Eradication of integrated HIV-1 genome from latently infected T-cells by targeting LTR sequences using CRISPR/Cas9 gene editing system.

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Abstract

Longevity, homeostatic proliferation and resistance to antiviral therapy of latently infected cells represents the principal challenges toward curing AIDS. The main latent HIV-1 reservoir resides in the subset of CD4⁺ T-cells called resting, memory T-cells. Using CRISPR/Cas9 genome editing technology to target two unique sequences in U3 region of HIV-1 LTR (called target A and B), we were able to completely eliminate proviral sequences from the genome of latently infected T-cell lines. We tested and validated two delivery approaches: plasmid DNA transfection/single cell clones selection and inducible lentiviral delivery system. Both strategies resulted in abrogation of HIV-1 reactivation as a result of removal of the proviral sequences from the host cell genome by long range PCR genotyping. This result was further confirmed by sequencing of cleavage lariat from integration locus in chromosome 16. Surveyor assay and sequencing of potential off target sites in the host genome showed no detectable off target effects. Furthermore, removal of the proviral sequence had no significant impact on the expression in neighboring genes. Finally, stable expression of Cas9/gRNA complexes targeting LTR was able to protect cells against new infections. Our results indicate that CRISPR/Cas9 system can be used to specifically remove integrated viral sequences from the genome of latently infected cells. This proof of concept study provides a new avenue to cure AIDS.

Using CRISPR/Cas9 gene editing tool to target integrated HIV-1 genome

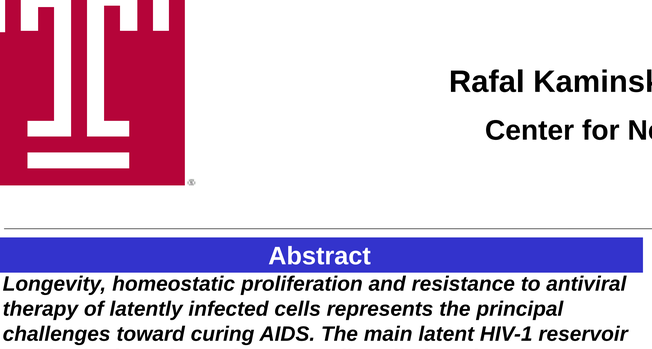
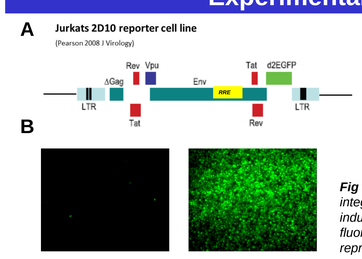


***Fig 1**. Schematic representation of HIV-1 genome **(A)**. Our strategy and predicted/possible result of successful targeting of viral LTR sequences (flanking integrated provirus) with Cas9/gRNA complexes **(B)**. Detailed structure of the LTR **(C)**. Sequence of target sites and their location in LTR **(D)**.*

Experimental model



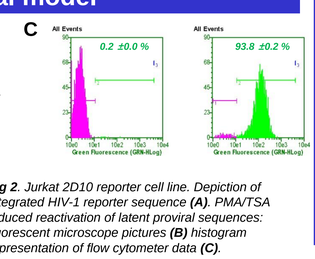


***Fig 2**. Jurkat 2D10 reporter cell line. Depiction of integrated HIV-1 reporter sequence **(A)**. PMA/TSA induced reactivation of latent proviral sequences: fluorescent microscope pictures **(B)** histogram representation of flow cytometer data **(C)**.*

Targeting viral LTR sequences with Cas9/gRNA expressing plasmids abrogates reactivation of latent reporter HIV-1 provirus

