Chapter 4

Gene Correction Technology and Its Impact on Viral Research and Therapy

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Additional information is available at the end of the chapter

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Abstract

Aims

1. To explain why gene correction technology is a useful tool for studying chronic/latent viral infections.

2. To explain how gene editing technology may facilitate or restrict virus replication and impact on future therapy.

3. To cite specific examples of how gene correction technology is being applied to target specific viruses, including HIV, hepatitis B virus (HBV), herpes simplex virus (HSV), and other viruses.

Methodology

We attempted to identify all scientific publications including basic science, translation research, and any clinical trials involving DNA correction technology [zinc finger endonuclease (ZFN), transcription activator-like endonucleases (TALENs), and CRISPR/Cas-based systems] and persistent viral infections [including but not limited to HIV, hepatitis B, C, and D viruses, herpes viruses, cytomegalovirus, Epstein–Barr virus (EBV), human papillomavirus (HPV), measles virus, and varicella-zoster virus] published on or before December 31, 2015. We conducted searches of MEDLINE, Cochrane Central Register, and EMBASE. The identified papers have been summarized and organized into relevant sections within the chapter.

Conclusion

Sequence-specific DNA endonucleases target and destroy DNA viruses, with early work describing the use of ZFNs, TALENs, or a third type of endonuclease, called a homing endonuclease (HE), to target HBV, HPV, and HSV-1 with varying degrees of success. The new CRISPR/Cas9 systems do not allow virologists to screen for host genes that affect the replication of pathogenic human viruses but to derive human cell lines that are genetically engineered to either facilitate or suppress viral replication. Scientists now

widely use adeno-associated virus (AAV)-based vectors to directly target chronic viruses that infect discrete organs/tissues in the human body, such as HBV and HSV, although the safety of such delivery system is still a concern. The eventual goal is to eradicate or to disable the entire population of latent viral DNA genomes within the infected cells, resulting in permanent cure for these viral infections, which remains elusive until now.

Keywords: antiviral gene therapy, CRISPR/Cas, gene editing, sequence-specific DNA cleavage, TALENs, zinc finger

1. Introduction

Sequence-specific DNA endonucleases were first identified in the 1960s as enzymes that restrict the ability of DNA bacteriophage to grow in particular bacterial isolates or species. However, it was many years later that the DNA editing technology for selective modification of large viral or cellular DNA genomes was developed with the discovery of zinc finger endonuclease (ZFN) [1]. It was originally developed as an artificial restriction endonuclease to replace or complement restriction enzymes, and the engineered nucleases have become a versatile and indispensable tool in research as well as in biotechnology. However, each zinc finger is not necessarily highly specific for the 3-bp target that it is designed to bind and zinc fingers specific for all possible 3-bp target sequences have not yet been derived [2].

To address some of the deficiencies of ZFN, transcription activator-like endonucleases (TALENs) were developed based on a distinct modular DNA-binding motif. TALENs are made up of four ~34 amino acid domains derived from a DNA-binding protein found in the pathogenic plant bacterium *Xanthomonas*, which each recognizes a single DNA base pair with higher specificity [3, 4]. Although being more specific than ZFNs, TALENs require a rather laborious exercise in genetic engineering and are quite large, thus limiting the ability to express TALENs using viral vectors.

The newly developed CRISPR/Cas-based systems, or RNA-guided engineered nucleases (RGENs), unlike ZFNs and TALENs that use protein motifs for DNA sequence recognition, depend on RNA-DNA recognition. These are both highly specific and allow facile retargeting to new genomic loci [5, 6] and may be superseding the two older technologies. A key step forward in making the CRISPR/Cas9 system more user-friendly is by demonstrating that the crRNA and tracrRNA could be linked by an artificial loop sequence to generate a fully functional small guided RNA (sgRNA) [5, 6].

To date, the ever-increasing list of organisms with genomes that have been modified successfully using engineered nucleases includes mosquitoes, crickets, silkworms, pigs, cows, rabbits, and nonhuman primates, among others [7, 8]. The ability to genetically manipulate human pluripotent stem cells and somatic cells using engineered nucleases opens new opportunities to develop novel therapies for patients with various genetic and acquired diseases. Genetic defect correction is now possible in cultured cells from patients with a number of genetic

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diseases, including sickle cell disease [9], cystic fibrosis [10], Down syndrome [11], Duchenne muscular dystrophy [12], α1-antitrypsin deficiency [13], dystrophic epidermolysis bullosa [14], chronic granulomatous disease [15], and infectious diseases, particularly persistent viral infections that were among the most attractive targets in both ex vivo and in vivo studies, which will be discussed in the rest of the chapter. Despite all these promising advances, we should also be aware that these DNA editing technologies have been shown to cut at off-target sites with mutagenic consequences. Therefore, issues such as efficacy, specificity, and delivery are likely to drive selection of reagents for particular purposes. Human therapeutic applications of these technologies will ultimately depend on risk-versus-benefit analysis [8].

2. Main text

2.1. Why is DNA editing technology important in chronic viral disease research?

Chronic viral infection underlies a wide variety of medically important diseases that either follow directly from primary infection or may require months, years, or even decades to develop. Diseases caused by persistent virus infections include AIDS, AIDS-related complexes, chronic hepatitis, subacute sclerosing panencephalitis (chronic measles encephalitis), chronic papovavirus encephalitis (progressive multifocal leukoencephalopathy), several herpes virus–induced diseases, and some neoplasias (see **Table 1**). Pathogens with worldwide impact, such as HIV, hepatitis B virus (HBV), and a number of herpes viruses remain uncontrolled. The pathogenic mechanisms by which these viruses cause diseases include disorders of biochemical, cellular, immune, and physiologic processes. The chronic nature of these infections limits the number of antiviral options and increases the risk of developing drug-resistant strains in the host. Some recent studies suggest that chronic viral infection also contributes to certain cancers as well as to diabetes and atherosclerosis. Ongoing studies are rapidly advancing our understanding of many persistent infections. Viruses have evolved a wide variety of strategies by which they maintain long-term infection of populations, individuals, and tissue cultures.

Most of our current understanding of the host immunity and viral virulence are from studies on the progression of acute infection. When a virus enters the host, there is an initial nonequilibrium phase of acute infection, in which viral and immune strategies compete for dominance. A transition point will be reached in a survivor, at which the infection either is cleared or becomes chronic. This transition point may be reached very early in infection for viruses that can establish a latent infection, in which case the infection is permanent regardless of the course of acute infection. If recovery occurs, the immune system must reset by clearing the antigen and reestablishing immune homeostasis. If the balance shifts toward chronic infection, a new set of viral and host strategies interact to define a metastable equilibrium in which viral replication is held in check, but the virus is not cleared [16].

