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Hepatitis B Virus cccDNA: Formation, Regulation and Therapeutic Potential

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Abstract

Hepatitis B virus (HBV) infection remains a major public health concern worldwide with about 257 million individuals chronically infected. Current therapies can effectively control HBV replication and slow down disease progress, but cannot cure HBV infection. Upon infection, HBV establishes a pool of covalently closed circular DNA (cccDNA) in the nucleus of infected hepatocytes. The cccDNA exists as a minichromosome and resists to antivirals, thus a therapeutic eradication of cccDNA from the infected cells remains unattainable. In this review, we summarize the state of knowledge on the mechanisms underlying cccDNA formation and regulation, and discuss the possible strategies that may contribute to the eradication of HBV through targeting cccDNA.

Keywords

host-virus interaction; HBV cure; HBV cccDNA minichromosome; transcriptional regulation; cccDNA eradication; drug target

1. Introduction

Hepatitis B virus (HBV) infection remains a major public health burden. While one third of the people worldwide have been exposed to HBV, about 257 million of them are chronically infected according to WHO report (Revill et al., 2019). Those people are at high risk of developing cirrhosis and hepatocellular carcinoma (HCC) (Liang et al., 2015; Polaris Observatory, 2018).

HBV is a small DNA virus specifically targets hepatocytes. The virus infection is initiated through a low-affinity interaction of viral envelope protein with heparan sulfate proteoglycans (HSPG), followed by a high-affinity specific interaction with its receptor

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sodium-taurocholate cotransporting polypeptide (NTCP) to facilitate viral entry (Schulze et al., 2007; Verrier et al., 2016; Yan et al., 2012). Furthermore, the epidermal growth factor receptor (EGFR) is involved in HBV internalization into hepatocytes(Iwamoto et al., 2019). Mediated by endocytosis, viral particle is then transported from early to late endosomes for releasing of nucleocapsid from the envelope (Macovei et al., 2013). Afterwards, viral nucleocapsid migrates along microtubules to the nuclear periphery (Rabe et al., 2006). Disassembly of capsid at the nuclear pore results in the release of viral relaxed circular DNA (rcDNA) genome into the nucleus, where the partially double-stranded rcDNA is converted into the covalently closed circular DNA (cccDNA). cccDNA serves as a template for transcription of viral RNAs through employing the cellular transcription machinery(Rall et al., 1983). In the cytoplasm, together with the viral polymerase, the 3.5 kb pregenomic RNA (pgRNA) is encapsidated by HBV core protein (HBc) to form viral capsid, inside of which pgRNA is reverse transcribed into negative-strand DNA, followed by an asymmetric synthesis of plus strand DNA to yield the viral genomic rcDNA. The rcDNA-containing capsids are either transported back to the nucleus to amplify the cccDNA pool or enveloped and released *via* multivesicular bodies as progeny virions. Additionally, HBV double stranded linear DNA (dslDNA), which is occasionally produced by reverse transcription of the pgRNA as an aberrant by-product, can be released as defective virions or integrated into host genome (Tu et al., 2017). Although HBV integration normally fails to transcribe precore mRNA and pgRNA due to the loss of upstream basal core promoter, it can still act as a template for hepatitis B surface antigen (HBsAg) expression(Sung et al., 2012; Wooddell et al., 2017).

The persistence of cccDNA in the infected hepatocytes is the major challenge to antiviral therapies. Currently, two classes of treatments are approved for chronic HBV infection, including interferon alpha (IFNα) or its PEGylated form PEG-IFNα and nucleos(t)ide reverse transcriptase inhibitors (NRTIs)(Xia and Liang, 2019). Both treatments are effective to some extent but have limitations(Tang et al., 2018). IFN-α is the only approved immunomodulatory drug, however, the response rate remains low and side effects are often difficult to tolerate(Ghany, 2017). NRTIs target the reverse transcriptase activity of viral polymerase to limit virus replication, but exhibit little or no effect on HBV cccDNA, thus cannot clear virus infection. Therefore, there is an urgent need to develop novel therapies that can cure HBV infection.

Complete sterilizing cure of hepatitis B, which is defined as undetectable HBsAg in serum and eradication of all forms of HBV DNA including cccDNA, can be hardly achieved at this moment(Lok et al., 2017). Current aim of chronic hepatitis B treatment is a “functional cure” of viral infection. This aim is defined as seroclearance of HBsAg, undetectable serum HBV DNA, normal liver enzymes and histology after stopping treatment, which requires complete blockade of HBV cccDNA(Lok et al., 2017). As the viral persistence reservoir which play a central role in HBV infection, HBV cccDNA is the key obstacle for a cure. Here, we summarize our current knowledge about the basic biology of HBV cccDNA, including its formation and regulation, and discuss potential therapeutic strategies to target cccDNA.

