Efficiency and Specificity of Targeted Integration Mediated by the Adeno-Associated Virus Serotype 2 Rep 78 Protein

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The adeno-associated virus serotype 2 (AAV2) Rep 78 protein, a strand-specific endonuclease (nickase) promotes site-specific integration of transgene sequences bearing homology arms corresponding to the AAVS1 safe harbor locus. To investigate the efficiency and specificity of this approach, plasmid-based donor vectors were tested in concert with nuclease encoding vectors, including an engineered version of the AAV2 Rep 78 protein, an AAVS1-specific zinc finger nuclease (ZFN), and the CRISPR-Cas9 components in HEK 293 cells. The Rep 78 and ZFN-based approaches were also compared in HEK 293 cells and in human induced pluripotent stem cells using integrase deficient lentiviral vectors. The targeting efficiencies involving the Rep 78 protein were similar to those involving the AAVS1-specific ZFN, while the targeting specificity for the Rep 78 protein was lower compared to that of the ZFN. It is anticipated that the Rep 78 nickase-based targeting approach may ultimately contribute to the reduction of risks associated with other genome editing approaches involving DNA double-strand breaks.

Keywords: AAVS1 locus, site-specific integration, site-specific nuclease, DNA nickase, integrase-defective lentiviral vectors, human iPSCs

INTRODUCTION

WILD-TYPE ADENO-ASSOCIATED VIRUS (AAV) has the capacity to integrate preferentially on human chromosome 19 (at 19q13.42), at a site that is referred to as the AAVS1 locus.^{1,2} The AAVS1 locus overlaps with the first exon of the gene (*PPP1R12C*) encoding the protein phosphatase 1 regulatory protein subunit 12C.³ AAV integration requires the viral nonstructural Rep 78/68 proteins in trans, as well as a cis-acting Rep binding site (RBS) and the terminal resolution site (*trs*) sequences located either in the AAV inverted terminal repeat (ITR) or within the AAV p5 promoter.^{4,5} AAV integration is also dependent on a 33 bp sequence present at the AAVS1 locus that bears similarities with the RBS and the *trs* sequences in the ITR sequence of the virus.⁶

The AAV Rep 78/68 proteins have been pursued as a means for transgene insertion at the AAVS1 locus, since this particular region is considered to

be a safe harbor for transgene insertion.^{7–9} Numerous reports have shown that recombinant plasmids and viral vectors bearing either the AAV ITRs or the AAV2-derived p5 integration efficiency element (p5IEE)¹⁰ have the capacity to site-specifically integrate at the AAVS1 locus, mediated by the AAV Rep 78/68 proteins.^{11,12}

The AAV2 Rep 78/68 proteins have also been shown to promote site-specific integration of transgene sequences flanked by homology arms derived from the AAVS1 locus in HeLa cells.^{13–15} The inclusion of homology arms may add to the specificity of this approach, since AAV virus integration events are not unique. For example, integration hot spots for AAV2 integration in addition to the canonical AAVS1 locus were revealed by integration capture sequencing¹⁶ and by linear amplification mediated polymerase chain reaction (PCR).¹⁷

The main objective of the work described in this report was to determine the efficiency and speci-

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ficity of transgene insertion at the AAVS1 locus through homology-directed repair (HDR), mediated by site-specific nucleases. A primary focus was on a modified Rep 78 protein, referred to as Rep 78-III,¹⁸ and its capacity to mediate transgene insertion compared to other site-specific nucleases, including an AAVS1-specific zinc finger nuclease (ZFN)⁸ and the CRISPR-Cas9 components involving the Cas9 nuclease and an AAVS1-specific single-guide RNA (sgRNA).

MATERIALS AND METHODS

Plasmid constructs

DNA sequences encoding Rep 78-III and the ZFNSh-L and ZFNSh-R proteins were synthesized by GenScript (Piscataway, NJ) based on the sequences described by Sitaraman *et al.*,¹⁸ Hockemeyer *et al.*,⁸ and Guo *et al.*,¹⁹ and were subcloned into pENTR2B (Invitrogen, Carlsbad, CA). The wild-type Rep 78 (Rep 78 WT) sequence was PCR amplified using pAAV-DJ Vector DNA (Cell Biolabs, Inc., San Diego, CA) as a template and Q5 and hot start high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA). The hCas9 plasmid (Addgene; plasmid # 41815), the hCas_D10A plasmid (Addgene; plasmid # 41816), and the gRNA_AAVS1-T2 plasmid (Addgene; plasmid # 41818) were gifts from George Church Department of Genetics, Harvard Medical School, Boston, MA 02115, USA.²⁰ The pAAVS1 SA-2A-puro-pA donor plasmid (Addgene; plasmid # 22075) was a gift from Rudolf Jaenisch The Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, USA.

