Molecular Therapy
Nucleic Acids
Original Article

Zinc-Finger Nucleases Induced by HIV-1 Tat Excise HIV-1 from the Host Genome in Infected and Latently Infected Cells

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Highly active anti-retroviral therapy (HAART) cannot clear infected cells harboring HIV-1 proviral DNA from HIV-1-infected patients. We previously demonstrated that zinc-finger nucleases (ZFNs) can specifically and efficiently excise HIV-1 proviral DNA from latently infected human T cells by targeting long terminal repeats (LTRs), a novel and alternative antiretroviral strategy for eradicating HIV-1 infection. To prevent unwanted off-target effects from constantly expressed ZFNs, in this study, we engineered the expression of ZFNs under the control of HIV-1 LTR, by which ZFN expression can be activated by the HIV-1 (Trans-Activator of Transcription) Tat protein. Our results show that functional expression of ZFNs induced by Tat excise the integrated proviral DNA of HIV-NL4-3-eGFP in approximately 30% of the population of HIV-1-infected cells. The results from HIV-1-infected human primary T cells and latently infected T cells treated with the inducible ZFNs further validated that proviral DNA can be excised. Taken together, positively regulated expression of ZFNs in the presence of HIV-1 Tat may provide a safer and novel implementation of genome-editing technology for eradicating HIV-1 proviral DNA from infected host cells.

INTRODUCTION

Highly active antiretroviral therapy (HAART) can efficiently inhibit the replication of HIV-1 and improve the status of HIV-infected patients, as previous studies have indicated.[1,2] Unfortunately, virus infection is refractory because of the presence of a viral reservoir integrating into the host DNA and forming latent transcriptionally silent HIV-1 proviruses.[1–3] In such cases, transcriptionally silent HIV-1 proviruses can evade host immune system control and the action of anti-retroviral drugs, followed by a new round of virus infection in the absence of HAART.[1,2] Thus, the presence of latent reservoirs is the key barrier to eradicate HIV-1 at present. The most widely discussed approach for eliminating the latent reservoir is the so-called "shock and kill" strategy involving reversing HIV-1 from latent reservoirs using activators initially. Although a number of small molecular activators have been applied in clinical trials in anti-HIV-1 latency, the results were unsatisfactory because of a lack of target specificity.[3–5] More importantly, existing drugs could not delete the integrated HIV-1 proviral genome.[6,7] Thus, innovative approaches involving reversing HIV-1 from latency and eradicating the HIV-1 provirus are urgently needed. The emergence of gene-editing tools would provide a realistic possibility to address these problems.

We previously designed and obtained one pair of zinc fingers (ZFNs) to target HIV-1 long terminal repeat (LTR) sequences, which are highly conserved in the viral genome. The results demonstrated that ZFNs can mediate integrated full-length proviral genome excision in latently infected and infected cell models, indicating antiretroviral activity.[8] However, problems related to unwanted off-target effects induced by constant expression of ZFNs needed to be addressed. Considering that the viral trans-activator Tat can stimulate viral gene expression and replication by binding to the transactivation-responsive element (TAR) located at LTRs, we hypothesized that the expression of ZFNs under the control of the HIV-1 LTR promoter could be activated by Tat protein and then mediate HIV-1 proviral DNA deletion from the host genome. Our results demonstrated that inducible ZFNs can delete the HIV-1 proviral genome from the host genome in HIV-1-infected and latently infected cells, indicating antiviral activity mediated by inducible ZFNs in the presence of Tat.

RESULTS

Analysis of the Activity of the Promoter

The HIV-1 LTR promoter is responsible for viral transcription initiation, whereas Tat protein, an HIV-1-expressed protein, can recruit transcription elongation complexes to the TAR of the HIV-1 5′-LTR to promote transcription elongation.[9–11] In our study, we first detected the effects of Tat protein on the LTR promoter by luciferase assay. Here we used one TAR and tandem TAR repeat (2 × TAR)

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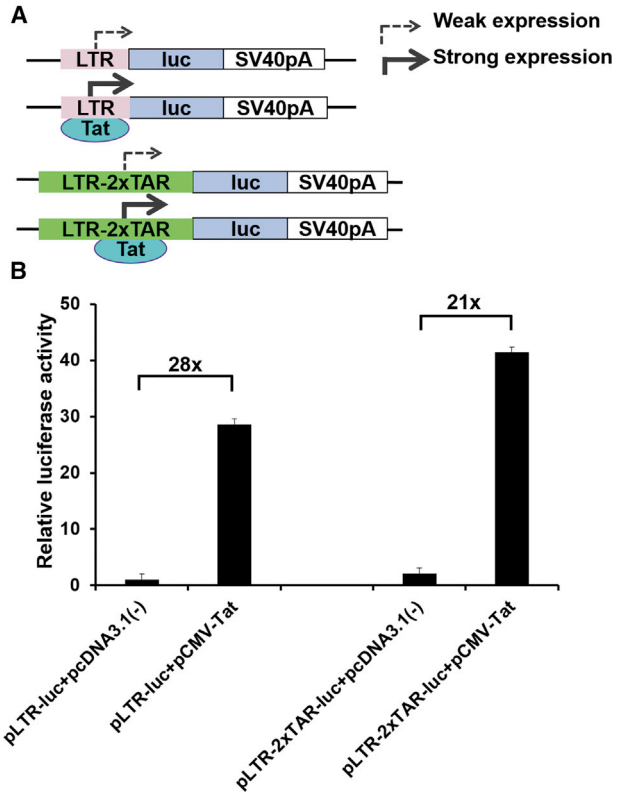

