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REVIEW

Epigenetic regulation of covalently closed circular DNA minichromosome in hepatitis B virus infection

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Abstract Hepatitis B is caused by hepatitis B virus (HBV), and persistent HBV infection is a global public health problem, with 257 million people as HBV chronic carriers. Viral covalently closed circular DNA (cccDNA) is a key factor to establish persistent infection in infected hepatocytes. Current antiviral therapies have no direct impact on pre-existing cccDNA reservoir, which can be assembled into minichromosome by hijacking host factors. Understanding the mechanisms of epigenetic regulation in cccDNA minichromosome is crucial to develop new therapy on cccDNA, an attractive target for HBV cure. This review summarizes the current advances in epigenetic regulation of cccDNA minichromosome, which might provide clues to novel druggable targets to cure hepatitis B by either silencing or eliminating cccDNA reservoir.

Keywords HBV, cccDNA, Minichromosome, Epigenetic regulation

INTRODUCTION

HBV infection remains a global health problem and results in approximately a million death annually. Although infection rate has decreased significantly due to effective vaccines, there are more than 257 million people worldwide suffering from HBV chronic infection, with high risk of developing to liver fibrosis, cirrhosis, and hepatocellular carcinoma (WHO 2017). Large number of HBV carriers can be asymptomatic for decades, because there is no therapy available to thoroughly eliminate HBV genome in patients. It has been reported that HBV chronic infection is dependent on the persistence of covalently closed circular DNA (cccDNA) in infected cellular nucleus (Köck *et al.* 2010). Currently, there are two categories of approved drugs for hepatitis B treatment. First, the immune modulator Peg-IFN can epigenetically control cccDNA minichromosome and regulate host antiviral immune responses (Belloni *et al.* 2012; Shi *et al.* 2018). Second, nucleos(t)ide analogues (NAs) exhibit the repression of HBV polymerase (Lai *et al.* 2017; Papatheodoridis *et al.* 2002; TAK *et al.* 2016; Wong *et al.* 2013). Unfortunately, cccDNA is insensitive to antiviral therapy, leading to rapid reoccurrence of HBV replication in most patients once drug withdrawal (Hu *et al.* 2019). Besides potential serious side effects, long-term treatment with NAs can lead to drug resistance (Aspinall and Pockros 2004; Fontana 2009), although tenofovir alafenamide approved in 2016 has an excellent resistance profile in about 2-year test period (Agarwal *et al.* 2018). Therefore, it is urgent to develop novel therapies.

The stable episomal cccDNA minichromosome in nucleus is one of the key obstacles for cure (Fig. 1). HBV is an enveloped DNA virus of the hepadnaviridae family, whose genetic information resides in a 3.2 kb partially double-stranded relaxed circular DNA (rcDNA). After entry to hepatocyte, rcDNA is transformed into cccDNA in nucleus (Dezhbord *et al.* 2019; Guo *et al.* 2007, 2010; Yeh *et al.* 1998). cccDNA serves as transcriptional

