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Genome editing strategies: potential tools for eradicating HIV-1/AIDS

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Abstract

Current therapy for controlling HIV-1 infection and preventing AIDS progression has profoundly decreased viral replication in cells susceptible to HIV-1 infection, but it does not eliminate the low level of viral replication in latently infected cells which contain integrated copies of HIV-1 proviral DNA. There is an urgent need for the development of HIV-1 genome eradication strategies that will lead to a permanent or "sterile" cure of HIV-1/AIDS. In the past few years, novel nuclease-initiated genome editing tools have been developing rapidly, including ZFNs, TALENs, and the CRISPR/Cas9 system. These surgical knives, which can excise any genome, provide a great opportunity to eradicate the HIV-1 genome by targeting highly conserved regions of the HIV-1 long terminal repeats or essential viral genes. Given the time consuming and costly engineering of target-specific ZFNs and TALENs, the RNA-guided endonuclease Cas9 technology has emerged as a simpler and more versatile technology to allow permanent removal of integrated HIV-1 proviral DNA in eukaryotic cells, and hopefully animal models or human patients. The major unmet challenges of this approach at present include inefficient nuclease gene delivery, potential off-target cleavage, and cell-specific genome targeting. Nanoparticle or lentivirus-mediated delivery of next generation Cas9 technologies including nickase or RNA-guided FokI nuclease (RFN) will further improve the potential for genome editing to become a promising approach for curing HIV-1/AIDS.

Keywords

Genome editing; CRISPR/Cas9; HIV-1 integration; latent reservoir; cure; animal models

Viral latency as a complex barrier to an HIV-1 cure

An effective cure for HIV-1 infection can only be achieved with the complete purging of the viral reservoirs within an infected individual in order to ultimately prevent the spread of the virus to healthy cells (Stone *et al*, 2013). Viral latency is a phenomenon common to several viruses including cytomegalovirus (CMV), herpes simplex virus (HSV), human T-lymphotropic virus 1 (HTLV-1), and human immunodeficiency virus (HIV-1). Latency has been defined as a state of non-productive infection (Siliciano and Greene, 2011) and it is

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thought to have evolved as a mechanism to evade triggering of an immune system response, therefore allowing viral persistence. In the context of HIV-1, latency is established within specific cell populations (latent reservoir) (Battistini and Sgarbanti, 2014; Kumar *et al*, 2014; Van Lint *et al*, 2013).

Several approaches have been proposed to eliminate the latent HIV-1 viral infection. One of the most studied consists of a "purging strategy" to induce reactivation of the virus in latently infected T-cells in order to make the latently infected cells susceptible to treatment without inducing T-cell activation (Shan and Siliciano, 2013). Indeed, small molecules that can target the DNA and impact viral gene expression offer alternatives that avoid activation of cell surface receptors (Matalon *et al*, 2011). Histone deacetylase inhibitors (HDACi), like valproic acid or voninostat (SAHA), are well suited to the purpose, with the ability to increase histone acetylation of the integrated viral promoter and therefore trigger reactivation of the latent reservoir. This strategy was tried in patients but unfortunately was unsuccessful in eliminating all latent viruses, likely due to only partial activation of the latent reservoir (Archin *et al*, 2014; Manson McManamy *et al*, 2014; Rasmussen *et al*, 2013; Siliciano and Siliciano, 2014).

Two different strategies have been described as potential "cures" for HIV-1 infection: a *functional* cure and a *sterilizing* cure (Van Lint *et al*, 2013). A functional cure would be obtained when the latent reservoir remains but there is permanent control of viral replication without the need for continuous anti-retroviral therapy. Indeed, CCR5 impairment is the most advanced approach for a functional cure for HIV-1 to date (Allers *et al*, 2011; Tebas *et al*, 2014; Ye *et al*, 2014). On the other hand, a sterilizing cure would only be obtained upon complete eradication of all replication-competent forms of HIV-1 by eliminating the latent reservoir and removing all traces of the virus from infected patients. At present, a path leading to a sterilizing cure has not been achieved, however, such a cure could be achieved by excision of the integrated viral genome from the host DNA utilizing one of several genome editing strategies which are still in the early stages of development (Hu *et al*, 2014; Manjunath *et al*, 2013; Stone *et al*, 2013).

Genome editing technologies and their application to HIV-1 infection

Genome engineering strategies have been largely explored in the past decade and a better understanding of their functions and mechanisms has been achieved. Traditional gene targeting, such as replacement of genomic DNA with exogenous DNA (donor DNA) by homologous recombination, has been extensively employed to manipulate the eukaryotic genome. In particular, gene targeting in mouse embryonic stem cells has generated a large number of transgenic knockin/knockout mice. However, several challenges for traditional gene targeting exist, including extremely low target efficiency, limited gene target size, species limitations, time-consuming and labor-intensive selection/screening, and uncertain germline transmission. These challenges have been improved by inducing site-specific DNA double-strand breaks (DSBs), which dramatically increase the recombination efficiency for gene targeting by utilizing the intrinsic cellular DNA repair machinery. DSBs stimulate restoration of the intact genome either by homologous recombination (HR) in the presence of DNA donor or, more commonly, via error-prone non-homologous end joining (NHEJ),

which has a high probability of gene alteration due to insertion and deletion mutations, known as indels. Several types of nucleases have been identified that induce DSBs (Fig. 1) and implicated in targeting HIV-1 virus and host cellular genes (Table 1).