Whereas acute viral infection represents a nonequilibrium process, chronic viral infection is a process in dynamic and metastable equilibrium. During acute infection, both the host and the virus change continuously until infection is resolved, kills the host, or becomes chronic. Certain genes in a virus or in the immune system function during acute but not chronic infection. The failure of these immune system genes to function effectively or the overly effective evasion of

immunity by the virus may eventually lead to multiorgan failure and death. In contrast, during chronic infection, viral and host genes balance each other [16]. The mechanisms of viral persistence despite the impressive immune armamentarium of the host, without causing overt disease, remain unclear, although the events leading to the establishment of chronicity were described.

Two events are fundamental to the establishment of chronic viral infection. First, the virus must evade sterilizing immunity (the complete elimination of a virus). Second, the immune system must adjust to the continuous presence of viral antigen-driven inflammatory responses to limit viral replication to an acceptable level without untoward damage to permanently infected tissues. If the immune system cannot eliminate the virus, unrestrained immune attack on virus antigen-bearing cells causes tissue injury. Thus, down-regulation of inflammation during chronic viral infections can result in decreased tissue damage, at least for noncytopathic viruses. Immunopathology can be severe in human chronic infections caused by HBV and hepatitis C virus (HCV) [46], which may remain noncytopathic for many decades initially. It is important to realize that viruses which rely on a living but chronically infected host for their own survival must carefully avoid mechanisms that overwhelm immunity and kill their hosts.

Viruses have evolved highly effective strategies for establishing chronic infection despite the presence of an active host antiviral immune response. There are three general strategies for chronic viral infection: continuous replication, latency and reactivation, and invasion of the genome followed by vertical spread from generation to generation. Individual viruses usually rely mostly on one strategy, but viruses can use more than one mechanism. For example, HIV effectively uses both continuous replication and establishment of latency [47–49], a dangerous combination. The differences between these strategies have profound implications for designing new ways to prevent or control harmful chronic viral infections.

The mechanisms of the viral persistence have not been completely understood, and some common factors are known [50]. Immune modulation is one of them. Many of these viruses managed to avoid the specific and nonspecific immune defenses in several different ways. These include (1) limitation of recognition molecules on infected cells; (2) altered lymphocyte and macrophage functions, including the modified production of cytokines and general immunosuppression [e.g., HIV-1 and -2, Epstein–Barr virus (EBV), and HBV]; (3) infection in immunologically privileged anatomic sites [e.g., herpes simplex virus (HSV) and VZV in central nervous system]; (4) compromised nonspecific defenses (e.g., suppress interferon production); and (5) immune tolerance (e.g., HBV).

Another common mechanism is the modulation of viral gene expression. Such examples include (1) down-regulation of some viral genes by viral or cellular regulatory gene products [e.g., HIV and human papillomaviruses (HPVs)], (2) specific latency-associated proteins (e.g., EBNA-1), and (3) synthesis of latency-associated transcripts (LATs; e.g., HSV-1 and -2) as well as viral variants (e.g., HIV and measles) [50].

Developing a successful cure for persistent or chronic viral infections remains a major challenge due to their ability to evade/suppress the immune system and their ability to incorporate viral sequences into the host genome and long inactive latent phases, which makes

targeting of active biological activities nearly impossible. However, recent concerted effort and improvement of drug design, leading to multiple new drugs that successful cure HCV, demonstrate that the complete clearance of chronic viral infections is an attainable goal. A particularly tantalizing application of programmable nucleases is the potential to directly correct genetic mutations in affected tissues and cells to treat diseases that are refractory to traditional therapies. A number of approaches targeting specific disease-causing viral infections will be discussed in the following sections.

			Disease during chronic infection		
Virus, primary nucleic acid, estimated percent of humans infected	**Major site of persistence (organ or cell)**	**Acute infection examples**	**Within normal hosts**	**Within immuno compromised hosts**	**References**
Hepatitis B virus (HBV), DNA, 350 million, ~5%	Hepatocytes	Hepatitis	Cirrhosis, hepatocellular carcinoma	Same diseases	McGovern [17]; Rehermann and Nascimbeni [18]
Hepatitis C virus (HCV), RNA, 170 million, ~2.5%	Hepatocytes	Hepatitis	Cirrhosis, hepatocellular carcinoma	Same diseases	Rehermann and Nascimbeni [18]; Lemon et al. [19]
Human immunodeciency virus (HIV-1 and HIV-2), RNA, 33 million, ~0.5%	CD4+ T cells, monocyte/ macrophages	Acute febrile illness	AIDS	AIDS	UNAIDS [20]; Kuritzkes and Walter [21]
Hepatitis D virus (HDV), RNA, 15 million, ~0.2%	Hepatocytes	Unknown	Exacerbation of chronic HBV infection	Unknown	Taylor et al. [22]
Human T cell leukemia virus type 1 (HTLV1), RNA, 10–20 million, ~0.2%	T cells	Unknown	Adult T cell leukemia (2–6% of carriers), tropical spastic para-paresis, myelopathy, uveitis, dermatitis	Unknown	Matsuoka and Jeang [23]; Lairmore and Franchini [24]
Xenotropic murine leukemia virus-related	Prostate	Unknown	Prostate cancer?	Unknown	Urisman et al. [25]; Dong et al. [26]

			Disease during chronic infection		
Virus, primary nucleic acid, estimated percent of humans infected	**Major site of persistence (organ or cell)**	**Acute infection examples**	**Within normal hosts**	**Within immuno compromised hosts**	**References**
virus (XMLV), RNA, unknown					
Rubella virus, German measles, RNA, rare	CNS	Rubella, arthritis	Progressive rubella panencephalitis	Unknown	Hobman and Chantler [27]
Parvovirus B19, DNA, rare	Bone marrow erythroid progenitors	Fifth disease, arthritis	Aplastic crisis in hemolytic anemia, hydrops fetalis, chronic bone marrow de ciency	Red cell aplasia	Berns and Parrish [28]; Norja et al. [29]
Human papillomavirus, DNA, <5%	Epithelial skin cells	Unknown	Papilloma, cervical and other mucosal carcinomas	Increased severity and incidence of same diseases	Leggatt and Frazer [30]; Howley and Lowy [31]
Measles virus, RNA, rare	Neurons and supporting cells in CNS	Measles	Subacute sclerosing panencephalitis, measles inclusion body encephalitis	Unknown	Griffin [32]
Coxsackie, RNA, rare	Myocardial cells	Hand foot and mouth disease, herpangina	Myocarditis	Unknown	Chapman and Kim[33]; Whitton and Feuer [34]
Human herpes virus 6 (HHV-6), DNA, >90%	Lymphocytes?	Roseola	Unknown	Meningoencephalitis, secondary infections, immunomodulatory?	Straus[35]; Yamanishi et al. [36]
Varicella zoster virus (VZV), DNA, >90%	Sensory ganglia neurons and/or satellite cells, lymphocytes	Chicken pox	Herpes zoster	Disseminated disease, hepatitis, pneumonitis	Zerboni and Arvin [37]; Straus [38]
Cytomegalovirus (CMV), DNA, 80%–90%	Myelomonocytic cells	Mononucleosis	Rare	Disseminated disease, vasculitis, pneumonitis, retinitis, hepatitis,	Mocarski et al. [39]

