REVIEW ARTICLE

Prospects of viral vector-mediated delivery of sequences encoding anti-HBV designer endonucleases

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Available treatment for chronic hepatitis B virus (HBV) infection offers modest functional curative efficacy. The viral replicative intermediate comprising covalently closed circular DNA (cccDNA) is responsible for persistent chronic HBV infection. Hence, current efforts have focused on developing therapies that disable cccDNA. Employing gene editing tools has emerged as an attractive strategy, with the end goal of establishing permanently inactivated cccDNA. Although anti-HBV designer nucleases are effective in vivo, none has yet progressed to clinical trial. Lack of safe and efficient delivery systems remains the limiting factor. Several vectors may be used to deliver anti-HBV gene editor-encoding sequences, with viral vectors being at the forefront. Despite the challenges associated with packaging large gene editor-encoding sequences into viral vectors, advancement in the field is overcoming such limitations. Translation of viral vector-mediated gene editing against HBV to clinical application is within reach. This review discusses the prospects of delivering HBV targeted designer nucleases using viral vectors.

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INTRODUCTION

Since its discovery in 1965, tremendous efforts have been made to control or eliminate Hepatitis B virus (HBV) infection. Despite this large body of work, approximately 1.5 million new infections still occur annually [1]. HBV is a highly transmissible pathogen that results in acute and chronic infections. Progression of the viral infection is determined by multiple viral and host factors. HBV chronicity is primarily mediated by: (i) the unique replication mechanism that results in the formation of a highly stable covalently closed circular DNA (cccDNA), and (ii) inability of the immune system to resolve the infection in chronic carriers of the virus [2–4].

The 3.2 kb HBV genome is organized in a remarkably compact arrangement, where all proteins are encoded by four overlapping open reading frames (ORFs). These ORFs comprise the surface (*S*), polymerase (*P*), core (*C*) and X (*X*) sequences. Additionally, the genome includes six start codons, four distinct promoters (pS1, pS2, pC, pX), two enhancers (Enh1 and Enh2) and other regulatory elements. All transcripts are generated from the negative strand of cccDNA and are similarly oriented. The viral transcripts are translated into seven functional proteins [5].

The sodium-taurocholate co-transporting polypeptide (NTCP) receptor and epidermal growth factor receptor are used by the hepatitis B virion to gain entry into hepatocytes [6, 7]. The mechanism by which HBV is endocytosed following viral entry is still poorly characterized [8]. After entry, nucleocapsids are transported to the nucleus, where they break down to release the relaxed-circular DNA (rcDNA) genome into the nucleus. The partly double-stranded rcDNA undergoes several processing steps and is "repaired" by host factors to form cccDNA. cccDNA associates with histone and non-histone proteins to create a highly stable minichromosome. In addition to protein-coding mRNAs, cccDNA encodes pre-genomic RNA (pgRNA), which is exported to the cytoplasm where it is incorporated into capsids. pgRNA is then reverse transcribed by the viral polymerase in a multistep process to form rcDNA. Nucleocapsids are enveloped and then secreted from the hepatocyte as mature virions. Alternatively, nucleocapsids may be recycled to the nucleus to provide an additional source of the cccDNA precursor. Even in the absence of observable viremia, the cccDNA reservoir may be maintained over long periods and this property limits efficacy of available therapeutic options [9–11].

Established techniques used to assess HBV infection in a clinical setting include quantitation of HBV DNA, hepatitis B surface antigen (HBsAg), circulating HBV core antigen (HBcAg), hepatitis B core-related antigen (HBcrAg) and HBV RNA. In some instances liver damage is assessed using alanine aminotransferase and aspartate transaminase (ALT and AST) [12–15]. Quantification of HBV cccDNA is challenging, owing to specific features of the viral genome e.g., low copy number relative to replication intermediates and the fact that replicative intermediates (relaxed-circular DNA, double-stranded linear DNA, and single stranded DNA) have identical sequences to cccDNA [16, 17]. A variety of methodologies for cccDNA detection and quantification, including Southern blot, various PCR-based quantitation, in situ hybridization and invader assays, has been employed with varying success [18]. However, there is still no universal method for detection and quantification of cccDNA, especially in samples from patients, that has been widely accepted.