Homing endonucleases

More than a decade ago, meganuclease families, also called homing endonucleases, were described which are sequence-specific endonucleases with recognition sites larger than 12 bp (usually 16-30 bp) (Karvelis *et al*, 2013; Pingoud and Silva, 2007). Homing endonucleases have been used for site-directed genome modification and for several years were considered to be a very promising tool for genome editing. As proof of concept, homing endonucleases were tested for their ability to disrupt integrated HIV-1 provirus DNA using an integrated lentiviral reporter assay. The main limitation of the technology is their size and their potential disruption of endogenous genes or transcriptional activation of neighboring genes (Arnould *et al*, 2011). An added challenge to the engineering of meganucleases is the fact that the DNA recognition and cleavage functions of these enzymes are combined within a single domain.

Zinc finger nucleases (ZFNs)

The zinc finger nucleases (ZFNs), however, are much more compact in size. ZFNs are nucleases engineered to contain a specific class of transcription factor that targets the DNA via zinc finger motifs and a non-specific cleavage domain from the Type IIS restriction endonuclease, *Fok*I. *Fok*I dimerization through the binding of paired zinc finger proteins activates the ZFN, leading to DSBs within the target region. Since their discovery in 1996 (Kim *et al*, 1996), ZFNs have been extensively used to manipulate the genomes of plants, animals, and humans. The ability of ZFNs to disrupt CCR5 and CXCR4 genes has largely been tested in HIV-1 infected CD4+ T cells (Mani *et al*, 2005; Perez *et al*, 2008; Yuan *et al*, 2012). Due to the reduced number of CCR5Δ32 donors, strategies using ZFNs have been employed to obtain engineered CCR5 knockout cells in HIV-1 patients (Holt *et al*, 2010) and a clinical trial has demonstrated that the ZFN technique appears to be safe and effective in humans (Tebas *et al*, 2014). For the first time, Tebas and colleagues used ZFNs to target and disrupt the CCR5 gene in CD4+ T-cells, inducing a “beneficial” mutation in the CCR5 gene within those cells. CCR5-modified autologous CD4+ T-cells were then infused into the patients to repopulate the immune system with CCR5-deficient central memory T cells. This approach increased resistance to HIV-1 virus in 12 patients with chronic aviremic HIV infection under cART (Tebas *et al*, 2014). Indeed, 6 of the 12 participants suspended cART, and their HIV levels rebounded more slowly than normal, suggesting that the attempt was not yet a “permanent cure” but a delay in progression.

Transcription activator-like effector nucleases (TALENs)

Similar to ZFNs, transcription activator-like effector (TALE) nucleases (TALENs) have been developed and tested for controlling HIV-1 infection. TALEs are natural proteins secreted by the bacteria *Xanthomonas spp.* to regulate gene transcription in host plant cells. TALEN is a synthesized nuclease composed of a nonspecific *Fok*I nuclease domain in fusion with a customizable TALE-derived DNA-binding domain with highly conserved

33-35 amino acid repeats. Each TALE repeat binds to a single nucleotide of DNA, which is dependent on the two hypervariable residues which are usually found at positions 12 and 13 of the repeat. The domain containing Asn/Ile for adenine (A), Asn/Gly for thymine (T), Asn/Asn for guanine (G) and His/Asp for cytosine (C) has been identified and is currently used. The number of repeats in the array corresponds to the length of the target site. The dimerization of the fused *Fok*I brought by the opposite pair of TALE repeats allows the cleavage of the target DNA at the spacer region (10-15 bp). In the past 3-4 years, TALEN has been extensively used in a large number of applications due to its easy design, high cleavage efficiency and seemingly unlimited targeting range (Guilinger *et al*, 2014a; Joung and Sander, 2013). For HIV-1 infection, both TALENs and ZFNs induced ~45% efficiency in disrupting the CCR5 gene but TALEN induced much lower cytotoxicity and off-target effects (Mussolino *et al*, 2014; Mussolino *et al*, 2011). In inducible pluripotent stem cells, TALEN is capable of inducing up to 100% targeting of CCR5 32 on one allele with ~14% biallelic targeting (Ye *et al*, 2014). TALEN knockout of the PSIP1 gene in HEK293T cells and Jurkat cells rendered them resistant to HIV-1 infection in a cell culture model (Fadel *et al*, 2014b). In contrast to ZFNs that recognize three nucleotides per finger, TALENs recognize only one nucleotide per domain. Therefore, the ability to construct functional TALEN pairs with large numbers of repeats may increase their potential specificity when compared to ZFNs (Holkers *et al*, 2013). However, the sequence homology between each of the repeat variable diresidues (RVDs) makes it very difficult to build constructs containing multiple TALENs. Furthermore, a crucial limitation to their potential therapeutic application is their delivery into host cells, due to TALEN's susceptibility to induce rearrangements resulting in genomic instability. Indeed, previous studies have shown that HIV-1-based lentiviral vector genomes containing TALEN sequences are prone to rearrangements in the target cells and new optimization strategies will likely be required if they are to be used in future clinical applications (Hockemeyer *et al*, 2011).

