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Application of Gene Editing Technologies to HIV-1

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Abstract

Purpose of Review—This review will highlight some of the recent advances in genome engineering with applications for both clinical and basic science investigations of HIV-1.

Recent Findings—Over the last year, the field of HIV cure research has seen major breakthroughs with the success of the first phase I clinical trial involving gene editing of CCR5 in patient-derived CD4+ T cells. This first human use of gene editing technology was accomplished using zinc-finger nucleases (ZFNs). ZFNs and the advent of additional tools for genome engineering, including TALENs and the CRISPR/Cas9 system, have made gene editing remarkably simple and affordable. Here we will discuss the different gene editing technologies, the use of gene editing in HIV research over the past year, and potential applications of gene editing for both *in vitro* and *in vivo* studies.

Summary—Genome engineering technologies have rapidly progressed over the past few years such that these systems can be easily applied in any lab for a variety of purposes. For HIV-1, upcoming clinical trials will determine if gene editing can provide the long-awaited functional cure. Additionally, manipulation of host genomes, whether *in vivo* or *in vitro*, can facilitate development of better animal models and culture methods for studying HIV-1 transmission, pathogenesis, and virus-host interactions.

Keywords

Gene editing; HIV-1; zinc finger nuclease; ZFN; TALEN; CRISPR/Cas9

Introduction

The HIV field has made significant advancements since the early days of the epidemic in understanding the virus biology and developing effective therapies to inhibit replication. Today, thanks to HAART, patients can live with HIV for decades before AIDS progression and transmission rates are on the decline in the United States. However, due to the HIV latent reservoir, HAART cessation unfailingly leads to viral outgrowth and viremia. Long-term HAART usage also has its own set of side effects including coronary artery disease¹, osteoporosis², and kidney failure³, furthering the need for curative strategies.

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The story of Timothy Brown, better known as the Berlin Patient, is an optimistic one for the community. Brown, suffering from acute myeloid leukemia and HIV-1 infection, required a bone marrow transplant and was given donor cells from a individual homozygous for a naturally occurring 32bp deletion in CCR5, an HIV-1 co-receptor required for entry⁴. Following the success of the transplant, Brown discontinued HIV-1 therapy and has no detectable viremia for over six years⁵. From this observation, CCR5 modification has become a gene therapy target for curative HIV research. In 2014, Tebas *et al* published the first Phase I clinical trial of reconstituting HIV-1 patients with autologous CD4+ T cells that had been subject to targeted CCR5 disruption using a designer zinc-finger nuclease (ZFN)⁶. While only intended to test safety of the intervention, the treatment had observable efficacy, when patient viral loads started to decrease during the HAART cessation period following the engraftment. These promising observations are driving additional clinical trails and the hope that a functional cure is in our future.

As illustrated by the ZFN-CCR5 trial, the field of genetic engineering is changing the way we think about gene therapy and treatment strategies. In a little over a decade since the completion of the Human Genome Project, the field of human genetics is again transformed by the development of tools for precise modification of genomes. While the ZFN used in the CCR5 trial was developed by Sangamo over several years, recent advances in designer nuclease technology have greatly reduced the time required to design and test these tools. Moreover, the cost of assembling designer nucleases has also decreased, making them widely available. In this review, we will compare the various designer nucleases available including their delivery methods and applications. Furthermore, we will discuss how gene editing is currently being applied in the search for a cure and how these tools can facilitate the development of systems to better study HIV *in vitro* and *in vivo*.

Designer Nucleases

There are three types of nucleases that are currently used for genome engineering (Figure 1). These include ZFNs, TALENs and CRISPRs. While each type of nuclease recognizes its DNA targets differently, the effective outcomes are all the same. Upon substrate (DNA target sequence) recognition, the endonuclease creates a double-stranded break (DSB) in the DNA that can be repaired with high fidelity by homologous recombination (HR) or more likely through the error-prone mechanism of non-homologous end joining (NHEJ). NHEJ repair can result in small insertions and deletions (indels) surrounding the break, which can subsequently lead to frameshift mutations that disrupt proper gene expression when occurring in coding exons. These designer nucleases can also be delivered to cells or accompanied by donor DNA that can serve as a template strand to preferentially induce HR-mediated repair, leading to insertion of new sequences or seamless replacement of single-nucleotide polymorphisms (SNPs) both *in vitro* and *in vivo*^{7–10}.

