Review

Laboratory Models of Hepatitis B and D Viruses: Search for Cell Permissiveness and Restriction Factors

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Laboratory Models of Hepatitis B and D Viruses: Search for Cell Permissiveness and Restriction Factors

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Abstract

Hepatitis B virus (HBV is a widely spread human pathogen that accounts for 250 mln cases of chronic hepatitis worldwide. Hepatitis delta virus (HDV) is its viroid-like satellite that substantially aggravates liver disease. Importantly, both HBV and HDV are oncogenic viruses. The development of new antivirals and investigation of virus pathogenesis is limited by absence of effective cellular and animal models. Currently used cell models are based on human primary hepatocytes, liver progenitor HepaRG cells, hepatocyte-like cells derived from iPS or various hepatoma cells overexpressing putative NTCP receptor. However, in most of these models a majority of cells are non-permissive. In this review we briefly review these models, summarize approaches to increase infection rates, and discuss data about proviral and antiviral (restriction) host factors. The data discussed suggest that rates of HBV and HDV infection are limited by hepatocyte polarization, insufficient expression of the NTPC receptor as well as existence of additional co-receptors and restriction factors that can act mostly on virus entry stage.

Keywords: hepatitis A virus; hepatitis B virus; hepatitis C virus; hepatitis delta virus; hepatitis E virus; cell models; permissiveness factors; restriction factors

1. Introduction

Hepatitis B, C and delta viruses (HBV, HCV, HDV) are the widely spread human pathogens that account for > 1/3 cases of end-stage liver diseases worldwide, while in certain regions such as Pacifica or Eastern Mediterranean region their input can be predominant [1]. They together with hepatitis A and E viruses (HAV, HEV) belong to different families, have different replication cycle but have one thing in common: they infect almost exclusively hepatocytes – the major type of liver epithelial cells. Acute HAV and HEV are spread by feacal-oral route with water quality being one of the major risk factors (Table 1) [2,3]. These infectious resolve with formation of life-lasting immune response with very few exceptions. However, they represent a threat for patients due to risk of fulminant hepatitis – acute liver necrosis [4]. In contrast, HBV, HDV and HCV are transmitted via non-sterile syringes and medical instruments, blood transfusions, and drug injections [5–9]. HBV is also transmitted vertically, i.e. from infected mothers to their newborn kids [10]. HBV, HCV and HDV are responsible for dozens of millions of cases of chronic infections. Acute hepatitis C establishes chronic hepatitis in

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approximately 80% cases, while rates of chronization of acute hepatitis B lie within a range of <5% in case of adults to >90% in case of infants and neonates [9]. In case of HCV infection the rate of chronization lies within a rather wide range from 55 to 85% [6,8], as a vast majority of patients with acute infection are asymptomatic and thus escape diagnosis. To date, despite existence of prophylactic vaccines against HBV [11,12] and direct acting antivirals (DAA) that can cure >95% patients with chronic hepatitis C [13], there are still 254 and 50 mln patients worldwide with these chronic infections [6,7]. Chronic viral hepatitis is accompanied by inflammation and hepatocyte death by various mechanisms which leads to the development of fibrosis as well as steatohepatitis. Both these conditions are the risk factors of end-stage liver disease – cirrhosis and hepatocellular carcinoma (HCC) [14,15]. This is profoundly aggravated by HDV infection which is a viroid-like HBV satellite [16–18]. Simultaneous HBV/HDV infection (co-infection) usually resolves but has a markedly elevated risk of fulminant hepatitis that presents a death threat for a patient [5]. In contrast, superinfection, i.e. infection with HDV of a chronic hepatitis B patient induces chronic hepatitis delta, with HDV increasing rate of incidence and rate of development of liver cirrhosis and cancer [5]. Liver cancer can also occur in hepatitis B and delta patients without cirrhosis [19]. In fact, HBV, HCV and HDV are currently classified as oncogenic viruses [20,21].

HCC is the predominant type of liver cancer (85-90%), with up to 1 mln new cases arising annually [22]. According to modern estimates, liver cancer is the primary cause of death of 507.7 thousand patients worldwide, with 343.8 thousands (i.e. 68%) of which are attributed to chronic viral hepatitis [4]. Importantly, incidence of liver cancer has increased by 56% from 2000 to 2023 [4]. This underlies the importance of eradication of hepatitis viruses, which requires the development of adequate cell and animal models for these infections.

Table 1. Natural course of viral hepatitis disease.

	HAV	HBV	HCV	HDV	HEV
Transmission route	Faecal-oral	Parenteral, perinatal			Faecal-oral
Annual number of new cases (thousands)	No global statistics [4]	1200 [7]	1000 [6]		19470 (2021) [3]
Rate of chronization	-	>90% (neonates/infants) <5% (adults) [9]	55-85% [6,8]	<5% (coinfection) [23] >90% (superinfection) [24]	Rare [3]
Number of patients with chronic hepatitis (mln)	-	254 [7]	50 [6]	From 12 [23] to 62-74 [5]	
Risk of cirrhosis development for chronic patients	-	8-20% in 5 years [3] [9]	15-30% in 20 years [6,8]	30-70% in 2-6 years	-
Risk of HCC development for non-cirrhotic patients		~ 3% in 5 years [25]		7.5% in 5 years [26]	
Risk of HCC development for patients with cirrhosis	-	2-5% in 5 years [3] [9]	2-4% in 20 years [8]	23.1% in 5 years [26]	-
Annual number of associated deaths (thousands)	From 7.1 (2016) [2] to 35.6 (2023) [4]	1100 (2022) [7]	242 (2022) [6]		4.4 (2023) [4]
Prophylactic vaccine	Yes	Yes [7]	No	Yes [1]	Yes [2]
Curable with antiviral drugs	NA	No [9]	Yes (for 95% cases) [6]	No	NA

[1] Prevented with anti-HBV vaccines. [2] The only available is HEV 293 vaccine (Hecolin) approved in China [3,12]. [3] in case of adult patients. [4] 4548 cases in the EU [https://www.ecdc.europa.eu/en/publications-data/hepatitis-annual-epidemiological-report-2022]; 1648 cases in the US in 2023 [https://www.cdc.gov/hepatitis-surveillance-2023/hepatitis-a/].

HCV is (+)-strand RNA enveloped virus of *Flaviviridae* family [27], reclassified recently to a newly defined *Hepaciviridae* family [28]. It enters hepatocytes by interaction with four different receptors [29–32] with concomitant clathrin-dependent endocytosis [33]. Following uncoating, viral RNA is translated, and proteolysis of the polypeptide leads to production of 3 structural and 7 non-structural proteins [27]. The nonstructural proteins assemble into replicase on ER membrane and ER-

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derived double-membrane vesicles, which are the sites of viral genome replication [34,35]. Subsequent capsid assembly on lipid droplets and their coating with envelope glycoproteins and lipids the virions are released from cells [36].

HCV was discovered in 1989 [37], and the progress in its research and the discovery of DAAs occurred only ten years later. The key events were the development of non-infectious replicon [38] and pseudovirus [39] particle models that allowed investigation of virus entry and replication. The discovery of infectious HCV clone from a patient with fulminant hepatitis C (JFH1 isolate) [40] gave rise not just to an efficient replicon model [41] but also to infectious cell culture model (HCVcc) that gave the researchers ability to study late stages of virus life cycle [42–44]. These discoveries were associated with identification of host proteins that serve either as (co)receptors [29–32] or factors of efficient genome translation (i.e. liver-specific miR122 [45]) or, in contrast, restrict the virus and confer resistance of cells to the infection (i.e. RigI dsRNA sensor that triggers production of type I and III interferons during the infection) [46].

