Published in final edited form as:

Hum Genet. 2016 September ; 135(9): 1071–1082. doi:10.1007/s00439-016-1686-2.

Genome editing and the next generation of antiviral therapy

Daniel Stone¹, Nixon Niyonzima^{1,3}, and Keith R. Jerome^{1,2}

Daniel Stone: dstone2@fredhutch.org; Nixon Niyonzima: nniyonzi@fredhutch.org; Keith R. Jerome: kjerome@fhcrc.org, kjerome@fredhutch.org

¹Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

²Department of Laboratory Medicine, University of Washington, Seattle, WA, USA

³Graduate Program in Molecular and Cellular Biology, University of Washington, Seattle, WA, USA

Abstract

Engineered endonucleases such as homing endonucleases (HEs), zinc finger nucleases (ZFNs), Tal-effector nucleases (TALENs) and the RNA-guided engineered nucleases (RGENs or CRISPR/Cas9) can target specific DNA sequences for cleavage, and are proving to be valuable tools for gene editing. Recently engineered endonucleases have shown great promise as therapeutics for the treatment of genetic disease and infectious pathogens. In this review, we discuss recent efforts to use the HE, ZFN, TALEN and CRISPR/Cas9 gene-editing platforms as antiviral therapeutics. We also discuss the obstacles facing gene-editing antiviral therapeutics as they are tested in animal models of disease and transition towards human application.

Introduction

Over the last decade, the tools available for DNA manipulation have advanced significantly. A wide array of powerful gene-editing systems is now available, and researchers new to the field can easily use these tools with little instruction. Gene manipulation is being widely used in both basic research and the development of novel therapies, and it has become commonplace to knock genes in and or out of cells, or to manipulate the genomes of viruses, single cell organisms, and even complex vertebrates. Subsequently, the number of gene-editing publications in recent years has soared.

The workhorses of the gene-editing field are the targeted endonucleases. Targeted endonucleases are engineered enzymes that can introduce DNA double strand breaks (DSBs) with high specificity into desired target sequences. Several site-specific endonuclease platforms are now available, and their ability to precisely cleave a desired locus is being used to disrupt genes via mutagenic non-homologous end joining (NHEJ) or to direct homologous recombination mediated gene insertion/correction. Endonuclease

Correspondence to: Keith R. Jerome, kjerome@fhcrc.org, kjerome@fredhutch.org.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

platforms including meganucleases/homing endonucleases (HEs), zinc finger nucleases (ZFNs), Tal-effector nucleases (TALENs), and the RNA-guided engineered nucleases (RGENs) such as CRISPR/Cas9 can be used for gene editing, and they are discussed in greater detail elsewhere (Mussolino and Cathomen 2012; Rahman et al. 2011; Sander and Joung 2014; Stoddard 2011). Importantly, gene-editing tools like the targeted endonucleases are now being developed as therapeutics that can impact many types of disease at the genetic level.

Despite advances in modern medicine, a broad spectrum of infectious diseases pose a serious global health burden. Many pathogens currently have no vaccine, and cannot be treated with antimicrobial drugs. Other pathogens may be preventable through vaccination or treatable with drugs, but remain a serious health concern due to the chronic nature of the illness, or the lack of access to basic health care within the populations most at risk. Subsequently, much effort is required to develop new or alternative therapies that can be used to prevent or treat numerous serious diseases.

In this review, we introduce the recent development of gene-editing technologies as therapeutics to eliminate persistent/chronic viral infections. Hundreds of millions of people are affected worldwide by debilitating or life-threatening persistent viral infections (Boldogh et al. 1996; Kane and Golovkina 2010), and gene-editing technology provides a method to target therapeutics towards distinct viral targets. Since the first report of an endonuclease therapy that targeted the hepatitis B virus in 2010 (Cradick et al. 2010), more than 30 subsequent publications have described gene-editing therapeutics targeting the long-lived forms of viral nucleic acid that enable viral persistence. These therapies now offer the potential to functionally cure several chronic viral infections when used either alone, or in combination with other novel or existing antiviral therapies. Here we review efforts directed towards persistent viruses other than HIV, which will be discussed elsewhere in this special issue (Benjamin et al. 2016).

The current status of gene-editing antiviral therapies

Hepatitis B virus

Despite the development of an effective vaccine over 30 years ago, it is estimated that more than 240 million people globally are chronically infected with the hepatitis B virus (HBV), and more than 780,000 people are thought to die each year due to complications of chronic HBV infection (Lozano et al. 2012; WHO 2015a). A significant number of patients with chronic HBV develop life-threatening cirrhosis, and the high incidence of hepatocellular carcinoma caused by HBV makes it the second most common cause of infection-related cancer (de Martel et al. 2012). Although several drugs can be used to suppress HBV replication, they are not curative and can only slow down disease progression. HBV is a partially double-stranded DNA enveloped virus that causes life-long infections in approximately 5–10 % of infected individuals. Chronic HBV infections are maintained by the covalently closed circular DNA (cccDNA) HBV ‘minichromosome’, which accumulates in infected hepatocytes and acts as a permanent template for virus replication. Due to the life-long persistence of cccDNA in chronically infected patients, cccDNA elimination is a requirement for potential curative therapies.

The first study to propose that a targeted endonuclease could be used to cleave and disrupt viral genomic DNA was performed by Cradick et al. (2010). They were able to demonstrate that designer ZFNs targeting HBV genes could introduce mutations at the target site in a HBV-containing plasmid and inhibit production of pregenomic viral RNA in an in vitro model of replication. Since this first study, numerous other groups have employed similar strategies to target HBV. The first follow-up studies showed antiviral activity in vitro by ZFNs delivered using adeno-associated virus (AAV) vectors (Weber et al. 2014b), or by TALENs in vitro and in a mouse hydrodynamic injection model of HBV lacking cccDNA (Bloom et al. 2013; Chen et al. 2014). Importantly, the in vitro studies using TALENs showed for the first time that HBV cccDNA could be cleaved and disrupted directly.

More recently the CRISPR/Cas9 system has been utilized as a therapeutic to target HBV. CRISPR/Cas9 is able to inhibit HBV replication and target cccDNA in a number of in vitro models (Karimova et al. 2015; Kennedy et al. 2014a; Liu et al. 2015a; Ramanan et al. 2015; Seeger and Sohn 2014; Wang et al. 2015). It has also shown the ability to suppress HBV antigen expression from plasmids in a mouse hydrodynamic injection model of HBV that lacks cccDNA (Lin et al. 2014; Zhen et al. 2015). More recently it was demonstrated that a recombinant form of cccDNA called rcccDNA could be cleaved, disrupted, and/or cleared from mouse liver when Cas9 and guide RNAs were delivered using plasmids (Dong et al. 2015). In this new hydrodynamic mouse model rcccDNA enables viral persistence (Qi et al. 2014), making the work of Dong et al. the most comprehensive in vivo demonstration of an efficacious gene-editing HBV-directed therapeutic.

As endonuclease-based approaches to treat HBV are developed further, it will be important to assess their antiviral activity in more relevant in vivo models of HBV infection. HBV cannot fully replicate in mice, and several humanized mouse models with chimeric livers have been developed to study human liver diseases including HBV (Dandri and Lutgehetmann 2014). In the future the efficacy of HBV-specific endonucleases should be tested in such a model with fully replicating HBV. However, it is important to note that humanized liver models of HBV are limited by the lack of a functional immune system, which plays a significant role in the control of (or lack of) natural HBV infections (Ferrari 2015; Yoshio and Kanto 2016). Other aspects of treatment will also need to be addressed such as enzyme delivery, enzyme dosing, development of cleavage resistance, and the levels of intrahepatic cccDNA (Schiffer et al. 2013).

Epstein–Barr Virus

The Epstein–Barr Virus (EBV) is a large linear dsDNA gamma herpesvirus, to which more than 95 % of adults have been exposed (Kutok and Wang 2006; Longnecker et al. 2013). EBV causes infectious mononucleosis and is associated with a number of malignant tumors including Burkitt lymphoma, nasopharyngeal carcinoma, Hodgkin lymphoma and non-Hodgkin lymphoma. After infection, lytic replication of EBV first occurs in epithelial cells on mucosal surfaces, but the main targets of infection are B-lymphocytes where EBV establishes a lifelong latent infection. In latently infected B cells, the linear genome circularizes to form a long-lived episome that provides a template for viral persistence. There is currently no cure for EBV, so a gene-editing strategy that targets the latent viral

episome for disruption would be an attractive therapeutic strategy to prevent reactivation from latency.

