	Disadvantages
Primary human hepatocytes	Gold standard for in vitro analysis of HBV infection and analysis of drug toxicity	Difficult to obtain, limited genetic variability, and rapid loss of infectivity
HepG2 cells	Produces HBV virions, polarized cells	Morphological and chromosomal differences from primary hepatocytes; does not support HBV entry
HepaRG cells	Supports replication of HBV and HDV; supports HBV entry	Requires time-consuming differentiation step; chromosomal differences
NTCP-expressing cells	Supports HBV entry and replication	Yield of HBs, HBe, and HBV DNA is low
Human hepatocytes isolated from chimeric mice	Has most advantages of human hepatocytes with few of the disadvantages, improved infectivity	Expensive, complex, requires proliferation in chimeric mice
2D/3D/microfluidic culture	More accurately reflect liver architecture and interactions among cell types	Complex; still shows differences compared to in vivo
Induced pluripotent stem cells	Supports HBV infection and analysis of innate immune response	Complex, heterogeneous, difficult to establish in culture

3.1 Primary Human Hepatocytes

Fresh primary human hepatocytes (PHHs) probably best recapitulate conditions in the liver and are a natural choice for in vitro analysis (Shimizu et al. 1986; Gripon et al. 1988, 1993; Ochiya et al. 1989; Galle et al. 1994), but there are a number of drawbacks in relying on them as an in vitro model of HBV infection. Not only are donor cells difficult to obtain and heterogeneous genetic backgrounds may introduce confounding, but the cells quickly begin to de-differentiate and lose the ability to support HBV infection due to changes in gene expression resulting in loss of hepatocyte-specific factors (Guillouzo et al. 2007; Wilkening and Bader 2003; Wilkening et al. 2003; Birkus et al. 2019). The strong tissue tropism exhibited by HBV is driven in part by liver-specific expression of NTCP as well as nuclear factors required for efficient transcription of the HBV genome. Treatment with dimethyl sulfoxide (DMSO) and dexamethasone and hydrocortisone helps to maintain hepatocyte differentiation and prolongs infectivity (Evripioti et al. 2019). Similarly, the use of polyethylene glycol and a high MOI helps to improve infection efficiency (Verrier et al. 2016a). Co-culture with mouse embryonic fibroblasts and establishment of 3D microfluidic liver culture also facilitates long-term infection by helping to recreate the functional architecture of the liver (Winer et al. 2017, 2020; Ortega-Prieto et al. 2018). However, availability remains a key limitation of PHHs. One solution is to further drive de-differentiation of PHHs to form liver progenitor cells then induce them to proliferate and re-differentiate into PHHs (Fu et al. 2019). Primary hepatocytes derived from chimpanzees can also support HBV infection but face many of the same issues as PHHs. Fortunately, tree shrew primary hepatocytes

also transiently support HBV infection and were instrumental in identifying NTCP as primary HBV receptor (Walter et al. 1996; Yan et al. 2014).

3.2 Hepatoma Cell Lines

Although PHHs provide an attractive HBV infection model, they are not ideal. Aside from being difficult to acquire, fresh PHHs rapidly de-differentiate and no longer support HBV infection. To overcome these limitations, hepatoma cell lines have been widely used to investigate mechanisms of viral replication, identify host factors, and evaluate drug candidates. Unfortunately, HepG2 and Huh7 cell lines do not support HBV entry due to lack of hNTCP expression, and instead HBV production is made possible by transfecting or integrating HBV genomes (Verrier et al. 2016a).

3.3 HepG2 Cells

Given the limited availability and short window of infectivity of PHHs, hepatoma cell lines offer a number of potential advantages. Derived from a hepatocellular carcinoma in an adolescent male, HepG2 cell lines such as HepG2.2.15 support HBV production using transfected HBV DNA (Sells et al. 1987; Ladner et al. 1997). HepG2 cells do not support HBV entry but retain important aspects of liver microarchitecture, including polarization into basolateral and apical domains (Glebe and Urban 2007). Nonetheless, HepG2 cells differ with respect to morphology, chromosome number, and the number of nuclei (Wilkening et al. 2003; Natarajan and Darroudi 1991). While housekeeping genes and some liver-specific genes are expressed at comparable levels to PHHs, several key transcription factors and enzymes, such as C/EBP-α and CYP3A, are expressed poorly in HepG2 cells (Wilkening et al. 2003; Knowles et al. 1980; Rodriguez-Antona et al. 2002; Jover et al. 2001). Such differences complicate drug development and identification of essential host factors.

3.4 HepaRG Cells

The HepaRG cell line was derived from an HCV-associated hepatocellular carcinoma (Guillouzo et al. 2007) and is characterized by an additional chromosome 7 and a translocation between chromosomes 12 and 22 that resulted in a deletion of the 12p region (Gripon et al. 2002). HepaRG cells support HBV and HDV infection and (Gripon et al. 2002; Hantz et al. 2009) but first require a time-consuming differentiation process. Addition of DMSO and hydrocortisone hemisuccinate induces differentiation into hepatocytes and biliary cells. Hepatocytes maintain stable expression of liver-specific factors for over 6 weeks, but albumin levels are variable, and CYP3A4 and CYP7A1 levels are strongly upregulated relative to PHHs (Kanebratt and Andersson 2008a, b).

3.5 HepCHLine-4 Cell

The key to improving HBV infectivity in vitro may be to combine the infectivity of primary human hepatocytes with the advantages of hepatoma cell lines. To this end, Jiang et al. fused primary human hepatocytes with HepG2 cells to create the HepCHLine-4 (Jiang et al. 2009). These cells remained susceptible to HBV infection even after a year of subculturing and produce cccDNA and viral particles.

