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Gene Editing: A New Tool for Viral Disease

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Keywords

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Abstract

The emergence of the CRISPR/Cas system of antiviral adaptive immunity in bacteria as a facile system for gene editing in mammalian cells may well lead to gene editing becoming a novel treatment for a range of human diseases, especially those caused by deleterious germline mutations. Another potential target for gene editing are DNA viruses that cause chronic pathogenic diseases that cannot be cured by using currently available drugs. We review the current state of this field and discuss the potential advantages and problems with using a gene editing approach as a treatment for diseases caused by DNA viruses.

INTRODUCTION

CRISPR: clustered regularly interspaced short palindromic repeat

PAM: protospacer adjacent motif

SpCas9: *Streptococcus pyogenes* Cas9

sgRNA: single-guide RNA

Clustered regularly interspersed short palindromic repeats (CRISPRs), found in a range of bacterial and archeal genomes, were first identified in 1987 as unusual genomic arrays in which a repeated identical sequence was separated by diverse interspersed "spacer" sequences (1). We now know that the spacers are, in fact, short sequence motifs excised from the genomes of pathogenic DNA bacteriophages that prey on the bacterium in question (2). The CRISPR arrays are transcribed and processed to produce CRISPR RNAs (crRNAs) that each contain one spacer and one copy of the adjacent constant region (3). The crRNA then binds a second, invariant RNA, the transactivating RNA (tracrRNA), and the resultant crRNA:tracrRNA complex, in the case of bacterial class II CRISPR/Cas systems, then binds to the CRISPR-associated (Cas) protein Cas9. Once this ribonucleoprotein complex is formed, it is targeted to the genome of the cognate, invading DNA bacteriophage by the sequence complementarity of the spacer sequence to that genome. Once bound, Cas9 cleaves the bacteriophage DNA, resulting in its elimination and thereby blocking productive infection (3, 4).

Although complementarity to the spacer sequence is a major determinant of DNA binding by Cas9, this interaction also requires an invariant sequence on the target DNA, located immediately 3′ to the region of homology to the spacer, called the protospacer adjacent motif (PAM), which is recognized and bound by the Cas9 protein itself (3, 5). In the most commonly used Cas9 protein, derived from *Streptococcus pyogenes* (SpCas9), the PAM sequence is 5′-NGG-3′ but the identity of the PAM varies widely between Cas9 proteins of different bacterial origin. Overall, one can consider the CRISPR/Cas machinery to be an adaptive immune response, comparable to an anamnestic immune response in mammals, that is used by bacteria to block reinfection by previously encountered bacteriophages (3). Bacteria that survive an initial bacteriophage infection acquire resistance to reinfection by excising a short sequence of bacteriophage DNA that is inserted into the CRISPR array as a new spacer sequence, which is then transcribed and processed to generate a Cas9-based endonuclease specific for the bacteriophage in question (2, 3).

The key step toward utilizing Cas9 as a programmable DNA endonuclease was the realization that a synthetic crRNA, containing a novel spacer sequence linked to the constant region of the crRNA, could be used to confer a novel DNA sequence specificity on Cas9. This result, obtained first in vitro (6) and soon after in cultured mammalian cells (7–9), opened the way for Cas9 to be repurposed as a facile tool for genome engineering, especially once it was realized that the crRNA and the tracrRNA could be linked together by an artificial RNA loop to generate a single-guide RNA (sgRNA) that could effectively program sequence-specific DNA cleavage by Cas9 (6–8). However, although the cleavage of bacteriophage DNA in prokaryotes results in the efficient destruction of the cleaved genome (4), eukaryotic cells possess mechanisms that can repair genomic DNA cleavages. Of these, the most prevalent is nonhomologous end joining (NHEJ), which simply rejoins the two ends of cleaved DNA substrates (5). If NHEJ repairs the cleaved site perfectly, restoring the previous DNA sequence, then Cas9 simply recleaves the substrate DNA (**Figure 1**). However, as NHEJ is somewhat error prone, it is expected that the repair will eventually introduce either a missense mutation(s) or an indel. At this point, the DNA is no longer fully complementary to the sgRNA loaded into Cas9 and is no longer subject to Cas9 cleavage. As a result, the introduced mutation becomes fixed in the target cell genome (5).

