Progress With Developing Use of Gene Editing To Cure Chronic Infection With Hepatitis B Virus

Abdullah Ely[1], Buhle Moyo[1] and Patrick Arbuthnot[1]

[1]*Wits/SAMRC Antiviral Gene Therapy Research Unit, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa*

Chronic infection with hepatitis B virus (HBV) occurs in approximately 6% of the world's population. Carriers of the virus are at risk for life-threatening complications, and developing curative treatment remains a priority. The main shortcoming of licensed therapies is that they do not affect viral covalently closed circular DNA (cccDNA), a stable intermediate of replication. Harnessing gene editing to mutate cccDNA provides the means to inactivate HBV gene expression permanently. Reports have described use of engineered zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated (Cas) nucleases. Although inhibition of viral replication has been demonstrated, reliably detecting mutations in cccDNA has been difficult. Also, the dearth of murine models that mimic cccDNA formation has hampered analysis *in vivo*. To reach a stage of clinical use, efficient delivery of the editors to HBV-infected hepatocytes and limiting unintended off-target effects will be important. Investigating therapeutic efficacy in combination with other treatment strategies, such as immunotherapies, may be useful to augment antiviral effects. Advancing gene editing as a mode of treating HBV infection is now at an interesting stage and significant progress is likely to be made in the immediate future.

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The year 2015 marked 50 years since the Nobel prize-winning discovery of the Australia antigen by Baruch Blumberg and his colleagues.[1,2] This protein, which was the first recognized marker of infection with hepatitis B virus (HBV), is now known as a surface antigen of HBV (HBsAg). Since this seminal finding, the considerable medical significance of HBV became apparent and research on the virus progressed at an impressive pace. It is estimated that 2 billion people have been infected with HBV and about 240 million of these individuals are currently chronic carriers of the virus.[3] It is these persistently infected individuals who are at high risk for the life-threatening complications of cirrhosis and hepatocellular carcinoma (HCC). The mechanism of HBV-induced hepatocarcinogenesis is incompletely understood. However integration of viral DNA into the host genome is thought to contribute to the process of malignant transformation.[4]

Spread of HBV occurs by parenteral transmission and when infection with the virus occurs at a young age, the risk for chronicity is high.[5–7] Available therapies to treat HBV infection, which include reverse transcriptase inhibitors and interferon-α (IFN-α) derivatives, rarely completely clear the virus from carriers.[5,8] Vaccines, comprising eukaryotic recombinant HBsAg, may be used to prevent HBV infection. However, incomplete global coverage and the inadequacy of current therapy indicate that complications arising from chronic HBV will remain a significant global health problem for some time. Research using gene editing to inactivate the virus permanently has shown that advancing this approach may lead to a cure for chronic HBV infection.

ESSENTIAL MOLECULAR BIOLOGY OF HBV

The virion of HBV has an envelope, with embedded large, middle and small HBsAgs that surround the viral nucleocapsid. The restricted host range of HBV and limited availability of cultured cells that are infectable with the virus have impeded research on the early stages of HBV replication. An initiating step of hepatocyte infection is the glycosaminoglycan-mediated clustering of viral particles on the surface of hepatocytes (**Figure 1**).[9] Thereafter virions enter hepatocytes following interaction of the myristolated large HBsAg with the sodium-dependent taurocholate cotransporting polypeptide (NTCP).[10,11] Other host factors are also likely to be involved in uptake of virions. The viral genome, comprising partly double-stranded relaxed circular DNA (rcDNA), is released in the nucleus where it is "repaired" to form covalently closed circular DNA (cccDNA).[12] This replication intermediate serves as the template for transcription of viral pregenomic RNA (pgRNA) and protein-coding mRNAs. Viral gene expression is maintained by the X protein of HBV, which exerts its effects by modulating DNA methylation and modifying cccDNA-associated histones.[13–15] Arrangement of the open reading frames (ORFs) and regulatory elements within the cccDNA is remarkably compact (**Figure 2**). The four ORFs, *surface* (*S*), *polymerase* (*P*), *core* (*C*) and *X*, overlap with each other. Moreover, promoters, enhancers, and other regulatory elements are embedded in the ORFs. This multifunctional nature of the cccDNA limits its sequence plasticity. Consequently, emergence of escape mutants is fairly unusual, and HBV is susceptible to inactivation by engineered mutation.