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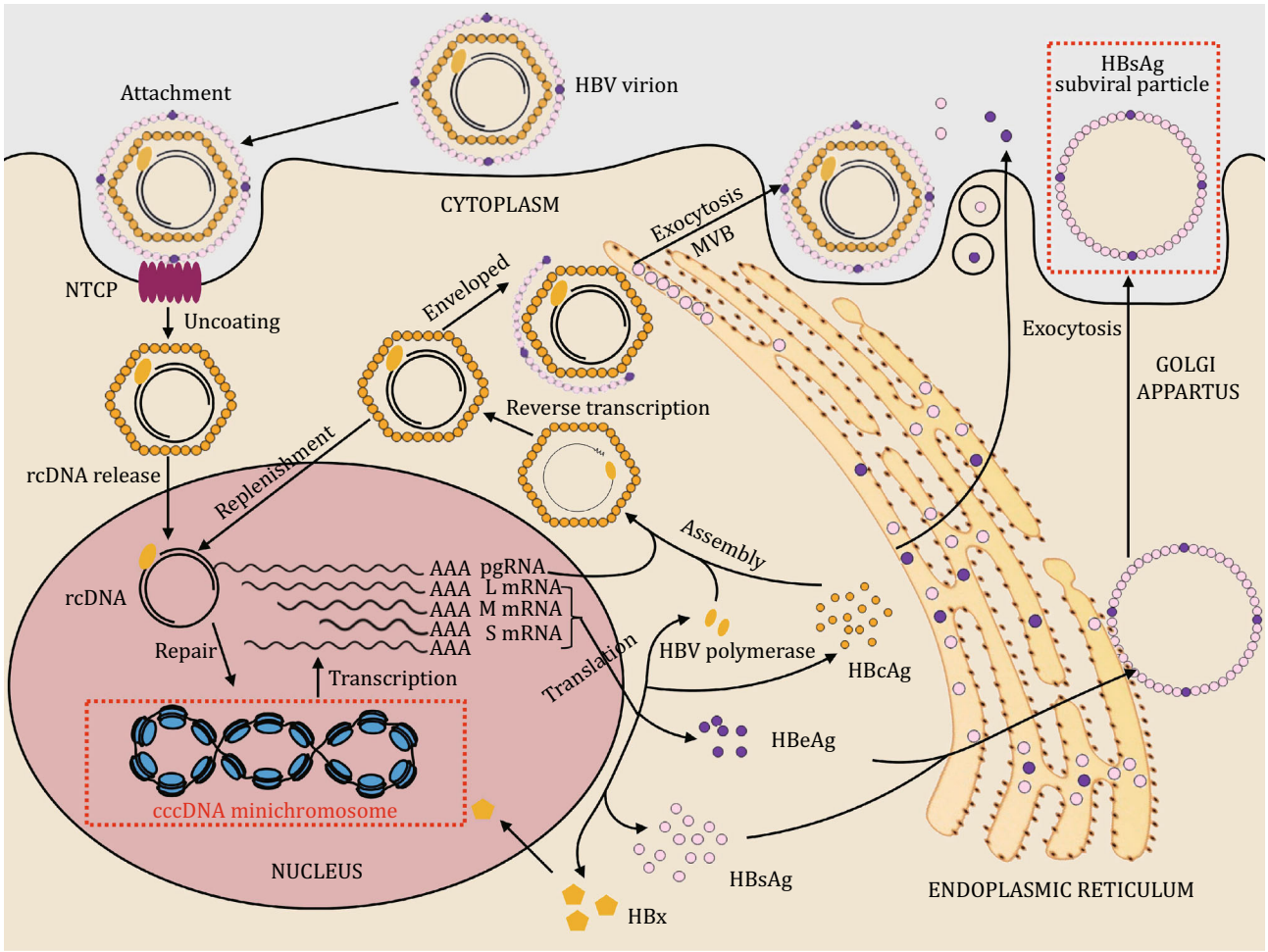

Fig. 1 cccDNA minichromosome—the key obstacle to cure Hepatitis B. HBV binds NTCP receptor and followed by entry to the hepatocyte. Upon uncoating, rcDNA is released into nucleus and repaired to form cccDNA. Then cccDNA is packaged into minichromosome and serves as template for viral RNAs, which are transported to cytoplasm and some of them are translated into viral proteins (*i.e.*, HBcAg, HBV polymerase, HBsAg). HBV polymerase and pre-genomic RNA (pgRNA) are packaged into HBcAg-formed capsid, where pgRNA is reverse-transcribed to form rcDNA. HBsAg proteins surround the capsid to form new virions. The clearance of cccDNA and HBsAg is rarely achieved with current therapies (in red frame), while the cccDNA minichromosome as the viral reservoir (in red) is the key obstacle to finally cure Hepatitis B

template for all viral RNAs, among which pre-genomic RNA (pgRNA) is reverse-transcribed into rcDNA to be further processed to replenish cccDNA reservoir (Beck and Nassal 2007). In nucleus, cccDNA takes a chromatin-like conformation, known as cccDNA minichromosome, which consists of both histones and non-histone host factors (Bock *et al.* 2001). cccDNA minichromosome has been considered under the regulation of nuclear transcription factors, transcriptional coactivators, chromatin-modifying enzymes, *etc*. However, the detailed regulatory mechanisms remain unclear. It has been proposed that rcDNA is recognized by host factors and transformed into cccDNA, which may be closely related to host DNA-damage-repair pathway (Gómez-Moreno and Garaigorta 2017; Königer *et al.* 2014). Results of CsCl density gradient ultracentrifugation and electron microscope observation show that HBV nucleoprotein complex displays a typical "beads-on-string" model (Bock *et al.* 1994). In conjunction with the results of nucleosome positioning, the evidence shows that the average number of nucleosomes is 18 and repeat unit comprises 180 bp in HBV minichromosome, which is smaller than host chromosome (Bock *et al.* 1994; Shi *et al.* 2012). That means cccDNA minichromosome may have a little more compact conformation. Therefore, it is reasonable to suppose that HBV cccDNA minichromosome might be transformed between closed conformation (inactive) and open conformation (active) to manipulate viral transcription and replication (Fig. 2). In this review, we