			Disease during chronic infection		
Virus, primary nucleic acid, estimated percent of humans infected	**Major site of persistence (organ or cell)**	**Acute infection examples**	**Within normal hosts**	**Within immuno compromised hosts**	**References**
				gastroenteritis, meningoencephalitis	
Epstein–Barr virus (EBV), DNA, 80–90%	Pharyngeal epithelial cells, B cells	Mononucleosis	Burkitt's lymphoma, nasopharyngeal carcinoma, non-Hodgkin's lymphoma	CNS lymphomas, oral hairy leukoplakia, lymphoproliferative disease	Rickinson and Kieff [40]; Straus [38]; Kieff and Rickinson [41]
Polyomavirus BK, DNA, 72–98%	Kidney	Unknown	Unknown	Hemorrhagic cystitis (post bone marrow transplantation), nephropathy (post kidney transplantation)	Zur [42]
Polyomavirus JC, DNA, 72–98%	Kidney, CNS	Unknown	Unknown	Progressive multifocal leukoencephalopathy	Zur [42]
Herpes simplex type 1 (HSV-1), DNA, 50–70%	Sensory ganglia neurons	Pharyngitis, encephalitis, keratitis	Cold sores, encephalitis, keratitis	Increased severity of same diseases, pneumonitis, hepatitis	Straus [38]
Adenovirus, DNA, up to 80%	Adenoids, tonsils, lymphocytes	Upper respiratory infection, gastroenteritis	Unknown	Enteritis, hemorrhagic cystitis, pneumonitis, hepatitis, others	Garnett et al. [43]; Wold and Horwitz [44]
Herpes simplex type 2 (HSV-2), DNA, 20–50%	Sensory ganglia neurons	Genital herpes	Genital herpes, encephalitis	Increased severity of same diseases	Straus [38]
Kaposi's sarcoma herpes virus (KSHV) or human herpes virus 8, DNA, 2–60%	Endothelial cells, B cells	Unknown	Castleman's disease, Kaposi's sarcoma	Kaposi's sarcoma, primary effusion lymphoma	Ganem [45]

Table 1. List of known human pathogenic chronic viral infections and their respective prevalence and clinical disease [16].

2.2. Defining the cellular factors that facilitate or restrict virus replication

Using sequence-specific DNA endonucleases to target and destroy DNA viruses had been attempted almost as soon as the technology was invented [51], with earlier work describing the use of ZFNs, TALENs, or an earlier HE, to target HBV, HPV, and HSV-1.

The newer DNA editing systems not only impact virology by allowing researchers to screen for human genes that affect the replication of pathogenic human viruses and are extremely useful in generating human cell lines that lack gene products that may facilitate or restrict specific virus replication. Adeno-associated virus (AAV)-based vectors offer the possibility of directly targeting DNA viruses that infect specific organs or tissues in the human body (e.g., HSV and HBV) to target and destroy the entire population of viral DNA genomes. Safety concerns, such as off-target genomic mutations, of such delivery system remains [52].

Viruses have very compact genomes and therefore rely on the host cell for many activities required to support their replication cycle. Ironically, viruses as pathogens put selective pressure on their host organisms, thus selecting for cellular restriction factors that can limit its own level of viral replication. One of the best studied viruses in this context is HIV, which requires a wide range of human cofactors for replication in $CD4^+$ T cells, several of which are lacking in other mammalian species, such as in mice. HIV is targeted by a wide range of human restriction factors and dedicates a substantial portion of its coding capacity to the neutralization of these restriction factors. For example, the Vif protein blocks the activity of the host APOBEC3 family of restriction factors, which otherwise interfere with the production of the HIV-1 provirus, whereas the HIV-1 Vpu protein neutralizes the cellular restriction factor tetherin, which blocks the release of progeny virions [53].

The identity of cellular factors that either facilitate or restrict viral replication is central to the search for novel targets for antiviral drug development. Researchers have been using RNA interference (RNAi) libraries to knock down the expression of human genes systematically to identify factors required for virus replication. Although this approach has led to some interesting insights, it is also clear that RNAi screens for viral cofactors in different laboratories have often led to very different lists of cellular proteins with this potential activity. This may be due to the use of different cell systems, different assays for viral replication, different RNAi reagents, and sometimes incomplete knockdown of target gene expression. As a result, most of the viral cofactors and restriction factors identified so far have required confirmation using biochemical approaches (e.g., by identification of cellular factors that specifically bind to a viral protein) or genetic approaches (e.g., by complementation of a human and/or animal cell line that lacks a particular cofactor or restriction factor) [52, 53].

RGEN systems appear highly suitable for use in screens for viral cofactors or restriction factors; indeed, several screens for cellular factors involved in cell transformation have been published [54–56]. This could now be extended to analysis of the replicative potential of viruses in a 96- or 384-well plate format, possibly in the form of comprehensive CRISPR/Cas9-generated libraries of cellular clones available for specific human cell lines. Each of these lines will lack a functioning gene that is dispensable for cell viability in a tissue culture setting. These clones should allow the reproducible and almost complete identification of cellular factors that either

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help or hinder virus replication in that human cell line in vitro. Factors required for human cell viability would be missed, but it seems likely that almost all host innate immune factors involved in restricting virus replication would not be essential. Essential factors required for host cell viability must also be essential for virus replication and clearly would not provide potential targets for antiviral drug development. A summary of studies using DNA editing technology to define the host factors that facilitate or restrict virus replication is listed in **Table 2**.