Correspondence: Patrick Arbuthnot, Wits/SAMRC Antiviral Gene Therapy Research Unit, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, Private Bag 3, Wits 2050, South Africa. E-mail: Patrick.Arbuthnot@wits.ac.za

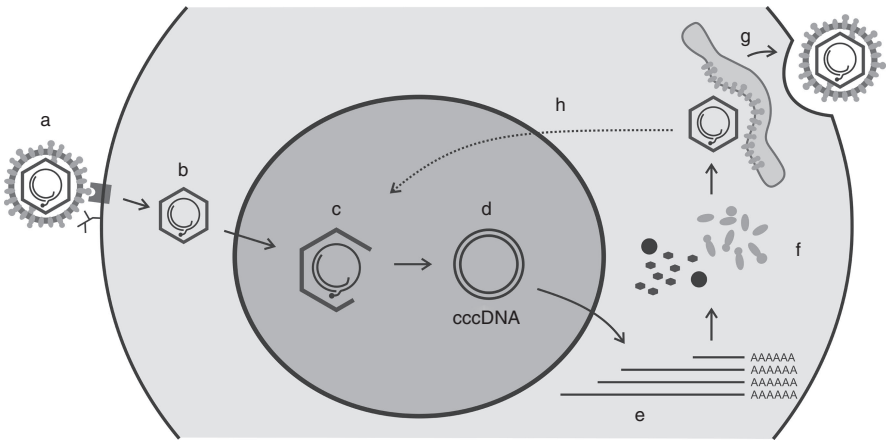

Figure 1 Schematic summary of the replication of HBV. (**a**) The small surface protein binds to glycosaminoglycans on the surface of hepatocytes. The virion attaches specifically and with high affinity to the sodium taurocholate cotransporting peptide receptor through interaction with the myristolated N-terminal region of the large surface protein. (**b**) After entry into the cytoplasm, probably by a process of endocytosis, the viral capsid containing relaxed circular DNA (rcDNA) is transported to the nucleus. (**c**) The rcDNA is released from the capsid and then (**d**) "repaired" to form covalently closed circular DNA (cccDNA). This stable and problematic replication intermediate is the target of strategies employing gene editing to disable HBV replication. (**e**) The cccDNA serves as template for transcription of viral pregenomic RNA (pgRNA) and protein-coding mRNAs. (**f**) Translation to form viral proteins leads to assembly of capsid particles containing packaged pgRNA, reverse transcription of this replication intermediate and (**g**) release via the secretory pathway. Licensed nucleoside and nucleotide analogs inhibit reverse transcription, and candidate therapeutic RNA interference activators disable viral RNAs. (**h**) Some rcDNA may be recycled from nascent core particles to generate cccDNA within the nucleus.

