Experimental models of hepatitis B and C — new insights and progress

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Abstract

Viral hepatitis is a major cause of morbidity and mortality, affecting hundreds of millions of people worldwide. Hepatitis-causing viruses initiate disease by establishing both acute and chronic infections, and several of these viruses are specifically associated with the development of hepatocellular carcinoma. Consequently, intense research efforts have been focusing on increasing our understanding of hepatitis virus biology and on improving antiviral therapy and vaccination strategies. Although valuable information on viral hepatitis emerged from careful epidemiological studies on sporadic outbreaks in humans, experimental models using cell culture, rodent and non-human primates were essential in advancing the field. Through the use of these experimental models, improvement in both the treatment and prevention of viral hepatitis has progressed rapidly; however, agents of viral hepatitis are still among the most common pathogens infecting humans. In this Review, we describe the important part that these experimental models have played in the study of viral hepatitis and led to monumental advances in our understanding and treatment of these pathogens. Ongoing developments in experimental models are also described.

Over the past century, major advances have occurred in the study of viral hepatitis. Specifically, advances in our understanding of the epidemiology, diagnosis, pathogenesis, treatment and prevention of viral hepatitis have all been achieved. In addition, viral hepatitis A, B, C, E and Delta are well defined[1]. With regards to prevention, safe and effective vaccines for hepatitis A and B are available, but not yet for hepatitis C, although major efforts to this end are currently underway[2]. By contrast, effective hepatitis E virus (HEV) vaccines have been developed but are not yet widely available[3]. For chronic viral hepatitis, therapy for HBV infection is effective in achieving viral suppression, leading to undetectable HBV DNA levels whilst stopping necroinflammation and often resulting in regression of fibrosis[4,5]. Importantly, historical advances in therapy for HCV infection have led to interferon-free, curative, all-oral combinations with direct-acting antiviral agents (DAAs)[6,7]. These new treatment regimens can be used with or without ribavirin and the end goal of

short-term, pangenotypic curative regimens for hepatitis C with minimal adverse effects is quickly approaching. In this Review, we highlight the expanding model systems to study viral hepatitis, focusing on available models of HCV and HBV infection as examples, which have contributed greatly to the advances in this field.

HBV and HCV biology

Overall, it has been challenging to develop models to study viral hepatitis and advances have been achieved through the generation of complex biological systems. A complete and exhaustive discussion pertaining to molecular virology and of the precise details under lying these models is beyond the scope of this Review but will be briefly discussed herein. We recommend more targeted references for precise virological and model-specific information. For hepatitis B, we recommend published papers specifically in the areas of *in vitro* culture systems and virology[8,9], animal models[10,11] and those that address these topics more broadly[12,13]. Similarly, for hepatitis C, we recommend published papers specifically in the areas of *in vitro* culture systems and virology[14,15], animal models[16,17] and those that address these topics more broadly[18,19].

HBV is a DNA virus with a partially double-stranded genome; it predominantly infects hepatocytes and, after entry, its relaxed circular genome is released from the virion at which points its genome translocates to the nucleus (FIG. 1). There, its genome is modified to the covalently closed circular (cccDNA) form that exists stably as an extrachromosomal viral genome. Viral RNA is made from the DNA genome and is translated into the major proteins that make up the virion. HBV surface antigens are extremely potent antigens that have facilitated the development of effective vaccines[20]. Clearly, a major hurdle is to cure HBV with finite therapy. This step will be achieved by facilitating the loss of HBV surface antigen (HBsAg) and by eliminating cccDNA, which is thought to be a key element of chronic HBV infection[13]. Thus, if this feat can be achieved, hepatitis Delta virus (HDV) prevalence will also decrease because HDV requires the HBV envelope protein for the assembly of infectious virus particles. Consequently, novel models to study HBV infection are desperately needed to investigate clearance of HBV cccDNA, which exists in the nucleus as a minichromosome and persists even in the absence of active viral replication[21]. Unfortunately, cccDNA is not eliminated by the potent suppressive therapies that are currently available[8].

HCV is a single-stranded positive-sense RNA virus; it completes its virus life cycle in the hepatocyte cytoplasm where its RNA genome is translated into the structural and nonstructural viral proteins (FIG. 2). Although antibody responses are generated that can neutralize these viral proteins, the extraordinary ability of the virus to mutate facilitates the generation of escape mutants that evade this host defence strategy. As a result, individuals who are cured of HCV with potent all-oral antiviral agents, some at great expense, can be reinfected. Thus, for HCV infection, a durable vaccine has been elusive and is desperately needed[2].

However, even with the eradication of HBV and HCV, progressive liver disease in some infected individuals and the risk of hepatocellular carcinoma (HCC) remains a clinical

challenge. Specifically, in the USA, the majority of HCV-infected individuals fall within a birth cohort born between 1945 and 1965 having acquired the virus several decades ago[22]; over many years, chronic HCV infection can result in progressive liver fibrosis and cirrhosis that predisposes to the development of HCC, and the ageing HCV-infected population is at substantial risk of the development of this deadly tumour. Even with cure, HCV-infected individuals with pre-existing advanced liver disease remain at increased risk of decompensation and other poor clinical outcomes including HCC[23]. Consequently, additional models are needed to understand progression of liver disease even after curing chronic viral hepatitis.

The early days

Modern viral hepatitis research began in 1963 with the discovery of the 'Australian Antigen' — now designated as HBsAg — by Baruch Blumberg and Harvey Alter[24]. Initially, much of the work to study viral hepatitis relied on biochemical methods, microscopy and primate models to characterize the agents of viral hepatitis. In addition, controversial experiments were carried out in populations in prisons[25] and individuals with intellectual disabilities at the Willowbrook State School in Staten Island, New York, USA[26]. These initial studies were successful in characterizing hepatitis A virus (HAV)[27], discovered by using electron microscopy on infected patient stool samples, and HBV, first identified by seroimmunoassay[28]. The identification and characterization of HAV and HBV subsequently led to the identification of HCV, formerly known as non-A, non-B hepatitis[29]. HDV was also identified by assessing liver samples from patients who were known to be positive for HBV but displayed peculiar viral protein staining patterns in the nucleus, and chimpanzees were utilized to demonstrate coexistence of HBV and HDV[30–32]. Similarly, HEV was characterized by using clinical samples from patients infected as a result of sporadic HEV infection epidemics[33,34]. Although effective in defining these aetiological agents of viral hepatitis, more sophisticated molecular studies were needed to develop targeted antivirals and vaccines for these viruses. The difficulty in generating an easily accessible animal model to study these pathogens prompted researchers to develop simpler systems that relied upon cultured cells for studies on viral hepatitis.