The oldest and most well characterized are zinc-finger nucleases (ZFNs) that are composed of an N-terminal zinc-finger DNA binding domain fused to one half of a FokI cleavage domain at the C-terminus. Two ZFNs must bind to an adjacent stretch of DNA for FokI to dimerize and become activated. Each zinc-finger domain recognizes 3 base pairs of DNA in a modular fashion with each ZFN arm containing 3–4 domains, adding additional levels of

context specificity. The protein-DNA interactions are empirically determined and as such, design of ZFNs can be time-consuming. To reduce off-target effects, the two FokI domains have been engineered as heterodimers that can only create DSBs when the two different arms come together¹¹.

More recently, transcription activator-like effector nucleases (TALENs) have functioned as a second-generation designer nuclease, whereby a modular protein arm is fused to one half of a FokI cleavage domain. In contrast to ZFNs, TALENs are made up of “fingers” that recognize a single base pair of DNA. While these constructs are less context specific, the protein-DNA interactions are much more predictable and do not have to undergo the same level of rigorous optimization as ZFNs. Additionally, since TALEN design follows a general code, groups have developed software tools to assist researchers in designing their own TALENs from commercially-available assembly kits. Although the design of TALENs is generally more straightforward than ZFNs, production of these nucleases can be challenging.

The most recent and likely to become the ubiquitous form of gene editing is the CRISPR/Cas9 system. CRISPRs (clustered regularly interspaced short palindromic repeats) were initially discovered as a bacterial adaptive immune system for incoming foreign phage/plasmid DNA, but were subsequently re-engineered by several groups to target DNA in eukaryotic cells^{12–15}. Unlike ZFNs and TALENs, DNA-binding activity is conferred through hybridization with a guide RNA (gRNA) and follows simple Watson-Crick base pairing. This gRNA has a short (20bp) complementary region fused to a larger RNA scaffold that is recognized and bound by the Cas9 endonuclease. The only stringent requirement for successful targeting is the presence of a protospacer adjacent motif (PAM) (typically NGG) in the target DNA immediately downstream (3' proximal) of the RNA binding region. While each of the designer nuclease is entirely customizable, ZFNs and TALENs require more time spent on design, assembly, and optimization than CRISPRs making the CRISPR system the easiest and cheapest gene editing platform, as illustrated by the sheer number of papers published in the two years since they were first described for eukaryotic use.

Delivery Mechanisms

Delivery of designer nucleases in tissue culture can be accomplished through multiple routes. Standard transfection and nucleofection methods can be used for plasmid DNA delivery, while delivery by viral transduction is also quite successful. Viral transduction is the most efficient for nuclease delivery and generally has less toxicity issues than nucleofection. Adenoviral vectors rather than lentiviral vectors must be used with TALEN constructs due to their highly repetitive elements, which have the potential to recombine. Lentiviral vectors are safe to use with CRISPRs and numerous groups have deposited into AddGene, a Cambridge, MA non-profit organization dedicated to making it easier for scientists to share plasmids, lentiviral constructs specifically for gRNA and Cas9 delivery. The method of delivery will also depend on the target cell, as some cell lines are not easily transfected.

Moving *in vivo*, there has been great success in generating transgenic animals using CRISPRs, including modification of multiple genes within the same organism^{16–20}. Delivery of gRNA and Cas9 is accomplished by microinjection of *in vitro* transcribed RNA into one-cell embryos. Since the RNA is eventually degraded, little toxicity is observed in manipulated embryos and long-term accumulation of off-target modifications (e.g. in the case of stable transduction) is mitigated. In terms of therapeutic applications, delivery of the nuclease needs to be efficient, occur at a large scale (10^8 – 10^9 cells), and be highly reproducible. In a CCR5-ZFN Phase I trial, the ZFN was delivered by a replication-defective Ad5/35 vector⁶. Delivery by non-integrating viruses will likely be the route of delivery in future studies.

Applications

An exciting utilization of designer nucleases has been in curative HIV research. The CCR5-ZFN trial is promising for the field, and subsequent trials are under way by Sangamo investigating the dosing of the modified CD4⁺ T-cells, with and without cyclophosphamide pre-treatment^{21–23}. Time will tell if this is a viable treatment option and whether or not HIV infected individuals will be able to live without daily HAART.

In addition to gene therapy trials, genome engineering can be applied to better understand virus-host interactions. A non-human primate (NHP) model for HIV-1 infection is still lacking in the field, but two groups have shown that transgenic monkeys can be made using CRISPR/Cas9²⁴ and TALENs²⁵. Manipulation of NHP to remove barriers to cross-species transmission (e.g. TRIM5 α , tetherin) has the potential to elicit HIV-1 susceptibility. Additionally, humanized mouse-models of HIV infection have been helpful for studying HIV pathogenesis *in vivo*. Gene editing has the ability to better these small animal models by targeted replacement of murine genes with the human homolog (e.g. cytokines) to better recapitulate the human adaptive immune system.