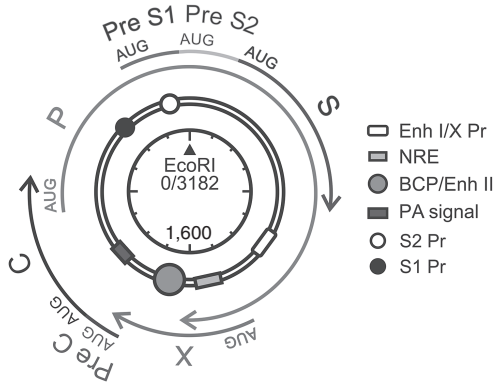

Figure 2 Arrangement of viral open reading frames and *cis* regulatory elements within the cccDNA of HBV. The cccDNA of HBV comprises approximately 3.2 kb. Nucleotide co-ordinates are typically calculated from the unique *Eco*RI site of the HBV DNA (top center). Approximate location of promoters, enhancers, and *cis* regulatory elements are indicated as circular and rectangular symbols on the cccDNA, which is shown as a double circle. The *core* (*C*), *polymerase* (*P*), *surface* (*S*), and *X* overlapping open reading frames are shown as arrows immediately surrounding the genome. Initiation of translation from the preS1 start codon generates the large surface antigen and the preS2 AUG begins synthesis of the middle HBsAg. The preC codon initiates formation of the secreted HBV e antigen (HBeAg).

cccDNA is very stable, and exists as a minichromosome in infected hepatocytes.[12] Difficulties with eradicating cccDNA have been the most important obstacle to developing an effective cure for HBV infection. Currently licensed treatments are capable of suppressing replication of HBV, but relapse following treatment withdrawal occurs frequently.[16] Ability to disable cccDNA permanently using gene-editing technology has therefore generated interest. A variety of gene-editing approaches is being developed to treat HBV infection and these are discussed below in more detail. Technologies include use of engineered clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated (Cas) systems, zinc finger nucleases (ZFNs), and transcription-activator-like effector (TALE) nucleases (TALENs). Homing endonucleases (HEs) have been investigated for antiviral therapeutic use, but complicated requirements for engineering these nucleases have restricted their widespread use. The rationale for employing designer nucleases to effect mutation is that repeated digestion of the target DNA, followed by error-prone repair mediated by the nonhomologous end joining pathway, eventually leads to mutation of the cccDNA. Epigenome editing, based on recruitment of transcriptional silencing elements to the cccDNA, is another approach that is being investigated and is discussed below. Dependence on use of an RNA guide to direct the Cas protein to cleave targets at specific sites is an advantage of the CRISPR/Cas system. These RNA-guided nucleases are simple to engineer and this has been the main reason for the popularity of CRISPR/Cas systems. Generating derivatives of zinc finger proteins (ZFPs) is complicated by the unpredictable context-dependent efficiency of the DNA-binding finger modules. Producing artificial gene editors containing sequences of TALEs is easier as the monomers interacting with each nucleotide are not influenced by neighboring sequences.

DIFFICULTIES WITH EVALUATING cccDNA INACTIVATION MEDIATED BY GENE EDITING

Advancing gene editing as a means of therapeutic inactivation of cccDNA has been hindered by the paucity of models that simulate natural HBV infection and difficulties with proving specificity of mutagenic effects on cccDNA. The problem of assessing therapeutic efficacy *in vivo* is further exacerbated by the inability of murine cells to generate cccDNA.[17] Probing viral sequences in rcDNA, integrated and free linear viral DNA, needs to be distinguished from analysis of cccDNA. Recent development of NTCP-expressing cells that are susceptible to infection by HBV[18] goes some way to enabling discrimination of an effect on

cccDNA. Many convenient and commonly used models of HBV replication do not depend on formation of cccDNA. For example, transient transfection of cultured liver-derived cells or murine hepatocytes *in vivo* supports production of HBV particles from replication-competent plasmids. Thus, although gene editors may cause inhibition of HBV replication in these models, the effect is a result of action on plasmid DNA and not cccDNA.

