A Novel and Promising Strategy of HIV Eradication via LTR-LTR Recombination

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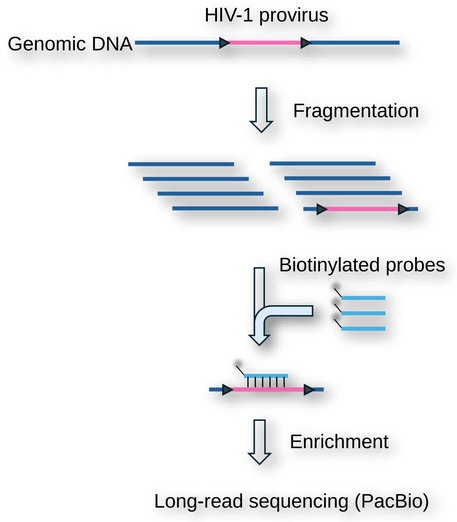
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Background Long-lived HIV-infected cells persist and undergo clonal expansion, preventing viral eradication during long-term antiretroviral therapy. The majority of infected cells contain defective proviruses with mutations, deletions, or insertions generated during reverse transcription . Defective proviruses are also generated post-integration by homologous recombination between LTRs, excising the entire coding sequence, leaving only a solo LTR at the integration site. *Little is known regarding solo HIV LTR formation and understanding mechanisms driving their formation will inform efforts to eradicate expression of HIV proteins*. Here we describe formation of solo HIV LTRs in vitro.

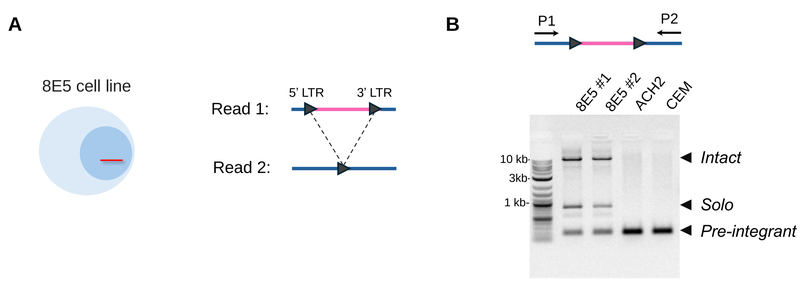
Method Cell lines containing HIV proviruses (ACH-2, 8E5, J-Lat; ATCC) and uninfected control (CEM) were maintained in vitro and subcloned. HIV DNA and RNA were quantified using multiplexed droplet digital PCR (ddPCR), and sequenced (Illumina). Proviruses were recovered from cell lines using enrichment through biotin-labeled probes targeting HIV sequence and characterized by long-read sequencing (PacBio and Nanopore). In addition, we used yeast genetic assay to screen factors required for the formation of solo-LTR.



Results We captured and characterized HIV-containing DNA fragments in latently HIV-1 infected cell lines, 8E5 and ACH-2, originally generated as survivor clones from in vitro infections of CEM cells. In 8E5 cells, we identified a full-length HIV provirus at a previously described integration site as well as a solo-LTR with the exact same integration site and target site duplication, demonstrating formation solo-LTR in vitro. Analyses of multiple single cell clones containing full-length or solo LTR HIV, including growth competition assays, revealed cells with solo LTR exhibit growth advantage over cells containing full-length provirus. Solo LTR clones contained few HIV LTR RNA copies (<10 copies/10e3 cells) and little LTR RNA read-through was detected. Importantly, with yeast genetic assay we identified that homologous recombination (HR) factors Rad50 and Rad52 are essential for the formation of solo-LTR, but not the Rad51. Beyond its primary role in HR, Rad52 has a unique function in single-strand annealing (SSA), which is independent of Rad51. This indicates that Rad52-mediated SSA primarily facilitates the deletion of intervening sequences and the generation of solo-LTRs.