HBV is an enveloped virus with short circular partially double-stranded DNA (rcDNA) genome covalently linked to viral P protein [47]. It enters the cells via initial binding to heparan sulfate proteoglycan (HSPG) [48], subsequent internalization via binding of pre-S1 domain of surface antigen with sodium taurinecholate receptor (NTCP) [49] and clathrin- and dynamin-dependent entry [50]. After uncoating and transporting to the nucleus, P protein is removed and viral DNA is ligated with following plus strand recovery and covalently closed DNA (cccDNA) formation (reviewed in [51]). Lately, cccDNA can be sensed by chromatin remodelers (such as HIRA 1) to form minichromosomal DNA, which persists in cells and serves as matrix for viral genome replication [52]. HBV genome has four partially-overlapping open reading frames that encode Pol (DNA polymerase), S (surface HBs), C (core, capsid, HBc) and X (regulatory, HBx) proteins [53]. Their transcription results in formation of viral protein mRNAs as well as pregenomic RNA (pgRNA) followed by core protein and viral polymerase translation. Then the association of HBc, Pol and pgRNA occurs and reverse transcription leads to synthesis of minus strand DNA with later partial plus strand formation due to presence of direct repeats in viral genome [54]. At the latest stages rcDNA containing particles associate with surface antigens, which are secreted as mature virions or empty capsids.

HDV is an RNA virus with circular RNA genome that has a single open reading frame [17]. Being HBV satellite hepatitis delta has same surface antigens and enter into hepatocytes in a similar manner. After internalization, HDV genome translocated to nucleus where its replication occurs via host cell RNA polymerase activity (with RNA pol II predominantly) [55]. At later stages of life cycle, adenosine deaminases (ADAR) catalyzes editing of stop codon sequence in a negative strand which leads to extension of the ORF which ultimately leads to translation of two versions of HDV antigens: small and large (S- and L-HDAg) [56]. S-HDAg promotes viral genome replication by displacing negative elongation factor from RNAP II and via interactions with histones and chromatin remodeling proteins [57–59]. L-HDAg plays is responsible for transporting of HDV genome from nucleus to cytoplasm, association with surface antigens of HBV on the endoplasmatic reticulum and finally for viral particles assembly and excreting [60,61]. At the same time, both small and large variants of HDAg are found in HDV virions [62] implying that both of them form ribonucleoprotein complex for subsequent coating with HBV surface antigens. It is worth mentioning that envelope proteins of other RNA viruses (such as HCV, Dengue or vesicular stomatitis viruses) can ensure formation and subsequent spread of HDV-like infectious particles [63], albeit so far this has not been shown in nature.

The state of HBV and HDV research resembles the state of HCV research in early 2000s – i.e. prior identification of a full list in indispensable pro-and antiviral cell proteins. It is well acknowledged that just few genomic equivalents (GE) of HBV is sufficient to establish infection in all liver hepatocytes [64]. In 2D culture primary human hepatocytes similar infection rates can also be achieved though up to 10,000 GE are required [64,65]. In contrast, in both tumor and non-tumor cell lines even overexpression the NTCP receptor HBV infection rates do not normally exceed 20-25%

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(discussed below). Permissivity to HDV is much lower, even in PHH [65–68]. This suggests that there are some yet undiscovered host cell pro- or/and antiviral factors.

So, the general goal of this review is to present an overview of available in vitro models, give account of approaches used to increase infection rates, and available data of host cell proteins that either promote these infections or act as antiviral factor, as well as to provide possible directions of their discovery.

2. Cell Culture Models of Hepatitis B and Delta Viruses

2.1. HepG2.2.15 and HepAD38 Cell Lines

Transfection of n-meric viral genome constructs is one of the most efficient way to reproduce infection in vitro. Although such approach may be non-physiological due to absence of viral fusion and entry, it fully reproduces HBV and HDV replication in such non-permissive cells as Huh7, Huh7.5 and HepG2. Back in 1987 M. Sells and colleagues used transfection of 4-meric HBV construct with following antibiotic selection to create HepG2.2.15 cell line which stably produces virus especially under DMSO treatment [69]. Apparently, careful selection allowed to pick a cell clone with successfully conversion viral DNA to a minichromosome with establishment of chronic infection. Thus, this cell line can be effectively used for drug screening, as a HBV positive control or as a source of viral particles with following concentration using 8% PEG for highly concentrated stock solution (for example, [70]). A similar cell line HepAD38 with inducible HBV production was created [71].

Finally, recently Blanchet M. and colleagues used HepG2.2.15 cell line to modify it for HDV coinfection studies [72]. They were able to conduct insertion of 2kb HDV cDNA under Tet-off control via CRISPR-Cas9 system and generate HepG2DB cell line. During the absence of doxycycline these cells demonstrate reliable HDV RNA production as well as virion production. Interesting detail, HBV pgRNA and viral particles production were decreased under HDV replication which is in line with studies of HBV/HDV dynamics in coinfected patients [73].

2.2. Primary Human Hepatocytes (PHH)

Primary human hepatocytes (PHH's) were the first identified cells to support HBV and HDV infection *in vitro* [74,75]. Even now PHH's are considered as the most physiological model due to their metabolic status and levels of expression of hepatocyte-specific genes [76]. They are typically maintained in Williams E medium supplemented with insulin, hydrocortisone and DMSO to support its differentiated state [65]. Under these conditions >90% cells could be infected with HBV [65], albeit it requires 10^3-10^4 GE per cell [65]. The infection in PHH is non-lytic and is stable for >1 month [77]. Moreover, PHH is the The drawbacks of this system are i) low availability of these cells for laboratory usage; ii) very high heterogeneity in infection rates between PHH batches from different donors, as reported from the very first paper on the subject [74]; short-term suitability due to dedifferentiation and decrease in NTCP expression shortly after isolation of the cells [78,79].

2.3. Differentiated HepaRG Cells (dHepaRG)

HepaRG cells were discovered by Guguen-Guillouzo team in 2002 [80]. It was initially isolated from a hepatocellular carcinoma of a patient with chronic hepatitis C. The authors observed that some isolated cells retained ability to grow and form a hepatocyte-like structure upon treatment with DMSO and hydrocortisone. They were also capable of dedifferention with subsequent differentiation. Importantly, these cells despite their origin were negative for a classical HCC marker – α-fetoprotein but in differentiated state demonstrated marked expression of hepatocyte markers such as albumin secretion, cytochrome P450 (CYP) expression resembling those of PHH. Therefore, HepaRG cells became one of the best models for toxicology [81].

HepaRG cells differentiate not just into hepatocytes: the resulting heterogenous culture is comprised of two different areas representing hepatocyte- and cholangiocyte-like cells [82]. The latter

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exhibits expression of typical markers of mature cholangiocytes. So, HepaRG cells are considered as liver bipotent progenitor cells [83].