2. The Formation of HBV cccDNA minichromosome

The formation of cccDNA in nucleus requires the nuclear transportation and uncoating of rcDNA, which is initially located in cytoplasmic viral capsid upon infection and *de novo* replication. Previous studies have demonstrated that viral envelope proteins regulate cccDNA formation through sorting the mature nucleocapsids into virion morphogenesis and egress route, hereby reducing the intracellular cccDNA amplification (Lentz and Loeb, 2011; Raney et al., 2001; Summers et al., 1990). Furthermore, recent studies have identified capsid protein residues interacting with viral envelope or serving as CDK2 phosphoacceptors play important roles in nucleocapsid maturation and/or rcDNA uncoating, which eventually affect the outcome of cccDNA formation in nucleus (Cui et al., 2015a; Cui et al., 2013; Liu et al., 2015; Luo et al., 2020b). The detailed molecular mechanisms underlying the conversion of rcDNA to cccDNA remain elusive. Many evidences suggest that a multi-step process, including cellular ATR-CHK1 DNA damage response, DNA repair and chromatinization, is involved (Guo and Guo, 2015; Hu et al., 2019; Luo et al., 2020a; Nassal, 2015). Presumably, this multi-step process involves the release of the covalently bound viral polymerase and RNA primer from the rcDNA negative strand and positive strand, respectively; the cleavage of terminal redundancy from the negative strand; repair of the incomplete positive strand and ligation of both DNA strands (Figure 1).

Removal of the viral polymerase from rcDNA is considered as a mandatory step in cccDNA formation, giving rise to a deproteinated (DP) (or protein-free) rcDNA as putative precursor for cccDNA biosynthesis (Gao and Hu, 2007; Guo et al., 2007; Guo et al., 2010). Previous studies suggested that cytoplasmic rcDNA deproteination is associated with a nucleocapsid conformation shift, which leads to the exposure of capsid protein nuclear localization signal (NLS) for karyopherin-mediated nuclear transportation of the DP-rcDNA-containing capsid (Guo et al., 2007; Guo et al., 2010). The mechanism of rcDNA deproteination remains largely unknown. A DNA repair enzyme tyrosyl-DNA phosphodiesterase 2 (TDP2) has been shown to unlink the attached viral polymerase from HBV and duck hepatitis B virus (DHBV) rcDNA *in vitro* (Jones et al., 2012; Koniger et al., 2014). However, TDP2-knockout cells still support human HBV infection, indicating that other protein(s) functionally close to TDP2 may contribute to this activity(Cui et al., 2015b). Alternatively, it has been shown that host factor flap structure-specific endonuclease 1 (FEN1) is involved in cccDNA formation, which perhaps cleaves the putative 5’ flap-like structure on the negative strand of rcDNA to remove the terminal redundant sequence (r) together with viral polymerase (Kitamura et al., 2018). However, it remains very possible that the 5’ r is removed by FEN1 after rcDNA deproteination as a previous study demonstrated that the 5’ r was maintained on DHBV DP-rcDNA (Guo et al., 2007), or the 3’ r is removed by other nuclease(s) during cccDNA formation.

Inhibition of HBV polymerase by NRTIs does not block cccDNA formation in various HBV *de novo* infection models, suggesting that the host DNA polymerase(s) rather than viral polymerase is responsible for cccDNA formation if DNA synthesis is required. (Hantz et al., 2009; Qi et al., 2016; Xia et al., 2017). In line with this, DNA polymerase κ and λ (Pol κ and λ) have been shown to play a critical role in cccDNA formation in HBV-infected HepG2-NTCP(Qi et al., 2016), while Pol α as well as Pol δ and ε are required for cccDNA

formation in HBV stable cell line HepAD38 (Tang et al., 2019), suggesting that the first round of cccDNA formation from incoming virus and the intracellular cccDNA amplification pathway may rely on different host polymerases. SAMHD1, which is traditionally considered as an antiretroviral host factor functions through depleting the cytoplasmic pool of dNTPs to block reverse transcription, has been recently reported to play a role in the early steps of cccDNA formation in HBV-infected cells (Wing et al., 2019). The detailed mechanism of how SAMHD1 promotes cccDNA formation is unclear. It has been reported that SAMHD1 can bind ssDNA and act as a scaffolding protein to facilitate both homologous recombination and DNA resection, thus may account for its proviral role in the rcDNA repair process (Daddacha et al., 2017; Goncalves et al., 2012). Recently, topoisomerase I (TOP1) and II (TOP2) have been shown to be required for both *de novo* synthesis and intracellular amplification of HBV cccDNA, probably during the circularization of negative-strand DNA (TOP1) or both strands of rcDNA (TOP2) (Sheraz et al., 2019). Finally, the ligation of both strands of rcDNA during cccDNA formation is mediated by DNA ligase 1 and 3 (Long et al., 2017). Considering the existence of a closed minus-strand rcDNA (cM-rcDNA) intermediate in HBV-replicating cells (Luo et al., 2017a), it is possible that the ligation of processed rcDNA may be strand- and/or ligase-specific during cccDNA formation. A recent study using yeast- and human-extract screenings identified the five core components of DNA lagging strand synthesis machinery for cccDNA formation, including the proliferating cell nuclear antigen (PCNA), the replication factor C complex (RFC), Pol δ, FEN1 and DNA ligase 1, which confirmed several aforementioned earlier studies (Wei and Ploss, 2020).

