Perspectives of Genome-Editing Technologies for HIV Therapy

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Abstract: *Background*: Current HIV antiretroviral therapies potently suppress virus replication and prevent patients from progressing to AIDS but are unable to completely eliminate HIV due to the existence of dormant viral reservoirs which threaten to reemerge at anytime. Recently, genome-editing technologies that can recognize specific DNA sequences, including viral DNA, are being touted as promising tools for curing HIV, owing to their specificity, ease of use, and ability to be custom designed.

Conclusion: Here, we introduce several novel strategies aimed at eradicating HIV proviruses with state-of-the-art genome-editing technologies and discuss perspectives of these approaches for curing HIV.

Keywords: CRISPR/Cas9, genome editing, HIV, latency, provirus, TALENs, ZFNs.

ISSUES WITH CURRENT HIV THERAPY

Persistent HIV infection causes a gradual loss of CD4+ T lymphocytes and leads to immunodeficiency syndrome approximately 8 years post infection in untreated patients. However, the advent of highly active anti-retroviral therapy (HARRT) has turned HIV infection from a death sentence into a controlled chronic condition. Anti-HIV drugs targeting viral enzymes, such as the reverse transcriptase, protease, and integrase, inhibit different stages of the virus life cycle. In addition, *maraviroc* binds to the HIV co-receptor CC chemokine receptor 5 (CCR5) and disrupts the membrane fusion between the virus and the cell (Fig. **1**). Consequently, while combinations of HAART have succeeded in controlling HIV disease progression, they are not considered a cure for HIV infection [1].

HIV has a distinctive feature that integrates its viral genome into the host genome, called a provirus, often leading to the integrated HIV provirus becoming transcriptionally silent. The latently HIV-infected cells make up the majority of long-lived viral reservoirs and later produce HIV particles upon stimulation [2]. Thus, current drug-based therapies effectively suppress the virus when patients are adherent but any interruptions to the drug regimen may result in an immediate rebound of viremia. Furthermore, a growing problem with drug-based therapy is that HIV associated neurocognitive disorders (HAND) may arise as side effects. Therefore, a novel method for eradicating latently infected cells is needed for the complete eradication of HIV infection.

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APPLICATION OF GENOME-EDITING TECHNOLOGIES

Genome-editing technologies facilitate editing of genomes by creating double strand brakes (DSB) in DNA at a specific locus. DSBs promote non-homologous end joining (NHEJ)-mediated insertion/deletion (indel) mutations or homology directed repair (HDR) in the presence of template DNA. Induction of target-specific DSBs in living cells have been performed with engineered nucleases such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), in which a designable zinc finger DNA-binding protein or TAL effector originating from *Xanthomonas bacteria* is fused to a FokI nuclease, respectively [3-5]. The most popular genome-engineering tool in recent years is an RNA guided nuclease, also known as the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system, which was originally identified as a prokaryotic immune defense system against foreign genetic elements [6]. The CRISPR/Cas9 system is a simple and user-friendly tool because the cleavage site can be designed as synthetic RNA [7]. Moreover, the simultaneous use of several single guide RNAs (sgRNAs) targeting multiple genes is possible using the CRISPR/Cas9 system [8]. Therefore, it has become a widely adopted genome-editing tool and has led to an accumulation of knowledge and expertise in the field. These genome-editing applications are used for creating gene knockout organisms and/or cells in various species. Furthermore, the scalability of these technologies is not only well suited for applications related to genetically engineered animals but clinical applications as well. These technologies have also attracted attention in the field of infectious disease therapy. In this article, we introduce and discuss about recent genome editing technology trends in HIV research.