Differentiated HepaRG cell culture supports HBV infection, though just 10-20% cell are permissive [65]. These cells represent hepatocyte-like cells, while cholangiocyte-like cells remain non-infected. Infection of HepaRG cells requires high MOI: typically 300-1000 genomic equivalents per cell are used.

2.4. HepaRG^{NTPC}

Discovery of NTCP protein as main entry receptor by H. Yuan and colleagues in 2012 significantly boosted all available cell models in HBV and HDV permissiveness [49]. Thus, HepaRG cells with stable ectopic expression NTCP under Tet-inducible promoter has become one of the most useful model. Typically, the most efficient infection can be established by adding 2.5% DMSO, 4% of PEG8000 with MGE 100 or higher [84]. Moreover, due to increased permissiveness, HepaRG^{NTCP} cells could be differentiated much faster than during 4-weeks, as according to the standard protocol. David Durantel's team proposed an alternative approach which takes just 10-day treatment with 1.8% DMSO and 5C cocktail [85].

2.5. Hepatocarcinoma Cell Lines (HepG2^{NTCP}, Huh7^{NTCP}, and Huh7.5^{NTCP})

Initially, Huh7 cells were introduced for hepatitis virus research by Ralf Bartenschlager's group who identified that these cells could support autonomous replication of HCV full-length and subgenomic replicons [38]. However, this was a feature of just few cells from a population [38], leading to isolation of various clones of HCV replicon-harboring Huh7 cells with subsequent clearance of infection with a recombinant interferon α. These clones, obtained by various groups, were labelled as Huh7cured [86] and Huh7.5 [87], respectively. The latter was previously shown to have a mutation in Rig-I (DDX58) sensor of dsRNA thus lacking ability to induce interferon response to RNA viruses [46], albeit lately the inactivation of this innate response pathway was attributed to its missing IRF7 component [88]. To date, there are different variants of Huh7 and Huh7.5 cells circulating in the community, as exemplified in [89]. We even encountered incorrectly labelled cells, i.e. Huh7 cells lacking interferon response indicating that they are actually a Huh7.5 or Huh7cured cell line. With this in mind, Ming Zhou et al screened Huh7 cells from various sources and identified the variant named Huh7D that demonstrated resistance to polyethylene glycol and DMSO with ability for partial differentiation in confluent state [90]. They obtained Huh7D^{NTCP} cell line and showed that it ensured higher levels of HBV infection than classical HepG2^{NTPC} cells. A Huh7.5^{NTCP} cell line was recently reported to support HBV, HCV, HDV and HEV infections after differentiation with DMSO for q week [91]. This was explained by higher levels of NTCP expression. Noteworthy, that in HCV field there is another cell line with the same name (Huh7D) generated by Feigelstock and coauthors [92].

Initially, HepG2 cells were isolated from liver of 15 years old patient with hepatocellular carcinoma in 1975 [93]. The key feature of this is the high-level production of some hepatocyte markers like Alb and AFP, which made them a very popular model in further studies. Due to its easy handling HepG2 were commonly used in Hepatitis C studies as well as for HBV replicon transfection [94,95]. Moreover, even before NTCP was described as HBV/HDV entrance receptor, some experiments demonstrated HepG2 permissiveness for HBV [96,97]. Though, the information on infection effectiveness and antigen expression are controversial and has poor reproducibility [80]. It is worth mentioning that ectopic expression of NTCP significantly improves HBV and HDV permissiveness. Infection of HepG2^{NTCP} cells require 10^2-10^3 genomic equivalents of HBV [98] and lower MOI for HDV are used, typically 20 to 100 particles per cell [99,100]. Additionally, multiple studies demonstrate effectiveness of colony-peaking approach. For example, A. König and colleagues generated HepG2-NTCP derivate cell clone that supports not only infection but also cell-to-cell transmission [101]. Also, in report of Zahoor et al one of subclones demonstrated significantly increased cccDNA content as well as antigens production with efficient infection even in PEG-free

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conditions [102]. Nevertheless, a lot of studies demonstrated multiple genomic rearrangements, transcriptome changes and poor cytochrome P450 expression compared to normal human hepatocytes [103]. As a result, HepG2, being a cancer cell line, has very low suitability in studies of HBV/HDV-driven pathogenesis.

To ensure production of infective HDV virions, a Huh7-HB2.7 cell line was developed by stable expression of HBs antigens together with HBx [104]. Although it is not permissive to the viruses itself due to absence of NTCP, its transfection with pSVLD3 plasmid (encoding a trimer of HDV genome) not only established replication of virus genome but also support late stages of HDV life cycle. In addition, its clone that provided higher titers of HDV virions was selected. Finally, to obtain cells that ensures complete HDV life cycle of HDV a similar hepatoma cell line was obtained from HepG2^{NTCP} by transfection of HB2.7 fragment of HBV genome (encoding HBs and HBx) [105].

Li23 is another HCV-permissive cell line [106,107] that was utilized in hepatitis B research. As in case of Huh7, NTCP overexpression gave Li23 ability to support HBV infection [108].

In 2014, a new hepatocarcinoma cell line - HLCZ01- was isolated [109]. It was shown to express the receptors for HBV (NTCP) and HCV (CD81, SR-BI, claudin 1, occudin) thus not requiring their overexpression. As a result, it can be infected with both viruses. But again, the infection rates for HBV do not exceed 30% of the population. However, HBV infection continues for at least three months. So, this cell line should allow to model HBV/HCV coinfection, although this was not studied by the authors.

2.6. Stem Cell Derived Hepatocyte-like Cells

Human hepatocyte-like cells (hHLCs) is a general term to describe cells with phenotype close to PHH which can be obtained from induced pluripotent stem cells or embryotic stem cells. Initial interest to such cell models dictated by difficulties with PHH availability for many research facilities. Thus, HLCs have become a valuable instrument in drug metabolism and liver diseases.

There are numerous protocols describing HLCs generation but most of them aim to replicate normal hepatocyte maturation in vivo. The description of common steps as well as key factors mediating phenotypical changes are given in Table 2.

Table 2. Steps of differentiation of stem cells into hepatocyte-like cells.

Stage	Cellular identity	Key signaling cues (small-molecule / growth-factor)	What the stage accomplishes
1. Pluripotent stem cell maintenance	hESC or iPSC (self-renewing)	mTeSR1 or equivalent basal medium; ROCK inhibitor (Y-27632) for survival [110,111]	Provides cell survival and pluripotency preservation
2. Definitive endoderm (DE)	Early endodermal progenitors that give rise to liver, pancreas, gut	High Activin A (≈100 ng*ml^{-1}); Wnt activation (CHIR99021) and/or BMP-4; often combined with low-dose FGF-2 [112]	Shift cell phenotype from pluripotent towards endoderm lineage
3. Foregut with hepatic specification	Cells of the foregut that are primed for hepatic fate	BMP-4, FGF-2/FGF-4 and/or low-dose CHIR99021 [113,114]	Establishes a liver-biased transcriptional program (e.g., HNF4α, FOXA2)
4. Hepatoblast / Early hepatic progenitor	Bipotent progenitors capable of becoming hepatocytes or cholangiocytes	HGF (20–50 ng ml^{-1}) and FGF-2, sometimes supplemented with DMSO [115]	Expands a population that can further differentiate into functional hepatocytes
5. Immature hepatocyte-like cells	Cells expressing early hepatic markers (ALB, AFP) but still fetal-like	Continued HGF, addition of Oncostatin M (OSM) and low-dose dexamethasone; small-molecules such as forskolin or vitamin C may be added	Initiates maturation, enhances metabolic enzyme expression

