Published in final edited form as:

AIDS. 2016 May 15; 30(8): 1163–1174. doi:10.1097/QAD.0000000000001079.

Functional screening of guide RNAs targeting the regulatory and structural HIV-1 viral genome for a cure of AIDS

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Abstract

Objective—There is an urgent need for the development of HIV-1 genome eradication strategies that lead to a permanent cure for HIV-1/AIDS. We previously reported that 4 guide RNAs (gRNAs) targeting HIV-1 long terminal repeats (LTR) effectively eradicated the entire HIV-1 genome. In this study, we sought to identify the best gRNAs targeting HIV-1 LTR and viral structural region and to optimize gRNA pairing that can efficiently eradicate the HIV-1 genome.

Design—Highly specific gRNAs were designed using bioinformatics tools and their capacity of guiding Cas9 to cleave HIV-1 proviral DNA was evaluated using high throughput HIV-1 luciferase reporter assay and rapid Direct-PCR genotyping.

Methods—The target seed sequences for each gRNA were cloned into lentiviral vectors. HEK293T cells were cotransfected with a pEcoHIV-NL4-3-*firefly*-luciferase reporter vector (1:20) over lentiviral vectors carrying Cas9 and single gRNA or various combinations of gRNAs. The EcoHIV DNA cleaving efficiency was evaluated by Direct-PCR genotyping and the EcoHIV transcription/replication activity was examined by a luciferase reporter assay.

Results—Most of the designed gRNAs are effective to eliminate the predicted HIV-1 genome sequence between the selected two target sites. This is evidenced by the presence of PCR genotypic deletion/insertion and the decrease of luciferase reporter activity. In particular, a combination of viral structural gRNAs with LTR gRNAs provided a higher efficiency of genome eradication and an easier approach for PCR genotyping.

Conclusion—Our screening strategy can specifically and effectively identify gRNAs targeting HIV-1 LTR and structural region to excise proviral HIV-1 from the host genome.

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Authors' contributions: CY, TZ, YZ, and FY carried out the sgRNA design, functional screening and PCR genotyping. CY, FL, FY and RP did the vector cloning, sequencing and cell cultures. CY, YZ, WH, KK, and WY did experimental design, data analysis and interpretation. WY constructed the EcoHIV_{NL4.3-eLuc}. CY, TZ and WH prepared figures. YZ and WH coordinated the study and drafted/revised the manuscript. The final manuscript was reviewed and approved by all authors.

Introduction

HIV-1 infection remains a major public health problem that affects more than 35 million people in the global world and more than 1.2 million people in the United States. The currently used combined anti-retroviral therapy (cART) has been successful in prolonging the lives of many AIDS patients due to effective suppression on the viral replication/production in HIV-1-infected cells. However, the cART cannot eliminate the integrated and transcriptionally silent HIV-1 provirus in latently infected cells. HIV-1 resurgence after cART withdrawal remains a main obstacle to a permanent or “sterile” cure for HIV-1/AIDS patients. Thus, novel strategies are urgently needed to eradicate the integrated proviral DNA in HIV-1 latent reservoir. Two promising strategies have been developed: proviral genome eradication[1] and latency-reversal/elimination in HIV-1 latent reservoir cells[2-6]. The challenge of HIV-1 genome eradication is the ability of HIV-1 to insert its proviral DNA into host cellular genome in a non-specific fashion, which prohibits the use of the integration site-specific knockout. Another challenge is the rapid emergence of mutations in HIV-1 genome, particularly in the envelope gene, among different cells in the same individuals or among different individuals with the same strain of HIV-1 infection. Nevertheless, the relatively high level of homology and conserved sequences within the viral long terminal repeats (LTR), an essential feature for HIV-1 replication at both ends of HIV-1 genome, and structural regions, such as Gag and Pol, allows a great opportunity to remove the entire HIV-1 proviral DNA. For example, engineered loxLTR site has been developed for Tre-mediated eradication of HIV-1 proviral genome[7-9].