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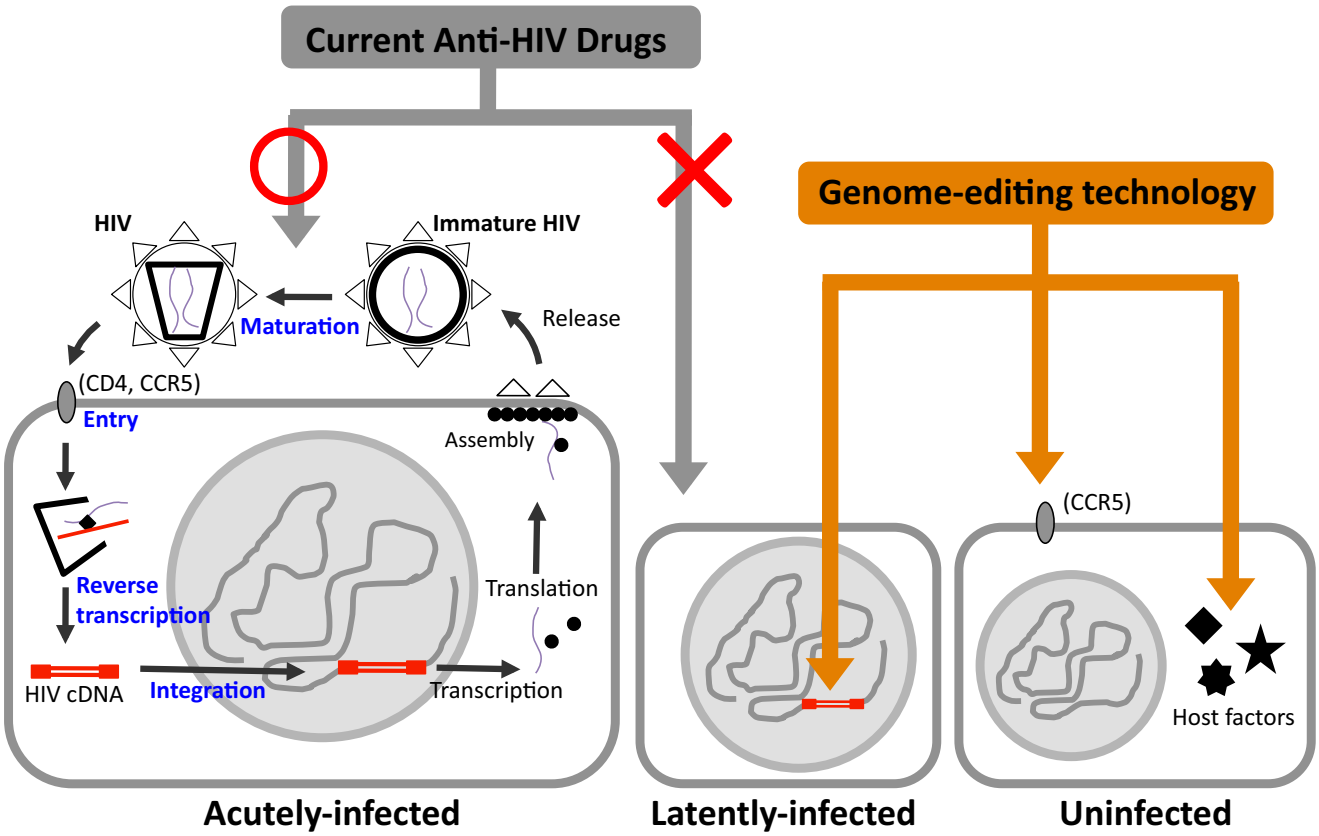

Fig. (1). Genome-editing technology is able to target latently HIV-infected cells. Current therapy using anti-HIV drugs inhibits reverse transcription, integration, maturation, and entry steps of HIV replication described as a circle (○) (Left, the target of HIV drugs is indicated as a blue character). However, those drugs are ineffective for latently HIV-infected cells described as a cross mark (x). Genome-editing technology is able to distinguish the latently-infected and uninfected cells by the genome sequence. In uninfected cells, CCR5 or other host factors can be targeted by the genome-editing technology (Right).

GENOME-EDITING TECHNOLOGIES FOR GENE THERAPY TARGETING HOST FACTORS OF HIV INFECTION

To date, there has been only one reported case of a patient successfully cured of HIV. Known as the "Berlin Patient", this HIV-positive patient had lymphoma and received a hematopoietic stem cell transplantation from a donor who possessed a bi-allelic 32-base pair deletion mutation in the *CCR5* gene (*CCR5-delta32*), which confers resistance to HIV infection. As a result, he was successfully treated for lymphoma and HIV was undetectable in the blood and tissue samples even in the absence of antiretroviral therapy [9, 10]. However, while the Berlin Patient demonstrated the efficacy of gene therapy targeting *CCR5*, applying this approach to other patients is complicated by difficulties associated with finding an HLA matching donor among a rare population with bi-allelic *CCR5-delta32*.