Defining the host factors that facilitate or restrict virus replication

Virus	Human disease	Type	Host factors studied	Cell/ animal/ human study	Delivery method	Reference
Cytomegalovirus (CMV)	Disseminated disease, vasculitis, pneumonitis, retinitis, hepatitis, gastroenteritis, meningoencephalitis	TALEN	Glucocorticoid receptor (GR) gene	CD8 + T cells	Electroporation	Menger et al. [57]
Hepatitis C virus (HCV)	Cirrhosis, hepatocellular carcinoma	TALEN	DGAT1	Huh7.5 cells	Transfection	Sung et al. [58]
		CRISPR	CLDN1, OCLN and CD81	Huh7.5 cells		Ren et al. [59]
		CRISPR	ISG15	Huh7 cells	Transfection	Domingues et al. [60]
Herpes simplex type 1 (HSV-1)	Core sores, encephalitis, pneumonitis, hepatitis	CRISPR	IFI16	U2OS cells	Transfection	Johnson et al. [61]
		CRISPR	LULL1	HeLa cells	Transfection	Turner et al. [62]
Human immunodeficiency virus	AIDS	ZFN	CCR5	CD4 + T cells, human	AAV	Perez et al. [63], Tebas et al. [64]

Defining the host factors that facilitate or restrict virus replication						
Virus	**Human disease**	**Type**	**Host factors studied**	**Cell/ animal/ human study**	**Delivery method**	**Reference**
(HIV-1)						
		CRISPR	CCR5	CD4 + T cells	Ad5F35 adenoviral vector	Li et al. [65]
Kaposi's sarcoma herpes virus (KSHV) or human herpes virus 8	Kaposi's sarcoma, primary effusion lymphoma	CRISPR	ORF45, RSK	SLK-iBAC cells	Lentiviral delivery method	Avey et al. [66]
Human papillo mavirus	Papilloma, cervical and other mucosal carcinoma	TALEN	E6, E7 ORFs	SiHa or HeLa cells, Mice	Transfection	Hu et al. [67]
		CRISPR	E6, E7 ORFs	HeLa cells	Transfection	Kennedy et al. [68]

Table 2. Summary of studies using DNA editing technology to define the host factors that facilitate or restrict virus replication.

2.3. Using engineered nucleases for gene therapy applications in specific viral pathogens

2.3.1. Human immunodeficiency virus (HIV)

In the last two decades, the availability of highly active antiretroviral therapy (HAART) capable of reducing HIV replication to undetectable level and prolonged survival has greatly improved the prognosis of infected patients. However, HIV-1 persists as a latent infection in a small number of resting $CD4^+$ memory T cells [47]. In these long-lived cells, intact integrated HIV-1 proviruses persist in a transcriptionally silent state that is refractory to both drugs and host immune responses. However, these memory T cells can be reactivated by an appropriate recall antigen, resulting in the induction of a productive viral replication cycle [47, 49]. If this occurs after drug treatment has been stopped, HIV-1 will rapidly spread through the available $CD4^+$ T cells and rekindle the same level of virus replication that was seen before antiviral drug treatment. Other disadvantages of such long-term therapy include limitations including high

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cost, patient compliance and side effects of long-term therapy, as well as emergence of drug resistance [84]. Therefore, there is a need to develop a more effective "cure" for HIV infection.

The approaches to purge the pool of latently infected cells have focused on two strategies. Some have attempted to activate latent HIV-1 proviruses using drugs, including histone deacetylase inhibitors and protein kinase C agonists [85]. However, so far, this strategy has not been proven able to activate HIV-1 in a high percentage of latently infected cells.

An alternative strategy would be to directly target and destroy latent proviruses using HIV-1-specific CRISPR/Cas combinations. Early attempts have shown that this is feasible and that latent proviruses can be excised from the host cell genome, and then destroyed, by cleavage in the HIV-1 long terminal repeat regions [86, 87]. In principle, the HIV-1 provirus is a perfect target for CRISPR/Cas, as there is only a single proviral copy in the infected cell, and in the presence of antiviral drugs, no spread of the virus is possible. The problem, however, is that latently HIV-1-infected T cells are scattered throughout the body and infecting all of these seems currently to be an insurmountable problem, especially as T cells are poor targets for AAV infection. This contrasts with HBV, HSV, and HPV, all of which are tightly localized in known tissues in the body that can be readily targeted by AAV [52]. Therefore, in the absence of novel vector delivery systems that can target latently HIV-1-infected cells throughout the body, HIV-1 is likely to remain a technically challenging target for elimination by CRISPR/Cas in vivo.

CCR5, which encodes a coreceptor for HIV entry [88, 89], has been a popular target for developing a new generation of HIV therapy for several reasons. First, its disruption seemed likely to increase the survival of CD4T cells; persons homozygous for a naturally occurring 32-bp deletion (delta32/delta32) in CCR5 are known to be resistant to HIV infection [90]. CD4 T cells from such persons are highly resistant to infection in vitro [91]. Persons who are heterozygous for CCR5 delta32 and HIV infection have slower progression to full-blown AIDS [92, 93]. The effectiveness of blocking or inhibiting CCR5 with the use of small interfering (siRNA) and other small-molecule inhibitors has been shown in humans [94]. Finally, there is the remarkable success story of HIV eradication in the so-called "Berlin Patient". This HIV-positive patient with lymphoma, who had been transplanted with bone marrow from a CCR5-Δ32 homozygous donor, became cured with no measurable virus (undetectable HIV RNA and proviral DNA in the blood, bone marrow, and rectal mucosa) even 5 years after transplantation, showing the potential benefits of CCR5 disruption [95, 96]. Although the mechanism responsible for the apparent cure associated with this procedure remains to be established, acquired CCR5 deficiency is one possibility [97]. Due to the low frequency of CCR5-Δ32 homozygotes in the general population and the difficulties of identifying suitable donors, alternative methods to artificially disrupt CCR5 are being sought [65]; in particular, gene editing methods have attracted a lot of attention as a potential therapy for HIV, as they allow permanent disruption of the selected gene(s).

This approach had recently led to a phase I clinical study published in the *New England Journal of Medicine* [64]. The researchers enrolled 12 patients in an open-label, nonrandomized, uncontrolled study of a single dose of ZFN-modified autologous CD4 T cells. Six of these 12 patients underwent an interruption in antiretroviral treatment 4 weeks after the infusion of 10

billion autologous CD4 T cells. Between 11% and 28% of these CD4 T cells were genetically modified by ZFN. The primary outcome was safety in terms of treatment-related adverse events. Secondary outcomes included measures of immune reconstitution and HIV resistance.

The only serious adverse event associated with infusion of the ZFN-modified CD4 T cells was attributed to a transfusion reaction. The median concentration of CCR5-modified CD4 T cells at 1 week was 250 cells/mm^3. This represented approximately 8.8% of circulating peripheral blood mononuclear cells and 13.9% of circulating CD4 T cells. These modified T cells had an estimated mean half-life of 48 weeks. During treatment interruption and the resultant viremia, the decline in circulating CCR5-modified cells (-1.81 cells/day) was significantly less than the decline in unmodified cells (-7.25 cells/day; p = 0.02; see **Figure 1**) [64]. HIV RNA became undetectable in one patient. The blood level of HIV RNA decreased in the majority of the study subjects. This provides one of the first evidence that CCR5-modified autologous CD4 T-cell infusions are safe and opens the door for applying DNA editing technologies for treating human chronic viral diseases, although many obstacles remain.