The use of SpCas9 to introduce genetic lesions therefore proceeds as outlined in **Figure 1**. Cells are engineered to express Cas9 and an sgRNA of choice, resulting in an RNA programmed SpCas9 complex. This complex then scans the target DNA for the 5′-NGG-3′ PAM sequence. Once a PAM has been identified, the SpCas9:sgRNA complex samples the immediately 5′ adjacent DNA sequence for complementarity to the "seed" sequence of the sgRNA, which forms the 5′ proximal ∼13 nucleotides (nt) of the ∼30-nt spacer sequence (5). If full complementarity to the

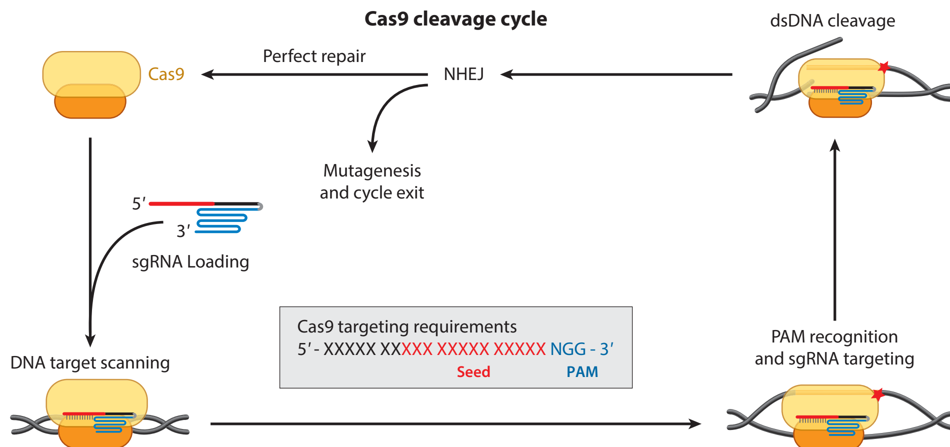

Figure 1

Cas9 cleavage cycle in mammalian cells. See text for detailed description. If nonhomologous end joining (NHEJ) repairs the cleaved site perfectly, then Cas9 simply recleaves the substrate DNA. If the repair introduces a mutation, then the DNA is no longer subject to Cas9 cleavage because it is not complementary to the single-guide RNA (sgRNA) loaded into Cas9. Red Xs represent the seed sequence in the sgRNA used for DNA target recognition. The invariant SpCas9 protospacer adjacent motif (PAM) is labeled.

sgRNA seed is detected, Cas9 then cleaves both strands of the target DNA three base pairs (bp) 5′ to the PAM. The cleaved DNA can then be repaired by NHEJ, resulting in mutagenesis of the target sequence or perfect repair. If the latter, then Cas9 will cleave the target DNA again, and this futile cycle will repeat until a mutation is introduced into the target DNA that destroys the required homology to the sgRNA seed. SpCas9 has now been used to introduce genome alterations in cultured cells from a wide variety of species, including humans, mice, zebrafish, pigs, etc., and several animals containing germline modifications generated by using CRISPR/Cas have now been reported (5).

CAS9-MEDIATED GENE EDITING AS A NOVEL ANTIVIRAL TOOL

Given that CRISPR/Cas naturally functions as an adaptive antiviral immune response in bacteria (1), it was clearly of interest to ask whether Cas9 could be also used as an antiviral tool in humans (10). For this to be achieved, the DNA target cleaved by Cas9 would have to be either part of the virus itself or a cellular gene required for virus replication but entirely dispensable in the human host. If the virus itself is the target, then a delivery strategy that can result in the expression of Cas9 and a virus-specific sgRNA(s) in all, or almost all, infected cells needs to be devised. Expression of SpCas9 clearly requires some form of expression vector containing, at minimum, an RNA polymerase II (pol II)–dependent promoter, the Cas9 coding sequence, and an mRNA polyadenylation site. In addition, this vector needs to express one or more ~110-nt-long sgRNAs specific for the viral target DNA, and these can be readily transcribed by using a pol III promoter such as the U6 promoter. These expression cassettes can be engineered into a single expression vector that can be transfected into cultured target cells. In vivo delivery, with the exception of the hydrodynamic injection technique in mice, would clearly require a more sophisticated delivery technology.