RNA interference strategies such as small interfering RNAs (siRNAs) or small hairpin RNAs (shRNAs) targeting CCR5 mRNA have been proposed as an anti-HIV gene therapy strategy to transiently knockdown CCR5 [11]. However, these approaches do not potently inhibit HIV entry nor do they sustain sufficient knockdown of CCR5 protein levels. In response to this problem, a genome-editing strategy utilizing ZFNs has garnered much attention in the field. In theory, genome-editing technology can mutate a target gene with a single round of editing, transiently. This consideration motivated scientists to develop ZFN technology targeting *CCR5*, yielding much progress in a relatively short period of time. Recently, it has been reported that human hematopoietic stem cells (HSC) modified by ZFNs targeting *CCR5* successfully produced HIV-resistant mature CD4+ T cells in humanized mice [12]. Furthermore, Sangamo BioSciences, Inc. has performed a Phase 1 trial of ZFN-based gene therapy targeting *CCR5* in autologous T cells and reported the therapy is safe and has long-lasting effects on the immune system of HIV-positive individuals, including increased white blood cell counts and potentially suppressed HIV replication [13]. This is a promising approach for curing HIV, however, the strategy has some disadvantages. Although the majority of HIV isolated from HIV-infected individuals is CCR5-tropic virus, CXCR4- or dual (CCR5 and CXCR4)-tropic viruses are found in some individuals who develop the disease. Therefore, modifying CCR5 will be ineffective against CXCR4-tropic viruses. Unfortunately, modifying CXCR4 is not an option because unlike CCR5, CXCR4 has a critical function in the immune response and hematopoiesis as a chemokine receptor. Furthermore, it is undetermined whether autologous transplantation facilitates elimination of cells infected with HIV, because there is a possibility that donor T cells

eliminate recipient cells infected with HIV as in the case of the allogeneic transplantation of the Berlin patient [14]. Alternative strategies which address the shortcomings of the nuclease-based gene therapy targeting *CCR5* should be developed.

In the past two decades, a number of host factors have been reported to be essential for HIV replication and may serve as alternative candidates for nuclease-based gene therapy. Of these host factors, lens epithelium-derived growth factor (LEDGF), which is important for HIV integration, has been proposed as a potential target for nuclease-based HIV therapy [15, 16]. Importantly, LEDGF seems to be expendable in CD4+ T cells and CD34+ hematopoietic cells, as it does not play a physiological role [17, 18]. Because even minor constituents of LEDGF protein are sufficient to facilitate proviral integration [19], nuclease-based gene knockout (KO) is advantageous to completely knockout gene expression. Recently, LEDGF KO cells, generated by TALENs reduced HIV infection [20]. A redundant protein, HRP2, that possesses an integrase-binding domain has also been identified recently and suggested to mediate HIV integration in the absence of LEDGF. However, even double LEDGF/HRP2 KO cells were still susceptible to HIV integration, implying that further redundant host mechanisms may exist [17, 21]. Even though a complete block of HIV integration may be impossible with a gene KO strategy, those host factors promote HIV cDNA integration at actively transcribed genes. Therefore, the reduced efficiency of integration in LEDGF/HPR2 KO cells might be sufficient for reducing HIV infections *in vivo*. Further studies are expected to evaluate whether the LEDGF KO strategy is sufficient for inhibiting HIV replication *in vivo*.

GENOME-EDITING TECHNOLOGIES TARGETING VIRAL GENOMES

Genome-editing technologies are not only useful as tools to cleave the genome of living organisms, animals, plants and microorganisms, they can also target the genomes of non-living and disease-causing agents. Recent reports have shown that genome of DNA viruses such as hepatitis B, human papilloma and Epstein-Barr virus, can be cleaved with ZFNs, TALENs and CRISPR/Cas9 systems [22, 23]. There is also an interest in using artificial nucleases as a tool to cure HIV by cleaving virus cDNA [24]. During the HIV life cycle, virus particle contents are released into the cytoplasm of an infected cell after entry. Once inside, a viral RNA genome is reverse transcribed into double stranded DNA in the cytoplasm, and subsequently integrated into the host genome after nuclear translocation (Fig. **1**, Left). Because the integrated HIV DNA assimilates into the host genome, latently infected cells in the clinical setting can be targeted. With this strategy in mind, our group validated the HIV proviral-editing strategy using the CRISPR/Cas9 system [25]. We designed sgRNA targeting the trans-activation response (TAR) region in the long terminal repeat (LTR) because it is essential for efficient elongation of HIV RNA (Fig. **2**, Top). The TAR region is relatively conserved among HIV subtypes [26] and many clinical isolates of HIV [27]. We demonstrated that the TAR-targeting CRISPR/Cas9 system i) induced indel mutations at the cleavage site and suppressed HIV expression; ii) was able to cleave and mutate provirus even in latently infected T cells; and iii) simultaneously cleaved LTRs located at the both end of provirus resulting in an excision of the internal HIV provirus sequence (Fig. **2**, bottom). Around the same time, Qu *et al.* constructed ZFNs targeting the TAR region in HIV LTR and reported efficient editing of HIV proviral DNA [27]. These results suggested that the TAR region in the LTR is an ideal target for nuclease-based therapy.