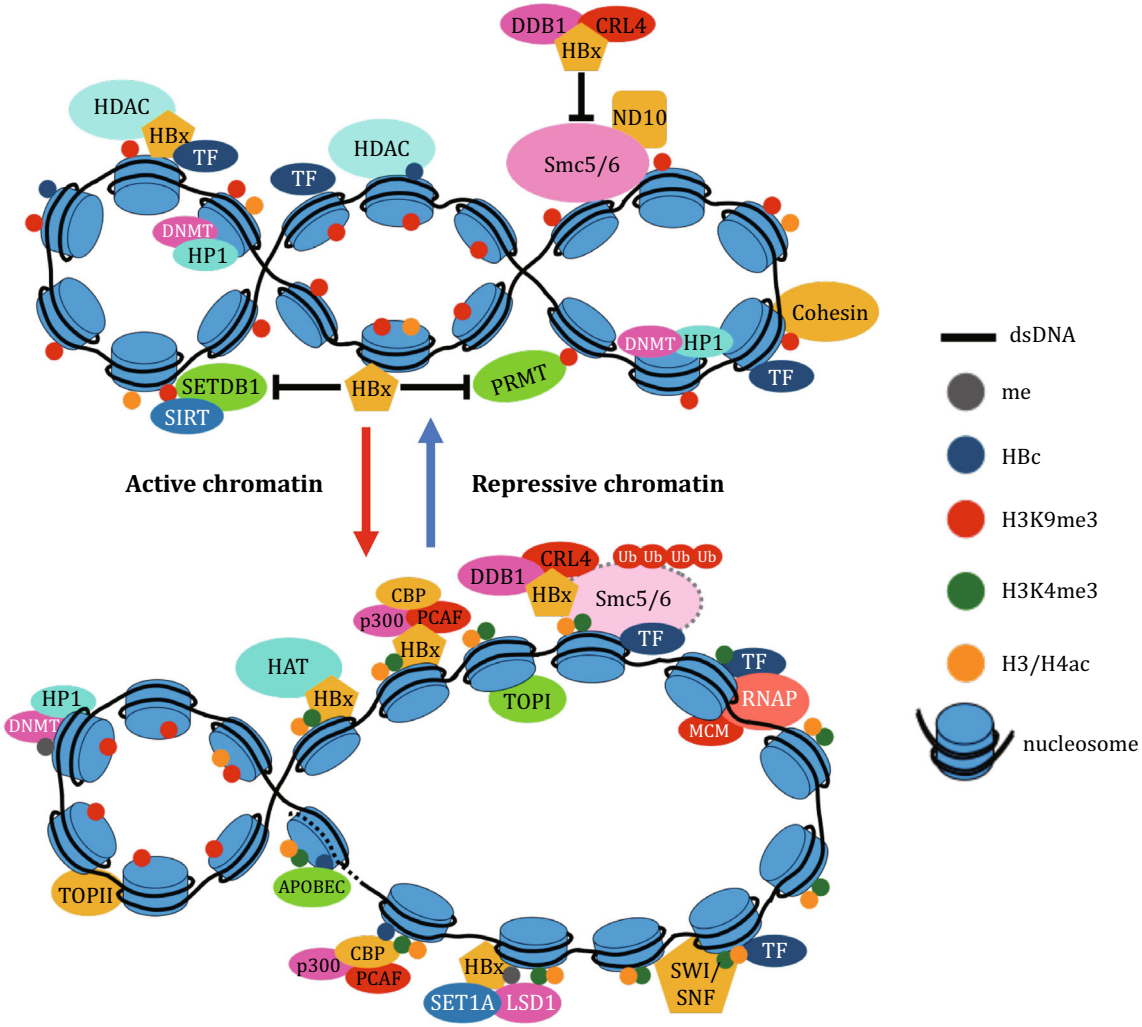

Fig. 2 Schematic representation of HBV cccDNA minichromosome under different epigenetic regulation. In the presence of host restriction factors and inhibitive modifiers, cccDNA minichromosome is in a closed configuration, and viral genes are transcriptionally repressed (upper). In the presence of HBx and other activators, cccDNA minichromosome is in an open configuration, and viral transcription is activated and leads to a high level of virus replication (down)

summarize the advances of epigenetic regulation of HBV cccDNA minichromosome and provide a distinct perspective through a structural view, which might provide clues to novel druggable targets to pave the way to cure hepatitis B.

