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Excision of HIV-1 DNA by Gene Editing: A Proof-of-Concept In Vivo Study

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Abstract

A CRISPR/Cas9 gene editing strategy has been remarkable in excising segments of integrated HIV-1 DNA sequences from the genome of latently infected human cell lines and by introducing InDel mutations, suppressing HIV-1 replication in patient-derived CD4+ T-cells, ex vivo. Here, we employed a short version of the Cas9 endonuclease, saCas9, together with a multiplex of guide RNAs (gRNAs) for targeting the viral DNA sequences within the 5′- LTR and the Gag gene for removing critically important segments of the viral DNA in transgenic mice and rats encompassing the HIV-1 genome. Tail vein injection of transgenic mice with a recombinant Adeno-associated virus 9 ($rAAV_9$) vector expressing saCas9 and the gRNAs, rAAV:saCas9/gRNA, resulted in the cleavage of integrated HIV-1 DNA and excision of a 940 bp DNA fragment spanning between the LTR and Gag gene in spleen, liver, heart, lung, and kidney as well as in circulating lymphocytes. Retro-orbital inoculation of $rAAV_9$:saCas9/gRNA in transgenic rats eliminated a targeted segment of viral DNA and substantially decreased the level of viral gene expression in circulating blood lymphocytes. The results from the proof-of-concept studies, for the

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CONFLICT OF INTERESTS

The authors declare that there is no financial conflict of interest

AUTHOR CONTRIBUTIONS

Conceived experiments: KK, JG, WH, RB
Performed experiments: RK, RB, CY, JO, RB, HL
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Prepared manuscript: KK, RK, JG, RB

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first time, demonstrate the in vivo eradication of HIV-1 DNA by CRISPR/Cas9 upon delivery by an $rAAV_9$ vector in a range of cells and tissues that harbor integrated copies of viral DNA.

Keywords

HIV-1; gene editing in vivo; in vivo HIV-1 eradication; saCas9/gRNA; AAV delivery

INTRODUCTION

HIV/AIDS remains a major public health problem, as over 40 million people worldwide are infected and new infections continue at greater than two million/year [1]. Combination antiretroviral therapy (cART) effectively controls ongoing viral replication and can restore lost numbers of CD4+ T-cells. However, treatment fails to eliminate virus from latently infected cells [2]. In subsets of resting CD4+ memory T-cells, integrated proviral DNA persists and can be reactivated to produce replication-competent virus. This can result in rapid viral rebound when cART ceases [3, 4]. Therefore, those infected must maintain life-long treatment due to viral persistence in cell reservoirs.

Elimination of latent proviral DNA remains enigmatic. During latency, HIV-1-infected cells produce little or no viral proteins, thereby avoiding host antiviral immune clearance or direct viral cytopathicity. Eradication of virus requires its clearance and prevention of re-infection of latently infected cell CD4+ T effector memory cells [5, 6] amongst other infected lymphocytes and monocyte-macrophages present in spleen, lymph nodes, brain, genitourinary tract and gut to achieve a disease “cure” [7]. Recently, gene editing techniques were developed and based on RNA-guided Cas9 (known as CRISPR/Cas9) to specifically target the HIV-1 genome and eliminate integrated copies of the proviral DNA. We identified several specific sequences within the U3 region of the HIV-1 long term repeat (LTR) that serve as targets for the creation of specific guide RNAs (gRNAs) to edit their target sequences by single and multiplex Cas9/gRNAs complexes. This strategy can lead to complete elimination of viral replication in latently infected CD4+ T-cells and mononuclear phagocytes (monocyte, macrophages, microglia and dendritic cells) [8, 9]. Our CRISPR/Cas9 had no genotoxic effects or off-target editing of the host genome, and for the first time, showed the ability of this technology to precisely excise a 9709 bp DNA fragment of the integrated proviral genome that spans the 5′ and 3′ HIV-1 LTRs. The presence of multiplex gRNAs and Cas9 also protects against subsequent HIV-1 infection and was used successfully in cell models of human disease [10–12]. All of these observations offer a novel pathway for the use of CRISPR/Cas9 as a strategy to permanently remove HIV-1 DNA from the host and to develop a potential means to cure HIV/ AIDS.