If nuclease-based HIV therapy is utilized for clinical applications, the goal will be to reduce off-target mutations and obtain higher nuclease activity. TALEN technology may be a better choice for the application because they exhibit significantly reduced off-target effects and cytotoxicity compared with ZFNs [28]. Furthermore, Sakuma *et al.* recently developed a TALEN system, called Platinum TALENs, which increased the specificity and activity by N- and C-terminal deletions of the PthXo1 TALE scaffold [29]. We constructed the platinum TALENs targeting the TAR region and verified the activity (Fig. **2**, Top) [30]. The newly designed TAR-targeting TALENs exhibited qualitatively higher activity during proviral editing than TAR-targeting CRISPR when plasmid DNA was used for the transfection. Notably, the proviral-editing efficiency was drastically improved to over 90% when mRNAs encoding TAR-targeting TALENs were used. Furthermore, off-target mutations by the TAR-targeting TALENs were not detected even though it exhibited high activity. These results suggest that TALEN-based therapy might be a promising strategy for an HIV cure.

Interestingly, constitutively expressed nucleases cleave unintegrated HIV DNA and protect against new HIV infection [31, 32]. These results suggested that the HIV provirus is cleaved before integration in the nucleus when the anti-HIV nuclease system is installed and functioning. However, a very recent study reported that the HIV cDNA is integrated at the genomic locus associating with the nuclear pore complex [33], meaning that immediate integration of HIV cDNA can occur after nuclear transport. Therefore, it is quite unlikely that the inhibition of the majority of HIV provirus occurs through cleavage before integration and, if so, complete inhibition of HIV replication *via* nucleases may be difficult to achieve (Fig. **3**). Further studies are necessary to uncover whether the pre-transduction of the anti-viral nuclease provides resistance for HIV replication.

As a point of note in nuclease-based HIV therapy, it is possible for mutant viruses to emerge that may be resistant to targeting by a designable nuclease (DN). It will be important to evaluate target sites with a low-risk to give rise to mutant viruses. A combined approach using DNs targeting different sites might reduce this risk. Similarly, simultaneous treatment of DNs with current anti-HIV drugs might be a simple and sound method. In sum, the DN-based strategy is the only method available to recognize and eliminate the provirus in latently infected cells and therefore further studies are needed.

Genome-editing technologies are not limited to just cleavage as they can be converted into tools for epigenetic regulation. The DNA binding modules of ZFNs, TALENs