Several novel systems for eradicating endogenous genes have been developed recently, including homing endonucleases (HE), zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and CRISPR-associated system 9 (Cas9) nucleases [10-15]. These genome editing technologies utilize site-specific double-strand DNA break (DSB)-mediated DNA repair machinery to enable genetic studies including targeted gene deletion, insertion, or modification. The feasibility of genetically excising the integrated HIV-1 provirus using HE to target the conserved viral protein sequences has been reported[16]. ZFNs targeting HIV-1 host co-receptor CCR5 gene have entered phase 2 clinical trials for the treatment of HIV/AIDS[17-19]. TALEN has been experimentally shown to effectively cleave CCR5 at the expected site[20, 21]. The novel and versatile Cas9 technology has also been used to disrupt HIV-1 entry co-receptors, e.g. CCR5 and CXCR4, and proviral structural proteins [20, 22, 23]. Genome editing at HIV-1 entry levels remains a promising therapeutic tool to prevent new HIV-1 infection through stem cell transplantation. However, it cannot eliminate the integrated HIV-1 genome, and CCR5 is not the sole receptor for HIV-1 infection and has many other cellular functions as well[20]. The highly conserved HIV-1 LTR has been targeted by ZFN and TALEN for the eradication of the integrated HIV-1 genome[24-26]. The major drawbacks for ZFNs and TALENs are the tedious engineering of custom DNA-binding fusion proteins for each target site, which limit their universal application and clinical safety[27-30]. In contrast, the novel RNA-guided Cas9 biotechnology has been developing rapidly due to its simplicity, specificity and versatility[10-15]. We previously reported that stable transfection of human cell cultures with plasmids expressing *Streptococcus pyogenes* Cas9 (SpCas9) and HIV-1 gRNAs

targeting 4 sites of HIV-1 LTR successfully eradicated part and/or the entire HIV-1 genome without compromising host cell function, and pre-existence of spCas9/LTR-gRNAs in cells prevented new HIV-1 infection[1]. Our finding was supported by several recent reports[31-33]. The aims of this study were to screen best gRNAs targeting conserved HIV-1 regulatory and structural regions and optimize a cocktail of gRNAs that efficiently eradicate HIV-1 genome.

Materials and Methods

Bioinformatics design of gRNAs with high efficiency and low off-target effects

We utilized the Broad Institute gRNA designer tool for highly effective gRNA design (<http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design>) and MIT's CRISPR Design for the off-target prediction (<http://CRISPR.mit.edu>).

Plasmids and cloning of gRNA expression vectors

The lentiviral vector (LV) pLV-EF1 α -spCas9-T2A-RFP was obtained from Biossetia Inc (San Diego, CA). The pNL4-3-firefly-luciferase was first constructed by inserting the eLuc reporter gene and P2A sequence with 5' end of Nef sequence, which was PCR amplified using fusion PCR approach, into the unique *XhoI* digestion site in the 5' region of the Nef of the pHIV-NL4.3 molecular clone. The pNL4-3-EcoHIV-firefly-luciferase (eLuc) vector (Fig. 1A) was then generated by replacing the coding region of gp120 Env with the ecotropic gp80 from pHCMV-EcoEnv (Addgene plasmid #15802, a gift from Dr. Miguel Sena-Estevés)[34]. A pair of oligonucleotides for each targeting site with 5'-CACC and 3'-AAAC overhang (Table 1) was obtained from AlphaDNA (Montreal, Canada). The target seed sequence was cloned via modified *BbsI* sites into pKLV-WG-U6(*BbsI*)-gRNA-PKG-Puro-2A-BFP LV (Fig. 1B) derived from pKLV-gRNA(*BbsI*)-Puro-2A-BFP LV (Addgene #50946)[35]. The LV was digested with *BbsI*, treated with Antarctic Phosphatase, and purified with a Quick nucleotide removal kit (Qiagen). Equal amount of complementary oligonucleotide was mixed in T4 polynucleotide kinase (PNK) buffer for annealing. These annealed seed pairs were phosphorylated with T4 PNK and ligated into the *BbsI*-digested LV using T4 ligase. The ligation mixture was transformed into *Stab13* competent cells. Positive clones were identified by PCR screening and verified by Sanger sequencing.

Cell culture, transient transfection and dual luciferase reporter assay

The HEK293T cells were cultured in high-glucose DMEM containing 10% FBS and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin) in a humidified atmosphere with 5% CO₂ at 37 °C. All cells were verified being free of mycoplasma contamination. For dual luciferase reporter assay, cells were cultured in a 96-well plate (1 $\times$ 10⁴ cells/well) and transfected with indicated plasmids by the standard calcium phosphate precipitation. After 2 days, the culture media was collected for *renilla* luciferase assay (Promega) and the cell lysate was prepared for the ONE-Glo eLuc assay (Promega). The luminescence was measured in a 2104 EnVision® Multilabel Reader (PerkinElmer).

