

## REVIEW

# Persistence of hepatitis B virus covalently closed circular DNA in hepatocytes: molecular mechanisms and clinical significance

Hung-Chih Yang<sup>1,2,3</sup> and Jia-Horng Kao<sup>2,3,4,5</sup>

Covalently closed circular DNA (cccDNA) is the transcriptional template of hepatitis B virus (HBV). Extensive research over the past decades has unveiled the important role of cccDNA in the natural history and antiviral treatment of chronic HBV infection. cccDNA can persist in patients recovering from acute HBV infection for decades. This explains why HBV reactivation occasionally occurs in patients with resolved hepatitis B receiving intensive immunosuppressive agents. In addition, although advances in antiviral treatment dramatically improve the adverse outcomes of chronic hepatitis B (CHB), accumulating evidence demonstrates that current antiviral treatments alone, be they nucleos(t)ide analogs (NAs) or interferon (IFN), fail to cure most CHB patients because of the persistent cccDNA. NA suppresses HBV replication by directly inhibiting viral polymerase, while IFN enhances host immunity against HBV infection. Viral rebound often occurs after discontinuation of antiviral treatment. The loss of cccDNA can be induced by non-cytolytic destruction of cccDNA or immune-mediated killing of infected hepatocytes. It is known that NA has no direct effect on viral transcription or cccDNA stability. Therefore, the long half-life of hepatocytes leads to a very slow decline in cccDNA in patients under antiviral therapy. Novel antiviral agents targeting cccDNA or cccDNA-containing hepatocytes are thus required for curing chronic HBV infection. *Emerging Microbes and Infections* (2014) 3, e64; doi:10.1038/emi.2014.64; published online 17 September 2014

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## INTRODUCTION

Hepatitis B virus (HBV) infection is a key global public health concern. More than 350 million people are chronically infected with HBV.<sup>1</sup> The advent of modern antiviral therapy has dramatically reduced the mortality and morbidity of patients with chronic hepatitis B (CHB). Currently, there are two types of antiviral therapy for HBV infection: nucleoside/nucleotide analogs (NAs) and interferon (IFN).<sup>2</sup> The former suppresses HBV replication by inhibiting its polymerase; the latter does so by its direct antiviral effects as well as indirect modulation of the host immune response. Only a small portion of patients can undergo HBsAg seroclearance with antiviral therapy; this response has been associated with several host and viral factors.<sup>3,4</sup> Unfortunately, viral rebound often occurs following discontinuation of antiviral therapy.<sup>5–7</sup> Accumulating evidence has proven that the persistence of covalently closed circular DNA (cccDNA) is the major barrier to eradicating chronic HBV infection.<sup>8</sup> In addition, persistent cccDNA can occasionally be detected even in the liver of patients with resolved hepatitis B infections.<sup>9–12</sup> More importantly, the persistent cccDNA appears to be replication competent at least in a significant portion of these patients, as evidenced by the fact that HBV reactivation occurs when they receive intensive immunosuppressive agents, such as chemotherapy. This review will discuss the molecular mechanisms of persistence of HBV cccDNA and its clinical significance as well as the potential strategies to cure chronic HBV infection.