The pAAVS1-p5IEE-SA-2A-puro-pA donor plasmid was constructed by inserting into pAAVS1 SA-2A-puro-pA donor plasmid a synthetic p5IEE DNA sequence¹⁰ (GenScript) upstream of the SA sequence. The Rep 78-III and ZFNSh coding regions were transferred into the pDEST 12.2 plasmid (Invitrogen) by Gateway recombination (Invitrogen). Alternatively, they were transferred into the pNL(CMV)EGFP/CMV/WPREΔU3 lentiviral vector plasmid,²¹ resulting in the pNL(CMV)Rep 78-III/CMV/WPREΔU3 and the pNL(CMV)AAVS1 ZFNSh-L/CMV/WPREΔU3 and pNL(CMV)AAVS1 ZFNSh-R/CMV/WPREΔU3 vector plasmids, respectively. The Rep 78 WT coding region and the Rep 78-III sequence were also subcloned into the pCDH-CMV-MCS-EF1-copGFP lentiviral expression vector plasmid (System Biosciences, Mountain View, CA), resulting in the pCDH-CMV-Rep 78 WT-EF1-copGFP and pCDH-CMV-Rep 78-III-EF1-copGFP plasmids, respectively. The Rep 78-III coding region was subcloned into pENTR2B/TRE Pitt plasmid²¹

and then transferred to the Dox-regulatable pSLIK-Venus plasmid²² Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA. (Addgene; plasmid # 25734; a gift from Iain Fraser) by Gateway recombination.

The pCD/NL-BH*D116N helper plasmid encoding a defective HIV-1 integrase has been described previously.²³

Cell lines

Human embryonic kidney (HEK) 293T cells (CRL-11268TM; American Type Culture Collection [ATCC], Manassas, VA), HEK 293 cells (CRL-1573TM; ATCC) and HeLa cells (CCL-2TM; ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS), 2.5 mM of L-glutamine, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin (all from Invitrogen, Grand Island, NY).

The human induced pluripotent stem cell (iPSC) line ND2.0 (NHLBI iPSC Core Facility) was maintained in Essential 8 medium (Gibco, Grand Island, NY). Puromycin-resistant mouse embryo fibroblasts (MEF Puro) were purchased from Globalstem (Rockville, MD) and were cultured in DMEM containing 10% FBS.

Transfection of HEK 293 cells, puromycin-selection, and analysis of gene editing events

HEK 293 cells were seeded at $4\text{--}5 \times 10^5$ cells per well on six-well tissue culture plates 1 day before transfection. Cells were transfected either with the donor plasmid alone (1 μg of pAAVS1-p5IEE-SA-2A-puro-pA donor) or the donor plasmid (1 μg) plus the p12.2/Rep 78-III plasmid (0.2 μg). Alternatively, cells were transfected with the donor plasmid alone (1 μg) or the donor (1 μg) plus the p12.2/AAVS1-ZFNSh-L and p12.2/AAVS1-ZFNSh-R plasmids (1 μg each). For the CRISPR-Cas9 approach, cells were transfected with the donor plasmid alone (1 μg) or the donor plasmid (1 μg) plus the hCas9 (1 μg) plasmid plus the gRNA-AAVS1-T2 plasmid (1 μg). All transfections were carried out by polyethylenimine-mediated transfection.²⁴ The transfected cells were selected using 0.5 μg/mL of puromycin (Invitrogen) for 10–14 days, starting 3 days post transfection. Puromycin-resistant clones were randomly picked and expanded, and genomic DNA was extracted using the DNeasy[®] Blood & Tissue Kit (Qiagen, Germantown, MD). PCR was performed to detect the integration at the AAVS1 locus using the AAVS1-F (5'-CTGCCGTCTCTCTCCTGAGT) and Puro-R (5'-GTGGGCTTGTA CTGCGTCAT) primers.²⁰ PCR was

carried out using Platinum Taq DNA polymerase (Invitrogen). The conditions used were as follows: 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 90 s, and a final elongation for 7 min at 72°C.

Integration deficient lentiviral vector production and concentration

Lentiviral vector production was carried out using 15 cm plates (Nunc; Thermo Fisher Scientific, Waltham, MA), as described.^{25,26} Vector stocks were titrated by quantitative PCR using genomic DNA extracted from transduced HEK 293 cells using GAG-specific or WPRE-specific primers.^{25,27} Vector titers are expressed as TU/mL.