Figure 1. Transient Luciferase Assay Detection of the Activity of the LTR or LTR-2 × TAR Promoter

(A) Schematics of reporter gene luciferase expression cassettes. Expression of the luc gene driven by LTR or LTR-2 × TAR was very low in the absence of Tat protein but robustly increased in the presence of Tat protein. (B) Transient luciferase assay detection. HEK293T cells were co-transfected with the pLTR-luc or pLTR-2 × TAR-luc plasmid with the pcDNA3.1(−) or Tat expression plasmid pCMV-Tat and the internal control pRL-SV40 plasmid at the indicated time. The relative luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, USA) 72 hr post-transfection. The data were analyzed by normalizing the Tat-transfected group to the pcDNA3.1(−)-transfected group. Data represented the mean ± SD of three independent experiments.

located in the LTR to promote reporter gene *luciferase* expression. The schematics of two luciferase gene expression cassettes driven by the LTR or LTR-2 × TAR in the presence or absence of Tat are shown in Figure 1A. We co-transfected HEK293T cells with a reporter gene vector comprising the *luciferase* (luc) gene driven by the LTR (pLTR-luciferase) or LTR-2 × TAR (pLTR-2 × TAR-luc) in the presence or absence of the Tat plasmid. The luciferase activity was measured after 72 hr transfection. The results showed that co-transfection of Tat and the pLTR-luc construct induced an approximately 28-fold increase of luc reporter expression, whereas an approximately 21-fold induction of reporter gene expression was observed with co-transfection of pLTR-2 × TAR-luc with the Tat expression plasmid pCMV-Tat (Figure 1B). Our data indicate that HIV-1 LTR or LTR-2 × TAR activation is dependent on Tat.

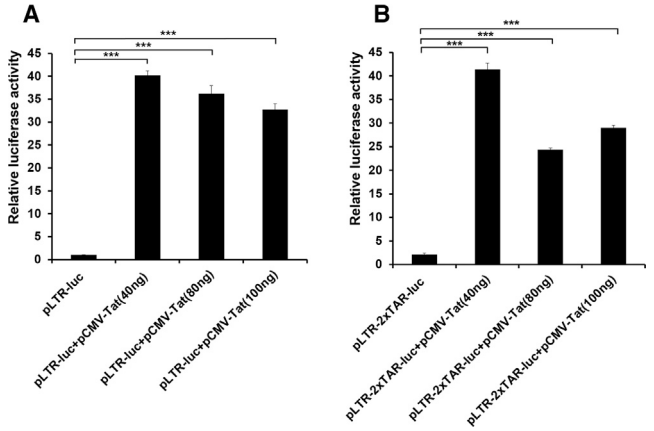

Figure 2. Analysis of Different Doses of Tat-Induced Effects on Luciferase Gene Expression

(A) Detection of LTR-driven luciferase gene expression when transfected with different doses of Tat. HEK293T cells were transfected with pLTR-luc in the presence of different doses of Tat at the indicated times. HEK293T cells transfected with pLTR-luc and pcDNA3.1(−) were used as controls. The relative luciferase activity was measured after 72 hr transfection. The data shown were normalized to the pcDNA3.1(−)-transfected group. Data represent the mean ± SD of three independent experiments. (B) Detection of LTR-2 × TAR-driven luc gene expression at different transfection amounts of Tat. Cell transfection methods were as indicated as above. Data represent the mean ± SD of three independent experiments. ***$p < 0.001$; paired t test.

Analysis of Different Amounts of Tat-Induced Effects on *luciferase* Expression

We further tested the dose-dependent effects of Tat on LTR or LTR-2 × TAR promoter activity. pLTR-luc or pLTR-2 × TAR-luc was transfected with the indicated amount of the Tat expression plasmid pCMV-Tat into HEK293T cells. After 72 hr transfection, cells were lysed and subjected to luciferase activity detection. Our results suggest that no increased luciferase expression occurred with co-transfection with higher amounts of Tat (Figure 2). The luc expression is highest only with co-transfection with 40 ng of Tat expression plasmid (Figure 2).