Hepatoma cell lines

As for most viruses, tumour-derived cells lines have been useful in increasing our understanding of viral hepatitis. General molecular biology methods, which are presented in detail in the forthcoming section, have been used to overexpress viral proteins and to generate cell lines that continuously express hepatitis viral genomes. Specifically, the HBV DNA and HCV RNA genomes and plasmids encoding viral proteins have been delivered intracellularly through various transfection techniques and viral transduction systems. In addition, with the generation of cell lines that express the viral genomes, small-molecule screens have been carried out to identify agents that could suppress viral replication[35–37]. Overall, the use of transformed cell lines has relied on the fact that several of them harbour defects in intrinsic innate immune antiviral pathways in cells that would normally restrict expression of viral genomes and proteins[38,39]. These cell lines, although abnormal, enabled

these viruses to be studied without major interference from host defence signalling pathways.

The first attempts at culturing HBV and HCV in cell lines relied on using serum from infected patients to introduce the virus into an *in vitro* model[40]. Even though investigators were able to detect viral replication in these models, the resulting infection was at a very low level and was challenging to detect using standard methods including western blot, immunofluorescence and PCR[41]. For HCV, much more success was achieved by *in vitro* transcription of HCV RNA from a DNA construct containing the viral genome and then transfecting the viral RNA into a cell line[14]. In addition, inoculation of these *in vitro* transcribed viral genomes into chimpanzees subsequently led to HCV infection[42]. For HBV, selection of cell lines that stably incorporated into the HBV DNA genome, in multiple chromosomal sites, was found to be a successful approach[43]. A summary of hepatoma cell line models to study HBV and HCV is presented in TABLE 1 and shown in FIGS 3 and 4.

HuH-7 cells

The HuH-7 cell line, created in 1982, was derived from a well-differentiated HCC tumour that was removed from a 57-year-old Japanese man who underwent a liver resection for the tumour[44]. This cell line was useful in studies of HBV[45] but has been more commonly used for *in vitro* models to study HCV. Initially, the HuH-7 cell line was successful in propagating luciferase-containing subgenomic mutants that only encoded HCV nonstructural proteins[46]. As a result, this cell line, described in detail in the next section, was utilized as the primary substrate for the generation of a replicon that would continuously express and replicate the HCV RNA genome, without the integration of a DNA intermediate in the cellular genome.

Replicon—The development of a cell line stably expressing the HCV RNA genome was a landmark achievement in the field[46], and was rapidly duplicated by other groups[47]. Many of the latest DAAs that have been tested in patients with hepatitis C were initially discovered through drug screens using this model for HCV genotype 1 infection, and use of this model is one of the reasons the new DAAs can efficiently target this HCV genotype[35]. Interestingly, it was clearly demonstrated that the DNA from the replicon plasmid used to generate the HCV RNA did not insert into the genome as was done for HBV-overexpressing cells[46]. This model was also particularly useful because in addition to a selection cassette, a reporter (for example, luciferase or green fluorescent protein) could be inserted into the replicon construct in several different configurations (for example, monocistronic or bicistronic) to easily monitor genome replication by luminescence or fluorescence microscopy[15]. Two main reasons led to the successful establishment of this model for HCV replication: first, mutations in the viral genome occurred during the selection process that favoured replication in cell culture; and second, the presence of cells that were more permissive to hepatitis viral replication owing to defects in innate immune pathways that usually restrict virus growth[14,15]. Understanding the role of these two aspects of replicon creation enabled the subsequent development of cell culture models for HCV infection and propagation.

One of the advances that contributed to development of the HCV *in vitro* infectious model involved curing replicon cells of the HCV genome through treatment with interferon. The removal of the HCV RNA genome from these cells resulted in the selection and development of cell lines (for example, HuH-7.5 and HuH-7-lunet) that subsequently harboured more notable defects in innate immune antiviral pathways, involving genes such as RIG-I, than that observed in the parental HuH-7 cell line[48,49]. These cells lines were subsequently used to establish more replicon cell lines using additional HCV genotypes (such as genotypes 3 and 4)[50,51] and to develop the first HCV *in vitro* model that could fully propagate infection. Furthermore, in a study published in 2015 (REF. 52), the HuH-7.5 cell line was utilized to support wild-type replicons for all HCV genotypes and even infection with clinical isolates through the overexpression of the *SEC14L2* gene (and subsequent protein), which can promote HCV infection by enhancing vitamin-E-mediated protection against lipid peroxidation. Overexpression of this gene can also confer other hepatoma cell lines, including HuH-7 and Hep3B–miR122 albeit at lower levels, with the ability to support multiple HCV replicons[52].

JFH1 strain and use of ribozymes—Although the replicon was a monumental breakthrough in the study of HCV and proved to be useful as a tool for high-throughput screening for antiviral drugs, it still failed to be quickly translated into a fully infectious *in vitro* system. Seminal studies demonstrated that mutations in the HCV genome, which conferred increased replication *in vitro* and were needed to establish the replicon cell lines, prevented the production of infectious virions in chimpanzees[53]. Thus, it was assumed that the best method to develop an *in vitro* model that fully recapitulates HCV infection would rely on wildtype HCV genomes. As replication-favouring mutations were not helpful toward this end, cell lines that were highly permissive for replication were also used. One of the first successful approaches to generating an *in vitro* model of HCV infection involved the JFH1 genotype 2a clone. This viral strain was generated after isolation from a Japanese patient who developed fulminant hepatitis[54]. Upon transfection of the HuH-7.5 cell line (which is more permissive for HCV replication[48]) with this cloned viral genome, it was found that infectious HCV virions were produced that could be used to infect chimpanzees and propagate the virus in this specific human hepatoma cell line[55]. Importantly, several other groups contributed to and confirmed this exciting achievement in HCV virology[56,57]. In 2006, additional infectious clones were developed for HCV genotype 1a[58], and several others genotypes including 1b, 3a and 4a[59,60]; yet none of these replicate as robustly as the JFH1-based viruses *in vitro*.

A second, less frequently utilized *in vitro* model was also generated by an alternative approach[61]. In this case, the HCV cDNA, initially from genotype 1b and later extended to other genotypes, was inserted between two ribozymes that were designed to generate the naturally occurring 5. and 3. ends of the viral genome. Transfection of this construct into HuH-7 cells resulted in the production of infectious virus that can be propagated in chimpanzees[62]. This system has been extended to facilitate production of infectious virus from other genotypes including JFH1 (REF. 62).

With regards to HBV-related experimentation, the HuH-7 cell line has been useful but it has had less of a prominent role than that seen with HCV. Similar to HCV, this cell line was

initially used to over express viral proteins from the HBV genome[45]. One of the important uses of HuH-7 cells to study HBV was the transfection of linear, full-length HBV cDNA into this cell line[63,64]. Because this model enabled the establishment of cccDNA during the HBV life cycle, this approach was used to study more complex aspects of HBV biology, including those related to epigenetic modification of the HBV episomal DNA[65]. In 2012, with the identification of the putative HBV entry receptor, *NTCP*, encoding sodium/bile acid cotransporter (also known as SLC10A1), a HuH-7 cell line stably expressing this gene was used as a model that can support HBV infection[66].