Upon the release of rcDNA into nucleus, chromatinization of rcDNA may occur concurrently with DNA repair. Loaded with histone and non-histone proteins, HBV cccDNA is stably maintained as a mini-chromosome in infected hepatocytes(Bock et al., 1994; Bock et al., 2001) (Figure 1). However, the dynamics of HBV cccDNA chromatinization is obscure. A numbers of DNA viruses, like herpes simplex virus, Epstein-Barr virus, Kaposi's sarcoma-associated herpesvirus and papillomavirus, become rapidly chromatinized upon infection(Knipe et al., 2013). While not completely understood, it is clear that this process is a dynamic interplay of cell-mediated deposition of chromatin and virally directed modulation of histone modification and nucleosome remodeling(Knipe et al., 2013). For HBV, after entry into the nucleus, the viral DNA genome is loaded with host histones to form a chromatin-like structure (Bock et al., 1994). The resulted HBV cccDNA minichromosome contains nucleosomes and histone post-translational modifications (PTMs) similar to cellular chromatin (Tropberger et al., 2015). In addition, viral core proteins which initially constitute the incoming capsid remain associated with cccDNA by binding to CpG islands within the viral genome(Chong et al., 2017; Guo et al., 2011b). HBx has been shown to activate HBV transcription through its recruitment onto cccDNA by host PCAF/GCN5, p300, and CBP acetyltransferases; and to inhibit cellular factors involved in chromatin regulation, such as PP1/HDAC1 complex (Belloni et al., 2009). A recent study examined the three-dimensional localization of cccDNA within higher-order chromatin architecture and found that cccDNA preferentially interacts with host genome at unmethylated CpG islands (CGI)-rich regions which are often associated with highly expressed genes(Moreau et al., 2018). Although little is known, studying how histones and other factors (like histone

chaperones, chromatin remodelers, transcription factors and other viral proteins) are temporally and spatially organized on HBV genome is crucial to understand the chromatin-based regulation of cccDNA.

Understanding the stability of the cccDNA pools in the infected hepatocytes is important to the design and evaluate antiviral therapies against HBV infection. In DHBV infection, 1–18 cccDNA molecules was found per infected cells(Li et al., 2018; Zhang et al., 2003). For HBV, 1 to 10 cccDNA copies per infected hepatocyte were reported(Allweiss et al., 2014; Ko et al., 2018; Lucifora et al., 2011; Lucifora et al., 2014). The half-life of DHBV cccDNA ranges from 35 to 57 days *in vivo*(Addison et al., 2002), while in NRTI-suppressed chronic hepatitis B patients, average half-life of cccDNA was estimated at 9.2 months (Boyd et al., 2016). A recent kinetic study on the replacement of wide type HBV with NRTI-resistant mutants in treated patients indicated that cccDNA turnover could occur in several months, raising a possibility of HBV cure with finite therapy through completely blocking cccDNA replenishment(Huang et al., 2020).

3. The regulation of HBV cccDNA

Mediated by host RNA polymerase II, HBV cccDNA serves as a template for transcription of all viral RNAs through the cellular transcription machinery(Rall et al., 1983). cccDNA transcription is under the control of two enhancers and four promoters (the pre-C/C, pre-S1, pre-S2/S, and X promoters), which contain binding sites for ubiquitous and liver-enriched transcription factors and nuclear receptors. Enhancer I is responsible for the activation of HBx mRNA transcription, while enhancer II is involved in the expression of other transcripts (Doitsh and Shaul, 2004). Enhancer 1 harbors binding sites for hepatocyte nuclear factor (HNF)1, HNF3, nuclear factor 1 (NF1) and CCAAT-enhancer-binding protein (C/EBP)(Chen et al., 1994; Ori and Shaul, 1995; Spandau and Lee, 1992; Trujillo et al., 1991). pre-S1 promoter contains binding sites for HNF1 and HNF3(Courtois et al., 1988; Guo et al., 1993; Lopez-Cabrera et al., 1990; Raney et al., 1995). Transcription from the pre-S2/S promoter is mediated by transcription factor SP1 and it is also responsive to retinoid X receptor alpha (RXRα), peroxisome proliferator- activated receptor alpha (PPARα) and HNF4α (Raney et al., 1992; Tang and McLachlan, 2001). In addition, the pre-C/C promoter and both enhancers contain binding sites for nuclear receptors including HNF4α, RXRα, PPARα, the chicken ovalbumin upstream promoter transcription factors (COUP-TF) 1 and 2 and human testicular receptor 2 (TR2) (Guo et al., 1993; Lopez-Cabrera et al., 1990; Yu and Mertz, 1997). Activating protein-1 (AP-1) has been shown to bind on X promoter(Choi et al., 1998). Recently, a Hi-C-based study identified the CpG-binding protein CXXC finger protein 1 (Cfp1) as a bridge linking cccDNA and the CGI-rich regions of host genome, suggesting a role of Cfp1 in cccDNA transcriptional regulation(Moreau et al., 2018).

