HBV cccDNA—A Culprit and Stumbling Block for the Hepatitis B Virus Infection: Its Presence in Hepatocytes Perplexed the Possible Mission for a Functional Cure

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ABSTRACT: Hepatitis B virus infection (HBV) is still a big health problem across the globe. It has been linked to the development of liver cirrhosis and hepatocellular carcinoma and can trigger different types of liver damage. Existing medicines are unable to disable covalently closed circular DNA (cccDNA), which may result in HBV persistence and recurrence. The current therapeutic goal is to achieve a functional cure, which means HBV-DNA no longer exists when treatment stops and the absence of HBsAg seroclearance. However, due to the presence of integrated HBV DNA and cccDNA functional treatment is now regarded to be difficult. In order to uncover pathways for potential therapeutic targets and identify medicines that could result in large rates of functional cure, a thorough understanding of the virus’ biology is required. The proteins of the virus and episomal cccDNA are thought to be critical for the management and support of the HBV replication cycle as they interact directly with the host proteome to establish the best atmosphere for the virus while evading immune detection. The breakthroughs of host dependence factors, cccDNA transcription, epigenetic regulation, and immune-mediated breakdown have all produced significant progress in our understanding of cccDNA biology during the past decade. There are some strategies where cccDNA can be targeted either in a direct or indirect way and are presently at the point of discovery or preclinical or early clinical advancement. Editing of genomes, techniques targeting host dependence factors or epigenetic gene maintenance, nucleocapsid modulators, miRNA, siRNA, virion secretory inhibitors, and immune-mediated degradation are only a few examples. Though cccDNA approaches for direct targeting are still in the early stages of development, the assembly of capsid modulators and immune-reliant treatments have made it to the clinic. Clinical trials are currently being conducted to determine their efficiency and safety in patients, as well as their effect on viral cccDNA. The influence of recent breakthroughs in the development of new treatment techniques on cccDNA biology is also summarized in this review.

LIFE CYCLE OF THE HEPATITIS B VIRUS (HBV) AND TREATMENTS

Blumberg and colleagues in 1965 discovered the replication cycle of the hepatitis B virus (HBV). Today, around 257 million individuals suffer from chronic HBV disease, which kills 650,000 people annually around the globe. According to WHO data, HBV caused 887,000 fatalities in 2015, the majority of which were due to complications (such as hepatocellular carcinoma and liver cirrhosis).

The genome of HBV is a circular, partially double-stranded DNA surrounded by an exterior lipoprotein with a nucleocapsid core. HBV-DNA is only 3.2 kb long and has four overlapping open reading frames called S, C, P, and X. Pre-S1, pre-S2, S, C, pre-C, X protein (often called HBX), and polymerase of HBV are the seven viral proteins coded by the four open reading frames. HBV is endocytosed and penetrated into hepatocytes of the host after interacting with the sodium taurocholate co-transporting polypeptide NTCP receptor molecule. The virus enters the cytoplasm and delivers its DNA, made up of nucleocapsid, which is then carried to the nucleus. The DNA of the virus is transformed from its relaxed circular form (rcDNA) to covalently closed circular DNA (cccDNA) in the nucleus. HBV pregenomic RNA (pgRNA) and various subgenomic RNAs are produced from cccDNA. HBV-DNA begins to integrate into the host genome as soon as hepatocytes are infected. In the nucleocapsid, PgRNA is packaged, assembled after transcription and translation and is then translated to rcDNA. Eventually, the nucleocapsid wraps around the virion, and the liver cells then release this replicative intermediate (Figure 1).
PEGylated interferon and nucleotide analogues (NAs) are currently used as the standard treatment for the infection of HBV. According to a recent study, for example, interferon produces a prolonged and sustainable reduction of transcription of cccDNA potentially through modifying the epigenetic alteration of cccDNA minichromosomes. However, determining the clinical value of presently licensed IFN-α-based medication has been difficult since few patients respond to treatment while others do not. The use of IFN-α has also been limited due to its high prices and negative side effects. Choices are being studied, and an improving strategy has resulted in the discovery of novel interferons (IFNs) with higher efficacy, like IFN-14, which was recently reported. The viral DNA polymerase, which is RNA-dependent, catalyzes the reverse transcription of pgRNA to develop viral DNA and is targeted by NAs, which inhibits HBV replication. Ever since 1998, lamivudine, entecavir, dipivoxil, adefovir, and tenofovir have all been licensed for clinical use. All viral RNAs use cccDNA as a transcriptional template, with pregenomic RNA (pgRNA) being reverse-transcribed into rDNA and then processed to refill the cccDNA reservoir. The cccDNA acts as a minichromosome, which is comprised of histones as well as nonhistone host proteins, and acquires a chromatin-like structure in the nucleus. Consequently, the best therapeutic strategy for curative therapies is to reduce or eliminate the entire cccDNA pool. To address this issue, we examine how emerging treatment tactics, such as editing of genomes, epigenetics, gene maintenance, host targeting approaches, assembly of nucleocapsids, immunology, and so on significantly affect cccDNA.

**CCCDNA AS A MINICHROMOSOME**

The robust episomal HBV cccDNA minichromosome in the nucleus is one of the major roadblocks to a functional cure. HBV is a hepadnaviridae family enclosed DNA virus with genetic material stored in a 3200 bp partially double-stranded rcDNA. In the nucleus of the liver cells, cccDNA is formed from rcDNA. All viral RNAs use cccDNA as a transcriptional template, with pregenomic RNA (pgRNA) being reverse-transcribed into rDNA and then processed to refill the cccDNA reservoir. The cccDNA acts as a minichromosome, which is comprised of histones as well as nonhistone host proteins, and acquires a chromatin-like structure in the nucleus.
factors identify rcDNA and convert it to cccDNA, which could be related to the host DNA damage-repair system. Previous research has discovered that episomal HBV cccDNA in the nuclei of infected hepatocytes is linked with chromatin and nonchromatin proteins. The complex HBV nucleoprotein exhibits a characteristic “beads-on-string” model, according to the findings of CsCl density gradient ultracentrifugation and electron microscopy study. The data suggest that the total count of nucleosomes is 18, and the repeat unit in the minichromosome of HBV which is shorter than the host chromosome is 180 bp, in line with the results of nucleosome orientation. This suggests that the conformation of the cccDNA minichromosome may be more compact. As a result, it is fair to believe that the HBV cccDNA could be switched between closed (inactive) and open (active) conformations to influence replication and viral transcription (Figure 3).