## cccDNA IS THE TRANSCRIPTIONAL TEMPLATE OF HBV

### The life cycle of HBV

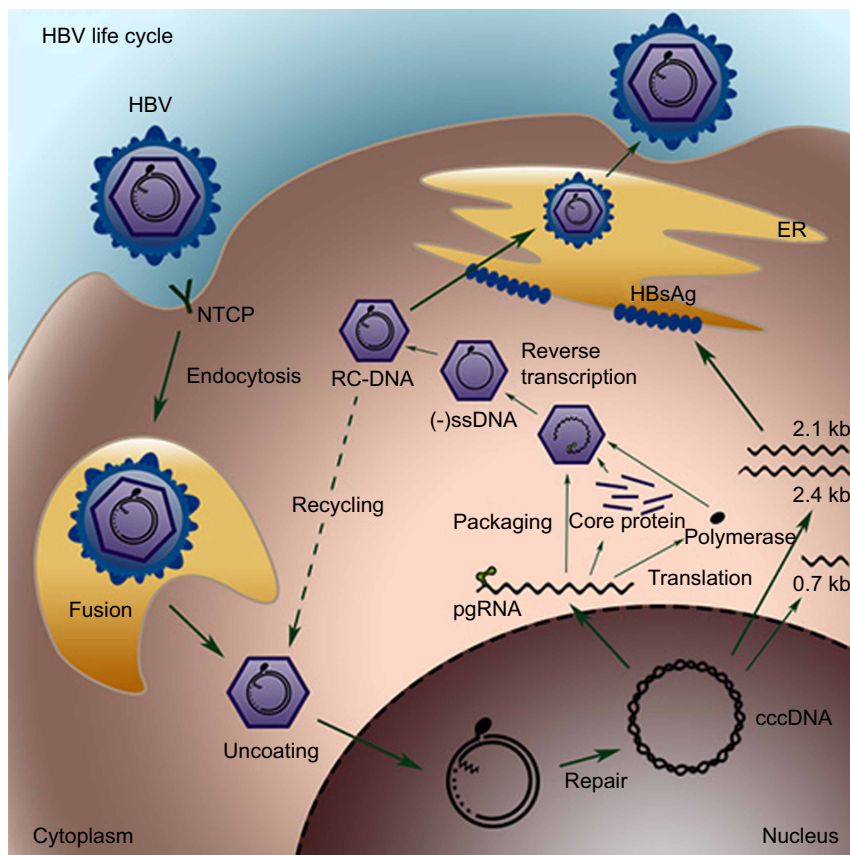
HBV is the prototype member of the family *Hepadnaviridae*. The genome of HBV is composed of partially double-stranded DNA, also known as relaxed-circular DNA (RC-DNA); it is 3.2 kb in length. Although HBV is a DNA virus, it replicates through reverse transcription.<sup>13,14</sup> The replication cycle of HBV in infected hepatocytes has been extensively studied (Figure 1).<sup>15</sup> Recently, sodium taurocholate cotransporting polypeptide (NTCP) has been identified as either the receptor or part of the receptor complex for HBV infection.<sup>16</sup> Following entry into hepatocytes, HBV undergoes uncoating of its capsid and releases its RC-DNA. The RC-DNA is then translocated to the nuclei of infected hepatocytes, where it is converted to cccDNA through a series of processes that have not been fully elucidated. The details of this process will be described below.

The resulting cccDNA is the template for transcription of the pregenomic RNA (pgRNA) and several subgenomic RNAs. The genome of HBV contains four open reading frames, precore/core, polymerase, preS1/S2/S and X domains, which are organized in an overlapping manner. The transcription of these four open reading frames is controlled by four distinct promoters and is regulated independently.<sup>17,18</sup> The pgRNA serves as the template for reverse transcription and translation of the core and polymerase proteins. The viral polymerase binds to the packaging signal of the pgRNA and subsequently initiates the

<sup>1</sup>Department of Microbiology, National Taiwan University College of Medicine, Taipei 10002, Taiwan, China; <sup>2</sup>Graduate Institute of Clinical Medicine, National Taiwan University College of Medicine, Taipei 10002, Taiwan, China; <sup>3</sup>Department of Internal Medicine, National Taiwan University Hospital, Taipei 10002, Taiwan, China; <sup>4</sup>Hepatitis Research Center, National Taiwan University Hospital, Taipei 10002, Taiwan, China and <sup>5</sup>Department of Medical Research, National Taiwan University Hospital, Taipei 10002, Taiwan, China

Correspondence: JH Kao  
E-mail: kaojh@ntu.edu.tw

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**Figure 1** The replication cycle of HBV. HBV virions bind to the receptor NTCP on hepatocytes and are internalized. Nucleocapsids are released into the cytoplasm and then translocated to the nucleus, where the genome is converted into cccDNA through a poorly understood mechanism, most likely via the DNA repair mechanism. The HBV cccDNA serves as the template for transcription of the pregenomic and subgenomic RNAs. The pregenomic RNA is the template for both reverse transcription and translation of polymerase and core proteins. The polymerase binds to the packaging signal of the pregenomic RNA, and both of them are incorporated into the viral capsid, inside which RC-DNA is generated through reverse transcription. The resulting RC-DNA can either be enveloped in ER and secreted as progeny virions or be recycled back to the nucleus for cccDNA amplification. ER, endoplasmic reticulum; NTCP, sodium taurocholate cotransporting polypeptide.