HepG2 cells

Another cell line that has been used to study agents of viral hepatitis is the HepG2 cell line. This cell line was derived from the liver tissue of a 15-year-old white male patient who had a well-differentiated hepatoblastoma. In contrast to the HuH-7 cell line, the HepG2 cell line is polarized and has proved to be much more useful for the study of HBV than HCV. Initial breakthroughs in HBV research were achieved as the HBV genome could be continually expressed in HepG2 cells. This feat was achieved by selecting for stably transfected HepG2 cells carrying the HBV genome through growth in selection media[43,67]. Although these transfected cells were able to support HBV replication and formation of cccDNA, albeit at a low level, they were unable to be readily infected by HBV virions, now known to be due to the absence of expression of the *NTCP* gene. Similar approaches have been used to generate additional cell lines that were selected for carrying the HBV genome and they are designated as the HepG2 H1.3 and HepAD38 cell[68–70]. These cell lines continue to be used to study aspects of the HBV life cycle, including late steps of HBV replication and clearance of cccDNA by host defence pathways[71]. An additional model that was subsequently developed involved HepG2 cells transduced with baculovirus and adenovirus constructs, used as delivery vehicles, to efficiently express the HBV genome in these cells[72,73]. These transduction models were an improvement over transiently transfected or stable cell lines because HBV gene expression and replication far exceeded that found in HepG2.2.15, and these models are more reproducible than the transfected cells. With the discovery of the putative receptor for HBV (NTCP), HepG2 cells that stably express *NTCP* are now available to study HBV infection with infectious virions[66]. This model has facilitated the routine and reliable study of the entire HBV life cycle in a renewable cell culture platform.

An advance in the past decade has also enabled HepG2 cells to be more useful for studies of HCV. HepG2 cells had been observed to lack miRNA 122 (miR-122), which is necessary to support the HCV lifecycle. Specifically, miR-122 might support HCV RNA stability, replication and translation through several distinct mechanisms by directly binding homologous sequences in the untranslated region of the virus genome[74,75]. In addition, HepG2 cells also demonstrate very low expression of CD81 (a known entry factor for HCV)[76,77]. Consequently, it was demonstrated that HepG2 cells that are selected to stably express both the CD81 receptor and miR-122 can be infected by HCV and support viral replication[78,79]. Interestingly, owing to the differences in antiviral innate immunity within this cell line when compared with HuH-7 cells, this model has proven to be useful in the study of host defence pathways and to validate results obtained in primary human hepatocytes (PHH). Specifically, the genetically altered cell line was capable of producing

large amounts of type III IFNλ in response to HCV infection, which has also been observed in primary human hepatocytes[78,80].

HepaRG cells

An additional cell line has proven to be useful for studies of both HBV[81] and HCV[82]: the HepaRG cell line. This cell line was developed from cells obtained from a resection of tumour from an HCV-infected individual with liver cancer, although the infection was not sustained *in vitro* after isolation of these cells. HepaRG cells are bipotent hepatic progenitor cells that can differentiate into both biliary and hepatocyte-like cells and can divide indefinitely[81]. To be fully capable of supporting infection with HBV and HCV, these cells must also be treated with dimethyl sulfoxide to foster additional maturation into more differentiated hepatocyte-like cells. According to head-to-head comparisons, HepaRG cells have many similarities to PHHs, which are considered to be the gold standard for hepatocyte studies *in vitro*, in terms of drug metabolism and ability to support infection by HBV and HCV[83,84]. Initially, this cell line was demonstrated to be capable of supporting HBV infection and replication[81]. Unfortunately, the use of polyethylene glycol is needed to facilitate viral entry by increasing interactions between the HBV virion and the cell membrane but the overall infection rate is low with minimal cell-to-cell virus spread[85]. Later, investigators were able to use this cell line as a substrate for HCV infection[82]. In this study[82], the investigators were able to utilize serum-derived HCV genotype 3 to validate susceptibility to HCV infection[55].

Other cell lines

Other cell lines that have been utilized for studies specifically related to viral hepatitis include PH5CH8 and Hep3B cells. Hep3B cells contain a mutation in the *P53* gene that renders the protein inactive; the cell line has thus been used to study the interaction between p53 and both HCV and HBV in the context of a non-functional p53 protein[86,87]. PH5CH8 cell lines have been utilized for additional studies because it has a functional Toll-like receptor 3 system (necessary for recognition of double-stranded RNA associated with some viral infections) that is present in PHHs, but absent in HepG2 and HuH-7 cells[88–90]. Consequently, specific studies focused on RNA-sensing pathways and their role in HCV infection have been facilitated through its use.

Primary human hepatocytes

As previously mentioned, PHHs are considered the gold standard for laboratory studies of hepatocyte function. These normal cells are obtained from patients undergoing liver resection (usually for a hepatic tumour) and they are isolated from the outer edges of the tumour margin. In addition, PHHs can also be routinely obtained from the fetal livers of aborted embryos and can also serve as a substrate for infection[91]. Given that these fetal hepatocytes (often a combination of both hepatocytes and hepatoblasts depending on the gestational age of the donor fetus) are utilized and discarded, their use is much less controversial than that of embryonic stem cells, which offer the prospect for long-term biomedical applications such as regenerative medicine[92]. Unlike HepaRG cells, PHHs once plated do not divide and they have a limited lifespan in tissue culture, usually between 1 and

2 weeks, although fetal hepatocytes can be stable for several weeks[91]. Once in culture, these cells rapidly dedifferentiate, concomitantly downregulating biological characteristics found in mature hepatocytes[93]. Although these cells have a limited lifespan in culture, they readily support infection by HCV and HBV and are thus useful for the study of viral hepatitis. However, viral replication is usually limited when compared with levels seen in cell lines such as HuH-7.5; these PHHs presumably have intact host defence pathways that combat the infection[80]. For HCV, many studies have demonstrated productive infection of PHHs after culture, including cells that are obtained from HCV-positive patients who continue to harbour replicating virus as opposed to *de novo* infections[80,94–96]. Many studies have also demonstrated productive infection of PHHs with serum-derived and cell-culture-derived HBV[97–100]. In addition, primary hepatocytes isolated from other species including chimpanzees and *Tupaia* have proven to be capable of supporting infection by HBV[101] and related hepadnaviruses that infect woodchucks and ducks[102,103].