cccDNA is present at low concentrations in HBV-infected cells, and naturally the number of copies per cell is estimated to be less than 1.5.[19] Assays to measure cccDNA and evaluate induced mutation at intended target sites are difficult and sometimes unreliable.[12] Use of primers that flank the gap of rcDNA was initially thought to provide the means for specific amplification of cccDNA. However, this polymerase chain reaction (PCR)-based assay is indeed not specific for cccDNA. Presence of the duplicated sequence of the "flap" at the 5' end of the minus strand of rcDNA results in synthesis of sequences with overlapping complementary 3' ends during PCR.[12] The benchmark for identifying cccDNA is Southern blot hybridization, and radioactive probes are generally required to detect the viral replication intermediate with sufficient sensitivity. Southern blot hybridization is however not amenable to identifying and quantifying mutagenesis at target sites. PCR coupled to treatment with endonucleases, such as T7E1, and next-generation sequencing are better to evaluate target mutation. To separate cccDNA from other viral DNA, such as rcDNA, linear DNA, and viral sequences that have been integrated into the host's genome, Hirt extraction coupled to treatment with ATP-dependent DNase (plasmid-safe) has been employed.[20,21] With this approach, non-cccDNA should be degraded to leave cccDNA for amplification. Of course appropriate controls and standards need to be included to verify reliability of individual assays. Digestion with plasmid-safe DNase has a disadvantage in that nicked cccDNA may also be removed during the extraction procedure. As a result, existing cccDNA may be diminished and any real effect of gene editors on cccDNA may be underestimated. Despite difficulties with reliable assay of mutation in cccDNA, progress has been made with using gene editing to disrupt cccDNA by gene editors, and this is described below and in **Table 1**.

ENGINEERING ZFPS TO DISABLE HBV REPLICATION

In one of the first studies to describe anti-HBV utility of engineered DNA-binding proteins, Zimmerman *et al.*[22] reported on the efficacy of ZFPs targeted to the enhancer region of duck HBV (DHBV). Significant nontoxic reductions in the core antigen, pgRNA, total viral RNA, and surface antigen were induced by the ZFPs following transfection of cells in culture. As the ZFPs did not possess nuclease domains, their mechanism of action is likely to have resulted from obstructing access of transcription factors required for DHBV gene expression. A later study employed target-cleaving ZFNs to inhibit HBV replication.[23] Although there was evidence for digestion of target sequences, proof of mutation of cccDNA was not provided. Subsequently, another group generated ZFNs that were delivered using self-complementary adeno-associated viruses in a cell culture model of HBV replication.[24] The three ZFN pairs targeted *P/X*, *P/C*, and *P* sequences. All three ZFNs showed targeted disruption in cultured cells but one of the gene editors caused significant cytotoxicity. Inhibitory effects of the lead ZFNs were sustained and high-throughput sequencing indicated that the ZFNs acted specifically on HBV sequences. The HepAD38 cells that were used in this study replicate HBV in a controllable manner: the virus proliferates on withdrawal of doxycycline from the culture medium, subsequently establishing a pool of cccDNA molecules in the liver-derived cells.[25] Reintroduction of doxycycline prevents replication from integrated sequences and consequently HBV proliferation should only be driven by the established pool of cccDNA. Weber *et al.*[24] attempted to exploit this property of HepAD38 cells but were unfortunately unable to obtain reproducible evidence of ZFN-induced mutation of cccDNA.

Zhao *et al.*,[26] investigated the use of a ZFP-based artificial transcription factor (ATF) to inhibit function of the enhancer I region and diminish *X* expression. Two three-fingered ZFP motifs were linked to form a six-fingered DNA binding domain that recognized an 18 base pair sequence. To confer transcriptional repression characteristics, the ZFP was coupled to a Krüppel associated box (KRAB) domain. Evaluation showed that the ATF suppressed transcription specifically and caused cell cycle arrest specifically in cells containing integrated *X*.