However, an important challenge relates to the *in vivo* delivery of functional CRISPR/Cas9 to tissues and cells that harbor viral DNA. In recent years, Adeno-Associated Virus Vectors (AAV) has captured much attention as a gene delivery system for treating human disease caused by a gene loss or mutation [13]. The advantages of the AAV delivery scheme include its low toxicity and sustained gene expression which can extend to twelve months after a single administration [14, 15]. The most common diseases targeted by AAV delivery

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systems include cancer, heart failure, neurodegenerative diseases, arthritis, muscular dystrophy, cystic fibrosis and Canavan's disease amongst others [13, 16, 17]. In this concise study we present results from our preliminary studies in two small animal species in which AAV serotype 9 was used as a delivery platform to bring HIV-1 targeted CRISPR/Cas9 to various cells and organs of two species of small animals for excising the integrated copies of HIV-1 from the host genome to edit integrated HIV-1 DNA as a strategy towards viral elimination in two species of small animals.

RESULTS

As a testing platform, first we used HIV-1 Tg26 transgenic mice, which carry a transgene derived from the genome of HIV-$1_{NL4–3}$ with a deletion of a 3.1 kb spanning the C-terminal of the Gag and the N-terminal of the Pol genes (Fig. 1A). While no productive HIV-1 replication is reported, expression of viral transcripts, at low levels, have been detected in various tissues prior to disease onset [18–20]. Select clinical features of HIV-1 infection and AIDS including HIV-associated nephropathy (HIVAN) are seen [21, 22]. Due to the early lethality of the mice we crossed Tg26 mice into the C57BL/6L background to create animals where secondary disease is limited and mice can survive to 12 months of age. For delivery of genes expressing Cas9 and gRNAs, we selected an AAV_9 vector. One of the major challenges associated with the use of AAV vectors relates to its genome accepting capacity. To alleviate this problem, we selected a smaller Cas9 or homologue, saCas9 (3.3 kDa), derived from Staphylococcus aureus that can edit the genome with efficiency similar to that of the original Cas9 from Streptococcus pyrogens (spCas9), while being more than 1 kb shorter [23]. The gene encoding saCas9 along with DNA sequences corresponding to the two gRNAs for targeting the HIV-1 LTR (gRNA LTR 1) and the Gag gene (gRNA Gag D) were cloned into the AAV_9 vector DNA. Fig. 1A illustrates the structural organization of the HIV-1 genome highlighting the region of the viral gene that was deleted for creating the transgene in Tg26 mice and the position of gRNA LTR 1 and gRNA Gag D. As a first step, we performed ex vivo studies by developing a culture of mouse embryo fibroblasts (MEFs) from Tg26 animals and using them to assess the ability of the recombinant AAV_9 ($rAAV_9$) containing saCas9/gRNAs, $rAAV_9$:saCas9/gRNAs, in excising portions of integrated HIV-1 DNA. Fig. 1B illustrates results from PCR gene amplification utilizing a pair of primers (P1 and P2) spanning −413/−391 for the LTR and +888/+910 for the Gag gene (Fig. 1A). In addition to the expected full-length amplicon of 1323 bp, a smaller fragment of 368 bp was detected in $rAAV_9$ treated cells. These were not seen in control untreated cells. Results from sequencing data verified excision of a 955 bp DNA fragment spanning the two guide RNAs and re-joining the residual viral DNA after excision (as schematized in Fig. 1A). For in vivo studies we selected two Tg26 animals and two age-matched (2 months) control mice for tail vein injections of 10^{12} functionally titered of $rAAV_9$:saCas9/gRNA. After five days, the injections were repeated and five days later the animals were sacrificed and the liver, heart, spleen, lung, kidney, brain and blood lymphocytes were harvested (Fig. 2A). In a first pass experiment DNA from liver was analyzed by PCR amplification using P1 and P2 primers. Consistent with the results from MEF cell culture, results of gel analysis of PCR products showed a full-length (1323 bp) and a truncated (368 bp) DNA fragment after amplification from $rAAV_9$:saCas9/gRNA treated animals but not in age-matched control animals (Fig. 2B