## HBV CCCDNA: FORMATION AND MODIFICATION

cccDNA is a virus transcription template that is stable. Per infected cell, 3–50 cccDNA copies are produced, and the count of copies reduces as the host cell multiplies. In order to keep a consistent copy number of cccDNA, the fresh relaxed circular form is recycled to the nucleus. The transformation of the relaxed circular form to cccDNA is divided into three phases: (1) The covalently bound protein to the 5′ end of the (−)-DNA is unlocked; (2) the (+)-strand of an RNA oligonucleotide is removed at the 5′ end; and (3) both strands are covalently ligated. The damage response of host DNA is implicated in the cccDNA development, according to recent research, but the exact process is yet unknown. The transcriptional activity of cccDNA is influenced by epigenetic changes, for instance, acetylation of histone and methylation of DNA. There are six CpG islands that have been identified in the genome of HBV. CpG islands in HBV genotypes are essential for the formation of cccDNA from dsDNA but not for replication. The discovery of a protein partner in vitro, and the reduction in TDP2 affected the metamorphosis of rcDNA to cccDNA minichromosomes in cells. Therefore, the rcDNA metamorphosis into cccDNA involves the release of P protein (covalently linked with rcDNA), RNA maturation of the plus strand, and ultimately ligation. An important step for the conversion of cccDNA from rcDNA is the cleavage of the P protein.

## CONTRIBUTION OF HOST FACTORS IN THE SYNTHESIS OF CCCDNA

The host factors meant for the production of cccDNA are poorly understood. The L-HBsAg (large surface antigen) is not directly implicated in the generation of cccDNA, but it is a component of a negative feedback process in which excessive amounts of surface protein prevent mature nucleocapsids from being shuttled into the nucleus and instead direct the cell to generate virions. The presence of HBcAg (core antigen) during the cccDNA production process has been hypothesized, which is supported by the evidence that capsid modifiers impede cccDNA synthesis. During the generation of HBV cccDNA, different host factors have been identified to bind with it and play diverse roles. In the nucleus, the endonuclease Flap endonuclease 1 (FEN1) is responsible for DNA replication and repair and has been found to bind with rcDNA, which supports in vitro synthesis of cccDNA. The discovery of a protein partner engaged in DNA damage repair is consistent with the prior findings that viruses take advantage of this mechanism for their own interest. Ku80 is associated with the DNA repair pathway in nonhomologous end-joining (NHEJ) and was essential for the formation of cccDNA from dsDNA but not rcDNA. Under the idea that HBx already exists in the cell when cccDNA is generated, HBx could act as an adapter to connect cccDNA synthesis with DNA damage response pathways in these mechanisms.

The DNA polymerases H (POLH), K (POLK), and L (POLL) of the host have all been shown to have a significant contribution to the generation of cccDNA, although the specific mechanism(s) is (are) unknown. Knockout tests revealed the importance of DNA ligase 1 and 2 of the cell in the cccDNA production in addition to DNA polymerases. It was recently discovered that the plus and minus strands need various biological proteins. DNA ligase 1 (LIG1), flap endonuclease 1 (FEN1), DNA polymerase delta (POL), PCNA, and the replication factor C (RFC) complex were necessary for the repair of the plus strand, while only FEN1 and LIG1 were important for minus-strand repair. For cccDNA synthesis and amplification, cellular DNA topoisomerases are also necessary. Finally, PRPF31 (pre-mRNA processing factor 31), part of the spliceosome complex, was discovered to be meant for the cccDNA synthesis.

## TDP2, A HOST DNA REPAIR ENZYME, IMPORTANT FOR CCCDNA SYNTHESIS

The tyrosyl-DNA-phosphodiesterase (TDP2) released the viral transcriptase from the RC-DNA, which plays a vital role in cccDNA production by interacting with the host DNA damage and repair machinery. A research study conducted in ref 31 in the hepatoma cell model reported that the cellular DNA repair enzyme tyrosyl-DNA phosphodiesterase 2 (TDP2) was important for the breakdown of the polymerase virus from the HBV rcDNA, hence acting as a fundamental phase in triggering the cccDNA genesis. The mechanism of biogenesis of cccDNA is poorly understood, but it is believed that TDP2 is physiologically necessary for cccDNA generation. It has been demonstrated that the host TDP2 is vital for cellular protein–DNA adduct repair. This step was carried out in vitro, and the reduction in TDP2 affected the metamorphosis of rcDNA to cccDNA in primary hepatocytes. TDP2 was identified as the first TDP enzyme with a strong 5′ substrate function. This catalytic enzyme was therefore a candidate for the functioning of cells, allowing the detachment of the 5′-phosphotyrosyl covalently bonded P protein from rcDNA.

## LONGEVITY OF CCCDNA

Despite the virtual loss of replicative intermediates, viral replication frequently resumes after discontinuation of NA treatment or immunosuppression, indicating that cccDNA can last for ages. Human patients make it challenging to obtain solid data on the kinetics of cccDNA depletion. Woodchucks infected with WHV and ducks infected with DHBV were studied, and half-lives between 33 and 57 days were persistently found. The corresponding values were obtained in primary hepatocytes. Throughout the acute infection’s clearance phase, the half-life of cccDNA was lowered to 3 days in chimpanzees despite the fact that copies of cccDNA existed for decades. These findings show the longevity of the cccDNA pool in HBV infection; moreover, because there are so many factors affecting the generation of new cccDNA molecules against the decline in existing cccDNA molecules,

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deconvolution is very difficult. As a result, we do not know how long a single cccDNA molecule persists at the moment, despite the fact that this is a critical question for emerging treatment approaches that attempt to target cccDNA directly.