ANTI-HBV TALE DERIVATIVES

The efficacy of anti-HBV TALENs was first demonstrated by Bloom *et al.*[21] in 2013. Four dimeric TALENs were designed to target within the *S* (S TALEN), *C* (C TALEN), and *P* (P1 TALEN and P2 TALEN) ORFs. Bioinformatic analysis revealed that the TALENs had few if any off-target binding sites within human and murine genomes. The S and C TALENs were the most effective and caused intended mutation in the stable HBV-producing HepG2.2.15 cell line. Analysis using a T7E1 assay, carried out on amplicons derived from putative cccDNA, revealed mutagenesis of up to 35 and 12% by the S and C TALENs respectively. Hydrodynamic injection (HDI) of mice, using plasmids encoding the TALENs and the HBV genome, was used to assess efficacy *in vivo*. With this model, the S and C TALENs reduced HBsAg levels by 90% and circulating viral particle equivalents (VPEs) by 70%. Targeted mutagenesis of plasmid HBV sequences by the S and C TALENs was between 58 and 87% respectively, and no associated cytotoxic effects were detected. Sequencing demonstrated that deletions were the commonest mutations. An investigation by Chen *et al.*[27] also provided evidence that TALENs are effective anti-HBV agents. In this study, three pairs of anti-HBV TALENs were designed against conserved regions within *C* (L1/R1 and L2/R2) and *P* (L3/R3) sequences of the HBV genome. As was previously reported,[21] the TALENs caused reductions in markers of viral replication in cell culture and following HDI of mice.[27] A decrease in the concentration of detectable putative cccDNA molecules, ranging from 10–50%, was observed following transfection of Huh7 cells with linear HBV-encoding DNA and plasmids expressing *C*-targeting TALENs. HDI-mediated transfection with similar sequences showed diminished hepatic HBV pgRNA and reduced serum concentrations of HBV e antigen, HBsAg, and VPEs in mice receiving the TALEN-encoding sequences. The authors analyzed target sequences from various HBV subtypes, and showed that efficacy of the TALENs was good across different viral isolates. A synergistic effect when the TALENs were coadministered with IFN-α was also demonstrated.

Table 1 Summary of selected studies using gene editing to disable HBV replication

Type of gene editor	Most effective viral target sites	Models used to evaluate efficacy	Efficacy		Evidence for efficacy against cccDNA	Reference
			Cultured cells	*In vivo*		
ZFN	*C, P*	ZFN-encoding sequences delivered with AAVs to HepAD38 cells in culture	Sustained complete inhibition of HBV replication. Little if any off-target effects	Not assessed	The question of cccDNA mutation was addressed, but reproducible evidence was difficult to obtain	Weber *et al.*[24]
TALEN	*S, C*	Cell culture. Murine hydrodynamic injection	Significant decrease of markers of viral replication in stable HBV-producing cells	HBsAg decreased by 90% and circulating VPEs by 90%	Mutagenesis of Hirt and plasmid-safe-treated DNA extracted from HepG2.2.15 cells	Bloom *et al.*[21]
TALEN	*C, P*	Cell culture. Murine hydrodynamic injection	Significant decrease of markers of viral replication in transiently transfected Huh7 cells	Significant decrease of serum HBeAg, HBsAg, VPEs and hepatic pgRNA	Mutagenesis of Hirt and plasmid-safe treated DNA extracted from Huh7 cells. (Target HBV DNA transfected as linear duplex)	Chen *et al.*[27]
CRISPR/Cas	*X, P*	Transient transfection of cultured cells. Murine hydrodynamic injection	Significant decrease of markers of viral replication in transfected cells. Efficacy good against different viral genotypes. Synergistic effect of two sgRNAs used in combination	Significant decrease of markers of viral replication. Decrease in intrahepatic plasmid DNA-containing HBV sequences	None provided	Lin *et al.*[31]
CRISPR/Cas	*X, C*	Stable and transiently transfected HBV-producing cells. Murine hydrodynamic injection to produce rcccDNA	Good inhibition of markers of viral replication and effective against different viral genotypes	Significant decrease of markers of viral replication: serum HBsAg, HBeAg and hepatic viral core protein	Reduction of rcccDNA and targeted mutation of 34–44%	Dong *et al.*[38]
CRISPR/Cas	*P, X, C*	Lentiviral vectors used to transduce cultured HBV-producing cells with CRISPR/Cas. Murine hydrodynamic injection	Good inhibition of secretion of HBsAg and decreased intracellular viral pgRNA	Significant decrease of markers of serum HBsAg and circulating VPEs	Reduction of cccDNA demonstrated using Southern blot hybridization	Ramanan *et al.*[39]