assembly of core proteins to a viral capsid, which incorporates the pgRNA–polymerase complex in the cytoplasm. Inside the capsid, the pgRNA undergoes reverse transcription, which is initiated by protein priming at the tyrosine residue of the polymerase. The resulting RC-DNA is partially double stranded. The minus strand is longer than a genome length, with a covalently bound polymerase and a redundant flap at the 5' end. The plus strand synthesis is incomplete, and a gap exists down to the 3' end. The mature capsid can be enveloped with HBV surface antigens in the endoplasmic reticulum (ER) and secreted to the blood. It can also re-enter the nuclei through a process called intracellular recycling to replenish the cccDNA pool.<sup>19,20</sup> It has been estimated that the cccDNA pool can reach an average of 5–50 copies per cell, most likely through this intracellular recycling replenishment process.<sup>19–22</sup>

#### Conversion of RC-DNA to cccDNA

By comparison of the structures of RC-DNA and cccDNA, several required steps are deduced.<sup>14</sup> First, the 3' downstream gap on the plus strand must be filled. Whether the viral polymerase or a cellular polymerase elongates the 3' end of the plus strand remains unclear. The covalently bound polymerase at the 5' end of the minus strand needs to be removed. The part of the cell in which the removal of the polymerase occurs is still debated; however, some previous studies have suggested that the bound polymerase is cleaved in the cytoplasm, and

the resultant protein-free RC-DNA is subsequently transported to the nucleus.<sup>23–25</sup> The flap DNA and the RNA primer, located at the 5' ends of the minus and plus strand DNA, respectively, have to be removed before ligation of the nicked DNA. It is generally assumed that DNA repair mechanisms play a role in this process. However, the host factors involved in this process remain elusive. Recently, it has been demonstrated that tyrosyl-DNA-phosphodiesterase 2 can cleave the bound polymerase at least *in vitro*, although its role in cccDNA formation *in vivo* is unclear.<sup>26</sup>

#### PERSISTENCE OF HBV cccDNA DURING THE NATURAL HISTORY OF HBV INFECTION

Given that cccDNA is the transcriptional template for HBV, a correlation between the levels of intrahepatic cccDNA and serum HBV DNA during the natural history of HBV infection is expected. A cross-sectional natural history study revealed that cccDNA levels are consistently significantly higher in HBeAg-positive patients compared to HBeAg-negative patients, inactive carriers, and patients who underwent HBsAg clearance.<sup>27</sup> This is also consistent with observations of higher levels of intrahepatic HBV replicative intermediates and serum HBV DNA in HBeAg-positive patients.

HBsAg seroconversion, defined by the loss of HBsAg and appearance of anti-hepatitis B antibodies, is a typical serological consequence in patients with transient HBV infection and can also occur in a small

proportion of patients with chronic hepatitis B.<sup>28</sup> It is generally believed that HBsAg seroconversion is closely related to the complete clearance of HBV infection. However, even after HBsAg seroconversion, HBV DNA is occasionally detectable in the liver and even the serum of patients. Previous studies have demonstrated that despite active maintenance of robust antiviral T-cell immunity, the HBV genome can persist in patients recovering from acute HBV infection for decades.<sup>9–11</sup> In particular, intrahepatic HBV cccDNA can sometimes be detected in these HBsAg-negative patients.<sup>12,27,29</sup> The intrahepatic cccDNA level in HBsAg-negative patients is usually lower than that in HBsAg-positive patients.<sup>26</sup> These patients are commonly denoted as having ‘occult HBV infection’, which is defined by the presence of the HBV genome in the liver and sometimes also the serum of HBsAg-negative patients.<sup>30,31</sup>