3.6 Recombinant cccDNA

Given the importance of cccDNA as a key target in ongoing drug development, reliance on HBV plasmids is not ideal. Instead site-specific DNA recombination and minicircle technology have made it possible to deliver recombinant cccDNA molecules into hepatoma cells (Yan et al. 2017). rcccDNA support stable HBV production, and use of a luciferase reporter system facilitates development of compounds that target cccDNA, but the model cannot be used to examine viral entry (Allweiss and Strick-Marchand 2020) and delivery of rcccDNA into mice through hydrodynamic injection or adenovirus is technically challenging and may induce inflammation in the liver (Yan et al. 2017; Li et al. 2018).

3.7 NTCP Expression as a Limiting Factor for HBV Infection

The revelation that NTCP serves as the primary HBV receptor went far to help understand the species- and tissue-specificity of HBV infection and revealed a potential approach to develop new infection models (Watashi et al. 2014). The lack of robust NTCP expression in hepatoma cell lines prevents HBV from binding and entering HepG2 cells, and the rapid decrease in NTCP expression in PHH cell culture limits the ability of cultured cells to maintain long-term infection (Chen and Ye 2012). While HepaRG cells do express NTCP, the orientation of the basolateral membrane limits physical access by the virus (Schulze et al. 2012).

3.8 NTCP-Expressing Cell Lines.

Once the identity of the HBV receptor was known, it became possible to modify existing hepatoma cells to support HBV entry and spread among cells (Li and Urban 2016), leading to the development of NTCP-expressing cell lines such as hNTCP-HepaRG, hNTCP-Huh, hNTCP-HepG2, and hNTCP-HEK293 (Yan et al. 2012; Iwamoto et al. 2014). For example, Iwamoto et al. transfected an NTCP expression plasmid into HepG2 (Iwamoto et al. 2014). The resulting HepG2-NTCP-C4 cells could be infected with serum-derived HBV with an infection rate close to 50%. Yan et al. transfected the pcDNA6-NTCP plasmid into HepG2 cells to produce the HepG2-NTCP12 line and improved the initially low infection rate using

centrifugation (Yan et al. 2015). Although NTCP expressing cell lines are promising, production of HBs antigen, HBe antigen, and HBV DNA remain low even with the use of a high viral titer (6000–18,000 GEq/cell) (Yan et al. 2012; Iwamoto et al. 2013; Ni et al. 2014). While NTCP is the primary receptor for HBV, co-receptors or other host factors that are deficient in hepatoma cells probably assist in viral entry or replication. For example, RNA silencing of glypican 5 (GPC5) hindered HBV binding and suppressed HBsAg and HBV pgRNA levels, suggesting that GPC5 plays an accessory role in HBV entry (Verrier et al. 2016b). Similarly, Iwamoto et al. recently showed that epidermal growth factor receptor (EGFR) is critical for internalization of bound virions (Iwamoto et al. 2019). It is likely to be necessary to induce expression of additional host factors in order to achieve efficient HBV replication in hepatoma cells (Tnani and Bayard 1999).

3.9 Human Hepatocytes Isolated from Humanized Mice

Given the variety of challenges of restoring hepatocyte-specific gene expression in hepatoma-derived cell lines, improving the cell culture properties of primary hepatocytes remains an important goal. While PHHs are known to rapidly lose infectivity in culture, Ishida et al. noted that human hepatocytes explanted from human hepatocyte chimeric mice tended to remain infective longer (Ishida et al. 2015). They proposed the humanized mouse model as a source of primary human hepatocytes (Fig. 2.1). Cryopreserved hepatocytes from a single donor are transplanted into uPA/SCID mice (Tateno et al. 2004), allowed to proliferate, and then isolated using a two-step collagenase perfusion method and cultured in hepatocyte clonal growth medium. While the need for an animal model as a first step in establishing an in vitro model is expensive and complex, this approach offers several advantages, including a 500–1000 fold increase in yield in the number of cells derived from the

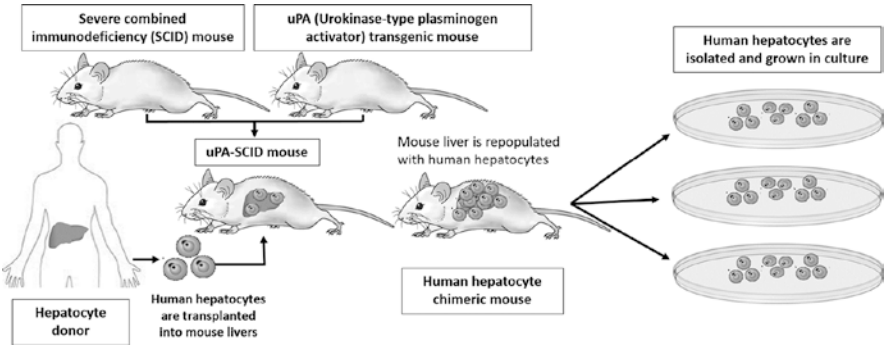

Fig. 2.1 Human hepatocyte chimeric mice as a source of primary human hepatocytes. Human hepatocyte chimeric mice are prepared by transplanting cryopreserved human hepatocytes from a donor into urokinase-type plasminogen activator-transgenic/severely combined immunodeficient (uPA/SCID) mice. Hepatocytes divide several times and repopulate the mouse liver. Human hepatocytes are then isolated from the mouse livers and grown in culture

same donor, as well as better homogeneity and higher rates of infection and virus production than PHHs, HepaRG, and NTCP-HepG2 cell lines.