RNA-guided CRISPR/Cas9 technology

In the past several years, ZFNs and TALENs have attracted extensive attention in the field of genome editing because of their advantages over traditional gene targeting technologies, including unlimited species application, higher target efficiency, rapid animal engineering, and faithful germline transmission. One major challenge for ZFNs and TALENs is the costly and time-consuming engineering of the target gene-specific fusion proteins. Thus, a new easy and versatile genome editing tool has recently entered the spotlight: the CRISPR-associated (Cas) RNA-guided endonuclease Cas9 technology (Hsu *et al*, 2014).

The clustered regularly interspaced short palindromic repeats (CRISPR) system has been detected in the majority of bacteria and archaea where it acts as a natural defense mechanism against bacteriophages. Due to the ubiquity and abundance of bacteriophages, prokaryotes have developed and refined this notable defense mechanism to neutralize their infectivity (Barrangou *et al*, 2007). The CRISPR machinery works as a kind of primitive yet highly selective and efficient immune system to preserve genetic integrity after exogenous DNA exposure and uptake. CRISPR consists of an array of small conserved repeat sequences interspaced by small DNA sequences called "spacers", which derive from the phage DNA. CRISPR loci, together with Cas genes, form the CRISPR-Cas adaptive immune system. The

type II system has been engineered to incorporate nucleic acids of the invading host into CRISPR loci and uses the corresponding CRISPR RNA (crRNA) or “guide” RNA (gRNA) to direct the degradation of homologous target sequences (protospacer).

Engineered gRNAs contain a 20 bp guide sequence (spacer, seed) that directs Cas9 to the genomic target using canonical base pairing which can be easily programmed (Ran *et al*, 2013). Hence, the multifunctional Cas9 protein and its appropriate gRNA are fundamental components for the formation of the functional Cas9/gRNA complex (Mali *et al*, 2013b). In order to recognize a target sequence (protospacer) and perform precise cleavage of the target DNA, the system requires complementarity between the spacer and the target sequence (protospacer) together with the presence of the protospacer-adjacent motif (PAM) sequence at 3′ end of the target sequence (Fig. 2). The PAM allows strict selectivity to the target array. The type II engineered system, mimicking the strategy of *Streptococcus pyogenes* (SpCas9), requires either NGG or NAG as the PAM sequence, in which N is any nucleotide. In just over a year from its first application in mammalian cells (Cong *et al*, 2013; Mali *et al*, 2013c), the engineered CRISPR/Cas9 system has been employed by thousands of laboratories for genome editing in a large number of cells and species including human cell lines, bacteria, zebrafish, yeast, mouse, fruit flies, roundworms, rats, common crops, pigs, and monkeys (Hsu *et al*, 2014). Since Cas9 works as a universal nuclease, researchers in any lab need only a tiny custom RNA molecule which, like small interfering RNA (siRNA), can be chemically synthesized or *in vitro* transcribed for direct RNA transfection or expressed from an RNA expression vector containing a U6 or H1 promoter. Thus, simple oligonucleotide synthesis and a single cloning step are all that is needed to utilize this versatile genome-editing tool. The simplicity of Cas9 targeting lends itself to the design of large gRNA libraries, which can be adapted to include multiple gRNAs covering the entire genome of a host organism, and thus can be employed for forward genetic screening and selection approaches. In addition gRNA libraries with the Cas9 nuclease have been used to induce knockout mutations in a variety of cells, in contrast with short hairpin RNA libraries which only induce gene knockdown (Sander and Joung, 2014; Zhou *et al*, 2014).

Similar to ZFNs and TALENS, Cas9 has been extensively tested for its ability to disrupt CCR5 due to its critical importance as a receptor for HIV-1 infection (Cho *et al*, 2013; Cradick *et al*, 2013; Niu *et al*, 2014; Yang *et al*, 2013). For biallelic targeting of the CCR5 32 transgene in induced pluripotent stem cells, Cas9 induced a better targeting efficiency (33%) than TALENs (14%) (Ye *et al*, 2014). Thus, extensive application of Cas9 to edit the HIV-1 genome and host cellular genes important in each step of the viral life cycle is now possible.