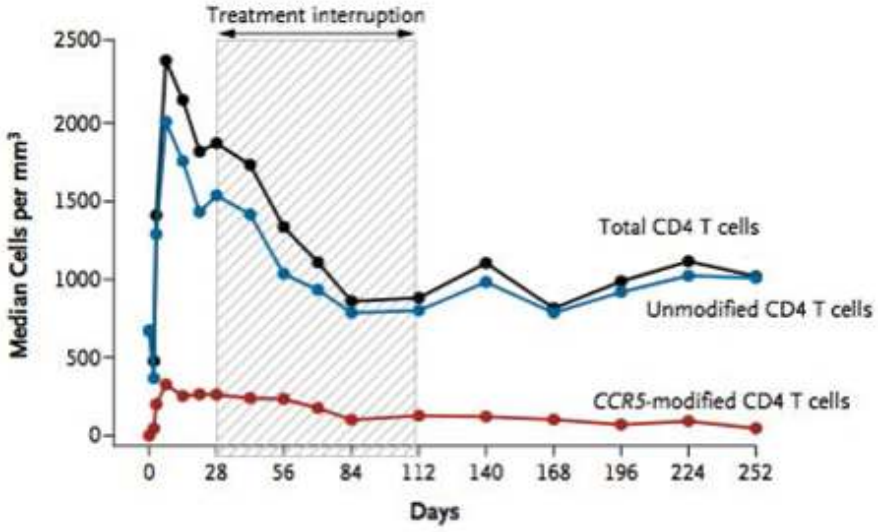

Figure 1. CCR5-modified CD4 T cells during treatment interruption [64].

2.3.2. Hepatitis B virus (HBV)

HBV remains a major public health problem, with more than 300 million people chronically infected worldwide [98]. These individuals have an approximately 25% risk of dying from the consequences of HBV infection, including hepatocellular carcinoma (HCC) and cirrhosis, and approximately 800,000 individuals are thought to die each year due to HBV. An effective vaccine for HBV is available, but this is not helpful in individuals with preexisting infection

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and is not fully effective at preventing vertical transmission, compounded by the problem that it is not given to all children in resource-limited regions timely. HBV polymerase can be effectively inhibited by nucleoside-based antiviral agents (lamivudine, adefovir, telbivudine, entecavir, tenofovir), but this does not cure this infection due to the extraordinary stability of the viral episomal cccDNA intermediate [99], which continue to persist and produce new viral particles, as soon as the antiviral agents were stopped.

Current research in HBV therapy focuses on finding new targets on the viral life cycle (see **Figure 2**) and trying to overcome immune tolerance through immunotherapy, with limited success. It is clear that a complete "cure" cannot be achieved unless the new therapy is able to remove or destabilize the HBV cccDNA, and genome editing is one of the most promising approaches. Some of the challenges that need to be overcome include toxicity, development of viral resistance, specificity, ensuring therapeutic effect of sufficient duration, and hepatocyte-targeted delivery. Significant progress has been made to overcome these obstacles.

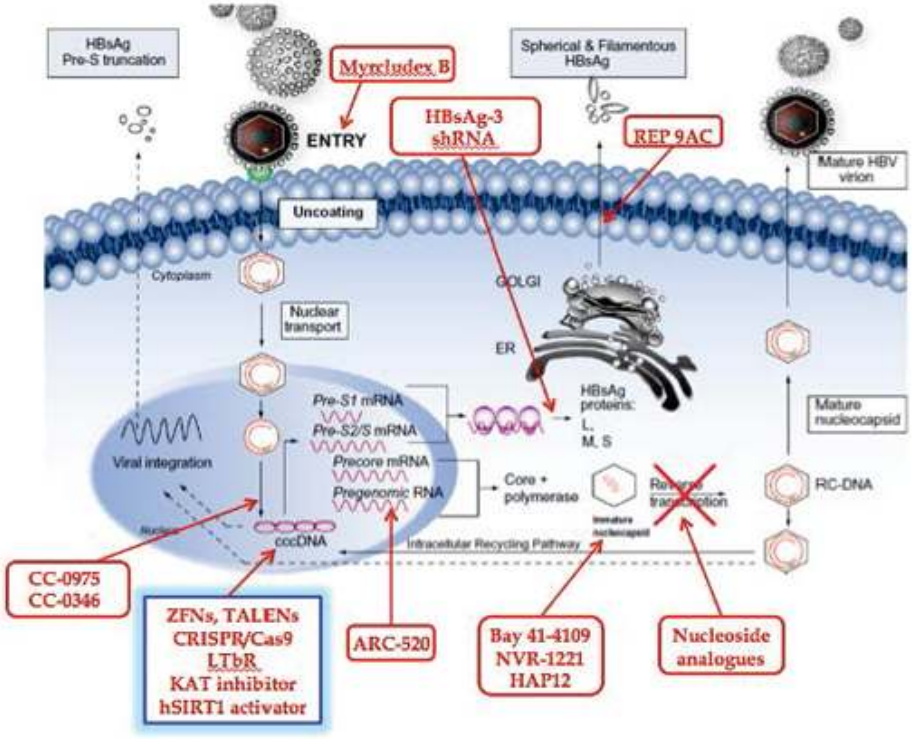

Figure 2. Replicative cycle of HBV and the respective targets of the current and experimental therapeutic agents (modified from Phyo et al. [100]).

Zimmerman et al. [101] were the first to advance a gene editing approach to countering HBV replication. The researchers used duck HBV (DHBV) as a model and designed six different zinc finger proteins (ZFPs) to target the DHBV enhancer sequences, which control the transcription of the core and surface sequences. Marked reduction in viral pgRNA and total viral RNA was observed. ZFPs significantly reduced viral core and surface protein production with no obvious cytotoxicity. As the ZFPs did not cause target DNA mutation or durable epigenetic changes, this inhibition was not lasting. Later, Cradick et al. [71] also demonstrated the effectiveness of using ZFNs to specifically cleave HBV episomal DNA. The team engineered nine pairs of HBV-specific ZFNs and cotransfected each of these ZFN pair plus an HBV genome target plasmid into a hepatoma cell line. Targeted cleavage of viral DNA was demonstrated. The cleaved fragments were misrepaired in a manner that could potentially inactivate HBV. Further, cotransfection with the ZFN pair 6 decreased HBV pregenomic viral RNA levels by almost 30%. However, it should be noted that the study did not clearly demonstrate that the HBV cccDNA was modified by the ZFNs.