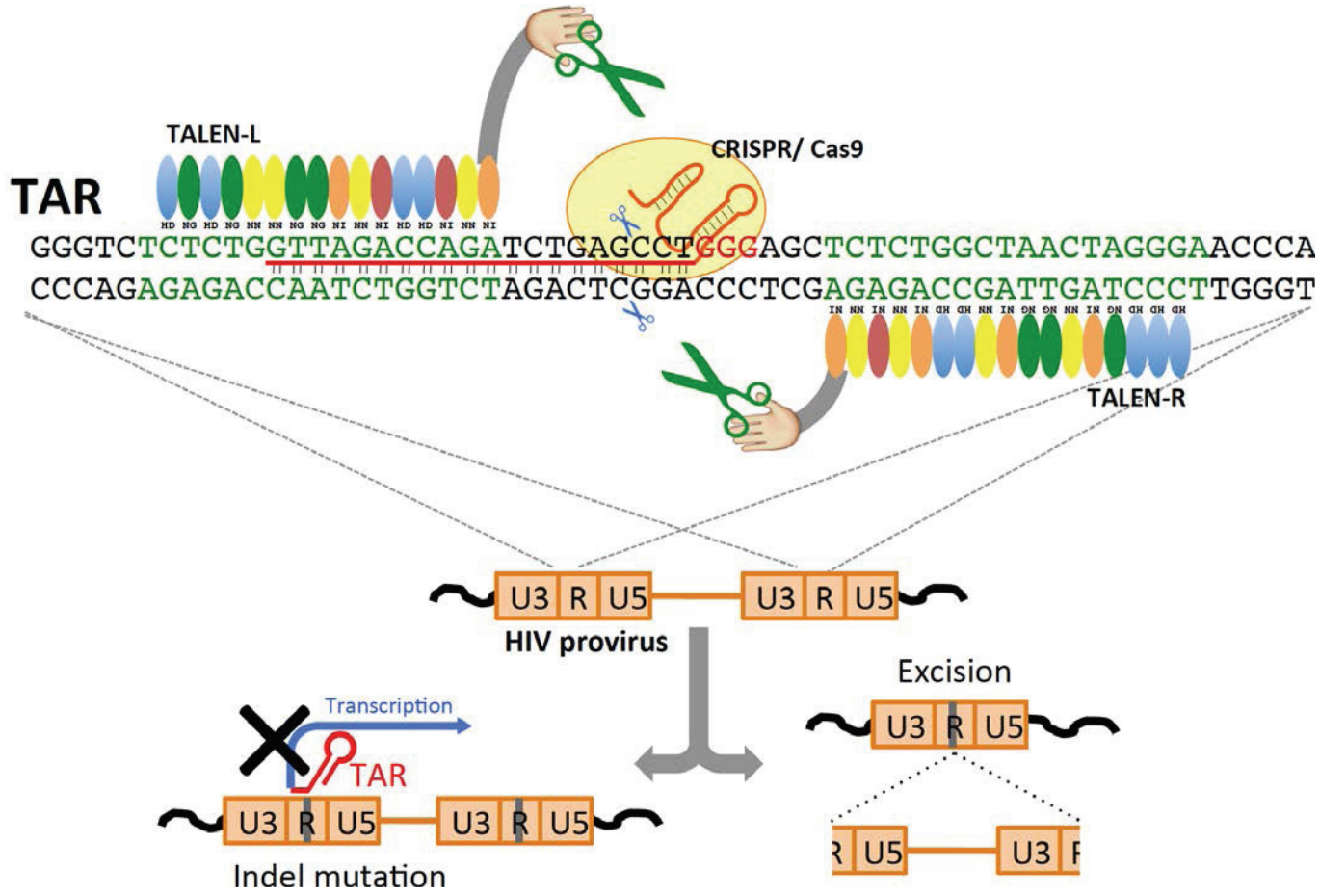

Fig. (2). Strategies of nuclease-based HIV therapy targeting HIV provirus. TAR region in LTRs are designed as target of CRISPR/Cas9 and TALENs. Designable nucleases targeted TAR is able to inactivate the LTR promoter and excise the internal sequence of HIV provirus.

and catalytically inactive Cas9 (dCas9) can be fused with a transcriptional activator such as VP64 or KRAB [34, 35]. Because latent HIV is transcriptionally repressed, the dCas9-VP64 system may be useful for reactivating the virus and flushing out dormant viral reservoirs. In fact, studies have been performed using a combination of HAART and HDAC inhibitors to cure HIV, in an approach called "shock and kill." The proposed method eliminates latently HIV infected cells by activating viral gene transcription while preventing new HIV infection at the same time. Although the activation is non-specific with HDAC inhibitors, the dCas9-VP64 system may serve as an HIV-specific "shocker" (Fig. **4**).

