Advance in Genome Editing of HIV-1 through CRISPR Technique

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Abstract: Although tons of great efforts and improvement have been made to treat HIV-1 patients, HIV-1/AIDS remain a big threat to global health. Although antiretroviral treatment (AT) yielded outstanding results in terms of its impressive effect in the suppression of HIV-1 replication and expression, latent viral reservoirs in HIV-1 patients still exist and potentially activate to disrupt human health in the future. Recent advance in the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9) system has been well-engineered with finely designed single-guide RNA (sgRNA) to effectively target cellular co-receptors CCR5 and CXCR4 or HIV-1 genome so that this genomic editing system can reduce HIV-1 reduction and eradicate integrated provirus. Such a versatile genome editing technology has been applied to several types of human cells or animal models to testify its value in the prevention of HIV-1. Here, the progress of the CRISPR/Cas9 -based approach in recent decades will be covered and discussed. In addition, its potential drawbacks are also analyzed for future perspectives of its full application in the HIV-1 treatment regimen.

1 INTRODUCTION

For centuries, human beings have been struggling against troubles from the human immunodeficiency virus (HIV), which causes acquired immunodeficiency syndrome (AIDS). At present, it has become one of the world's most severe public health issues in terms of its high incidence and prevalence. HIV is normally spread through wounds, injured skin, or blood containing HIV and sexual intercourse, etc., which makes it omnipresent in daily life. Indeed, its nearly ubiquitous presence has caused a lot of death and fear around the world. Such a depressing impact continues inflicting both psychological and physiological pains on everyone else in recent years, especially in the developing countries in Africa and the Asia Pacific. In 2018 an estimated 37.9 million people were living with HIV (including 1.7 million children), with a global HIV prevalence of 18% among adults. Roughly 21% of those people do not realize that they carry the virus (Global HIV and AIDS Statistics 2020). Statistically, an estimated 74.9 million people have become infected with HIV and 32 million people have died of HIV-related illnesses since the start of the epidemic (Global HIV and AIDS Statistics 2020). According to the data gathered by Kaiser Family Foundation (KFF), which focused on adults ages 15-49, global prevalence has leveled since 2001 and was 0.7% in 2019 (Global HIV and AIDS Statistics 2020). A lot of countries are still suffering from the pains it has brought. Of those countries, Eastern and Southern African countries are populated by most people living with HIV, which is about 20.7 million, accounting for 54% of the total global amount. Although Asia and the Pacific, Western and Central Europe, and North America do not have the same amount of infected population as those African countries, the situation is far from being good or optimistic since they collectively account for 21% of the infected global population (Global HIV and AIDS Statistics 2020).

As stated above, the situation in developed countries like the U.S. is not optimistic as well. In the U.S., roughly 1.2 million people are living with HIV today. About 14 percent of those people are not aware of it and need testing (U.S. Statistics 2021). It is obvious that HIV is hard to be tested in the early stage and can lay dormant for several months or a couple

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of years. This is indeed the case; most people are living with HIV before being diagnosed, and others are diagnosed right after HIV infection. According to the latest CDC data, in 2018, 37,968 people received an HIV diagnosis in the United States and dependent areas. From 2014 through 2018, the annual number and rate of diagnoses of HIV infection in the United States decreased. However, trends are mostly disparate due to different groups of people (U.S. Statistics 2021). In 2018, the gay or bisexual population are the most vulnerable groups since they accounted for 69% of new HIV diagnoses. Heterosexual people accounted for 24% and the remaining part all comes from people who inject drugs (U.S. Statistics 2021). By race, the Blacks or African American population is 13% of the U.S. population in 2018, but they sadly contributed to 42% of new HIV diagnoses. When it comes to age, it reveals that people aged 25-44 held the highest number of new HIV diagnoses (U.S. Statistics 2021). As stated above, it is not quite hard to realize that HIV prefers certain groups of people. It reminds people to be careful of their choices on occupations, daily habits, or ethnicity. Furthermore, there were 15,820 deaths among adults and adolescents with diagnosed HIV in the U.S. in 2018 (U.S. Statistics 2021).