The X gene is thought to play a major role in the development of HCC [102]. Zhao et al. [103] designed ZFPs to inhibit the expression of integrated sequences of the X gene. An artificial transcription factor (ATF) was synthesized to target a sequence in the enhancer I region, which is upstream of the X promoter. The ATF comprised a DNA-binding domain of a ZFP that was linked to a KRAB repressor domain. X repression was demonstrated on a luciferase reporter assay. Another study by Weber et al. [72] aimed to prevent viral reactivation by targeting three HBV protein-coding sequences with ZFNs in HepAD38 cells, a tet-regulated cell line. AAV vectors containing sequences encoding the ZFNs were used for gene delivery. Site-specific mutagenesis with low cytotoxicity was confirmed for two of three engineered ZFNs. Inhibition of HBV replication and virion production over a period of 14 days could be achieved after a single treatment with ZFN targeting the viral polymerase gene.

Bloom et al. [73] first applied TALENs to disrupt hepatitis B replication in a cell line and a mouse model. HBV-specific TALENs were generated, targeting conserved sequences in the surface, core, and pol open reading frames (ORFs). The TALEN targeting surface and core ORFs were the most effective. The viral cccDNA were isolated using Hirt's extraction and plasmid-safe DNase. T7 endonuclease I assay was used to verify targeted mutation in cultured cells. The TALEN targeting surface ORF reduced HBsAg by more than 90% and circulating viral particle equivalents were diminished by approximately 70% by the S and C TALENs. T7E1 assays and deep sequencing confirmed the targeted disruption. Chen et al. [74] confirmed the successful targeting and inactivation of HBV genomic sequences by TALENs. The researchers also showed significant knockdown in markers of viral replication. Interestingly, when used in combination with interferon-α, synergistic antiviral effects were observed. Although promising, a limitation of using mice to simulate HBV replication in vivo is that these animals do not produce HBV cccDNA.

Some key studies employing CRISPR/Cas9 systems recently demonstrated the utility of RGEN cleavage of HBV DNA [75–78]. Lin et al. [75] designed eight HBV-targeting sgRNAs to target different conserved regions of the HBV genome. A significant decrease in the production of viral proteins was observed. Cotransfection with more than one sgRNA-encoding sequence

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augmented antiviral efficacy. This effect was corroborated by an increase in indels at the targeted sites. However, the efficacy against HBV cccDNA was not evaluated. Seeger and Sohn [76] investigated the targeted disruption of HBV cccDNA and confirmed the efficient cleavage of viral sequences with all five of their sgRNA constructs. Approximately eightfold reduction of HBcAg expression was achieved in HBV-infected HepG2-NTCP cells. The cells were transduced using recombinant lentiviral vectors. Targeted deletions from single nucleotide up to 2.3 kb was produced. This demonstrated the potential of CRISPR/Cas to target and excise host-integrated HBV genomes. Another similar study by Kennedy et al. [77] also showed suppression of HBV replication by lentiviral vector-delivered Cas9 and sgRNA sequences. Dong et al. confirmed the efficacy of sgRNA-Cas9 against HBV. In addition, they demonstrated the disruption of artificial cccDNA in a murine hydrodynamic model [78] that was based on the use of engineered recombinant cccDNA precursor plasmid (rcccDNA) [104].

These studies suggest that HBV-specific Cas9/sgRNA combinations can block HBV replication and eliminate the cccDNA pool if they can be effectively delivered to hepatocytes. At the moment, AAV may be the best carrier for this task, as several AAV serotypes are naturally hepatotropic and even more highly hepatotropic AAV vectors have recently been isolated by "shuffling" AAV sequences in vivo [105]. The next step is to examine whether AAV-delivered Cas9/sgRNA combinations can effectively cure HBV in the humanized, immunodeficient mouse liver model system.

2.3.3. Herpes simplex virus (HSV)

HSV-1 infects approximately 70% of the U.S. population and about one third of affected individuals suffer from recurrent, primarily oral, cold sores. HSV-1 most commonly initially infects the oral mucosal epithelium, leading to a local productive infection, and then undergoes retrograde transport to the trigeminal ganglia, where it establishes a latent infection in a small number of sensory neurons, which persists after the initial, productive infection is cleared by the host immune response [106]. During latency, the HSV-1 DNA genome is maintained as a nuclear episome, with 1 to ~50 copies per latently infected neuron. At this point, the only region of the genome that is actively transcribed encodes the LAT, which is processed to give rise to a single long noncoding RNA of 2.1 kb, as well as eight virally encoded miRNAs, which together are thought to regulate exit from latency [107]. Because no viral proteins are made, there is no immune recognition of latently infected cells. Occasionally, one or more latently infected neuron is activated to produce infectious virions that migrate down the axons of the reactivating neuron to the original site of infection, where they reestablish a transient productive infection that can lead to the formation of cold sores. Although often no more than an irritation, HSV-1 infections can also lead to serious morbidity and HSV-1 keratitis represents the most common form of infectious blindness in the West [108]. Infection of the central nervous system may also cause fatal encephalitis. A closely related virus, HSV-2, which is found in approximately one fifth of the U.S. population, has a similar replication cycle but generally is sexually transmitted and infects the genital mucosa. Latency is established in sensory neurons of the sacral ganglia and reactivation can lead to genital ulcers. Again, serious

morbidity is rare but does occur in some individuals and neonatal HSV-2 infections acquired during vaginal delivery can be fatal [106].

Although there are several drugs that can treat productive HSV-1 or -2 infections, generally by targeting viral DNA synthesis, latent HSV genomes are entirely refractory to current treatment regimens and it remains impossible to cure these infections. What is clearly needed is an approach that directly targets HSV-1 or HSV-2 episomal DNA for cleavage and elimination from latently infected neurons. AAV-delivered HSV-specific engineered endonucleases appear ideal for this purpose. Aubert et al. [79] introduced DNA double-stranded breaks in an HSV latency model using the engineered HE HSV1m5, which targets a sequence in the HSV-1 gene UL19, encoding the viral protein VP5. Coexpression of the 3′-exonuclease Trex2 with HEs increased the mutagenesis frequencies by up to sixfold. Following HSV1m5/Trex2 delivery with AAV vectors, the target site within latent HSV genomes was mutated. There was no detectable cell toxicity. The viral production by latently infected cells after reactivation was significantly decreased. Prior HSV1m5/Trex2 treatment followed by exposure to histone deacetylase inhibitors increased mutagenesis frequencies of latent HSV genomes by another twofold to fivefold. This indicates that chromatin modification may be a useful adjunct to gene-targeting methods. Using CRISPR/Cas9-mediated genome engineering to create single- and double-knockout (KO) cell lines, Turner et al. [62] discovered that the Torsin Activator LULL1 is required for efficient growth of HSV-1, whereas Johnson et al. [61] reported that the interferon-γ-inducible factor 16 (IFI16) restricts HSV-1 replication by accumulating the HSV-1 genome and repressing HSV-1 gene expression, and modulates histone modifications. Given the tight localization of HSV-1 and -2 to the trigeminal and sacral ganglia, respectively, low level of viral DNA genomes present in these cells, and the ability to efficiently transduce sensory neurons with AAV8-based vectors, this seems like an ideal viral candidate for cure using RGENs.