Analysis of the Activity of Inducible ZFNs by Transient Luciferase Assay

Previous study have suggested that ZFN expression plasmids targeting viral LTRs (ZFN-LTRs) can specifically and efficiently excise HIV-1 proviral DNA from infected and latently infected human T cells.[8] In this study, we used previously reported ZFNs to target LTRs under the control of a viral promoter. For this purpose, we constructed two sets of regulated ZFNs under the control of the HIV-1 LTR or LTR-2 × TAR promoter, termed pLTR-ZFN and pLTR-2 × TAR-ZFN (Figure S1) and then monitored the protein expression of the two sets of regulated ZFNs by western blot. Because the ZFN-LTR pair (ZFN-LTR-L and ZFN-LTR-R) under the control of the HIV-1 LTR or LTR-2 × TAR promoter was isolated by T2A "self-cleaving" peptide in both constructs, the result demonstrated two bands in both constructs after detection of the infused FLAG tag,

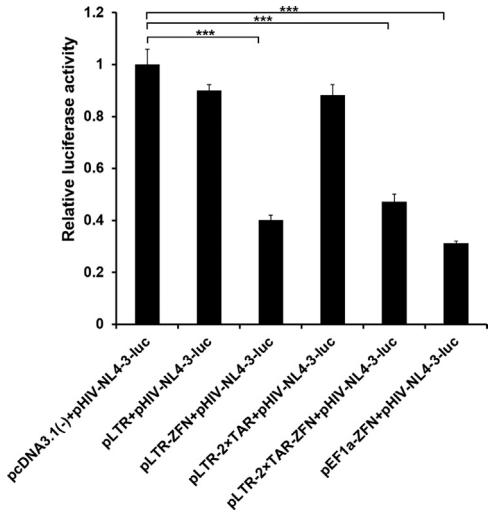

Figure 3. Inducible ZFNs Could Induce HIV-1 Proviral DNA Excision by Transient Luciferase Assay

HEK293T cells were co-transfected with pLTR-ZFN or pLTR-2 × TAR-ZFN with the HIV-1 gene expression construct carrying the luc gene pHIV-NL4-3-luc and pRL-SV40 at the indicated time. HEK293T cells transfected with ZFN expression plasmids pEF1a-ZFN, pHIV-NL4-3-luc, and pRL-SV40 were used as a positive control; HEK293T cells transfected with pcDNA3.1(−) or the ZFN empty expression plasmid pLTR or pLTR-2 × TAR, pHIV-NL4-3-luc, and pRL-SV40 were used as a negative control. The relative luciferase activity was measured after 72 hr transfection. The data shown were normalized to the pcDNA3.1(−)-transfected group. Data represent the mean ± SD of three independent experiments. ***$p < 0.001$; paired t test.

indicating left and right ZFP protein expression in both cassettes (Figure S2). Next, we examined the inducible ZFN-induced viral DNA excision by luciferase assay. An HIV-1 pseudovirus vector carrying luc (pHIV-NL4-3-luc) was transfected with pcDNA3.1(−), pLTR-ZFN, or pLTR-2 × TAR-ZFN and the internal control pRL-SV40 vector into HEK293T cells. The plasmid expressing ZFNs isolated by T2A driven by elongation factor 1α (EF1α), named pEF1α-ZFN, was used as a positive control. After 72 hr transfection, cells were collected, lysed, and subjected to a luciferase assay. The results revealed a significant decrease in luciferase activity upon transfection of pLTR-ZFN or pLTR-2 × TAR-ZFN, up to 60% and 55%, respectively. No reduction was observed in cells transfected with pcDNA3.1(−) with ZFN empty vector pLTR or pLTR-2 × TAR (Figure 3), suggesting that the HIV-1 proviral DNA excision was induced by the inducible ZFNs.

Evaluation of the Biological Activity of ZFN Treatment and Cytotoxicity in HIV-1-Infected Cells

To determine whether ZFN treatment of HIV-1-infected cells could decrease viral gene expression, we used Jurkat-derived HIV-1 pseudovirus-infected cells (YA), which express EGFP as a marker for viral infection, as studied previously.[8] We could monitor the change in the percentage of $EGFP^+$ cells to determine the activity of ZFNs. To this end, YA were left untransfected (mock) or nucleofected with pcDNA3.1(−), pLTR-ZFN, or pLTR-2 × TAR-ZFN at specified times. The percentage of $EGFP^+$ cells was measured by flow cytometry at different time points. The results showed a loss of EGFP expression up to 20% after treatment with pLTR-ZFN or pLTR-2 × TAR-ZFN on day 5 post-transfection compared with pcDNA3.1(−)-treated cells (Figure 4). We further detected a loss of 30% of $EGFP^+$ cells after 7-day transfection. However, no reduction of the percentage of $EGFP^+$ cells was observed in the mock- and pcDNA3.1(−)-transfected group up to 7-day transfection, indicating that the inducible ZFNs could excise proviral DNA in HIV-1-infected cells. We also evaluated the effect of the inducible ZFNs on YA viability at the indicted times. The results revealed no effects on cell viability in cells transfected with the inducible ZFNs compared with the pcDNA3.1(−)-transfected group (Figure 5).