Terra™ Direct PCR genotyping

Cells were seeded in 96-well plate and transfected with indicated vectors. After 2 days, the media was removed and the cells were treated with 22.5 µl of 50 mM NaOH per well and incubate for 10 min at 95°C. After neutralization with 2.5 µl of 1 M Tris-HCl (pH 8.0), 0.5 µl of the DNA extract was used in a 10 µl PCR reaction using the Terra™ Direct PCR Polymerase Mix (Clontech) and the indicated primers. After denaturation at 98°C for 3 min, two steps of standard PCR were carried out for 35 cycles with annealing/extension at 68°C for 1 min/kb and final extension at 68°C for 10 min. The PCR products were resolved in a 2% agarose gel. The gel images were collected and analyzed.

TA cloning and Sanger sequencing

The bands of interest were gel-purified and directly cloned into the pCRII T-A vector (Invitrogen), and the nucleotide sequence of individual clones was determined by sequencing at Genewiz using universal T7 and/or SP6 primers.

Statistical analysis

The quantitative data represented mean $\pm$ standard error from 3-6 independent experiments, and were evaluated by Student's *t*-test. A *p* value that is < 0.05 or 0.01 was considered as a statistically significant difference.

Results

Bioinformatics screening of sgRNAs with high efficiency and low off-target

The efficiency and specificity of target gRNAs are critical concerns for Cas9/gRNA application in infectious diseases. Several computing programs have been developed for the design and selection of gRNAs for the spCas9-gRNA system, wherein the 20 bp seed sequence and NRG PAM were used. While most of the gRNA design programs were developed to predict off-target effects, very few programs were able to predict cleaving efficiency. We designed 20 gRNAs targeting the HIV-1 LTR with a high score of cleaving efficiency and specificity against the human genome (Table 1) utilizing the following criteria: (1) Targeting -18 to -418 bp region of LTR-U3 promoter to disrupt HIV-1 initial transcription (and suppress virus production), and this 400 bp region is precluded in most LVs, thus avoiding LV self-cleavage; (2) Avoiding transcription factor binding sites that may affect the expression of host cellular genes due to high homology (Fig. 1B); (3) Matching both end LTRs to enable elimination of entire proviral DNA between LTRs; (4) Off-target score at more than 50%; and (5) Applicability to the double spCas9 nickase or dimeric RNA-guided *FoKI* nucleases. We also designed a few gRNAs targeting the structural region Gag and Pol (Fig. B) with a hope of obtaining the best combination of gRNAs to eradicate HIV-1 entire genome. The Env structural region was not selected due to lower conservation in this structural sequence between different strains. We chose LV gene delivery system separately expressing spCas9 and gRNA (Fig. 1C) for the following reasons: (1) LV itself provides many benefits for high efficient gene therapy in hard-to-transfect HIV-latent cell lines, animal studies and potential clinical application (integration-free LV)[36, 37]; (2) The separate spCa9 LV ensures good packaging efficiency for the large size of spCas9 gene; (3)

Separate gRNA expressing LV can be used for cloning multiplex gRNA expressing cassettes into one vector for good packaging efficiency.

Functional screening in HEK293T cells to identify effective gRNAs

For a rapid functional screening of the best targets, we performed EcoHIV-eLuc reporter assay using a high-throughput Envision multiple plate reader. The EcoHIV-eLuc reporter was selected because (1) it contains all the components needed for HIV-1 replication except for the HIV Env, (2) convenient to be handled at biosafety level II containers due to Env deletion and (3) bioluminescence is more sensitive than fluorescence and the eLuc reporter can be used to detect less than 10 single cells[38]. We chose the HEK293T cell line because of the high transfection efficiency with the cost-effective calcium phosphate precipitation method. With single gRNA transfection, we found that most gRNAs targeting the LTR promoter and the structural region could only result in marginal reduction of EcoHIV proviral reporter production but some increased the promoter activities or had no effect (Fig. 2A, B). The increase in promoter activity is consistent with a recent report that spCas9/gRNA-induced DSB within the promoter of neuronal early response genes stimulates their expression[39]. A single gRNA is expected to induce a single cut of the target sites, generating InDel mutations in the targeted regions and/or the deletion of the entire proviral DNA between both end LTRs. The mutation in the promoter may affect the functional activities of transcriptional activators and/or repressors, which may lead to an increase or decrease in the transcriptional activity. Mutation in the structural region may result in the shift of the open-reading frame of the HIV-1 structural proteins and thus decrease the expression of eLuc reporter.