PROBLEMS OF GENOME-EDITING TECHNOLOGY AS A TOOL FOR HIV THERAPY

A major issue related to the application of genome-editing technologies for anti-HIV therapy is the risk of generating off-target mutations by unforeseen cleavage at similar genomic sequences. The risk of off-target mutations is invariably present as long as genome-editing technologies are used. For instance, Cas9 was reported to have off-target effects in commonly used cancer cell lines but the degree of off-target cleavage was also dependent on the targeting sequence [36]. Thus, the design of the target site is the most critical point to consider for lowering risks. Fortunately, many databases and methods for selecting unique target sequences are publicly available for all researchers to use to lower off-target cleavage [7]. As an alternative to wild type Cas9, a mutant variant Cas9 (D10A), lacking catalytic activity in the RuvC domain, generates a single nick in the DNA as opposed to a double stranded break [7]. Single-strand DNA breaks generated by the Cas9 D10A mutant are predominantly repaired by the high-fidelity base excision repair pathway (BER) and does not result permanent genome modification [37]. Therefore, a double nickase strategy that generates single-strand DNA breaks at a specific locus on opposite strands of ssDNAs cleaves either 3' or 5' overhangs will offer a lower risk of off-target mutations. With rapid technical innovations, approaches to reduce off-target mutations will ultimately help to make genome-editing technologies safer.

Advantages and disadvantages of these genome-editing strategies for an HIV cure are summarized in Table **1**. In addition to off-target mutations, another serious challenge facing the use of this technology in the therapeutic setting is the delivery method of the nucleases as shown in Table **1**. Current clinical studies often use lentiviral vectors for gene transduction in CD34+ hematopoietic stem cells or CD4 + T cells [38-40]. However, there are several drawbacks with using lentiviral transduction. First, constitutive expression of nucleases after transduction may increase the risk for

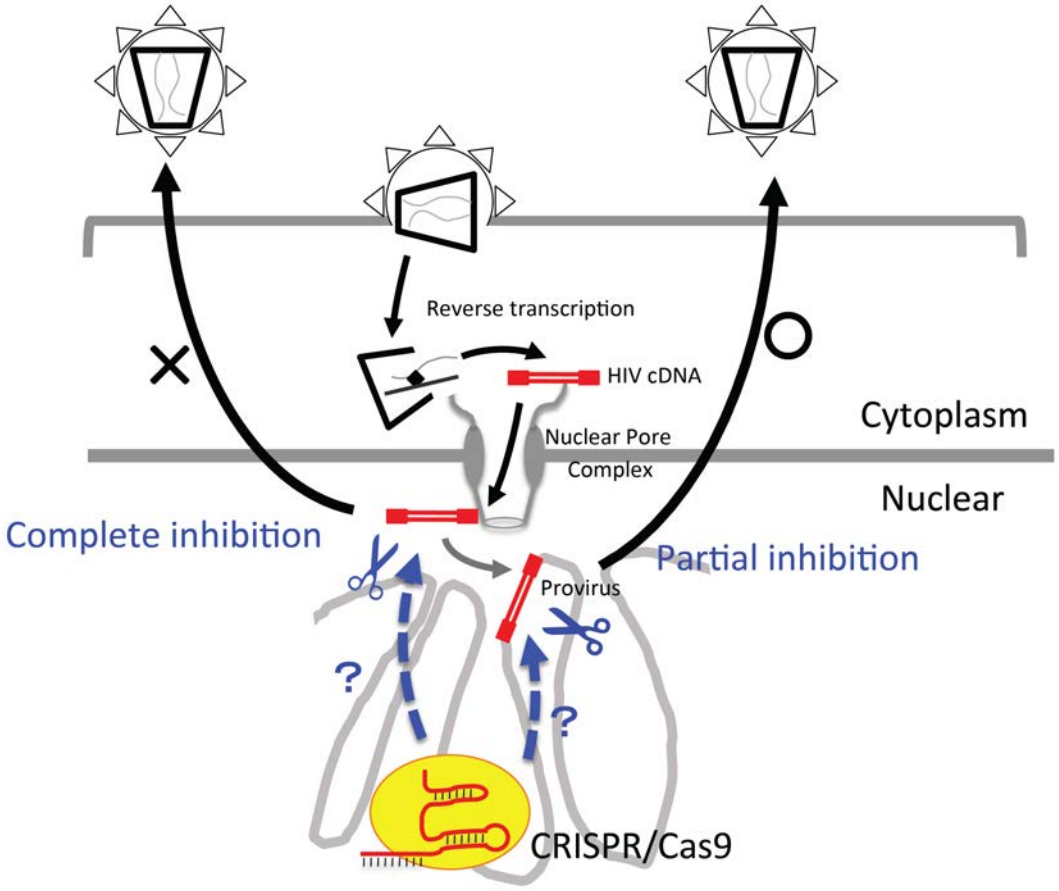

Fig. (3). Inhibition of HIV replication with anti-HIV nuclease. HIV cDNA is proposed to be integrated at the host genome interacting with nuclear pore complex immediately after nuclear import. If HIV cDNA is cleaved before integration, HIV replication can be inhibited. But if anti-HIV nuclease cleaves HIV cDNA after integration, the integrated provirus produces viruses until it is digested.