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left). Extension of DNA amplification in the presence of a pair of nested primers, P1′ (−375/−354) and P2′ (+755/+763) (depicted in Fig. 1A) yielded an additional smaller DNA fragment of 183 bp (Fig. 2B, middle and right panels). Results from DNA sequencing of the 183 bp DNA verified excision of the 955 bp DNA sequence spanning between gRNA LTR 1 and gRNA Gag D (Fig. 2C). We next assessed the effect of $rAAV_9$:saCas9/gRNA on HIV-1 DNA in other tissues. As seen in Fig. 2D, a distinct 183 bp DNA fragment, indicative of excision of the HIV-1 DNA between the LTR and Gag genes by gRNAs LTR1 and gRNAs Gag D, as verified by DNA sequencing, was observed in all tissues of $rAAV_9$:saCas9/gRNA treated animals (lane 2). These were not observed in control untreated animals (lane 1). The 183 bp amplicon produced upon treatment of MEFs with $rAAV_9$ served as an additional control (Lane 3). In parallel studies we assessed the effect of our excision strategy in another small animal model, i.e. rat, that encompasses the identical transgene used for the development of the mouse model. Here, we employed a retro-orbital route of inoculation of $rAAV_9$. Thirty day old rats injected twice with 2.73 x 1012 of $rAAV_9$:saCas9/gRNA at 5 day intervals led to the excision of segments of HIV-1 DNA spanning the 5′-LTR and the Gag gene from circulating lymphocytes, as examined by direct sequencing of the amplicon that was generated by target specific PCR. As illustrated in Fig. 3, a detailed analysis of the sequencing data from several PCR products of the excised fragments and their alignment with the reference HIV-1 DNA verified excision of the viral DNA with some variations in tissues from animals treated with $rAAV_9$:saCas9/gRNA. We also examined the level of viral gene expression by measuring the levels of Gag and Env transcripts using quantitative RT-PCR. As seen in Fig. 4, the level of viral RNA was drastically decreased in circulating blood cells obtained from animals treated with $rAAV_9$:Cas9/gRNA, indicating that excision of the viral genome has a significant impact on the level of viral gene expression from the integrated copies of HIV-1 DNA. Examination of viral RNAs in lymph nodes also showed suppression of viral RNAs in the treated rats (data not shown).

DISCUSSION

In recent years, methodologies have been developed that are specifically able to target nucleotide sequences within precise regions of DNA and represent a remarkable addition to the armamentarium for genetic manipulation [24]. Perhaps the best such methodology is the clustered regulatory interspaced short palindromic repeat (CRISPR)-associated 9 (Cas9) system, which is specific, effective, versatile and provides unprecedented control over genome editing for a variety of purposes, e.g. correction of inborn genetic errors [25] and genetic approaches to use against human viruses [26] and cancers [27]. Crucially, application of CRISPR/Cas9 to the clinic will require a method of safe and efficient delivery in vivo. A promising possibility is to use adeno-associated virus (AAV), which is only mildly immunogenic, but is a small virus unable to fit the 4.1 Kbp Cas9 gene. One solution is to use proteins with a similar function but smaller size such as Cas9 form Staphylococcus aureus (saCas9), which has a similar efficiency while being more that 1 kb shorter [27].

In this communication, we demonstrate that the AAV_9-based saCas9/gRNA gene editing delivery system can excise a segment of integrated copies of the HIV-1 genome in transgenic mice and rats carrying HIV-1 DNA sequences. Further, our results demonstrate suppression of viral RNA production in the animals that were treated with the therapeutic rAAV and

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exhibit excision in the designated segments of the viral genome by saCas9/gRNAs in blood and lymph nodes. These findings are an important advance from earlier cell culture work demonstrating CRISPR/Cas9 excision of viral infection from latently infected cells, in vitro [8, 9] or suppression of ongoing HIV-1 replication, ex vivo [28]. The work is a step forward towards the utilization of CRISPR/Cas9 platform technology as a robust HIV-1 gene elimination strategy in an in vivo setting. In principle, this technology can be further refined and used in tandem with an antiretroviral regimen to enhance the efficacy of editing and as such lead to a similar favorable outcome in infected animals and inevitably in humans. The ease of AAV delivery for the CRISPR/Cas9 technology and the flexibility of CRISPR/Cas9 in developing new gRNAs for targeting DNA sequences of HIV-1 with minor nucleotide variations brings additional values to this therapeutic platform for personalizing the cure strategy, if deemed appropriate.