Studying HIV-host interactions *in vitro* can also be greatly enhanced by the use of genome editing. With the ease that CRISPRs can be assembled and delivered, one can study the effects of knocking out genes of interest using the standard Cas9 nuclease, or modulate gene expression with catalytically inactive Cas9 fused to transcription activators or repressors^{26–28}. Similar systems are also available with TALENs^{29,30} however the more laborious process of producing TALENs suggests that the CRISPR systems will be more commonly utilized. Additionally, the Cas9 nickase³¹ (which creates a single-stranded break) delivered with a donor template to promote HR can be used to recapitulate interesting SNPs or polymorphisms that may be important modulators of susceptibility/resistance to HIV infection or replication.

Experience and Perspectives for Human Use

The therapeutic potential of genome editing is already evident with the current trials involving patient-derived CD4⁺ T Cells in HIV-1 infected individuals⁶. Many studies have been done to look at the off-target effects of ZFNs, and while there is risk involved, the potential benefit is much greater. Furthermore, the use of CD4⁺ T cells mitigates the risk of tumor development when compared to proposed studies in which hematopoietic stem cells will be edited. In contrast to ZFNs, TALENs have not been looked at closely in regards to

their off-target potential. The timing of TALENs commercial availability nearly coincided with the first publications involving CRISPR/Cas9 use in eukaryotic cells, leading most labs to focus on the latter. Several studies looking at CRISPR/Cas9 off-target effects have been published, although reports are somewhat conflicting. The relatively extensive use of CRISPRs in the generation of transgenic animals with seemingly normal development is encouraging for their therapeutic use.

An additional HIV-interacting host factor has been targeted for preliminary curative research. Two groups used TALENs³² or ZFNs³³ generate deletions in LEDGF, also known as PSIP1, resulting in impaired integration and virus spread. While LEDGF knock-out is tolerated in mice, it is unclear if this is a feasible target in humans and if any additional benefit would be provided in combination with ZFN-CCR5 therapy. Moving away from targeting host-factor determinants for infection (e.g. CCR5), three groups have recently published a method for disrupting the HIV provirus using ZFNs, TALENs and CRISPRs targeting the HIV LTR^{34–36}. These studies were done *in vitro* in HIV-transduced cell lines. While targeting the HIV reservoir to effect an HIV cure is an interesting concept, clinical trials attempting to “flush out” the reservoir using HDAC inhibitors have been ineffectual thus far^{37,38}. Using a gene therapy approach to disrupt the HIV reservoir has several obstacles to overcome in terms of identifying and successfully targeting the entirety of the reservoir, which would seem to be required for a functional cure.

Conclusions

Recent advances in generation of designer site-specific nucleases, specifically the advent of the CRISPR/Cas9 systems, have the potential to revolutionize many studies on HIV-1 biology. Immediate applications of this technology include *in vitro* studies of required host genes or of innate restriction systems and development of genetically altered animal models for HIV-1 infection. Longer term it seems likely that the ease with which gene editing systems can be developed and tested will lead to safe and efficient production of gene edited terminally differentiated or hematopoietic stem cells that are resistant to HIV-1 infection for re-introduction into patients. Less clear is the path to deployment of gene editing technologies for destruction of the HIV-1 reservoir in patients.

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Key Points

- Advances in genome editing technologies, particularly the CRISPR/Cas9 system, have made genome engineering widely accessible and rapidly attainable.
- Genome editing can be used not only to create genetic knockouts, but also can be used to generate genetic variants (e.g. alternative exon usage or SNPs) or modify expression levels (e.g. transcription factor binding, epigenetic modification).
- The use of gene editing in HIV cure research has had promising clinical trial results thus far and the community is eager to see how these studies progress.

Figure 1.

Designer nuclease systems for genome engineering. **(A)** Zinc-finger nucleases (ZFNs) are composed of tandem zinc finger domains fused to one-half of a FokI domain. Each zinc-finger domain binds three nucleotides. The ZFN arms must be positioned 5–6 bases apart for efficient FokI heterodimerization, activation, and double-stranded break formation. **(B)** TALENs are formed by the modular assembly of TALE domains, with each domain recognizing a specific nucleotide via two variable amino acids (Repeat Variable Diresidue). Like ZFNs, the two arms must be in close proximity for cleavage to occur. **(C)** The CRISPR/Cas9 system utilizes an ~85bp RNA-guide molecule (gRNA) to target the Cas9 endonuclease to the site of cleavage. The 5' 20-bp of the gRNA recognizes the target sequence, while the remaining structure serves as a scaffold for Cas9 binding. DSB formation occurs 5–10 nucleotides upstream of the PAM.