■ CURRENT EXPERIMENTAL APPROACHES FOR STUDYING CCCDNA: SCOPE AND LIMITATIONS

The production of cccDNA is required for productive hepadnavirus infection. Convenient in vitro cell culture methods would thus give way to exploring cccDNA production; however, because of a limited host range of HBV, only less and complicated cell culture models were accessible until recently. One acceptable system is primary hepatocytes from a matched host, i.e., humans; the accessibility and extremely fluctuating quality of these cells are serious limitations. Another option is primary hepatocytes derived from tree shrews (*Tupaia belangeri*). However, properly caring for these animals is time-consuming and expensive. Astonishingly, most monkey hepatocytes appear to be resistant to HBV infection, despite the fact that these animals are all far more strongly associated with humans than Tupaias. A recent finding demonstrated the increased resemblance of NTCP of tupia to the human proteins that are important for the binding with PreS1 of HBV. Searching for these sequences in other monkeys’ NTCP could lead to new infection models. The quick loss of susceptibility in primary hepatocytes, which is correlated with the reduction of NTCP expression, is a major disadvantage.

Xenotransplanting hepatocytes of tupia or humans into immunodeficient mice can result in longer-term HBV vulnerability. It was a milestone when the HepaRG cell line generated from the human liver was developed, which makes it prone to HBV and HDV upon a prolonged isolation process that stimulates inter alia expression of NTCP but with no visible viral dissemination. On the other hand, noninfection-based methods, for instance, transfection of HBV plasmids into hepatoma cells or hydrodynamic transfection with plasmid-harboring genomes of HBV into mice, all support poor or no production of cccDNA. Likewise, regardless of the extended replication of the virus at high levels, transcription of the virus in HBV-transgenic mice seems to be limited to the integrated HBV sequences. To establish an HBV infection in the mouse model, expressing human NTCP is obligatory. Unlike HBV, DHBV produces measurable quantities of cccDNA in all investigated systems, including avian and human hepatoma cell lines. Detecting HBV cccDNA without ambiguity is still difficult. Furthermore, DHBV infection in primary duck hepatocytes and ducks for *in vivo* investigations is readily available; the rare chimpanzee studies using human HBV that were essential in revealing HBV immunobiology are now mostly prohibited (Table 1).

| Animal models | CccDNA formation | Ref. |
|---------------|------------------|------|
| Chimpanzee    | yes              | 47   |
| Tupia         | yes              | 48   |
| Woodchuck     | yes              | 49   |
| Duck          | yes              | 50   |
| Woolly monkey | yes              | 51   |
| HBV transgenic mouse model | no | 52 |
| Human liver chimeric mouse model | yes | 53 |

| Cell culture models | CccDNA formation | Ref. |
|---------------------|------------------|------|
| HepG2 cell line     | yes              | 54   |
| HuH-7 cell line     | yes              | 55   |
| HepG2.2.15 cell line| yes              | 56   |
| HepRG cell line     | yes              | 57   |
| HepAD38 cell line   | yes              | 58   |
| Primary human hepatocytes (PHHs) | yes | 59 |
| Hepatocyte derived from the stem cell | yes | 60 |

■ MODERN APPROACHES FOR HBV CCCDNA TERMINATION

HBV treatment requires termination of HBV cccDNA, which is extremely cumbersome due to the fact that cccDNA persists in the nucleus as an episomal minichromosome that produces virus offspring. To deactivate HBV cccDNA, several gene therapy techniques have been developed. The exploitation of gene therapy to inhibit the replication of HBV has gained a lot of attention recently. The active clearance of existing cccDNA appears to be the most suitable method considering the turnover kinetics is not developed. The cccDNA, as previously stated, is a major replicative intermediate in the replication cycle of HBV, important for the stubbornness of chronic HBV disease. Gene editing approaches, as well as silencing cccDNA expression through epigenetic alterations, have been used to prevent its production and silence cccDNA expression.

Another concern is the dearth of standardized tests for quantification of specific cccDNA in cells, rcDNA pool discrimination and indicators of cccDNA functioning to evaluate the efficiency of therapy.

■ SUPPRESSION OF THE CCCDNA GENERATION

ccc-0975 and ccc-0346 are substituted sulfonamides (DSS) that have been shown to impede the transformation of rcDNA to cccDNA in cell culture, most likely through blocking deproteination. Because cccDNA has an extended half-life, these molecules could be useful in the early stages of infection or when hepatocyte turnover is high. Small-molecule inhibitors that block the transformation of rcDNA into cccDNA in the infected nucleus of liver cells may impede the buildup of fresh replication-competent viral genomes, but they will not stop de novo cccDNA synthesis.

■ IMMUNE-MEDIATED CLEARANCE OR CCCDNA ERADICATION

Immune-mediated cccDNA elimination is a new impressive approach. For clearance of the virus, which includes both cytolytic and noncytolytic elimination, the interplay of innate and adaptive immune responses is required. The two methods for clearing cccDNA from hepatocytes are noncytolytic removal (“curing”) and killing of all cells containing cccDNA by T lymphocytes (“killing”) and substitution with noninfected cells. Research has demonstrated that, in chimpanzees and replicating HBV-transgenic mice, inflammatory cytokines, for example, type I interferon and TNF alpha, can effectively inhibit replication of the virus via immune-mediated mechanisms that also help to reduce cccDNA pools in infected cells by causing deamination *in vitro*. However, these procedures may be incompetent in the chronic phase, and due to a dearth of cell division, the hepatocytes’ lengthy half-life will ensure the stubbornness and longevity of cccDNA in infected hepatocytes. Both innate and adaptive immune responses, as well as the molecular processes, which might alter.
the replication of the virus, are mediated by Kupffer cells and dendritic cells.\textsuperscript{68} Different viruses have developed strategies to directly reduce the defense of the host by modifying multiple pathways associated with innate immune response, according to a growing body of research.\textsuperscript{69} Chronic hepatitis B disease, in particular, has been linked to a considerable loss in dendritic cell activity and a weakened innate immune response. Noncytolytic removal ("curing") or damage of almost all cells containing cccDNA by T lymphocytes ("killing") and substituting with noninfected cells are the two extreme possibilities for the elimination of cccDNA from liver cells.\textsuperscript{73}

Regardless of these challenges, cytokines like α-interferons and their downstream effectors seem to produce a significant impact, albeit the exact mechanisms are unknown. On the other hand, IPN-reliant treatments are restricted to considerable adverse effects, and only a small number of patients are successfully treated. As a result, complementary treatments based on antiviral immune-mediated pathways are being developed. A new investigation\textsuperscript{70} demonstrated that different stages of the HBV life cycle may be influenced.\textsuperscript{65} It further suggested that an extremely high dose of interferon or more effectively lymphotactic receptor activation could attack cccDNA integrity directly through APOBEC3A and 3B-mediated deamination of the (\(\sim\))-strand and resulting destruction. In other words, lymphotactic-receptor agonists cause noncytolytic cccDNA degradation by activating the apolipoprotein B mRNA editing enzyme and catalytic polypeptide 3A and 3B (APOBEC3A and APOBEC3B) cytidine deaminases in infected cells of HBV.\textsuperscript{64}