2.3.4. Human papillomavirus (HPV)

HPV infection, although normally innocuous, can also give rise to warts on the skin or genitalia [31]. Most HPV variants replicate as episomes in the basal epithelial layer of the skin, where the virus expresses exclusively nonstructural proteins. When the infected precursor epithelial cell migrates toward the surface of the epidermis and undergoes differentiation into a keratinocyte, the productive HPV replication cycle is activated leading to the release of infectious HPV virions (**Figure 3**) [31].

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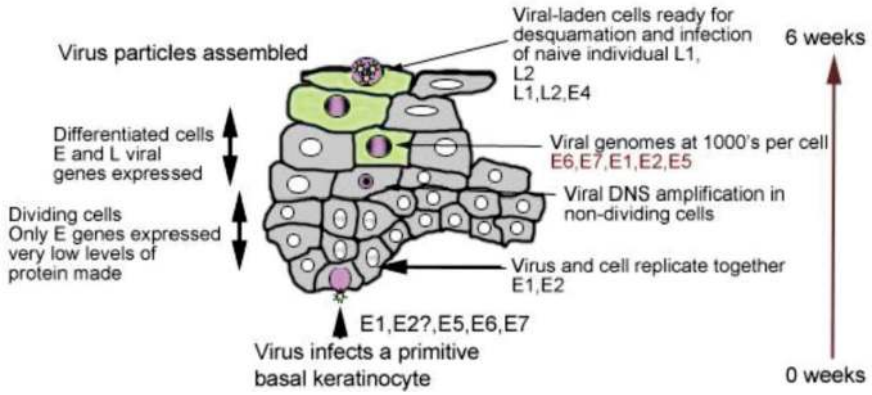

Figure 3. HPV penetrating the basal layer and released at the epithelial surface (Medscape [109]).

Although most HPVs are nonpathogenic, there are a small number of high-risk HPV serotypes, especially HPV-16 and -18, which together cause approximately 70% of all cervical cancers. In most HPV-induced cancers, the HPV episome is found clonally integrated into the cell genome in a manner that destroys or deletes the viral E2 gene (E for early) [31]. The role of the E2 protein is to bind to the HPV origin of replication, where it functions to ensure the distribution of HPV episomes to both daughter cells after cell division, and E2 also acts to regulate HPV early gene transcription. One key activity of E2 is to limit the expression of the HPV oncogenes E6 and E7, and disruption of E2 during integration into the host cell genome can lead to high constitutive levels of E6 and E7 expression [110]. E6 functions to bind and destabilize the p53 tumor suppressor [111], whereas E7 similarly binds and destabilizes the Rb tumor suppressor [112], and these two functions play a critical role in the maintenance of HPV-transformed cells. Cancers associated with HPV infection include cervical carcinoma, which is almost always HPV positive, as well as a fraction of head and neck (H&N) carcinoma and anal cancer, all of which are related to sexual transmission of HPV. Novel treatment for chemoresistant HPV-positive tumors will be important for treating recurrent disease. Of note, almost all HPV-positive H&N and anal cancers are HPV 16 positive, thus restricting the required sequence range for engineered endonuclease-based therapy.

Both HPV E6 and E7 proteins play a crucial role in HPV tumorigenesis by blocking the action of p53 and Rb, respectively [112]. Consistent with this idea, the inactivation of the E6 gene in the HPV-18-positive cervical carcinoma cell line HeLa or the HPV-16-positive cell line SiHa using Spy CRISPR/Cas has been found to result in the induction of p53 expression followed by the expression of downstream targets of this cellular transcription factor, including the CDK inhibitor p21 and several activators of apoptosis, leading to cell cycle arrest and cell death [68, 82]. Similarly, disruption of the E7 gene using CRISPR/Cas results in the increased expression of Rb, formation of Rb/E2F heterodimers, and then the induction of cellular genes that induce senescence and cell death [68, 113]. Mino et al. [81] improved the design of a ZFN-based hybrid

nuclease to the single-chain FokI dimer (scFokI) and demonstrated that it inhibited HPV-18 DNA replication in transient replication assays using mammalian cells more efficiently. By linker-mediated PCR analysis, the researchers confirmed that AZP-scFokI cleaved an HPV-18 ori plasmid around its binding site in mammalian cells. These studies suggest that targeted endonucleases specific for HPV E6 and/or E7 has the potential to serve as a novel, highly specific, and effective therapy for chemoresistant HPV-16 induced anal and H&N tumors.

2.3.5. Other viruses

A number of other chronic virus infections are associated with serious human diseases including EBV, cytomegalovirus, HCV, Kaposi's sarcoma-associated herpes virus (KSHV), human T-cell leukemia virus type 1 (HTLV-1), and Merkel cell polyomavirus (MCPyV) (see **Tables 2** and **3**). Of these, perhaps the most relevant in relation to DNA editing technology is EBV. EBV is the etiologic agent of several cancers, including an epithelial cell tumor called nasopharyngeal carcinoma (NPC), which is highly prevalent in southern China and Southeast Asia [40, 52]. In NPC cells, EBV is found in a form of viral latency that nevertheless involves the expression of several viral nonstructural proteins and microRNAs [114]. EBV-positive NPCs share a number of characteristics with HPV-16-positive H&N cancers, and as in the latter case, the continued presence and transcription of the viral (in this case, EBV) genome is thought to be essential for tumor survival. Wang and Quake [70] used the CRISPR/Cas9 system for antiviral therapy in human cells, specifically targeting the EBV genomes of latent viral infections. Patient-derived Burkitt's lymphoma cells with latent EBV infection showed significant proliferation arrest and decrease in viral load after treatment with specific CRISPR/Cas9 vector targeting the viral genome. It seems likely that NPC cells would be excellent targets for transduction in vivo using Sau Cas9/sgRNA-based AAV vectors specific for the EBV genome.