MATERIALS AND METHODS

Construction of the AAV_9 delivery vector

The background plasmid for creating the AAV delivery system, px601-AAV-CMV::NLS-saCas9-NLS-3xHA-bGHpA;U6::Bsa1-SgRNA (briefly, pX601) was a gift from Feng Zhang (Addgene #61591) [23]. The oligonucleotide encompassing the DNA sequences corresponding to the LTR and Gag targets were cloned ahead of the U6 promoter at the Bsa1 site in pX601. After confirmation by sequencing, 20 ng of pX601 saCas9 gRNA HIV-1 LTR1/GagD plasmid was provided to the Penn Vector Core, Gene Therapy Program (Perelman School of Medicine, University of Pennsylvania) for plasmid DNA production, DNA structure/sequence analysis, packaging in AAV serotype and high titer production.

Mouse embryo fibroblasts (MEFs)

HIV-1 Tg26 Mouse embryo fibroblasts (MEFs) were prepared from 17 day gestation embryos by mechanical and enzymatic dissociation and maintained in DMEM supplemented with 10% fetal bovine serum. MEF cells were prepared as previously described [29] and genotyped by PCR using primers specific for the HIV transgene [19, 22].

In vitro transduction of Tg26 MEF

Tg26 MEF cells were transduced with AAV-CRISPR/Cas9 at MOI 10^5 and 10^6. Viral inoculum was prepared in Opti-Mem, cells were incubated with the virus for 1 hour in minimal volume (0.5 ml/well in a 6 well plate) then 1 ml of growth medium was added and left overnight. The next day, inoculum was removed, cells were washed with PBS and fed fresh growth medium. Cells were harvested for DNA analysis one week after transduction.

In vivo $rAAV_9$:saCas9/gRNA administration

For studies with transgenic mice, 100 μl of AAV_9: Cas9/gRNA (2.73×10^{12}) or 100 μl of PBS for control animals was injected via tail vein into mice at day 0 and day 5. At day 5, one pair (AAV and PBS) animals were subjected to a retro orbital bleed for a blood sample, euthanized and tissues harvested. The second pair of animals received a second tail vein injection of AAV-Cas9 or PBS, and were euthanized for harvest of tissue 7 days later after retro orbital bleed. Tissues harvested were brain, heart, lung, liver, kidney, spleen and

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peripheral blood for lymphocyte recovery. For studies with rats, we followed a similar approach as described above and the tissues were harvested two weeks after the initial injection for DNA and RNA analysis.

DNA analysis

Genomic DNA was isolated from cells/tissues using NucleoSpin Tissue kit (Macherey-Nagel) according to the manufacturer's protocol. 25ng of genomic DNA was subjected to PCR using Terra PCR direct polymerase mix (Takara, Clontech) under the following PCR conditions: 98 °C for 3 minutes, 35 cycles (98 °C for 10s, 68 °C for 1.30 minutes), 68 °C for 5 minutes and resolved in 1% agarose gel. Nested PCRs were performed under the same conditions using 5 μl of first PCR reaction. PCR using genomic DNA extracted from Tg26 rat blood samples was performed in one step using LTR F and nested Gag R primers. PCR products were purified, cloned into TA vector (Invitrogen) and sent for Sanger sequencing (Genewiz) and aligned in Clustal Omega software using HIV-1 NL_{4-3} sequence as a reference.