Intracellular pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), trigger immune responses by encouraging the release of mediators and antiviral cytokines like interferons (IFNs) as well as natural killer and T cell stimulation.\textsuperscript{71} TLR activation can lower HBV replication in mice models. TLR3, 7/8, and 9 are receptors that identify viral nucleic acids in endosomes and trigger a type 1 interferon response.\textsuperscript{72} In cell culture models, the TLR7 agonist GS-9620 has shown antiviral effectiveness,\textsuperscript{73} in chimpanzees, there was a decrease in viremia and HBsAg.\textsuperscript{74} In the woodchuck model, there was a decline in cccDNA production,\textsuperscript{75} and in humans, it did not have a strong anti-HBV activity.\textsuperscript{76} Other TLR7 agonists, for example, RO7020531 and JNJ-4964, are being tested in experimental clinical trials for HBV infection.\textsuperscript{77} In the woodchuck model, the TLR8 agonist GS-9688 significantly decreased viral nucleic acid and productions of cccDNA and antigen\textsuperscript{78} and is currently in clinical trials.\textsuperscript{77} Recent research has found that IL-2 as a key immunotherapeutic approach important to prevent CD8+ T cells is rendered dysfunctional by hepatocellular carcinoma.\textsuperscript{79} Besides from CHB-affected individuals, TRL2 was downregulated in hepatocytes, peripheral blood mononuclear cells, and Kupffer cells,\textsuperscript{79} with a loss of cytokine release.\textsuperscript{80} On the other hand, an augmentation in TLR2-positive monocytes was linked with a better response to PEG-IFN-α therapy,\textsuperscript{81} and higher expression of TLR2 on monocytes led to IL-6 release.\textsuperscript{82} In an in vitro cell culture system, a TLR2 ligand exhibited a robust anti-HBV potential.\textsuperscript{83} Other ligands Pam3CSK4 and TLR3 are also competent enough to decrease cccDNA formation.\textsuperscript{83}

As a checkpoint for immunity, proteins like PD-1 are being studied to see if they can help restore the anti-HBV immune response and break immunological tolerance. In a woodchuck model, the PD-1 ligand (programmed death ligand 1)\textsuperscript{84} combined with DNA vaccines caused full viral elimination,\textsuperscript{85} demonstrating therapeutic efficacy of a revived T cell response.\textsuperscript{86} Undoubtedly, blocking PD-1 has been demonstrated to revive the activity of HBV-specific T cells to some extent.\textsuperscript{87} For example, anti-PD-1 mAb nivolumab is a type of antibody that is used to treat HBV that is now in progress in clinical trials.\textsuperscript{87} Overstimulation of the immune system, which can lead to serious side effects such as autoimmunity, which is a major challenge.\textsuperscript{88} Furthermore, during check point therapy, HBV infection has been observed to reactivate.\textsuperscript{89,90} Though clinical investigations in infected HBV individuals have shown broad-spectrum tolerability so far, there is a definite risk of potentially fatal or life-threatening side effects.\textsuperscript{90} To fully comprehend the function of check point inhibitors in the treatment of chronic HBV infection, more research is needed.

Despite the fact that immunotherapy’s current outcomes in CHB treatment are disappointing, it remains the most appealing strategy for eradicating the virus as long as the right patient population is identified and ideal study designs are executed. However, a small percentage of cccDNA may evade immune-mediated clearance and destruction (Table 4).

### NUCLEOCAPSID ASSEMBLY MODULATORS PREVENT HBV CCCDNA FORMATION

The HBV core protein (HBc), which governs numerous activities in the HBV life cycle including assembly of capsid, reverse transcription, and secretion of a virion, is a well-studied intervention target.\textsuperscript{91} As a result, modulators of capsid assembly (CAMs) have been made to disturb the activities of HBcs, preventing the production of cccDNA (Table 4. CAMs influence cccDNA levels through various mechanisms: (1) restrict the reimport of a freshly generated nucleocapsid, preventing cccDNA pool amplification;\textsuperscript{92} (2) prevent cccDNA production in freshly infected cells (likely by inhibiting the import of nuclear capsid);\textsuperscript{92} and (3) contribute to the cccDNA construction (because HBc is linked with cccDNA). For example, in CHB patients, HBc is linked with cccDNA CpG Island\textsuperscript{29}, and APOBEC3A may be recruited to cccDNA in order to degrade it.\textsuperscript{94}

Various CAMs have been produced and are divided into two categories. The primary, heteroaryldihydropyrimidines (HAPs), cause the assembly of capsid to be misdirected (CAM-A), where A refers to aberrant because these CAMs cause empty capsids with abnormal structures), while the next, indicated by phenylpropenamides (PPAs) and sulfamoylbenzimides (SBAs), causes empty capsid assembly (CAM-N where N represents normal because these CAMs cause empty capsids with a normal form).\textsuperscript{92} The antiviral activity of HAPs, PPAs,\textsuperscript{95} and SBAs\textsuperscript{97} was reported in vivo along with the long-term decrease of HBV-DNA levels. In addition, JNJ-6379 has been demonstrated to suppress the synthesis of cccDNA in vitro.\textsuperscript{98} HAPs (Bay 41-4109 and GLS4) as well as an SBA ENAN-34017 hinder de novo cccDNA synthesis.\textsuperscript{92} The discovery of new CAMs has accelerated dramatically in recent years.\textsuperscript{99} Indeed, CAMs have been demonstrated to interfere with cccDNA amplification and production. Various CAMs are now being tested in clinical trials, including JNJ56136379,\textsuperscript{100} JNJ-6379,\textsuperscript{101} ABI-H0731,\textsuperscript{102} and NVR 3-778.\textsuperscript{103}

In a cell culture system, new tools like zinc-finger nucleases (ZFNs), transcription activator-like endonucleases (TALENs), and the RNA-directed clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system have been investigated for targeting and cleaving cccDNA and are discussed below.
Using Gene Editing Approaches to Silence cccDNA Expression