To extend the length of viability in culture and to maintain the differentiation status of PHHs, several approaches have been developed each with some success. These approaches include the growth of PHHs with additional so-called feeder cells such as mouse embryonic fibroblast cell lines (for example, STO) and non parenchymal liver cells including Kupffer and endothelial cells that support hepatocyte viability[104]. Hepatocytes have also been cultured in 3D organoids[105]. In their 'natural' environment, hepatocytes and other epithelial cells exist and function in a highly structured system. The organization of cells into an ordered microenvironment facilitates optimal viability by facilitating efficient nutrient uptake and waste removal. In addition, complex biological interactions with tissue matrix proteins and surrounding non parenchymal cells and vessels promotes functional polarization. Unfortunately, these critical properties of the tissue microenvironment are absent in standard *in vitro* cell culture systems. To provide more physiological culture conditions, micro patterning approaches facilitate the creation of a tissue-like microenvironment. This step is largely achieved by imposing a defined cell adhesion pattern that promotes the formation of islands of cells. Current micropatterning approaches also utilize feeder cells to produce growth factors and provide adhesion molecules and extracellular matrix components for cell attachment. For HCV, this approach has led to the establishment of infection of PHHs in culture for several weeks while the cells maintain functional characteristics of mature hepatocytes[104]. Although micropatterning of hepatocytes offer several advantages over traditional 2D cell culture, organization of hepatocytes into 3D organoids also presents additional benefits. This approach includes the ability to facilitate the organization of all three of the major nonparenchymal cells in the liver (for example, hepatic stellate cells, Kupffer cells and endothelial cells) in self-assembled networks with hepatocytes and to allow the laminar-flow of fresh media through channels, using sophisticated perfusion apparatuses, over cells that are plated in scaffolds. The design and optimization of these devices, also called bioreactors, represent an exciting area of future use for models of viral hepatitis[106,107]. In addition, traditional approaches to immortalize primary hepatocytes by introducing oncogenes, such as human papilloma virus encoded E6 and E7, continue to be utilized with increasing success to extend the length of utility of PHHs in culture[108].

Stem cells

Progress in the propagation of stem cells in culture has led to exciting models that can be used to study viral hepatitis. Methods are now available to differentiate stem cells toward the endodermal lineage and then forward to foster development into mature hepatocytes[109]. Stem cells of embryonic origin are a more controversial source because of the need to derive these cells from discarded embryos and because of the possibility of long-term biomedical applications (including cloning)[92]. Induced pluripotent stem cells (iPSCs) are a newly developed source of hepatocytes that can be used for studies on viral hepatitis. These cells, once generated, can be a reliable source of cells that can be differentiated into mature hepatocytes. The advantages of stem-cell-derived hepatocytes over PHHs include the ability to obtain an unlimited supply of primary hepatocytes and that these cells are less variable when compared with PHHs that are obtained from different donors, who can vary by gender, age, exposure to medications and/or chemicals, genetic polymorphisms and the presence or absence of underlying liver disease[110,111]. These human stem-cell-derived hepatocytes have proven to be a useful substrate for infection by JFH1 and HCV obtained from infected patient serum[112–114] and cells from other species such as pigtail macaques have also been utilized for HCV infection and propagation[115]. An interesting finding in one particular study utilizing stem-cell-derived hepatocytes was that the ability to support HCV infection was dependent upon the subsequent expression of miR-122 that was induced during the differentiation process. A similar approach has been used to successfully support infection by HBV[116]. Unfortunately, stem-cell-derived hepatoctyes do not reach the phenotype of fully functional mature hepatocytes; however, various approaches have been taken to overcome hurdles in function and other drawbacks of this model, including the use of small molecules that promote differentiation, and manipulation of the microenvironment used in the *in vitro* culture systems[117,118]. An exciting prospect for this line of inquiry has also been developed through the use of stem cells to develop vascularized and functional human liver *in vitro*[119]. This model might offer opportunities to study viral hepatitis in a 3D system with a functional vasculature. A summary of *in vitro* models to study HBV and HCV is presented in TABLE 1.

Animal models

Mouse models

Mouse models were initially developed using animals that overexpressed HBV viral proteins or the full-length replication competent viral genome so that the immune response to HBV viral antigens could be studied in an *in vivo* setting[120]. Subsequently, complete replication of HBV was accomplished using hydrodynamic injection of the HBV genome into both immunocompetent and immunodeficient mice, demonstrating an important role of the cellular immune system in controlling HBV infection *in vivo*[121]. An improvement of this method was achieved using adenoviral delivery of the HBV genome to the liver of mice, which established chronic infection and facilitated studies on adaptive immune responses and antiviral drug testing[73,122].

With respect to HCV, as the virus has an RNA genome, it was much more difficult to develop stable mouse models[16]. Initial efforts to overexpress HCV proteins in mice were

achieved much later than the corresponding HBV models[123]. As is the case with HBV, mouse hepatocytes are a poor substrate for natural HCV infection because of differences in multiple host factors present in human hepatocytes[124]. One method that has been successful in overcoming this species-specific barrier is through the generation of humanized mice through xenotransplantation of PHHs. Through this method, PHHs can be delivered into a mouse to subsequently repopulate the mouse liver. For this process to be successful, the mouse needs to be immunodeficient so as not to reject the transplanted human hepatocytes and the normal mouse hepatocytes need to be replaced. The replacement of the mouse hepatocytes can be specific ally achieved by genetically inducing liver injury in the mouse, thereby creating a growth advantage for the engrafted human hepatocytes. The first successful utilization of this approach was achieved using the SCID (severe combined immunodeficiency) mice carrying a urokinase plasminogen activator transgene that is activated by a liver-specific albumin promoter (Alb-uPA)[125]. Subsequent expression of the transgene results in acute hepatotoxicity that can be rescued by the engraftment of injected PHHs, which are then the substrate for HCV infection.

Unfortunately, the frailty of these mice has limited the widespread use of this model. Utilizing a promoter that is activated later in mouse development, such as the major urinary protein promoter, the development of a healthier and more robust animal model (MUP-uPA) proved to be effective to support both HCV and HBV infection[126]. An additional hepatotoxic model uses *Fah*-knockout mice for which the liver remains healthy with the administration of the small molecule 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione also called NTBC or nitisinone. Upon discontinuation of this agent, mouse hepatocytes specifically accumulate toxic metabolites and PHHs can be introduced into the mouse liver, facilitating infection by both HCV and HBV[127]. A more complex mouse model (AFC8) has been developed that offers both a humanized liver and rudimentary immune system. This model utilizes the caspase 8 transgene that can be activated with administration of FK506 to cause mouse hepatocyte apoptosis[128]. When PHHs were infected with HCV in this model, these animals developed liver fibrosis as a result of the immune response to HCV[128]. Similar approaches have also been used to create chimeric mice that support HBV infection and also develop liver fibrosis[129]. Similarly, HDV infection has also been achieved in this model[130]. Excitingly, humanized mice have now been developed that utilize iPSC-derived human hepatocytes (iHeps) to repopulate the livers of MUP-uPA mice to support HCV infection and this approach might be amenable to support infection with other hepatitis viruses[131].