The current goal of HBV therapy is to achieve a "functional cure" which is characterized by a sustained loss of serum HBsAg and undetectable levels of circulating HBV DNA with or without

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cccDNA elimination [19]. However, the ultimate goal is a "sterilizing cure", which would be achieved when the virus is purged and cccDNA reservoirs are eradicated [20]. Presently there are two main types of licensed HBV therapies approved by the U.S. Food and Drug Administration (FDA). These are: (i) interferon (IFN) alpha derivatives (standard IFN-α and pegylated IFN-α) which have several antiviral actions that include immunomodulation, and (ii) five oral nucleoside/nucleotide analogues (NAs) (lamivudine, entecavir, telbivudine, tenofovir and adefovirdipivoxil). The NAs suppress viral replication by inhibiting reverse transcription of HBV pgRNA. The standard first line monotherapies include PEGylated IFN-α and NAs [21]. Although these compounds can control infection and alleviate progression to more severe disease, they rarely eliminate the cccDNA, and relapse following treatment withdrawal is common. Several direct- or indirect-acting novel small molecule therapies that target various steps of the viral replication cycle, such as entry inhibitors and capsid assembly modulators are in clinical testing [22–24].

In recent years, advancing therapies based on engineered nucleases that disable cccDNA has gained momentum, and therapeutic application against HBV shows promise. Although engineered Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the RNA-guided clustered regulatory interspaced short palindromic repeats (CRISPR) and CRISPR associated (Cas) protein based therapies have shown efficacy in preclinical evaluations [25–28], progress to clinical testing has not been achieved. Finding a safe and efficient system to deliver these gene editors remains a challenge, and viral vector-based delivery systems potentially offer a solution. Recent advances have resulted in development of viral vectors with good liver tropism, which are suited for treatment of HBV infection. High packaging capacity to accommodate gene editor expression cassettes and good safety profiles are additional positive features.

ENGINEERED NUCLEASES AGAINST HBV

Feasibility of inducing targeted genomic changes was first demonstrated in yeast and mice in the 1980s [29]. More recently, gene editing technologies have been improved by development of designer nucleases that are capable of inducing double-strand breaks (DSBs) at specific target sites. Host DNA repair machinery is then responsible for repairing DSBs by error prone non-homologous end joining (NHEJ) or homology directed repair (HDR) if a donor sequence is present [30, 31]. In the case of HBV infection, targeted cccDNA cleavage with subsequent NHEJ is exploited to disrupt viral gene expression [32]. ZFNs, TALENs and CRISPR/Cas 9 nucleases currently spearhead the field of gene editing and have been applied to disabling HBV cccDNA.

ZFNs comprise a DNA-binding zinc finger protein domain fused to a sequence-independent endonuclease, typically of the *Fok*I type IIS restriction enzyme. ZFNs work in pairs by binding to opposite strands of DNA flanking the target site to induce DSBs. Engineered zinc finger proteins are usually made up of three fingers and each finger recognizes three nucleotides [33–35]. ZFNs are however limited in that the α-helix of one finger determines the orientation of the α-helix side chains in the remaining fingers which affects the overall DNA-binding efficiency [35].

Cradick et al. were among the first to use ZFNs against HBV [36]. Although their study demonstrated inhibition of viral replication markers in cultured cells, no evidence of targeted mutagenesis against cccDNA was reported. Another study reported an anti-HBV ZFN with improved inhibition of HBV replication and higher mutation rates, but was shown to be cytotoxic in cultured cells [27]. Despite disappointing results against HBV, ZFNs were successfully used in a phase I clinical trial to assess action against human immunodeficiency virus-1 (HIV-1) -infected patients (ClinicalTrials.gov, NCT00842634) [37].

Analogous to ZFNs, TALENs also comprise a DNA-binding domain tethered to the nuclease domain of *Fok*I [38]. In the case of TALENs, the DNA-binding domain is a transcription activator-like effector (TALE) [39]. TALEs typically contain 17.5 tandem repeats, each comprising 34 amino acids. Amino acid positions 12 and 13, referred to as repeat variable diresidues, recognize and bind single nucleotides of the target DNA. TALENs, like ZFNs also function as dimers to cleave both DNA strands at targeted sequences. TALEN design is however more versatile than ZFNs, because neighboring monomers of the TALE domain do not interact with each other and therefore do not affect binding efficiency [40–42].