RNA analysis

Total RNA was prepared from tissues using TRIzol Reagent (Ambion) according manufacturer's protocol followed by DNAse I treatment and RNA cleanup using RNeasy Mini Prep Kit (Qiagen). Next 1 ug of RNA was used for M-MLV reverse transcription reactions (Invitrogen). cDNA was diluted and quantified using TaqMan qPCR specific for HIV-1 Gag and Env genes and cellular rat beta-actin gene as a reference (for primers see table). qPCR conditions: 98 °C 5 minutes, 45 cycles (98 °C 5 minutes, 45 cycles (98 °C 15s, 62 °C 30s with acquisition, 72 °C 1 minute). Reactions were carried out and data analyzed in a LightCycler480 (Roche) using relative quantification mode.

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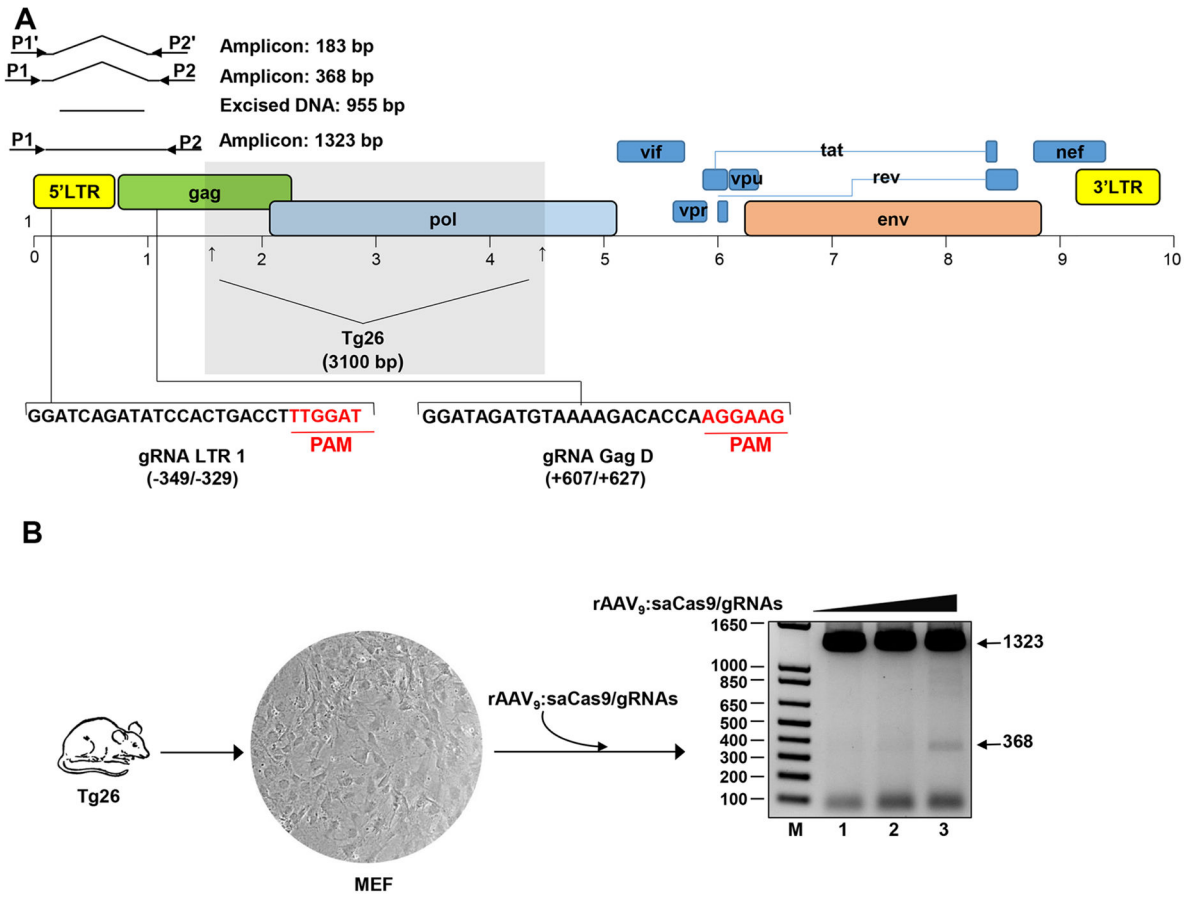