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6. Mature HLCs	Cells resembling adult hepatocytes (high albumin secretion, CYP activity, glycogen storage)	High-dose OSM, dexamethasone, and sometimes additional maturation factors (e.g., HGF boost, nicotinamide, insulin-transferrin-selenium)	Produces functional HLCs suitable for disease modeling, drug screening, or therapeutic applications

Additionally, some protocols describe direct overexpression of factors which promotes iPSCs hepatic commitment. For example, K. Takayama and colleagues used Ad vector for transduction of HNF4α, SOX17 and HEX [116]. HNF4α is known as one of key regulator during liver development responsible for organ architecture formation and epithelium development [117]. Therefore, its overexpression during hepatoblast stage (day 9) (i) increases hepatocyte markers expression (ii) decrease levels of pancreatic and cholangiocytic markers and (iii) promotes mesenchymal-to-epithelial transition which ultimately leads to hepatocyte phenotype development. Moreover, the same group of authors used the established approach for HLCs generation for HBV infection simulation [114]. On the day 25 iPS derived HLCs demonstrated similar to PHH NTCP expression and supported HBV infection. Remarkable that unlike in HepG2-NTCP cells, infection in iPS-HLCs demonstrate a gradual increasement in HBV RNA and antigen proteins levels over time. Nevertheless, no experiments on HDV infection were perfomed using these cells.

Numerous studies demonstrate that iPSC or hESC derived HLCs in monolayer or organized in 3D structures can be used for HBV infection [118,119] but limited information available for hepatitis D. Nevertheless, in the study of H. Chi and colleagues hESC derived HLCs were infected with HDV and demonstrated viral genome replication (both genomic and antigenomic RNAs) as well as HDAg translation [67]. Remarkably that unlike common HCC cell lines, ectopic expression in HLC via AVV transduction has minor effect on infection rate. But the most interesting observation is that coinfection of HBV and HDV (around 8% of HBc positive cells are coinfected with HDV) or monoinfection of HDV followed by AAV-HBsAg boosted hepatitis D viral particle formation and progeny which is consistent with infection *in vivo*. Lastly, it's important to highlight the diverse nature HLCs-based models due to variety of protocols. Nevertheless, some attempts have been made to create transcriptomic-based approach for research guidelines [120].

2.8. Three-Dimensional (3D) Cell Models

Marcus Dorner's team used an alternative approach which was a cultivation of PHH in a microfluidic collagen coated polysterene scaffold [79]. It allowed formation of 3D microtissues in which PHH were functional for at least 40 days and exhibited 5-6 fold higher levels of albumin secretion than 2D PHH cultures. Importantly, these hepatic microtissues could be effectively infected even at ultra-low MOI (0.05 genomic equivalents per cell) without additional reagents. This system was characterized by high levels of HBV cccDNA, pgRNA and HBsAg secretion.

2.9. Other Cell Lines

Primary hepatocytes of chimpanzees are permissive for HDV infection but do not support full infection of HBV [121,122]. In contrast, productive HBV infection can be established in primary hepatocytes of Tupaia [123–125]

*K*ato group reported HBV infection in the NTCP-overexpressing NKNT-3 cell line (NKNT-3^{NTCP}) [126,127], representing human hepatocytes immortalized by transduction of SV40 T-antigen-encoding retrovirus [128]. The parental NKNT-3 cell line was not permissive to HBV [126]. Overexpression of the NTCP receptor conferred ability to support HBV infection but its levels were markedly lower than those in HepG2^{NTPC} cells.

2.11. Co-Culture Systems

One of the major drawbacks of current in vitro models of HBV infection is the absence or very low level of virus spread after initial infection. This can be enhanced by co-cultivation of hepatocytes

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with non-parenchymal liver cells. The first report on the subject characterized a co-culture of human fetal hepatocytes with co-isolated non-parenchymal cells that supported prolonged (up to 10 weeks) HBV infection [129]. This paper did not assess the spread of virions. This approach was later used by Alexander Ploss lab who also demonstrated a prolonged HBV infection upon co-cultivation of PHH with liver stromal murine fetal fibroblasts (3T3-J2 line) [130]. Such co-cultivation was earlier reported to enhance hepatocyte functions and ensure switch to oxidative phosphorylation, as evidenced by increased oxygen uptake[131]. Percentage of infected cells, assessed in the subsequent paper of Ploss lab [132], reached 40% for HBV and 80% for HDV. Importantly, such co-culture system ensured prolonged replication of both viruses in coinfection and superinfection settings. A similar approach from other group could demonstrate HBV spread upon co-cultivation of human PHHs with human fibroblasts [133]. The highest levels of spread were observed at fibroblast:PHH ratio of 1:5. Although the role of fibroblasts in enhancement of infection is not completely understood, they do upregulate expression of the NTPC receptor. A recent paper [134] shows that portal fibroblasts increase functions of hepatocytes in 2D and 3D cultures partially via insulin-like growth factor binding protein-5 signaling but mostly via direct contracts. Additional enhancement of PHH functions can be achieved by inhibiting Notch pathway. However, it should be emphasized that co-culture approach to our knowledge has never been validated in other HBV-permissive cell lines as well as for HDV.

Interestingly, the same paper [133] reports mutations in HBV genome that enhance infection rate and ensure prolonged infection. They include substitutions in HBx (G1764A and C1766T), HBc (A2062T) and P/HBs proteins (G225A). The same mutations are found in patients with chronic hepatitis B, conferring high replication levels and poor prognosis with a high risk of HCC. For *in vitro* models, the mutations allowed to achieve a complete infection at very low MOI (5-25 genomic equivalents per cell).

3. Approaches to Increase Infection Rates

3.1. Low Affinity Binding Enhancers: Polyethylene Glycol (PEG) and Heparin

One of the main approaches to increase rates of HBV and HDV infection is the addition of PEG-6000 or PEG-8000. This compound is widely used for precipitation of HBV [] and other viruses for concentration of virion stocks or to increase sensitivity of their detection in diagnostics [135]. PEG is also a reagent with high fusogenic activity, thus it can enhance entry of enveloped viruses (for example, [136]). Initially it was proposed for infection of PHHs [137] and later the approach was translated for infection of other permissive cell lines []. The standard protocol includes removal of virions (and PEG) containing conditioned medium the next day after transfection to avoid toxicity. However, in recent years Rice group suggested adding PEG 2-3 days post-infection and keeping it afterwards medium to provide a possibility for HBV spread in culture [109]. This approach may increase percentage of infected cells up to 90%. PEG was detrimental for high spread of both HBV and HDV in the settings of PHH-nonparenchymal cell fibroblast co-culture [132] that are extensively discussed below above.

Finally, heparin was also described as agent capable to promote HBV infection [138]. It is interesting because earlier reports demonstrated that NTCP binding with its natural substrates (bile acids salts) can impar HBV and HDV infection [139]. But physiological concentration of heparin, which is capable to bind HSPG, allows to infect cells effectively with lower PEG concentrations. This can be beneficial as the whole model becomes closer to real infection conditions.