Notably, the HBV cccDNA that persists in the infected hepatocytes is replication competent rather than replication defective. This is evidenced by the fact that HBV reactivation occasionally occurs in patients with occult HBV infection who receive intensive immunosuppressive chemotherapy, particularly in lymphoma patients with rituximab-containing regimens.<sup>32,33</sup> In addition, occult HBV infection carriers may be a source of HBV transmission through blood transfusion and orthotopic liver transplantation.<sup>34</sup> Therefore, the persistent intrahepatic replication-competent cccDNA in these patients provides a mechanistic explanation for these alarming events.

## PERSISTENCE OF HBV cccDNA IS THE PRIMARY BARRIER TO ERADICATING CHRONIC HBV INFECTION

### Experimental evidence from model systems

Curing chronic HBV infection requires elimination of HBV cccDNA from infected hepatocytes; therefore, it is important to know the stability and half-life of HBV cccDNA. By inhibition of viral replication with NAs, it is possible to estimate the stability and half-life of cccDNA *in vitro* and *in vivo*. Initially, the stability of cccDNA was determined in a tissue culture system by using non-dividing primary hepatocytes or hepatoma cell lines. However, the estimated half-life of cccDNA in culture systems is often confounded by the death of cultured cells, resulting in inconsistent conclusions from different systems. For example, using the congenitally infected primary duck hepatocyte culture system, the half-life of duck hepatitis B virus (DHBV) cccDNA was found to be very short, ranging from 3 to 5 days.<sup>35</sup> In contrast, studies in primary woodchuck hepatocytes and HepaRG cells showed that cccDNA was quite stable under the inhibition of NAs.<sup>36–38</sup>

The half-life of hepadnavirus has also been investigated *in vivo*. Using inhibition of viral replication by NAs, the half-lives of the cccDNAs of DHBV and woodchuck hepatitis virus (WHV) *in vivo* were estimated to be 33–50 days and 35–57 days, respectively.<sup>39,40</sup> Short-term antiviral NA treatment fails to cure hepadnavirus infection, and withdrawal of NAs inevitably leads to rebound viremia.<sup>41,42</sup> Additionally, the use of the potent reverse transcriptase inhibitor entecavir in DHBV-infected ducks further suggested that the cccDNA is maintained by its stability rather than ongoing replication.<sup>43</sup> Notably, the half-life measurements in these studies are confounded by uncertainties. For example, NAs alone may not be able to completely inhibit viral replication *in vivo*. In addition, the turnover and division of infected hepatocytes may also affect the decline rate of the cccDNA. All these factors should be taken into consideration when the half-life of cccDNA is measured.

### Reduction of HBV cccDNA in patients during current antiviral treatments

Current antiviral treatment involves two types of agents, IFN and NAs. NAs only block reverse transcription and have no direct effect on the

existing cccDNA minichromosome, which is the template for viral transcription. This is analogous to the observation that highly active antiretroviral therapy has no effect on the integrated provirus of HIV.<sup>44</sup> No cure for HIV has been achieved by highly active antiretroviral therapy alone. Likewise, NA alone does not cure CHB. However, in contrast to highly active antiretroviral therapy for HIV, NA also fails to prevent the *de novo* formation of cccDNA from incoming virions.<sup>45–47</sup> Despite its lack of a direct effect on cccDNA, NA treatment can partially and transiently restore the immune response to HBV infection and may thus indirectly promote viral clearance.<sup>48,49</sup>