Figure 1. Excision of HIV-1 DNA by rAAV$_9$:saCas9/gRNA Tg26 MEF

A. Schematic illustration of HIV-1 highlighting the position between the Gag and Pol genes (3100 bp) that was removed to create a transgene for developing Tg26 animals. The positions of gRNAs LTR 1 and Gag D, and their nucleode compositions are shown. Red letters indicate the PAM sequence. The top left of the diagram shows the positions of the primers used for PCR (P1 and P2) and nested PCR (P1′ and P2′) with the expected amplicons before and after excision. B. Depiction of MEFs derived from Tg26 and results from gel analysis of PCR amplification of HIV-1 DNA after treatment with increasing amounts of rAAV:Cas9/gRNAs. The position of full-length (1323 bp) and the truncated (368 bp) PCR products are shown.

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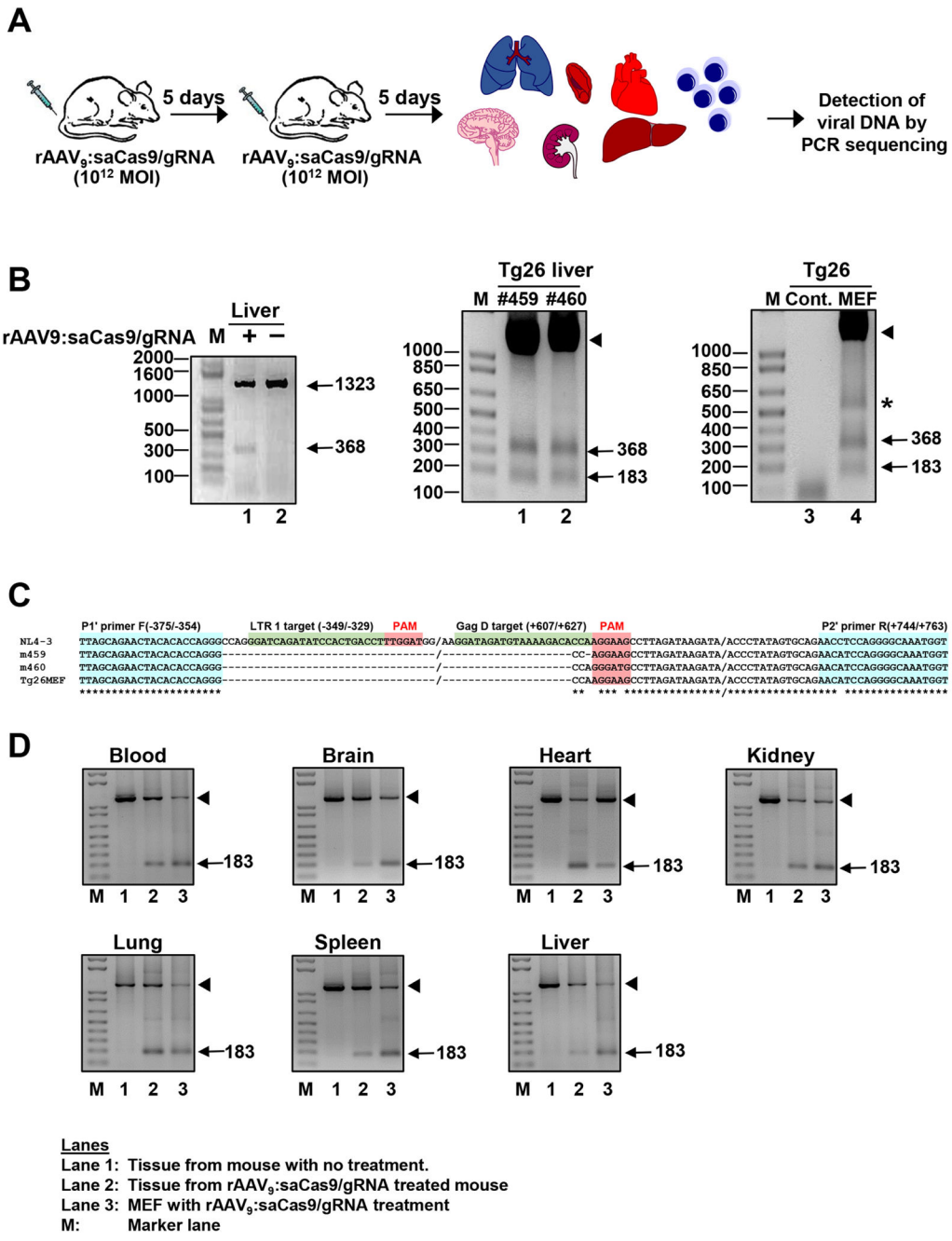