Transduction of HEK 293 cells and human iPSCs using integration deficient lentiviral vectors, puromycin-selection, and analysis of gene editing events

For transduction, 1×10^5 of HEK 293 cells per well were seeded onto six-well plates. The cells were transduced with the integration deficient lentiviral vector (IDLV) donor vector NL8/AAVS1-SA-2A-p5IEE-PuroΔU3 alone or in concert with the NL(CMV)Rep78-III/CMV/WPREΔU3 vector or the NL(CMV)AAVS1 ZFNSh-L/CMV/WPREΔU3 plus NL(CMV)AAVS1 ZFNSh-R/CMV/WPREΔU3 vectors at the multiplicities of infection (MOIs) specified in the figure legends. The transduced cells were selected using 0.5 μg/mL of puromycin (Invitrogen) for 10–14 days starting 2 days post transduction. Puromycin-resistant clones were randomly picked and expanded, and genomic DNA was extracted using the DNeasy® Blood & Tissue Kit (Qiagen). PCR was performed to detect the integration at the AAVS1 locus using AAVS1-F (5'-CTGCCGTCTCTCTCCTGAGT) and Puro-R (5'-GTGGGCTGTACTCGGTCAT) primers.²⁰ PCR was carried out using Platinum Taq DNA polymerase, as described above.

For transduction of human iPSC ND2.0 cells, ND2.0 cells were separated into single cells using StemPro Accutase (Life Technologies, Rockville, MD). Single cells were then re-suspended in Essential 8 medium supplemented with 10 μM of Rock inhibitor Y27632 (Tocris Bioscience, Bristol, United Kingdom) and plated onto Matrigel-coated six-well plates at a density of 1×10^5 cells/well. Twenty-four hours later, ND2.0 cells were transduced with the IDLV donor vector NL8/AAVS1-SA-2A-p5IEE-PuroΔU3 alone or together with the IDLV NL-Rep78-III/CAG/WPREΔU3 vector at MOIs of 50 or 100. Cells were then dispersed using

0.5 mM of EDTA and plated onto 2×10^5 of MEF Puro cells 2 days post transduction and maintained in Essential 8 medium for another 2 days. Cells were selected using 0.3 μg/mL of puromycin for 7–10 days with daily medium change.

Surveyor assay

To amplify the sequence spanning the trs/RBS sequence, the TRS-RBS B-Fwd (5'-GGCCACTTC TGCTAATGGAC) and TRS-RBS B-Rev (5'-CAG CAGAGGGTGGACCTG) primers were used. The CEL-1 primer set⁸ was used to amplify a sequence spanning the AAVS1 ZFNSh and Cas9 cleavage sites. The PCR was done using OneTaq Master Mix with GC buffer (New England Biolabs). The conditions used were as follows: 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min, and a final elongation for 7 min at 72°C.

The PCR products were purified using a DNA Clean & Concentrator™-5 kit (Zymo Research, Irvine, CA). A 10 μL aliquot of the PCR reaction was transferred to a new tube and mixed with 1.1 μL of Platinum Taq 10× HiFi Buffer (Life Technologies). The DNA was denatured, slowly annealed to allow the formation of DNA heteroduplexes, and then digested with 2 μL of Surveyor Nuclease S (IDT, Coralville, IA). Digested products were run on a 10% Mini-PROTEAN TBE gel (Bio-Rad Laboratories, Hercules, CA), stained with ethidium bromide, and quantified using the Bio-Rad ChemiDoc MP Imaging System and Image Lab v5.2.1 software.

Deep sequencing of amplicons

Short amplicons spanning the trs/RBS sequence or the sequence recognized by AAVS1-specific ZFNSh or the CRISPR-Cas9 nuclease were prepared as above using the trs-RBS 1L (5'-GGGG ACCGGGAGATCCTT) and trs-RBS 1R (5'-CATC CTCTCCGGACATCGC) primers and the AAVS1 1L (5'-CGGTTAATGTGGCTCTGGTT) and AAVS1 1R (5'-ACAGGAGGTGGGGGTTAGAC) primers, respectively. Deep sequencing was performed by the CCIB DNA Core Facility at Massachusetts General Hospital (Cambridge, MA). Indel rates were analyzed using a browser-based CRISPResso instance with default settings.²⁸