Given those discouraging statistical data, scientists and pharmacists have been doing tons of tests and related research to search for a pharmacologically or medically efficacious regime to tackle the problems it causes. To date, there is still no efficacious cure for HIV-related diseases like AIDS. Antiretroviral therapy (ART) is being incorporated into the treatment of AIDS, along with other daily medications. This treatment regimen yields outstanding results due to its observable effect in declining HIV-related deaths and simultaneously protecting CD4 cells. It normally suppresses the viral load to an undetectable level in the peripheral blood of HIV patients. However, ART cannot eradicate the integrated HIV-1 genome from latently infected cells. This means that ART is not a desirable cure for HIV infection and necessitates a lifelong dependence on this therapy (Prevention 2021, HIV treatment overview 2021). Accordingly, a promising gene-editing tool, which is CRISPR/CAS9, has been tested and investigated both in vivo and in vitro to resolve those problems in the ART treatment regimen.

2 THE CRISPR/CAS9 ADVANCE IN HIV-1 TREATMENT

2.1 Life Cycle of HIV

Based on the previous findings, two types of HIV viruses are identified, which are HIV-1 and HIV-2. They both can give rise to AIDS in human bodies, but they do have a lot of distinctions regarding pervasiveness, geographical distribution, and lethality. Specifically, HIV-1 is the most common HIV seen in clinical practices, and it is more fatal and spreading more quickly than HIV-2. Therefore, HIV-1 has been regarded as a focal point for the treatment of AIDS. Here, all the materials and discussion centers around it. Below is the shared mechanism by which both HIV-1 and HIV-2 make their invasion come true.

Here, as shown in Figure 1, In the first stage, HIV normally enters the human body, and it strives to flow around CD4 T lymphocytes (CD4 + cells) and prepares for an invasion by binding its glycoprotein 120 (gp120) to the CD4 receptor on the surface of CD4 cells, and subsequently to the co-receptors C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4). In the second stage, the binding from the first stage normally gives rise to a fusion between HIV and cell membrane, which provides easy access for HIV to enter and release its viral RNA. In the third stage, HIV viral RNA was converted into double-stranded DNA (dsDNA) with the help of reverse transcriptase. This stage is also named after reverse transcription. The conversion of HIV RNA to HIV DNA makes HIV genetic material penetrate easily into the nucleus of CD4 cells, and subsequently, HIV DNA goes through the fourth stage where it employs integrase to embed its viral DNA into the genome of CD4 cells. Then, new HIV RNA, which is derived from proviral HIV DNA, can serve as genomic RNA to manufacture viral proteins. In the fifth stage, those viral proteins will aggregate and fuse with HIV viral RNA, and then they come to the cell surface to generate immature (noninfectious) HIV particles. Then, it comes to the last stage of its life cycle, which is budding. During budding, immature HIV particles are released out of CD4 cells, and simultaneously it releases a type of HIV protease, which will generate several mature (infectious) viruses by breaking the long protein chains of those immature HIV particles.

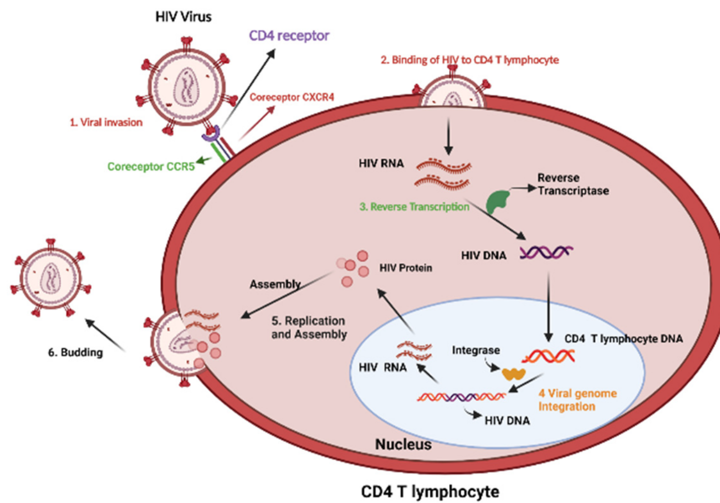

Figure 1: Life Cycle of HIV.

2.2 CRISPR/Cas9 Technology

On earth, to defend against the outside attack from bacterial and archaeal viruses, prokaryotes have developed a new defensive immune system clustered regularly interspaced short palindromic repeats & CRISPR-associated proteins (CRISPR-Cas). Given its viral genomic editing ability, such a defense system caught the attention of the scientific community, especially the pharmaceutical and medical field. Its biological value is highly evaluated not only because of its adaptive nature, but also its therapeutic potential. In medical applications, it serves as a gene-editing tool by recognizing and cleaving foreign RNA or DNA in a targeted sequencing manner. This defense system consists of three stages, which are adaptation, crRNA biogenesis, and target interference, respectively.