Although the use of some form of liposome or nanoparticle is potentially feasible, the best-studied method of in vivo gene delivery is viral vectors, especially lentiviral vectors and vectors

based on adeno-associated virus (AAV). In the context of Cas9 delivery, lentiviral vectors have the major advantage that their packaging size of up to ~7 kilobases (kb) readily accommodates the SpCas9 gene (~4.2 kb) as well as one or more sgRNAs and the *cis*-acting regulatory sequences required for efficient expression. However, titers of lentiviral vectors cannot exceed $\sim 10^9$ infectious units per milliliter, which is not adequate for the efficient infection of an entire population of virally infected cells in vivo (10), such as HIV-1-infected T cells, even though lentiviruses are highly lymphotropic. In contrast, AAV-based vectors can be generated at titers up to 10^{14} genome equivalents per milliliter. However, two limitations stand in their way.

AAV: adeno-associated virus

SaCas9: *Staphylococcus aureus* Cas9

HBV: hepatitis B virus

HPV: human papillomavirus

HSV: herpes simplex virus

AAV has a packaging capacity of only ~4.8 kb. This makes it impossible to express the ~4.2-kb SpCas9 gene, together with an sgRNA, from a single AAV vector. One approach is to use two AAV vectors: one to express SpCas9 from a minimal pol II promoter element while the other encodes one or more sgRNAs (11). A second approach is to use a different, smaller Cas9, for example, the ~3.2-kb Cas9 gene encoded by *Staphylococcus aureus* (SaCas9) (12, 13). SaCas9 has a somewhat less common PAM (5′-NNGRRT-3′, where R is purine) but otherwise appears to have a cleavage capacity and targeting accuracy comparable to SpCas9. Single AAV vectors able to express SaCas9 and one or two sgRNAs have been described and appear potentially very useful for in vivo gene editing (12, 13). A second concern with AAV vectors is their limited tissue tropism, although this has gradually expanded with the identification of additional AAV variants from different species and the derivation of AAV recombinants with enhanced tropism for specific tissues (14). AAV serotypes with a strong tropism for hepatocytes, neurons, and epithelial and endothelial cells have been described, but AAV variants able to efficiently infect lymphoid cells remain to be identified, which is a particular problem if editing of the HIV-1 genome is envisioned (see below).

One final issue with viral vectors relates to their potential to integrate and, hence, cause mutations that could inhibit or enhance the expression of specific cellular genes. Lentiviral vectors include integration as a key step in their life cycle, so this is clearly a concern. In contrast, AAV vectors do not normally integrate into target cell genomes, but integration of wildtype AAV (2) has been reported in a small number of hepatocellular carcinomas (15). Whether this represents a real concern with AAV vectors, which have gained wide popularity as tools for introducing genes into hepatocytes in vivo, remains to be established.

In considering which viruses might represent appropriate direct targets for elimination using CRISPR/Cas, two criteria are paramount. First, the virus must be a DNA virus or exist in a DNA form as part of its life cycle. Second, the virus must replicate in a discrete, well-defined region of the body that can be accessed by vectors expressing a virus-specific Cas9/sgRNA combination. In our view, the list of potential target viruses (**Figure 2**) is therefore rather limited and consists primarily of hepatitis B virus (HBV), human papillomavirus (HPV), herpes simplex virus (HSV) types 1 and 2, and human immunodeficiency virus type 1 (HIV-1).

CRISPR/CAS TARGETING OF HUMAN DISEASE VIRUSES

Hepatitis B Virus

HBV virions contain a small, partially double-stranded DNA (dsDNA) genome of ~3.2 kb that is converted to covalently closed circular DNA (cccDNA) in the cell nucleus shortly after infection (16). The resultant cccDNA episome serves as the template for transcription of the viral pregenomic RNA (pgRNA) as well as mRNAs that encode the viral structural proteins. These are the HBV core and surface antigen (HBsAg), the viral polymerase (pol) gene, and the nonstructural X protein. Because of the small size of the HBV genome, the open reading frames encoding these viral gene products extensively overlap, such that introduced mutations have the potential to inactivate more than one viral gene simultaneously.