3.10 Non-Cancer-Derived Immortalized Human Hepatocytes

The use of cancer cells has made it possible to analyze the HBV life cycle in detail, but cancer cell lines differ from primary hepatocytes in a number of ways. E/NtG8 cells are immortalized NTCP expressing human hepatocytes that do not derive from a cancer cell line (Akahori et al. 2020). When cultured under three-dimensional conditions, the cells support infection with HBV from blood as well as from recombinant HBV from culture, suggesting that E/NtG8 cells may support investigation of the HBV life cycle under conditions that better replicate the liver environment.

3.11 Improvements to Primary Hepatocyte Culturing

One disadvantage of cell culture is that normal interactions among cells of the same and different types cannot be fully recreated. Hepatocytes are by far the most common cells in the liver (80%), but the remaining 20% of cells are also important in establishing the functional architecture of the sinusoid. A number of approaches have been made to more accurately model the liver microenvironment, including 2D, 3D, or microfluidic culture, and co-culture with non-parenchymal cells (Shlomai et al. 2014; Godoy et al. 2013; Petropolis et al. 2016). Although complex, these approaches might yield insight into early steps in infection that are difficult to examine by other means, such as passing through the sinusoidal endothelial barrier and interaction with Kupffer cells and hepatic stellate cells (Petropolis et al. 2016).

3.12 Hepatic Cell Lines Derived from Human Induced Pluripotent Stem Cells

Unraveling the formation and maintenance of cccDNA represents a key goal in the development of HBV therapies. While PHHs recapitulate some aspects of the cellular environment within the liver, PHHs are in limited supply and lose infectivity rapidly. An alternative approach is to induce pluripotency in somatic cells and then drive hepatocyte-specific cell differentiation. For example, stem cell-derived hepatocyte-like cells (HLCs) possess characteristics of mature hepatocytes and can be used for drug testing (Xia et al. 2017). Kaneko et al. developed two induced pluripotent stem cell (iPS) models of HBV infection: immature proliferating progenitor-like cells (iPS-HPCs), and differentiated hepatocyte-like cells (iPS-Heps) (Kaneko et al. 2016). HBV replicates successfully and induces a primary hepatocyte-like innate immune response in both cell lines. While iPS-Hep cells supported higher infection efficiency, iPS-HPC cells were more homogeneous and easier to culture. Overexpression of NTCP helped to improve infection efficiency in iPS-HPC cells. cccDNA is detectable in these cells

and is maintained by inhibition of the Janus-kinase pathway. Therefore, iPS cells can serve as a suitable substitute for primary human hepatocytes for large-scale applications such as drug screening (Kaneko et al. 2016).

4 Conclusions

In hindsight, it is remarkable how much progress has been made in unraveling the HBV life cycle and developing antiviral therapies given the lack of animal models and limitations in the ability to infect hepatocyte-derived cells in vitro. Nonetheless, infection with related but distinct viruses or delivery methods that omit part of the viral life cycle, as well as the use of immunocompromised non-primate animal models, has in some cases led to confusion in interpreting the role of host factors and identifying drug targets, suggesting that candidate drugs must be evaluated using more than one model (Allweiss and Strick-Marchand 2020). Most notably, this ambiguity long delayed the discovery of the primary HBV receptor. The lack of NTCP in hepatoma cell lines and small animal models explained much of the species and tissue specificity of HBV, and it became possible to modify existing cell lines to gain or improve infectivity. While overexpression of NTCP can allow HBV to enter previously non-susceptible cells, infection rates are typically far below maximum levels observed in vivo, suggesting that additional factors may be involved that are lacking in cultured cells. Improvements in primary hepatocyte cell culture have also helped to overcome problems due to use of immortalized cell lines. Recent attempts to recreate the microarchitecture and cell-to-cell interactions of the liver help to bridge the gap between in vivo and in vitro experiments and provide insight into aspects of HBV binding and viral spread that are difficult to address by other methods. While the chimpanzee is no longer a viable option as an animal model in many countries, several other primates and small animal models have been established, including tree shrews and macaques. Similarly, human hepatocyte chimeric mice, as well as immunocompetent mouse models, can be used to examine different aspects of the immune response in a mouse model. Each experimental system has advantages and disadvantages, and no single system is currently useful for all purposes, but the current arsenal of models and delivery methods are suitable for a wide range of research questions. The stage is finally set for a new era in research into the treatment of chronic HBV infection.

Conflict of Interest Kazuaki Chayama has received honoraria from Bristol-Myers Squibb and MSD K.K., AbbVie, Gilead Science, Dainippon Sumitomo Pharma, and Mitsubishi Tanabe Pharma and research funding from Gilead Science, Dainippon Sumitomo Pharma, MSD K.K., AbbVie, Eisai, TORAY, Otsuka Pharma, Chugai Pharma, Takeda Pharma, and Roche.

Financial Support The study was supported in part by research funding from the Research Program on Hepatitis from the Japan Agency for Medical Research and Development, AMED (grant number: 19fk0210020h0003).

References

Akahori Y, Kato H, Fujita T, Moriishi K, Tanaka Y, Watashi K, et al. Establishment of a novel hepatitis B virus culture system using immortalized human hepatocytes. Sci Rep. 2020;10(1):21718. https://doi.org/10.1038/s41598-020-78655-x.