EPIGENETIC REGULATION BY HBV ENCODED PROTEINS

HBV core protein (HBc) forms viral nucleocapsid and belongs to non-histone proteins associated with minichromosome (Bock *et al.* 2001). Scientists revealed that HBc may promote transcription due to its preferentially binding to CpG island 2 on minichromosome, where HBc associates with HDAC1 and CBP to regulate histone acetylation and DNA methylation (Chong *et al.* 2017; Guo *et al.* 2011). Moreover, HBc recruits DNA polymerase coordinator PCNA to minichromosome to promote viral proliferation (Feng *et al.* 2019). HBc also facilitates APOBEC3 family members A3A and A3B to colocalize with cccDNA minichromosome to induce deamination and subsequently Apurinic/Apyrimidinic (AP) sites for degradation, which is, respectively, upregulated by IFN-α and LTβR activation (Lucifora *et al.* 2014). Furthermore, core protein allosteric modulators (CpAMs) can regulate viral nucleocapsid assembly or disassembly. For

instance, Bay 41-4109 and GLS4 are representative of heteroaryldihydropyrimidines (HAPs) and can introduce significant conformational changes to core protein subunits to inhibit *de novo* cccDNA synthesis and transcription, while ENAN-34017 of sulfamoylbenzamide (SBA) can only induce a subtle conformational change that renders viral particle susceptible to DNase degradation (Guo *et al.* 2017; Zhou *et al.* 2017). However, these CpAMs can accelerate intracellular cccDNA formation from existed progeny rcDNA.

HBV X protein (HBx) is a regulatory protein (Ramakrishnan *et al.* 2019). It is essential for transcription initiation, maintenance, and epigenetic regulation of cccDNA by interacting with various factors including histone acetyltransferases p300, CBP and PCAF, as well as histone deacetylases HDAC and hSirt1 (Cougot *et al.* 2007; Guerrieri *et al.* 2017; Lucifora *et al.* 2011). Recently, it has been shown that HBV minichromosome associated with HBx can be enriched around host transcriptionally active chromatin with host RNA Polymerase II (Pol II) and other hallmarks such as H3K36me3, H3K36ac, and H3K4me3, while silencing of HBx can decrease viral minichromosome stability (Hensel *et al.* 2018; Jin *et al.* 2019). Besides, nuclear HBx can bind plasmid-encoded HBV minichromosome through the C-terminal domain (Hensel *et al.* 2018), which may imply that HBx is involved in nuclear localization of viral minichromosome. Meanwhile, HBx can recruit coactivators (CBP, p300, and PCAF), and transcription factors (ATF/CREB, ATF3, c/EBP, NF-IL-6, Ets, Egr, SMAD4, Oct1, RXR receptor, p53) to HBV minichromosome to regulate cccDNA epigenetically (Belloni *et al.* 2009; Levrero *et al.* 2009). HBx is also able to bind CUL4-DDB1 ubiquitin ligase through its H-box motif (Landsberg *et al.* 2018; Li *et al.* 2010), which facilitates degradation of chromosome structure maintenance complex 5/6 (Smc5/6) (Rivière *et al.* 2019). In absence of HBx, Smc5/6 functions as a restriction factor to inhibit HBV transcription (Decorsière *et al.* 2016). However, the transcription is resumed in the presence of functional HBx (Abdul *et al.* 2018; Murphy *et al.* 2016). Additionally, viral replication and transcription are highly repressed by utilizing anti-HBx 2A7 epitope antibody, which specifically blocks the interface between HBx and DDB1 (Tao *et al.* 2019). Moreover, IFN-induced TRIM interacts with the C-terminal of HBx and inhibits the formation of Smc5/6-HBx–DDB1 complex, leading to suppressive HBV replication (Tan *et al.* 2018). Therefore, HBx-DDB1 interaction interface becomes an attractive target for drug development to silence HBV transcription (Sekiba *et al.* 2019). HBx-targeted siRNA can also inhibit viral replication at both mRNA and protein level (Xie *et al.* 2012). Besides Smc5/6, HBx interacts with other host restriction factors to regulate minichromosome. For instance, HBx is responsible for the upregulation of E3 ubiquitin ligase MSL2, which contributes to HBV cccDNA activation by inducing APOBEC3B degradation via ubiquitylation of Lys320 site of APOBEC3B (Gao *et al.* 2017). Similarly, HBx/STAT3 signaling is stimulated by lncRNA HULC to facilitate production of miR-539 to downregulate cytidine deaminase APOBEC3B to maintain cccDNA stability (Liu *et al.* 2019). Additionally, HBx can facilitate viral DNA replication through restraining MDM2-mediated ubiquitination and degradation of RNA helicase DHX9 (Shen *et al.* 2020). Moreover, HBx can activate Notch-CREB signaling to facilitate cccDNA replication, but it is restricted by E3 ubiquitin ligase (Gao *et al.* 2016; Wang *et al.* 2010). In addition, HBx binds lncRNA DLEU2 to modulate minichromosome transcription and relieve repression induced by chromatin-modifying enzymes like EZH2 and PRC2 (Salerno *et al.* 2020). Furthermore, HBx can colocalize with host transcription factor Sp110 and drive it out of ND10 complex via Sp110 deSUMOylation, which facilitates viral persistence by downregulating factors (IRF9, STAT1, and STAT2) in IFN-I-response pathway and altering epigenetic landscape via coactivator p300 (Sengupta *et al.* 2017). Another study revealed host pre-mRNA processing factor PRPF31 in spliceosome associates with HBx to promote cccDNA formation (Kinoshita *et al.* 2017). In hepatocellular carcinoma cells, 14-3-3ζ protein binds to Akt-induced RPLpS^{31}GP motif of HBx and significantly inhibits ubiquitination and degradation of HBx, while 14-3-3ζ itself can activate Akt pathway, indicating the altered structure and biological function of phosphorylated HBx (Tang *et al.* 2018). In summary, both HBc and HBx are essential in cccDNA accumulation and multiple epigenetic regulatory pathways in cccDNA minichromosome. Thus, they are becoming potential virus-encoded drug targets for silencing viral transcription or eliminating the cccDNA reservoir to achieve a cure eventually.