The potential to directly remove or suppress HBV cccDNA has been regarded as the “holy grail” of HBV treatment. Specific destruction of cccDNA is the most efficient approach against cccDNA, which has recently been made possible with the help of new genome-editing technologies. To destabilize HBV cccDNA, scientists used a number of tools for editing genomes, such as the clustered regularly interspaced short palindromic repeat/CRISPR-associated (CRISPR/Cas) system, zinc finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs). All of these editing methods cause a nick in double-stranded DNA at a specific target region and then change the DNA sequence to repair the cleavage sites. ZFNs are a type of tailored DNA endonuclease that is employed to nick a double-stranded DNA at a specific target site and then repair it by altering at the cleavage site sequence.\(^{103}\) For example, cognates 6L and 6R are a ZFN pair, which acts as a novel therapeutic approach targeting cccDNA. It has been reported that in a cell culture setting it degraded 36% of plasmid-derived sequences of the virus, according to a proof-of-concept investigation. Weber et al.\(^ {105}\) developed three ZFNs that target gene X and the core, and HBV polymerase used self-complementary adeno-associated viral vectors to introduce them to HepAD38 cells. Over the course of the experiment, they discovered that these anti-HBV ZFNs decrease the levels of HBV for a long span of time. ZFNs and TALENs are similar. On the other hand, the TALEN DNA-binding domain which is generated from transcription activator-like III effectors is extremely repetitive as are xanthomonas bacteria-secreted proteins.\(^ {105}\) Bloom et al. in 2013 were the first to describe the effectiveness of TALENs in lowering HBV production in an \textit{in vitro} cell culture system.\(^ {106}\) The researchers discovered that targeting the C and S cccDNA domain reduced the number of molecules of cccDNA in commonly used hepatoma cell lines HepG2 by 35%. Furthermore, genome editing can be combined with technologies of gene silencing to treat HBV infections. TALENs could be used to deliver artificial primary miRNAs into the genome of HBV, potentially increasing TALENs’ anti-HBV potency.\(^ {107}\)

The CRISPR/Cas9 technique is the most famous, garnering considerable interest because of its ease of use and versatility.\(^ {65,108}\) Type II has been employed widely for research purposes out of the three types of CRISPR mechanisms that have been identified. Only one protein, the Cas protein, is used in the type II CRISPR process. Through RNA–DNA complementary base pairing, the Cas9 protein forms a complex with a single guide RNA and generates site-specific DSBs. The CRISPR/Cas tool is a type of adaptive immunity seen in archaea and bacteria that uses RNA-guided DNA cleavage to defend against invading foreign DNA.\(^ {109}\)

The CRISPR/Cas9 method is a newly generated programmable technique for editing genomes. It causes the breakage of DNA at particular sequences. The CRISPR/Cas9 method has been shown in several experiments to effectively remove HBV cccDNA.\(^ {110}\) Researchers have successfully eliminated a complete length of 3175 bp integrated DNA of HBV from the genome of the host and degraded the HBV cccDNA in a stable HBV cell line using the CRISPR/Cas9 technology.\(^ {111}\) As a result, gene editing appears to be a potential method for removing HBV cccDNA. These three methods of genome editing all function by employing designed nucleases to target and change DNA sequences, resulting in a rupture of targeted double-stranded DNA that promotes cellular DNA repair systems. Nevertheless, the molecular mechanism, identification domain, and designed nucleases are all dissimilar.\(^ {112}\) A binding domain of HBV and a Fok I nuclease motif are found in engineered ZFNs and TALENs. A nonspecific CRISPR-linked endonuclease and a “guide” RNA comprise the CRISPR/Cas9 genome editing mechanism (the Cas9). Zink finger proteins, NLS, and sgRNA are the prominent domains of the ZFNs, TALENs, and CRISPR/Cas systems, respectively. The Fok I enzyme is used by ZFNs and TALENs to break target DNA, whereas the Cas protein is used by CRISPR/Cas. The sizes of the three genome-editing target techniques differ as well: for ZFNs and TALENs, (9–12bp)*2 and 20bp+ NGG for CRISPR/Cas.\(^ {37}\) These technologies have some drawbacks. First, context-dependent effects were generated through contact between neighboring DNA binding domain zinc fingers which affect specificity; next, TALENs are challenging to administer due to their huge size; and finally, the CRISPR/Cas9 technology has a number of caveats and limitations, such as off-target unintended incision and delivery efficacy \textit{in vivo}. CRISPR/Cas9 editing of integrated HBV genomes also presents severe issues, as this manipulation can lead to genomic instability. As a result, we believe the CRISPR/Cas9 approach has great promise for treating chronic HBV once a few hurdles are overcome.\(^ {113}\)

Despite their potential, these molecules must first be tested in various animal models of chronic HBV disease to determine their efficacy as well as analyze issues such as off-target effects on the genome of the host. Off-target effects, such as linearized viral DNA chromosomal integration, could have a negative impact on hepatocyte function, especially when the effector nucleases must be present for a long time. Furthermore, the repair of nuclease-induced DSBs by NHEJ is susceptible to errors,\(^ {114}\) and a small percentage of repair activities will result in intact cccDNA reformation. Last but not least, it is unknown how in the same cells extra RC-DNA impacts the targeting efficiency of cccDNA.

Since the genome of HBV is so compressed, with several regulatory cis - elements and overlapping open reading frames (ORFs), genome editing that disrupts most of the target sites can have serious pleiotropic consequences on the stubbornness and replication of HBV-DNA. It is always a good idea to identify many target sites that are evolutionarily conserved among the various genotypes of HBV when designing experiments for editing the genome.\(^ {115}\)

The main impediment in the elimination of HBV infection is the stability of cccDNA under existing antiviral treatment.\(^ {116}\) For example, despite that nucleos(t)ide analogues significantly impede the replication of HBV, they are unable to eradicate cccDNA, which accumulates in hepatocyte nuclei.\(^ {117}\) As a result, HBV has become a popular target for CRISPR/Cas9 in the lab, and treating HBV infection will necessitate modern approaches for targeting cccDNA preferentially.\(^ {117}\) Treatment of HBV with CRISPR/Cas9 was first described by Lin et al.\(^ {118}\) In addition, many studies have shown that antiviral gRNAs can be induced to expression in hydrodynamics HBV \textit{in vivo} models by coinjecting HBV-expressing plasmid DNA and CRISPR/Cas9 vectors into the animal’s tail vein, thus eliminating virus production.\(^ {118}\) Dong et al. then employed the CRISPR/Cas9 target to develop an \textit{in vivo} model of cccDNA expression.\(^ {118}\) Furthermore, during de novo HBV...