Alioto TS, Cruz F, Gomez-Garrido J, Triyatni M, Gut M, Frias L, et al. The Genome Sequence of the Eastern Woodchuck (*Marmota monax*) - a preclinical animal model for chronic hepatitis B. G3 (Bethesda). 2019;9(12):3943–52. https://doi.org/10.1534/g3.119.400413.

Allweiss L, Dandri M. Experimental in vitro and in vivo models for the study of human hepatitis B virus infection. J Hepatol. 2016;64(1 Suppl):S17–31. https://doi.org/10.1016/j.jhep.2016.02.012.

Allweiss L, Strick-Marchand H. In-vitro and in-vivo models for hepatitis B cure research. Curr Opin HIV AIDS. 2020;15(3):173–9. https://doi.org/10.1097/COH.0000000000000616.

Araki K, Miyazaki J, Hino O, Tomita N, Chisaka O, Matsubara K, et al. Expression and replication of hepatitis B virus genome in transgenic mice. Proc Natl Acad Sci U S A. 1989;86(1):207–11.

Barker LF, Chisari FV, McGrath PP, Dalgard DW, Kirschstein RL, Almeida JD, et al. Transmission of type B viral hepatitis to chimpanzees. J Infect Dis. 1973;127(6):648–62.

Billerbeck E, Mommersteeg MC, Shlomai A, Xiao JW, Andrus L, Bhatta A, et al. Humanized mice efficiently engrafted with fetal hepatoblasts and syngeneic immune cells develop human monocytes and NK cells. J Hepatol. 2016;65(2):334–43. https://doi.org/10.1016/j.jhep.2016.04.022.

Birkus G, Snyder C, Jordan R, Kobayashi T, Dick R, Puscau V, et al. Anti-HBV activity of retinoid drugs in vitro versus in vivo. Antivir Res. 2019;169:104538. https://doi.org/10.1016/j.antiviral.2019.104538.

Bramson JL, Graham FL, Gauldie J. The use of adenoviral vectors for gene therapy and gene transfer in vivo. Curr Opin Biotechnol. 1995;6(5):590–5.

Bukh J, Lanford RE, Purcell RH. Persistent human hepatitis B virus infection in cynomolgus monkeys: a novel animal model in the search for a cure? Hepatology. 2013;58(5):1533–6. https://doi.org/10.1002/hep.26560.

Burwitz BJ, Wettengel JM, Muck-Hausl MA, Ringelhan M, Ko C, Festag MM, et al. Hepatocytic expression of human sodium-taurocholate cotransporting polypeptide enables hepatitis B virus infection of macaques. Nat Commun. 2017;8(1):2146. https://doi.org/10.1038/s41467-017-01953-y.

Chen ZJ, Ye J. Getting to grips with hepatitis. Elife. 2012;1:e00301. https://doi.org/10.7554/eLife.00301.

Dandri M, Burda MR, Torok E, Pollok JM, Iwanska A, Sommer G, et al. Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. Hepatology. 2001;33(4):981–8. https://doi.org/10.1053/jhep.2001.23314.

Douam F, Ploss A. The use of humanized mice for studies of viral pathogenesis and immunity. Curr Opin Virol. 2018;29:62–71. https://doi.org/10.1016/j.coviro.2018.03.003.

Douglas DN, Kawahara T, Sis B, Bond D, Fischer KP, Tyrrell DL, et al. Therapeutic efficacy of human hepatocyte transplantation in a SCID/uPA mouse model with inducible liver disease. PLoS One. 2010;5(2):e9209. https://doi.org/10.1371/journal.pone.0009209.

Dupinay T, Gheit T, Roques P, Cova L, Chevallier-Queyron P, Tasahsu SI, et al. Discovery of naturally occurring transmissible chronic hepatitis B virus infection among *Macaca fascicularis* from Mauritius Island. Hepatology. 2013;58(5):1610–20. https://doi.org/10.1002/hep.26428.

Dusseaux M, Masse-Ranson G, Darche S, Ahodantin J, Li Y, Fiquet O, et al. Viral load affects the immune response to HBV in mice with humanized immune system and liver. Gastroenterology. 2017;153(6):1647–61. e9. https://doi.org/10.1053/j.gastro.2017.08.034.

Evripioti AA, Ortega-Prieto AM, Skelton JK, Bazot Q, Dorner M. Phosphodiesterase-induced cAMP degradation restricts hepatitis B virus infection. Philos Trans R Soc Lond Ser B Biol Sci. 2019;374(1773):20180292. https://doi.org/10.1098/rstb.2018.0292.

Farza H, Hadchouel M, Scotto J, Tiollais P, Babinet C, Pourcel C. Replication and gene expression of hepatitis B virus in a transgenic mouse that contains the complete viral genome. J Virol. 1988;62(11):4144–52.

Fu GB, Huang WJ, Zeng M, Zhou X, Wu HP, Liu CC, et al. Expansion and differentiation of human hepatocyte-derived liver progenitor-like cells and their use for the study of hepatotropic pathogens. Cell Res. 2019;29(1):8–22. https://doi.org/10.1038/s41422-018-0103-x.

Galle PR, Hagelstein J, Kommerell B, Volkmann M, Schranz P, Zentgraf H. In vitro experimental infection of primary human hepatocytes with hepatitis B virus. Gastroenterology. 1994;106(3):664–73.

Glebe D, Urban S. Viral and cellular determinants involved in hepadnaviral entry. World J Gastroenterol. 2007;13(1):22–38.