Figure 2. In vivo excision of HIV-1 DNA by rAAV$_9$:saCas9/gRNA in various tissues of Tg26 mice

A. Diagram of the procedure used for the inoculation of rAAV$_9$:Cas9/gRNAs and the organs used for the analysis. B. Results from PCR of DNA obtained from lines of Tg26 mice with and without rAAV$_9$ show the positions of 1323 bp and the truncated 368 bp in the rAAV$_9$ treated samples (left). Gel analysis of PCR products using two pairs of primers; P1/P2 for detection of 368 bp fragment and the nested primers P1′/P2′ for detection of the 183 bp fragment. The full-length PCR product is shown by an arrowhead(middle). Results from double primer PCR amplification of the MEF DNA again showing the expected two

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truncated HIV-1 DNA fragments. The position of the full-length amplicon (arrowhead) and a non-specific band (asterisk) are shown. Control (Cont.) illustrates PCR reaction in the absence of primers (right). C. Results from sequencing of the 183 bp DNA fragment shown in Panel B (middle and right panels). The positions of the primers as well as gRNAs LTR 1 and Gag D are shown. D. Results from PCR amplification of DNA from various tissues (lanes 1 and 2) and MEFs (lane 3) demonstrating the absence of the 183 bp truncated DNA fragment in animals with no inoculation of $rAAV_9$:saCas9/gRNAs and the detection of this band in the animals and cells that received $rAAV_9$:saCas9/gRNAs.

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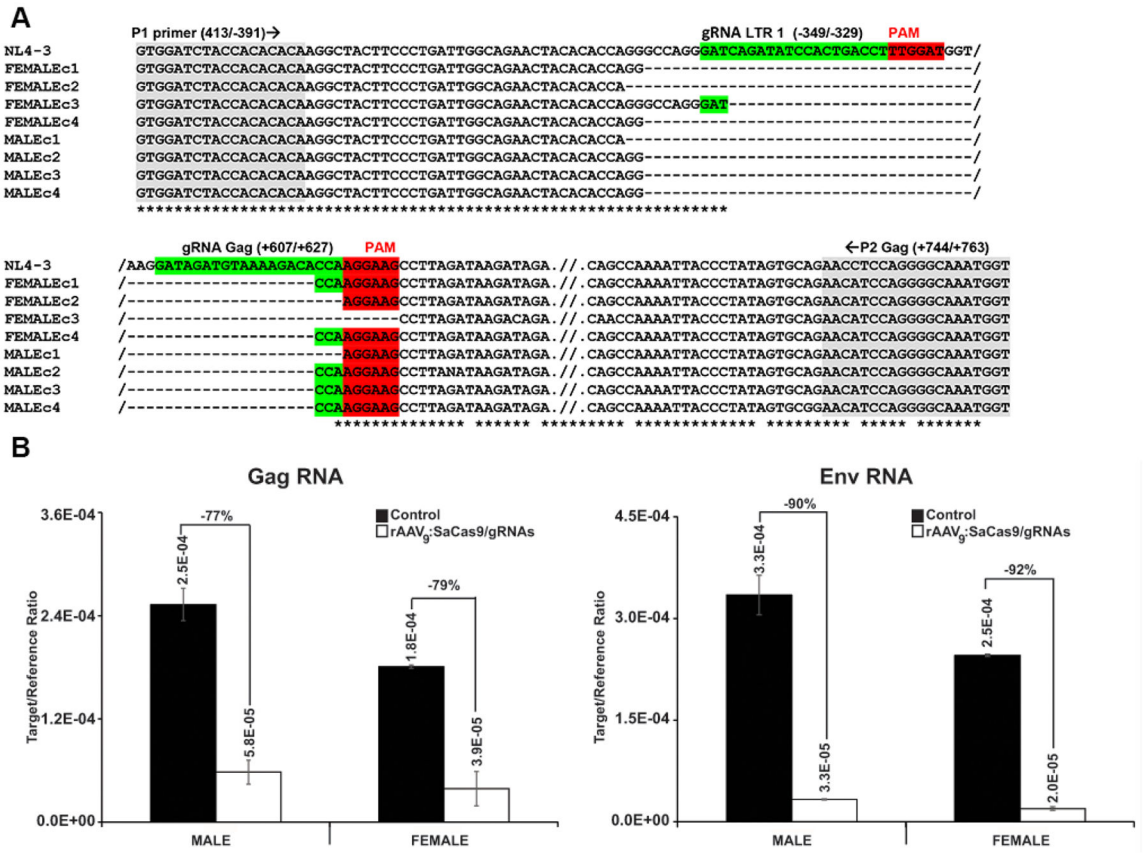