The intrahepatic level of cccDNA has been shown to be an indicator for treatment response.<sup>50</sup> To monitor the reduction of cccDNA during antiviral therapy, several assays to quantify intrahepatic cccDNA were developed.<sup>27,50,51</sup> The quantification of HBV cccDNA in infected cells is a challenging task. The cccDNA quantitative assays typically rely on a plasmid-safe DNase treatment and cccDNA-specific primers for amplification and detection of cccDNA.<sup>27</sup> However, the specificity of the quantitative PCR for cccDNA is always a concern because a vast excess of RC-DNA is present in infected hepatocytes.<sup>52</sup> A more reliable cccDNA quantitative assay is clearly needed. Interestingly, HBsAg levels are correlated with intrahepatic cccDNA levels in HBeAg-positive patients.<sup>8,27,53</sup> Recent studies also suggested that the serum HBsAg level can predict clinical outcomes in natural history and antiviral treatment of CHB.<sup>54,55</sup> Therefore, quantification of HBsAg can serve as a surrogate marker of intrahepatic cccDNA. By using the cccDNA quantitative assay, it has been found that treatment with adefovir, entecavir or lamivudine for 48 weeks may result in the reduction of intrahepatic cccDNA by 0.8–1.0 log.<sup>27,56</sup> Although significant, this reduction appears to be insufficient for the eradication of HBV cccDNA because viral rebound often occurs after withdrawal of NAs. Therefore, cccDNA persists even under long-term NA therapy.

In contrast, IFN is an immune modulatory agent. Despite a higher rate of adverse effects, IFN is an attractive therapeutic option because it provides higher rates of off-therapy immune control of HBV as well as HBsAg/HBeAg loss or seroconversion.<sup>4,57</sup> The mechanisms by which IFN controls HBV infection include direct inhibition of HBV replication and clearance of infected hepatocytes through stimulation of cell-mediated immunity. Furthermore, using human chimeric severe combined immunodeficiency mice that lack adaptive immune cells, it was found that pegylated IFN- $\alpha$  reduced serum HBsAg and HBeAg levels by inhibiting viral transcription independent of immune cells; however, it did not significantly reduce intrahepatic cccDNA.<sup>58</sup> Interestingly, a recent study reported that IFN could result in the specific and non-hepatotoxic degradation of intrahepatic cccDNA.<sup>59</sup> However, data are scarce regarding the effects of IFN treatment on the reduction of intrahepatic cccDNA in patients, partly because a reliable intrahepatic quantitative cccDNA assay was not available until recently. A combination treatment with pegylated IFN- $\alpha$ 2b and adefovir has been shown to induce a strong cccDNA decline of 2.4 logs and HBsAg reduction.<sup>60</sup> Another study demonstrated that combination therapy with pegylated IFN- $\alpha$ 2b plus entecavir led to a cccDNA decrease of 1.4 logs.<sup>61</sup> Collectively, these findings suggest that IFN may result in more significant reduction of intrahepatic cccDNA than NA in CHB patients.

### Viral kinetics during antiviral therapy

Several previous studies investigating viral kinetics in CHB patients receiving antiviral treatment provided new mechanistic insights into the persistence of cccDNA. In brief, CHB patients receiving lamivudine reported a high rate of viral production up to 10<sup>11</sup> virions/day. They also found that the half-life of a virion was approximately 1 day,



but the half-life of producing cells varied, ranging from 10 to 100 days.<sup>62</sup> A further study in patients receiving adefovir revealed an interesting biphasic decline profile of viral kinetics, which included an initial faster phase and a second slower phase.<sup>63</sup> The former may reflect the clearance of HBV particles from plasma with a half-life of approximately 1 day, whereas the latter most likely represents the slow destruction of the infected hepatocytes, with a half-life of approximately 18 days. Based on the deduced rate of cccDNA reduction, it is estimated to take at least 14.6 years to eradicate persistent cccDNA. Even with the use of a potent antiviral agent, such as entecavir or tenofovir, treatment still fails to achieve complete clearance of CHB within a finite treatment duration. A biphasic decline pattern could also be observed in studying the viral kinetics in CHB patients treated with entecavir or a combination of antiviral agents, indicating that NAs have little to no effect on the second slow decline of virion-producing cccDNA-containing cells.<sup>64,65</sup>