The CRISPR/Cas9 system has been used as a therapeutic to target EBV. Wang and Quake were able to target latent EBV genomes in a Burkitt Lymphoma cell line and demonstrated inhibition of viral replication and cellular proliferation (Wang and Quake 2014). Guide RNAs were targeted to EBNA1 to excise EBNA1 or inhibit gene regulation and replication, to EBV repeats to promote genome shredding, or to EBNA3C and LMP1 to inhibit cellular transformation. By combining a total of 7 sgRNAs they demonstrated a 65–85 % reduction in EBV viral loads in Raji cells. They also demonstrated restoration of apoptosis in treated cells and arrest of cellular proliferation. CRISPR/Cas9 treatment caused shredding of the viral genome, repressed functions supplied by targeted genes, and inhibited viral replication. In another study, Yuen et al. (2014) targeted the EBV BART region using CRISPR/Cas9. They were able to delete a segment of the BART promoter region and this led to a reduction in the miRNAs produced from BART, and also led to a reduction in the viral load. The miRNAs and protein products from the BART region are involved in cellular transformation and inhibition of apoptosis (Marquitz et al. 2011). Disruption of these viral genes can prevent re-activation from latency, cellular transformation and can potentially be curative. The encouraging data from these two studies suggests that the dsDNA EBV episome could be effectively targeted with an antiviral gene-editing therapeutic.

Herpes simplex virus

Herpes simplex viruses (HSV-1 and HSV-2) are neurotropic alpha herpesviruses that cause oral and anogenital ulceration, ocular disease, and in rare cases encephalitis. In different parts of the world an estimated 60–90 % of the population has been exposed to HSV-1 (Smith and Robinson 2002) and 10–80 % have chronic HSV-2 infections (Looker et al. 2015; Xu et al. 2002). Upon initial infection, HSV undergoes lytic replication in epithelial cells at mucosal surfaces. From there, HSV enters sensory nerve endings and travels via retrograde axonal transport to neuronal cell bodies in the trigeminal or dorsal root ganglia where it establishes life-long latency (Nicoll et al. 2012; Subak-Sharpe and Dargan 1998). In sensory neurons and autonomic ganglia, the HSV genome forms a long-lived circular viral episome that acts as the template for viral reactivation. HSV is able to sporadically reactivate from latency, which can lead to symptomatic disease at the peripheral site of infection. There is also frequent subclinical HSV shedding that is asymptomatic, but may be important in disease transmission (Mertz et al. 1992). There is currently no vaccine for HSV (Koelle and Corey 2003), but HSV can be treated with acyclovir, which inhibits lytic HSV replication, but does not cure latent HSV infections or prevent reactivation from latency. Therefore, a treatment for HSV that prevents reactivation from latency would be highly beneficial.

Curative therapies for HSV must eliminate or inactivate the HSV episome in sensory ganglia. Recently, three groups demonstrated that engineered meganucleases targeting the UL19 gene of HSV could disrupt the HSV genome and inhibit viral replication. Grosse et al. (2011) first demonstrated that meganuclease-mediated disruption of the HSV genome could inhibit lytic replication of HSV in vitro. Aubert et al. (2014) then showed that latent HSV genomes could be targeted for disruption by an HSV-specific meganuclease using a primary

human fibroblast model of HSV latency. In this model of latency, disruption of the HSV genome was concomitant with reduction in viral titers following HSV reactivation. Finally, Elbadawy et al. used a HSV-specific meganuclease to introduce mutations into HSV genomes in explanted human corneas (Elbadawy et al. 2014). A 23.4 % reduction in HSV viral load was seen after treatment with HSV-specific meganuclease compared to controls. These data suggest that targeted disruption of latent HSV genomes in sensory ganglia could prevent reactivation from latency and offers hope for a sterilizing cure for HSV.

Human papillomavirus

The human papillomavirus (HPV) is a member of the extensive papillomavirus family of DNA viruses. Papilloma viruses infect basal cells of stratified epithelium within skin or mucous membranes, causing benign papillomas or pre-malignant lesions. HPV is the leading cause of infection-related cancer in humans (de Martel et al. 2012), and is directly responsible for more than 70 % of some prevalent cancers such as cervical cancer (WHO 2015c). An effective preventive vaccine that targets several high-risk types of HPV has been available for approximately 10 years, but vaccine use worldwide has been low and there remains a serious need for therapeutic interventions in patients who have already been exposed to HPV. After initial infection, the HPV genome forms a highly stable double-stranded DNA episome within an infected cell, and this episome enables HPV to establish a latent infection. As latent HPV infections can be present for years before viral reactivation occurs, it is important that new therapies efficiently target the viral episome for clearance or disruption.

As a virus that is responsible for a substantial disease burden with a well-characterized life cycle, HPV represents an attractive target for therapeutic gene editing. The HPV E6 and E7 viral proteins have long been known to be oncogenic, and therefore represent excellent targets for therapeutic endonucleases. The first attempts to target HPV with endonucleases used ZFNs that were designed to target the E6 or E7 genes of the high-risk HPV types 16 or 18 (Ding et al. 2014; Mino et al. 2013, 2014). These studies demonstrated that ZFNs could be used to disrupt HPV genomes in in vitro culture models and HPV-positive cervical cancer cell lines. Importantly, one of these studies also showed that ZFNs could be used to inhibit growth of HPV-positive tumor xenografts (Ding et al. 2014), demonstrating their potential uses as anti-cancer therapeutics. In another comprehensive study, it was demonstrated that TALENs could also be used as anti-HPV therapeutics (Hu et al. 2014a). TALENs targeting the E6 and E7 genes of HPV-16 and HPV-18 showed efficacy in several HPV in vitro models, and importantly were able to reverse the HPV-driven malignant phenotype of K14-HPV16 transgenic mice that are immune competent.

Recently the CRISPR/Cas9 system has also been used as an anti-HPV therapeutic, and reagents have been developed to target the E6 or E7 genes of the high-risk HPV types 6, 11, 16 or 18. CRISPR/Cas9 has shown anti-HPV efficacy in a number of in vitro cell culture models, and in an HPV-positive tumor xenograft model (Hu et al. 2014b; Kennedy et al. 2014b; Liu et al. 2015b; Yu et al. 2015; Zhen et al. 2014). Efficient restoration of the p53 pathway and up-regulation of pRb was shown, indicating that the functions of the HPV E6

and E7 viral proteins were being disrupted. Taken together this data suggests that the persistent HPV episome could be a viable target for antiviral endonuclease therapy.

Human T cell leukemia virus

Human T-cell leukemia virus (HTLV) is a delta retrovirus that can cause adult T-cell leukemia/lymphoma (ATLL) and is also linked with tropical spastic paresis (TSP), also known as HTLV-associated myelopathy (Gessain et al. 1985; Kondo et al. 1989; Osame et al. 1986; Poiesz et al. 1980). HTLV has also been associated with a variety of other inflammatory disorders including myositis, uveitis, and dermatitis. Upon infection of T lymphocytes, HTLV integrates its proviral genome into the host cell genome and establishes viral latency, only undergoing sporadic stochastic reactivation. To enable it to establish a life-long infection, the latent HTLV proviral genome does not express any viral proteins, and this helps prevent HTLV-infected cells from being cleared by the immune system. There are currently no treatment interventions for either acute or chronic HTLV infections.

The HTLV proviral genome is an attractive target for gene-editing enzymes, as disruption of the integrated HTLV viral genome could prevent reactivation from latency and potentially be curative. HTLV has been targeted for disruption by Tanaka et al, who developed ZFNs that target highly conserved regions of the HTLV long terminal repeats (Tanaka et al. 2013). They were able to demonstrate that ZFNs could introduce mutations in the LTR that inhibited LTR-driven HTLV gene expression. Furthermore, ZFN expression inhibited cellular proliferation in HTLV-1 transformed cell lines, and inhibited tumor growth in a xenograft model that used the HTLV positive adult T cell leukemia ED cell line. These results demonstrated the potential of endonucleases as therapeutics targeting integrated proviruses during retrovirus infections.

JC polyoma virus

JC polyoma virus (JC virus) is a double-stranded DNA virus of the family Polyomaviridae that was first isolated from an immunocompromised patient with progressive multifocal leukoencephalopathy (Padgett et al. 1971). JC virus is not known to cause disease pathology in immune-competent hosts, but is associated with disease in immunocompromised hosts including patients with cancer, HIV, inflammatory disorders, or receiving immunomodulatory therapies. The global prevalence of JC virus ranges between 20 and 60 % depending on the serological assay used (Egli et al. 2009; Gossai et al. 2016; Knowles 2006), and there are currently no curative therapies or vaccine for JC virus.