Figure 3. Elimination of segments of the integrated HIV-1 DNA from rat blood cells after inoculation with rAAV$_9$:saCas9/gRNA and expression of viral RNAs

A. Total DNA from circulating lymphocytes of the control (untreated) and experimental (treated with rAAV$_9$:saCas9/gRNA) rats was prepared and used for PCR amplification using a set of primers derived from the 5′-LTR and Gag gene (shown in Materials and Methods). After gel electrophoresis, a short DNA fragment of approximately 221 bp was detected in the treated, but not the control samples, which were then purified and cloned in a TA vector. Several clones were selected for DNA sequencing. Four representative DNA sequences (C1–C4) obtained from each animal were aligned to the reference LTR-Gag region of the HIV-1 pNL$_{4-1}$ sequence. The positions and nucleotide composition of LTR 1 and Gag D target sequences are highlighted in green, PAM in red and LTR specific primers using PCR are highlighted in blue. B. Total RNA prepared from circulating lymphocytes and lymph nodes of transgenic rats, control (untreated) and treated with rAAV$_9$:saCas9/gRNA for 10 days were prepared and used for quantatitive RT-PCR for detection of Gag RNA and Env RNA. Expression of β-actin in each assay was determined and the values were used as reference for quantification of viral RNA expression.

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Table

primer	sequence
LTR-Gag PCR	
P1 [LTR F [−413/−391](T361)]	5′-GATCTGTGGATCTACCACACACA-3′
P2 [Gag R (+888/+910) (T458)]	5′-CCCACTGTGTTTAGCATGGTATT-3′
P1′ [nested LTR F (−375/−354)]	5′-TTGGCAGAACTACACACCAGGG-3′
P2′ [nested Gag R (+744/+763)]	5′-ACCATTTGCCCCTGGAGGTT-3′
Taqman qPCRs	
HIV-1 Env F	5′-TCCTTGGGATGTTGATGATCT-3′
HIV-1 Env R	5′-TGGCCCAAACATTATGTACC-3′
HIV-1 Env Probe	5′-FAM-TGGTGGTTGCTTCTTTCCACACA-ZEN-IowaBlackFQ-3′
HIV-1 Gag F	5′-AAGTAGTGTGTGCCCGTCTG-3′
HIV-1 Gag R	5′-TCGAGAGATCTCCTCTGGCT-3′
HIV-1 Gag Probe	5′-FAM-CTGTTCGGGCGCCACTGCTA-ZEN-IowaBlackFQ-3′
Rn b-actin F	5′-AGCGCAAGTACTCTGTGTGG-3′
Rn b-actin R	5′-AACAGTCCGCCTAGAAGCAT-3′
Rn b-actin probe:	5′-FAM-CCTCCATCGTGCACCGCAA-ZEN-IowaBlackFQ-3′