Cellular miRNAs can also regulate HBV replication either indirectly, by targeting cellular proteins that are essential for HBV replication, or directly, by targeting viral RNAs. Several miRNAs, including miRNA-1, 15b, 18a, 26b, 125b, 141, 148a, 152, 210, 372/373, 449a and 501, are involved in the regulation of HBV replication, mainly by targeting host factors (Braconi et al., 2010; Dai et al., 2014; Guo et al., 2011a; Hu et al., 2012; Jin et al., 2013; Liu et al., 2009; Zhang et al., 2009; Zhang et al., 2011; Zhang et al., 2014; Zhao et al., 2014). A

number of miRNAs can directly target HBV transcripts. miR-122, the most abundant liver-specific miRNA, is able to suppress HBV replication in HBV-replicating hepatoma cells through binding to pgRNA sequence (Chen et al., 2011; Wang et al., 2012). miR-199a-3p and miR-210 efficiently reduced HBsAg expression and HBV replication in HepG2.2.15 cells(Zhang et al., 2010). miRNA-125a-5p, which can be induced by HBx, inhibits HBV translation by binding to the 2.1-kb RNA in HBV plasmid transfected cells (Mosca et al., 2014; Potenza et al., 2011). miR-1231 targets HBV core sequence, resulting in reduced HBV replication(Kohno et al., 2014). miR-15a/miR-16–1 target the coding region for HBV polymerase and the overlapping region between HBV polymerase and HBx and exhibited antiviral effect in HBV-transfected HepG2 cells(Wang et al., 2013). However, most these studies were conducted with overexpressing system in hepatoma cells. The physiological roles of these microRNA in HBV infection in primary hepatocytes should be further evaluated.

Epigenetic modifications of HBV cccDNA minichromosomes, such as DNA methylation and histone modifications, have been implicated in regulating the transcriptional activity of HBV cccDNA (Hong et al., 2017; Mitra et al., 2018). HBV has been shown to induce methylation of both host and viral DNA *in vitro* through the induction of DNA methyltransferases(Vivekanandan et al., 2010). Hypoacetylation of the cccDNA-associated H3 and H4 histones and the recruitment of cellular HDAC1 onto cccDNA are associated with low HBV replication *in vitro* and *in vivo* (Pollicino et al., 2006). Similarly, HDAC11 inhibits HBV transcription and replication in both HBV-transfected and -infected cells through deacetylating cccDNA-associated histone H3 (Yuan et al., 2019). Various host factors involved in epigenetic modifications, including cAMP response element-binding protein (CREB), CREB-binding protein (p300/CBP), p300/CBP-associated factor (PCAF), CREB-regulated transcriptional coactivator 1 (CRTC1), general control nonderepressible 5 (GCN5) and Yin Yang 1 (YY1), bind to cccDNA and promote its transcription (Belloni et al., 2009; Hayashi et al., 2000; Tang et al., 2014). On the other hands, signal transducer and activator of transcription (STAT) 1 and 2, HDAC1, HDAC11, sirtuin (SIRT1) 1 and 3, protein arginine methyltransferase (PRMT) 1 and 5, enhancer of zeste homolog (EZH)2, heterochromatin protein 1 (HP1) and chromosome 5/6 complex (Smc5/6) interact with cccDNA to silence HBV cccDNA transcription (Belloni et al., 2012; Belloni et al., 2009; Benhenda et al., 2013; Decorsiere et al., 2016; Guo et al., 2011b; Ren et al., 2018; Riviere et al., 2015; Yuan et al., 2019; Zhang et al., 2017).

To counteract the host restriction mechanisms of cccDNA transcription, HBV encodes a transactivator protein HBx for this purpose (Lucifora et al., 2011; Slagle and Bouchard, 2016). A key function of HBx is to redirect the DNA-damage binding protein 1 (DDB1)-CUL4 E3 ubiquitin ligase to target Smc5/6 for degradation and thus relieve this suppression (Decorsiere et al., 2016; Murphy et al., 2016). In addition, HBx counteracts SETDB1-mediated histone 3 di- and tri-methylation (H3K9me3) and HP1 recruitment that represses cccDNA transcription (Riviere et al., 2015). Another study revealed that a host long non-coding (lnc) RNA HOTAIR forms complex with RNA helicase DDX5 and recruits polycomb repressive complex 2 (PRC2) to suppress cccDNA transcription, but HBx antagonizes this mechanism by activating polo-like kinase 1 (PLK1) to phosphorylate PRC2 subunit SUZ12 and downregulating DDX5. This is followed by SUZ12 ubiquitination by

HOTAIR-binding E3 ligase Mex3b and subsequent proteasomal degradation, leading to reactivation of cccDNA transcription (Zhang et al., 2016). Recently, Parvulin 14 and Parvulin 17 were discovered to bind to HBx and cccDNA and promote HBV replication in an HBx-dependent manner (Saeed et al., 2019). Thus, HBx itself and HBx involved protein-protein interactions are considered as new molecular targets for therapeutic development.

4. HBV cccDNA as a therapeutic target

Because HBV cccDNA is responsible for viral persistence, the removal, destruction, or inhibition of cccDNA represent the key to virus eradication. However, HBV cccDNA clearance as a hallmark of complete sterilizing cure has been very difficult to achieve in chronically infected livers.