3.2. Dimethylsulfoxide (DMSO)

DMSO critically enhances infection of HBV and HDV in primary human hepatocytes [74], HepaRG [140], HepaRG^{NTCP} and HepG2^{NTPC} cells [141]. The only exception are Huh7.5^{NTPC} cells [142]. Its addition increases percentage of infected PHH from 5% [75] to 20% in case of HDV and from 10% to 90% in case of HBV. Its effect is associated with induction of the NTCP receptor on PHH and HepaRG cells as well as with suppressed proliferation and enhanced cell differentiation. So, it is not

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surprising that DMSO primarily enhances entry of viruses rather than their consequent replication [74].

DMSO is an organic solvent that is often used as a differentiation agent for cell lines of various origin including stem and progenitor cells, blood cells, liver cells (for example, [143,144]). DMSO is used during last two weeks of differentiation of HepaRG cells according to the standard protocol. It profoundly increases levels of expression of hepatocyte-specific genes such as albumin, α-antitrypsin, and cytochromes P450 [81,145,146]. In HepaRG^{NTCP} cells it triggers moderate/partial differentiation, as the levels of expression of hepatocyte-specific genes remain far from those in PHH or naïve HepaRG^{diff}. This is currently attributed to suppression of differentiation by NTPC-encoding lentivirus transduction. DMSO is known to trigger partial differentiation of hepatocarcinoma cell lines as well, as revealed by expression of the same hepatocyte markers [147,148].

DMSO becomes dispensable for HBV and HDV infection in experimental system that ensure other techniques for keeping hepatocytes differentiated, such as PHH-nonparenchymal fibroblast cocultures [132]. However, even in this case it does increase rates of infections, although less pronouncedly as monocultures.

Trancing changes in gene expression during DMSO treatment may help to identify pro- and antiviral factors. Apart from induction of the NTCP receptor [149], DMSO in HepaRG cells increases expression of genes regulated by PXR and PPARα, and the genes controlled by histone acetylation [145]. It also induces remodeling of cytoskeleton and extracellular matrix. This points to these genes as potential proviral factors.

DMSO triggers reprogramming of cell metabolism. In HepG2 cells it suppresses glycolysis and lactate production, as well as biosynthesis of fatty acids / triglycerides [150]. Reduction of lipid levels in DMSO-treated cells is also achieved via induction of autophagy [151]. This does not necessarily imply shift from glycolytic to respiratory phenotype, as such data are missing for liver cells, and in breast cancer cell lines DMSO was reported to suppress oxidative respiration [152]. In contrast, differentiated HepaRG cells demonstrate oxidative respiratory phenotype with extremely low glycolytic capacity [153]. Transcriptomic analysis also shows suppression of biosynthetic and activation of catabolic pathways which is contrary to the phenotype of hepatocarcinoma Huh7.5 cells [154]. However, metabolic reprogramming is unlikely to affect HBV/HDV infection rates, as these viruses have rather similar replication levels in HepaRG, HepaRG^{NTCP} and Huh7^{NTCP} cells [66].This conclusion is also confirmed by our recent paper showing absence of anti-HBV or anti-HDV activity of pharmacological inhibitors of central metabolic pathways (glycolysis, glutaminolysis, serine and asparagine biosynthesis), proviral effect of DMSO is unlikely be mediated by metabolic reprogramming [153]. However, this has to be verified with inhibitors of pathways that were not tested in this paper such as respiration/oxidative phosphorylation.

Interestingly, DMSO significantly enhances infection rates in HepaRG cells of not only HBV or HDV but also of human adenovirus [155]. Its effect was much more pronounced in Williams E medium rather than in classical DMEM. Importantly, DMSO enhanced entry of adenovirus and not its subsequent replication. This implies that its role is likely to be associated with elevated expression of virus receptor and/or enhanced endocytosis. However, the authors could not totally exclude partial effect of this solvent on repression of antiviral proteins such as PML.

3.3. Culture Medium and Extracellular Matrix

Optimization of culture medium is less widely used approach. To date most studies are performed in PHH or HepaRG cells maintained in Williams E medium supplemented with insulin (suppressor of glyconeogenesis and activator of PI3K/AKT cascade) and hydrocortisone (differentiation inducer) as well as DMSO as the differentiation agents [156,157], or in NTCP-overexpressing hepatocarcinoma cell lines in DMSO-supplemented DMEM or DMEM/F12 [98,158]. As mentioned above, in the first case, Williams E medium is required at least for a more protent effect of DMSO on cell differentiation process. However, there are also scarce reports on attempts to enhance infection rates by adjusting medium composition. One of the most successful attempts was

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reported by Zodgdi Feng group [159]. They showed that cultivation of HepG2^{NTPC} cells in commercial human inducible pluripotent stem cell (iPSC)-derived hepatocyte maintenance medium (HMM) from Takara Bio significantly enhanced expression of the NTCP receptor and enhanced rates of HBV infection. This medium potentiated virus entry rather than replication, as the increase required at least 24 h preincubation prior the infection. The authors also showed that the medium did not affect virus spread after the initial infection. Transcriptomic analysis identified differential expression of various genes. An additional approach to increase rates of HBV infection is the usage of reagents that enhance differentiation of cells towards mature hepatocytes. It was reported that a mixture of 5 small molecules supports long-term polarization of PHH with high-level expression of hepatocyte markers and significantly prolongs HBV infection [160]. This mixture includes an adenylate cyclase activator forskolin (FSK), an inhibitor of TGFβ1 receptor SB43, an inhibitor of Notch signaling DAPT, inhibitor of Wnt/βcatenin pathway IWP2, and BMP1 receptors LDN193189. Later, Lucifora and colleagues reported that the same C5 mix can accelerate HepaRG^{NTCP} differentiation, boost NTCP expression and thus allow efficient HBV and HDV infection [85]. Importantly, this medium allowed to differentiate HepaRG cells just within one week, in contrast to four-week standard protocol.

Tyrell's group reported substitution of human serum instead of classical fetal bovine serum [142]. Such change resulted in two-fold increase in cccDNA levels and up to tenfold increase in pgRNA levels and HBsAg secretion. The effect is at least partially mediated by enhanced glycosylation of the NTCP receptor with unaltered levels of this protein. Noteworthy, earlier the same group demonstrated enhancement of HCV infection rates in Huh7.5 cells by FBS-to HS substitution [161]. In case of HCV the enhancement was mediated by production of more infectious virions due to better differentiation of hepatoma cells in the presence of human serum and higher production of lipoproteins that associate with HCV virions and define their infectivity. In case of HBV infection, HS can also promote infection via enhanced differentiation of Huh7.5^{NTCP} cells, as they require 21-day differentiation [142].

Selenite is another trace nutrient added to HBV-permissive cell lines. It was initially used as one of components of serum-free in experiments with non-adherent HepaRG cells (see below) [162]. Selenite is required for expression of selenoproteins many of which act as antioxidant enzymes (such as glutathione peroxidase 4). HBV, HCV and HDV induce oxidative stress [163,164], therefore, antioxidant enzymes could be required for their efficient replication and survival of infected cells. HCV was previously shown to induce GPx4 expression to protect infectivity of its virions against lipid peroxidation-mediated inactivation [165]. Moreover, Rice group identified a selenoprotein Sec14L2 as a pan-genotype proviral factor in Huh7.5 cells [166]. In virology-unrelated studies, selenite was shown to protect cells seeded at low density against ferroptosis – a programmed variant of necrosis [167]. However, in case HBV and HCV research, the role of selenite remains doubtful, as the only paper reports its moderate antiviral activity at rather high (micromolar) concentrations [168].