Gene-editing technologies offer a potential therapy for the eradication of persistent JC virus infections. Wollebo et al. (2015) demonstrated that JCV T-antigen expression could be inhibited using the CRISPR/Cas9 system and three guide RNAs that target segments of the JC virus genome encoding the T antigen. They were able to suppress T-antigen expression in SV40 or JC virus transformed cell lines and inhibit JC virus replication in permissive cell lines. The observations from this study are important, as some polyoma virus T-antigens are involved in cell transformation, and gene-editing therapeutics could therefore prevent the pathogenesis of polyoma virus associated tumors (Gupta et al. 2016).

Hepatitis C virus

Hepatitis C virus (HCV) is a positive-sense single-stranded RNA virus from the flavivirus family that is associated with hepatitis, liver fibrosis, cirrhosis and development of hepatocellular carcinoma. Almost 80 % of people who get infected with HCV go on to develop chronic infections, and worldwide an estimated 110–180 million people have chronic HCV (Gower et al. 2014; Messina et al. 2015; WHO 2015b). It is estimated that approximately 500,000 people die each year from complications that develop during chronic HCV infection. Until recently the mainstay of treatment for HCV was ribavirin and pegylated interferon alpha, but new direct acting antiviral (DAA) therapies were recently approved by the FDA that have enabled high cure rates in patients receiving therapy. Unfortunately, even after cure the chance of re-infection remains high for those with recurrent risk of exposure. Therefore, a therapy against HCV with long-lasting activity would be beneficial.

Unlike the other viruses described in this review, HCV is an RNA virus. Subsequently, the targeting of viral genomes in HCV infected cells with endonucleases would seem problematic, because of the DNA-specific nature of nucleic acid cleavage performed by most classes of endonuclease. However, a recent study targeting the highly conserved 5'UTR and 3'UTR regions of HCV with gene-editing therapeutics offered a viable treatment alternative. Price et al. (2015) investigated the impact of HCV UTR disruption on virus replication with the CRISPR/Cas9 system. Using a modified Cas9 from *Franciscella novicida* (FnCas9) they generated guide-RNAs that are complementary to the 5'UTR regions of the HCV genome. They showed up to a 40 % reduction in the translation of HCV proteins following treatment in Huh-7.5 human hepatocellular carcinoma cells. They also demonstrated that RNA targeting with CRISPR/Cas9 does not have the same requirements for a PAM sequence as DNA targeting, and that a nuclease-deficient FnCas9 with target guide RNAs could disrupt translation of viral products through binding to target RNA species. Although the antiviral effects seen were RNA cleavage independent and not strictly caused by gene editing, this study has opened up the possibility of using gene-editing reagents as antiviral therapeutics for RNA viruses. In the light of recent advances in DAA therapies that cure a high proportion of infected patients but not all, a gene-targeting therapy with efficacy against DAA-resistant HCV genotypes could provide a therapeutic pathway forward.

Hurdles to human application

New gene-editing reagents are developed almost daily, and the outlook for future therapeutic interventions is bright. However, a number of hurdles still remain before reagents that target persistent/chronic viral infections can be transitioned into the clinic.

Off-target endonuclease cleavage

One of the major obstacles to gene editing is the possibility that off-target cleavage can occur at a similar or identical site within the human genome. In theory off-target cleavage at an undesired location could introduce deleterious mutations in the genome, or lead to chromosomal translocation. Either of these events can cause cellular toxicity or

transformation, although the outcome depends on whether an essential housekeeping gene or a gene involved in cell cycle regulation is altered. Due to the size of the human genome, probability suggests that an endonuclease target site of less than 17 base pairs has a 50 % or higher chance of being present within the human genome (Schiffer et al. 2012). Therefore, much effort has been put into analyzing the target-site cleavage specificity of each gene-editing platform when single or multiple nucleotide differences are present within homologous off-target sites in the host cell genome.

Some of the earliest efforts to assess the off-target cleavage activity of targeted endonucleases focused on HEs. Unlike other targeted endonuclease platforms, HEs use a single protein domain for target site binding and cleavage, and it was suggested that any off-target activity would be more limited and predictable because potential off-target sites need to be substrates for both binding and cleavage. Although off-target cleavage by HEs can be observed (Barzel et al. 2011), the levels seen depend on the particular HE and in general tend to be low. Subsequently, newer HE platforms have been developed that increase enzyme specificity, and subsequently the levels of HE-mediated off-target cleavage can be significantly minimized (Boissel et al. 2014; Wolfs et al. 2014).

Like TALENs and RGENs, ZFNs contain a DNA-binding domain, and a DNA-cleavage domain that is independent of the DNA target sequence. Therefore, the off-target activity of TALENs, RGENs, and ZFNs is defined by the specificity of their DNA-binding domains. For ZFNs this is complicated by the intricate context dependence of DNA binding for individual zinc finger domains when they are linked (Cornu et al. 2008; Sander et al. 2011). A number of studies have demonstrated that ZFNs can have significant off-target cleavage activity, although the extent of off-target cleavage is highly dependent on the ZFN being tested (Gabriel et al. 2011; Pattanayak et al. 2011). To limit the risk of off-target cleavage, modified ZFNs with altered FokI-derived cleavage domains have been developed (Miller et al. 2007; Szczepek et al. 2007). These ZFNs will only cleave DNA when the two ZFN subunits bind next to each other at their target site and an obligate FokI heterodimer forms. By using this approach the level of ZFN off-target activity can be significantly reduced, although some modified ZFNs can still be significantly cytotoxic (Weber et al. 2014b).

Like HEs and ZFNs, TALENs are able to cleave homologous off-target sites within the host cell genome (Guilinger et al. 2014). However, TALENs appear to have higher specificity for target-site binding and cleavage than ZFNs, as they are less likely to cleave off-target sites in the host cell genome and cause cytotoxicity (Mussolino et al. 2014). This improved specificity is likely because TAL-effector DNA-binding domains are less tolerant of local target sequence mismatches than ZFNs. It has also been suggested that TALENs have a favorable safety profile when compared to CRISPR/Cas9 (Frock et al. 2015), although cleavage at multiple off-target sites was seen, along with evidence of cleavage-induced chromosomal translocations.

Despite being the newest gene-editing tools available, RGENs have been studied extensively for their ability to mediate off-target cleavage. A number of studies have shown that the CRISPR/Cas9 system can cleave off-target sites, even when multiple target site mismatches are present (Fu et al. 2013; Kim et al. 2016; Tsai et al. 2015). Therefore, substantial work

has gone toward limiting the off-target activity of the CRISPR/Cas9 system. Work from the Church and Zhang labs has shown that CRISPR/Cas9 systems that use larger PAM sequences have improved off-target cleavage profiles (Esvelt et al. 2013; Ran et al. 2015). Alternatively, the Joung lab has shown that guide RNAs with shorter sequences are less likely to cleave at off-target sites (Fu et al. 2014). More recently both the Joung and Zhang labs were able to engineer the spCas9 protein so that off-target cleavage was either significantly reduced or not detectable (Kleinstiver et al. 2016; Slaymaker et al. 2016). These latest advances in CRISPR/Cas9 reagents provide hope that off-target activity may not be a problem for gene-editing technologies in the future.

Endonuclease safety in vivo

Aside from toxicity caused by off-target cleavage, other safety risks must also be addressed before endonucleases are used in humans. All of the gene editing platforms described here and elsewhere (Mussolino and Cathomen 2012; Rahman et al. 2011; Sander and Joung 2014; Stoddard 2011) are at least partially derived from non-human organisms, so they represent potential immunogens if delivered to humans. Of major concern is the risk that expression of an engineered endonuclease within a target cell may make it a target for immune-mediated clearance in vivo.