TALEN technology is currently in clinical trials for treatment of various blood cancers [43], but is not yet being assessed in humans for treatment of HBV infection. The first reported study using anti-HBV TALENs against the *C*, *S* and *P* ORFs resulted in >90% hepatitis B surface antigen (HBsAg) knockdown in vivo and demonstrated targeted disruption of HBV DNA of up to 87% [25]. This was confirmed by T7 endonuclease 1 (T7E1) and next-generation sequencing (NGS). Chen et al. subsequently designed TALENs targeting the *C* ORF and demonstrated significant knockdown of viral replication markers and also targeted disruption of cccDNA in cultured cells [44]. TALENs containing wildtype *Fok*I domains, referred to as first generation TALENs, can result in unwanted homodimeric binding and targeting which may lead to off-target effects. A recent study focused on using second generation TALENs with engineered *Fok*I domains to ensure binding and targeting of HBV DNA as obligate heterodimeric pairs [26]. Second generation TALENs were reported to be just as effective at targeting and suppressing viral replication as those reported on in earlier studies. More impressively, second generation TALENs demonstrated better target specificity. This was supported by analysis of mutations at a predicted off-target site located in the intronic region of the *phenylalanine hydroxylase* gene. The second generation TALENs caused fewer mutations at this sequence when compared to first generation TALENs, and highlights the importance of targeted specificity [26].

Unlike ZFNs and TALENs, which depend on protein-DNA recognition of specific targets, CRISPR/Cas is an RNA-guided nuclease. Naturally the CRISPR loci, found in various bacteria and Archea, express CRISPR RNAs (crRNAs) that associate with trans-activating crRNAs (tracrRNAs) to guide the Cas nuclease to complementary DNA for targeted cleavage. Type II Cas nuclease 9 (Cas 9) from *Streptococcus pyogenes* (*S. pyogenes*) or *Staphylococcus aureus* (*S. aureus*) is the most commonly used nucleases [45, 46]. To simplify application to gene editing, crRNA and tracrRNA are typically combined to produce a single guide RNA (gRNA). The only requirement for directed cleavage is the presence of a protospacer adjacent motif (PAM) sequence upstream from the target sequence recognized by Cas 9. gRNAs function by binding to target sites downstream of the PAM which then signal Cas nuclease to cleave at the target site [47–49].

Multiple studies demonstrated use of CRISPR/Cas 9 to achieve targeted disruption of cccDNA in cell culture [50–52]. An impressive study by Seeger and Sohn demonstrated efficient targeting and disruption of cccDNA using gRNAs spanning Enh2 and the pre-core regions of the HBV genome [53]. This region encodes the HBV X protein (HBx) which is required for transcription of pre-core and core mRNA from cccDNA templates [54]. Effective targeting in a liver-derived HepG2 cell line expressing NTCP (HepG2-hNTCP) infected with HBV, resulted in truncated *HBx* which inhibited HBV replication up to eight-fold. A subsequent study by Wang et al. demonstrated that dual gRNAs targeted against *C*, *S*, *P*, and *X* ORFs across HBV genotypes A-D efficiently reduced HBsAg or HBeAg as well as cccDNA reservoirs. Furthermore, dual gRNAs were found to be more efficient compared to single gRNAs [55].