AAV, adeno-associated virus; Cas, CRISPR-associated; cccDNA, covalently closed circular DNA; CRISPR, clustered regularly interspaced short palindromic repeats; HBsAg, hepatitis B virus surface antigen; HBeAg, hepatitis B virus e antigen; pgRNA, pregenomic RNA; rcccDNA, recombinant cccDNA; TALEN, transcription activator-like effector nuclease; VPEs, viral particle equivalents; ZFN, zinc finger nuclease; *C*, HBV *core* DNA; *P*, HBV *polymerase* DNA; *S*, HBV *surface* DNA; *X*, HBV *X* DNA.

USE OF CRISPR/CAS9 TO MUTATE HBV DNA

Since the first descriptions of how CRISPR/Cas9 may be employed for gene editing,[28–30] numerous reports have shown the versatility of the system. The facile nature of CRISPR/Cas design has led to this technology becoming the most commonly utilized genome engineering approach. Application as a therapeutic to limit viral infections was immediately evident, and of course utility as a cure for chronic HBV infection has been investigated. Lin *et al.*[31] were the first to demonstrate the feasibility of CRISPR/Cas-mediated inhibition of HBV replication in cultured mammalian cells and mice following HDI. Eight small guide RNAs (sgRNAs) were designed against regions of the HBV genome that encompassed all four ORFs. The authors showed suppression of markers of viral replication in cultured cells and *in vivo*. Zhen *et al.*[32] provided further support for the utility of CRISPR/Cas by demonstrating suppression of viral replication in HBV-producing HepG2.2.15 cells and in HBV transgenic mice. In addition to having a mutagenic effect, the engineered nucleases appeared to be capable of destroying the target plasmids.[31]

A useful feature of the CRISPR/Cas system is that it is readily amenable to targeting of multiple viral sequences. Lin *et al.*[31] also demonstrated that combining two of the most effective sgRNAs caused a greater rate of mutation in viral targets than did each individual guide. Wang *et al.*[33] also employed a pair of sgRNAs to target two sites on the HBV genome simultaneously and demonstrated good efficacy in transfected cells. In a subsequent study, Seeger and Sohn assessed CRISPR/Cas-mediated targeting of HBV in an infectable HepG2-derived cell line stably expressing NTCP.[34] These cells were transduced using lentiviruses expressing sgRNAs and Cas9 under the inducible control of doxycycline, and then infected with HBV. Since this model depends on cccDNA formation for viral replication, the authors could verify that the CRISPR/Cas system is capable of targeting this crucial viral replication intermediate. Sequencing revealed single-nucleotide indels

as well as large deletions of up to 2,301 nucleotides in length. These findings were corroborated by Kennedy *et al.*[35] who used HepAD38 cells and the HBV-infectable HepaRG line. Reductions in cccDNA of up to 100-fold were observed after lentivirus-mediated delivery of Cas9 and a sgRNA targeted to sequences encoding the YMDD motif of the *P* ORF. This result is significant as it supported the notion that cccDNA targeted by CRISPR/Cas could be destroyed, as was reported by Lin *et al.*[31] Sequence analysis confirmed extensive targeted mutation of a large proportion of the remaining viral DNA. Evidence for CRISPR/Cas-mediated degradation of target DNA was provided in another study, which demonstrated reduction in concentrations of plasmid DNA containing HBV sequences.[36] The authors posited that nonhomologous end joining repairs cleaved viral DNA inefficiently, which leads to degradation of the target and the significant drop in viral replication. Although destruction of viral targets by the nucleases is desirable, an immune response to the Cas protein may play a role in eliminating viral sequences.