Genome editing to eradicate the whole HIV-1 genome

As described above, all three well-established genome editing tools (ZFN, TALEN and Cas9) have been tested for their efficiency in disrupting CCR5 because of the very promising cure of HIV-1 in the “Berlin” patient with a CCR5 32 mutation (Burke *et al*, 2014). However, CCR5 is not uniquely a receptor for HIV-1 infection as it exhibits many other cellular functions as well. Targeting the CCR5 gene will not offer protection against viruses using alternate co-receptors, such as CXCR4. Also, cells already infected with the

virus still persist within the host organism after CCR5 or CXCR4 disruption which affects only the entry of HIV-1 into the host cells. Therefore, manipulation of the integrated HIV-1 genome in latently infected cells is critical to achieve a permanent or sterilizing cure for HIV-1/AIDS. Such proof of principal is also applicable to other latently-integrated chronic viral infections such as hepatitis B virus, and herpes simplex virus (Bi *et al*, 2014; Kennedy *et al*, 2014; Schiffer *et al*, 2012; Suenaga *et al*, 2014; Wang and Quake, 2014; Zhen *et al*, 2014).

Targeting genes encoding HIV-1 proteins within the proviral DNA has been tested using homing endonuclease (Aubert *et al*, 2011) and ZFN (Wayengera, 2011), and is anticipated using TALEN and Cas9. However, such a strategy has limitations in that it will not eliminate the entire genome, and the remnant long terminal repeat (LTR) sequences may continue promoting transcription of potential toxic proteins after cleavage. Therefore, targeting the highly conserved LTRs of the HIV-1 proviral DNA is an ideal approach to eradicate the entire viral genome between the 5′ and 3′ LTRs while simultaneously disrupting the LTR sequences. We have tested this proof of concept using the novel Cas9/gRNA technology in several types of latently infected cells (Hu *et al*, 2014). During the course of our investigation, two labs reported the successful editing of the HIV-1 LTR by ZFN and Cas9 targeting a single conserved transcription factor binding site (Ebina *et al*, 2013) or the HIV-1 trans-activation response (TAR) element (Qu *et al*, 2013), which may potentially affect the host genome. In both studies, single cleavage of the targeted 5′ and 3′ LTRs induced excision of the entire integrated HIV-1 proviral genome in latently infected T cells as determined by PCR genotyping, Sanger sequencing, and functional assays (Ebina *et al*, 2013; Qu *et al*, 2013). In our studies, we demonstrated higher efficiency of HIV-1 genome eradication by single and multiplex Cas9/LTR-gRNAs not only in latently infected T cells but also in myeloid lineage cells (microglia and monocyte/macrophage) which are major cell types that can serve as HIV-1 reservoirs. Most interestingly, the HIV-1 Cas9/gRNA system is capable of eliminating more than one copy of the viral genome in an infected cell, even when the virus is integrated within different regions of the host genome. For example, we demonstrated the ability of the HIV-1 Cas9/gRNA system to delete both copies of the HIV-1 proviral DNA contained within the monocytic U1 cell line, one located on chromosome X (Hu *et al*, 2014) and the other on chromosome 2 (Fig. 3), suggesting that this novel genome editing system can alter the DNA sequences of HIV-1 in latently infected cells containing multiple copies of the proviral DNA. Despite the high promise of engineered nuclease-induced genome editing, a key barrier to its clinical and basic research applications is potential off-target effects due to permanent host genome modifications. The specificities of ZFN, TALEN and Cas9 have been extensively investigated using a combination of *in vitro* and *in vivo* experimental assays, computational characterization, and next generation sequencing. Although unbiased assays for off-target effects and continuous improvement of genome editing tools are still required, apparent genotoxicity, cytotoxicity, and off-target effects were not observed in most cases, particularly for Cas9-induced genome editing in both cells and animals (Cho *et al*, 2014; Fu *et al*, 2013; Gabriel *et al*, 2011; Hsu *et al*, 2014; Hsu *et al*, 2013; Pattanayak *et al*, 2013; Wang *et al*, 2013; Wu *et al*, 2013). In addition, long-term stable expression of Cas9 and/or gRNAs in cells (Heckl *et al*, 2014; Koike-Yusa *et al*, 2014; Zhou *et al*, 2014) and the viability of transgenic mice which

constitutively express Cas9 show no signs of toxicity (Platt *et al*, 2014 and Hu et al., unpublished observations). However, reliable whole genome sequencing for careful assessment of genome stability and potential genotoxicity is warranted. In terms of foreign viral DNA editing, the potential off-target effects of Cas9/gRNAs on the host genome may not be a big concern because of the fairly low homology between the exogenous viral genome and endogenous human genomes including human endogenous retrovirus (van der Kuyl, 2012), although the transcription factor binding sites within the HIV-1 LTR are highly homologous to sites within the host cellular genome and can be avoided by selective gRNA design. In addition, careful bioinformatics screening of HIV-1 gRNA target sites against the host genome could eventually improve the genome editing specificity of Cas9/HIV-1 gRNAs. Nevertheless, whole genome sequencing of homogeneous individual cells with deep sequencing coverage and higher calling confidence will be urgently needed to evaluate the potential risks of off-target effects in clinical applications. Recent whole genome sequencing in human iPS cells revealed high specificity and very rare off-target effects of Cas9/gRNA technology (Smith *et al*, 2014; Veres *et al*, 2014).