Godoy P, Hewitt NJ, Albrecht U, Andersen ME, Ansari N, Bhattacharya S, et al. Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. Arch Toxicol. 2013;87(8):1315–530. https://doi.org/10.1007/s00204-013-1078-5.

Gripon P, Diot C, Theze N, Fourel I, Loreal O, Brechot C, et al. Hepatitis B virus infection of adult human hepatocytes cultured in the presence of dimethyl sulfoxide. J Virol. 1988;62(11):4136–43.

Gripon P, Diot C, Guguen-Guillouzo C. Reproducible high level infection of cultured adult human hepatocytes by hepatitis B virus: effect of polyethylene glycol on adsorption and penetration. Virology. 1993;192(2):534–40. https://doi.org/10.1006/viro.1993.1069.

Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, et al. Infection of a human hepatoma cell line by hepatitis B virus. Proc Natl Acad Sci U S A. 2002;99(24):15655–60. https://doi.org/10.1073/pnas.232137699.

Guillouzo A, Corlu A, Aninat C, Glaise D, Morel F, Guguen-Guillouzo C. The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. Chem Biol Interact. 2007;168(1):66–73. https://doi.org/10.1016/j.cbi.2006.12.003.

Hantz O, Parent R, Durantel D, Gripon P, Guguen-Guillouzo C, Zoulim F. Persistence of the hepatitis B virus covalently closed circular DNA in HepaRG human hepatocyte-like cells. J Gen Virol. 2009;90(Pt 1):127–35. https://doi.org/10.1099/vir.0.004861-0.

Heckel JL, Sandgren EP, Degen JL, Palmiter RD, Brinster RL. Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. Cell. 1990;62(3):447–56.

Hirschman RJ, Shulman NR, Barker LF, Smith KO. Virus-like particles in sera of patients with infectious and serum hepatitis. JAMA. 1969;208(9):1667–70.

Huang LR, Wu HL, Chen PJ, Chen DS. An immunocompetent mouse model for the tolerance of human chronic hepatitis B virus infection. Proc Natl Acad Sci U S A. 2006;103(47):17862–7. https://doi.org/10.1073/pnas.0608578103.

Ilan E, Burakova T, Dagan S, Nussbaum O, Lubin I, Eren R, et al. The hepatitis B virus-trimera mouse: a model for human HBV infection and evaluation of anti-HBV therapeutic agents. Hepatology. 1999;29(2):553–62. https://doi.org/10.1002/hep.510290228.

Ishida Y, Yamasaki C, Yanagi A, Yoshizane Y, Fujikawa K, Watashi K, et al. Novel robust in vitro hepatitis B virus infection model using fresh human hepatocytes isolated from humanized mice. Am J Pathol. 2015;185(5):1275–85. https://doi.org/10.1016/j.ajpath.2015.01.028.

Iwamoto M, Watashi K, Tsukuda S, Aly HH, Fukasawa M, Fujimoto A, et al. Evaluation and identification of hepatitis B virus entry inhibitors using HepG2 cells overexpressing a membrane transporter NTCP. Biochem Biophys Res Commun. 2013; https://doi.org/10.1016/j.bbrc.2013.12.052.

Iwamoto M, Watashi K, Tsukuda S, Aly HH, Fukasawa M, Fujimoto A, et al. Evaluation and identification of hepatitis B virus entry inhibitors using HepG2 cells overexpressing a membrane transporter NTCP. Biochem Biophys Res Commun. 2014;443(3):808–13. https://doi.org/10.1016/j.bbrc.2013.12.052.

Iwamoto M, Saso W, Sugiyama R, Ishii K, Ohki M, Nagamori S, et al. Epidermal growth factor receptor is a host-entry cofactor triggering hepatitis B virus internalization. Proc Natl Acad Sci U S A. 2019;116(17):8487–92. https://doi.org/10.1073/pnas.1811064116.

Jiang Y, Wang AH, Shao LH, Wang G, Yao YY, Sai LT, et al. A new cell culture system for infection with hepatitis B virus that fuses HepG2 cells with primary human hepatocytes. J Int Med Res. 2009;37(3):650–61.

Jover R, Bort R, Gomez-Lechon MJ, Castell JV. Cytochrome P450 regulation by hepatocyte nuclear factor 4 in human hepatocytes: a study using adenovirus-mediated antisense targeting. Hepatology. 2001;33(3):668–75. https://doi.org/10.1053/jhep.2001.22176.

Kanebratt KP, Andersson TB. HepaRG cells as an in vitro model for evaluation of cytochrome P450 induction in humans. Drug Metab Dispos. 2008a;36(1):137–45. https://doi.org/10.1124/dmd.107.017418.

Kanebratt KP, Andersson TB. Evaluation of HepaRG cells as an in vitro model for human drug metabolism studies. Drug Metab Dispos. 2008b;36(7):1444–52. https://doi.org/10.1124/dmd.107.020016.

Kaneko S, Kakinuma S, Asahina Y, Kamiya A, Miyoshi M, Tsunoda T, et al. Human induced pluripotent stem cell-derived hepatic cell lines as a new model for host interaction with hepatitis B virus. Sci Rep. 2016;6:29358. https://doi.org/10.1038/srep29358.

Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science. 1980;209(4455):497–9.

Kock J, Nassal M, MacNelly S, Baumert TF, Blum HE, von Weizsacker F. Efficient infection of primary tupaia hepatocytes with purified human and woolly monkey hepatitis B virus. J Virol. 2001;75(11):5084–9. https://doi.org/10.1128/JVI.75.11.5084-5089.2001.