To date very little has been done to investigate the potential in vivo toxicity or immunogenicity of engineered endonucleases. Nevertheless, the existing data suggests that targeted endonuclease expression in vivo may not be associated with significant toxicity. No report of in vivo toxicity was seen when HEs were expressed in transgenic mice (White et al. 2013), or delivered to mice by plasmids (Riu et al. 2005) or adenovirus vectors (Gouble et al. 2006). No ZFN-related in vivo toxicity or immunogenicity was seen in human clinical trials, where patients who received ZFN-treated T cells retained gene modified cells for at least 252 days post-transplant (Tebas et al. 2014), although T-cell expansion prior to infusion likely limited ongoing levels of ZFN expression. Similarly, no in vivo toxicity was observed when TALENs were used as therapeutics in mouse models of HBV and HPV (Bloom et al. 2013; Chen et al. 2014; Hu et al. 2014a). For CRISPR/Cas9 no associated toxicity was seen in CRISPR knock-in mice (Platt et al. 2014), or when saCas9 (Ran et al. 2015) or spCas9 (Swiech et al. 2015) were delivered to the liver and brain, respectively. Despite these observations, the effect of long-term exposure to targeted endonucleases remains unknown, and 'safety switches' like those being developed for cell-based therapies may be needed (Barese et al. 2015; Di Stasi et al. 2011). Conditional expression of gene-editing endonucleases from tissue-specific promoters, drug-inducible promoters or non-constitutive viral promoters may also improve treatment safety. Further studies are warranted to fully investigate the safety and immunogenicity of targeted endonucleases following long-term expression in vivo.

Delivery challenges

Possibly the greatest challenge facing endonuclease-mediated antiviral therapies is the need to deliver each virus-specific enzyme to all infected cells. For each persistent/chronic viral infection the hurdles to achieving this vary greatly, and have been discussed in detail elsewhere (Weber et al. 2014a). Essentially two main questions need to be addressed for

each virus. Is there a therapeutic delivery system that can reach all of the target cell population? And can a given enzyme therapeutic be packaged into this delivery system?

The choice of delivery system used in all gene therapies is primarily dictated by the cell type or organ that must be transduced. Some viruses, such as HBV or HSV, are primarily found within a single cell type that is only found in a particular anatomic location, and this makes gene delivery easier. Other viruses, such as EBV and HTLV, target cell types that are distributed throughout the body, complicating delivery of therapeutics to all infected cells. A large number of non-viral and viral systems are available to achieve gene delivery to different virus-infected cell populations (reviewed in Pereyra and Herenu 2013), and for each target cell population more than one gene delivery system may enable efficient gene transfer. Each delivery system must have its efficacy, safety, and cost assessed on a per-virus basis.

For each gene-editing platform the requirements for delivery vary. HEs are derived from a single small gene so they can be efficiently delivered by most gene delivery systems. ZFNs and TALENs have two separate subunits that may be small (ZFNs) or large (TALENs), and the size constraints imposed by this restricts which gene delivery platform can be used. For example, ZFNs have been delivered by AAV vectors (Anguela et al. 2013; Li et al. 2011; Weber et al. 2014b), whereas TALENs have not. Additionally, the repeat domains present in ZFNs and TALENs make it difficult to incorporate separate subunits into a single vector because of the risk of recombination during production. CRISPR/Cas9 is limited by the need to deliver both a large Cas9 gene and a guide RNA, which makes their incorporation into some gene delivery vectors difficult. The discovery of smaller Cas9 genes has made this easier (Hou et al. 2013; Ran et al. 2015), but delivery of CRISPR/Cas9 to certain cell types may still be challenging. Ultimately each researcher must identify a gene delivery system that efficiently targets the correct target cells, and can accommodate their endonuclease of choice.

Treatment resistance

A large obstacle to any antiviral therapy is the potential to develop treatment resistance (Fig. 1). For gene-editing antiviral therapies, resistance could be caused by the acquisition of target site mutations that allow viral replication but prevent endonuclease binding or cleavage. Such mutations could be attained either during replication of the viral genome, or when a deleterious mutation is introduced following targeted gene editing. Alternatively, resistance could be gained when a defective viral gene target is restored to its wild-type state by a recombination event with a functional viral genome. Although de novo replication-derived resistance would be less likely to occur for the less error-prone DNA viruses compared to RNA viruses, any of these potential outcomes would be an unwanted consequence of a gene-editing antiviral therapy.

De Silva Felix et al. (2016) were the first to demonstrate that viral resistance can occur following endonuclease therapy. They showed that an insertion mutation introduced into the HIV provirus following ZFN therapy enabled virus replication and ZFN cleavage resistance. The mutant virus replicated at close to wild-type levels, and was infectious in activated primary T cells. However, resistance could be overcome when multiple regions of the viral

genome were targeted for disruption with separate endonucleases. Wang et al. (2016a) further demonstrated that treatment resistant virus can emerge when HIV cultures are treated with CRISPR/Cas9 reagents that individually target different regions of the provirus. Resistance-yielding mutations were found in HIV target sites that were likely generated during the process of DSB repair after Cas9 cleavage. This CRISPR/Cas9 study was soon followed by another that also demonstrated resistance to cleavage at target sites present in HIV pol, env and LTR (Wang et al. 2016b). Although the high reverse transcriptase error rate of HIV makes the likelihood of de novo replication-derived resistance greater than for other DNA viruses, these three studies demonstrate that endonuclease-derived resistance could be a significant concern. Future studies should determine whether endonuclease resistance can occur for other viruses, and if so, whether combinations of endonucleases (De Silva Feelixge et al. 2016) or sgRNAs (Kennedy et al. 2015) can avoid the development of resistance.

Treatment cost

A major impediment to the widespread application of gene-editing antivirals is the potential cost to the populations most at risk of infection. Although in their relative infancy, gene therapeutics have been used widely in clinical trials, but the cost of reagent production has been extremely high, and the pricing of the recently approved gene therapy product Glybera suggests high costs will trickle down to patients (Morrison 2015). For many chronic diseases a one-off high-cost curative therapy might ultimately be cheaper than the cost of life-long noncurative treatment, but this is not true for all diseases. Subsequently, new models of gene therapy pricing are being discussed (Brennan and Wilson 2014; Touchot and Flume 2015).

For gene-editing reagents against viruses the large-scale production of plasmids, recombinant proteins or viral vectors that deliver antiviral therapeutics is also likely to be high. For example, Glybera, a therapeutic based on an AAV viral vector, was initially priced as high as \$1.5 million. With some of the virus targets discussed here, mathematical modeling also suggests that multiple treatments will be likely, which would significantly increase treatment costs (Schiffer et al. 2013). In some cases, persistent viral infections can be adequately controlled with relatively inexpensive antiviral drugs, so this option would likely be preferred in many settings, particularly as cheap antiviral drugs become more readily available globally. Therefore, diligence will be required to ensure that gene-editing therapies will be affordable for populations in developing nations who are disproportionately affected by chronic and persistent infections.

Conclusions

Over the last 5 years advances in gene-editing technology have made it possible to target both DNA and RNA viruses with endonuclease-based antiviral therapeutics. This has opened the door for future therapies targeting chronic/persistent viral infections for which there are currently no or poor treatments. Although a fast-growing body of evidence suggests that endonucleases can be effective antiviral agents in vitro and in vivo, significant efforts are still needed to improve the efficacy, safety, and delivery of individual treatments.

Nevertheless, the rapid advances in gene-editing technologies bring hope that this approach to treating persistent viral infections can 1 day lead to human application.

Acknowledgments

We thank Chelsea Spragg for critical reading of this manuscript. This work was funded by a philanthropic Grant from the Caladan Foundation, by NIH supported Martin Delaney Collaboratory Grant U19 AI 096111, NIH Grants R21 AI117519 and R21 AI107252, and in part by a developmental Grant from the University of Washington Center for AIDS Research (CFAR), an NIH funded program under Award Number P30 AI 027757 which is supported by the following NIH Institutes and Centers (NIAID, NCI, NIMH, NIDA, NICHD, NHLBI, NIA, NIGMS, NIDDK).