To obtain more reliable and sensitive screening of the effective gRNAs for functional cleavage, we cotransfected the paired gRNAs: each LTR gRNA v.s. one of the gRNAs targeting the structural region. With this strategy, we expect to observe more dramatic reduction of reporter virus due to a large fragment deletion between either 5' or 3' LTR and the structural region. As an example shown in Fig. 2C, all combinations between GagD and any one of LTR-gRNAs reduced the eLuc expression significantly (64-96%), which is more robust than using a single gRNA. Half of LTR-gRNAs (10/20) showed >90% reduction in eLuc activity. Similarly, as another example shown in Fig. 2D, LTR-R gRNA paired with any one of GagA-D or PolA-B significantly reduced luciferase reporter activity to 7-23%. Selection of GagD or LTR-R for the pairing was also based on their PAM site applicable to *Staphylococcus aureus* Cas9 system and their targeting sites applicable to HIV-1 latent cell lines[40] and Tg26 transgenic mice[41] wherein the partial Gag and entire Pol sequences were deleted. These data suggested that a combination of LTR-gRNA with structural gRNAs provided a better and easier strategy to screen the effective gRNAs using high-throughput HIV-1 eLuc reporter assay. All the designed gRNAs are functional to reduce the expression of EcoHIV-eLuc reporter, which is consistent with the high score of the efficiency prediction by the bioinformatics analysis.

Identification of effective gRNAs using Direct-PCR genotyping

To validate whether these candidate gRNAs are functional to cleave the appropriate targets as designed, we performed Direct-PCR genotyping analysis using the DNA samples with the

paired gRNAs and corresponding PCR primers as indicated (Fig. 3A). The Direct-PCR approach does not require DNA extraction and purification and is thus more convenient for genotype screening. When one of the gRNAs targeting structural regions was used to pair with LTR targeting sites, PCR genotyping apparently generated new fragments (designated Deletion for convenient description) derived from the remaining (residual) viral LTR and Gag sequence after the deletion of fragments between 5' LTR to Gag (Fig. 3B, primer T361/T458). Consistent with the eLuc reporter assay, almost all the gRNAs induced various degrees of reduction in the wild-type band which can be easily amplified by the standard PCR condition on the 5'-LTR/Gag because of the size (1.3 kb). After cleavage, various degrees of Deletion as designed were detected in most combinations (Fig. 3B). Interestingly, additional fragments (designated Insertion) larger than the predicted Deletion were observed in most combinations on the 5'-LTR-Gag cleavage (Fig. 3B). Quantification of wild-type band intensity showed that LTR-O possesses the highest efficacy, followed by I, C, A as shown in highlighted red (Fig. 3B). This wild-type band cleaving efficiency pattern was not completely correlated with the reduction pattern of eLuc reporter activity (Fig. 2C), likely because the amplification of PCR product in a mix population usually prefers the small size product. On the other hand, weak reduction of the wild-type band in some pairs might result from various degrees of small InDel mutations (within a few nucleotides) within the gRNA target sites without any fragmental Deletion or Insertion. To avoid the potential influence of the PCR preferential amplification, we performed PCR genotyping using primers covering 3'-LTR and Gag (Fig. 3C, S1), which is predicted to generate 7 kb wild-type PCR product that is unlikely to be amplified by the PCR setting used. The fragmental Deletion pattern among the gRNAs detected by the single PCR product (Fig. 3C, S1) was consistent with that revealed by the relative ratio changes (Fig. 3B). The pair of LTR-K and GagD exhibited no Deletion or Insertion fragmental band in all the four sets of PCR genotyping reactions (Fig. 3B, C, S1A, B), correlating to only 7% reduction in the wild-type band (Fig. 3B). The pair of LTR-F vs. GagD showed weak deletion band in one set of PCR reaction (Fig. S1A), correlating to 17% reduction in the wild-type band (Fig. 3B). The pairs of LTR-G, P vs. GagD showed around 50% reduction in the wild-type bands, resulting from the deletion in either 5'-LTR-Gag (Fig. 3B) or Gag-3'-LTR cleavage (Fig. 3C, S1A, B). When the LTR-R was used to pair with any one of GagA-D and PolA-B, PCR genotyping with indicated corresponding primers also generated predicted new fragments (Deletion) (Fig. 3D, E) and additional insertions on the 5'-LTR/Gag (Fig. 3D) to various extent in all the tested gRNAs except for Gag-B gRNA, which exhibited very weak editing capacity (Fig. 3D). However, all these combinations with weak or no deletion genotyping still showed dramatic reduction in EcoHIV-eLuc reporter activity (Fig. 2C). This might be attributed to none or only one of the two gRNA plasmids transfected into the same cells wherein the single gRNA remains highly effective in inducing small InDel mutation. Taken together, these data suggest that the Direct-PCR genotyping provide a reliable and fast tool to validate the presence of fragmental Deletion and/or Insertion. However, evaluation of the efficacious HIV-1 eradication by various gRNAs requires a combination of functional reduction by the virus reporter assay and proviral DNA fragmental excision by the 5'-LTR or 3'-LTR-directed PCR genotyping.