Efficacy of the CRISPR/Cas system was also assessed using the recently described recombinant cccDNA (rcccDNA) model. This model employs a plasmid (prcccDNA) containing a full length HBV sequence with flanking *loxP* sites.[37] Removal of plasmid sequences by Cre-recombinase and intramolecular ligation of HBV sequences leads to formation of replication-competent rcccDNA. HDI of prcccDNA with a vector expressing Cre-recombinase enables replication *in vivo* from the cccDNA-like intermediate. Using this model, Dong *et al.*[38] demonstrated disruption by CRISPR/Cas of rcccDNA *in vivo*.

A recent study reported on efficacy of CRISPR/Cas against clinical isolates of HBV.[39] Efficient inhibition of viral replication and reduction of cccDNA in infected cells in culture were demonstrated following administration of a panel of sgRNAs. Lentiviruses were used to deliver the sgRNA/Cas9 cassettes and the transduction achieved long-term expression of the components of the anti-HBV nucleases. Next-generation sequencing revealed no mutations at sequences predicted to be likely nonspecific targets. Although impressive, it may be premature to conclude that CRISPR/Cas directed against viral sequences is devoid of off-target effects. Limiting off-target effects by using nickase Cas9 (Cas9n) against HBV has also been investigated.[40,41] As Cas9n cuts only one strand, specificity is improved by a requirement for two juxtaposed but offset guides to introduce a double-strand break at target DNA. Karimova *et al.*[42] evaluated the use of a Cas9n in conjunction with sgRNAs targeted against the *S* and *X* ORFs of HBV. Although the study demonstrated efficient silencing of viral replication there was no data on off-target effects using the Cas9n system.

CONCLUSIONS AND PROSPECTS OF THERAPEUTIC GENE EDITING TO TREAT CHRONIC HBV INFECTION

Gene editing has justifiably attracted attention for its potential to provide the means to achieve cure from infection with HBV. ZFNs, TALENs, and CRISPR/Cas derivatives have all been used with some success to inactivate viral DNA. Despite the enthusiasm, several challenges need to be met before the technology is applied in a clinical setting. One such challenge is validation of efficacy *in vivo* using models of HBV infection that accurately simulate replication of the virus in humans. Woodchucks infected with woodchuck hepatitis virus (WHV) and xenografted uPA-SCID mice infected with HBV reproduce all stages of hepadnavirus replication, but have some limitations. uPA/SCID mice are difficult to work with and their immunocompromised state means that the contributions of innate or adaptive immune responses to anti-HBV efficacy of gene editors are not assessed. In the case of WHV infection, the virus has some distinguishing genetic and immunological features that may make extrapolation to the human condition of HBV infection unreliable. Nevertheless assessing efficacy in these models will be important to inform utility of gene editors as inactivators of cccDNA. Ensuring specificity of action, efficient delivery, attenuation of an immune response to the gene editors and improving insights into the basic biology of cccDNA dynamics are other challenges that are crucial to advancing therapeutic gene editing for HBV infection. It is also not yet established whether gene editing alone will be capable of eliminating HBV infection. Since a poor immunological response to HBV infection is central to failure of chronic carriers to eliminate the virus, augmenting anti-HBV immunity may well be required to enhance efficacy of gene editing. However, improving innate, cell-mediated and humoral immune responses to the virus are not easily achieved. Recently described strategies, such as Toll-like receptor 7 stimulation[43] and IFN-induced degradation of cccDNA by APOBEC3A and 3B,[44] are interesting and may be amenable to synergistic combination with antiviral gene editing.