References

- Anguela XM, Sharma R, Doyon Y, Miller JC, Li H, Haurigot V, Rohde ME, Wong SY, Davidson RJ, Zhou S, Gregory PD, Holmes MC, High KA. Robust ZFN-mediated genome editing in adult hemophilic mice. *Blood*. 2013; 122:3283–3287. DOI: 10.1182/blood-2013-04-497354 [PubMed: 24085764]
- Aubert M, Boyle NM, Stone D, Stensland L, Huang ML, Magaret AS, Galetto R, Rawlings DJ, Scharenberg AM, Jerome KR. In vitro inactivation of latent HSV by targeted mutagenesis using an HSV-specific homing endonuclease. *Mol Ther Nucleic Acids*. 2014; 3:e146.doi: 10.1038/mtna.2013.75 [PubMed: 24496438]
- Barese CN, Felizardo TC, Sellers SE, Keyvanfar K, Di Stasi A, Metzger ME, Krouse AE, Donahue RE, Spencer DM, Dunbar CE. Regulated apoptosis of genetically modified hematopoietic stem and progenitor cells via an inducible caspase-9 suicide gene in rhesus macaques. *Stem Cells*. 2015; 33:91–100. DOI: 10.1002/stem.1869 [PubMed: 25330775]
- Barzel A, Privman E, Peeri M, Naor A, Shachar E, Burstein D, Lazary R, Gophna U, Pupko T, Kupiec M. Native homing endonucleases can target conserved genes in humans and in animal models. *Nucleic Acids Res*. 2011; 39:6646–6659. DOI: 10.1093/nar/gkr242 [PubMed: 21525128]
- Benjamin R, Berges BK, Solis-Leal A, Igbinedion O, Strong CL, Schiller MR. TALEN gene editing takes aim on HIV. *Hum Genet*. 2016; doi: 10.1007/s00439-016-1678-2
- Bloom K, Ely A, Mussolino C, Cathomen T, Arbuthnot P. Inactivation of hepatitis B virus replication in cultured cells and in vivo with engineered transcription activator-like effector nucleases. *Mol Ther J Am Soc Gene Ther*. 2013; 21:1889–1897. DOI: 10.1038/mt.2013.170
- Boissel S, Jarjour J, Astrakhan A, Adey A, Gouble A, Duchateau P, Shendure J, Stoddard BL, Certo MT, Baker D, Scharenberg AM. megaTALs: a rare-cleaving nuclease architecture for therapeutic genome engineering. *Nucleic Acids Res*. 2014; 42:2591–2601. DOI: 10.1093/nar/gkt1224 [PubMed: 24285304]
- Boldogh, I.; Albrecht, T.; Porter, DD. Persistent viral infections. In: Baron, S., editor. *Medical microbiology*. 4. Galveston (TX): 1996.
- Brennan TA, Wilson JM. The special case of gene therapy pricing. *Nat Biotechnol*. 2014; 32:874–876. DOI: 10.1038/nbt.3003 [PubMed: 25203033]
- Chen J, Zhang W, Lin J, Wang F, Wu M, Chen C, Zheng Y, Peng X, Li J, Yuan Z. An efficient antiviral strategy for targeting hepatitis B virus genome using transcription activator-like effector nucleases. *Mol Ther J Am Soc Gene Ther*. 2014; 22:303–311. DOI: 10.1038/mt.2013.212
- Cornu TI, Thibodeau-Beganny S, Guhl E, Alwin S, Eichtinger M, Joung JK, Cathomen T. DNA-binding specificity is a major determinant of the activity and toxicity of zinc-finger nucleases. *Mol Ther J Am Soc Gene Ther*. 2008; 16:352–358. DOI: 10.1038/sj.mt.6300357
- Cradick TJ, Keck K, Bradshaw S, Jamieson AC, McCaffrey AP. Zinc-finger nucleases as a novel therapeutic strategy for targeting hepatitis B virus DNAs. *Mol Ther J Am Soc Gene Ther*. 2010; 18:947–954. DOI: 10.1038/mt.2010.20
- Dandri M, Lutgehetmann M. Mouse models of hepatitis B and delta virus infection. *J Immunol Methods*. 2014; 410:39–49. DOI: 10.1016/j.jim.2014.03.002 [PubMed: 24631647]

- de Martel C, Ferlay J, Franceschi S, Vignat J, Bray F, Forman D, Plummer M. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncol.* 2012; 13:607–615. DOI: 10.1016/S1470-2045(12)70137-7 [PubMed: 22575588]
- De Silva Felixge HS, Stone D, Pietz HL, Roychoudhury P, Greninger AL, Schiffer JT, Aubert M, Jerome KR. Detection of treatment-resistant infectious HIV after genome-directed antiviral endonuclease therapy. *Antiviral Res.* 2016; 126:90–98. DOI: 10.1016/j.antiviral.2015.12.007 [PubMed: 26718067]
- Di Stasi A, Tey SK, Dotti G, Fujita Y, Kennedy-Nasser A, Martinez C, Straathof K, Liu E, Durett AG, Grilley B, Liu H, Cruz CR, Savoldo B, Gee AP, Schindler J, Krance RA, Heslop HE, Spencer DM, Rooney CM, Brenner MK. Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med.* 2011; 365:1673–1683. DOI: 10.1056/NEJMoa1106152 [PubMed: 22047558]
- Ding W, Hu Z, Zhu D, Jiang X, Yu L, Wang X, Zhang C, Wang L, Ji T, Li K, He D, Xia X, Liu D, Zhou J, Ma D, Wang H. Zinc finger nucleases targeting the human papillomavirus E7 oncogene induce E7 disruption and a transformed phenotype in HPV16/18-positive cervical cancer cells. *Clin Cancer Res Off J Am Assoc Cancer Res.* 2014; 20:6495–6503. DOI: 10.1158/1078-0432.CCR-14-0250
- Dong C, Qu L, Wang H, Wei L, Dong Y, Xiong S. Targeting hepatitis B virus cccDNA by CRISPR/Cas9 nuclease efficiently inhibits viral replication. *Antiviral Res.* 2015; 118:110–117. DOI: 10.1016/j.antiviral.2015.03.015 [PubMed: 25843425]
- Egli A, Infanti L, Dumoulin A, Buser A, Samaridis J, Stebler C, Gosert R, Hirsch HH. Prevalence of polyomavirus BK and JC infection and replication in 400 healthy blood donors. *J Infect Dis.* 2009; 199:837–846. [PubMed: 19434930]
- Elbadawy HM, Gailledrat M, Desseaux C, Salvalaio G, Di Iorio E, Ferrari B, Bertolin M, Barbaro V, Parekh M, Gayon R, Munegato D, Franchin E, Calistri A, Palu G, Parolin C, Ponzin D, Ferrari S. Gene transfer of integration defective anti-HSV-1 meganuclease to human corneas ex vivo. *Gene Ther.* 2014; 21:272–281. DOI: 10.1038/gt.2013.82 [PubMed: 24430237]
- Esvelt KM, Mali P, Braff JL, Moosburner M, Yaung SJ, Church GM. Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. *Nat Methods.* 2013; 10:1116–1121. DOI: 10.1038/nmeth.2681 [PubMed: 24076762]
- Ferrari C. HBV and the immune response. *Liver Int Off J Int Assoc Study Liver.* 2015; 35(Suppl 1): 121–128. DOI: 10.1111/liv.12749
- Frock RL, Hu J, Meyers RM, Ho YJ, Kii E, Alt FW. Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. *Nat Biotechnol.* 2015; 33:179–186. DOI: 10.1038/nbt.3101 [PubMed: 25503383]
- Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, Sander JD. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol.* 2013; 31:822–826. DOI: 10.1038/nbt.2623 [PubMed: 23792628]
- Fu Y, Sander JD, Reyon D, Cascio VM, Joung JK. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat Biotechnol.* 2014; 32:279–284. DOI: 10.1038/nbt.2808 [PubMed: 24463574]
- Gabriel R, Lombardo A, Arens A, Miller JC, Genovese P, Kaeppl C, Nowrouzi A, Bartholomae CC, Wang J, Friedman G, Holmes MC, Gregory PD, Glimm H, Schmidt M, Naldini L, von Kalle C. An unbiased genome-wide analysis of zinc-finger nuclease specificity. *Nat Biotechnol.* 2011; 29:816–823. DOI: 10.1038/nbt.1948 [PubMed: 21822255]
- Gessain A, Barin F, Vernant JC, Gout O, Maurs L, Calender A, de The G. Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. *Lancet.* 1985; 2:407–410. [PubMed: 2863442]
- Gossai A, Waterboer T, Nelson HH, Michel A, Willhauck-Fleckenstein M, Farzan SF, Hoen AG, Christensen BC, Kelsey KT, Marsit CJ, Pawlita M, Karagas MR. Seroepidemiology of human polyomaviruses in a US population. *Am J Epidemiol.* 2016; 183:61–69. DOI: 10.1093/aje/kwv155 [PubMed: 26667254]
- Gouble A, Smith J, Bruneau S, Perez C, Guyot V, Cabaniols JP, Leduc S, Fiette L, Ave P, Micheau B, Duchateau P, Paques F. Efficient in toto targeted recombination in mouse liver by mega-nuclease-induced double-strand break. *J Gene Med.* 2006; 8:616–622. DOI: 10.1002/jgm.879 [PubMed: 16475243]