Pre-existing genome editing tool to vaccinate cells against HIV-1 infection

Given that the Cas9 genome editing system originated in bacteria as an immune defense system against invading bacteriophages, we hypothesized that pre-existing genome editing in cells may immunize them against new HIV-1 infection. To test this hypothesis, we established Cas9/gRNA-expressing stable cell lines from TZM-bI (Hu *et al*, 2014) and CHME5 cells (Fig. 4) by puromycin selection and limiting dilution. The target genome editing efficiency for the subclones depends upon the simultaneous expression of both Cas9 and the gRNA, which varied with different subclones (Fig. 4) and cell types (Hu *et al*, 2014). Using these effective subclones, we demonstrated that Cas9/gRNA genome editing effectively immunizes cells against new HIV-1 infection. Most interestingly, the preexistence of the Cas9/gRNA system in cells leads to a rapid elimination of the new HIV-1 before it integrates into the host genome (Hu *et al*, 2014). Similarly, the vaccinating system would be functional in eradicating newly packaged proviruses from the post-integrated HIV-1 genome in cells already infected with the virus. Further investigation of such HIV-1 vaccination in various latent reservoir cells and transgenic animals with stable or inducible expression of Cas9/LTR-gRNAs (Platt *et al*, 2014) is urgently needed. In clinical practice, the delivery of Cas9 protein plus LTR gRNAs (in the form of nanoparticles, for example) in healthy subjects (including vaginal, rectal, or systemic deliveries) would be a promising approach to prevent HIV-1 infection, because efficient *in vitro* genome editing using a mixture of Cas9, gRNA, and target genome has been well established (Chen *et al*, 2014; Jinek *et al*, 2013; Karvelis *et al*, 2013; Kim *et al*, 2014). Like any gene delivery, it is possible that the promoters for stably-expressing Cas9 and/or gRNA could be silenced in some cells, particularly in memory T cells and macrophages. The likelihood of promoter silencing, although very low, may dampen the therapeutic application of Cas9/gRNA approach.

Paired Nickase or Cas9-FokI strategies for genome editing to reduce off-target effects

CRISPR-dependent targeting of crucial genetic loci is a highly selective method to degrade foreign DNA with great versatility due to its ability to target multiple gene locations. In order to obtain Cas9 specificity for editing of a target gene, Zhang and colleagues have described four golden rules to minimize potential 'off-target' effects within the host genome (Hsu *et al*, 2013). A powerful computational tool (http://crispr.mit.edu/) has been designed to aid in the selection of gRNAs and to predict off-target loci for specificity analyses (Hsu *et al*, 2013). Also, several other programs are available for designing specific gRNAs with various levels of scores, such as the DNA2.0 CRISPR gRNA design tool (https://www.dna20.com/eCommerce/cas9/input) and the E-CRISP (http://www.e-crisp.org/E-CRISP/designcrispr.html), sgRNAcas9 software package (www.biootools.com) (Xie *et al*, 2014), Cas-OFFinder (Bae *et al*, 2014) and CasOT (Xiao *et al*, 2014). However, the specificity of the gRNAs in host cells remain to be explored and, so far, three human cell types have acquired bona fide off-target mutations induced by Cas9 (Fu *et al*, 2013; Kuscu *et al*, 2014). A better understanding of gRNA-mediated off-target effects in eukaryotic cells is essential for this approach to find potential therapeutic applications in the clinic. As mentioned above, the wild-type Cas9 nucleases are able to induce site-specific DSBs through the activity of their RuvC and HNH nuclease domains. Catalytically inactivating the RuvC or HNH nuclease domains via point mutations can convert Cas9 into a DNA "nickase" (Gasiunas *et al*, 2012; Jinek *et al*, 2012; Sapranauskas *et al*, 2011). Nickase activity typically induces repair either seamlessly or through high-fidelity homology directed repair (Cong *et al*, 2013; Gasiunas *et al*, 2012), therefore its action causes less off-target effects. Recently, a 'paired nickase' strategy has been developed in which adjacent off-set nicks are generated at the target site using two offset gRNAs and Cas9 nickases (Cho *et al*, 2014; Mali *et al*, 2013a; Ran *et al*, 2013). This strategy is similar to the dimeric ZFNs or TALENs, leading to the doubling of the target sequence (from 23 bp to 46 bp) and thus significantly increasing the editing specificity (by up to 1500-fold) (Ran *et al*, 2013; Shen *et al*, 2014). The cooperative nicks with tail-to-tail offset (−10 to +30 bp) mimic DSBs and mediate efficient indel formation. The nickase strategy has been shown to be effective for gene knockout in mouse zygotes without reducing on-target cleavage efficiency (Mali *et al*, 2013a; Ran *et al*, 2013; Shen *et al*, 2014).