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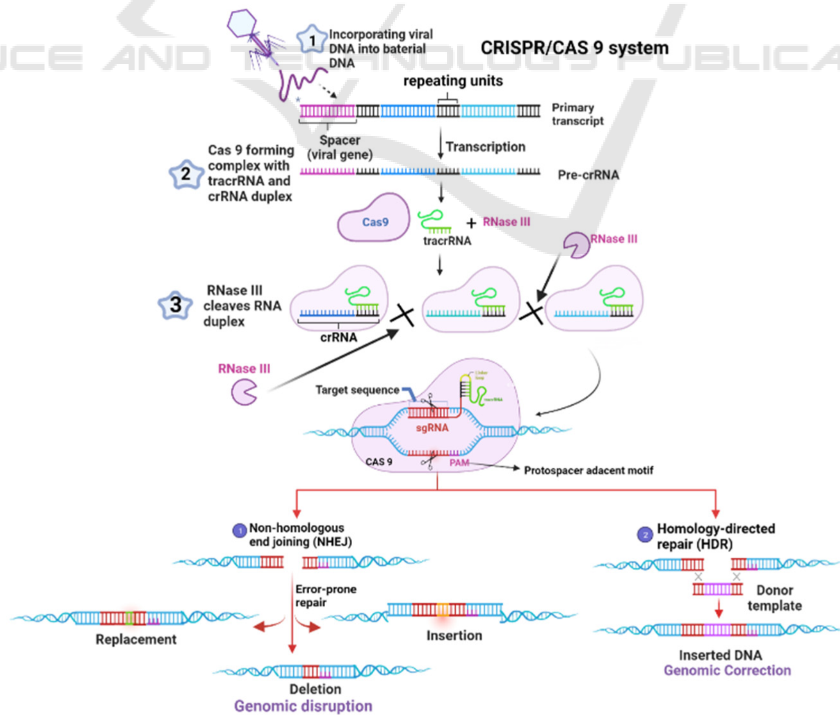

Figure 2: CRISPR/Cas9 Technology in Genomic Editing of HIV-1.

As shown in Figure 2, there are three essential stages CRISPR systems must go through, which are spacer integration (adaptation), expression, and interference. In the first stage, the bacteriophage invades prokaryotic cells and releases its viral gene. To defend against such an attack, prokaryotic cells give that bacteriophage permission to get access to its DNA sequence, and simultaneously cell itself integrates such an invading nucleic acid into its DNA sequence. Such a foreign viral gene is normally derived from a phage of plasmid DNA, which is called a spacer. By structure, CRISPR is comprised of arrays of short repeating units interspaced by spacers. The acquisition of foreign spacer paves a way for prokaryotic cells in further defense against invasion by phages or plasmids carrying similar sequences.

In the expression stage, the CRISPR arrays, which are generated from the spacer acquisition stage, turn themselves into long primary transcripts via transcription. Those transcripts are precursor crRNAs (pre-crRNAs), which play a vital role in subsequent stages. Subsequently, Pre-crRNA matures into crRNA by virtue of Cas9 proteins, tracrRNA, and RNase III. TracrRNA is a trans-encoded small RNA base-paired with the repeating region located on the Pre-crRNA.

Then, in the interference stage, an effector complex is formed by binding of Cas9 proteins into a single guide RNA (sgRNA), which is formed by the linkage between tracrRNA and targeting crRNA. The formation of this effector complex can directly initiate and control target DNA cleavage in the Protospacer adjacent motifs (PAMs). Such cleavage is directed by two essential domains within Cas9 nuclease, which are the histidine-asparagine-histidine (HNH) domain, and RuvC domain, respectively. Target DNA strand, which matches the base pairs with sgRNA, is normally cleaved by the HNH domain, while the non-target DNA strand is broken apart by the RuvC domain. Such a cleavage will give rise to double-stranded DNA break (DSB), which is subsequently going to be repaired by non-homologous end-joining (NHEJ) without any homologous template or homology-directed repair (HDR).

2.3 CRISPR/CAS9 Technique in Genome Editing of HIV-1

Currently, ART is still being used as a major treatment regimen for HIV-1/AIDS patients in the clinic. However, it cannot easily eradicate latent viral reservoirs in HIV-1 patients. To achieve this, more and more attention was paid to recently popular new genome editing technology, which is the CRISPR system. Such a functional system, along with nuclease 9 (Cas9), is engineered to target certain genomic regions in the HIV genome or cellular co-factors, which can give rise to a big reduction in HIV infection and an elimination of internal viral genes.