- Gower E, Estes C, Blach S, Razavi-Shearer K, Razavi H. Global epidemiology and genotype distribution of the hepatitis C virus infection. *J Hepatol.* 2014; 61:S45–S57. DOI: 10.1016/j.jhep.2014.07.027 [PubMed: 25086286]
- Grosse S, Huot N, Mahiet C, Arnould S, Barradeau S, Clerre DL, Chion-Sotinel I, Jacqmarcq C, Chapellier B, Ergani A, Desseaux C, Cedrone F, Conseiller E, Paques F, Labetoulle M, Smith J. Meganuclease-mediated inhibition of HSV1 infection in cultured cells. *Mol Ther J Am Soc Gene Ther.* 2011; 19:694–702. DOI: 10.1038/mt.2010.302
- Guilinger JP, Pattanayak V, Reyon D, Tsai SQ, Sander JD, Joung JK, Liu DR. Broad specificity profiling of TALENs results in engineered nucleases with improved DNA-cleavage specificity. *Nat Methods.* 2014; 11:429–435. DOI: 10.1038/nmeth.2845 [PubMed: 24531420]
- Gupta T, Robles MT, Schowalter RM, Buck CB, Pipas JM. Expression of the small T antigen of lymphotropic papovavirus is sufficient to transform primary mouse embryo fibroblasts. *Virology.* 2016; 487:112–120. DOI: 10.1016/j.virol.2015.10.003 [PubMed: 26517398]
- Hou Z, Zhang Y, Propson NE, Howden SE, Chu LF, Sontheimer EJ, Thomson JA. Efficient genome engineering in human pluripotent stem cells using Cas9 from *Neisseria meningitidis*. *Proc Natl Acad Sci USA.* 2013; 110:15644–15649. DOI: 10.1073/pnas.1313587110 [PubMed: 23940360]
- Hu Z, Ding W, Zhu D, Yu L, Jiang X, Wang X, Zhang C, Wang L, Ji T, Liu D, He D, Xia X, Zhu T, Wei J, Wu P, Wang C, Xi L, Gao Q, Chen G, Liu R, Li K, Li S, Wang S, Zhou J, Ma D, Wang H. TALEN-mediated targeting of HPV oncogenes ameliorates HPV-related cervical malignancy. *J Clin Investig.* 2014a; doi: 10.1172/JCI78206
- Hu Z, Yu L, Zhu D, Ding W, Wang X, Zhang C, Wang L, Jiang X, Shen H, He D, Li K, Xi L, Ma D, Wang H. Disruption of HPV16-E7 by CRISPR/Cas system induces apoptosis and growth inhibition in HPV16 positive human cervical cancer cells. *Biomed Res Int.* 2014b; 2014:612823.doi: 10.1155/2014/612823 [PubMed: 25136604]
- Kane M, Golovkina T. Common threads in persistent viral infections. *J Virol.* 2010; 84:4116–4123. DOI: 10.1128/JVI.01905-09 [PubMed: 19955304]
- Karimova M, Beschorner N, Dammermann W, Chemnitz J, Indenbirken D, Bockmann JH, Grundhoff A, Luth S, Buchholz F, Schulze zur Wiesch J, Hauber J. CRISPR/Cas9 nickase-mediated disruption of hepatitis B virus open reading frame S and X. *Sci Rep.* 2015; 5:13734.doi: 10.1038/srep13734 [PubMed: 26334116]
- Kennedy EM, Bassit LC, Mueller H, Kornepati AV, Bogerd HP, Nie T, Chatterjee P, Javanbakht H, Schinazi RF, Cullen BR. Suppression of hepatitis B virus DNA accumulation in chronically infected cells using a bacterial CRISPR/Cas RNA-guided DNA endonuclease. *Virology.* 2014a; 476C:196–205. DOI: 10.1016/j.virol.2014.12.001 [PubMed: 25553515]
- Kennedy EM, Kornepati AV, Goldstein M, Bogerd HP, Poling BC, Whisnant AW, Kastan MB, Cullen BR. Inactivation of the human papillomavirus E6 or E7 gene in cervical carcinoma cells by using a bacterial CRISPR/Cas RNA-guided endonuclease. *J Virol.* 2014b; 88:11965–11972. DOI: 10.1128/JVI.01879-14 [PubMed: 25100830]
- Kennedy EM, Kornepati AV, Mefferd AL, Marshall JB, Tsai K, Bogerd HP, Cullen BR. Optimization of a multiplex CRISPR/Cas system for use as an antiviral therapeutic. *Methods.* 2015; 91:82–86. DOI: 10.1016/j.ymeth.2015.08.012 [PubMed: 26291065]
- Kim D, Kim S, Park J, Kim JS. Genome-wide target specificities of CRISPR-Cas9 nucleases revealed by multiplex Digenome-seq. *Genome Res.* 2016; doi: 10.1101/gr.199588.115
- Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, Joung JK. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature.* 2016; doi: 10.1038/nature16526
- Knowles WA. Discovery and epidemiology of the human polyomaviruses BK virus (BKV) and JC virus (JCV). *Adv Exp Med Biol.* 2006; 577:19–45. DOI: 10.1007/0-387-32957-9_2 [PubMed: 16626025]
- Koelle DM, Corey L. Recent progress in herpes simplex virus immunobiology and vaccine research. *Clin Microbiol Rev.* 2003; 16:96–113. [PubMed: 12525427]
- Kondo T, Kono H, Miyamoto N, Yoshida R, Toki H, Matsumoto I, Hara M, Inoue H, Inatsuki A, Funatsu T, et al. Age- and sex-specific cumulative rate and risk of ATLL for HTLV-I carriers. *Int J Cancer.* 1989; 43:1061–1064. [PubMed: 2732000]