\(^{103}\) Cognates 6L and 6R are a ZFN pair, which acts as a novel therapeutic approach targeting cccDNA. It has been reported that in a cell culture setting it degraded 36% of plasmid-derived sequences of the virus, according to a proof-of-concept investigation. Weber et al.\(^ {105}\) developed three ZFNs that target gene X and the core, and HBV polymerase used self-complementary adeno-associated viral vectors to introduce them to HepAD38 cells. The researchers discovered that these anti-HBV ZFNs decrease the levels of HBV for a long span of time. ZFNs and TALENs are similar. On the other hand, the TALEN DNA-binding domain which is generated from transcription activator-like III effectors is extremely repetitive as are xanthomonas bacteria-secreted proteins.\(^ {105}\) Bloom et al. in 2013 were the first to describe the effectiveness of TALENs in lowering HBV production in an \textit{in vitro} cell culture system.\(^ {106}\) The research...
infection, cccDNA synthesis is aided by DNA polymerase (POLK), a Y-family DNA polymerase with the highest function in nondividing cells. CRISPR/Cas9 knockdown of POLK gene expression reduced the transformation of rcDNA to cccDNA, decreasing cccDNA production and viral infection. Replication of HBV markers was significantly decreased following the delivery of SaCas9 and S gene targeting gRNAs into HepG2.2.15 cells, according to a new study (Figure 2 and Table 2).

EPIGENETIC ALTERATIONS SILENCE CCCDNA TRANSCRIPTION

Another treatment option is to use epigenetic changes to silence cccDNA transcription. The process of epigenetic gene splicing is utilized to cure HBV infection. Epigenetic therapy may be able to suppress the cccDNA minichromosome indefinitely, leading to a functional cure. This method includes chemically altering DNA without affecting the genetic code. cccDNA methylation and cccDNA acetylation are examples of epigenetic changes. Histone acetyltransferases and lysine/DNA/arginine methyltransferases are two of the most important epigenetic regulators of HBV-DNA. Histone deacetylase inhibitors decrease cccDNA in duck HBV, according to studies.

The cccDNA of the hepatitis B virus (HBV) undergoes epigenetic modulation which is critical for infection establishment and maintenance. HBV accomplishes this through manipulating many biological pathways, resulting in a complicated and nuanced interaction between the host and the virus. cccDNA epigenetic silencing could prevent transcription of the virus indefinitely. Modulators of the immune system, minute compounds, and tools for engineering of epigenome can be used in silencing of HBV-DNA and diminish the concentration of cccDNA.

Three CpG islands have been identified in HBV cccDNA. These CpG islands are properly positioned in the regulatory domain of the HBV genome. The ATG start site overlaps

Table 2. Uses of CRISPR/CAS9 in Hepatitis Virus Infection

| Species | Target | Cell type | Effect | Function |
|---------|--------|-----------|--------|----------|
| hCas9   | HBV1.2, S1 (3028–3039), P1 (1292–1314), PS (261–283) | Huh7 cells | Reduce the synthesis of cccDNA and rcDNA block | HBV |
| pX330-U6-Chimeric_BB-CBh-hSpCas9 | HBV1.3X (1523–1542; 1661–1700; 2338–2357; 2416–2435), ORF X/L | Huh7 and HepG2.2.15 cells | Reduce the level of cccDNA | HBV |
| SaCas9  | Huh7 and HepG2.2.15 cells | Huh7 and HepG2-NTCP cells | CccDNA inactivation and selective destruction | HBV |
| Cas9    | FEN1 | Huh38.7-Tet cells, HepG2-hNTCP-C4 cells, Hep38.7-Tet cells, 293FT cells, and PXB primary human hepatocyte | Hamper transformation of rcDNA to cccDNA and diminishes the concentration of cccDNA | HBV |

Figure 2. CRISPR/CAS9 targets in hepatitis B virus infection.
Table 3. Factors Associated with Epigenetic Regulation of Host Cells Associated with the cccDNA Minichromosomes

| Chromatin remodeling enzymes | Effect of epigenetics | Impact on HBV | Ref. |
|-----------------------------|----------------------|--------------|------|
| Histone acetyltransferases p300/CBP | Increases H3K12a | Activation | 146 |
| P300/CBP-associated factor (PCAF) |  | Activation | 136, 147 |
| Histone deacetylase 1 (HDAC1) | IFN inhibits acetylation of H3K9 and H3K27 | Inhibition |  |
| Sirtuin 1 and 3 (SIRT1/3) | Decreases H3K9me3 | Inhibition | 131, 148 |
| Sisitone acetyltransferases 1 (HAT1) | HAT1 overexpression enhances, H4K5, H4K12, and H3K27 acetylation | Activation | 149 |
| Mixed lineage leukemia protein 3 (MLL3) | Enhances H3K4me3 | Activation | 145, 150 |
| Protein arginine methyltransferase (PRMT) | H4R3me2s are upregulated when PRMT3 binds with HBc | Inhibition | 138 |
| Demethylases (KDMs) | SIRT1-mediated silencing of chromatin promotes H3K79me and contributes to transcriptional suppression |  |  |
| Histone methyltransferase suppressor of variegation 3–9 homologue 1 (SUV39H1) | Enhances levels of H3K9me3 | Inhibition | 152 |
| DNA methyltransferases (DNMTs) |  | Inhibition | 153 |
| Methyl-CpG binding protein (MBPs) | At methylated domains a histone-modifying complexes and recruits chromatin remodeling, contributing to methylation of histone | Inhibition | 154 |

| Different transcription factors of cells | Effect of epigenetics | Impact on HBV | Ref. |
|------------------------------------------|----------------------|--------------|------|
| Activating transcription factor 2 (ATF2) | Suppression of transcription of virus | Inhibition | 155 |
| cAMP response element binding protein (CREB) | Increases transcription | Activation | 156 |