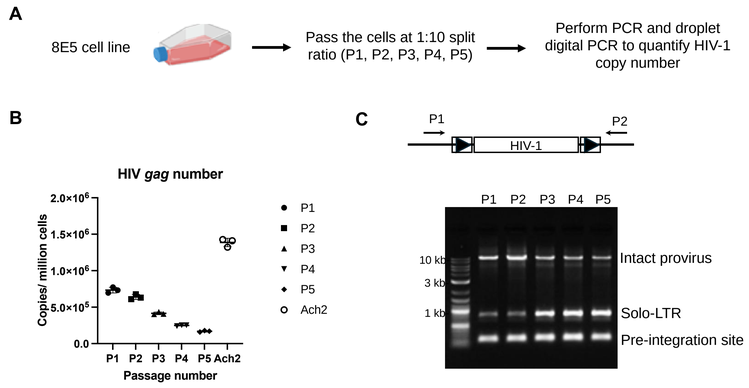
RESULTS

· A solo-LTR Provirus Captured by Long-read Sequencing in 8E5 Cell Line



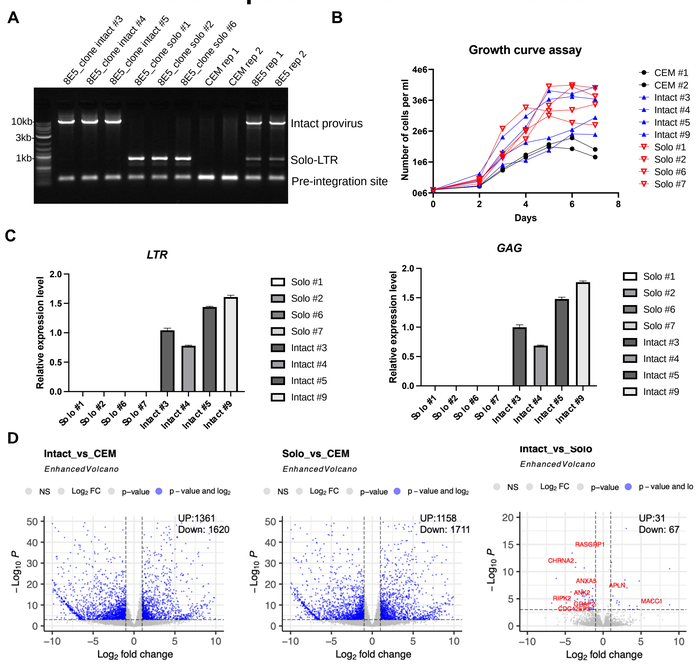
(A) 8E5 cell line was used to validate the pipeline. Two reads containing intact HIV-1 and solo-LTR were captured. (B) Primers located upstream and downstream of the provirus were used to perform PCR in the cell lines. CEM is the parent of latently infected 8E5 and ACH-2 cell lines.

Cells with Solo-LTR Dominate 8E5 Population After Successive Passages



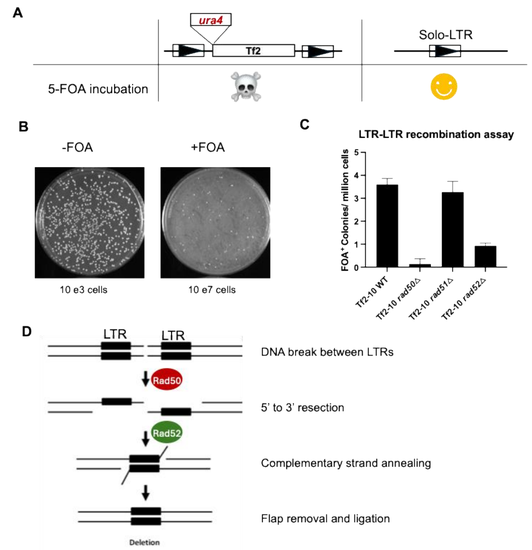
(A) 8E5 cell line were passed for five generations and genomic DNA were collected to perform PCR or droplet digital PCR (ddPCR). (B) The probe targeting HIV-1 gag was used to quantify HIV-1 DNA copies through ddPCR. (C) The same PCR strategy in Fig 2C was used here to monitor the ratio of intact provirus versus solo-LTR provirus in the passaged 8E5 cell line.

Intact Clones Exhibit Distinct Growth Patterns and Transcriptome Profiles Compared with Solo-LTR clones



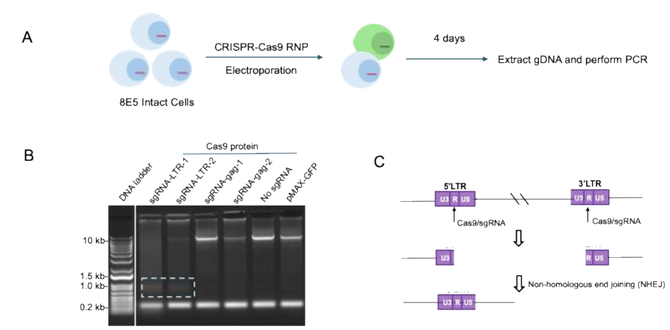
(A) The intact and solo-LTR clones were purified by limited dilution and validated by PCR (B) Four intact and solo-LTR clones were isolated to perform growth curve assay. Two replicates of CEM cell line were also included in the assay. (C) The HIV-1 RNA expression in the clones were tested by RT-ddPCR with primers targeting *LTR or gag*. (D) RNA-seq analysis was conducted in the parent CEM cell line (three replicates), intact and solo-LTR clones (four replicates).

Solo-LTR formation is mediated by Rad52-dependent single strand annealing (SSA) in *S. pombe*



(A) Scheme for monitoring the formation of solo-LTR in the LTR-retrotransposon Tf2 in *S. pombe*. Recombination between intra-element LTRs yields solo LTR and loops out *ura4* gene, allowing cells to survive in the presence of 5-FOA. (B) Results of LTR-LTR recombination assay using colonies of cells from Tf2-10-*ura4* with FOA (2e7 cells) or without 5-FOA (1e3 cells). (C) The frequency of solo-LTR formation was quantified by the number FOA-resistant colonies divided by the number of colonies without 5-FOA treatment. LTR-LTR recombination frequencies were tested in the wild-type, *rad50, rad51 and rad52* deletion strains. (D) Proposed model of SSA-mediated solo-LTR formation.

Formation of solo-LTR in 8E5 by Cas9/gRNAs targeting HIV-1 LTR



(A) Experimental procedure layout of CRISPR/Cas9 editing in the 8E5 intact cells. (B) PCR analysis of the intact and solo-LTR provirus after 4 days of recovery from the editing. (C) Diagram showing a model of solo-LTR formation promoted by double strand cuts and non-homologous end joining (NHEJ).

CONCLUSIONS

- A solo-LTR provirus was identified in the 8E5 cell line (not ACH-2)
- Cells with solo-LTR provirus exhibit growth advantage against the cells with intact provirus
- Clone stability of cells containing intact HIV-1 genome is tested and maintained for three months
- *RT, vpr, vpu* in the HIV-1 provirus have either frameshift or nonsense mutation (contribute to the survival of the 8E5 cells after acute infection)
- Ongoing LTR-LTR recombination not observed in 8E5 by measuring level of 1-LTR circles
- RNA-seq results show that the transcriptome profile of intact clones is slightly different from the profile of solo clones
- HR factors, such as Rad50 and Rad52, are required for the solo LTR formation in fission yeast
- Solo-LTR generation can be achieved by CRISPR/Cas9 editing that targets LTR

ACKNOWLEDGEMENTS

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