HBV cccDNA and Its Potential as a Therapeutic Target

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Abstract

Chronic hepatitis B virus infection continues to be a major health burden worldwide. It can cause various degrees of liver damage and is strongly associated with the development of liver cirrhosis and hepatocellular carcinoma. Covalently closed circular DNA in the nucleus of infected cells cannot be disabled by present therapies which may lead to HBV persistence and relapse. In this review, we summarized the current knowledge on hepatitis B virus covalently closed circular DNA and its potential role as a therapeutic target.

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Hepatitis B virus (HBV) life cycle and therapies

HBV was discovered by Blumberg and colleagues1 in 1965. Today, there are about 257 million people chronically infected with HBV, which causes 650000 deaths worldwide every year. According to World Health Organization reports, HBV caused 887000 deaths, mostly from complex diseases (including cirrhosis and hepatocellular carcinoma) in 2015. HBV infection brings a huge burden to people and even society.

The HBV genome is a circular, partially double-stranded DNA, which is enveloped by an outer lipoprotein with an inner nucleocapsid. HBV DNA is only 3.2kb in length and contains four overlapping open reading frames, known as S, C, P and X. The four open reading frames code for 7 viral proteins: pre-S1, pre-S2, S, C, pre-C, X protein (commonly known as HBX), and HBV polymerase. After infection, HBV enters host hepatocytes by binding to the sodium taurocholate cotransporting polypeptide NTCP and being endocytosed. After entry, the virus releases its DNA-containing nucleocapsid into the cytoplasm, which is then transported to the nucleus. In the nucleus, the viral DNA is converted from its relaxed circular form to closed covalent circular DNA (cccDNA). Integration of the HBV DNA into the host genome begins immediately after infection of hepatocytes. After transcription and translation, pgRNA is packaged and assembled in the nucleocapsid, where it is transcribed to relaxed circular DNA. Finally, the nucleocapsid is enveloped and the virion is secreted from the hepatocyte.

The current standard therapy for HBV infection includes PEGylated interferon α and nucleotide analogues (NAs). For example, a recent study showed that interferon α induces a long-term and sustainable suppression of cccDNA transcription, possibly by altering epigenetic modification of cccDNA minichromosomes. The inhibition of HBV replication by NAs is mediated by targeting the viral RNA-dependent DNA polymerase which catalyzes the reverse transcription of pgRNA to mature viral DNA. Five NAs have been approved for clinical use since 1998, including lamivudine, entecavir, telbivudine, adefovir dipivoxil and tenofovir. Although NAs have been considered as first-line therapy for the treatment of chronic HBV infection due to their high efficiency, they have failed to cure HBV in most cases because they cannot clear cccDNA. As such, virus rebound occurs following the termination of NAs treatment. Obviously, disabling the cccDNA is key in terms of curing HBV infection.

HBV cccDNA: Formation and modification

cccDNA is stable and acts as a virus transcription template. There are 3–50 copies of cccDNA per infected cell, and the number of copies decreases when the host cell divides. Thus, recycling of the new relaxed circular form to the nucleus occurs in order to maintain the relatively stable cccDNA copy number. There are three main steps in conversion of the relaxed circular form to cccDNA: (1) unlocking of the protein, which is covalently linked to the 5′ end of the (−)-DNA; (2) removal of the 5′ end of the (−)-strand consisting of an RNA oligonucleotide; and (3) covalently ligation of both strands. Recent studies have shown that the host DNA damage response is involved in the formation of cccDNA, but the exact mechanism remains to be clarified. Epigenetic modifications, like histone acetylation and DNA methylation, play an important role in the transcriptional activity of cccDNA. Six CpG islands have been reported in the HBV genome. The CpG islands distribute differently in HBV genotypes, overlapping some functional genes. Three conventional CpG islands (I, II, III) are potential targets for HBV DNA methylation (Fig. 1).

Novel strategies for eradicating HBV cccDNA

HBV cure depends on disabling of the HBV cccDNA, which is very difficult because cccDNA resides in the nucleus as an...
episomal plasmid-like molecule to produce progeny virus.15,26 Several gene therapy strategies have been proposed to disable HBV cccDNA.