Different transcription factors of cells | Effect of epigenetics | Impact on HBV | Ref. |
---|---|---|---|
| Nuclear factor 1 (NF1) |  | Activation | 157, 158 |
| Transcription factor Yin Yang 1 (YY1) |  | Inhibition | 158 |
| Specificity protein 1 (SP1) | Enzymes that acetylate and methylate histone proteins should be recruited | Activation | 159 |
| Nuclear transcription factor Y (NF-Y) |  |  |  |
| Activator protein 1 (AP-1) |  | Activation | 161 |
| TATA binding protein (TBP) |  | Activation | 162 |
| Nuclear factor kappa-B (NF-kB) |  | Inhibition | 163 |
| Hepatocyte factors | Effect of epigenetics | Impact on HBV | Ref. |
| Retinoic X receptors (RXRs) | Binding of p300 with the cccDNA minichromosome, promotes acetylation of histones H3 and H4 | Activation | 164 |
| CAAT enhancer-binding protein | C/EBP in low doses; C/EBP in high doses | Activation and inhibition | 165 |
| Hepatocyte nuclear factor 1α and β (HNF1) | HNF1 and Oct 1 are important transcriptional coactivators; elevated levels of HNF1 boost the expression of NF-kB and lead to suppression of transcription | Activation and inhibition | 166 |
| Hepatocyte nuclear factor 3α, β, and γ (HNF3) | Plays an important role in chromatin remodeling | Activation | 167 |
| Hepatocyte nuclear factor 4 (HNF4) |  | Activation | 168 |
| Interferon type I (IFNα) | Decreased acetylation of H3K27 and H3K9 | Inhibition | 147 |

with the first CpG island (CpG I) of the short sequence of HBsAg, the enhancers I and II, the core promoter, and the sequence of HBx promoter overlap in the second (CpG II) island, and the Spl promoter overlaps the third (CpG III) island with the ATG start codon of the polymerase gene.21 In the ten genotypes (A–J) of HBV, CpG I is found in five different genotypes (A, B, D, E, and I); on the other hand, CpG II and III have been reported in all genotypes.21 The three CpG islands have been shown to have varying degrees of methylation. Within CpG I, II, and III, methylation frequencies of 14, 0.6, and 3.7 percentage were found in CHB patients, respectively.128 However, different computational research found that 50% of the genomes of HBV have a dearth of CpG I, but CpG II and II were genotype-independent.21 This means that patterns of methylation between genotypes possibly vary.

Interferon suppresses replication of HBV via modulating the epigenetic alteration of cccDNA, which is a therapeutic target. Interferon inhibits HBV cccDNA transcription by recruiting the hSirt1 and histone deacetylases HDAC1 and lowering cccDNA-bound histone acetylation (Table 3).129 PTMs of histones regulated by HBx are linked with hypomethylated CpG islands and are considered to initiate replication of the virus and stabilize the cccDNA minichromosome (mainly GpG I and III).130 HATs, for example p300, CBP, and the p300/CBP-associated factor (PCAF), are recruited to the cccDNA, allowing viral transcription to be upregulated (Figure 3 and Table 3).131 Belloni et al.131 demonstrated an increase in sirtuin 1 (SIRT1) and HDAC1 recruitment to cccDNA, which was associated with a decrease in cccDNA acetylation bound H4 and transcription of the virus in the existence of an HBx mutant. These findings imply that HBx plays a vital role in enhancing viral transcription by boosting cccDNA histone acetylation.132 However, SIRT1’s role in promoting or suppressing HBV replication appears to be fluctuating. SIRT1 was discovered to positively regulate HBV replication.
by deacetylating PGC1 and FXR in other investigations and encouraging activator protein 1 (AP-1) to connect with the core promoter. Nevertheless, these investigations did not examine the involvement of SIRT1 in cccDNA-reliant viral replication models. According to Deng et al., SIRT1 binds with HBx and stabilizes it in HepG2-NTCP cells. It boosts HBx and other coactivating factors recruited to cccDNA. This resulted in a deacetylase-independent increase in viral transcription. These findings suggested that SIRT1 has a dual function in the maintenance of replication of HBV, depending on the existence or lack of HBx protein.

In human hepatoma cells, short hairpin RNA promoted methylation of HBV cccDNA, which inhibited its transcription. In CHB disease, methylation of CpG controlled HBV cccDNA transcription. Methylation of CpG island II has been associated with reduced blood HBsAg titers, while methylation of CpG island III has been linked with decreased transcription of cccDNA and consequent replication of HBV-DNA. Interestingly, HBx can diminish SETDB1 histone methyltransferases. Transcriptional silencing of HBV cccDNA induced by chromatin makes the chromatin protein active. Furthermore, it was documented that arginine amino acid residues in the HB core carboxyl-terminal motif decrease the acetylation of histones bound to cccDNA and therefore lower the HBc cccDNA binding. Methylation has been reported to be modulated by two enzymes. The protein arginine methyltransferase 5 regulates the symmetric dimethylation of arginine 3 on histone 4 of cccDNA. Replication of HBV and transcription are restricted by the silent mating type information regulation of homologue 3 SIRT3 via epigenetic control of cccDNA, which involves the histone methyltransferases SUV39H1 and SETD1A. The acetylation state of HBV cccDNA-bound histone 3 and histone 4 also influences the replication of HBV. The Np95/ICBP90-like RING finger protein NIRF, a novel E3 ubiquitin ligase, has been reported to decrease the replication of HBV-DNA and production of the hepatitis B antigen in HepG2 cells with significantly decreasing HBV cccDNA-bound histone 3. The natural compound curcumin inhibits replication of HBV by means of lowering acetylation of histone on cccDNA-bound histones. HBV replication has been shown to be regulated by retinoid X receptor a, which can also epigenetically alter HBV cccDNA. Furthermore, the mutations in the basal core promoter were reported to impede replication of HBV by altering the state of deacetylation and acetylation of cccDNA-bound histones, whereas changes in the preCore promoter had no significant impact on replication of the virus. These results suggest that control of histone acetylation and methylation of DNA might restrict HBV replication by inactivating cccDNA transcription. Peg interferon’s potential to suppress cccDNA transcription is based on the acetylation of cccDNA-bound histones being reduced (Figure 3). Because it appears to be important to suppress cellular components that impede cccDNA transcription, direct-acting antivirals may be able to target HBx. The involvement of the HBx protein in associating with the “structural regulation of chromosome” Smc complex Smc5/6 has recently been discovered, which suppresses extra chromosomal transcription of HBV-DNA. By eliminating the Smc5/6 complex, HBx relaxes the inhibition of HBV gene expression. Further investigations in hepatocytes of humans found that when the Smc5/6 complex is bound to ND10 (Nuclear Domain 10) it inhibits hepatitis B