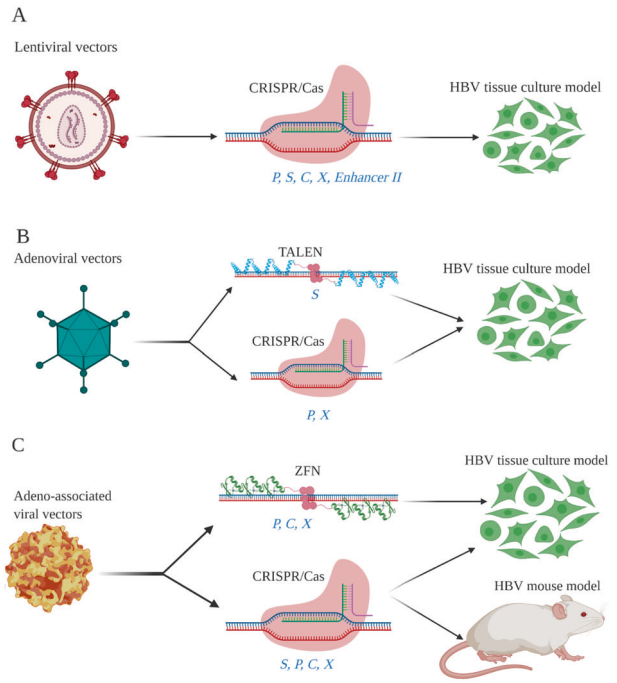

Fig. 1 Viral vectors expressing anti-HBV gene editing nucleases. **A** Lentiviral vectors expressing anti-HBV clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated (Cas) sequences targeting *P*, S, *C*, *X*, and *Enhancer II* sequences were characterized in HBV tissue culture model. **B** Adenoviral vectors expressing transcription activator-like effector nucleases (TALENs) targeting the *S* ORF or the CRISPR/Cas targeting *P* or *X* ORF were characterized in HBV tissue culture model. **C** Adeno-associated viral vectors have been used to deliver *P*, *C* or *X* specific Zinc finger nucleases (ZFNs) in HBV tissue culture model or CRISPR/Cas against *S*, *P*, *C* or *X* ORF in HBV tissue culture model and mouse model of HBV replication. In blue is the HBV targets (Created with Biorender.com).

Using CRISPR/Cas 9, Lin et al. were the first to demonstrate targeted cleavage of HBV DNA in vivo [48]. Mice were given an HBV-replicating plasmid as well as CRISPR/Cas 9 expression vectors by hydrodynamic injection (HDI). Targeted cleavage reduced serum levels of HBsAg and intrahepatic HBV templates in vivo. Several other studies have also shown significant HBV viral inhibition when using similar HDI murine models [50, 56–58]. Although these studies demonstrate the efficacy of CRISPR/Cas 9 against HBV DNA in vivo, HDI models do not result in HBV infection or production of cccDNA. Humanized mice can produce cccDNA and CRISPR/Cas 9 has been studied using these models (see below). Recently CRISPR/Cas-derived base editors were developed to inactivate target genes without cleavage of DNA. In principle, the approach is based on use of catalytically inactive Cas 9, commonly referred to as dead Cas 9 (dCas 9), which is tethered to a base-modifying enzyme. Two classes of base editors have been well characterized as either cytosine base editors (CBEs) which converts C · G to T · A or adenine base editors (ABEs) which convert A · T to G · C [59]. A study by Yang et al. developed CRISPR/Cas 9 tethered to enhanced CBEs and demonstrated successful introduction of point mutations in cccDNA in an in vitro model [60].

Although CRISPR/Cas 9 has lately been at the forefront of gene editing applications, it has also been associated with off-target cleavage [61–63]. To enhance targeted cleavage, Cas 9 nickases (Cas9ns) were developed to act as dimers [64]. In principle, two Cas9ns in juxtaposition on opposite strands of DNA should improve specificity of target mutation. This approach has led to impressive rates of HBV inhibition and fewer off-target effects have been reported [65–67].

VIRAL VECTOR-MEDIATED DELIVERY OF ANTI-HBV GENE EDITING NUCLEASES

Successful gene therapy requires safe, stable and efficient transfer of nucleic acids to target cells. Viruses possess unique characteristics that can be harnessed to deliver therapeutic sequences. However, the suitability of a viral vector for any given application depends on multiple factors. These include tropism, packaging capacity and potential for host genome integration. Nevertheless, availability of diverse viruses and viral serotypes provides versatility to the viral vector toolbox. Lentiviral vectors (LVs), Adenoviral vectors (AdVs) and Adeno-associated viral vectors (AAVs) are popular and have been widely used.

Lentiviral vectors

Well characterized LVs are derived from HIV-1. In addition to the *cis*-acting elements, the 9.2–9.6 kb HIV-1 genome encode structural, enzymatic, regulatory and accessory proteins. After entering the target cell, single stranded genomic RNA is released and reverse transcribed to form double-stranded DNA (dsDNA), which is then integrated into host DNA as a provirus [68]. LVs are grouped in three generations based on viral genes and number of plasmids used for viral production. All LVs are devoid of viral genes, and only the non-coding *cis*-acting elements remain in the vectors. LVs are produced using a combination of plasmids carrying the transgene and *cis*-acting elements (vector genome plasmid) or the envelope gene (usually the vesicular stomatitis virus G protein (VSV-G) encoding gene, thus pseudotyping the vectors, Env plasmid) or other viral genes (packaging plasmid) [69].