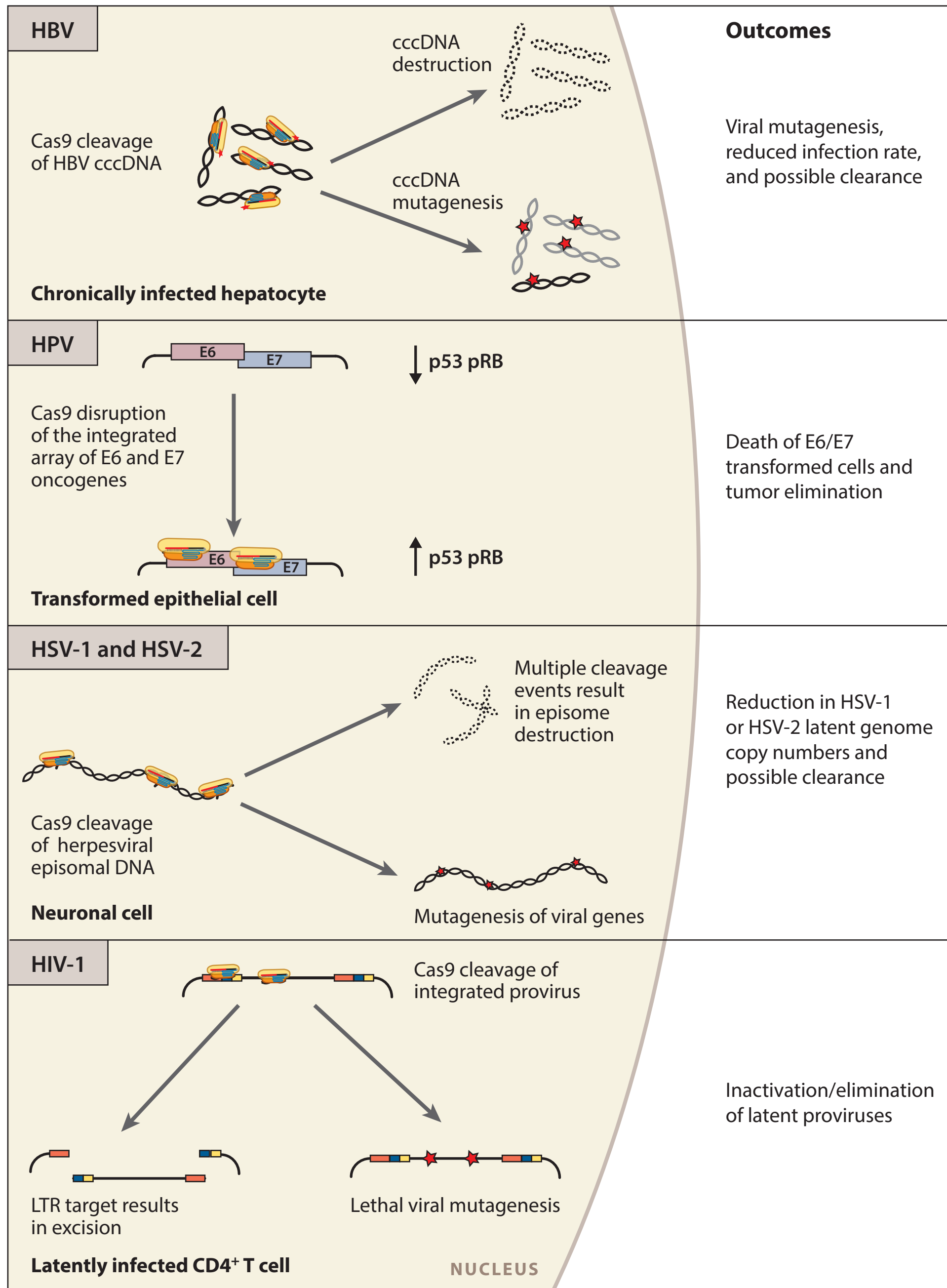

Figure 2

Overview of potential approaches to the elimination of the listed viruses using CRISPR/Cas. See text for detailed discussion. Abbreviations: cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; HPV, human papillomavirus; HSV-1 and -2, herpes simplex virus types 1 and 2; HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; pRB, retinoblastoma protein.

A key feature of the HBV life cycle is that the pgRNA is packaged into the viral core in the cytoplasm of infected cells and is then reverse transcribed to give the viral DNA genome (16). The resultant virion particles can bud into the surrounding environment after assembling the HBsAg onto their surface, or can instead enter the nucleus to form more cccDNA episomes.