Figure 3. Graphical illustrations of chromatin modifications on cccDNA in association with HBV replication. Regarding treatment of IFNα and the presence of HBx, the acetylation state of cccDNA-bound histones and the deployment of chromatin-modifying enzymes onto cccDNA are altered. Histones associated with cccDNA are hyperacetylated. The chromatin associated with cccDNA is in an open state. PgRNA is aggressively transcribed in the presence of strong viral replication or in the absence of IFNα therapy. The employment of the p300 acetyltransferase is significantly hindered in cells reproducing an HBx mutant and in IFN-treated cells, but the recruitment of the histone deacetylases (HDACs) hSirt1 and HDAC1 including the polycomb protein enhancer of zeste homologue 2 (Ezh2) is elevated. In the absence of HBx, hypoacetylation is followed by the recruitment of heterochromatin protein 1 factors (HP1) and the SET domain, bifurcated 1 (SETDB1). Adapted and modified with permission from ref 135.
viral transcription, and this interaction is critical for cccDNA transcriptional silencing in the absence of HBx. Tiny molecules can limit transcription of cccDNA and thus viral replication by inducing PTMs on cccDNA-bound histones, suggesting the potential of cccDNA epigenetic silencing as a new method. This method could result in a functional cure, although it must be proven in vivo. Hepatitis B can only be cured if cccDNA is disabled. In cultured cells and hydrodynamic injected mice, studies have revealed that genome editing tools have an enormous ability for altering cccDNA, although there are certain drawbacks to adopting gene editing applications. The above-mentioned epigenetic changes are linked to HBV cccDNA replication and transcription. They can control the cccDNA, but they are unable to remove it.

APPLICATION OF ANTI-HBV MIRNA AND SIRNA FOR SILENCING HBV CCCDNA

Silencing RNAs (siRNA) are a strong approach for silencing genes. Likewise, miRNAs are short noncoding RNAs that can shut down the expression of many genes at the same time. MiRNAs play some vital function in the genesis, development of cancer, and transmission of infectious diseases. There are around 30 siRNA potential compounds and at least three miRNA molecules which have been approved in clinical studies for the remedy of various ailments to date. For example, Miravirense can seize mature miR-122, which is required for replication of HCV. In HCV-affected individuals, Miravirense therapy resulted in long-term dose-dependent decreases in HCV-RNA levels with no dose-dependent side effects or drug resistance. Crohn’s disease affected individuals were also given Mongersen, which is an antisense oligonucleotide for SMAD7. Patients of Mongersen show a substantial clinical implications. Previous research has shown that HBV reactivation is caused by persistent cccDNA in individuals with hepatitis B that rely on immunosuppressive drugs. Chronic HBV infection is also hampered by HBV cccDNA. Although there has been an emergence of effective NAs, HBV is unable to be treated alone with NAs. Various affected individuals with chronic hepatitis B need to take NAs for a long time, if not forever. The molecular pathways that regulate cccDNA formation and regulation must be understood. The molecular mechanism gives a mechanistic understanding of its stability and its maintenance. Novel compounds that target the stable cccDNA reservoir or hepatocytes having cccDNA may be promising in eradicating persistent cccDNA for the remedy of CHB disease.

CONCLUDING REMARKS

The majority of current treatment goals for a functional cure is demonstrated with the dearth of HBsAg. The ideal purpose of an HBV cure with cccDNA impairment is advantageous, but it is extremely cumbersome to achieve this feat. In this context, significant development has been achieved in the biology and molecular mechanism of HBV cccDNA, for example, the recent breakthroughs of novel factors affecting the host as well

| Target | Concept | Developmental stage | Potential difficulties |
|--------|---------|---------------------|-----------------------|
| CccDNA genome editing | Elimination of cccDNA in a direct manner | Discovery/ preclinical | Delivery and off-target effects |
| Host-dependent cccDNA factors | Suppression of cccDNA synthesis and/or regulation | Discovery/ preclinical | Adverse effects |
| Modifiers of chromatin and preclinical/clinical transcription factors | Inhibition of cccDNA transcription | Preclinical/ clinical | CccDNA specificity, adverse effects, and potential long-term cure |
| Capsid assembly CAM* | Degradation of Hbc inhibits amplification of cccDNA and synthesis and plays a vital function in the structure of cccDNA itself | Clinical | Possible resistance, long-term responses unknown |
| Immune-mediated pegylated interferon-alpha | Change of immune responses to antiviral destruction of cccDNA | Approved | Inadequate response, side effects |
as hitherto enigmatic mechanisms of cccDNA transcription epigenetic maintenance. Several cccDNA-targeting techniques are in the preclinical or early clinical stages of research (examples shown in Table 4). Considering the heterogeneity of the replication cycle of HBV and combination therapy, amalgamation of immune targeting and direct-acting antiviral approaches are likely to be vital for HBV treatment, together with the removal of HBV cccDNA. More research is required to fully comprehend and evaluate the efficiency and safety of treatment possibilities in clinical trials. Developing therapeutic medicines is a realistic aim that can naturally remove HBV disease. A better knowledge of these mechanisms in patients may open up new avenues for curative treatments, such as cccDNA eradication.

In conclusion, the goal of HBV treatment is to disable or completely inactivate cccDNA. However, overcoming the off-target issue and discovering routes to in vivo delivery are the most pressing concerns that must be emphasized before adopting tools of gene editing for an HBV cure. Furthermore, the lack of solid, trustworthy, and experimental models of HBV cccDNA has slowed the progression of cccDNA therapeutics for a long time. Scientists recently developed a cell line from HepG2-derived cell lines by integrating 2–60 copies of the monomeric HBV genome, allowing for the production of cccDNA and detection with gene-specific primers. The cell line development will give a suitable in vitro model for testing molecules that target cccDNA. However, both genome editing techniques and epigenetic alteration treatments have hurdles, and there is still a long way to go from fundamental research to clinical relevance. It is quite possible that we will be able to cure HBV in the future, comparable to the hepatitis C virus cure.

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