Based on the life cycle of HIV-1, there are two pathways to reach our goal. The first one is to directly target the HIV-1 genome to inactivate and eliminate the HIV provirus. The second one is to disrupt co-receptors CCR5 and CXCR4 by CRISPR/Cas9 technology. Tests or assays have been conducted to verify the feasibility and effectiveness of CRISPR/Cas9 in the suppression of HIV-1 expression and elimination of provirus within the host cell. The CRISPR/Cas9-based approach was first tested in HIV-1/AIDS treatment in 2013 (Ebina et al. 2013). Their test results revealed that the CRISPR/Cas9 system successfully targets HIV long terminal repeat (LTR) and then suppresses the expression of HIV genes. The target sites in their tests were the NV-kB binding cassettes and TAE sequences, which were situated in the U3 region of LTR and R region, respectively. By targeting those sites, HIV-1 provirus transcription and replication were effectively restrained (Ebina et al. 2013). Most importantly, the ability of CRISPR/Cas9 to eliminate internal integrated provirus from the host cell genome was proved. Such a desired ability is not something we cannot see from ART treatment. The HIV-1 patients treated with ART mostly have a risk of HIV-1 rebound since it cannot eradicate the latent provirus inside the host cells. That is why such a functional elimination of internal viral genes is really valued in the clinic. Soon afterwards, Wenhui Hu and his colleagues conducted another research to apply CRISPR/Cas9 based approach to excise genome of HIV-1, and they succeeded in getting some good results showing that inactivating viral gene expression and suppression of viral replication in a HIV-1 latently infected T cell line, microglial cell line with minor genotoxicity, pro-monocytic cell line, and no detectable level of off target genomic editing, was achieved (Hu et al. 2014). In their research, they designed four different LTR Guide RNA (gRNA) to separately test out their specificity and efficacy in editing HIV-target genome. It turned out all those gRNA worked well with different cell lines during the assay, but not all of them were functional, which indicates their individual specificity in genomic editing.

Later, Liao Hsin-Kai and his group members also selected several potential gRNA target sites located

inside the genome of HIV-1, and they identified optimal target sites that can provide the human body with long-term and effective protection against HIV-1. The host cells they used here were primary T cells and human pluripotent stem cells (hPSC). By analyzing mean fluorescence intensity (MFI) of enhanced green fluorescent protein (EGFP) via FACS analysis and the percentage of green fluorescence protein (GFP) cells, it indicated targeting LTR sequences in the R region was more efficient than the other gRNA target sites (Liao et al. 2015). Then, they experimentally concluded that different targeting/disruption strategies and efficiencies in different stages (pre-integration and provirus) and different targeting sites as well (coding and non-coding regions) (Liao et al. 2015).

When double-stranded breaks (DSBs) happen, exonucleases inside host cells are capable to degrade the viral genome near the DSBs, and the Coding region in the genome of HIV-1 was targeted and its viral genes were disrupted via non-homologous end joining (NHEJ), which includes replacement, insertion, and deletion. On the contrary, the structural disruption can be triggered in a pre-integration stage when targeting non-coding regions like LTR regions[7]. Most importantly, they found out multiplexed CRISPR/Cas9 systems, which involve different gRNAs, can increase the level of disruption and excision of the pre-integrated proviral genome. Additionally, targeting multiple conserved sites instead of single one can effectively disrupt the genome of HIV-1 while clearing the concern about HIV-1 variants forming a resistance to singly guided CRISPR/Cas9 (Liao et al. 2015).

Besides directly targeting the genome of the HIV-1 virus, CRISPR/Cas9 can also serve as a useful tool to prevent HIV-1 from invading primary T cells or other cell lines by editing of co-receptor, which are CCR5 and CXCR4. As stated above, HIV-1 makes its entry into CD4 T lymphocytes by binding to the CD4 receptor, as well as CCR5 and CXCR4. If either CCR5 or CXCR4 was genetically modified or engineered, a negative impact on entry of HIV-1 will be expected. In the research of Liu et al, they designed two types of sgRNAs, which served as a tool to target CXCR4 and CCR5 at the same time. What they were trying to do was apply CRISPR-sgRNAs-Cas9 to trigger the genomic editing of CXCR4 and CCR5 in different cell lines including CD4 T cells (Liu et al. 2017). Then, by off-target and apoptosis assays to figure out if such editing can result in any non-specific editing or cytotoxic effect on cell viability. The final result turned out to be very impressive because both CXCR4 modified and CCR5 modified cells exhibited a selective advantage over unmodified cells during HIV-1 infection. Most importantly, there weren't any nonspecific editing or cytotoxic symptoms, which concluded that CRISPR/Cas9 based approach in editing both co-receptors are quite safe and effective to suppress X4-or/and R5-tropic HIV-1 infection (Liu et al. 2017).