Although HBV infection can be prevented by a vaccine, ~260 million people worldwide are chronically infected with the virus, which significantly increases their risk of dying from hepatocellular carcinoma or cirrhosis. HBV can be treated with nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), which block the formation of new viral DNA genomes and the release of infectious virions (16). However, the existing pool of HBV cccDNAs is highly stable, persisting for decades in NRTI-treated patients. Moreover, these cccDNAs continue to serve as templates for viral mRNA production, resulting in the continued high-level production and secretion of viral proteins, which is thought to inhibit host antiviral immune responses that might otherwise eliminate HBV-infected cells (16). As a result, the spontaneous cure rate for HBV patients on NRTI treatment is low. Clearly, what is required is a method to cleave and destroy the pool of cccDNAs in the liver, and CRISPR/Cas seems ideal for this task.

Evidence that CRISPR/Cas could indeed represent an effective treatment for chronic HBV infection comes from studies conducted in cultured cells of hepatic origin or in mice (17–22). Studies conducted in cultured human cells detected a profound decline in the level of total DNA and cccDNA of HBV origin and also reported a significant but more modest drop in the level of viral protein expression, including reduced HBsAg production (17–19). Of note, cleavage of HBV episomal DNAs appears to frequently result in their complete loss, although the mechanism remains to be defined (**Figure 2**). Moreover, HBV episomal DNAs that survive cleavage (a small percentage) are generally mutationally inactivated by frameshift mutations introduced at predicted Cas9 cleavage sites (**Figure 2**) (17–19). In vivo studies reported so far have relied on immunodeficient mice subjected to hydrodynamic injection with HBV genomic DNA and vectors encoding Cas9 along with control or HBV-specific sgRNAs (20–22). Again, a significant drop in the level of HBV virus production in vivo was observed. While these initial studies are therefore encouraging, it will be important to perform studies that evaluate HBV infections in animal models that more accurately mimic human infections, for example, humanized liver mice that can support a productive HBV infection in vivo.

HBV seems a particularly promising target for treatment using a CRISPR/Cas approach because viral infection is confined to the liver and new infections can be blocked using NRTIs. Therefore, the problem is reduced to simply delivering Cas9 and HBV-specific sgRNAs to infected hepatocytes in order to destroy viral cccDNAs. Fortunately, the liver is a particularly good target for infection by AAV vectors, including especially AAV8 and derivatives thereof (14), so it is in principle entirely feasible to transduce every HBV-infected hepatocyte in vivo. Even if not every cccDNA molecule is destroyed, a substantial reduction in viral load should greatly decrease the production of viral proteins, including HBsAg, with the resultant possibility that the immune system will escape from the repression caused by high levels of circulating viral proteins and destroy residual HBV-infected cells—as is indeed observed at low levels in NRTI-treated patients (16). Overall, therefore, HBV appears to be the most promising viral infection for treatment using CRISPR/Cas.

Human Papillomavirus

Whereas HBV pathogenesis occurs as a function of high levels of virus replication, pathogenesis in HPV occurs as a result of an abortive infection (23). Humans are subject to infection by >150 different HPV serotypes, most of which are harmless or cause benign warts. However, a small number of HPV serotypes, termed high risk, have been associated with several human cancers. For

example, HPV16 and -18 account for ~75% of all cervical cancer, and HPV31 and -45 account for a further ~10%. Cervical cancer linked to HPV infection can be detected early by routine diagnostic tests, and progression to serious disease is therefore almost always avoided, at least in first-world countries. However, HPV infection, especially by HPV16, is also associated with a significant percentage of anal and oropharyngeal cancers. Although ~80% of patients presenting with HPV-positive anal or oropharyngeal cancer can be cured by chemoradiation, ~20% are not cured and may require some form of salvage surgery, which can have highly debilitating aftereffects.