ZFN-Mediated Excision of Integrated HIV-1 Proviral DNA in Human Primary T Cells

To determine whether ZFNs could induce integrated HIV-1 proviral DNA excision in HIV-1-infected human primary $CD4^+$ T cells, $CD4^+$ T cells were infected with HIV-1 NL4-3 viruses and then nucleofected with pLTR-ZFN or pLTR-2 × TAR-ZFN vector, followed by detecting virus p24 antigen content in the culture supernatants at the indicated time by ELISA. We found that HIV-1 p24 production decreased up to ~40% in HIV-1-infected $CD4^+$ T cells transfected with pLTR-ZFN or pLTR-2 × TAR-ZFN compared with the pcDNA3.1(−)-transfected group (Figure 6). Collectively, the data confirmed the excision of integrated HIV-1 proviral DNA mediated by ZFNs in HIV-1-infected human primary $CD4^+$ T cells.

Analysis of ZFN Treatment-Induced Proviral DNA Deletion in HIV-1 Latently Infected Cells

To further validate that the treatment of the inducible ZFNs could excise proviral DNA from the host genome in latently infected cells, we performed a study in C11 cells, which have been found to carry a single integrated HIV-1 vector at position Ch16p13.3.[8] To probe for the deletion of the proviral DNA, we used primers located at the outsides of the integrated HIV-1 genome separated by 10.4 kb when the provirus is integrated into the host genome (Figure 7A). The PCR product size we observed after co-treatment with pLTR-ZFN or pLTR-2 × TAR-ZFN and pCMV-Tat was approximately 1 kb (Figure 7B), a band size expected if the DNA segments between the ZFN target sites were deleted from the chromosome. No 1-kb band in cells co-transfected with pLTR-ZFN or pLTR-2 × TAR-ZFN and pcDNA3.1(−) was observed (Figure 7B), indicating that HIV-1 proviral genome deletion occurred only in the presence of pLTR-ZFN or pLTR-2 × TAR-ZFN with pCMV-Tat. To ensure that the 1-kb band was generated by rejoining DNA at the breakpoint induced by ZFNs, we ligated the PCR product into the pMD18-T vector and then performed a sequence analysis. The results demonstrated that the ZFN half target site at the 5′ LTR locus is indeed linked to the ZFN half target site at 3′ LTR locus, suggesting that the integrated HIV-1 5′ and 3′ LTR sites had been joined and that the intervening 9.8-kb DNA segment had been deleted (Figure S3).