- Kutok JL, Wang F. Spectrum of Epstein–Barr virus-associated diseases. *Annu Rev Pathol.* 2006; 1:375–404. DOI: 10.1146/annurev.pathol.1.110304.100209 [PubMed: 18039120]
- Li H, Haurigot V, Doyon Y, Li T, Wong SY, Bhagwat AS, Malani N, Anguela XM, Sharma R, Ivanciu L, Murphy SL, Finn JD, Khazi FR, Zhou S, Paschon DE, Rebar EJ, Bushman FD, Gregory PD, Holmes MC, High KA. In vivo genome editing restores haemostasis in a mouse model of haemophilia. *Nature.* 2011; 475:217–221. DOI: 10.1038/nature10177 [PubMed: 21706032]
- Lin SR, Yang HC, Kuo YT, Liu CJ, Yang TY, Sung KC, Lin YY, Wang HY, Wang CC, Shen YC, Wu FY, Kao JH, Chen DS, Chen PJ. The CRISPR/Cas9 system facilitates clearance of the intrahepatic HBV templates in vivo. *Mol Ther Nucleic Acids.* 2014; 3:e186.doi: 10.1038/mtna.2014.38 [PubMed: 25137139]
- Liu X, Hao R, Chen S, Guo D, Chen Y. Inhibition of hepatitis B virus by the CRISPR/Cas9 system via targeting the conserved regions of the viral genome. *J Gen Virol.* 2015a; 96:2252–2261. DOI: 10.1099/vir.0.000159 [PubMed: 25904148]
- Liu YC, Cai ZM, Zhang XJ. Reprogrammed CRISPR–Cas9 targeting the conserved regions of HPV6/11 genes inhibits proliferation and induces apoptosis in -transformed keratinocytes. *Asian J Androl.* 2015b; doi: 10.4103/1008-682X.157399
- Longnecker, RM.; Kieff, E.; Cohen, JI. Epstein–Barr virus. In: Knipe, DM.; Howley, PM., editors. *Fields virology.* 6. Lippincott, Williams, and Wilkins; Philadelphia: 2013. p. 1898-1959.
- Looker KJ, Magaret AS, Turner KM, Vickerman P, Gottlieb SL, Newman LM. Global estimates of prevalent and incident herpes simplex virus type 2 infections in 2012. *PLoS One.* 2015; 10:e114989.doi: 10.1371/journal.pone.0114989 [PubMed: 25608026]
- Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY, Alvarado M, Anderson HR, Anderson LM, Andrews KG, Atkinson C, Baddour LM, Barker-Collo S, Bartels DH, Bell ML, Benjamin EJ, Bennett D, Bhalla K, Bikbov B, Bin Abdulhak A, Birbeck G, Blyth F, Bolliger I, Boufous S, Bucello C, Burch M, Burney P, Carapetis J, Chen H, Chou D, Chugh SS, Coffeng LE, Colan SD, Colquhoun S, Colson KE, Condon J, Connor MD, Cooper LT, Corriere M, Cortinovis M, de Vaccaro KC, Couser W, Cowie BC, Criqui MH, Cross M, Dabhadkar KC, Dahodwala N, De Leo D, Degenhardt L, Delossantos A, Denenberg J, Des Jarlais DC, Dharmaratne SD, Dorsey ER, Driscoll T, Duber H, Ebel B, Erwin PJ, Espindola P, Ezzati M, Feigin V, Flaxman AD, Forouzanfar MH, Fowkes FG, Franklin R, Fransen M, Freeman MK, Gabriel SE, Gakidou E, Gaspari F, Gillum RF, Gonzalez-Medina D, Halasa YA, Haring D, Harrison JE, Havmoeller R, Hay RJ, Hoen B, Hotez PJ, Hoy D, Jacobsen KH, James SL, Jasrasaria R, Jayaraman S, Johns N, Karthikeyan G, Kassebaum N, Keren A, Khoo JP, Knowlton LM, Kobusingye O, Koranteng A, Krishnamurthi R, Lipnick M, Lipshultz SE, Ohno SL, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet.* 2012; 380:2095–2128. DOI: 10.1016/S0140-6736(12)61728-0 [PubMed: 23245604]
- Marquitz AR, Mathur A, Nam CS, Raab-Traub N. The Epstein–Barr virus BART microRNAs target the pro-apoptotic protein Bim. *Virology.* 2011; 412:392–400. DOI: 10.1016/j.virol.2011.01.028 [PubMed: 21333317]
- Mertz GJ, Benedetti J, Ashley R, Selke SA, Corey L. Risk factors for the sexual transmission of genital herpes. *Ann Intern Med.* 1992; 116:197–202. [PubMed: 1309413]
- Messina JP, Humphreys I, Flaxman A, Brown A, Cooke GS, Pybus OG, Barnes E. Global distribution and prevalence of hepatitis C virus genotypes. *Hepatology.* 2015; 61:77–87. DOI: 10.1002/hep.27259 [PubMed: 25069599]
- Miller JC, Holmes MC, Wang J, Guschin DY, Lee YL, Rupniewski I, Beausejour CM, Waite AJ, Wang NS, Kim KA, Gregory PD, Pabo CO, Rebar EJ. An improved zinc-finger nuclease architecture for highly specific genome editing. *Nat Biotechnol.* 2007; 25:778–785. DOI: 10.1038/nbt1319 [PubMed: 17603475]
- Mino T, Mori T, Aoyama Y, Sera T. Gene- and protein-delivered zinc finger-staphylococcal nuclease hybrid for inhibition of DNA replication of human papillomavirus. *PLoS One.* 2013; 8:e56633.doi: 10.1371/journal.pone.0056633 [PubMed: 23437192]
- Mino T, Mori T, Aoyama Y, Sera T. Inhibition of DNA replication of human papillomavirus by using zinc finger-single-chain FokI dimer hybrid. *Mol Biotechnol.* 2014; doi: 10.1007/s12033-014-9751-3

- Morrison C. \$1-million price tag set for Glybera gene therapy. *Nat Biotechnol.* 2015; 33:217–218. DOI: 10.1038/nbt0315-217 [PubMed: 25748892]
- Mussolino C, Cathomen T. TALE nucleases: tailored genome engineering made easy. *Curr Opin Biotechnol.* 2012; 23(5):644–650. [PubMed: 22342754]
- Mussolino C, Alzubi J, Fine EJ, Morbitzer R, Cradick TJ, Lahaye T, Bao G, Cathomen T. TALENs facilitate targeted genome editing in human cells with high specificity and low cytotoxicity. *Nucleic Acids Res.* 2014; 42:6762–6773. DOI: 10.1093/nar/gku305 [PubMed: 24792154]
- Nicoll MP, Proenca JT, Efstathiou S. The molecular basis of herpes simplex virus latency. *FEMS Microbiol Rev.* 2012; 36:684–705. DOI: 10.1111/j.1574-6976.2011.00320.x [PubMed: 22150699]
- Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A, Matsumoto M, Tara M. HTLV-I associated myelopathy, a new clinical entity. *Lancet.* 1986; 1:1031–1032. [PubMed: 2871307]
- Padgett BL, Walker DL, ZuRhein GM, Eckroade RJ, Dessel BH. Cultivation of papova-like virus from human brain with progressive multifocal leucoencephalopathy. *Lancet.* 1971; 1:1257–1260. [PubMed: 4104715]
- Pattanayak V, Ramirez CL, Joung JK, Liu DR. Revealing off-target cleavage specificities of zinc-finger nucleases by in vitro selection. *Nat Methods.* 2011; 8:765–770. DOI: 10.1038/nmeth.1670 [PubMed: 21822273]
- Pereyra, A.; Herenu, C. Gene delivery Systems. In: Romamowski, V., editor. Current issues in molecular virology—viral genetics and biotechnological applications. INTECH; Rijeka: 2013.
- Platt RJ, Chen S, Zhou Y, Yim MJ, Swiech L, Kempton HR, Dahlman JE, Parnas O, Eisenhaure TM, Jovanovic M, Graham DB, Jhunjhunwala S, Heidenreich M, Xavier RJ, Langer R, Anderson DG, Hacohen N, Regev A, Feng G, Sharp PA, Zhang F. CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell.* 2014; 159:440–455. DOI: 10.1016/j.cell.2014.09.014 [PubMed: 25263330]
- Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci USA.* 1980; 77:7415–7419. [PubMed: 6261256]
- Price AA, Sampson TR, Ratner HK, Grakoui A, Weiss DS. Cas9-mediated targeting of viral RNA in eukaryotic cells. *Proc Natl Acad Sci USA.* 2015; 112:6164–6169. DOI: 10.1073/pnas.1422340112 [PubMed: 25918406]
- Qi Z, Li G, Hu H, Yang C, Zhang X, Leng Q, Xie Y, Yu D, Gao Y, Lan K, Deng Q. Recombinant covalently closed circular hepatitis B virus DNA induces prolonged viral persistence in immunocompetent mice. *J Virol.* 2014; 88:8045–8056. DOI: 10.1128/JVI.01024-14 [PubMed: 24807718]
- Rahman SH, Maeder ML, Joung JK, Cathomen T. Zinc-finger nucleases for somatic gene therapy: the next frontier. *Hum Gene Ther.* 2011; 22(8):925–933. [PubMed: 21631241]
- Ramanan V, Shlomai A, Cox DB, Schwartz RE, Michailidis E, Bhatta A, Scott DA, Zhang F, Rice CM, Bhatia SN. CRISPR/Cas9 cleavage of viral DNA efficiently suppresses hepatitis B virus. *Sci Rep.* 2015; 5:10833.doi: 10.1038/srep10833 [PubMed: 26035283]
- Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA, Zhang F. In vivo genome editing using *Staphylococcus aureus* Cas9. *Nature.* 2015; 520:186–191. DOI: 10.1038/nature14299 [PubMed: 25830891]
- Riu E, Grimm D, Huang Z, Kay MA. Increased maintenance and persistence of transgenes by excision of expression cassettes from plasmid sequences in vivo. *Hum Gene Ther.* 2005; 16:558–570. DOI: 10.1089/hum.2005.16.558 [PubMed: 15916481]
- Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol.* 2014; 32(4):347–355. [PubMed: 24584096]
- Sander JD, Dahlborg EJ, Goodwin MJ, Cade L, Zhang F, Cifuentes D, Curtin SJ, Blackburn JS, Thibodeau-Beganny S, Qi Y, Pierick CJ, Hoffman E, Maeder ML, Khayter C, Reyon D, Dobbs D, Langenau DM, Stupar RM, Giraldez AJ, Voytas DF, Peterson RT, Yeh JR, Joung JK. Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). *Nat Methods.* 2011; 8:67–69. DOI: 10.1038/nmeth.1542 [PubMed: 21151135]