**Silencing cccDNA expression by gene editing techniques**

Several genomic editing technologies, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats/CRISPR associated (CRISPR/Cas) system, have been used to disrupt HBV cccDNA. ZFNs, a custom DNA endonuclease, are used to create a DNA double-strand break in a specific target site and repair by creating sequence alterations at the cleavage sites.27–29 Through a proof-of-concept experiment, scientists found that anti-HBV ZFNs (cognate 6L and 6R ZFN pair) disrupted 36% of plasmid-derived viral sequences in a cell culture model. Weber et al.30 designed three ZFNs targeting HBV polymerase, gene X and core, and delivered them into the HepAD38 cells by self-complementary adeno-associated viral vectors, respectively. They found these HBV-targeted ZFNs produced a sustained suppression of HBV levels over the course of the experiment.31 TALENs are similar to ZFNs. However, the DNA-binding domain of TALENs is highly repeated and derived from transcription activator-like III effectors, which are proteins secreted by Xanthomonas bacteria.32,33 The efficiency of TALENs in reducing HBV productions in cell culture was first described by Bloom et al.34 in 2013. The investigators found that targeting the S and C regions of cccDNA led to 35% decrease in cccDNA molecules in HepG2 cell lines. Moreover, gene editing can be used in combination with gene-silencing technologies for antiHBV therapy. Using TALEN-mediated homology directed recombination to introduce artificial primary miRNAs into HBV genome could boost the antiviral efficacy of TALENs.35

The CRISPR/Cas system is the adaptive immunity of bacteria and archaea, and acts against invading foreign DNA via RNA-guided DNA cleavage.36–40 The CRISPR/Cas9 system is a newly developed programmable genome-editing tool and allows for sequence-specific cleavage of DNA. Compared to other genome-editing tools, the advantage of the CRISPR/Cas9 system lies in its simplicity and flexibility in design. Several studies have demonstrated that the CRISPR/Cas9 system can efficiently destroy HBV cccDNA.41–45 Using the CRISPR/Cas9 system, scientists have completely excised a full-length 3175bp integrated HBV DNA from the host genome and disrupted the HBV cccDNA in a stable HBV cell line.44,45 Therefore, gene editing seems highly promising for disabling HBV cccDNA.29,34

These three genome-editing technologies work similarly by targeting and modifying DNA sequences using engineered nucleases, thereby inducing a targeted DNA double-strand break that stimulates the cellular DNA repair mechanisms. However, the engineered nucleases, recognition area, and the molecular mechanism are different.46 Engineered ZFNs and TALENs are composed of a DNA binding domain and a Fok I nuclease motif. The CRISPR-Cas9 genome editing system consists of two components: a “guide” RNA and a nonspecific CRISPR-associated endonuclease (the Cas9). The recognition areas of the ZFNs, TALENs, CRISPR/Cas system are zinc-finger proteins, NLS, and sgRNA, respectively. ZFNs and TALENs use the Fok I enzyme to cleave target DNA, while CRISPR/Cas uses the Cas protein. The target sizes among the three genome-editing tools are also different, that is (9-12bp)*2 for ZFNs and TALENs, 20bp+ NGG for CRISPR/Cas.29,46,47

There are some limitations for these technologies. First, specificity can be altered by context-dependent effects caused by interactions among neighboring zinc fingers of the DNA binding domain; second, the large size of TALENs makes it difficult to deliver. Finally, the CRISPR/Cas9 system faces several challenges like in vivo delivery efficiency and off-target cleavage.48,49 Cutting integrated HBV genomes by CRISPR/Cas9 also raises serious concerns, because this manipulation can cause genome instability. Compared to ZFNs and TALENs, the two advantages of the CRISPR/Cas9 system have contributed to the advancement of the new technology and generated widespread interest, according to its simplicity and flexibility. Thus, we believe that the CRISPR/Cas9 system is very promising for curing chronic HBV once several challenges are solved.50