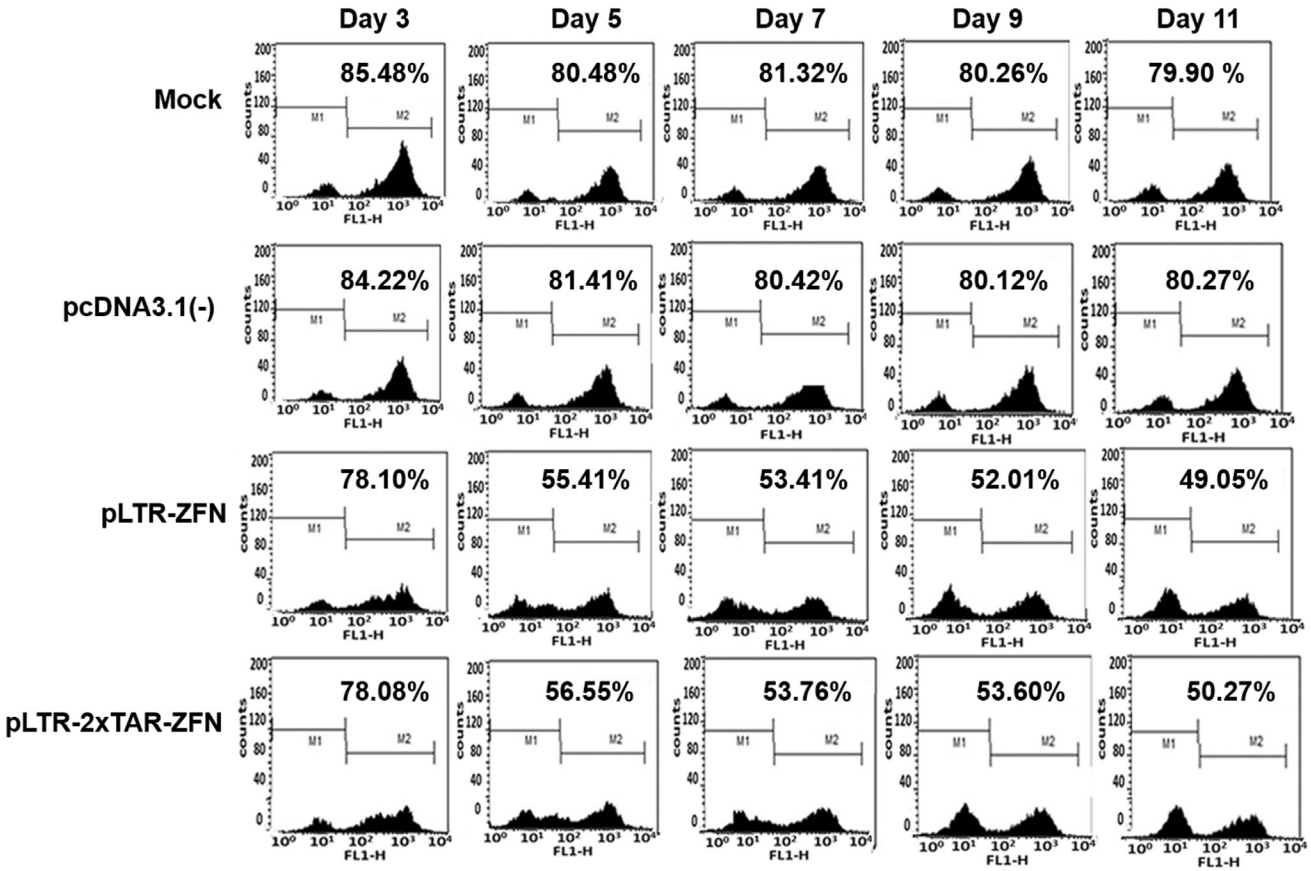

Figure 4. Inducible ZFNs Mediated HIV-1 Proviral DNA Excision in HIV-1-Infected Cells YA

YA were left untransfected (mock) or nucleofected with pcDNA3.1(−), pLTR-ZFN, or pLTR-2 × TAR-ZFN at specified times. The percentage of $EGFP^+$ cells was detected at different times post-transfection by flow cytometry. Data are representative of three independent experiments.

DISCUSSION

The viral trans-activator Tat binding to the *trans*-activating response element TAR on the LTR recruits intracellular transcription-related proteins to promote viral gene expression. These transcription factor complexes phosphorylate the positive transcription elongation factor b (P-TEFb) and RNA polymerase II, which, in turn, promotes the transcription of viral genes.[12–15] Additionally, nuclear factor κB (NF-κB), p300 and CBP (CREB binding protein), and GCN5, which bind to LTR, interact with Tat to promote transcriptional elongation of the HIV-1 virus gene.[16–19] Thus, Tat protein plays a vital role in the transcriptional regulation of HIV-1. Previous studies have shown that inhibition of Tat binding to TAR or blocking Tat binding to intracellular transcription factors by inhibitors suppressed viral replication.[20] In this study, we used the transactivation of Tat protein to induce ZFNs-LTR and established a inducible method for deleting viral genes by ZFNs. To this end, we first tested the biological activity of the inducible promoter by the luciferase reporter assay system. The results showed that LTR or LTR-2 × TAR-regulated luciferase expression was significantly increased in the presence of Tat protein, indicating that the activation of LTR or LTR-2 × TAR promoter requires Tat protein.

After confirming the biological activity of the LTR or LTR-2 × TAR promoter, we constructed inducible ZFN expression cassettes under the control of the LTR or LTR-2 × TAR promoter. First we tested the biological activity of ZFNs by transient luciferase assay. The results showed that the ZFN-transfected group could mediate the decrease of luciferase expression, suggesting that ZFNs had biological activity. Next we demonstrated that inducible ZFNs could mediate the reduction of the number of $EGFP^+$ cells to 30% in HIV-1-infected cells without affecting cell viability. We also performed a similar study to investigate inducible ZFN-mediated HIV-1 proviral DNA excision in human primary T cells. The results from this study revealed a significant suppression of p24 production (Figure 6). Moreover, the inducible ZFN-mediated HIV-1 provirus genome excision from HIV-1 latently infected cells was also validated, consistent with the results from Kaminski et al.[21] using CRISPR/Cas9 for ablation of HIV-1.

However, there are still many unknown problems that need to be explored if this inducible ZFNs expression vector is to be used in the clinical treatment of HIV. Safe and effective gene delivery

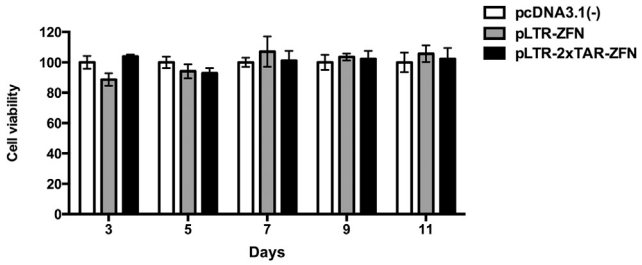

Figure 5. Inducible ZFNs Had No Effects on Cell Viability

HIV-1-infected cells YA were transfected with pcDNA3.1(–), pLTR-ZFN, or pLTR-2 × TAR-ZFN at the indicated time. At different times post-transfection, YA viability was measured using the CCK-8 kit. The data show the cell viability of the pcDNA3.1(–)-treated group divided by that of the pLTR-ZFN or pLTR-2 × TAR-ZFN- transfected groups. The data represent the mean ± SD of three independent experiments.