### EPIGENETIC REGULATION OF THE REPLICATIVE ABILITY OF HBV cccDNA

In contrast to the naked DNA of a prokaryotic genome, the eukaryotic genome is packaged into highly ordered chromatin, which contains a repeating unit, known as the nucleosome, composed of histones and approximately 200 bp of DNA. Each nucleosome contains two copies of each of the four core histones: H2A, H2B, H3 and H4. Epigenetic alterations, including DNA methylation at dinucleotide CpG sites and post-translational covalent histone modifications (i.e., acetylation, methylation, phosphorylation, *etc.*), can regulate chromatin accessibility and thus control eukaryotic gene transcription.<sup>66–68</sup> Previous studies found that episomal HBV cccDNA is associated with chromatin and non-chromatin proteins in the nuclei of infected hepatocytes. The cccDNA is organized into a typical beads-on-a-string nucleosomal DNA and exists as a minichromosome.<sup>21,69</sup> The HBV core protein has also been shown to preferentially bind to HBV double-stranded DNA, leading to a reduction in the nucleosomal spacing of the HBV nucleoprotein complex by 10%, from 200 bp to 180 bp.<sup>69,70</sup>

Recently, epigenetic regulation, including histone modification and DNA methylation, has been shown to play a role in the regulation of the transcriptional activity of cccDNA.<sup>71</sup> By using a novel chromatin immunoprecipitation assay for cccDNA, Pollicino *et al.*<sup>72</sup> demonstrated that the acetylation status of the cccDNA-bound H3 and H4 histones regulates the transcriptional activity of cccDNA. Additionally, histone hypoacetylation and histone deacetylase 1 recruitment to the cccDNA in liver tissue are correlated with low HBV viremia in CHB patients. Using the same approach with a cccDNA-specific chromatin immunoprecipitation, Belloni *et al.*<sup>73</sup> suggested that several non-histone proteins, including histone acetyltransferases and deacetylases, bind to the cccDNA minichromosome. Furthermore, viral protein HBx regulates the cccDNA transcription by prevention of hypoacetylation of cccDNA, illustrating the function of a non-chromatin protein in the regulation of cccDNA transcription.<sup>73</sup>

DNA methylation also occurs in cccDNA. Three CpG islands have been identified in the HBV genome.<sup>74</sup> Some variations have also been found in the cccDNA CpG islands in different genotypes of HBV.<sup>75</sup> Methylation of cccDNA reduces the expression of viral proteins.<sup>76</sup> Interestingly, the levels of cccDNA methylation in HBeAg-negative patients are higher than those in HBeAg-positive patients.<sup>77</sup> Consistent with this observation, the intrahepatic HBV cccDNA in HBeAg-negative patients exhibits a lower replication capacity compared to that in HBeAg-positive patients.<sup>78,79</sup> This indicates not only a decreased quantity of cccDNA, but also an impairment in its quality in HBeAg-negative

patients. In addition, IFN- $\alpha$  can inhibit HBV transcription by altering the epigenetic modification of cccDNA minichromosomes,<sup>80,81</sup> suggesting antiviral treatment may suppress HBV replication through epigenetic regulation.

### MECHANISMS FOR THE DESTRUCTION OF HBV cccDNA

Curing chronic HBV infection requires elimination of the persistent HBV cccDNA. Several mechanisms may be required to work cooperatively to achieve this goal: killing of the infected hepatocytes by the cytolytic immune response, primarily by cytotoxic CD8 T cells; non-cytolytic clearance of cccDNA by the inflammatory cytokines, i.e., IFN- $\gamma$  and tumor necrosis factor (TNF)- $\alpha$ ; and dilution of cccDNA by hepatocyte proliferation and replacement with regenerating uninfected cells.<sup>8,82</sup>

In HBV transgenic mice and HBV-infected chimpanzees, HBV replication can be suppressed without destruction of the HBV genome-containing hepatocytes, which is so-called non-cytolytic clearance of HBV.<sup>83–85</sup> In chimpanzees with acute HBV infection, the HBV DNA declines before the peak of alanine transaminase (ALT), supporting the notion of non-cytolytic suppression of HBV replication. This non-cytolytic control of viral replication is dependent on virus-specific CD8 T cells and is primarily mediated by the pro-inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$ .<sup>83,85,86</sup> Additionally, IFN- $\alpha$  also contributes to non-cytolytic clearance in HBV transgenic mice.<sup>87</sup> Further investigation into the molecular mechanisms involved in non-cytolytic clearance of virus revealed that IFN- $\gamma$  and TNF- $\alpha$  suppress viral replication by eliminating the HBV nucleocapsid particles inside viral genomes and destabilizing viral RNA.<sup>88</sup> However, similar non-cytolytic clearance of virus could not be demonstrated in WHV or DHBV animal models. Delivery of IFN- $\gamma$  did not provide any extra benefit nor lead to further destruction of cccDNA.<sup>89,90</sup> The causes of these inconsistent observations across model systems remain unclear.