The loss or partial loss of cccDNA can be achieved through different mechanisms (Figure 2). First, hepatocyte division can result in cccDNA loss. Experiments with HBV-related viruses indicated that cell division can lower cccDNA amount in the infected hepatocytes(Addison et al., 2002; Lutgehetmann et al., 2010). In the liver of human chimeric mice, *in vivo* proliferation of HBV-infected primary human hepatocytes leads to a strong cccDNA reduction(Allweiss et al., 2018). These results suggest that curative therapeutic approaches should suppress HBV replication and involve destruction of infected hepatocytes. This would accelerate cccDNA clearance also in the surviving proliferating hepatocytes, while strategies aiming at suppressing HBV replication would prevent the intracellular cccDNA amplification and virus spread.

Another important mechanism for viral clearance is killing of the infected hepatocytes by cytotoxic T cells. Spontaneous viral clearance of HBV infection is characterized by vigorous and sustained multi-epitope-specific CD4+ and CD8+ T-cell responses during the acute phase of infection(Schmidt et al., 2013). However, HBV-specific T cells in chronic hepatitis B patients are scarce and functionally defective, and this exhaustion state is a key determinant of virus persistence(Schmidt et al., 2013). This suggests that T cells related therapies may be promising options for chronic hepatitis B treatment. It has been demonstrated that T cells expressing a chimeric antigen receptor binding HBV envelope proteins specifically eliminated HBV-infected hepatocytes(Bohne et al., 2008; Krebs et al., 2013). Moreover, a significant reduction of HBV infection in humanized mice was recently demonstrated after repeated adoptive transfers of human T cells engineered to express HBV-specific T cell receptor(TCR) via mRNA electroporation(Kah et al., 2017). In an HBV-related HCC patient who had undergone liver transplantation, the gene-modified T-cells targeting HBsAg survived *in vivo*, expanded, and mediated a reduction in HBsAg levels without exacerbation of liver inflammation or other toxicity(Qasim et al., 2015). This encourages the development of therapies restoring T-cell responses in chronic hepatitis B by therapeutic vaccination, adoptive T-cell transfer, redirection of T-cells, or the use of checkpoint inhibitors.

In HBV-infected chimpanzees, HBV cccDNA loss was observed without elevation of liver enzymes, which is known as non-cytolytic clearance of HBV(Guidotti et al., 1999). Antiviral cytokines are believed to play a role. In HBV-infected human hepatocyte models, it

has been shown that high doses of IFN-α or lymphotoxin-β receptor-agonists are able to trigger non-cytolytic degradation of cccDNA from infected hepatocytes through induction of nuclear deaminase APOBEC3(A3)A or A3B, respectively (Lucifora et al., 2014). Interestingly, upregulation of A3A in the liver is correlated with antiviral response from IFN-α treated patients(Li et al., 2017b). Unfortunately, due to the side effects, it may not be feasible to increase the dose of IFN-α treatment to achieve this goal, but a novel, longer acting IFN-α may be an option. In HBV transgenic mice, PASylated IFN-α showed a profoundly increased antiviral effect compared to the non-modified version without toxicity, providing a proof-of-concept that an improved IFN-α can achieve higher rates of HBV antiviral and immune control(Xia et al., 2019). In cell cultures, basal line level of A3A and A3B negatively correlate with cccDNA amount(Brezgin et al., 2019; Xia et al., 2016). Overexpression of A3A in a Cre-mediated HBV recombinant cccDNA cell line resulted in cccDNA loss, suggesting an alternative way to induce cccDNA degradation without IFN-α treatment(Wu et al., 2018). Furthermore, it has been showed that IFN-β, IFN-λ1, and IFN-λ2 induce cccDNA deamination and degradation at least as efficiently as IFN-α, indicating that these antiviral cytokines are interesting candidates for the design of new therapeutic strategies aiming at cccDNA reduction and HBV cure(Bockmann et al., 2019). T cell-derived cytokines IFN-γ and TNF-α also induce A3A and A3B in a synergistic fashion, an effect becoming obvious also during acute or fulminant hepatitis B(Xia et al., 2016). This study identified the molecular mechanism of how T-cells not only control HBV through direct killing but even induce cccDNA degradation in a non-cytolytic fashion. *In vivo*, TCR-reprogrammed nonlytic T cells are capable of activating A3B in cell cultures and in HBV-infected human hepatocytes in mice, limiting viral infection(Koh et al., 2018). These engineered T cells with limited cytotoxicity could be further developed for treatment of chronic hepatitis B.

The use of genetic editing technology has shown promise in inactivating or eliminating cccDNA from infected cells. Several tools, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats associated nuclease (CRISPR/Cas) system, have been tested to target HBV cccDNA. ZFNs are used to create a DNA double-strand break in a sequence-specific target site and repair by creating sequence alterations at the cleavage sites. ZFNs targeting HBV were first described by using HBV plasmid transfection model(Cradick et al., 2010). After 3 days treatment, 26% of the target plasmid remained linear, whereas ~10% was cleaved and misjoined tail-to-tail. (Cradick et al., 2010). AAV-mediated delivery of ZFNs targeting different regions of HBV sequence was also tested in HepAD38 cells(Weber et al., 2014). TALENs are dimeric engineered nucleases that comprise a DNA-binding protein fused to an endonuclease domain. In HepG2.2.15 cells, where HBV DNA exists as integrated DNA, rcDNA, and cccDNA forms, as well as hydrodynamic injection mouse model, engineered TALENs showed inhibitory effect on viral replication (Bloom et al., 2013). DNA mutations were found in approximately 31% of cccDNA in HepG2.2.15 cells(Bloom et al., 2013). Furthermore, TALENs can specifically target and inactivate the HBV genome and are potently synergistic with IFN-α in cell cultures, resulted in a decreased cccDNA level and misrepaired cccDNAs without apparent cytotoxic effects. (Chen et al., 2014). The promise of CRISPR/Cas as a tool for the cleavage