Growing hepatocytes on various matrixes is known to promote their long-lasting survival and correct differentiation [169]. Moreover, the best results are observed when hepatocytes are kept between two layers of collagen [170]. This approach has been successfully applied for hepatitis assays. For example, HepG2.2.15 cell are maintained on collagen-coated plates/dishes for high-level infection [171]. Even higher yields of HBV virions are obtaine, if HepG2.2.15 cells are cultivated on Cytodex-3 substrate [172].

Another approach for enhanced and more rapid differentiation of HepaRG or hepatoma cells is coating of cell culture with Matrigel [82,173]. However, to our knowledge, coating of 2D cultures of hepatic cells with Matrigel has never been studied in the context of hepatitis viruses.

3.4. Infecting Cells in Suspension

Another approach that has been reported by several groups is the infection of cells in suspension. To date there are two variants of such infection. The one, reported by Okuyama-Dobashi et al, involved cultivation of HepG2^{NTPC} cells un serum-free media (Williams E medium supplemented with classical insulin/hydrocortisone together with dexamethasone, selenite, transferrin, and

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recombinant human epidermal growth factor (EGF) [162]. In such conditions the authors could achieve similar levels of HBV infection at considerably lower infection MOI (as low as just 100 GE per cell). Importantly, they achieved infection of a majority of cells in population, which is never observed in adherent cells. A detailed analysis suggested that virus entry was mediated through pre-S1 region of the HBs antigen.

An independent study of Ran Yan and colleagues proposed a "spinoculation" protocol of infection, in which adherent HepG2^{NTPC} cells are harvested by classical trypsin-ETDA procedure and are infected during centrifugation (up to 1,000 g) with subsequent cultivation in adherent state [141]. This allowed to increase infect up to 60-70% cells, while standard procedure yields up to 25%.

These two quite similar approaches suggest that virus attachment to cells remains one of the most critical steps in the infection process. On one hand, suspension cells can be more permissive also via different gene expression profile (with possible more favourable expression of host proviral factors), as they are cultivated in serum-free medium. On the other hand, the success of spinoculation protocol may show that the cells in suspension could merely be more "open" for correct HBV attachment, which could be additionally enhanced by centrifugation. One possible explanation could be a polarization of HepG2 or HepaRG cells, as the virus seems to infect via basolateral membrane which is not readily accessible in differentiated HepaRG cells [65]. Urban group demonstrated that disruption of cell-cell contacts (by EGTA) increased the rate on HBV infection [65]. Supporting evidence come from a microfluidic 3D PHH model, in which HBV spread towards a majority of cells can be achieved with just 0.05 genomic equivalents of the virus [79]. These approaches still have to be verified by other groups and be evaluated for hepatitis delta virus.

4. Host Cell Proteins as Permissiveness Factors to HBV and HDV

HBV entry occurs via clathrin- and dynein-dependent endocytosis [50]. There are three stages in early steps of virus life cycle that are required for establishment of production infection. They are: i) initial attachment of virions to low-specific factors of cell surface, ii) binding to receptor(s) and subsequent internalization and iii) conversion of rcDNA into cccDNA [50].

The initial step of HBV entry in binding of virions to heparan sulfate proteoglycans (HSPGs) (Table 3) [48,174]. Their glucosaminoglycan side chains mediate binding to the large HBV surface antigen (L-HBs) [174,175]. To date, two members of HSPGs were identified as pro-HBV/HDV factors: Glipican 5 [176] and Syndecan 2 [177]. Bartenschlager's group also revealed that HBV binds lipoptoteins and can reach hepatocytes via import into Kupffer cells and subsequent re-export by cholesterol transport system [178]. However, this is not likely to affect infection of hepatocytes in monoculture models.

The major host cell protein that ensures HBV/HDV entry is the sodium/taurocholate cotransporting polypeptide (NTCP) expressed in mature hepatocytes [49,66,139]. It was discovered in HepaRG cell, as various hepatocarcinoma cell lines do not express this receptor. However, its overexpression in hepatoma cells is not sufficient for virus entry, as it does not necessarily results in correct localization [179]. E-cadherin ensures correct localization of NTCP on cell membrane through their direct association [180]. Moreover, NTCP is the glycoprotein, and status of its posttranslational modification also significantly affects permissiveness of liver cells to HBV [108,181]. These modifications are required for NTCP-E-cadherin binding [180]. Noteworthy, NTCP is likely to be not the sole receptor for HBV and HDV, as its overexpression on cell surface in non-liver cells does not make them permissive [181].

Table 3. Host cell permissiveness factors to HBV and HDV.

Permissiveness Factor	Role
NTCP	Putative receptor for HBV and HDV
EGFR	Mediates clathrin-dependent entry after binding of viruses to NTPC

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IFITM3	Facilitates entry in NTPC-dependent manner
Heparan sulfate proteoglycans (HSPGs): Glypican 5 and Syndican 2	Serve as low-affinity attachment factors that prime HBV entry.
Nrp1	Binds HBV prior NTCP and is required for subsequent attachment of virions to NTPC
E-cadherin	Is required for correct localization of NTCP
NTCP-interacting proteins (e.g., EGFR, E-cadherin, IFITM3)	Modulate NTCP localization and entry efficiency.

Epidermal growth factor receptor is a pivotal cofactor required for HBV entry [182]. It mediates uptake of virions after their initial binding to NTCP. EGFR knockdown does not abolish attachment of HBV particles on cell surface but blocks their entry via clathrin-dependent endocytosis [182–184]. Importantly, interaction between NTPC and EGFR is required for this process [182], with NTPC oligomerization after initial association of these two receptors[185]. Its cofactor – EGF which is secreted by liver sinusoid cells modulates HBV entry by regulation of EGFR uptake route: low EGF levels support clathrin-dependent endocytosis, while high concentrations switch it to the independent utilization to lysosomes [183].

Another proviral factor for HBV and possibly for HDV is Neurolipin 1 (Nrp1) [186]. It was discovered as a differentially-expressed gene in HBV^+ and HBV^- cells in liver biopsies of children with chronic hepatitis B. Detailed analysis revealed that Nrp1 binds to pre-S1 domain of L-HBs prior to its attachment to NTCP. Nrp1 inhibition abolishes efficient binding of HBV virions to NTCP and their subsequent entry. However, it remains unclear if its overexpression can substantially increase infection rates in cell models.

The last known to date co-receptor for HBV is the Interferon-induced transmembrane protein 3 (IFITM3) [187]. Though it belongs to classical members of innate immune response against viral infections, Palatini and coauthors revealed that IFITM3 can bind to NTCP receptor and facilitate HBV uptake. Moreover, its knockout significantly reduced levels of HBV infection.

McKeating group clearly demonstrated that most bound virions are internalized but in most cells no cccDNA is not formed [50]. It points to cleavage of the P-protein and subsequent formation of double stranded viral genome by host cell reparation enzymes as host factors required for the infection. The enzymes involved in this process include tyrosilphosphodiesterase 2 (TDP2), FEN1 nuclease, and various other members of DNA replication and reparation machinery [47,188–190]. They are reviewed in details by Wei and Ploss [51].