Recently, it has been suggested that apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 (APOBEC3)-family proteins form an innate defense mechanism that inhibits HBV replication. The previous study showed that APOBEC3G inhibits the packaging of pregenomic RNA in the HBV capsid and thus, promotes the clearance of HBV DNA in a non-cytolytic manner.<sup>91</sup> A recent study also discovered that APOBEC3A is induced by IFN following DNA detection. It deaminates foreign double-stranded DNA cytidines to uridines, which are then converted by the uracil DNA glycosylase UNG2 to abasic lesions, and results in foreign DNA degradation. In contrast, IFN-induced APOBEC3A does not exert effects on genomic DNA.<sup>92</sup> The APOBEC3G also exerts its hypermutation effect on the HBV cccDNA, which is counteracted by UNG because inhibition of UNG extensively enhances the G-to-A or C-to-T hypermutation in cccDNA.<sup>93</sup> A recent paper also described that IFN- $\alpha$  and lymphotoxin- $\beta$ -receptor activation can upregulate APOBEC3A and 3B cytidine deaminases, respectively, in HBV-infected cell lines and primary hepatocytes. The HBV core protein mediates the interaction of APOBEC3A and 3B with nuclear cccDNA, ultimately resulting in cccDNA degradation.<sup>59</sup>

Another important mechanism for viral clearance is killing of the infected hepatocytes by cytotoxic CD8 T cells. CD8 T cells have been recognized to be an important factor in control of HBV infection. Experiments involving depletion of CD8 T cells confirmed their role in cytolytic and non-cytolytic effects on viral clearance.<sup>94</sup> In ducks chronically infected with DHBV, hepatocyte turnover was required for rapid viral clearance during antiviral therapy.<sup>95</sup> In an investigation of the transient WHV infection in woodchucks, apoptosis of the infected cells and subsequent replacement by the regenerated uninfected cells were responsible for the recovery from transient WHV infection.<sup>96</sup>

**Table 1** Current antiviral treatments and potential strategies that may eliminate persistent cccDNA

	Mode of action	Specific cccDNA degradation	Killing of infected hepatocytes
NA	Inhibition of HBV polymerase	No	No
IFN- $\alpha$	Immune modulation Inhibition of HBV replication Epigenetic regulation of the transcriptional activity of cccDNA Upregulation of APOBEC activity for cccDNA hypermutation and degradation	Yes (in a high dose)	Yes (indirectly in some patients)
LT $\beta$ R agonist	Upregulation of APOBEC3B activity for cccDNA hypermutation and degradation	Yes	No
Adoptive T-cell therapy (T cells expressing chimeric antigen receptor)	T cell immune response against HBV-infected hepatocytes	No	Yes
Genome editing tools (ZFNs, TALENs, CRISPR/Cas9)	Site-specific cleavage of HBV cccDNA	Yes	No

Abbreviations: LT $\beta$ R, lymphotoxin- $\beta$ -receptor; TALEN, transcription activator-like effector nuclease; ZFN, zinc-finger nuclease; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) systems.

To further analyze hepatocyte turnover for viral clearance, Summers *et al.*<sup>97</sup> developed a quantitative assay for the integrated genome of WHV, which serves as a cell lineage marker, to determine the proportion of hepatocyte turnover. The results indicated that during the resolution of transient infections, a large fraction of the infected hepatocyte population was killed and replaced by hepatocyte cell division.