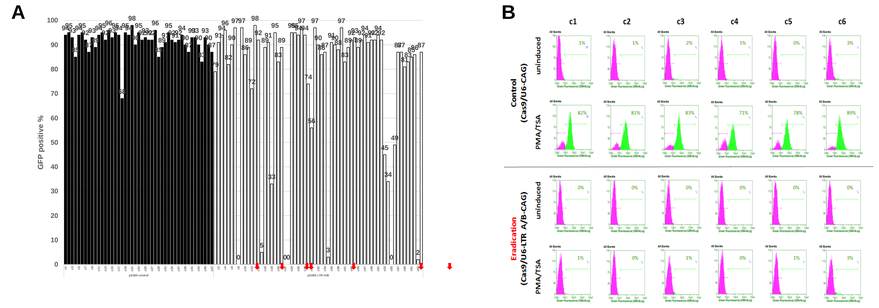


***Fig 3**. Jurkats 2D10 reporter cells (2x10⁶/condition) were electroporated with 10ug of control pX260 plasmid or pX260 LTR-A and pX260 LTR-B plasmids, 5ug each (Neon System, Invitrogen, 3 times 10ms/1350V impulse). 48h later medium was replaced with one containing puromycin 0.5ug/ml. After one week selection puromycin was removed and cells were allowed to grow for another week. Next, cells were diluted to a concentration of 10 cells/ml and plated in 96 well plates, 50ul/well. After 2 weeks, single cell clones were screened for GFP tagged HIV-1 reporter reactivation (12h PMA 25nM/TSA 250nM treatment) using Guava EasyCyte Mini flow cytometer**. Results of single cell clones screen*(A)*. Confirmation of the best clones **(B)**. Example of Western blot for FLAG-Cas9 (top) and RT-PCR/agarose gel electrophoresis (bottom) for gRNA expression in pair of single cell clones **(C)**.*

Analysis of HIV-1 integration site in host genome confirms successful excision of proviral sequences

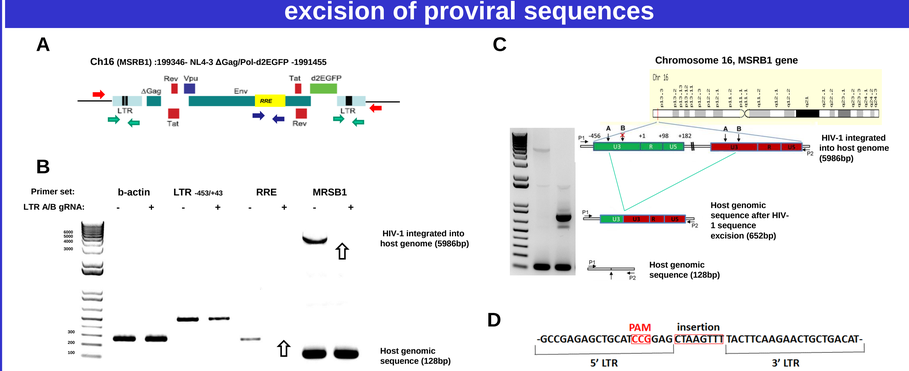


***Fig 4**. HIV eradication in positive clones was confirmed by PCR analysis of genomic DNA using primers specific for: proviral Env gene sequence motif (RRE), genomic sequences flanking integrated reporter provirus (chromosome 16, MSRB1 gene), LTR and control b-actin gene. Location of primers used in analysis **(A)**, agarose gel picture of PCR reactions, arrows point lack of eradicated HIV-1 sequences **(B)**. Long range PCR data under conditions optimized for shorter products allowing detection of proviral lariat sequences present at the integration site **(C)**. Sequencing results of proviral lariat **(D)**.*

Stable expression of Cas9/gRNA protects cells against new HIV-1 infection

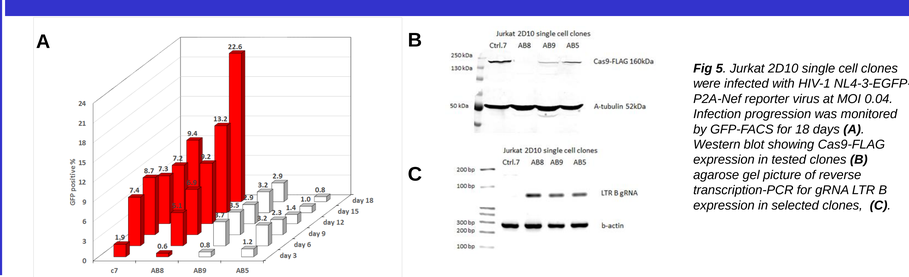


***Fig 5**. Jurkat 2D10 single cell clones were infected with HIV-1 NL4-3-EGFP-P2A-Nef reporter virus at MOI 0.04. Infection progression was monitored by GFP-FACS for 18 days **(A)**. Western blot showing Cas9-FLAG expression in tested clones **(B)** agarose gel picture of reverse transcription-PCR for gRNA LTR B expression in selected clones, **(C)**.*