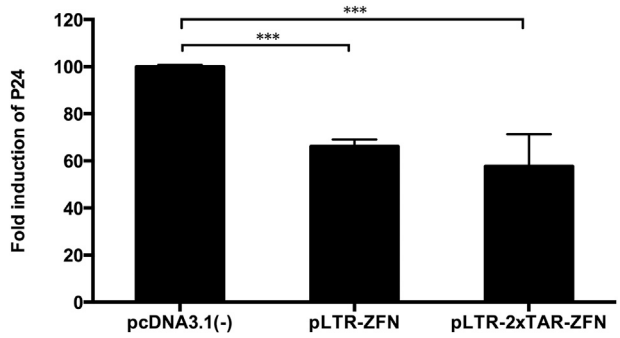

Figure 6. Inducible ZFNs Mediated Excision of Integrated HIV-1 Proviral DNA in Human Primary T Cells

HIV-1 NL4-3 infected $CD4^+$ T Cells were nucleofected with 5 μg of pcDNA3.1(–), pLTR-ZFN, or pLTR-2 X TAR-ZFN at the indicated times. Culture supernatant was harvested for detecting HIV-1 p24 production after 72 hr transfection. Data are representative of three independent experiments, and error bars represent SD. ***$p < 0.001$; paired t test.

methods are important issues in the field of HIV-1 gene therapy. At present, viral vectors are widely used in gene therapy; lentiviral vectors can achieve the sustained expression of genes, but the potential risk of random integration is under consideration. However, in our study, we constructed the inducible ZFNs expression vector to solve the problem of potential off-target risks by persistent expression of ZFNs. Therefore, a lentiviral vector was not suitable for this study; The adeno-associated viral vector is limited by packaging capacity;[22] baculoviruses could have enough capacity, but the infection efficiency is low.[23,24] Integrase-deficient lentiviral titers are relatively low.[25,26] Conclusively, the application of an adenovirus vector delivering the inducible ZFNs is much better.

Taken together, we conducted antiviral activity studies by inducing ZFNs. This technology could not only prevent the unwanted off-target but also provide a new direction for gene therapy of AIDS.

MATERIALS AND METHODS

Cells

HEK293T cells purchased from the American Type Culture Collection (Manassas, VA, USA) were maintained in DMEM supplied with 10% (v/v) fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin at 37°C with 5% CO_2. C11 cells are a type of HIV-1 latently infected cells constructed by our lab.[8,27–32] YA were derived from Jurkat T cells infected with HIV-1 pseudovirus carrying the *eGFP* reporter gene.[8,27] Both of them were cultured in RPMI 1640 medium with 10% FBS (Gibco, Grand Island, NY, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Shanghai, China) at 37°C under 5% CO_2.

Plasmid Constructs

To obtain the HIV-1 LTR-driven luciferase reporter gene expression plasmid (LTR-luc), a full-length LTR fragment was amplified from the HIV-1 NL4-3-EGFP backbone with the forward primer F-LTR (5′-CGGGGTACCTGGAAGGGCTAATTCACTCCCAAAG-3′) and the reverse primer R-LTR (5′-CCGCTCGAGTGCTAGA GATTTTCCACACTGACTA-3′), followed by purification and digestion by KpnI and XhoI and then ligation into the pGL3-basic plasmid (Promega, Madison, WI, USA) double-digested by KpnI and XhoI sites. The luciferase reporter gene driven by the HIV-1 LTR-2 × TAR promoter was constructed as follows. The LTR-2 × TAR fragment was amplified from plasmid pSH1-Tre[33] (kindly provided by Joachim Hauber) using the forward primer F-LTR (5′-CGGGGTACCAATT CACTCCCAAAGAAGACAAGATA-3′) and the reverse primer R-LTR (5′-CCGCTCGAGGAGGCTTAAGCAGTGGGTTCCCTAG TTAGCC-3′), followed by purification, digestion by KpnI and *Xho* I, and ligation into the KpnI-*Xho* I clone site of the pGL3-basic plasmid (Promega, Madison, WI, USA). All expression plasmids were confirmed by sequencing.

In our study, the expression of one ZFN-LTR pair (ZFN-LTR-L and ZFN-LTR-R) was isolated by T2A "self-cleaving" peptide in one plasmid under the control of the HIV-1 LTR or LTR-2 × TAR promoter. The FLAG tag was introduced at the N terminus of the left and right ZFP (Zinc-Finger Protein), respectively. Briefly, the LTR or LTR-2 × TAR promoter was cloned into the pEbackbone vector (constructed by our lab) by KpnI and *Xho* I. Then the bGHpA fragment amplified from the PX260 vector (Addgene, 42229) was digested by *EcoR* I and *Xho*I and ligated into the plasmid pLTR or pLTR-2 × TAR comprising the LTR or LTR-2 × TAR promoter. Next we synthesized T2A oligonucleotides and inserted them into the digested plasmid pZFN-LTR-L by BglII and *Xho* I. The ZFN-LTR-L plasmid containing the T2A fragment (ZFN-LTR-L-T2A) was then used to amplify ZFN-LTR-L-T2A, including *Sfu* I and *Xho* I cut sites, by forward primer (5′-AATTTTCGAAATGGAC TACAAAGACCATGACGGTGATTAT-3′) and reverse primer (5′-AAATCTCGAGCGGACCGGGATTTTCTTCCACAT-3′). Then we purified the PCR product and digested it with *Sfu* I and *Xho* I. The ZFN-LTR-R fragment, including the *Xho* I and *EcoR* I sites, was amplified from pZFN-LTR-R using the forward primer (5′-ATCTCTCGAGATGGACTACAAAGACCATGACGGTGA-3′)