However, the paired nickase approach requires two single monomeric nickases that may induce potential off-target indel mutations through an undefined mechanism (Mali *et al*, 2013a; Ran *et al*, 2013; Tsai *et al*, 2014). Recently, a dimerization-dependent RNA-guided *Fok*I-fused catalytically-dead Cas9 (dCas9) nuclease (RFN) has been established (Guilinger *et al*, 2014b; Tsai *et al*, 2014). Similar to ZFN and TALEN, this novel RFN takes advantage of the non-specific cleavage function of the well-characterized, dimerization-dependent *Fok*I nuclease domains that are brought together by two RNA-guided dCas9 monomers that simultaneously bind opposite target sites separated by approximately 14-17 bp or 25 bp (Guilinger *et al*, 2014b; Tsai *et al*, 2014). In contrast to the co-localized, paired Cas9 nickases, the cleavage activity of RFN is largely dimerization-dependent, requiring simultaneous binding of two gRNAs to the target DNA with stringent spacing and

orientation, and substantially increasing the specificity of genome editing. RFNs guided by a single gRNA generally induce very little or no off-target effects (Guilinger *et al*, 2014b; Tsai *et al*, 2014). However, the very stringent requirements of the off-set size (only 14-17 bp or 25 bp) between two gRNA target sites dramatically reduces the number of candidate gRNAs, limiting the opportunity to identify effective paired gRNAs. Therefore, new approaches to improve the specificity remain to be developed.

Conclusions and future directions

In conclusion, DSB-mediated nuclease-initiated DNA editing tools that can be used as novel genome surgical knives have extensive applications in biomedical science. The facile and versatile Cas9/gRNA technology platform (Ran *et al*, 2013) is growing rapidly, and shows promise for successful development into novel therapeutic platforms for treating human genetic diseases, infectious diseases, and cancer (Cho *et al*, 2013; Manjunath *et al*, 2013; Zhang *et al*, 2014). By targeting both 5′ and 3′ HIV-1 LTRs with single or multiplex gRNAs, this genome editing tool is capable of excising the entire genome spanning between the LTRs from the host genome, leading to disruption of latent provirus and protecting cells against new HIV-1 infection (Fig. 4). There are, however, several important issues that need to be considered. First, whether or not the Cas9/gRNA system will have any effect on episomal DNA. In this respect, one may consider several recent studies illustrating the capability of this novel genome editing system in cleaving several other viral genes including adenovirus, herpes simplex virus, Epstein-Barr virus and human papillomavirus (Bi *et al*, 2014; Kennedy *et al*, 2014; Suenaga *et al*, 2014; Wang and Quake, 2014; Zhen *et al*, 2014), leading to the belief that this strategy can also be effective in targeting episomal HIV-1 genomes. This is supported by our observation that expression of Cas9/LTR-gRNA in cells significantly prevents new HIV-1 infection before integration (Hu *et al*, 2014). The second issue concerns the ability of the Cas9/gRNA to effectively target every single cell which harbors a complete HIV-1 genome. While at present time, we can offer no conclusive evidence in the absence of in vivo data, one may hope that the elimination of a large fraction of activatable HIV-1 genomes from infected cells by Cas9/gRNA may provide a strong head start for the host immune system to overcome any remaining cells harboring HIV-1. Thus, a genetic approach for eliminating HIV-1 in some population of latently infected cells may rejuvenate immune cells to combat viral infection as a whole. Furthermore, rapid developing technologies such as nanoparticle and lentivirus delivery systems hold promise for highly efficient HIV-1 eradication in latently infected cells. Another important issue that needs attention relates to the robust expression of therapeutic genes, i.e. Cas9 and gRNAs in cells which are not actively replicating virus. In this respect, one may consider to employ an inducible promoter to actively control the expression levels of these genes in the target cells. Two other major barriers need to be overcome to bring this powerful genome editing tool into the clinic, including the potential off-target effects as well as the design of a safe and efficient delivery system for cell-specific distribution and genome editing within recipient organisms. Several promising strategies are under development to surmount these hurdles. In terms of HIV-1 genetic variation and quasispecies in individual patients, one may develop personalized treatment modalities based on the data from deep sequencing of the patient-

derived viral genomes prior to engineering therapeutic gRNA molecules, which can be generated quickly and easily.

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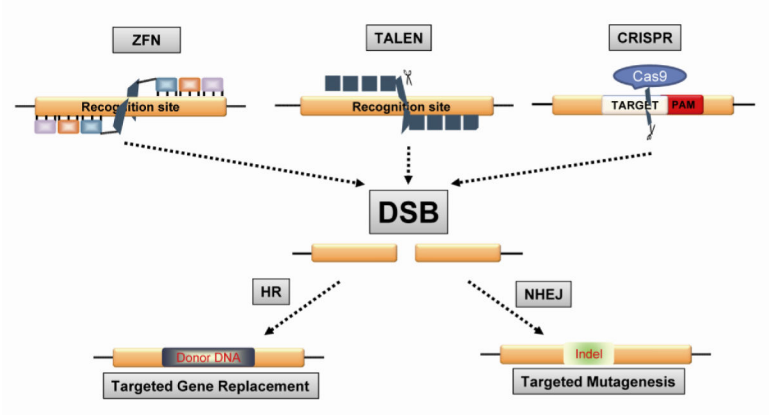

Figure 1. Nuclease-induced double strand breaks (DSBs) initiate the cellular homologous recombination (HR) and non-homologous end joining (NHEJ) DNA repair pathways exploited by the ZFN, TALEN, and CRISPR genome editing technologies

Yellow squares indicate target genome. PAM, protospacer adjacent motif. Indel, insertion or deletion mutants.