EPIGENETIC REGULATION BY HISTONE MODIFICATIONS

Histones with various modifications are associated with transcriptionally active or repressive HBV minichromosome to regulate genomic transcription as found in host chromosome. Current studies have shown that HBV minichromosome is regulated by histone post-translational modifiers, which have a significant impact on viral infection, replication, and maturation (Gong *et al.* 2011; Tropberger *et al.* 2015). Histone

modifications are generally reversible, including acetylation, methylation, phosphorylation, SUMOylation, ubiquitylation, ADP-ribosylation, *etc*. Here, we emphasize histone acetylation and methylation due to recent booming studies related to HBV.

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) manipulate histone acetylation to regulate minichromosome epigenetically. HAT can transfer acetyl to lysine, which is advantageous to dissociate histone octamers from loose chromatin. Previous studies revealed that HBV replication parallels acetylation of cccDNA-associated histone H3/H4 (Pollicino *et al.* 2006; Wei *et al.* 2017). Inhibitors of histone deacetylases can promote viral replication, while H3/H4 is highly acetylated under HBx activation. In a human liver-chimeric mouse model, HAT1 can be activated by the HBx coactivating transcription factor Sp1 and recruited to minichromosome through lncRNA HULC-mediated interaction with HBc (Yang *et al.* 2019). The overexpression of HAT1 can promote the acetylation of H3K27, H4K5 and H4K12 in minichromosome, while downregulation of HAT1 can impair the assembly of histone H3/H4 and recruitment of HBx and p300 to impede the formation of minichromosome (Yang *et al.* 2019). On the contrary, HDAC can remove acetyl from specific site, causing histone positively recharged to strengthen interaction between histones and DNA, which promotes chromatin to be condensed. Acetylation of H3K9 and H3K27 is specifically downregulated by HDAC11 to limit viral replication, while acetylated H4 is not affected (Yuan *et al.* 2019). Moreover, HDACs can also decrease the occupancy of Pol II in transcribing minichromosome (Balakrishnan and Milavetz 2008). The relationship between HDAC and antiviral therapy has been clarified that the inhibition of HBV replication and transcription is associated with histone deacetylation of H3K9/H3K27 and recruitment of inhibitors to cccDNA in the IFN treatment (Liu *et al.* 2013; Zhang *et al.* 2019). Moreover, IL6 inhibits cccDNA transcription by enhancing the recruitment of HDAC to render hypoacetylation of cccDNA-associated histone and reducing the binding of essential transcriptional factors (HNF1α, HNF4α, and STAT3) to cccDNA (Palumbo *et al.* 2015). In addition, a novel E3 ubiquitination ligase NIRF reduces acetylation of H3 and acts as a negative regulator of HBc to inhibit viral replication (Qian *et al.* 2015). Furthermore, Retinoid X receptor α (RXRα) can increase acetylation of histones H4 and H3 to promote viral replication and transcription by recruiting p300 to cccDNA minichromosome (Nkongolo *et al.* 2019; Zhang *et al.* 2017b).