Komiya Y, Katayama K, Yugi H, Mizui M, Matsukura H, Tomoguri T, et al. Minimum infectious dose of hepatitis B virus in chimpanzees and difference in the dynamics of viremia between genotype A and genotype C. Transfusion. 2008;48(2):286–94. https://doi.org/10.1111/j.1537-2995.2007.01522.x.

Kremsdorf D, Strick-Marchand H. Modeling hepatitis virus infections and treatment strategies in humanized mice. Curr Opin Virol. 2017;25:119–25. https://doi.org/10.1016/j.coviro.2017.07.029.

Kwon H, Lok AS. Hepatitis B therapy. Nat Rev Gastroenterol Hepatol. 2011;8(5):275–84. https://doi.org/10.1038/nrgastro.2011.33.

Ladner SK, Otto MJ, Barker CS, Zaifert K, Wang GH, Guo JT, et al. Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication. Antimicrob Agents Chemother. 1997;41(8):1715–20.

Lempp FA, Wiedtke E, Qu B, Roques P, Chemin I, Vondran FWR, et al. Sodium taurocholate cotransporting polypeptide is the limiting host factor of hepatitis B virus infection in macaque and pig hepatocytes. Hepatology. 2017;66(3):703–16. https://doi.org/10.1002/hep.29112.

Li Y, Di Santo JP. Modeling infectious diseases in mice with a "humanized" immune system. Microbiol Spectr. 2019; https://doi.org/10.1128/microbiolspec.BAI-0019-2019.

Li W, Urban S. Entry of hepatitis B and hepatitis D virus into hepatocytes: basic insights and clinical implications. J Hepatol. 2016;64(1 Suppl):S32–40. https://doi.org/10.1016/j.jhep.2016.02.011.

Li L, Shen H, Li A, Zhang Z, Wang B, Wang J, et al. Inhibition of hepatitis B virus (HBV) gene expression and replication by HBx gene silencing in a hydrodynamic injection mouse model with a new clone of HBV genotype B. Virol J. 2013;10:214. https://doi.org/10.1186/1743-422X-10-214.

Li X, Liu G, Chen M, Yang Y, Xie Y, Kong X. A novel hydrodynamic injection mouse model of HBV genotype C for the study of HBV biology and the anti-viral activity of lamivudine. Hepat Mon. 2016;16(2):e34420. https://doi.org/10.5812/hepatmon.34420.

Li G, Zhu Y, Shao D, Chang H, Zhang X, Zhou D, et al. Recombinant covalently closed circular DNA of hepatitis B virus induces long-term viral persistence with chronic hepatitis in a mouse model. Hepatology. 2018;67(1):56–70. https://doi.org/10.1002/hep.29406.

Lichter EA. Chimpanzee antibodies to Australia antigen. Nature. 1969;224(5221):810–1.

Liu CJ, Kao JH. Global perspective on the natural history of chronic hepatitis B: role of hepatitis B virus genotypes A to J. Semin Liver Dis. 2013;33(2):97–102. https://doi.org/10.1055/s-0033-1345716.

Lopez-Lastra S, Di Santo JP. Modeling natural killer cell targeted immunotherapies. Front Immunol. 2017;8:370. https://doi.org/10.3389/fimmu.2017.00370.

Luangsay S, Gruffaz M, Isorce N, Testoni B, Michelet M, Faure-Dupuy S, et al. Early inhibition of hepatocyte innate responses by hepatitis B virus. J Hepatol. 2015;63(6):1314–22. https://doi.org/10.1016/j.jhep.2015.07.014.

Lucifora J, Salvetti A, Marniquet X, Mailly L, Testoni B, Fusil F, et al. Detection of the hepatitis B virus (HBV) covalently-closed-circular DNA (cccDNA) in mice transduced with a recombinant AAV-HBV vector. Antivir Res. 2017;145:14–9. https://doi.org/10.1016/j.antiviral.2017.07.006.

Maini MK, Burton AR. Restoring, releasing or replacing adaptive immunity in chronic hepatitis B. Nat Rev Gastroenterol Hepatol. 2019;16(11):662–75. https://doi.org/10.1038/s41575-019-0196-9.

Maynard JE, Hartwell WV, Berquist KR. Hepatitis-associated antigen in chimpanzees. J Infect Dis. 1971;123(6):660–4.

McAuliffe VJ, Purcell RH, Gerin JL. Type B hepatitis: a review of current prospects for a safe and effective vaccine. Rev Infect Dis. 1980;2(3):470–92.

McKinney DM, Erickson AL, Walker CM, Thimme R, Chisari FV, Sidney J, et al. Identification of five different Patr class I molecules that bind HLA supertype peptides and definition of their peptide binding motifs. J Immunol. 2000;165(8):4414–22.

Menne S, Tumas DB, Liu KH, Thampi L, AlDeghaither D, Baldwin BH, et al. Sustained efficacy and seroconversion with the Toll-like receptor 7 agonist GS-9620 in the Woodchuck model of chronic hepatitis B. J Hepatol. 2015;62(6):1237–45. https://doi.org/10.1016/j.jhep.2014.12.026.

Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, et al. Hepatitis C virus replication in mice with chimeric human livers. Nat Med. 2001;7(8):927–33. https://doi.org/10.1038/90968.

Meuleman P, Libbrecht L, De Vos R, de Hemptinne B, Gevaert K, Vandekerckhove J, et al. Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. Hepatology. 2005;41(4):847–56. https://doi.org/10.1002/hep.20657.