Another approach for developing humanized mice is to genetically introduce human genes to express human proteins required for HCV infection. This approach was facilitated by the discovery of specific human viral entry factors for HCV, most importantly CD81 and occludin, that are needed to enable infection of mouse hepatocytes[77,132,133]. By genetically engineering mice with human homologues of these two HCV entry factors, viral entry was first reported *in vivo* in an important proof-of-concept study[134] and HCV infection was success fully demonstrated in a subsequent study[135]; however, viral replication was quite low and no liver disease was observed in this model[135]. Interestingly, a similar approach was utilized in mice with a different genetic background, and in this model sustained viraemia and infectivity was achieved for >12 months post-infection with HCV, alongside the

development of fibrosis and subsequent cirrhosis[136]. Expression of the *NTCP* gene in mice has also been developed and this model has been successfully utilized to support infection with HDV but not with HBV, for which it is more difficult to achieve productive HBV infection, possibly owing to other human host factors being needed[137].

Primate models

Historically, the study of viral hepatitis has greatly benefited from the use of non-human primates as a model of both acute and chronic infection beginning with landmark experiments conducted in the 1960s[138]. These initial experiments were performed as a result of strong evidence that hepatitis viruses could be accidentally transmitted from primates to humans[139]. Although there was already strong evidence supporting the existence of both HAV and HBV from epidemiological studies[26], experiments performed in chimpanzees substantiated the existence of HCV in the 1970s[140,141].

Until the past decade and the advent of humanized mice susceptible to infection with hepatitis viruses, the only acceptable model for vaccine development was the chimpanzee. However, the limited supply of these animals and their associated high cost has precluded their routine use. In addition, although the US Institute of Medicine report, published in 2012, on the use of chimpanzees for biomedical research has deemed these animals suitable for vaccine studies[142], increased scrutiny with regards to the ethical implications of these studies has dampened their continued use and many nonhuman primate facilities have since stopped supporting chimpanzee research[143]. Regardless, chimpanzees, owing to their high genetic similarity with humans, have proven to be the most useful model for the study of viral hepatitis mainly because they are susceptible to infection with hepatitis viruses. However, they do not develop a similar spectrum of liver disease as humans do[144,145]. Another interesting caveat is that these primates are monomorphic for several of the polymorphisms in the *IL28B* gene that have been linked to HCV clinical outcomes[146,147]; thus, chimpanzees might not be suitable for this line of investigation. Specific questions that have been addressed through the use of this model have included vaccine efficacy[148], antiviral responses (including both innate and adaptive responses[149]), and the evaluation of novel antiviral strategies[150]. A summary of these *in vivo* models to study HBV and HCV is presented in TABLE 2.

Other models for Hepadnavirus

Although chimpanzees have had a large contribution in terms of findings for studies of viral hepatitis, other animal models have been successfully developed. Specifically for HBV, owing to the lack of robust cell culture and mouse models historically and due to the high cost of chimpanzees, several models have been developed and have provided useful insights into the virus life cycle. Because of the known genetic similarity to primates, the tree shrew (*Tupaia belangeri*) has been used[151]. Specific beneficial aspects of this organism for studying HBV include the ability to support HBV entry and release from hepatocytes and establishment of chronic infection and cccDNA pools[152,153].

Additional models of hepadnavirus infection that have been invaluable to understanding HBV biology include the duck hepatitis B virus (DHBV) and woodchuck hepatitis B virus

(WHBV). Both of these animal models have proven useful because the biology of infection in each of these is similar to that seen in human HBV infection[13]. Subsequently, these models have been used for studies to increase our understanding of all aspects of HBV, including the virus life cycle, immune responses and for antiviral development. Specifically, WHBV has been informative towards our understanding of the liver disease and carcinogenesis as a result of hepadnavirus infection and the resulting pathology that is elicited[154]. DHBV has proven to be more useful in our understanding of the viral life cycle of hepadnaviruses mainly due to the fact that these animals are relatively easy to handle, can be infected easily and develop high viraemia[21,155]. However, there are numerous differences between these viral orthologues and HBV, such as the spectrum of comparable liver disease, transcriptional regulation of the viral genomes and substantial DNA sequence divergence[156,157]. The difficulty in working with these species, which are not routinely found in biomedical laboratories, limits their utility to the general viral hepatitis research community. In addition, the lack of specific research tools to study immune responses in these outbred models is also a major hurdle[152].

Other models for Hepacivirus

Similar to that observed for hepadnaviruses, additional animal models of hepaciviruses have been discovered[158,159]. However, many of these models have only just emerged and have yet to yield a substantial amount of insight for the study of HCV as has been achieved using animal models for HBV. However, it is anticipated that these models could offer new insight into HCV virology and pathogenesis as well as insight into the evolution of this virus. The pigtail macaque might be a useful model to study HCV as cells from this primate have been shown to be capable of supporting HCV infection *in vitro*[115]. GB viruses, closely related to HCV phylogenetically, are non-human primate hepaciviruses[160]. The GBV-B virus has been shown to cause hepatitis in experimentally infected tamarins (genus *Saguinus*)[161,162]. Specifically, infection of New World primates with GBV-B recapitulates the phenotypes of acute viral clearance and chronic pathological disease, and this model has been used for drug testing[163–165]. In this regard, GBV-B seems to be a viable surrogate model for the study of HCV infection with possible utility in the testing of novel vaccine strategies. In the past few years, newly discovered hepaciviruses in bats[166], dogs[158] and horses (most similar to HCV)[159], might also provide unique models for study. However, research on these newly discovered viruses is only just beginning and which of these animal models will prove to be most relevant to HCV remains to be seen.

Future directions

General

The mechanisms underlying how hepatitis viruses manifest as chronic infection and cause liver disease remain to be fully characterized. The means by which the genomes of these viruses persist intracellularly despite the presence of a functional innate immune response are still poorly understood. Precise insights pertaining to the strategies by which the agents of viral hepatitis are specifically able to subvert these antiviral responses and establish chronic infection are needed. Studies in PHHs and stem-cell-derived hepatocytes (iHeps) might be particularly useful in the case of HCV for which polymorphisms in the *IL28B*

locus, an established determinant in spontaneous viral clearance[146], can be specifically studied in normal cells from hepatocytes with different genotypes. In addition, improvements in these *in vitro* models utilizing normal hepatocytes are needed to facilitate robust infection from serum-derived HCV to facilitate studies with all genotypes; notably, major progress in this area has now been achieved[52]. Identifying the molecular pathways that are perturbed by chronic infection with HBV and HCV to facilitate the development of HCC is also an urgent area of unmet need. For HCV, the use of models that develop liver fibrosis and cirrhosis is important[128,136] because HCC usually develops in patients with cirrhosis[167]. These models might be particularly useful for the identification of biomarkers to predict the development of HCC because even after achieving sustained virologic response, patients with cirrhosis because of HCV infection are still at an increased risk of developing HCC[23]. Although mouse models of HBV infection have been established that develop liver disease[129], they need to be improved and further refined to capture the full spectrum and pathogenesis of HCC development[167].