and elimination, or at least inactivation, of HBV cccDNA and HBV genome integration has prompted a considerable number of studies, that provide a clear proof of concept that this approach indeed has the potential to treat or even cure chronic hepatitis B(Li et al., 2017a; Ramanan et al., 2015; Seeger and Sohn, 2014). Using next generation sequencing, the efficiency of Cas9 cleavage was determined as over 90%, which is more efficient than A3A-mediated degradation induced by IFN-α in HepG2 cells (Seeger and Sohn, 2016). Although data acquired from experimental models looks promising, challenges which are broadly associated with genetic editing therapies need to be met for the approach to be successful against chronic HBV infection. Efficient hepatic-specific delivery using viral or non-viral vehicles remains a challenge. Improving DNA-targeting specificity and defining off target effects are vital to limit unintended side effects. Additionally, potential immune responses raised by the transduced editing proteins could be harmful or even lethal to a patient if the transduction frequency is high. Finally, since HBV DNA often integrates, undesirable gene cleavage of host chromosome may occur.

Screening small molecule compound libraries for cccDNA inhibitors has been conducted in HBV stable cell lines using HBeAg as a cccDNA surrogate marker, and a handful of preclinical drug candidates have been reported, including the disubstituted sulfonamide compounds and hydrolyzable tannins as cccDNA formation inhibitors (Cai et al., 2012; Liu et al., 2016), and a cccDNA destabilizer compound ccc_R08 (Wang et al., 2019). However, the viral or host targets of these cccDNA inhibitors remain unknown. It is envisioned that the identification of compound target(s) would turn the phenotypic assay into target-based screening for small molecule cccDNA inhibitors. It is worth noting that the aforementioned host DNA repair enzymes involved in cccDNA formation can be considered as drug target(s) for cccDNA inhibition, but potential cytotoxicity and the redundant effect of host DNA repair systems on cccDNA formation may limit the development of such drugs into therapeutics (Fanning et al., 2019; Guo and Guo, 2015; Schreiner and Nassal, 2017). As above mentioned, HBV nucleocapsid maturation and uncoating are the essential prerequisites for cccDNA formation, thus, the capsid assembly modifiers (CpAMs) hold promise for reducing cccDNA biosynthesis through depleting the rcDNA precursors, especially in combination with NRTIs (Fanning et al., 2019). However, though HBV core protein has been shown to be associated with cccDNA minichromosome, CpAM treatment did not induce the reduction of preexisting cccDNA copy number or transcription in HBV-infected humanized mice (Klumpp et al., 2018). The ongoing clinical trials of CpAMs will reveal the long-term effect of these capsid inhibitors on cccDNA metabolism and activity.

Drugs that modify epigenetic regulation have been developed to treat patients with cancer or viral infections(Gherardini et al., 2016; Khan et al., 2018). With the goal of silencing cccDNA in infected hepatocytes, epigenetic therapy might be a promising therapeutic strategy for a functional cure (Hong et al., 2017). Some observations have been made in cell culture and mouse models. HDAC inhibitors have been shown to suppress cccDNA transcription in tissue culture under noncytotoxic conditions(Yu et al., 2018). A small molecule C646 inhibits cccDNA transcription by specifically inhibiting p300 and CBP histone acetyltransferases(Tropberger et al., 2015). GS-5801, an oral liver-targeted prodrug of a lysine demethylase-5 inhibitor, demonstrated antiviral activity in HBV-infected primary human hepatocytes with significant declines in viral proteins and HBV RNA(Gilmore et al.,

2017a). In addition, *in vivo* data demonstrated the pharmacodynamic response of GS-5801 within the liver in animal models(Gilmore et al., 2017b). However, in 2018, Gilead Sciences terminated a phase I trial that was designed to evaluate the safety, tolerability, pharmacokinetics and pharmacodynamics of GS-5801, and the effect of food on GS-5801 pharmacokinetics in healthy subjects (WHO International Clinical Trials ID: ACTRN12616001260415p). The limitation of tissue-specific drug delivery and systemic off-target effects substantially hamper the clinical application of epigenetic therapies. Thus, further research is needed to elucidate the favorable effects and drawbacks of anti-HBV epigenetic therapies in different experimental models.

5. Perspective

Our current knowledge of cccDNA formation, transcriptional regulation and turnover in HBV-infected hepatocytes is still limited. A greater understanding of the mechanisms regulating these processes will not only advance our knowledge of HBV basic biology, but also assist the discovery of new antiviral targets for future development of novel HBV therapeutics.