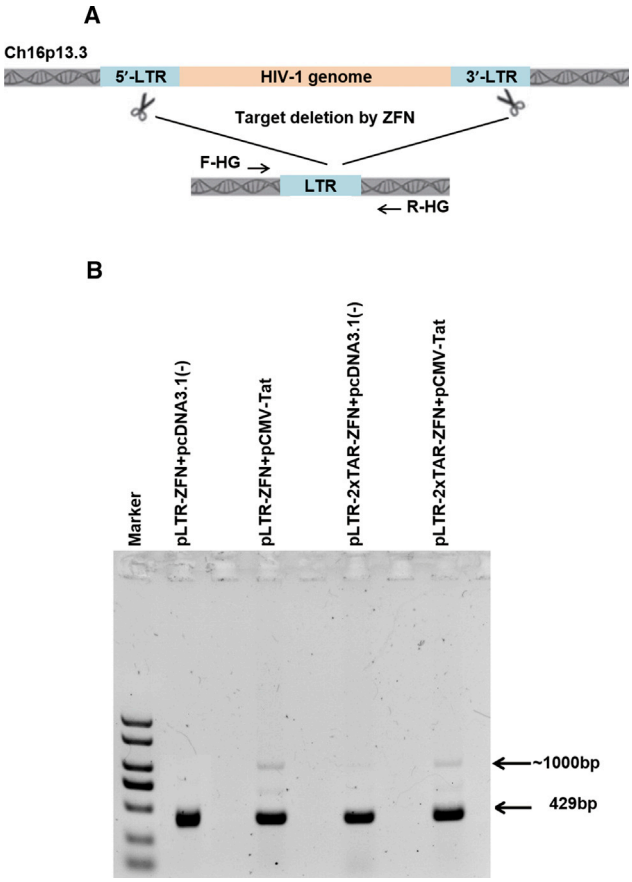

Figure 7. Inducible ZFNs Induced HIV-1 Proviral DNA Excision in HIV-1 Latently Infected Cell Clone C11 Cells

(A) Schematic representation of regulated ZFN-mediated genome deletions. The light blue box in the 5′ and 3′ LTR of the HIV-1 genome (10.4 kb) indicates ZFN target sites. F-HG and R-HG (arrows), located outside of the integrated HIV-1 genome, were used for amplifying genome deletion events. (B) PCR products validated regulated ZFN-induced HIV-1 proviral genome deletion in the presence of Tat. C11 cells were nucleofected with pLTR-ZFN or pLTR-2 × TAR-ZFN with pcDNA3.1(−) or pCMV-Tat at the specified time. Genomic DNA was extracted 72 hr post-transfection and then subjected to PCR using the F-HG and R-HG primers.

and reverse primer (5′-CATCGAATTCTTAGAAGTTGATCTCG CCGTTGTTGAACTT-3′) and then digested by *Xho* I and *EcoR* I. Finally, fragments ZFN-LTR-L-T2A and ZFN-LTR-R were both ligated into the vector cut by *Cla* I and *EcoR* I. The final positive clones were named pLTR-ZFN and pLTR-2 × TAR-ZFN.

Luciferase Reporter Assay

To examine LTR or LTR-2 × TAR promoter activity, pLTR-luc or p LTR-2 × TAR-luc (200 ng) with or without the Tat expression plasmid (40 ng) was co-transfected into HEK293T cells with the internal control pRL-SV40 (50 ng) in a 24-well plate using ViaFect reagent (Promega) according to the manufacturer's instructions. After 72 hr transfection, cells were harvested and lysed and then subjected to detection of luciferase activity using the Dual-Luciferase Reporter Assay system (Promega, USA). We also tested whether LTR or LTR-2 × TAR promoter activity depends on the dose of Tat plasmid. For this purpose, pLTR-luc or pLTR-2 × TAR-luc (200 ng) with different doses of Tat plasmid was transfected into HEK293T cells at the indicted time. 72 hr post-transfection, cells were lysed and subjected to detection of relative luciferase activity using the Dual-Luciferase Reporter Assay system (Promega, USA). Each experiment was performed in triplicate.

Next we detected the effect of ZFNs on HIV-1 proviral DNA deletion by luciferase assay. The pLTR-ZFN (600 ng) or pLTR-2 × TAR-ZFN (600 ng) vector with pHIV-NL4-3-luc (200 ng) and pRL-SV40 (50 ng) were co-transfected into HEK293T cells. Cells transfected with pEF1α-ZFN (600 ng) with pHIV-NL4-3-luc (200 ng) and pRL-SV40 (50 ng) were used as controls. Each experiment was performed in triplicate.