There is a paucity of studies describing use of LVs to deliver anti-HBV ZFNs and TALENs, but use of LVs to deliver CRISPR/Cas-based HBV-targeting editors to cultured cells is well documented (Fig. 1). LVs encoding Cas 9 and four gRNAs targeted to the basic core promoter (BCP/ Enh2) regions and pre-core regions of the HBV genome resulted in cleavage of the HBV DNA and a 10-fold decrease in HBcAg expression [53]. A similar study using three gRNAs targeting the *P*, *S,* and *C* regions also reported reduced levels of both intracellular and secreted HBV DNA levels. The *P*-specific gRNAs suppressed cccDNA formation by approximately 10- fold. [51]. Subsequently, another study reported transduction of HepG2 cells containing stably integrated replicating HBV DNA (HepG2.2.15) with LVs. The vectors encoding Cas 9 and gRNAs against conserved regions within the *S, C, P*, and *X* ORFs induced efficient cccDNA mutagenesis and significant decreases in HBV DNA [70].

Adenoviral vectors

In transduced cells, the 26–45 kb linear dsDNA adenoviral genome predominantly exists as an episome. Ad serotype 5 species C (Ad5), with a 36 kb genome, is most commonly used as a gene delivery vector. High liver tropism by Ad5 makes it attractive for delivering anti-HBV sequences, and in vitro and in vivo studies have demonstrated good potencies against HBV [71–74]. AdVs are divided into first generation, second generation and third generation adenoviral vectors. Residual viral gene expression in the first- and second generation vectors may induce T-cell responses and clearance of transduced cells. Temporary expression is desirable to limit off-target activities, which occur more commonly when expression of gene editors is sustained [75, 76]. Although smaller gene editors can be delivered with first and second generation AdVs, these vectors may not have adequate capacity for co-delivering subunit pairs or multiple gene editors, which may be required to limit emergence of HBV resistance [77]. Third generation AdVs, also termed gutless/high capacity/ helper-

dependent adenoviral vectors (HdAdVs), offer a great potential for delivery of bigger or multiple gene editor cassettes. Expression of all essential viral genes in a producer cell line is toxic, hence production of HdAdVs requires use of helper adenoviruses [73, 74]. The current body of literature on use of AdVs to deliver HBV-targeting gene editors is small, however useful information can be derived from studying application of these vectors against other diseases or viral infections. A summary of reported work using AdVs to deliver HBV-targeting gene editors is summarized in Fig. 1 and described below.

AdVs have demonstrated highly efficient delivery of TALENs to mediate targeted gene disruption in human cells [78, 79]. Holkers et al. compared AdV to LV delivery of TALENs. Following transduction of cells with LVs, DNA analysis revealed heterogeneous populations of vector genomes with truncations within the repetitive TALE regions. Interestingly, first and second generation AdVs delivered full length genomes and achieved significant disruption of the target locus [80]. A recent study used HdAdVs to deliver sequences encoding TALEN subunits targeting the *S* ORF in one vector [28]. However, the study showed that HBV gene expression was not affected after treatment of HBV-infected HepG2-hNTCP cells. When delivered as plasmids, mutation rates of up to 7.3% were observed, suggesting that TALEN expression in the context of HdAdVs was compromised.

Application of AdVs to delivery of CRISPR/Cas system sequences has been extensively explored in recent years, and several studies have shown therapeutic potential [76, 78, 81, 82]. Schiwon et al. demonstrated the feasibility of targeting HBV using HdAdV carrying CRISPR/Cas 9 and three gRNA sequences targeting *P* and *X* ORFs [28]. The approach reduced HBV gene expression and replication markers by up to 78% in HBV-infected HepG2-hNTCP cells. A recent study demonstrated stable expression of 8 gRNAs from a first generation AdV. Co-infection of this vector with an AdV expressing Cas 9 resulted in up to 92% *HBx* deletion efficiency in culture [83].

Although publications reporting the potential of AdVs to deliver anti-HBV ZFNs are limited, there is a body of work describing strategies to target HIV-1 using serotype 5 AdVs to transduce $CD4^+$ T-cells [79, 84–86]. Results from these studies, albeit ex vivo, provide good evidence that AdVs are a valuable tool for delivering ZFNs against chronic viral infections. The approach employing these ZFN-expressing AdVs to mutate CCR5 in $CD4^+$ T cells has progressed to clinical testing, and was the first clinical application of designer nucleases to treat a viral infection (NCT00842634) [37].