Validation of fragmental Insertion/Deletion mutation by TA-cloning and Sanger sequencing

To further validate cleaving efficiency of spCas9/gRNAs and examine the pattern of Deletion/Insertion mutation after cleavage, we selected three representative samples of PCR genotyping for TA-cloning and Sanger sequencing. Paired expression of LTR-R/GagA caused a Deletion of a 519-bp fragment between LTR-R and GagA target sites (Fig. 4A). Co-expression of LTR-L/GagD (Fig. 4B) and LTR-M/GagD (Fig. 4C) led to a Deletion of a 772-bp or 763-bp fragment between each pair of target sites respectively. Furthermore, they caused various extents or types of small InDels. In some cases, a large Insertion of additional sequences (e.g. 159-359 bp) was identified (Fig. 3B, 4B, 4C). NCBI Blast analysis showed that these additional sequences derived from the exogenous vectors instead of endogenous host cellular genes. These results indicate that most of our candidate gRNAs can efficiently mediate targeted disruption of integrated HIV genome by either excision or insertion/deletion.

Discussion

We demonstrated previously that multiplex gRNAs could induce a deletion of large fragments between the target sites[1], which provides a reliable remedy to evaluate the DNA cleavage efficiency of Cas9/gRNAs. In this study, we further validated this proof of concept by screening various multiplexes of 26 gRNAs. We demonstrate that most of the designed gRNAs are highly effective at eradicating the predicted HIV-1 genome sequence between the two selected targeting sites leading to significant excision of HIV-1 reporter virus. In particular, a combination of viral structural gRNAs with one or two LTR gRNAs provided a much higher efficiency of genome eradication and an easier approach with Direct-PCR genotyping and high throughput reporter screening. The effectiveness and specificity of the gRNAs selected in this study for excising HIV-1 proviral DNA promise a success in the preclinical animal and clinical patient studies using Cas9/gRNA technology, because: (1) These gRNAs can serve as a ready-to-use selection source to develop viral and non-viral gene therapy; (2) For individual HIV-1 patient, these gRNAs can be used as a master to screen new gRNAs designed specifically for any HIV-1 isolates despite of high mutation rate of HIV-1; (3) Easy gRNA cloning, rapid reporter screening and reliable Direct-PCR genotyping provide a feasibility for practical application of Cas9/gRNAs to the personalized medicine.

Not all the designed gRNAs exhibit needed activities in cleaving the expected target sites. Several approaches have been developed thus far to evaluate the efficiency of genome editing induced by Cas9/gRNAs technology. Continuously improving computational programs for efficiency predictions have been tested using host cellular genomes as the design target [42-44] but may not be reliable for applying to the exogenous genomes such as infectious viruses. The Sanger sequencing of the target region via PCR cloning provides high sensitivity and specificity for determining genome editing efficiency[45], however it is labor-intensive for high throughput screening. Mismatch-based Surveyor assay[46-48] and high resolution melt analysis [49] are sensitive to detect the small InDel mutations but the poor specificity makes them prone to produce false positive results. The restriction fragment length polymorphism (RFLP) assay requires the presence of a restriction enzyme site with

the target region, which is limited in most cases[47]. Next generation sequencing provides a reliable and specific measure but is expensive and time-consuming[50]. Recently several PCR-based assays provide an easy and reliable method to quantify editing efficiency but they require robust primer design, trace decomposition or capillary sequencer[51-53]. Here we established a fast, cost-effective and reliable screening platform to identify effective gRNAs using highly sensitive high-throughput bioluminescent reporter assay along with a fast Direct-PCR genotyping. The reporter assay relies on the eradication of large fragments between two gRNA target sites as well as the small InDel mutations at each gRNA site. The fragmental eradication abolishes promoter activity or reporter expression while the InDel mutations may change the promoter regulation or induce open read frame shift of viral proteins. All these events will subsequently affect the activity of the reporter. The PCR genotyping relies on the fragmental cleavage and efficient re-ligation between the remaining end DNAs. The presence of the re-ligated PCR fragments provides an affirmative evidence for efficiency of both gRNAs. The re-ligation efficiency depends upon the cell dividing, thus the PCR genotyping may be limited in the case of non-dividing cells. In addition, the PCR condition for some primers needs optimization to achieve best efficiency of genotyping.