HPV normally replicates in basal keratinocytes of the skin or mucosal epithelium (23). The viral genome initially replicates in the nucleus as a DNA episome that exclusively expresses the viral early (E) proteins. As the infected basal keratinocytes undergo differentiation and move toward the surface of the epithelial layer, the expression of the viral late (L) structural genes is activated, resulting in the shedding of HPV virions from the surface of the epithelium. Rarely, the HPV episome is integrated into the genome of an infected basal cell, which actually blocks viral replication. If the integration event disrupts the HPV E2 gene, which regulates the expression of the other E genes, overexpression of the HPV E6 and E7 proteins can result. Both E6 and E7 function to inhibit cellular tumor suppressors, including p53 in the case of E6 and retinoblastoma protein (pRB) in the case of E7. This represents the first step in the transformation of infected cells by HPV. Malignant transformation also requires the activation of cellular oncogenes, such as *ras* or *fos*; nevertheless, continued expression of E6 and E7 is required for the continued growth of malignantly transformed cells, and inactivation of either the E6 or E7 gene is sufficient to induce tumor cell death by apoptosis and/or senescence, due to reactivation of cellular p53 or pRB expression, respectively (**Figure 2**) (23). Therefore, the CRISPR/Cas-mediated cleavage of copies of the HPV E6 and/or E7 gene integrated into the tumor cell genome has the potential to function as a highly effective and highly specific approach to the elimination of transformed cells, especially in the case of HPV16-positive anal and oropharyngeal cancers. Several groups have in fact demonstrated that the Cas9 cleavage, and resultant mutational inactivation, of E6 or E7 in cervical cancer cell lines transformed by HPV16 or HPV18 results in their rapid demise, whereas HPV-negative tumor cells are unaffected (24–27). Similarly, in an immunodeficient mouse model bearing transplanted human cervical cancer cells, tumor growth was strongly inhibited upon Cas9 cleavage of the HPV E6 or E7 gene (25). Therefore, HPV-specific Cas9/sgRNA combinations, possibly delivered using AAV vectors, have the potential to emerge as a new approach to the treatment of HPV-positive anal and oropharyngeal cancers.

Herpesviruses

Humans are subject to infection by a number of pathogenic herpesviruses, all of which have DNA genomes and hence could potentially be targets for CRISPR/Cas. Here, we focus on the human herpes simplex viruses HSV-1 and HSV-2.

HSV-1 infection occurs via small lesions in the epithelium, especially around mucous membranes (28). Once inside the body, the virus establishes a productive infection resulting in a localized lesion. These often occur around the lips, where they are referred to as cold sores. HSV-1 infection induces a strong immune response that eventually blocks viral replication and leads to healing of the lesion. However, during the phase of active replication, HSV-1 virions migrate up the trigeminal ganglion (TG) that innervates the lip area and establish latent infections in a small number of neurons in the TG. During latency, HSV-1 expresses no viral proteins, and latently HSV-1 infected neurons are invisible to the immune system. Occasionally, often in response to some form of cell stress, one or more of the latent HSV-1 genomes are reactivated to establish

a productive infection. Virions produced from the reactivated viral genomes migrate down the TG to the site of initial infection, where a productive infection is reinitiated. This again results in a local lesion, which releases infectious virions and provokes an anamnestic immune response that normally rapidly destroys the infected cells (28). In the United States, ~70% of all adults are HSV-1 infected, and one-third of these individuals suffer from recurrent cold sores that might occur once a year or once a week. For most people these are only a painful inconvenience, but in others they can be an almost constant irritation. Moreover, HSV-1-induced keratitis can occur, which can lead to blindness. No cure for HSV-1 infection exists, although active viral replication can be blocked by inhibitors of the viral DNA polymerase, such as acyclovir. Infection by HSV-2 is quite similar to HSV-1 but most commonly occurs in the genitalia, leading to latent HSV-2 infections in the sacral ganglia and recurrent genital ulcers.

In latently infected neurons, HSV-1 and HSV-2 genomes are found as circular, nonreplicating DNA episomes in the cell nucleus with from 1 to perhaps as many as 50 copies per latently infected cell (28). These dsDNA molecules are in principle excellent targets for cleavage and either destruction or mutational inactivation by CRISPR/Cas (29–31) (**Figure 2**). Moreover, because the likelihood of reactivation of HSV-1 or HSV-2 directly correlates with the viral DNA load in the ganglion, even an incomplete ablation of the pool of viral genomes might have significant benefits. Neuronal cells are good targets for infection by AAV, and it seems possible that AAV-mediated delivery of SpCas9 or SaCas9, together with possibly multiple sgRNAs specific for HSV-1 or HSV-2 to shatter or mutationally inactivate the viral genome (**Figure 2**), would represent a potential strategy to cure this bothersome disease. Indeed, recent data suggest that HSV-1, even during lytic infection, may be an excellent target for treatment using a CRISPR/Cas-based approach (32).

Human Immunodeficiency Virus Type 1

HIV-1 in many ways represents a perfect target for inactivation using CRISPR/Cas. HIV-1-infected cells generally contain a single integrated provirus flanked by the viral long terminal repeats (LTRs). The integrated virus could be deleted by cleavage in both LTRs or mutationally inactivated after cleavage of an essential structural or regulatory protein. Indeed, several groups have now reported that Cas9/sgRNA complexes specific for HIV-1 can not only destroy integrated HIV-1 proviruses but also effectively block de novo infection by HIV-1 (33–37). Yet, to a greater extent than for HBV, HPV, or HSV-1, there are severe technical challenges to the effective use of CRISPR/Cas to cure HIV-1 infections.