Adeno-associated viral vectors

Data from clinical trials have shown that AAVs are safe and efficient gene therapy vectors. AAVs have also been used successfully to deliver anti-HBV nucleases to cultured cells and live animals [87, 88]. Of the 12 currently identified AAV serotypes, AAV-2 is the most extensively studied. The 4.7 kb single stranded linear DNA AAV genome is flanked by two inverted terminal repeats (ITRs) that play an important role in replication and packaging of the AAV genome. The genome contains *rep* and *cap* genes encoding replication and structural proteins, respectively. *rep* and *cap* genes are typically eliminated in AAV-derived vectors but transgene-flanking ITRs are retained. Functions of *rep* and *cap* are provided in trans during vector production. Production of AAVs also requires helper functions of Ad genes, namely *E1A, E1B, E2*, and *E4* [89].

There are two types of AAVs that are most used: single stranded AAVs (ssAAVs) and self-complementary AAVs (scAAVs). Transgene expression from scAAVs occurs more quickly than from ssAAVs. The structure of the genome of scAAVs is such that the DNA folds back on itself to form a dsDNA-like structure, which is amenable to transcription. Conversely, ssAAVs genomes require conversion to dsDNA before transcription can occur. Another important difference between these AAVs is their transgene capacity: ssAAVs may accommodate transgenes of up to 4.7 kb whereas the capacity of scAAVs is half of that (2.35 kb) [89, 90]. The success of using AAVs for liver-targeted delivery of gene editing nucleases has been demonstrated by transducing cells with sequences encoding ZFNs and a corrective donor sequence in adult mice [91]. In this study, stable expression of human factor IX was achieved in the murine model. A subsequent study co-transduced two scAAVs, each expressing one anti-HBV ZFN subunit in cultured cells. ZFNs against HBV *P*, *C*, or *X* genes resulted in up to 38% on-target mutation rates [27]. As a result of the small DNA packaging capacity of AAVs, delivery of TALENs with AAVs has not yet been achieved. Two-component strategies, where a pair of ssAAVs carry each TALEN subunit expression cassette, are under investigation. In a study conducted by Scott et al, sequences encoding the smaller Cas 9 protein derived from *Staphylococcus aureus* (saCas 9) together with gRNA cassettes were incorporated into an "all-in-one" ssAAV vector [92]. A gRNA directed towards the *S* ORF resulted in efficient inactivation of HBV replication and mutation of cccDNA in HepG2-hNTCP cells. Off-target analysis did not reveal non-specific mutagenesis. In a similar study, three gRNAs targeting different regions of the HBV genome decreased HBsAg and HBeAg secretion from cultured cells [93]. Recent studies have illustrated the utility of AAVs to deliver CRISPR/Cas 9 to achieve anti-HBV effects in vivo. Yan et al. observed effective reduction of HBeAg, HBV DNA and HBV RNA in vivo by one of the lead gRNAs [94]. More recent studies overcame the reduced packaging capacity of AAVs by dividing the Cas 9 cassette into two parts: Cas 9 up and Cas 9 down, with the gRNAs present in the latter. Using this approach CRISPR/Cas 9 targeting of the *C* and *X* ORFs of the HBV genome resulted in significant reduction of HBV DNA, HBsAg, and HBeAg levels in humanized mice or HBV transgenic mice [95, 96]. The further demonstration that AAV-mediated expression of HBV-targeting gRNAs can reduce cccDNA in humanized mice reinforced the suitability of AAV vectors for anti-HBV gene editor delivery [97].

CHALLENGES AND PROSPECTS OF VIRAL VECTOR-BASED ANTI-HBV DESIGNER NUCLEASE THERAPIES

Although designer nucleases are capable of successfully mutating and reducing cccDNA, it is important to note that off-target cleavage remains a major concern. Second generation TALENs and modified CRISPR/Cas 9 such as CRISPR/Cas derived base editors or Cas9ns offer improved targeting and therefore increased safety profile of these gene editors. Off-target effects require further investigation before these gene editing technologies can be translated to humans. Another major concern is that *S. pyogenes* and *S. aureus* are known to infect the human population at high frequencies. Recent studies have demonstrated that human serum harbors preexisting humoral and cell-mediated immunity against the Cas 9 protein [98, 99]. This is an important finding as preexisting immunity could impede the progression of CRISPR/Cas 9 to clinical trials.