Overall, the CRISPR/Cas9 systems have an apparent advantage over the other gene-editing techniques, such as ZFN and TALEN. CRISPR/Cas9 can change the cleavage target by simply changing the gRNA sequence. ZFN and TALEN systems need a redesigned protein binding domain to make changing of cleavage target sequences occur (Gaj et al. 2012, Khalili et al. 2017, Ousterout et al. 2016). Such an advantage makes CRISPR/CAS9 impressive from a design perspective. Besides, Yin Chaoran et al demonstrated the effective excision of HIV-1 proviral DNA from the host-host genome in pre-clinical animal models using saCas9 and multiplex sgRNAs by AAV-DJ/8 vector (Yin et al. 2017). Their research further supports the application of CRISPR/CAS9 in HIV treatment by animal model validation, which really lowers the sounds of those skeptics.

Despite the promising aspects of genomic editing of CRISPR/CAS9, challenges still exist. At present, one major concern is probably the off-target effect, which may give rise to genetic mutation or chromosol translocation. If CRISPR/Cas9 enters the clinic field, the reduction of the off-target effect would be necessary and urgent. Some researchers have already proved that the off-target effect found in CRISPR/Cas9 system was very limited compared with other editing techniques such as TALENs, ZFNs, or homing endonucleases by ChIP-seq (Duan et al. 2014). However, improvement is still needed for CRISPR/Cas9 to eliminate mismatches as many as possible.

Another challenge would be what kind of vectors should be utilized to deliver CRISPR/Cas9. As we know, CRISPR/Cas9 system is normally introduced into host cells through transfection. Normally, a successful transfection does need an appropriate delivering vector. The main vector being used consists of lentiviral, adenoviral, and adeno-associated viral vectors. Of those vectors, lentiviral vectors are widely utilized to deliver CRISPR systems with high efficiency and stable expression, but simultaneously the chances of off-target effect can be even higher (Wang et al. 2014). It is apparent that finding a vector with less off-target would be a big concern we should always keep in mind.

Also, HIV-genetic variation is highly likely to badly influence the efficacy of a CRISPR/Cas9 based treatment regimen. To resolve this problem, researchers designed dual-gRNA/Cas9 strategy, and their results demonstrated that although such a strategy can cure T cells infected by distinct HIV-1 isolates, the efficacy of this strategy is really compromised by sequence variation of the target sites in HIV-1 (Darcis et al. 2014). Later on, a quadruplex sgRNAs/saCas9 strategy was applied and tested. The results demonstrated maximization of the possibility of multiple indel mutations on six on-target sites and fragmental deletions among these sites Furthermore, this strategy can offer additional advantages, such as reducing the potential of HIV-1 escape (Wang et al. 2016), the high possibility of HIV-1 excision despite the continuous proviral mutation in the clinical HIV-1 patient population, a reliable loss-of-function achievement due to removal of a substantial portion of the target gene or genome (Bauer et al. 2015), and optimal efficiency of excision (Yin et al. 2016).

3 CONCLUSIONS

Currently, ART is still being used as the major treatment regimen for HIV-1/AIDS patients in the clinic. By the long-term effect of ART, there would be an obvious decrease in HIV-1 expression and symptoms can be alleviated to an undetectable level. However, meanwhile, a lot of side effects, such as appetite loss, or lipodystrophy, become a big concern. Although there has been a lot of improvement in ART to minimize its possible side effects, some severe side effects are still out there. Additionally, ART does require a long-term intake regularly. If patients forget or skip one of the doses of the day or the week, the HIV-1 virus would take full advantage of this chance to replicate and copy itself in their bodies again. At worst, it could result in drug-resistant, and then there would be few feasible HIV-1 treatment regimens for HIV patients to choose from. Hence, genomic editing technologies start to get more and more attention due to their potential in the suppression of HIV-1 viral gene and elimination of integrated HIV-1 viral gene within CD4 lymphocytes. CRISPR/Cas9 is one of them and the one with no cytotoxic effect and less off-target effect. However, there is still a long way ahead until this technique can be fully applied to the clinic field. Off-target and delivery vectors need more time and effort to deal with and eventually this technique is going to be more mature and feasible to be clinically qualified enough in the near future.

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