Lentiviral delivery of Cas9/gRNA allows efficient and time controlled targeting of proviral sequences

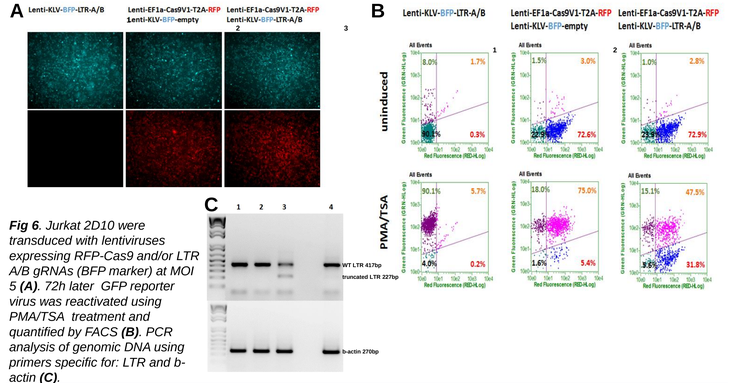


***Fig 6**. Jurkat 2D10 were transduced with lentiviruses expressing RFP-Cas9 and/or LTR A/B gRNAs (BFP marker) at MOI 5 **(A)**. 72h later GFP reporter virus was reactivated using PMA/TSA treatment and quantified by FACS **(B)**. PCR analysis of genomic DNA using primers specific for: LTR and b-actin **(C)**.*

No detectable off-target effects and minimal changes in adjacent gene expression

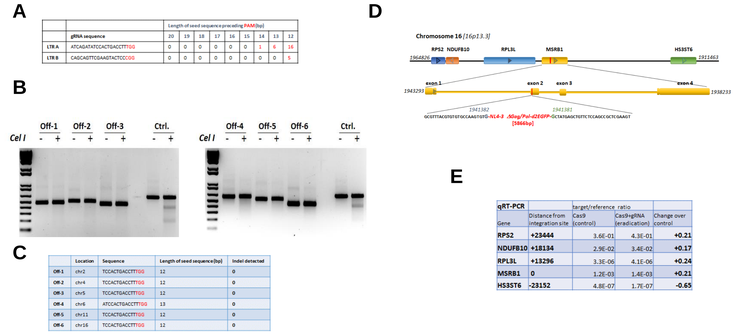


***Fig 7.** Predicted/possible off-target site frequency in human genome for LTR A and B protospacer + PAM sequences **(A)**. Agarose gel pictures of SURVEYOR assay reactions **(B)** and Sanger sequencing results **(C)** from PCR amplified, six predicted/possible off-target sites in genome of successfully eradicated Jurkat cells clone showing lack of indel mutations. Localization of HIV-1 reporter integration site in second exon of MSRB1 gene in chromosome 16 and neighboring genes **(D)**. Comparison of qRT-PCR results for mRNA expression of genes adjacent to integration site before and after HIV-1 sequence eradication **(E)**.*

Conclusions

- *Targeting LTR sequences with Cas9/gRNA gene editing machinery successfully eradicates HIV-1 provirus from the genome of latently infected T cells*
- *Stable expression of Cas9/gRNA provides resistance to new infections*
- *Lentiviral delivery provides faster, easier and more versatile Cas9/gRNA mediated gene targeting*

Future Directions

- *Testing Cas9/gRNA mediated HIV-1 eradication in primary cells from infected patients*
- *Checking efficiency of Cas9/gRNA approach in animal models for HIV-1 disease*
- *Improving Cas9/gRNA delivery systems*

Acknowledgements

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