Extensive efforts has gone into developing viral vectors that are suitable for delivery of sequences encoding HBV cccDNA-targeting designer nucleases. The field has however faced significant challenges (Table 1). Most LVs are derived from HIV-1 and have the potential to integrate into the host genome. Whereas this feature can be exploited for gene therapy, it carries the risk of disrupting essential genes. Development of non-integrating vectors by inactivating the integrase can avoid this challenge. Self-inactivating LVs designed by mutating the 3'LTR can avoid emergence of replication-competent recombinants [100–102]. However, the fact that LVs do not transduce adult liver cells in vivo very efficiently make them less favored for HBV targeted gene therapies.

Expression of all required subunits or components of gene editors in one AdV is appealing. However, a complication is that

Table 1. Major strengths, limitations and possible solutions to viral vector-mediated delivery of anti-HBV gene editors.

Viral vector	Major strength	Major limitation	Possible solution/s to the limitation	References
LVs	Large transgene capacity (~8 kb)	• Life-long nuclease expression from integrating vectors may result in off-target action and toxicity • Risk of essential genes disruption • Inefficient transduction of adult liver cells.	Non-integrating LVs Self-inactivating LVs	[100–102]
AdVs	Large transgene capacity (up to 37 kb) Higher transduction efficiency	• Strong capsid induced immunostimulatory effects	Polymer or chemical modification of capsids Immunosuppressive drugs Using serotypes with low seroprevalence	[108, 109, 117]
AAVs	Well-established safety profile	• Small transgene capacity limits ability to accommodate components of designer nucleases	Using smaller saCas 9 proteins Expressing nuclease monomers as a single polypeptide connected by a ribosome skipping 2 A peptide.	[92–94, 111, 112]

high transgene expression during vector production may result in self-targeting in vectors that carry substrates of homology directed repair and rearrangements of vector genomes in vectors carrying repetitive sequences like those encoding TALENs. Strategies to circumvent this include suppression of nuclease expression by use of vectors that carry sequences targeted by microRNAs that are specific to vector producer cell lines [75, 76, 103, 104]. The immunogenicity of AdVs makes them the excellent choice for designing vaccines against emerging viruses [105, 106]. However, a potentially toxic induction of the innate and adaptive immune response following systemic administration of high AdV doses remains a concern in gene therapy [107]. This may reduce transduction efficiency and diminish therapeutic benefits. Using low vector doses, vectors with low seroprevalence and immunosuppressive drugs may overcome limitation by Ad immunity [108, 109].

Low packaging capacity of AAVs limit delivery of bigger or multiple anti-HBV gene editors. Several studies have explored application of oversized and dual AAV vectors. These strategies take advantage of the potential for AAV genomes to concatemerize and serve as substrates for homologous recombination, which enables transgene reconstitution upon entry of vector DNA into cells. However, reconstitution is inefficient and high doses are required to achieve therapeutically relevant transduction efficacies. Two-component systems with two vectors each carrying a subunit or component of a gene editor are promising [110]. Although their application has not been demonstrated, AAV genome modifications, such as expressing nuclease monomers as a single polypeptide connected by a ribosome skipping 2 A peptide, may be applicable to anti-HBV strategies [111, 112]. Inflammatory responses to AAVs and preexisting immunity have greatly limited their utilization in clinical settings [113–116]. Several approaches to avoid inflammatory responses and preexisting immunity have been described, and capsid modification is one that offers great potential. Random mutagenesis, DNA shuffling, chemical modification and ancestral capsid reconstruction are some of the promising strategies [110].

Most work to date that describes use of viral vectors to edit HBV genes has been carried out in cultured cells (Fig. 1), and progress to analysis in vivo is a priority. Advances in the field will be dependent on overcoming challenges in several different topics. These include: (i) efficient delivery of candidate therapeutic sequences to HBV-infected hepatocytes, (ii) availability of mechanisms to regulate designer nuclease expression, (iii) precise definition of on-and off-target editing of sequences, (iv) accessible animal models that simulate HBV infection in humans and (v) methods to assay cccDNA during viral replication in vivo. Although studies using viral vectors to deliver HBV-targeting gene editors are scarce, the field is steadily growing and efficacy in vivo is becoming apparent. With the significant milestones in viral vector development, progression of viral vector-mediated HBV DNA editing to clinical application is within reach.

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AUTHOR CONTRIBUTIONS

RJ wrote the sections on LVs and AAVs, put the first draft together and revised all the drafts. PS wrote the introduction section on HBV biology and revised all drafts. TS wrote the introduction section on anti-HBV designer nucleases and revised all drafts. PA revised the final draft. MBM conceptualized the idea, wrote the section on Ads, revised all the drafts and finalized the manuscript for submission.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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