A functional or complete sterilizing cure for chronic HBV infection requires innovative therapeutic approaches aiming at silencing or eliminating HBV cccDNA minichromosome. Though there are challenges, treatments that act directly on the HBV cccDNA, such as designer nucleases editing, cccDNA destabilizers, and epigenetic modifiers, possess the potential to disable viral replication permanently. Restoration of the anti-HBV immune response may also facilitate the decay of cccDNA pool through cytokine induced non-cytolytic degradation of cccDNA and direct killing of infected hepatocytes. Taken together, a combination of direct cccDNA-targeting agent and immune therapy may serve as the effective means to achieve a cure of chronic hepatitis B.

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Highlights

- HBV establishes a pool of covalently closed circular DNA (cccDNA) minichromosome in the nucleus of infected hepatocytes.
- HBV cccDNA is responsible for viral persistence and resistance to current antiviral treatments.
- The formation of cccDNA involves host DNA repair machinery.
- HBV hijacks host ubiquitous and liver-enriched transcription factors for cccDNA transcriptional regulation.
- Elimination or transcriptional silencing of cccDNA is essential for HBV cure.

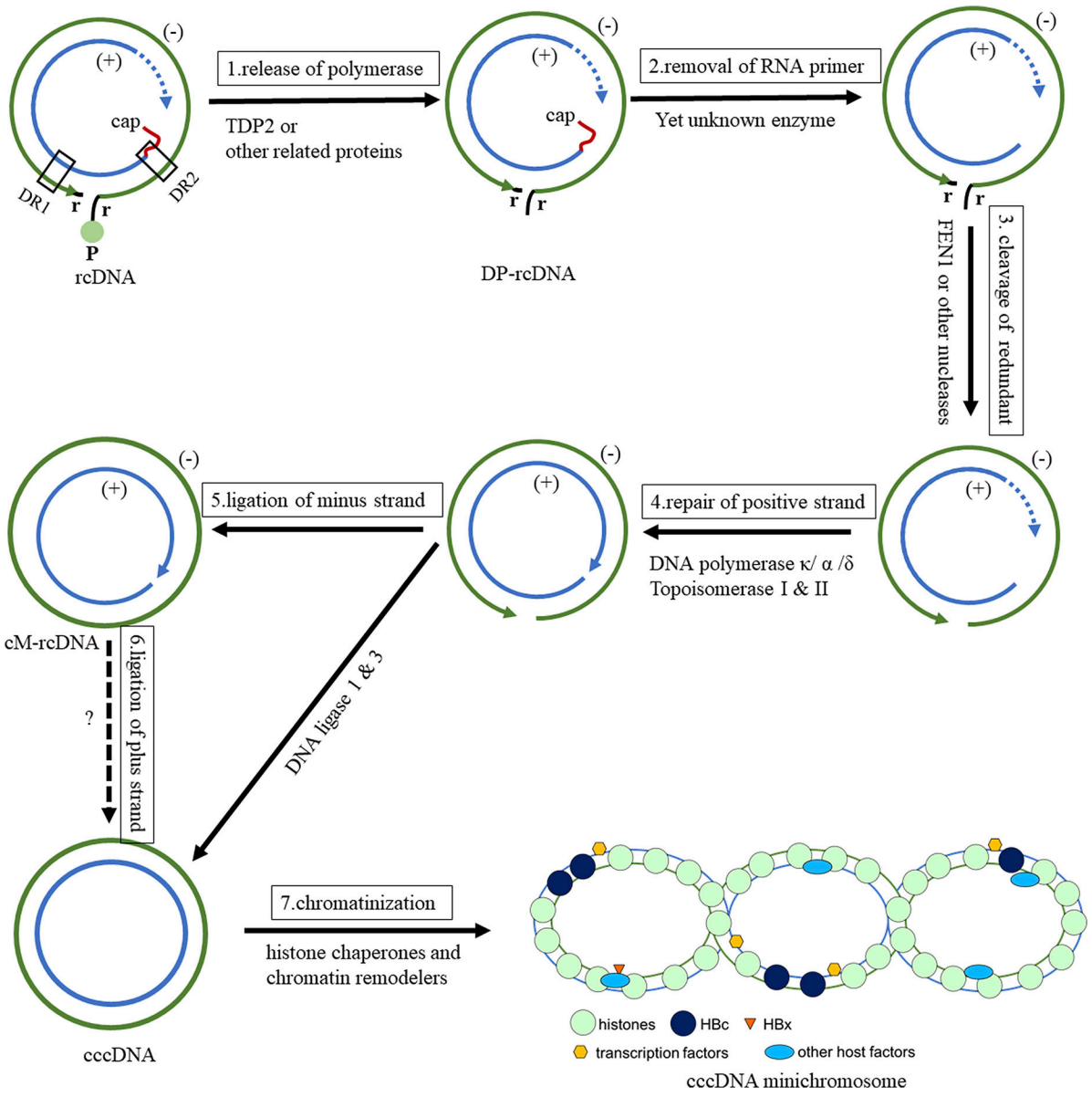

Figure 1. The formation of HBV cccDNA

The formation of HBV cccDNA involves: 1. the release of viral polymerase, which may mediated by tyrosyl-DNA-phosphodiesterase 2 (TDP2) or its related proteins (Koniger et al., 2014) (Cui et al., 2015b).; 2. removal of RNA primer from the positive strand by some yet unknown enzymes; 3. cleavage of terminally redundant sequences (r) from the negative strand, which may require flap structure-specific endonuclease 1 (FEN1) activity (Kitamura et al., 2018); 4. repair of the positive strand, with the help of DNA polymerase κ(Qi et al., 2016) or polymerase α, δ and ε (Tang et al., 2019), and DNA topoisomerase I and II (Sheraz et al., 2019); 5. ligation of minus strand (Luo et al., 2017b) and 6. Plus strand DNA separately or simultaneously by DNA ligase 1 and 3 (Long et al., 2017); 7. chromatinization, which involves histone chaperones, chromatin remodelers, transcription factors and viral proteins.