**Silencing cccDNA transcription by epigenetic modifications**

HBV cccDNA exists in the nucleus as multiple copies of nucleosome-decorated minichromosomes, which indicates that epigenetic modifications may influence HBV replication and persistent infection.51 It has been shown that DNA methylation and histone acetylation are required for cccDNA formation.21 Therefore, regulation of DNA methylation or histone acetylation is a potential method to reduce cccDNA.21,52–55 Interferon α treatment has been adopted to silence cccDNA through epigenetic modification. It has been
found that interferon α inhibits HBV replication by regulating the epigenetic modification of HBV cccDNA. Interferon α represses the transcription of HBV cccDNA through recruitment of the histone deacetylases HDAC1 and hSirt1 and decreasing the acetylation of cccDNA-bound histones. Hyun et al. found that short hairpin RNA induced HBV cccDNA methylation to inhibit its transcription in human hepatoma cells. HBV cccDNA transcription was regulated by CpG methylation during chronic HBV infection. CpG island II methylation has been shown to significantly decrease cccDNA transcription and subsequent viral core DNA replication, while CpG island III methylation has been shown to be associated with low serum HBsAg titers (Fig. 1).21,53,54,57

Interestingly, HBx can reduce chromatin-mediated transcriptional repression of HBV cccDNA caused by SETDB1 histone methyltransferase and therefore allow the establishment of active chromatin. Recently, it has also been reported that HB core carboxyl-terminal domain arginine residues reduced acetylation of cccDNA-bound histones and thus reduced the interaction of HBC with cccDNA.61 Two enzymes have been found to regulate the methylation.52,62 Protein arginine methyltransferase 5 can regulate symmetric dimethylation of arginine 3 on histone 4 of cccDNA.62 The silent mating type information regulation 2 homolog 3 SIRT3 restricts HBV transcription and replication via epigenetic regulation of cccDNA, involving SUV39H1 and SETD1A histone methyltransferases.63 HBV replication is also regulated by the acetylation status of HBV cccDNA-bound histone 3 and histone 4.64 The Np95/ICBP90-like RING finger protein NIRF, a novel E3 ubiquitin ligase, has been found to inhibit HBV DNA replication and hepatitis B e antigen secretion in HepG2 cells through reducing HBV cccDNA-bound histone 3.64 Curcumin can inhibit HBV replication via down-regulation of cccDNA-bound histone acetylation.65 Retinoid X receptor α was also reported to be able to regulate the replication of HBV and modulate the HBV cccDNA epigenetically.66 In addition, basal core promoter mutations were found to inhibit viral replication through modulating the acetylation and deacetylation of cccDNA-bound histones, while preCore mutations have been shown to have no effect on viral replication.67 These collective findings indicate that regulation of the DNA methylation and histone acetylation may inactivate cccDNA transcription and thus inhibit HBV replication.

Disabling cccDNA is the key to curing hepatitis B. Studies have shown the great potential of gene editing technologies for disrupting the cccDNA in cultured cells and in hydrodynamically-injected mice. However, in the case of HBV infection, there are some disadvantages of using gene editing technologies. First, HBV DNA often integrates into the host genome and undesirable gene cleavage may occur. Second, off-target effects has been observed in some studies. Finally, the means by how to deliver in vivo remains a challenge for therapeutic application of these technologies. The epigenetic modifications mentioned above are related to the replication and transcription of HBV cccDNA. While they can regulate the HBV cccDNA, they cannot eliminate the HBV cccDNA.

Conclusions
In conclusion, disabling or complete inactivation of cccDNA is the desired end-point of HBV treatment. However, the most urgent issues should be addressed before using gene editing technologies for HBV treatment include solving the off-target problem and finding ways to deliver in vivo. What is more, for a long time, lack of robust, reliable and quantifiable HBV cccDNA models has delayed the development of cccDNA therapies. Recently, Yuan et al.68 established a cell line through integrating 2–60 copies of the monomeric HBV genome into HepG2-derived cell lines, where the cccDNA could be produced and detected with specific primers. The establishment of a cell line will provide a proper model for evaluation of drugs or therapies targeting on cccDNA. Though there are challenges to overcome for both gene editing- and epigenetic modification-based strategies and it there remains a long road from basic research to clinical application, it is very likely that we will conquer HBV similar to the hepatitis C virus cure in the future.

Conflict of interest
The authors have no conflict of interests related to this publication.

Author contributions
Collected the data and wrote the manuscript (AZ, XD), revised the manuscript and answered the reviewers questions (AZ, MX), directed and wrote the manuscript with comments (LC), helped to edit the manuscript (XL, SL, HZ). All authors have seen and approved the content of this manuscript.

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