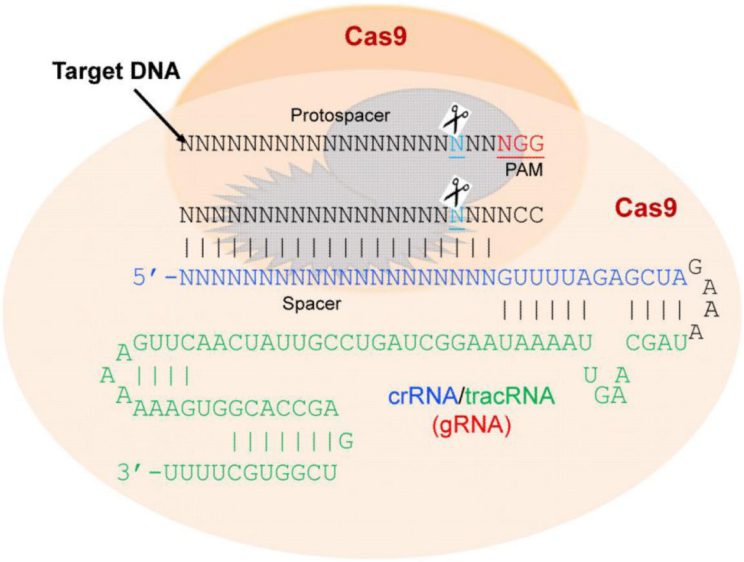

Figure 2. Precise cleavage of the third nucleotide from protospacer adjacent motif (PAM) by RNA-guided Cas9 dual nucleases induces double strand breaks in the target DNA

The guide RNA (gRNA) is a chimeric stem-loop structure consisting of CRISPR RNA (crRNA) and trans-activating crRNA (tracRNA) with a GAAA tetraloop.

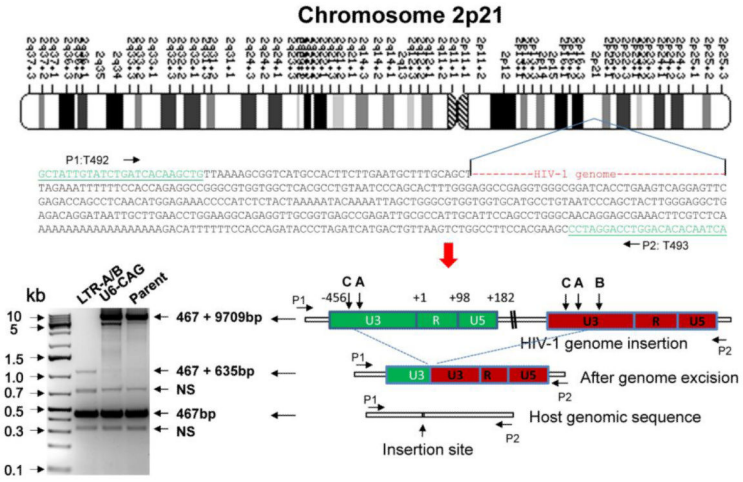

Figure 3. Eradication of the entire HIV-1 genome spanning between the 5′- and 3′-LTRs by Cas9/LTR-A/B gRNAs in the human U1 monocytic cell line

The integration site of the HIV-1 genome within chromosome 2 of the U1 cell line is shown. Cells were transfected with plasmids encoding Cas9 and gRNAs targeting two sequences within the 5′ and 3′ HIV-1 LTRs, called LTR-A and LTR-B. Sanger sequencing of a 1.1 kb fragment from long-range PCR using a primer pair (T492/T493) targeting the flanking sequences of the HIV-1 integration site (467 bp) validated the elimination of the entire HIV-1 genome (9,709 bp). NS, non-specific band. Similar results were seen for the second copy of the HIV-1 genome integrated into chromosome X of the U1 cell line. (See Hu et al., 2014 for a detailed description of the LTR-A and -B sequences and the results for chromosome X).

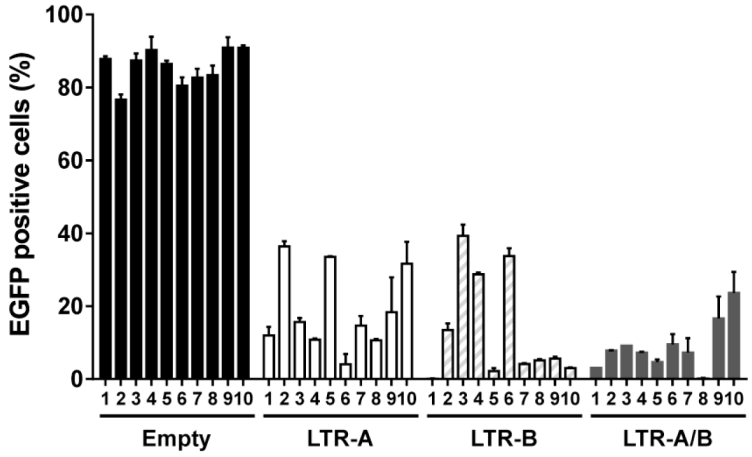

Figure 4. Stable expression of Cas9 plus LTR-A/B efficiently eradicates the HIV-1 genome in latently infected CHME5 microglial cells