Beyond acetylation, the methylation of H3/H4 is associated with chromatin structure, which regulates HBV transcription (Kallestad *et al.* 2013; Peng and Karpen 2007). The Sirtuin family members (SIRT1 and SIRT3) can deacetylate histones of minichromosome and regulate the recruitment of histone methyltransferase suppressor of variegation 3–9 homolog 1 (SUV39H1) to facilitate formation of heterochromatin by increasing the chromatin repressive marker H3K9me3 and reducing the chromatin active marker H3K4me3 (Peng and Karpen 2007, 2009; Ren *et al.* 2014, 2018; Vaquero *et al.* 2007), while HBx can relieve inhibition of viral transcription by not only impairing expression and recruitment of SIRTs (Deng *et al.* 2017), but also recruiting LSD1 and Set1A to establish active chromatin (Alarcon *et al.* 2016). Recent research suggested that HBx colocalizes with the core subunit WDR5 of SET domain containing 1 (SET1)/mixed lineage leukemia (MLL) histone methyltransferase complex and inhibits DDB1-induced degradation of WDR5 to promote viral transcription by H3K4me3 modification on minichromosome (Gao *et al.* 2019). Moreover, protein arginine methyltransferase 5 (PRMT5) may preferentially bind to cccDNA through interaction with HBc to elevate H4R3me2 on minichromosome and disrupt HBV Pol–pgRNA interaction to abrogate pgRNA encapsidation to inhibit viral replication (Zhang *et al.* 2017a). It has been reported that SETDB1-mediated H3K9me2/H3K9me3 and heterochromatin protein factor 1 (HP1) can induce viral transcriptional silence by rearranging chromatin structure, but HBx can antagonize this process to allow synthesis of active chromatin (Rivière *et al.* 2015). Histone methylation is involved in formation of heterochromatin by recruiting HP1, which may also recruit DNA methyltransferases to methylate DNA. Modified histones can not only regulate chromatin structure directly, but also serve as binding sites for other regulatory proteins to function indirectly. Besides acetylation and methylation, the roles of various histone modifications in epigenetic regulation of HBV minichromosome remain unclear and are urgent to be fully addressed in the future.

EPIGENETIC REGULATION BY DNA METHYLATION

DNA methylation is a major epigenetic regulation on gene activities and introduced by DNA methyltransferases (DNMTs) responsible for addition of methyl groups to the CpG islands of DNA. Methylated DNA may serve as a signal recognition site to specifically recruit corresponding factors to cccDNA minichromosome and result in allosteric effects. HBV cccDNA can be methylated to various extent, which is mostly associated with the replicative repression of cccDNA (Kim *et al.* 2011;

Zhang *et al.* 2014). Generally, HBV DNA methylation by DNMTs is closely related to transcriptional silence as in mammalian cells (Guo *et al.* 2009; Vivekanandan *et al.* 2009). DNMTs (DNMT1, DNMT2, DNMT3a, DNMT3b) upregulated by HBV can promote viral genome-wide methylation and reduce pgRNA production to inhibit HBV replication (Vivekanandan *et al.* 2010). However, further investigation indicated that DNMT inhibitors can activate host innate immune response through IFN signaling pathway, and thus inhibit both viral replication and transcription (Chiappinelli *et al.* 2015). Evidence also revealed that DNA methylation alone may not be efficient for inhibition, while methylated and condensed chromatin is required to repress gene transcription (Deuschle *et al.* 2016). DNA methylation on cccDNA seems to participate in inhibition of HBV, but meanwhile the host genes can be also methylated by elevated expression of DNMTs. Consequently, the detailed mechanism of DNA methylation during viral defense needs further exploration.

EPIGENETIC REGULATION BY CHROMATIN REMODELING

Chromatin remodelers play significant roles in regulating viral transcription in the context of minichromosome. Some chromatin remodelers do regulate HBV minichromosome in the similar ways as for host chromosome, which involves sliding, replacing, reassembling, or exchanging nucleosomes. Members of human SWI/SNF family are recognized as chromatin remodelers (such as BAF and PBAF), displaying an essential role in transcriptional regulation. For example, the core ATPase subunit Brg1 of the PBAF complex can antagonize the suppression induced by PRMT5 (Zhang *et al.* 2017a). Meanwhile, the core ATPase subunit Brm of the BAF complex also has a promotion on viral transcription (Chen *et al.* 2016). In addition, HBx-associated protein HBXAP/RSF1, a component of a ISWI chromatin remodeling complex, interacts with HBx as a transcription coactivator (Shamay *et al.* 2002). Furthermore, it was shown that inactivating mutation of ARID2 from human SWI/SNF family is closely related to cancer genesis through genomic analysis of hepatocellular carcinoma (Li *et al.* 2011).