Another obstacle to use of gene-editing technologies may be the emergence of viral escape mutants. Such mutants could arise following selection or may be induced *de novo* by gene editors themselves. However, as HBV exhibits limited sequence plasticity this scenario is less likely to occur than it is with other viruses such as HIV-1. Schiffer *et al.*[45] developed mathematical models to simulate selection of viral mutants following action of nucleases that targeted cccDNA. The models predicted that simultaneous or sequential targeting of different regions of the cccDNA should prevent emergence of endonuclease resistance. They also showed that the dynamics of cccDNA persistence are unlikely to influence the probability of cure from HBV infection, as long as antiviral therapy is administered concurrently. Although not yet shown to be the case with HBV, emergence of nuclease-induced replication-competent mutants has been demonstrated using models of HIV-1 replication.[46] This was displayed after administration of ZFNs that targeted the reverse transcriptase (RT) domain of the HIV-1 *pol* gene. However, introducing mutations at other regions of the same mutant HIV-1 abrogated replication, which highlights the importance of simultaneously targeting different viral sequences when escape is a concern.

Ensuring specificity without unintended mutation in the host genome is vital to the ultimate success of therapeutic gene editing. Problematic off-target mutagenesis by CRISPR/Cas has successfully been addressed by rational modification of Cas proteins,[47] use of Cas9n[40,41] and shortening of sgRNAs. A recent study has shown that engineering sites of Cas9 that make contact with target DNA may be employed to reduce nonspecific effects. A quadruple substitution variant, termed SpCas9-HF1, had on-target activity that was similar to that of the wild-type Cas9 while off-target mutagenesis was reduced to undetectable levels.[48] Evidence

indicates that TALENs have good specificity for cognates, which compares favorably to that of ZFNs.[49,50] Although progress aimed at minimizing off-target effects is promising, sensitive detailed characterization of unintended mutation is difficult. Rare small sequence changes, which may only occur in a minority of treated cells, are difficult to detect. Progress with next-generation deep sequencing and droplet digital PCR will be important to facilitate assessment of this aspect of the safety of therapeutic gene editing. Although computer-based prediction and subsequent interrogation of potential off-target sites is important for the characterization of off-target effects, the risk of overlooking some sites always exists. Tsai *et al.*[51] recently described a novel technique, termed GUIDE-seq, to detect genome-wide cleavage events induced by CRISPR/Cas nucleases. Approaches such as these eliminate bias associated with target prediction and may ultimately be used to assess off-target effects more accurately.

Another potentially important consideration for use of engineered nucleases against HBV is that the gene editors may act on viral integrants. Introducing double-strand breaks at sites of the host genome may predispose to chromosomal instability and translocation. Induction of epigenetic changes to cccDNA by promoting DNA methylation and histone modification should overcome this problem. Utility of repression as a mechanism of silencing HBV was demonstrated through use of ZFPs[22,26] and alluded to by demonstrating efficacy of an enzymatically inactive TALEN.[21] A TALEN targeting *P* suppressed markers of viral replication without evidence of causing mutation.[21] Coupling a repressor, such as the KRAB domain, to the TALE or ZFPs should improve durability of inhibition of transcription from the viral targets.

As with most gene therapies, achieving efficient delivery of the therapeutic effectors is essential. To advance gene editing to treat HBV infection, vectors that are economical to produce in large scale, free from side effects and efficiently hepatotropic will be required. Ensuring that the gene editors reach all HBV-infected cells and act on cccDNA molecules will be a significant achievement. The issue is exacerbated by large size of nucleases and a need for delivering two components making up the ZFNs, TALENs and CRISPR/Cas derivatives for them to act on their targets. In the long term, it seems likely that synthetic nonviral vectors (NVVs) will be preferable to recombinant viral vectors for application to treatment of HBV infection. Suitability of NVVs for large-scale preparation, amenability to chemical modification to alter biological properties and compatibility with mRNA delivery are advantageous for clinical application. Advances that are being made in research on basic molecular biology of HBV and other topics of gene therapy are likely to facilitate progress towards curative gene editing for chronic HBV infection. The field is now at an interesting stage and progress in the immediate future is enthusiastically awaited.

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