It is important to consider why the potential use of CRISPR/Cas in HIV-1-infected patients has become of interest. Large numbers of drugs that effectively block different steps in the HIV-1 life cycle, including infection, reverse transcription, integration, and proteolytic processing of the viral polyproteins, have been described and are clinically available. Highly active antiretroviral therapy (HAART), involving the simultaneous use of two or more drugs that target different steps in the viral life cycle or different binding sites on the same viral protein, has made it impossible for the virus to evolve resistance as long as patients adhere to the correct dosage regimen, yet HIV-1 is still not cured. The reason for this is thought to be the presence of a small number of memory $CD4^+$ T cells, perhaps 10^7 or so, that contain an integrated, intact HIV-1 provirus that is nevertheless transcriptionally inactive (38). Such a cell would not be killed by a viral cytopathic effect, nor does the cell present any virus-derived epitopes that could serve as targets for $CD8^+$ cytotoxic T cells. Rather, the integrated provirus remains inert until the memory T cell is activated upon encountering a recall antigen. The provirus then becomes transcriptionally active and can proceed to generate all the viral RNAs and proteins required to complete the viral life cycle. If antiviral drug treatment has been suspended, then the resultant virions are able to rekindle a fully

pathogenic infection. Unfortunately, these latently HIV-1-infected T cells are extremely long lived, so although currently available therapies entirely block virus replication, they nevertheless do not lead to a cure. Initial efforts to deal with these latently HIV-1-infected T cells have largely focused on a so-called "kick-and-kill" strategy whereby the latently infected cells would be induced to reactivate the integrated provirus after treatment with one of several different classes of drugs, such as protein kinase C agonists or histone deacetylase inhibitors (38). However, it now appears that treatments that do not induce unacceptable toxicity are incapable of inducing all latently HIV-1-infected cells in vivo.

Attention has therefore turned to the use of gene editing as a way of permanently inactivating the latent HIV-1 pool. Indeed, although actively replicating HIV-1 is able to rapidly escape from inhibition by specific Cas9/sgRNA combinations (39, 40), patients undergoing HAART, which in principle blocks all HIV-1 replication, should not be able to generate cleavage-insensitive escape mutants. Therefore, targeting Cas9/sgRNA combinations to highly conserved viral sequences, such as the transactivation response element or the tRNA primer binding site, is a potentially effective strategy to eliminate latent HIV-1 proviruses. The problem, however, is delivery. Humans contain $\sim 10^{11}$ $CD4^+$ T cells, of which, in HIV-1-infected patients with undetectable viral replication, perhaps 10^7 are latently HIV-1 infected. However, the location of these latently infected cells is not clear, and they are likely distributed in lymphoid tissues throughout the body. Unfortunately, AAV only very poorly infects lymphoid cells, which suggests that lentiviral vectors based on HIV-1, which are tropic for $CD4^+$ cells, represent the only possible approach. However, the maximal achievable titer for HIV-1-based vectors is $\sim 10^9$/ml (10). Therefore, although the idea of using CRISPR/Cas to eliminate latent HIV-1 proviruses by proviral excision or lethal mutagenesis (**Figure 2**) is attractive, its successful application requires a major technical breakthrough in vector design and/or production (10).

CONCLUSION

The continued development and optimization of bacterial CRISPR/Cas systems as tools for genome editing, not only in vitro and in animals but soon in humans, promises to lead to a new era in which manipulation of the human genome can be used to avoid or cure various diseases. Among the more obvious targets for CRISPR/Cas targeting are pathogenic DNA viruses that establish long-term, chronic infections in humans. As noted above, strategies to target viruses such as HBV and HSV-1 appear to be available and, once appropriate preclinical studies are completed, we believe that clinical trials in humans will soon follow. Whether viral treatment approaches based on CRISPR/Cas will ever become widely available is more difficult to predict, given the likely cost of any gene therapy–based approach. Nevertheless, over time, we predict that the use of CRISPR/Cas to treat certain chronic viral diseases will become increasingly commonplace.

DISCLOSURE STATEMENT

The authors have licensed technology related to the antiviral use of CRISPR/Cas to Cocrystal Pharma.

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