The objective of this study is to screen and identify the effective gRNAs by establishing reliable and sensitive high-throughput assays. We chose transient transfection of EcoHIV-eLuc reporter in HEK293T cells as a testing platform because a small amount of the reporter plasmid over spCas9/gRNA components (1:20) can ensure the target cleavage in all the reporter-expressing cells and thus maximize the detection efficiency of luciferase reporter assay and PCR genotyping. In contrast, the EcoHIV-eLuc stable cell line based on HEK293T cells (Fig. S2, S3), which may be closer to the real situation of HIV-1 latency, showed a poor detection sensitivity in both luciferase reporter assay and PCR genotyping. This is because the EcoHIV-eLuc-expressing cells without any gRNA plasmid always exist after transfection and thus eLuc reporter is constantly expressed, even while the transfection efficiency can be as high as 80-90%. Additional advantages of the transient reporter transfection include easy setup, cost-effective transfection and high-throughput luminescence measurement. Importantly, the identified gRNAs remain effective in the real HIV latently-infected cells or cell lines and can be further used for animal studies and clinical applications. Although the transiently transfected EcoHIV-eLuc reporter (episomal DNA) does not reflect the latent HIV proviral DNA in the host genome (nucleus), the spCas9/gRNA-mediated gene editing works in a similar efficiency between episomal and nuclear DNA of HIV provirus[1] and other viruses[54, 55]. Furthermore, the effective cleavage of the episomal DNA in addition to integrated HIV-1 proviral DNA allows for a novel preventative treatment for new infection of HIV[1] and other infectious viruses[56].

Some confounding factors may affect the transient transfection efficiency and transgene expression for the comparative analysis of different gRNAs. To minimize this, we have taken several precautions: 1) We prepared a master mixture of the reporter and spCas9 plasmids to ensure equal amount of these shared plasmids in each group of gRNAs; 2) We used *renilla* luciferase reporter (1:100) for normalization of transfection efficiency; 3) We performed a large scale of transfection in 96-well plate for all the gRNAs in 4-6 replicates at the same time; and 4) All the data were expressed as relative changes compared with the empty gRNA control in each experiment.

One gRNA targeting the LTR region may eliminate the entire proviral DNA due to the cleavage of both end LTRs but the eradication efficiency was not apparent as shown by the EcoHIV-eLuc reporter assay. It also requires long-range PCR to verify the eradication of entire HIV-1 proviral DNA because standard PCR with primers covering the LTR cannot distinguish 5'-LTR from 3'-LTR after deletion of a fragment between two LTR target sites[1]. Two gRNAs targeting LTR region induced fragmental cleavage within each LTR region that will suppress LTR promoter activity and reduce HIV-1 RNA stability, thus improving the entire eradication efficiency as we have demonstrated previously[1]. In this study, we tested a new proof of principle that any pair of gRNAs between the LTR and structural regions provides a better approach to evaluate HIV-1 eradication efficiency. By this method, the dramatic functional reduction in HIV-1 reporter virus production results from the three possible cleavages of 5'/LTR+Gag, Gag+3'/LTR and both end LTRs and can be easily monitored by the sensitive and high-throughput bioluminescence reporter assay. These cleavages can be efficiently and reliably detected by the standard and fast Direct-PCR genotyping using primers covering the LTR and structural regions. Similarly, a cocktail of two LTR gRNAs plus one or two structural gRNAs may provide an optimal and economical remedy to eradicate HIV-1 genome in the preclinical and clinical setting.

The potential for off-target effects involving the Cas9/gRNA technology has been a big concern in the field of genome editing. Stringent gRNA design, functional screening and Cas9 technology modification have been developing to increase the specificity of genome editing. Very rare instances of off-target effects related to spCas9/gRNAs in cultured cells have been validated by whole genome sequencing (WGS)[1, 57-60]. Newly developed unbiased profiling techniques further validate the high specificity of this spCas9-gRNA system[61-63]. *In vivo* off-target is expected to be much lower due to epigenetic protection. In our study, the exogenous viral DNA was analyzed against the host genome for best score of efficiency and specificity. No cellular toxicity was observed during gRNA screening. Double spCas9 nickases and RNA-guided *FokI* nucleases have shown to reduce potential off-target effects by up to 1500-fold[64-67]. Utilizing the identified effective gRNAs in these two systems remain to be investigated.