(Note: the numbers (1–7) are indicative of each specific step involved in cccDNA formation, but not the time sequence of these reactions, which remains obscure, and some reactions/steps may occur simultaneously.)

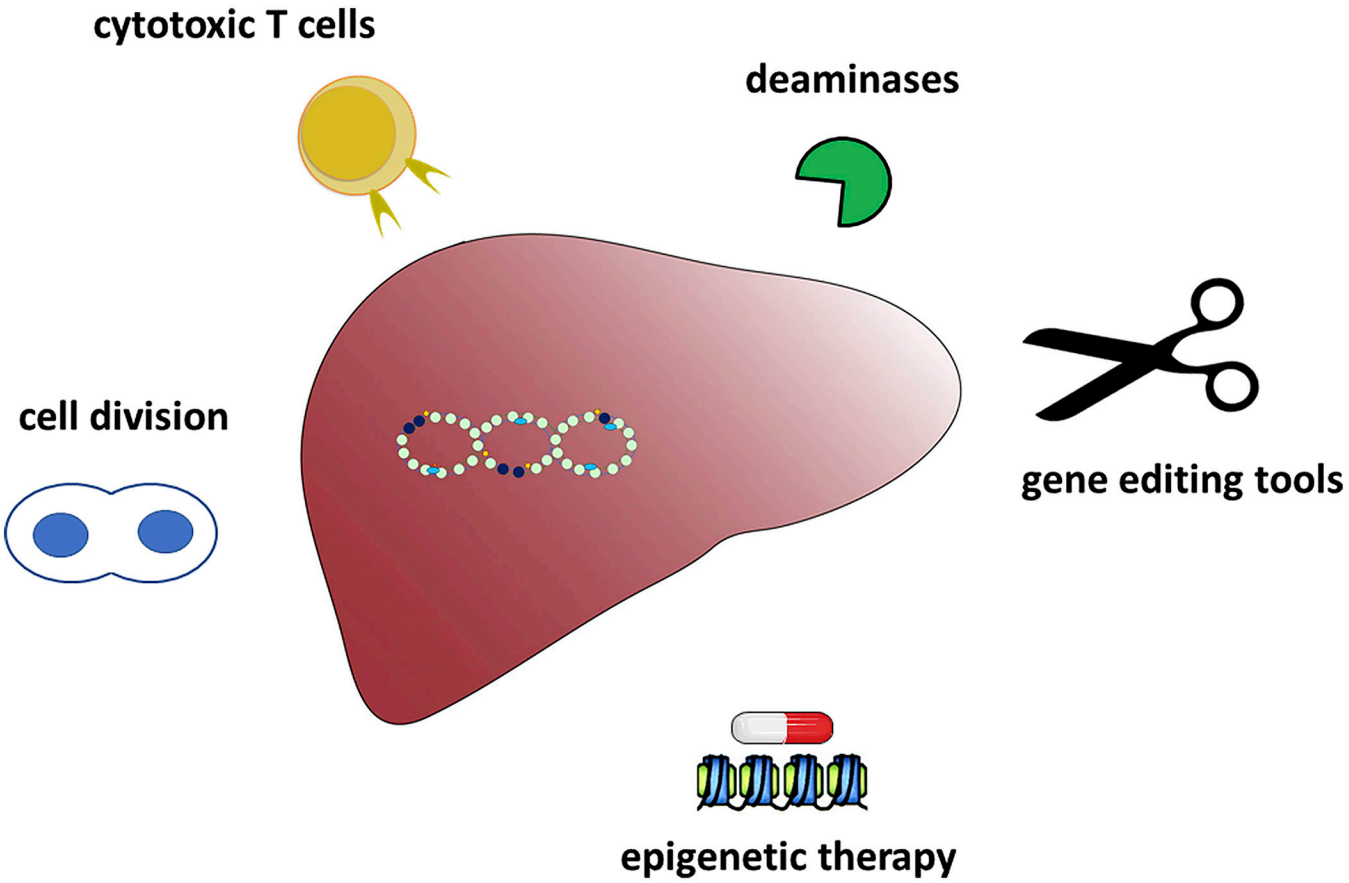

Figure 2. Mechanisms of cccDNA loss or inactivation

Cell division, or direct killing of infected cells by T cells can cause HBV cccDNA loss. Additionally, cccDNA destabilizer, cytokine-induced deamination or gene editing tools can affect the integrity of cccDNA. Furthermore, epigenetic drugs may be able to silence cccDNA transcription, resulting in cccDNA inactivation.