Cells were transfected with plasmids expressing Cas9 and LTR-A, -B, or -A/B. After a 2-week selection with puromycin (1 g/ml), subclones were cultured by limiting dilution and single cell clones were treated with trichostatin A (TSA, 250 nM) for 2 d to induce viral reactivation before EGFP flow cytometry. A dramatic reduction in TSA-induced reactivation of latent pNL4-3- Gag-d2EGFP reporter virus was detected in most of the subclones with single or duplex LTR-gRNAs as compared with the empty pX260 control vector. (See Hu et al., 2014 for further details.)

Table 1

HIV genomic and cellular targets by genome editing technology

Viral targets	Nuclease	Sequence/Strategy	Cells/Organism	Gene delivery	Efficiency	Reference
LTR	Cas9	Single, U3 region Duplex, U3 region	TZMbI, U1, J-Lat	Transient or Stable transfection	30-90%	(Hu *et al*, 2014)
	Cas9	Single: TAR and U3	HEK293T cell, Hela cells, Jurkat cell line	Repeated transient transfection	30-96%	(Ebina *et al*, 2013)
	ZFN	Single, R and U3 region	Primary T cells Jurkat T cell line	Transient transfection	46%	(Qu *et al*, 2013) (Qu *et al*, 2014)
	Tre	34 bp sequence (loxLTR)	T cells, HSC, Humanized mice	Lentivirus Cell transplantation		(Hauber *et al*, 2013; Mariyanna *et al*, 2012)
Gag/Pol/Env	ZFN	Disruption	T cells, TZMbI	Lentivirus	80%	(Wayengera, 2011)
Vpr	Meganuclease	Disruption	CHO	Lentivirus		(Izmiryan *et al*, 2011)
Cellular targets	**Nuclease**	**Sequence/Strategy**	**Cells/Organism**	**Gene delivery**	**Efficiency**	**Reference**
CCR5 (Too many reports, only representatives are cited)	Cas9	Disruption	HEK293T	Transfection	18-50%	(Cradick *et al*, 2013) (Fu *et al*, 2014)
		Disruption	iPSC	Stable transfection	33- 100%	(Ye *et al*, 2014)
		Disruption	K562	Plasmid transfection	11%	(Cho *et al*, 2013)
	ZFN	Delta32	T cells	Transfection Adenovirus Lentivirus NILV	10-54%	(Mani *et al*, 2005) (Lee *et al*, 2010) (Perez *et al*, 2008) (Maier *et al*, 2013) (Lombardo *et al*, 2011)
		Disruption	ESC	Transfection		(Yao *et al*, 2012)
		Disruption	HSC	Transfection Adenovirus Transplantation	10-50%	(Lombardo *et al*, 2007) (Holt *et al*, 2010) (Li *et al*, 2013)
		Disruption	iPSC	Transfection		(Lombardo *et al*, 2011) (Yao *et al*, 2012) (Ye *et al*, 2014)
		Disruption	Neural stem cells	Transfection	5%	(Lombardo *et al*, 2011)
		Delta32	Humanized mice	Adenovirus NILV Cell transplantation		(Lee *et al*, 2010) (Perez *et al*, 2008) (Yi *et al*, 2014)
		Delta32	Patients	Adenovirus Cell transplantation		(Maier *et al*, 2013) (Tebas *et al*, 2014)
	TALEN	CCR5delta32	HEK293T	Transfection		(Nerys-Junior *et al*, 2014) (Liu *et al*, 2014)
		CCR5 knockin	Rabbit animal model	Oocyte injection		(Tang *et al*, 2014)
		CCR5	iPSC	Transfection		(Ramalingam *et al*, 2014)
		Disruption	HEK293T NuFF cells	Plasmid	15-45%	(Mussolino *et al*, 2011) (Mussolino *et al*, 2014)

Viral targets	Nuclease	Sequence/Strategy	Cells/Organism	Gene delivery	Efficiency	Reference
CXCR4	ZFN	Disruption	T cells Humanized mice	Ad5/F35 Cell transplantation	30-34%	(Wilen *et al*, 2011)
CCR5 and CXCR4	ZFN	Disruption	T cells, Humanized Mice	Plasmid, Adenovirus, Cell transplantation		(Yuan *et al*, 2012)
		Disruption	T cells Humanized mice	Ad5/F35 Cell transplantation		(Didigu *et al*, 2014)
PSIP1	Talen	whole-gene deletion and exon deletion	HEK293T Jurkat	Transfection		(Fadel *et al*, 2014a)
Restriction factors	ZFN	TRIM5α and APOBEC3G	T cells	Transfection		(Voit *et al*, 2013)

Note: HSC, hematopoietic stem/progenitor cells; ESC, embryonic stem cells; iPSC, induced pluripotent cells; Tre, Tailored site-specific recombinase. NILV, non-integrated lentivirus.