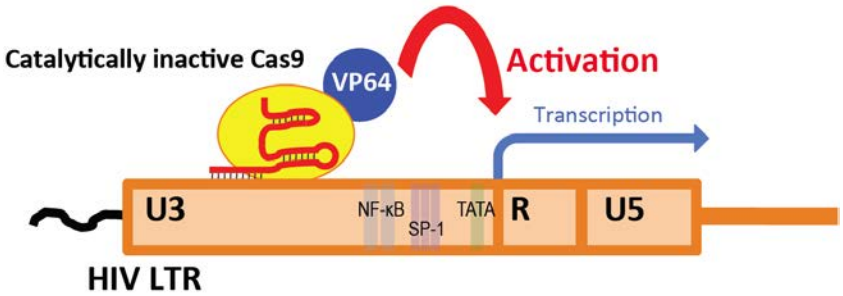

Fig. (4). Shock and Kill strategy using genome-editing technology. The CRISPR/dCas9-VP64 system can be used as an HIV promoter-specific activator.

generating off-target mutations. Integrase-deficient lentiviral vectors can be used instead to avoid toxicity from the persistent gene expression of nucleases [41], albeit at the cost of lower transduction efficiency. Second, lentiviral vectors cannot be used for TALEN delivery because the repeat sequence in the TAL module are missed during the reverse transcription step of the lentiviral transduction [42]. Third, the limited size of the lentivirus vector insert results in large inserts suppressing the titer of lentiviral vector. Unfortunately, alternative viral vector systems are unable to home in on target cells in the body for delivering a transgene. Thus, the development of alternative delivery systems is urgently needed in order to take advantage of genome-editing technologies for therapeutic use. In recent trends for DN delivery, methods introducing them as recombinant proteins or ribonucleoprotein complexes have been developed. Other delivery options into cells include the conjugation of nucleases with a cell-penetrating peptide or chemical methods such as bioconjugation and lipid nanoparticles [43-45]. Especially, lipid nanoparticle method may be an appropriate vehicle of DN for HIV therapy because it is possible to customize with peptide conjugation and provide CD4 selectivity [46].

Genome editing technologies are promising tools for achieving an HIV cure but it is still early to tell whether its potential can be realized in the clinical setting. Importantly, developing an understanding of the whole picture on latent infection in HIV infected patients such as identifying where

Table 1. Summary of genome-editing strategies for HIV cure.

Target Genes for Genome-Editing	Target Cell	Mechanism	Tool	Benefits	Problems
CCR5	Hematopoietic stem cell CD4+ T cell	➢ DSB mediated indel mutation	ZFNs, TALENs, CRISPR	➢ Genome-editing of CCR5 in hematopoietic stem cell is proposed to promise persistent resistance for HIV infection.	➢ No effect for X4 virus. ➢ There is no conclusive evidence for HIV cure with this strategy.
HIV provirus	Latently infected T cell	➢ DSB mediated indel mutation ➢ Proviral excision with simultaneous cleavage of LTRs	ZFNs, TALENs, CRISPR	➢ Only DNs strategy enables targeting latently infected cells. ➢ Disruption and excision of latent provirus theoretically enable HIV cure.	➢ It is difficult to deliver DNs to all of latently infected cells in vivo.
HIV cDNA	Uninfected T cell	➢ Cleave HIV cDNA before and/or after the integration in Nucleus	Constitutive expression of DNs	➢ This strategy may give a resistance for HIV infection.	➢ Cytotoxicity from constitutive nuclease expression is proposed. ➢ Emergence of resistant virus.
HIV provirus	Latently infected T cell	➢ HIV provirus specific transcriptional regulation	dCas9-VP64, dCas9-KRAB, etc	➢ This strategy is just concept. ➢ However, HIV provirus specific gene activation or suppression might be achieve HIV cure. ➢ Low risk of off-target mutation	➢ The inefficient delivery *in vivo* seems to be a major problem.

latent reservoir pools reside *in vivo* and how to access those deep recesses will be critical for eradicating the virus. New studies from basic and clinical studies will help to discover a practical method for utilizing these technologies.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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