HBV

With regards to HBV, studies to increase the development of strategies to eradicate cccDNA from infected cells are currently underway[71], but improved models are needed. Although HBsAg levels might be an indirect measure of cccDNA levels[13], a more precise and easily accessible biomarker is needed to monitor the endogenous cccDNA pool in infected patients. Unfortunately, the lack of robust laboratory models that accurately recapitulate *in vivo* HBV infection has hampered the study of cccDNA persistence and removal[152]. With the discovery of the *NTCP* gene, it is anticipated that a mouse model will be developed that will support HBV infection, as has been achieved for HDV[137], to aid in careful studies on the establishment and clearance of cccDNA pools. Such a model would also aid in the discovery of novel antiviral agents and new therapeutic strategies, including the use of CRISPR/Cas technology to inhibit HBV DNA replication[168–170] and to purge the cccDNA reservoir in infected individuals by eliciting target specific mutations and deletions in the virus genome. As cccDNA quantification requires access to liver tissue, human studies are difficult and reliable animal models would be much more amenable to study due to the ease of access for obtaining liver tissue.

HCV

With regards to HCV, an effective vaccine is needed to prevent further spread of the virus and reinfection in those who are cured. Although chimpanzee models are useful to aid in the development of vaccines, their cost is prohibitive for routine studies and tight restrictions have been placed to limit their use. Humanized mouse models might represent a reasonable alternative in the future to evaluate vaccine efficacy, but additional effort is needed to replicate human-specific immune responses to HCV[17] and facilitate their widespread use in the vaccine development community[2].

Conclusions

Since the discovery of the aetiological agents of viral hepatitis B and C[24,29], development of highly effective HBV vaccines and therapeutic breakthroughs that now enable successful

treatment with a durable cure for HCV infection have been achieved. These efforts will promote the eradication of these viruses. Although chronic HBV infection can now be treated with potent suppressive treatment regimens, this viral illness still necessitates improvement in therapeutic intervention to facilitate a durable cure as has now been achieved for HCV. Similarly, vaccines are needed to prevent the spread of HCV infection and also to prevent reinfection in those who have achieved a sustained virologic response. In addition, even with cure, chronically infected patients are still at an increased risk of developing HCC, which remains a cancer type associated with high mortality[23]. Continued effort is, therefore, needed toward developing new models to study viral hepatitis to further our understanding of the mechanisms by which these viruses manifest as chronic infection and the molecular pathways that are ultimately involved in the development of liver disease and HCC.

Acknowledgments

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Key points

- Early work on viral hepatitis relied on biochemical methods and microscopy, but the field greatly benefited from the use of primates and other animal models of both acute and chronic infection

- Use of tumour-derived cells lines has increased our understanding of viral hepatitis; small molecule screens were performed in cell lines expressing viral genomes to identify agents that could suppress viral replication

- Primary human hepatocytes are considered the most biologically relevant *in vitro* model for viral hepatitis infections; advances in propagation of stem cells in culture have led to exciting new stem-cell-derived hepatocyte models

- Hepatocytes and other epithelial cells exist and function in a highly structured system and *in vitro* models are moving to those that incorporate multiple nonparenchymal cells and 3D lattices to preserve full hepatocyte function

- Mouse models were initially limited by species-specific barriers, but transgenic animals can used to study immune responses and species-specific barriers can be overcome through the generation of humanized mice

- Further progress in recapitulating human immune responses in mouse models might facilitate the development of an HCV vaccine, with *in vitro* models of HBV cccDNA persistence aiding discovery of anti-HBV small molecules
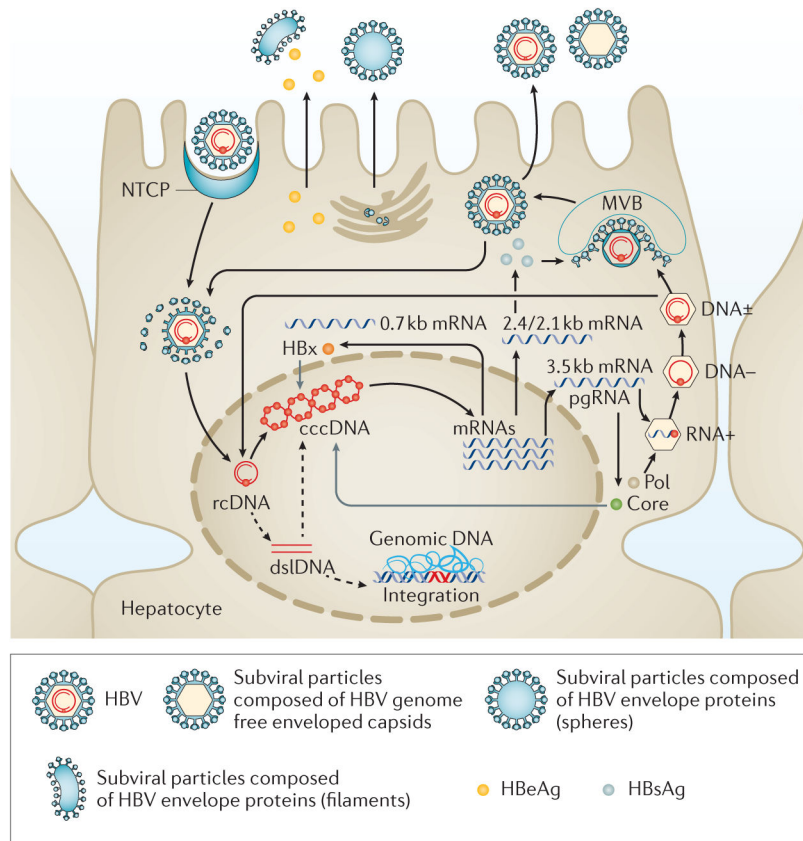
Figure 1. HBV biology