In conclusion, most of the designed gRNAs are highly effective to eradicate the predicted HIV-1 genome sequence between selected two targeting sites and affect eLuc reporter activities. In particular, a combination of viral structural gRNAs with one or two LTR gRNAs provided a higher efficiency of genome eradication and an easier approach for PCR genotyping. The screening with HIV-1 eLuc reporter assay and Direct-PCR genotyping provides a reliable, rapid and convenient approach to screen effective HIV-1 gRNAs. This can be utilized to set up high throughput gRNA library screen for any new HIV-1 isolates and other infectious viruses during new era of the personalized/precision medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by R01NS087971 (W.H., K.K.) and P30MH092177 (K.K.). The authors have no conflicting financial interests. A patent application has been filed relating to this work.

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Fig. 1. EcoHIV-firefly luciferase reporter construct and bioinformatics gRNA design

A. Diagram of EcoHIV reporter vector containing enhanced *firefly* luciferase (eLuc) derived from human HIV-1_{NL4-3} vector. The eLuc gene was inserted between Env and Nef with a self-cleaving 2A peptide before Nef, while the gp120 of HIV-1 was replaced with gp80 from the ecotropic murine leukemia virus. **B.** Selected gRNAs targeting HIV-1 LTR, Gag and Pol regions. The seed sequences targeting 400 bp within U3 region with red/underlined PAM at the sense strand and black/underlined PAM at the antisense strand. Most of them can be also paired for Cas9 nickase and RNA-guided *FoKI* nuclease. The TATA box and transcriptional factor binding motifs are shadowed and labeled in green. **C.** Lentiviral reporter vectors for spCas9 and gRNA cloning.

Fig. 2. Screening of effective gRNAs by EcoHIV-*firefly* luciferase reporter assay in a single (A, B) or paired (C, D) gRNA manner

HEK293T cells in 96-well plate were cotransfected with EcoHIV-*firefly* luciferase reporter (10 ng/well), pCMV-*renilla* luciferase reporter (2 ng/well), pLV-EF1a-Cas9-T2A-RFP (80 ng/well), and indicated gRNA expression vectors (80 ng/well for single gRNA or 60 ng/well each for paired gRNAs). After 2 days, the *firefly*-luciferase activity in the cell lysates was measured with ONE-Glo™ Luciferase Assay System and the *renilla*-luciferase activity was measured with Renilla Luciferase Assay System. Data represent mean $\pm$ SEM of 4-6 independent transfections with percentage changes in firefly luciferase after *renilla*-luciferase normalization compared with the empty gRNA LTR-0 group.

Fig. 3. Validation of effective gRNAs by PCR genotyping

A. Location of PCR primers and Gag/Pol gRNA targeting sites. **B, C.** GagD paired with various LTR-gRNAs. **D, E.** LTR-R paired with various gRNAs targeting Gag and Pol. Deletion of 5'LTR-Gag or Gag-3'LTR was detected. The band density of wild-type (WT), deletion and insertion was quantified with NIH Image J program. The number under the gel indicates WT band change (%) related to the empty gRNA control after normalization with Cas9 PCR product using Cas9 specific primers T477/T478, as well as fold changes in the insertion and deletion bands compared with WT band. The dramatic changes induced by corresponding gRNA were highlighted as red. The red boxes indicate the selected samples for TA-cloning and Sanger sequencing.

Fig. 4. TA-cloning and Sanger sequencing of representative samples confirmed the deletion of predicted fragments between corresponding gRNA target sites (A-C) and various additional insertions (B, C)

The PAM sequences are highlighted red and the scissors indicate the third nucleotide from PAM. The red arrow points the junction site after cleavage and ligation.