Michailidis E, Vercauteren K, Mancio-Silva L, Andrus L, Jahan C, Ricardo-Lax I, et al. Expansion, in vivo-ex vivo cycling, and genetic manipulation of primary human hepatocytes. Proc Natl Acad Sci U S A. 2020;117(3):1678–88. https://doi.org/10.1073/pnas.1919035117.

Natarajan AT, Darroudi F. Use of human hepatoma cells for in vitro metabolic activation of chemical mutagens/carcinogens. Mutagenesis. 1991;6(5):399–403.

Ni Y, Lempp FA, Mehrle S, Nkongolo S, Kaufman C, Falth M, et al. Hepatitis B and D viruses exploit sodium taurocholate co-transporting polypeptide for species-specific entry into hepatocytes. Gastroenterology. 2014;146(4):1070–83. https://doi.org/10.1053/j.gastro.2013.12.024.

Ochiya T, Tsurimoto T, Ueda K, Okubo K, Shiozawa M, Matsubara K. An in vitro system for infection with hepatitis B virus that uses primary human fetal hepatocytes. Proc Natl Acad Sci U S A. 1989;86(6):1875–9.

Ortega-Prieto AM, Skelton JK, Wai SN, Large E, Lussignol M, Vizcay-Barrena G, et al. 3D microfluidic liver cultures as a physiological preclinical tool for hepatitis B virus infection. Nat Commun. 2018;9(1):682. https://doi.org/10.1038/s41467-018-02969-8.

Ortega-Prieto AM, Cherry C, Gunn H, Dorner M. In vivo model systems for hepatitis B virus research. ACS Infect Dis. 2019;5(5):688–702. https://doi.org/10.1021/acsinfecdis.8b00223.

Paulk NK, Pekrun K, Zhu E, Nygaard S, Li B, Xu J, et al. Bioengineered AAV capsids with combined high human liver transduction in vivo and unique humoral seroreactivity. Mol Ther. 2018;26(1):289–303. https://doi.org/10.1016/j.ymthe.2017.09.021.

Petropolis DB, Faust DM, Tolle M, Riviere L, Valentin T, Neuveut C, et al. Human liver infection in a dish: easy-to-build 3D liver models for studying microbial infection. PLoS One. 2016;11(2):e0148667. https://doi.org/10.1371/journal.pone.0148667.

Rhim JA, Sandgren EP, Palmiter RD, Brinster RL. Complete reconstitution of mouse liver with xenogeneic hepatocytes. Proc Natl Acad Sci U S A. 1995;92(11):4942–6.

Rodriguez-Antona C, Donato MT, Boobis A, Edwards RJ, Watts PS, Castell JV, et al. Cytochrome P450 expression in human hepatocytes and hepatoma cell lines: molecular mechanisms that determine lower expression in cultured cells. Xenobiotica. 2002;32(6):505–20. https://doi.org/10.1080/00498250210128675.

Schulze A, Mills K, Weiss TS, Urban S. Hepatocyte polarization is essential for the productive entry of the hepatitis B virus. Hepatology. 2012;55(2):373–83. https://doi.org/10.1002/hep.24707.

Sells MA, Chen ML, Acs G. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. Proc Natl Acad Sci U S A. 1987;84(4):1005–9.

Shimizu Y, Nambu S, Kojima T, Sasaki H. Replication of hepatitis B virus in culture systems with adult human hepatocytes. J Med Virol. 1986;20(4):313–27.

Shlomai A, Schwartz RE, Ramanan V, Bhatta A, de Jong YP, Bhatia SN, et al. Modeling host interactions with hepatitis B virus using primary and induced pluripotent stem cell-derived hepatocellular systems. Proc Natl Acad Sci U S A. 2014;111(33):12193–8. https://doi.org/10.1073/pnas.1412631111.

Spangenberg HC, Lee HB, Li J, Tan F, Skidgel R, Wands JR, et al. A short sequence within domain C of duck carboxypeptidase D is critical for duck hepatitis B virus binding and determines host specificity. J Virol. 2001;75(22):10630–42. https://doi.org/10.1128/JVI.75.22.10630-10642.2001.

Sugiyama M, Tanaka Y, Kato T, Orito E, Ito K, Acharya SK, et al. Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. Hepatology. 2006;44(4):915–24. https://doi.org/10.1002/hep.21345.

Tabor E, Purcell RH, Gerety RJ. Primate animal models and titered inocula for the study of human hepatitis A, hepatitis B, and non-A, non-B hepatitis. J Med Primatol. 1983;12(6):305–18.

Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol. 2004;165(3):901–12. https://doi.org/10.1016/S0002-9440(10)63352-4.

Tennant BC, Gerin JL. The woodchuck model of hepatitis B virus infection. ILAR J. 2001;42(2):89–102.

Tnani M, Bayard BA. Evidence for IRF-1-dependent gene expression deficiency in interferon unresponsive HepG2 cells. Biochim Biophys Acta. 1999;1451(1):59–72.

Tsuge M, Hiraga N, Takaishi H, Noguchi C, Oga H, Imamura M, et al. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. Hepatology. 2005;42(5):1046–54. https://doi.org/10.1002/Hep.20892.

Tsukiyama-Kohara K, Kohara M. *Tupaia belangeri* as an experimental animal model for viral infection. Exp Anim. 2014;63(4):367–74.

Tuttleman JS, Pugh JC, Summers JW. In vitro experimental infection of primary duck hepatocyte cultures with duck hepatitis B virus. J Virol. 1986;58(1):17–25.