The life cycle of HBV, including attachment, entry, uncoating, trafficking to nucleus, covalently closed circular (cccDNA) formation, integration, transcription, translation, encapsidation and secretion, is depicted. Initially, HBV particles are taken up by hepatocytes through mechanisms that involve NTCP (sodium/bile acid cotransporter, also known as SLC10A1). After internalization, viral capsids are released and subsequently directed to the nucleus where the HBV genomes are liberated. In the nucleus, relaxed circular DNA (rcDNA) genomes are converted into cccDNA that can persist in the nucleus of infected cells as a minichromosome and which serves as template for viral RNA transcription. Double-stranded linear DNA (dslDNA) is also produced that can be integrated into the cellular genome or also converted into cccDNA. Viral mRNAs are transported to the cytoplasm where they are translated into viral proteins and together with the viral polymerase, the pregenomic RNA (pgRNA) is encapsidated and reverse transcribed within the nucleocapsid into progeny rcDNA. Mature nucleocapsids are then either directed to the multivesicular body pathway for envelopment with HBV envelope proteins or re-directed to the nucleus to establish a cccDNA pool. HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBx, hepatitis B x protein; MVB, multivesicular body. Adapted with permission from Nature Publishing Group © Revill, P. *et al. Nat. Rev. Gastroenterol. Hepatol.* http://dx.doi.org/10.1038/nrgastro.2016.7 (2016).
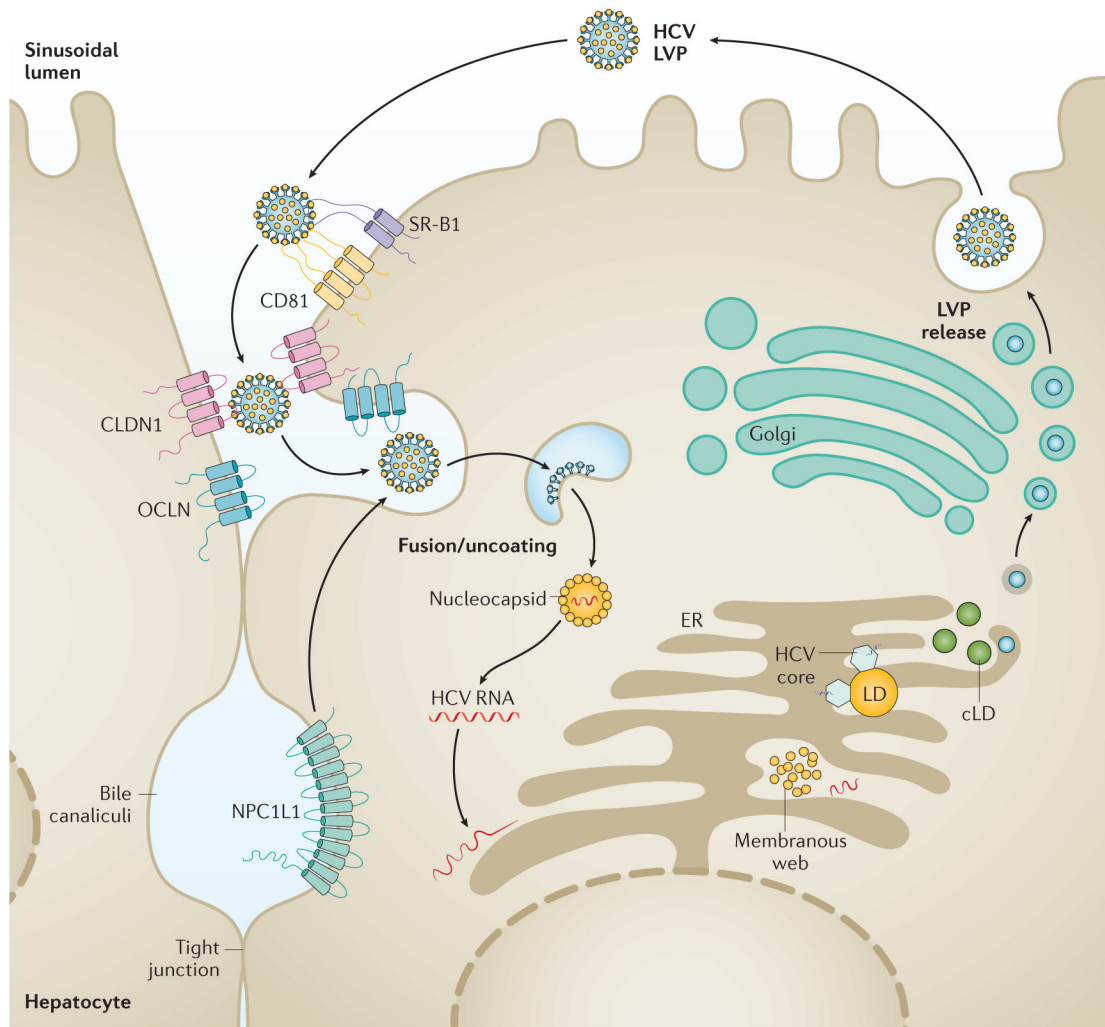
Figure 2. HCV biology
The life cycle of HCV, including attachment, entry, uncoating, encapsidation and secretion, is depicted. Interaction of extracellular HCV with cellular surface receptors initiates the entry process, which can also occur from direct cell-to-cell transmission. After pH-dependent fusion and uncoating, the incoming HCV genome is translated and the resulting polyprotein processed. Replication takes place in ER-derived membrane spherules (membranous web). In the assembly and secretion release process, the core protein is transferred from the lipid droplets to form nucleocapsids that, assisted by NS5A, are loaded with RNA. The replicase proteins can bind to the HCV genomic RNA during transfer from replication to packaging in close proximity, but eventually are removed from the maturing nucleocapsids, the intracellular sites of which might converge. HCV virion morphogenesis is coupled to the VLDL pathway, and particles are produced as lipoviral particles (LVPs). CLDN1, claudin-1; cLD, cytoplasmic lipid droplets; ER, endoplasmic reticulum; LD, lipid droplets; NPC1L1, Niemann-Pick C1-like protein 1; OCLN, occludin; SR-B1, scavenger receptor class B member 1. Adapted with permission from Nature Publishing Group © Scheel, T. K. H. & Rice, C. M. *Nat. Med,* **19**, 837–849 (2013).
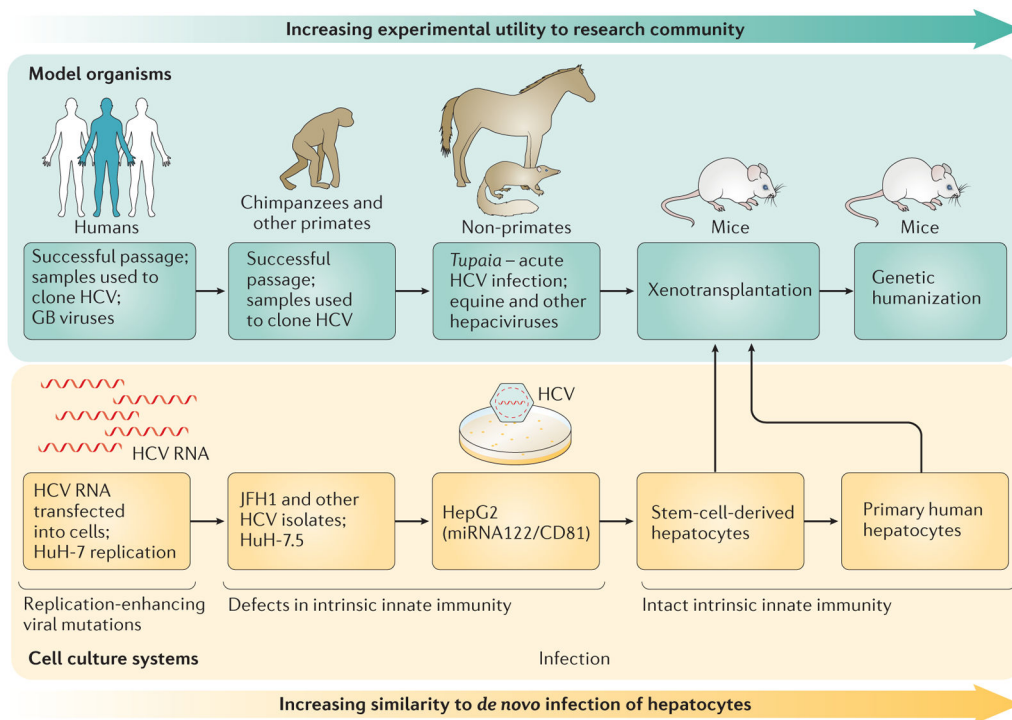
Figure 3. The evolving models to study the HCV life cycle and pathogenesis