DNA topoisomerases (TOPs) can modulate chromatin structure and catalyze distinct steps of cccDNA formation (Halmer *et al.* 1998; Sheraz *et al.* 2019), and meanwhile DNA topoisomerases are believed to function in PJA1-mediated viral inhibition (Xu *et al.* 2018). Both TOP1 and TOP2 are involved in the repair of negative-strand DNA gap, while TOP2 also participates in the repair of positive-strand DNA gap. In addition, it was shown that human minichromosome maintenance (MCM) protein heterocomplexes (MCM2, MCM4, MCM6, and MCM7) with high affinity to histone H3 play essential roles in the replication initiation, which may make structural change once replication initiates (Ishimi *et al.* 1996, 1998; Méndez and Stillman 2000). Moreover, researchers found that MCMs can initiate transcription by recruiting RNA Pol II holoenzyme to minichromosome (Holland *et al.* 2002). Furthermore, MCM7 can be inhibited by simvastatin (SIM) to downregulate HBV replication (Li *et al.* 2016). Therefore, MCMs might be a potential target for novel antiviral treatment. Besides acetylation regulation, SIRT3 can also restrain the binding of host Pol II and transcription factor YY1 to cccDNA, indicating that SIRT3 participates in establishment of repressive chromatin structure and transcriptional silencing of cccDNA (Ren *et al.* 2018). Additionally, Parvulin (Par14 and Par17) can bind and stabilize HBx through HBx RP motif, and may bind cccDNA minichromosome through S19/44, respectively, to upregulate HBV replication in a chromatin remodeling way (Saeed *et al.* 2019).

Host Smc5/6 suppresses HBV transcription when localized to nuclear domain 10 (ND10) without inducing a detectable innate immune response (Niu *et al.* 2017). Moreover, it has been reported that PJA1 can promote Nse4 to bind viral or episomal DNA in a synergistic way through competitive substitution of Nse1 in Smc5/6 complex, and thus represses HBV proliferation (Xu *et al.* 2018). Another SMC family member cohesin is highly affiliated with minichromosome and severing its SMC ring domain causes cohesin dissociating from minichromosome (Ivanov and Nasmyth 2005), which indicates a topological association between cohesion and minichromosome. However, whether there is a direct link between cohesion and HBV minichromosome needs to be further investigated. Recent studies of the mechanisms of DNA compaction by cohesion provide us a new insight into the formation of cccDNA minichromosome (Davidson *et al.* 2019; Kim *et al.* 2019, 2020). In general, non-histone proteins can modulate transcription factor's accessibility to cccDNA in either transcriptional repressive or active states. The mechanisms of some host canonical chromatin remodelers have been elucidated. However, how these remodelers participate in anti-HBV defense remains poorly understood.

POTENTIAL FOR THE DEVELOPMENT OF NOVEL THERAPIES

Since both Southern blot and cccDNA-specific PCR have their limitations for the detection of HBV cccDNA, different methods for quantification of cccDNA vary considerably. Therefore, it raises the possibility of using cccDNA surrogates to develop novel detection methods (Zhou *et al.* 2006). HBx can recruit transcription factors to transcriptionally active domain of cccDNA minichromosome and promote transcription of viral episome as well as transiently transfected plasmid (Reeves *et al.* 1985; van Breugel *et al.* 2012). However, HBx has no regulatory impact on HBV genes integrated into host chromosome (van Breugel *et al.* 2012). It suggests that HBx may apply a special mechanism to specifically activate expression of episome. Interestingly, researchers recently revealed that the expression of mitotic Aurora kinase A enhances viral replication in an Akt-dependent but HBx-independent manner, and DDB1 can also stimulate viral transcription via HBx-independent mechanism (Jeong and Ahn 2019; Kim *et al.* 2016), indicating that Aurora kinase A may be a potential substitution of HBx that would allow transcriptional stimulating of the CUL4/DDB1 complex. This property of Aurora kinase A elucidates that it might be a potential HBx surrogate and share similar signal transduction pathway as well as similar structural conformation. cccDNA is not naked but wrapped with large number of histones and non-histone proteins, which protect cccDNA from destruction by other factors, giving rise to its high stability and a long life-span. HBx or Aurora kinse A may be good potential targets for developing not only episomal DNA-targeted detection methods to improve the sensitivity and accuracy, but also for new antiviral therapies.

Gene editing enzymes comprising TALEN (Bloom *et al.* 2013), ZFN (Cradick *et al.* 2010), CRISPR/Cas9 (Moyo *et al.* 2018), and APOBEC (Lucifora *et al.* 2014) have been applied to reduce cccDNA stability to achieve the therapeutic eradication. According to the accuracy and efficiency among these gene therapies, rapidly-updated CRISPR/Cas9 tools would come to the frontline of antiviral therapeutic combat. With the use of combinations of HBV-targeting nucleases, cccDNA can be cleaved at more than one site and thus become unstable. However, *in vivo* precise delivery challenge and off-target effects of CRISPR/Cas9 system remain to be solved. In addition, RNA interference (RNAi) is an alternative gene therapy, including microRNAs (miRNAs), short hairpin RNAs (shRNAs), and small interfering RNAs (siRNAs) (Ely and Arbuthnot 2015; Moyo *et al.* 2018). Although RNAi can achieve a sustained HBV inhibition by knocking down viral transcripts, the major drawback of RNAi therapy for HBV is the failure to eliminate established cccDNA leading to HBV reoccurrence after withdrawal of gene inhibitors similar to current therapies with IFN or NAs. Hence the combination of unique viral epigenetic traits can be utilized to improve accuracy and efficiency of gene therapy tools.