Tzeng HT, Hsu PN, Chen PJ. Immunocompetent nontransgenic mouse models for studying hepatitis B virus immune responses. J Gastroenterol Hepatol. 2013;28(Suppl 1):116–9. https://doi.org/10.1111/jgh.12035.

Verrier ER, Colpitts CC, Schuster C, Zeisel MB, Baumert TF. Cell culture models for the investigation of hepatitis B and D virus infection. Viruses. 2016a; https://doi.org/10.3390/v8090261.

Verrier ER, Colpitts CC, Bach C, Heydmann L, Weiss A, Renaud M, et al. A targeted functional RNA interference screen uncovers glypican 5 as an entry factor for hepatitis B and D viruses. Hepatology. 2016b;63(1):35–48. https://doi.org/10.1002/hep.28013.

Walter E, Keist R, Niederost B, Pult I, Blum HE. Hepatitis B virus infection of tupaia hepatocytes in vitro and in vivo. Hepatology. 1996;24(1):1–5. https://doi.org/10.1002/hep.510240101.

Watashi K, Urban S, Li W, Wakita T. NTCP and beyond: opening the door to unveil hepatitis B virus entry. Int J Mol Sci. 2014;15(2):2892–905. https://doi.org/10.3390/ijms15022892.

Wieland SF. The chimpanzee model for hepatitis B virus infection. Cold Spring Harb Perspect Med. 2015; https://doi.org/10.1101/cshperspect.a021469.

Wieland SF, Chisari FV. Stealth and cunning: hepatitis B and hepatitis C viruses. J Virol. 2005;79(15):9369–80. https://doi.org/10.1128/JVI.79.15.9369-9380.2005.

Wieland S, Thimme R, Purcell RH, Chisari FV. Genomic analysis of the host response to hepatitis B virus infection. Proc Natl Acad Sci U S A. 2004;101(17):6669–74. https://doi.org/10.1073/pnas.0401771101.

Wilkening S, Bader A. Influence of culture time on the expression of drug-metabolizing enzymes in primary human hepatocytes and hepatoma cell line HepG2. J Biochem Mol Toxicol. 2003;17(4):207–13. https://doi.org/10.1002/jbt.10085.

Wilkening S, Stahl F, Bader A. Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. Drug Metab Dispos. 2003;31(8):1035–42. https://doi.org/10.1124/dmd.31.8.1035.

Winer BY, Huang TS, Pludwinski E, Heller B, Wojcik F, Lipkowitz GE, et al. Long-term hepatitis B infection in a scalable hepatic co-culture system. Nat Commun. 2017;8(1):125. https://doi.org/10.1038/s41467-017-00200-8.

Winer BY, Gaska JM, Lipkowitz G, Bram Y, Parekh A, Parsons L, et al. Analysis of host responses to hepatitis B and delta viral infections in a micro-scalable hepatic co-culture system. Hepatology. 2020;71(1):14–30. https://doi.org/10.1002/hep.30815.

Xia Y, Carpentier A, Cheng X, Block PD, Zhao Y, Zhang Z, et al. Human stem cell-derived hepatocytes as a model for hepatitis B virus infection, spreading and virus-host interactions. J Hepatol. 2017;66(3):494–503. https://doi.org/10.1016/j.jhep.2016.10.009.

Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, et al. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. elife. 2012;1:e00049. https://doi.org/10.7554/eLife.00049.

Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, et al. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. elife. 2014;3 https://doi.org/10.7554/eLife.00049.

Yan R, Zhang Y, Cai D, Liu Y, Cuconati A, Guo H. Spinoculation enhances HBV infection in NTCP-reconstituted hepatocytes. PLoS One. 2015;10(6):e0129889. https://doi.org/10.1371/journal.pone.0129889.

Yan Z, Zeng J, Yu Y, Xiang K, Hu H, Zhou X, et al. HBVcircle: a novel tool to investigate hepatitis B virus covalently closed circular DNA. J Hepatol. 2017;66(6):1149–57. https://doi.org/10.1016/j.jhep.2017.02.004.

Yang PL, Althage A, Chung J, Chisari FV. Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection. Proc Natl Acad Sci U S A. 2002;99(21):13825–30. https://doi.org/10.1073/pnas.202398599.

Yang D, Liu L, Zhu D, Peng H, Su L, Fu YX, et al. A mouse model for HBV immunotolerance and immunotherapy. Cell Mol Immunol. 2014;11(1):71–8. https://doi.org/10.1038/cmi.2013.43.

Yoneda M, Hyun J, Jakubski S, Saito S, Nakajima A, Schiff ER, et al. Hepatitis B virus and DNA stimulation trigger a rapid innate immune response through NF-kappaB. J Immunol. 2016;197(2):630–43. https://doi.org/10.4049/jimmunol.1502677.

Yuan L, Liu X, Zhang L, Li X, Zhang Y, Wu K, et al. A chimeric humanized mouse model by engrafting the human induced pluripotent stem cell-derived hepatocyte-like cell for the chronic hepatitis B virus infection. Front Microbiol. 2018a;9:908. https://doi.org/10.3389/fmicb.2018.00908.

Yuan L, Liu X, Zhang L, Zhang Y, Chen Y, Li X, et al. Optimized HepaRG is a suitable cell source to generate the human liver chimeric mouse model for the chronic hepatitis B virus infection. Emerg Microbes Infect. 2018b;7(1):144. https://doi.org/10.1038/s41426-018-0143-9.