Progressive multifocal leukoencephalopathy (PML) is a fatal demyelinating disease of the central nervous system caused by human polyomavirus JC (JCV) reactivation. JCV replicates in oligodendrocytes, the myelin-producing cells in the brain. Previously a rare disease seen in patients with lymphoproliferative and myeloproliferative disorders, PML is now seen more frequently in HIV-1-positive/AIDS patients and patients undergoing immunomodulatory therapy due for rheumatological/autoimmune disorders [83]. At this time, there is no cure for PML, and in most cases, disease progression leads to death within 2 years. The JCV genome is a small circular double-stranded DNA that includes coding sequences for the viral early protein, T-antigen, which is critical for directing viral reactivation and lytic infection. Wollebo et al. [83] applied CRISPR/Cas9 system to introduce mutations in the viral genome to inactivate the gene encoding T-antigen and inhibit viral replication. Transient or conditional expression of Cas9 and gRNAs specifically targets the N-terminal region of T-antigen on integrated genetic and functional studies. The mutation introduced interferes with the expression and function of the viral protein, suppressing viral replication in vitro [83]. There was no off-target effect of the JCV-specific CRISPR/Cas9 editing apparatus observed. These studies provide the first evidence for the employment of a gene editing strategy as a promising tool for the elimination of the JCV genome and a potential cure for PML.

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HTLV-1, which causes adult T-cell leukemia (ATL) in humans, establishes a lifelong latent infection. Current therapies are not very effective against HTLV-1-associated disorders. In a proof-of-concept study, Tanaka et al. [80] developed a targeted endonuclease based on zinc finger nuclease (ZFN) that specifically recognized a conserved region of HTLV-1 long terminal repeat (LTR) and introduced it into various HTLV-1-positive human T-cell lines, including HTLV-1-transformed and ATL-derived cell lines [80]. ZFN disrupted the promoter function of HTLV-1 LTR and specifically killed HTLV-1-infected cells. The researchers showed the first evidence of the removal of the proviral genome from HTLV-1-infected cells. The therapeutic effect of ZFN was confirmed in an in vivo model of ATL. This strategy may form the basis of a therapy that can eradicate HTLV-1 infection, and similar approaches can be used to target other malignancy-associated viruses.

Using DNA editing technology to inactivate or mutate DNA virus genomes

Virus	Human disease	Type	Target	Cell/animal/ human study	Delivery method	Reference
Endogenous retroviruses (ERV)	Unknown	ZFN, CRISPR	Pol	ATCC CRL-33, PK15 Pig ATCC CRL-1573, human	Transfection	Semaan et al. [69], Mali et al. [6], Cong et al. [5]
Epstein–Barr virus (EBV)	Burkitt's lymphoma, NPC, non-Hodgkin's lymphomas, oral hairy leukoplakia, lymphoproliferative disease	CRISPR	EBNA1	Raji cells, human	Transfection	Wang et al. [70]
Hepatitis B virus (HBV)	Liver cirrhosis, HCC	ZFN	Pol, Core, X	Huh7 cells, HepAd38 cells	Transfection, AAV vectors	Cradick et al. [71], Weber et al. [72]
		TALEN	Surface, Core, Pol	HepG2.2.15, mouse	Transfection, HDI	Bloom K et al. [73]
		TALEN	Core	Huh7 cells	Transfection	Chen et al. [74]
		CRISPR	Core, X	HepG2/NTCP cells	Lentiviral vectors	Lin et al. [75], Seeger et al. [76], Kennedy et al. [77], Dong et al. [78]
Herpes simplex type 1 (HSV-1)	Core sores, encephalitis, pneumonitis, hepatitis	Homing endonuclease (HE) HSV1m5	HSV-1 gene UL19	Primary human fibroblast	AAV vectors	Aubert et al. [79]

Using DNA editing technology to inactivate or mutate DNA virus genomes						
Virus	**Human disease**	**Type**	**Target**	**Cell/animal/ human study**	**Delivery method**	**Reference**
Human T cell leukemia virus type 1 (HTLV1)	Adult T cell leukemia (2–6% of carriers), tropical spastic paraparesis, myelopathy, uveitis, dermatitis	ZFN	HTLV-1 LTR	T-cell and ATL-derived cell lines		Tanaka et al. [80]
Human Papilloma virus	Papilloma, cervical and other mucosal carcinoma	ZFN	HPV-18 ori	293H cells	Transfection	Mino et al. [81]
		CRISPR	E6, E7 ORFs	SiHa cells, mice	Transfection	Zhen et al. [82]
Polyomavirus JC	Progressive multifocal leukoencephalopathy	CRISPR	JCV T-antigen gene	HJC-2, Hamster	Transfection	Wollebo et al. [83]

Table 3. Summary of studies using DNA editing technology to inactivate or mutate DNA virus genomes.

3. Conclusion

The development of effective gene editing technologies has the potential to lead to the global identification of almost all cellular factors that regulate virus replication in culture, leading to a wealth of new insights into viral molecular biology and producing numerous potential targets for antiviral drug development. If such screens can indeed identify cellular factors that are required for virus replication but entirely dispensable for the health of the adult target organism, then it might be possible to also treat viral infections, including RNA virus infections, via the localized ablation of a cellular gene as described above for direct targeting of DNA virus genomes. A similar approach has previously been reported using ZFNs to inactivate the HIV-1 coreceptor CCR5, to prevent the infection of $CD4^+$ T cells, by transduction of hematopoietic stem cells followed by an analysis of the production of HIV-1-resistant $CD4^+$ human T cells in engrafted immunodeficient mice [115, 116]. The challenges remain in applying engineered nucleases in the clinic setting, including immunogenicity of transduced proteins, with repeated application, delivery of the genes into the correct tissue/cell types, side effects of off-target mutagenesis, limitations in the design of the nuclease target sites, ethical issues, including tumorigenicity, undesired integration of nucleases or donor templates, and the germline transmission of the modified genome. One interesting and novel approach is to deliver engineered nuclease using modified mRNA, which is nonintegrating and provides a transient pulse of protein expression, as an alternative to traditional viral vectors. A team of researchers had recently applied this nuclease-encoding, chemically modified (nec) mRNA to deliver site-specific nucleases in a transgenic mouse model of SP-B deficiency, resulting in successful site-specific genome editing in vivo [117, 118]. Although several technical challenges and uncertainties remain, the promise of using gene correction technology to study and treat

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chronic viral infections is tremendous. Further advances in understanding and improvements in technology will open the next era of therapy against currently difficult-to-treat viral diseases.

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