The same research group also studied chronically WHV-infected woodchucks receiving antiviral therapy with the nucleoside analog 1-(2-fluoro-5-methyl-beta-L-arabino-furanosyl)uracil (L-FMAU). They found that although viral cccDNA declined 20- to 100-fold, the frequency of the integrated WHV remained relatively constant over the course of treatment; this result argued for the notion that the uninfected hepatocytes are derived from the infected hepatocyte population.<sup>98</sup> They also found clonal expansion of hepatocytes during chronic WHV infection. Approximately half of the liver is derived from the high copy number (i.e., >1000 cells) clones, indicating that a high degree of hepatocyte proliferation and selection occurred during the chronic period of WHV infection.<sup>99</sup> Taken together, clearance of hepadnavirus cccDNA in persistent hepadnavirus-infected animals and in CHB patients appears to require a high proportion of hepatocyte turnover, indicating a critical role for killing of the infected hepatocytes and regeneration of uninfected hepatocytes derived from the infected hepatocyte population.

#### A NOVEL AGENT TARGETING HBV cccDNA OR cccDNA-CONTAINING HEPATOCYTES IS A POTENTIALLY PROMISING STRATEGY TO ERADICATE HBV INFECTION

As discussed above, current antiviral treatments fail to eradicate chronic HBV infection. An ideal strategy for curing chronic hepatitis B should specifically destroy HBV cccDNA or eliminate the infected hepatocytes without overt hepatotoxicity. A recent study showed that IFN- $\alpha$  and lymphotoxin- $\beta$ -receptor activation can induce non-cytolytic destruction of cccDNA without hepatotoxicity by stimulating APOBEC3A and APOBEC3G, respectively.<sup>59</sup> Therefore, lymphotoxin- $\beta$ -receptor activation may serve as a therapeutic alternative for destruction of cccDNA. Although this is an exciting observation, several critical issues have been raised, including the potential uncertainty of cccDNA quantification.<sup>52</sup> Further studies are required to confirm the conclusions. Another approach is to use the sequence-specific genome-editing DNA endonucleases, which bear the potential to specifically disrupt HBV cccDNA.<sup>100</sup> Previous studies have shown that zinc-finger nucleases and transcription activator-like effector nucleases can specifically destroy HBV cccDNA.<sup>101–103</sup> Our preliminary data also demonstrated that the CRISPR/Cas9 system, another genome-editing tool,<sup>104</sup> can also be

utilized to specifically disrupt HBV cccDNA. However, delivery of these large systems to target the cccDNA-containing hepatocytes *in vivo* remains challenging.

In addition to specific disruption of cccDNA, an alternative strategy for curing chronic hepatitis B is to eliminate cccDNA-containing hepatocytes. A cytotoxic T-cell response is responsible for viral clearance and liver injury during HBV infection.<sup>91</sup> Patients who recover from acute HBV infection possess a polyclonal and multispecific T-cell response,<sup>105–107</sup> indicating the role of T cells in viral clearance. Protzer *et al.*<sup>108,109</sup> have engineered T cells with chimeric receptors directed against HBV surface proteins; these proteins allow HBV-infected hepatocytes to be specifically targeted and destroyed *in vitro* and *in vivo*. The effects of all the aforementioned strategies, including the currently used IFN and NA, on persistent cccDNA are summarized in Table 1.

#### FUTURE PERSPECTIVES

Persistence of cccDNA in HBV infection has significant clinical consequences. Previous studies have clearly demonstrated that persistent cccDNA is responsible for HBV reactivation in patients with resolved hepatitis B receiving immunosuppressives. HBV cccDNA is also the major barrier to eradicating chronic HBV infection. Despite the introduction of potent NAs, HBV still cannot be cured by NAs alone. Many patients with chronic hepatitis B require long-term or even life-long use of NAs. Understanding the molecular mechanisms regulating the synthesis and maintenance of cccDNA provides mechanistic insight into its stability and possible weak points. Novel drugs targeting the stable pool of cccDNA or cccDNA-containing hepatocytes may allow the realization of the hope of eliminating persistent cccDNA for the purpose of curing chronic HBV infection in a manner parallel to the clearance of hepatitis C virus infection.<sup>110</sup>

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