Western Blot

HEK293 T cells were plated in a 6-well plate and then transfected with 1 μg of pLTR-ZFN or pLTR-2 × TAR-ZFN plasmid with or without Tat using ViaFect reagent (Promega). After 72 hr transfection, cells were harvested, lysed, and subjected to western blot. The membrane was detected by primary antibody against FLAG for detecting ZFN protein expression. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as a reference. The Immun-Star WesternC chemiluminescence Kit (Bio-Rad) was used to detect the aforementioned proteins, and the chemiluminescence signal was captured using the ChemiDoc XRS+ System and analyzed using Image-Lab software (Bio-Rad).

Nucleofection and Flow Cytometry Assay

To assess the change in $EGFP^+$ cells number after ZFN-LTR treatment in HIV-1-infected cells, YA were nucleofected with 2 μg of pcDNA3.1(−) vector, pLTR-ZFN, or pLTR-2 × TAR-ZFN using the Amaxa Cell Line Nucleofector Kit V (Lonza, Gaithersburg, MD, USA). The percentage of EGFP-negative cells indicated the treatment of the regulated ZFNs. At the indicated time, each group of cells was collected and washed with PBS. EGFP expression was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA), and the data were analyzed using CellQuest software (Macintosh, Sunnyvale, CA, USA).

Cell Viability

Briefly, HIV-1-infected cells YA were seeded at approximately 1×10^6 cells/well in a 6-well plate and then transfected with 2 μg of pLTR-ZFN or pLTR-2 × TAR-ZFN constructs the following day. The pcDNA3.1(−)-transfected group was used as a control. 4×10^4 cells in each group were reseeded into a 96-well plate after different times of transfection, and then we added 10 μL of CCK-8 solution to each well, followed by incubation for 4 hr at 37°C. The absorbance at 450 nm was measured using a microplate reader. Each experiment was performed independently in triplicate.

Isolation of Primary CD4+ T cells

Peripheral blood mononuclear cells (PBMCs) from two blood units (400 mL) isolated from healthy donors were purchased from the Shanghai Blood Center (Shanghai, China). $CD4^+$ T cells were further purified from PBMCs by negative selection according to the manufacturer's instructions (Miltenyi Biotec). The $CD4^+$ T cells were maintained in RPMI 1640 medium containing 5 ng/mL interleukin-2 (IL-2) supplemented with 10% FBS (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin and then activated by beads coated with anti-CD3 and CD28 (Gibco, Thermo Fisher Scientific, USA) for 72 hr, followed by washing off and then culture at 37°C under 5% CO_2.[34]

Virus Infection of Human Primary T Cells and ELISA Detection of Antigen p24 Levels

Replication-competent HIV-1 NL4-3 viruses (CXCR4 tropism) were kindly provided by Dr. Wang (Institute Pasteur of Shanghai, China). For infection, Beads coated with anti-CD3- and CD28-activated $CD4^+$ T Cells were inoculated with 10 ng p24 of HIV-1 NL4-3 for 5 hr and then washed off cell-free viruses, followed by changing the medium. On day 3 and day 5 infection, we moved half of the volume of medium and then supplied the corresponding volume of medium.[35] For nucleofection, 5×10^6 $CD4^+$ T Cells were transfected with 5 μg of pLTR-ZFN or pLTR-2 × TAR-ZFN plasmid using the Amaxa Human T Cell Nucleofector Kit (Lonza, Gaithersburg, MD, USA). After 72 hr transfection, viral replication was monitored by quantifying the amounts of p24 produced in the culture supernatant by using the HIV-1 p24 Antigen ELISA Kit (XpressBio) according to the manufacturer's instructions.

PCR and Sequencing Analysis

To detect HIV-1 genomic deletion in HIV-1 latently infected cells (C11) after treatment with pLTR-ZFN or pLTR-2 × TAR-ZFN, PCR analysis was performed. Briefly, genomic DNA was extracted using the Blood and Cell Culture DNA Midi Kit (QIAGEN, China) according to the manufacturer's instructions and then subjected to PCR analysis. The primers F-HG (5′-TGCCACCCGAAACTAT TCACAAG-3′) and R-HG (5′- CCGGCATGGATTCCAGTTCT TAG-3′) were used for amplifying excised proviral DNA from C11 cells un-transfected or transfected with pLTR-ZFN or pLTR-2 × TAR-ZFN in the absence or presence of Tat. PCR products were analyzed by agarose gel electrophoresis and further sequencing.

Statistical Analysis

The Data represent the mean ± SD (SE) of three independent experiments in triplicate. Paired samples t tests were performed using SPSS version 13.0 (SPSS, Chicago); $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 20.001$ indicate statistical significance.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.04.014.

AUTHOR CONTRIBUTIONS

H.Z. conceived and designed the experiments. H.J. carried out most experiments. P.L., B.L., X.Q., Y.W., Z.J., X.Y., Y.Z., H.Y., H.P., and L.Z. participated in some of the experiments. J.X. and H.L. kindly provided some suggestions for the revised manuscript. H.Z. directed and supervised the experiments and interpretation of data. The manuscript was prepared by H.J. and H.Z.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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