5. HBV and HDV Restriction Factors in Host Cells

The information about possible factors that may restrict HBV and especially HDV infections is scarce. With the only exception, the data were obtained from the bulk population of infected and uninfected cells thus not allowing to discriminate between permissive and non-permissive cells. In addition, influence of HBV/HDV co-infection on expression and role of restricting factors remains mostly unexplored.

5.1. HBV-Restricting Host Factors

5.1.1. Interferon-Stimulated Genes (ISGs)

The main innate immune response against viral infections is represented by production and consequent signaling of type I and III interferons [191]. IFNs are produced in response to sensing viral RNA molecules (dsRNA or 5′-pppRNA) by a set of sensors including Rig-I, MDA5, and TLR3/7 [192,193]. In response to interferon production, the neighboring cells strongly induce an array of genes that encode proteins that interfere with various stages of life cycle of RNA viruses. They are referred to as interferon-stimulated genes (ISGs) [194]. Indeed, there are data showing that both viral RNA-sensing proteins and ISGs suppress HBV replication. Such inhibition is attributed to ISGs, as

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similar suppression can be induced by exogenous dsRNA molecules, as shown in PHH-mouse fibroblast co-cultivation system [132].

Typically, type I and III interferons are induced simultaneously, as in case of influenza virus [195] and HDV [68]. In contrast, two groups reported that HBV induces selectively interferons λ1 (IL29) and λ2/3 (IL28A/B) with concomitant production of ISGs. The data were obtained from PHH [196] and the above-mentioned non-tumor immortalized NKNT-^{3NTCP} hepatoma cell line [126]. In the first case this was mediated via Rig-I sensor that recognized ε-region of viral pgRNA [196], whereas in the second – by sensing HBV DNA by Cyclic GMP-AMP synthase (cGAS) with concomitant activation of STING [126]. A clone of NKNT-^{3NTCP} cells with lower sensitivity to the virus demonstrated higher levels of cGAS and induction of ISG56 suggesting that cGAS can act as an HBV-restricting factor that is not expressed in classical HBV-permissive cell lines such as HepG2^{NTCP} [127]. It should also be mentioned that HBV can overcome sensing by cGAS via down-regulation of its expression as well as by packaging its DNA into capsids [197].

Sato et al showed that different genotypes of the virus may have different capacity to induce these cytokines [196]. Unfortunately, their study did not include genotype D of the virus that is predominantly used in HBV models such as HepG2.2.14 and HepAD38 cell lines used to produce virus stock. Moreover, a list of other reports showed no induction of interferons and ISGs in chronic hepatitis B virus patients [198] as well as during acute HBV infection in chimpanzees [199] or humanized mice model [200]. Zoulim's lab also demonstrated that HBV does induce IFNs in HepaRG cells if delivered with baculovirus vector but suppresses their production and subsequent response during natural infection [201,202]. This could be explained by inhibition of RigI/MDA3 and TLR3 sensing systems by HBV [201] by HBx [203–205] and P [206,207]. Metabolic reprogramming could be another mechanism by which the virus evades induction of type I interferons, as demonstrated in PHH [208]. This is mediated by disruption of HK2/MAVS/Rig-I interaction on the outer membrane of mitochondria leading to enhanced aerobic glycolysis and increased lactate production. Lactate binds free MAVS adaptor protein preventing its interaction with Rig-I and subsequent interferon induction.

Interferon lambda can suppress HBV replication [209]. This is restricted by expression of different isoforms of the respective receptor – IFNLR1 [209], as it is expressed in rather low levels in differentiated HepaRG cells and hepatoma Huh7.5 cells, as revealed by RNA-seq by our group for instance [146]. So, this receptor could be regarded as another anti-HBV host factor.

Various ISGs repress HBV replication predominantly by interfering with post-transcriptional stages of virus life cycle. They include not only the abovementioned ISG56 [127], but also Mx1 (MxA) [210,211], Mx2 (MxB) [212], zinc finger antiviral protein (ZAP) [213], ISG20 [214,215], Bst-2 (tetherin) [216–218], and SAMHD1. MxA scavenges HBc in the perinuclear space preventing capsid assembly [211] as well as inhibits export of viral RNA from the nucleus [210]. However, we should acknowledge the ability of HBc to suppress MxA expression [219]. In contrast, MxB is believed to suppress conversion of rcDNA into cccDNA not allowing to establish productive infection [212]. ISG20, a host cell RNAse with broad spectrum of antiviral activity [220], binds HBV pgRNA and targets it to degradation [214,215]. ZAP is another ISG with ant-HBV effect that acts at post-transcription stage of virus life cycle [213]. Presumably, it scavenges pgRNA and Bst-2 in a complex with the regulatory ATP2B2 subunit of Na/K-ATPase inhibits HBV replication by preventing virion secretion [216–218]. Two other ISGs, namely Galectin-9 and viperin, suppress HBV replication by scavenging HBc antigen into cytoplasmic puncta and subsequent targeting to degradation [221].

SAMHD1 is the dNTPase [222] that is induced by type I interferons [223]. It can suppress and activate HBV replication by different mechanisms. On one hand, this enzyme suppresses HBV replication by decreasing nucleotide pools [224]. On the other, it enhances cccDNA formation [223]. One can speculate that its action during the infection can be modulated by CDK2 [225].

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5.1.2. Transcription Regulators

To date, several host cell proteins were identified as factors that suppress HV transcription and therefore its efficient replication. They could be divided into three groups: structural maintenance of chromosomes' (Smc) complex, Tripartite motif (TRIM) proteins and NFκB. In 2016 a Nature paper [226] with several consequent papers from other groups [227–229] reported that Sms5/6 can act as a repressor of HBV transcription, with HBx protein that targets Smc5/6 to ubiquitin-dependent degradation. Antiviral activity of Sms5/6 is achieved via targeting cccDNA to promyelocytic leukemia (PML) bodies, in which SMC5-SMC6 localization factor 2 (SLF2) acts as a transcription repressor [230].

Another group of anti-HBV factors is presented by members of TRIM proteins. They include TRIM5 [231], TRIM6 [231], TRIM11 [231], TRIM14 [231], TRIM21 [232], TRIM22 [233], TRIM25 [231], TRIM26 [231,234], TRIM31 [231], TRIM28 [235], TRIM41 [231], and TRIM56 [236]. The most active are TRIM5 and -11 isoforms [231]. They inhibit activity of core promoter and thus suppress HBV transcription [233,236]. Antiviral effect of TRIM21 and TRIM26 is likely to be mediated by targeting HBx to degradation [232,234].

The third repressor of HVB transcription is NFκB [237]. It act by bindings to Sp1 sites in HBV genome. However, NFκB cannot be considered as a bona fide restriction factor, as it is present in all cells and can be activated by various stimuli.