The use of model organisms has shifted from the use of primarily humans and primates to that of mouse models that are 'humanized' through xenotransplantation or genetic modification. Cell systems that initially relied upon the use of hepatoma-derived cell lines and transfected RNA genomes have transitioned to *in vitro* infection of primary hepatocytes and stem-cell-derived hepatocyte-like cells with functional cell intrinsic antiviral responses. JFH1, Japanese fulminant hepatitis 1.
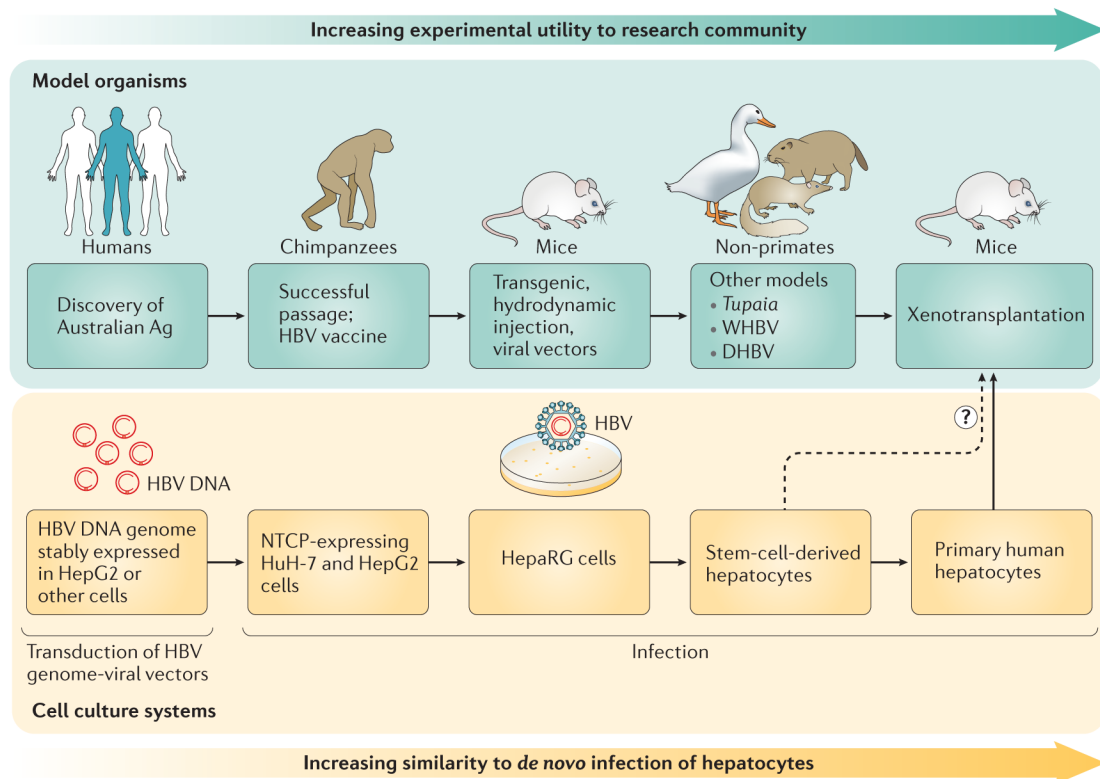
Figure 4. The evolving models to study the HBV life cycle and pathogenesis
The use of model organisms has shifted from the use of primarily humans and primates to alternative models including *Tupaia* and woodchuck (WHBV) and duck (DHBV) hepatitis viruses. Xenotransplantation in mouse models has more recently been developed with animals that are susceptible to HBV infection. Cell systems that initially relied upon the use of hepatoma-derived cell lines and transfected or virally transduced DNA genomes have transitioned to infectious systems including primary hepatocytes and stem-cell-derived hepatocyte-like cells with functional cell intrinsic innate antiviral responses. Ag, antigen; DHBV, duck hepatitis B virus; NTCP, sodium/bile acid cotransporter (also known as SLC10A1); WHBV, woodchuck hepatitis B virus.

Table 1

Commonly used cell culture models for the study of HBV and HCV

Cell biology	HuH-7 cell	HepG2 cell	HepaRG cell	Stem-cell-derived hepatocytes	Primary human hepatocytes
Functional innate immunity	Minimal	Moderate	Fully functional	Fully functional	Fully functional
Length of propagation	Indefinite	Indefinite	Indefinite	<1 month	<1 month
Polarized	No	Yes	Yes	Yes	Yes
Immortalized	Yes	Yes	Yes	No	No
Transformed	Yes	Yes	No	No	No
Availability	High	High	Medium	Low	Low
Lot variability *	Minimal	Minimal	Moderate	Moderate	High
Supports HBV infection	No, requires NTCP	No, requires NTCP	Yes, requires differentiation	Yes	Yes
Supports HCV infection	Yes	No, requires CD81 and miR-122	Yes, requires differentiation	Yes	Yes

miR-122, microRNA 122; NTCP, sodium/bile acid cotransporter (also known as SLC10A1).

*
Lot variability is susceptibility to variation in experimental results obtained between different laboratories.

Table 2

In vivo models for the study of HBV and HCV

Applicability of model	Chimpanzees	*Tupaia*	Mouse models			
			Genetically humanized model	**Stem-cell-derived human hepatocytes***	**Human immune system****‡**	**Mouse immune system****‡**
HBV infection	Yes	Yes	Not reported	Not reported	Yes	Yes
HCV infection	Yes	Yes	Yes	Yes	Yes	Yes
Liver disease	Yes, but mild	Yes, but transient	Possible	No	Possible	No
Development of immunotherapeutics	Yes	Yes	No	No	Possible	No
Antiviral testing	Yes	Yes	Yes	Yes	Yes	Yes
Vaccine testing	Yes	No	No	No	Possible	No
Availability	Very low	Low	Moderate	Low	Very low	Moderate

Not including HBV-related or HCV-related viruses in other animals models.

*Xenotransplantation.

‡Engrafted with primary human hepatocytes.