Table 1
Oligonucleotides for gRNAs targeting HIV-1 LTR, Gag and Pol and PCR primers

Target name	Direction	Sequences (5' to 3')
LTR-A	T353: Forward	aaac AGGCCCAGGGATCAGATATCCACTGACCTT g
	T354: Reverse	aaac AAGGTCAGTGGATATCTGATCCCTGGGCGCT
LTR-B	T355: Forward	aaac AGCTCGATGTCAGCAGTCTTGAAGTACT g
	T356: Reverse	aaac GAGTACTTCAGAACTGCTGACATCGAGCT
LTR-C	T357: Forward	caacc GATTGGCAGAACTACACACC
	T358: Reverse	aaac CGGTGTGTAGTTCTGCCAATC
LTR-D	T359: Forward	caacc GCCTGGCCTGGCGGGACTG
	T360: Reverse	aaac CAGTCCCGCCAGGCCACGC
LTR-E	T361: Forward	caacc GATCTGTGGATCTACCACACA
	T362: Reverse	aaac TGTGTGGTAGATCCACAGATC
LTR-F	T363: Forward	caacc GCTGCTTATGACAGCATCTGAG
	T364: Reverse	aaac CTCAGATGCTGCATATAAGCAGC
LTR-G	T530: Forward	caacc GTGTGGTAGATCCACAGATCA
	T531: Reverse	aaac TGATCTGTGGATCTACCACAC
LTR-H	T532: Forward	caacc GCAGGGAAGTAGCCTTGTGTG
	T533: Reverse	aaac CACACAAGGCTACTTCCCTGC
LTR-I	T534: Forward	caacc GATCAGATATCCACTGACCTT
	T535: Reverse	aaac CAAGGTCAGTGGATATCTGATC
LTR-J	T536: Forward	caacc GCACACTAATACTTCTCCCTC
	T537: Reverse	aaac GAGGGAGAAGTATTAGTGTGC
LTR-K	T538: Forward	caacc GCCTCCTAGCATTTCGTCA
	T539: Reverse	aaac TGTGACGAAATGCTAGGAGGC
LTR-L	T540: Forward	caacc GCATGGCCCGAGAGCTGCATC
	T541: Reverse	aaac GATGCAGCTCTCGGGCCATGC

Target name	Direction	Sequences (5' to 3')
LTR-M	T542: Forward	caccGCAGCAGTCTTTGTAGTACTC
	T543: Reverse	aaacGAGTACTACAAAGACTGCTGC
LTR-N	T544: Forward	caccGCTGACATCGAGCTTTCTACA
	T545: Reverse	aaacGTAGAAAGCTCGATGTCAGC
LTR-O	T546: Forward	caccGTCTACAAGGGACTTTCCGCT
	T547: Reverse	aaacAGCGGAAAGTCCCTTGTAGAC
LTR-P	T548: Forward	caccGCTTCCGCTGGGGACTTTCC
	T549: Reverse	aaacGGAAAGTCCCAGCGAAAGC
LTR-Q	T687: Forward	caccGCCTCCCTGGAAGTCCCAG
	T688: Reverse	aaacCTGGGACTTTCAGGGAGGC
LTR-R	T689: Forward	caccGCCTGGGCGGACTGGGGAG
	T690: Reverse	aaacCTCCCAGTCCCGCCAGGC
LTR-S	T691: Forward	caccGTCCATCCCATGCAGGCTCAC
	T692: Reverse	aaacGTGAGCCTGCATGGGATGGAC
LTR-T	T548: Forward	caccGCGGAGAGAGAAGTATTAGAG
	T549: Reverse	aaacCTCTAATACTTCTCTCTCCGC
Gag-A	T687: Forward	caccGGCCAGATGAGAGAACAAG
	T688: Reverse	aaacCTTGGTTCTCTCATCTGGCC
Gag-B	T714: Forward	caccGCCTTCCACAAGGGAAGCCA
	T715: Reverse	aaacTGCCCTTCCCTTGTGGGAAGGC
Gag-C	T758: Forward	caccGCGAGAGCGTCGGTATTAGCG
	T759: Reverse	aaacCGCTTAATACCGACGCTCTCGC
Gag-D	T760: Forward	caccGGATAGATGTAAAAGACACCA
	T761: Reverse	aaacTGGTGTCTTTACATCTATCC
Pol-A	T689: Forward	caccGCAGGATATGTAAGTACAG
	T690: Reverse	aaacCTGTCAAGTTACATATCCTGC

Target name	Direction	Sequences (5' to 3')
Pol-B	T716: Forward	cacc GCATGGGTACCAGCACAA
	T717: Reverse	aaac TTGTGTGCTGTACCCATGC
PCR	T422	cacc GCTTTATTGAGGCTTAAGCAG
	T425	aaac GAGTCACACAACAGACGGGC
	T645	TGGAATGCAGTGGCGCGATCTTGGC
	T477	CACAGCATCAAGAAGAACCTGAT
	T478	TGAAGATCTCTTGCAGATAGCAG