5.1.3. Cytidine Deaminases

Cytidine deaminases APOBECA3 isoforms represent another group of host factors that restrict HBV replication. APOBEC3G blocks assembly and production of HBV virions *in vitro* and *in vivo* [238,239]. In addition, APOBEC3G [240–242] as well as A3B [241–243], A3C [240–242], A3F [242], and A3H [241] edit HBV DNA either after incorporation into HBV capsids or at cccDNA level. This is achieved by interaction with APOBEC1 members [244]. HBx protein is capable of decreasing levels of APOBEC3G by promoting its secretion from cells [245]. However, its effect on levels of other isoforms of the enzyme remains unknown. APOBEC3 enzymes represent a valuable target for developing antiviral drugs. We showed that CRISPR-based induction of APOBEC3 expression leads to a two-log reduction of HBV replication [246]. Therefore usage of cytosine base editors is considered as a potential approach to suppress HBV infection in vitro and in vivo [178]. Antiviral activity of APOBEC3 members can be also decreased by host cell proteins such as DHX9 that prevents their binding to pgRNA of the virus [247].

5.1.4. DEAD-Box Helicases (DDX)

DEAD-box helicases comprise a large family of proteins with numerous functions that are involved in innate immune response [248,249]. Several of its members exhibit anti-HBV activity. The most notable example is DDX3 which is known to suppress replication of various viruses [250]. It suppresses HBV transcription [251] as well as reverse transcription of HBV pgRNA [95]. DDX17 is another member of the family that blocks packaging of pgRNA into capsids [252] where it is reversely transcribed into rcDNA [253]. Noteworthy, HBV infection upregulates DDX17 via HBx protein [254]. DDX17 as well as DDX5 inhibit HBV transcription termination leading to decreased stability of HBV RNA adding both enzymes as restriction factors of host cells [255].

5.1.5. Inflammatory and Profibrotic Cytokine-Signaling

Inflammatory cytokines produced by Kupffer cells (KC) also pronouncedly suppress HBV replication, as shown by Dorner group in a microfluidic 3D PHH-KC co-culture model [79]. Also, the discussed above cGAS/STING pathway also leads to induction of proinflammatory cytokines such as IL-6 in NFκB-dependent manner, which also contributes to suppression of HBV replication [126]. This could be achieved via IL-6-mediated suppression of NTCP expression in hepatocytes [256]. Other possible mediators of such inhibition remain undiscovered. One of them could be transforming

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growth factor β-activated kinase 1 (TAK1) which suppresses HBV transcription [257]. This protein is a mediator of TNFα, IL-1, and TGFβ1 [258]. However, this protein is unlikely to act as a restriction factor, as its degradation was also shown to down-regulate HBV replication [259]. DDX5 also acts as a repressor of HBV transcription by [260,261]. We should mention that DDX5 expression ensures interferon signaling in HBV-infected cells [262]. And of course, interferon signaling is dependent on DDX58 – Rig-I [196].

5.1.2. Miscellaneous

Other proteins that can restrict HBV infection are SERINK5 [263]. SERINK5 prevents secretion of HBV particles [263]. Its role is confined in inhibiting HBs glycosylation. Another study revealed SRSF10 protein, a regulator of alternative splicing machinery, as an HBV-restricting factor [264]. It is likely to control turnover of nascent HBV RNA.

5.2. HDV-Restricting Host Factors

At the same time, type I and III interferons are strongly induced by HDV that may accompany HBV infection [68,153]. The key sensor for HDV RNA is MDA5 [68]. Data on antiviral activity of type I interferons are contrary: The above-mentioned paper reports no changes in HDV replication, whereas Durantel's group evidenced suppression of HDV infection [68], although at non-physiologically high dose of IFNα [265]. However, proinflammatory NFκB signaling could restrict HDV spread, as its induction using various stimuli was shown to down-regulate release and, more importantly, infectivity of HDV virions [265]. This correlates with only moderate suppression of HDV replication by exogenous dsRNA in PHH-mouse fibroblast co-cultivation assay [132].

A recent study from Arnand Carpentier and colleagues identified IRF1 as a factor that restricts HDV spread [266]. They analyzed HDV-infected hepatocyte-like cells differentiated from iPS by single-cell transcriptomic analysis, paying special attention to levels of (+)- and (-)-copies of viral genome as well as to their correlations with expression of host proteins. This led to identification of IRF1, a member of ISGs, as a protein that suppresses HDV replication and spread during division of infected cells. Of note, IRF1 was not expressed in Huh7^{NTPC} cells, thus this host defense mechanism cannot be studied in in the latter cell line. However, as Huh7^{NTPC} cells are not fully permissive to HDV, there are other restriction factors that have to be identified.

Of note, the same paper [266] also revealed elevated levels of DDIT4 in infected cells that harbor genomic RNA of the virus (demonstrating active HDV replication). Although this excludes DDIT4 from a list of restricting factors, it suggests reprogramming cell metabolism by the virus. DDIT4 that suppresses mTOR pathway [267] and a regulates autophagy [268], is known to be induced various types of stress including ER stress [267,269,270]. As previously we showed that HDV antigens can induce ER stress and unfolded protein response [164], this paper supports our data, not verified in infectious cell model.

6. Future Directions

The data discussed above show that current laboratory in vitro models are inefficient, as in majority they do not allow to infect most cells. The exception is primary human hepatocytes. However, their limited accessibility and high heterogeneity make them not available for many research groups. In addition, infection rates for HDV are also not high. This requires more endeavors to improve these systems.

As presented in the Section 3, there are approaches to increase infections rates. They are the usage of DMSO and other inducers of hepatocyte differentiation, PEG as a reagent that enhances virus entry. However, they still do not ensure efficient infection in non-primary cells. The exact reasons are not fully understood. On one hand, low infection rates could be due to cell polarization making the NTPC receptor not available for HBV/HDV virions. This is supported by enhanced entry of cells in suspension during centrifugation as well as if maintained on in 3D conditions on matrixes.

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An additional supporting piece of evidence comes from increased levels of infection of cells maintained in hepatocyte maintaining medium.

On the other hand, changes in cell maintaining can also lead to changes in expression of yet undiscovered proviral or virus-restricting factors. Permissiveness of cells to HBV is more likely to be affected by host dependency factors rather than restricting factors [271]. As transfection of cccDNA into non-permissive cells leads to expression of viral proteins, the limiting host factors are rather upstream of cccDNA formation. Nrp1 can act a co-receptor, yet this assumption should be verified by its overexpression. Search for additional factors should be continued.

Such search can be made by various approaches such as development of reporter cell lines to visualize infected cells or single-cell RNAseq analysis. Both of them can yield a list of potential proviral (as well as antiviral) factors. The list can be narrowed by comparison of list of differentially-expressed (DE) genes with DE genes in cells maintained in media that enhance infection (such as HMM).

Another direction of future research can include optimization of culture medium for maintaining cells. As HMM is likely to include growth factors and regulators of signaling cascades, as well as 5C reagent mix, we should reveal which cascades increase HBV and HDV infection. Specifically, we should assess the role of Wnt/βcatenin, Notch, and Yap/Taz cascades. We should also apply more physiological media such as Plasmax or HPLM that resemble human blood plasma [167,272–274]. Lastly, impact of oxygen level should be assessed, probably by decreasing its levels to 5-6% [275], as oxygen is detrimental for maintaining healthy hepatocytes, their attachment and differentiation. However, we cannot exclude that its level should be increased, as it was shown earlier that an increase in oxygen tension to 30-40% supports metabolic performance of hepatocytes [276]. Similar enhancement of hepatocyte functions can be ensured by usage of specific matrixes that carry oxygen levels within the settings of collagen [277], so such studies should be carried out not only in standard 2D cell cultures but also in 3D cultures or when using extracellular matrixes.

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