- Schiffer JT, Aubert M, Weber ND, Mintzer E, Stone D, Jerome KR. Targeted DNA mutagenesis for the cure of chronic viral infections. *J Virol*. 2012; 86:8920–8936. DOI: 10.1128/JVI.00052-12 [PubMed: 22718830]
- Schiffer JT, Swan DA, Stone D, Jerome KR. Predictors of hepatitis B cure using gene therapy to deliver DNA cleavage enzymes: a mathematical modeling approach. *PLoS Comput Biol*. 2013; 9:e1003131.doi: 10.1371/journal.pcbi.1003131 [PubMed: 23861664]
- Seeger C, Sohn JA. Targeting hepatitis B virus with CRISPR/Cas9. *Mol Ther Nucleic Acids*. 2014; 3:e216.doi: 10.1038/mtna.2014.68 [PubMed: 25514649]
- Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX, Zhang F. Rationally engineered Cas9 nucleases with improved specificity. *Science*. 2016; 351:84–88. DOI: 10.1126/science.aad5227 [PubMed: 26628643]
- Smith JS, Robinson NJ. Age-specific prevalence of infection with herpes simplex virus types 2 and 1: a global review. *J Infect Dis*. 2002; 186(Suppl 1):S3–S28. DOI: 10.1086/343739 [PubMed: 12353183]
- Stoddard BL. Homing endonucleases: from microbial genetic invaders to reagents for targeted DNA modification. *Structure*. 2011; 19(1):7–15. [PubMed: 21220111]
- Subak-Sharpe JH, Dargan DJ. HSV molecular biology: general aspects of herpes simplex virus molecular biology. *Virus Genes*. 1998; 16:239–251. [PubMed: 9654678]
- Swiech L, Heidenreich M, Banerjee A, Habib N, Li Y, Trombetta J, Sur M, Zhang F. In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. *Nat Biotechnol*. 2015; 33:102–106. DOI: 10.1038/nbt.3055 [PubMed: 25326897]
- Szcepek M, Brondani V, Buchel J, Serrano L, Segal DJ, Cathomen T. Structure-based redesign of the dimerization interface reduces the toxicity of zinc-finger nucleases. *Nat Biotechnol*. 2007; 25:786–793. DOI: 10.1038/nbt1317 [PubMed: 17603476]
- Tanaka A, Takeda S, Kariya R, Matsuda K, Urano E, Okada S, Komano J. A novel therapeutic molecule against HTLV-1 infection targeting provirus. *Leukemia*. 2013; 27:1621–1627. DOI: 10.1038/leu.2013.46 [PubMed: 23411465]
- Tebas P, Stein D, Tang WW, Frank I, Wang SQ, Lee G, Spratt SK, Surosky RT, Giedlin MA, Nichol G, Holmes MC, Gregory PD, Ando DG, Kalos M, Collman RG, Binder-Scholl G, Plesa G, Hwang WT, Levine BL, June CH. Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. *N Engl J Med*. 2014; 370:901–910. DOI: 10.1056/NEJMoa1300662 [PubMed: 24597865]
- Touchot N, Flume M. The payers' perspective on gene therapies. *Nat Biotechnol*. 2015; 33:902–904. DOI: 10.1038/nbt.3332 [PubMed: 26348952]
- Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar VV, Thapar V, Wyvekens N, Khayter C, Iafrate AJ, Le LP, Aryee MJ, Joung JK. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol*. 2015; 33:187–197. DOI: 10.1038/nbt.3117 [PubMed: 25513782]
- Wang J, Quake SR. RNA-guided endonuclease provides a therapeutic strategy to cure latent herpesviridae infection. *Proc Natl Acad Sci USA*. 2014; 111:13157–13162. DOI: 10.1073/pnas.1410785111 [PubMed: 25157128]
- Wang J, Xu ZW, Liu S, Zhang RY, Ding SL, Xie XM, Long L, Chen XM, Zhuang H, Lu FM. Dual gRNAs guided CRISPR/Cas9 system inhibits hepatitis B virus replication. *World J Gastroenterol*. 2015; 21:9554–9565. DOI: 10.3748/wjg.v21.i32.9554 [PubMed: 26327763]
- Wang G, Zhao N, Berkhout B, Das AT. CRISPR-Cas9 can inhibit HIV-1 replication but NHEJ repair facilitates virus escape. *Mol Ther J Am Soc Gene Ther*. 2016a; doi: 10.1038/mt.2016.24
- Wang Z, Pan Q, Gendron P, Zhu W, Guo F, Cen S, Wainberg MA, Liang C. CRISPR/Cas9-derived mutations both inhibit HIV-1 replication and accelerate viral escape. *Cell Rep*. 2016b; 15:481–489. DOI: 10.1016/j.celrep.2016.03.042 [PubMed: 27068471]
- Weber ND, Aubert M, Dang CH, Stone D, Jerome KR. DNA cleavage enzymes for treatment of persistent viral infections: recent advances and the pathway forward. *Virology*. 2014a; 454–455:353–361. DOI: 10.1016/j.virol.2013.12.037
- Weber ND, Stone D, Sedlak RH, De Silva Felixge HS, Roychoudhury P, Schiffer JT, Aubert M, Jerome KR. AAV-mediated delivery of zinc finger nucleases targeting hepatitis B virus inhibits

- active replication. PLoS One. 2014b; 9:e97579.doi: 10.1371/journal.pone.0097579 [PubMed: 24827459]
- White RR, Sung P, Vestal CG, Benedetto G, Cornelio N, Richardson C. Double-strand break repair by interchromosomal recombination: an in vivo repair mechanism utilized by multiple somatic tissues in mammals. PLoS One. 2013; 8:e84379.doi: 10.1371/journal.pone.0084379 [PubMed: 24349572]
- WHO. Hepatitis B Fact Sheet Number 204. 2015a. <http://www.who.int/mediacentre/factsheets/fs204/en/>
- WHO. Hepatitis C Fact Sheet Number 164. 2015b. <http://www.who.int/mediacentre/factsheets/fs164/en/>
- WHO. Human papillomavirus (HPV) and cervical cancer Fact Sheet Number 380. 2015c. <http://www.who.int/mediacentre/factsheets/fs380/en/>
- Wolfs JM, DaSilva M, Meister SE, Wang X, Schild-Poulter C, Edgell DR. MegaTevs: single-chain dual nucleases for efficient gene disruption. Nucleic Acids Res. 2014; 42:8816–8829. DOI: 10.1093/nar/gku573 [PubMed: 25013171]
- Wollebo HS, Bellizzi A, Kaminski R, Hu W, White MK, Khalili K. CRISPR/Cas9 system as an agent for eliminating polyomavirus JC infection. PLoS One. 2015; 10:e0136046.doi: 10.1371/journal.pone.0136046 [PubMed: 26360417]
- Xu F, Schillinger JA, Sternberg MR, Johnson RE, Lee FK, Nahmias AJ, Markowitz LE. Seroprevalence and coinfection with herpes simplex virus type 1 and type 2 in the United States, 1988–1994. J Infect Dis. 2002; 185:1019–1024. DOI: 10.1086/340041 [PubMed: 11930310]
- Yoshio S, Kanto T. Host-virus interactions in hepatitis B and hepatitis C infection. J Gastroenterol. 2016; 51:409–420. DOI: 10.1007/s00535-016-1183-3 [PubMed: 26894594]
- Yu L, Wang X, Zhu D, Ding W, Wang L, Zhang C, Jiang X, Shen H, Liao S, Ma D, Hu Z, Wang H. Disruption of human papillomavirus 16 E6 gene by clustered regularly interspaced short palindromic repeat/Cas system in human cervical cancer cells. Onco Targets Ther. 2015; 8:37–44. DOI: 10.2147/OTT.S64092 [PubMed: 25565864]
- Yuen KS, Chan CP, Wong NH, Ho CH, Ho TH, Lei T, Deng W, Tsao SW, Chen H, Kok KH, Jin DY. CRISPR/Cas9-mediated genome editing of Epstein–Barr virus in human cells. J Gen Virol. 2014; doi: 10.1099/jgv.0.000012
- Zhen S, Hua L, Takahashi Y, Narita S, Liu YH, Li Y. In vitro and in vivo growth suppression of human papillomavirus 16-positive cervical cancer cells by CRISPR/Cas9. Biochem Biophys Res Commun. 2014; 450:1422–1426. DOI: 10.1016/j.bbrc.2014.07.014 [PubMed: 25044113]
- Zhen S, Hua L, Liu YH, Gao LC, Fu J, Wan DY, Dong LH, Song HF, Gao X. Harnessing the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated Cas9 system to disrupt the hepatitis B virus. Gene Ther. 2015; doi: 10.1038/gt.2015.2

Fig. 1.

Generation of endonuclease-resistant virus. Upon endonuclease cleavage of an essential viral gene the DNA double strand break can be repaired perfectly (*A*), mutated to yield a replication incompetent virus (*B*), mutated to yield a treatment-resistant virus (*C*), or treated with a second endonuclease to prevent treatment resistance (*D*)