SUMMARY AND PERSPECTIVES

HBV cccDNA minichromosome may utilize similar epigenetic regulative mechanism as the host chromatin. Various types of histone modifications may rearrange the charge of histones to affect interactions among chromatin constituents. Moreover, multiple sites in one histone can be modified, while the same residue can be modified in various types, which dominates the intricate regulative network through antagonism or synergism. Chromatin remodeling generally results from minichromosome-associated non-histones. Due to the similarities and the differences in the catalytic ATPases, chromatin remodelers can be divided into four subfamilies: ISWI, CHD, INO80 and SWI/SNF. Besides, there are some non-canonical host remodelers such as ATRX, CSB, *etc*. Generally, chromatin remodelers directly bind to nucleoprotein complexes to slide, exchange, or replace nucleosome along the DNA string in an ATP-dependent manner, causing rearrangement of the relative position of histone octamer to DNA, which in consequence regulates transcription of relevant genes (Sundaramoorthy 2019). Unfortunately, there are limited studies on the mechanism of host chromatin remodelers regulating HBV genomic transcription, because the HBV episome is less abundant in infected cells and the episomal structure is quite dynamic.

Intriguingly, the recent technique advances of structural biology provide a major boost in determination of the structures of multinucleosomal complexes with linear dsDNA, which makes it feasible to determine the structures of HBV cccDNA minichromosome or other episomes in various states as well. Schalch *et al.* reported the 9-Å resolution crystal structure of tetranucleosomal chromatin fiber and Garcia-Saez *et al.* reported the 9.7-Å resolution crystal structure of hexanucleosomal chromatin fiber, which showed the advanced chromatin structure is arranged into two-start nucleosome stacks in a zigzag helix (Garcia-Saez *et al.* 2018; Schalch *et al.* 2005). Song *et al.* reported the 11-Å resolution cryo-EM structure of the dodecanucleosomal 30-nm chromatin fiber and ~25-Å resolution cryo-EM structure of tetracosanucleosomal 30-nm chromatin fiber, which confirmed that higher-order chromatin

fibers apply a left-handed twist of the repeating tetranucleosomal units (Song *et al.* 2014). As expected, we could stabilize the HBV minichromosome via diverse epigenetic regulations to capture the high-resolution structures of certain conformational states to uncover the intricate regulatory mechanisms.

Novel perspective links HBV cccDNA with extrachromosomal circular DNA (ecDNA) and other viral episomal DNA. Recently, ecDNA (size range from 1 to 3 Mb or larger) found in eukaryotic species has been redefined in intimate relation to cancer pathogenesis (Verhaak *et al.* 2019; Wu *et al.* 2019). Although ecDNA can be packaged into chromatin, ecDNA chromosome lacks higher-order compaction and displays significantly enhanced chromatin accessibility compared to canonical chromatin (Wu *et al.* 2019). Despite intensive research concerning cccDNA formation, the mechanisms of cccDNA formation remain unclear. But there is no doubt that rcDNA would fail to be transformed into cccDNA without host DNA repair system (Guo *et al.* 2012), as ecDNA formation may also rely on the canonical homologous recombination (HR) or nonhomologous end joining (NHEJ)-like pathway (van Loon *et al.* 1994). But it can be reasonably assumed that DNA repair mechanism can be used to form higher-order chromatin due to the topological change of chromatin during rcDNA/cccDNA transformation, which can provide a novel insight into the establishment of stable minichromosome. Although cccDNA minichromosome is smaller than ecDNA chromatin or other viral episomes, the similar chromatin-like composition may indicate that the current epigenetic regulation for HBV cccDNA minichromosome might also be applied to the regulation of cancer-related ecDNA chromatin and other viral episomes in CMV (Olszewski *et al.* 1982), MVM (Ben-Asher *et al.* 1982), SV40 (Crémisi *et al.* 1978; Varshavsky *et al.* 1977), and EBV (Castán *et al.* 2017; Kumala *et al.* 2012), *etc*.

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Compliance with Ethical Standards

Conflict of interest Lanfeng Wang, Weiwei Wang, and Zhaoning Wang declare that they have no conflict of interest.

Human and animal rights and informed consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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