Western blot analysis

HeLa cells were transduced using the SLIK-Venus-based Rep 78-III vector at a MOI of 2 and treated with Dox (1 μg/mL) or left untreated. Cell extracts were prepared 72 h later using RIPA buffer (Sigma-Aldrich, St. Louis, MO). Aliquots (15 μg) were run on a 4–12% sodium dodecyl sulfate polyacrylamide gel (NuPAGE; Thermo Fisher

Scientific), and the proteins were transferred onto a polyvinylidene fluoride membrane. The blot was probed using mouse monoclonal antibody to the AAV Rep protein (Clone 303.9; American Research Products, Inc., Waltham, MA). The blot was then stripped and re-probed with anti-GAPDH antibody (mAbcam 9484; Abcam, Cambridge, MA).

RESULTS AND DISCUSSION

Analysis of non-homologous end joining events in HEK 293 cells expressing engineered nucleases

DNA double-strand breaks are powerful inducers of HDR, but single-strand breaks (nicks) have also been shown to promote HDR.^{29,30} The AAV2 Rep 78 protein was shown before to produce single-strand breaks (nicks) at its DNA target sites.³¹ This is an attractive feature in that it may help prevent the formation of unintended mutations, such as insertions and deletions (indels), resulting from non-homologous end joining (NHEJ) repair of double-strand breaks, introduced by nucleases at off-target genomic sites.³² To confirm these earlier findings, indels caused by NHEJ in HEK 293 cells expressing different endonucleases targeting the AAVS1 locus were investigated using the Surveyor assay³³ and by deep sequencing. The endonucleases tested included the AAV2-encoded Rep 78 DNA nickase¹⁴ (referred to as Rep 78 WT), an engineered version of Rep 78 involving synonymous codon pair recoding (referred to as Rep 78-III),^{18,34} and the Cas9 D10A nickase.²⁰ The Cas9 nuclease²⁰ and a modified ZFN (referred to as ZFNSh)⁸ bearing an improved FokI nuclease cleavage domain, referred to as Sharkey,¹⁹ served as positive controls to detect indels. Genomic DNAs from HEK 293 cells transfected with plasmid constructs encoding the above nucleases were prepared and subjected to PCR amplification. A 596 bp amplicon spanning the trs and RBS sequences recognized by the AAV Rep protein⁶ (Fig. 1A) and a 468 bp amplicon spanning the cleavage site for the other AAVS1-specific nucleases/nickases, including the ZFNSh nuclease, the Cas9 nuclease, and the Cas9 D10A nickase (Fig. 1A), were prepared and analyzed using the Surveyor assay and by deep sequencing. The Surveyor results presented in Fig. 1B show that indel formation was not apparent with genomic DNA from HEK 293 cells expressing either the Rep 78 WT (lane 3) or the Rep 78-III protein (lane 4), under conditions where the ZFNSh and Cas9 endonucleases (lanes 2 and 5, respectively) produced the expected cleavage products. A bar graph of the indel frequencies from three indepen-

dent sets of Surveyor results is presented in Supplementary Fig. S1A (Supplementary Data are available online at www.liebertpub.com/hgtb). The frequency of cleavage events was highest with the ZFNSh and Cas9 samples, but low levels of apparent cleavage events were also detected in the CasD10A sample.

Amplicon sequencing detected contigs with the expected indels for the ZFNSh and Cas9 treated samples. The analysis with CRISPResso²⁸ of the results involving two independent sets of deep sequencing data are presented in Supplementary Fig. S1B. Overall, the results were in agreement with the Surveyor assay data shown in Supplementary Fig. S1A, except that the frequency of indels observed by amplicon sequencing was lower for the ZFNSh sample compared to that of the Cas9 sample, possibly because the HEK 293 genomic DNAs used for the Surveyor assay were different from those used for deep sequencing. It is also apparent from Supplementary Fig. S1B that the Cas9D10A sample did not reveal any indels by deep sequencing, indicating that the apparent cleavage events seen using the Surveyor analysis (Supplementary Fig. S1A) are not real.

Directed insertion of transgene sequences at the AAVS1 locus mediated by the AAV2 Rep 78 protein

Recombinant plasmid constructs bearing the p5IEE element and homology arms corresponding to the AAVS1 locus in concert with the AAV2 Rep 78 and Rep 68 proteins were shown before to trigger HDR-directed gene targeting events in HeLa cells.^{14,15} Next, the specificity of this approach was investigated, and it was compared to an HDR approach involving other nucleases that are specific for the AAVS1 locus. To do this, a plasmid-based donor construct bearing homology arms corresponding to the AAVS1 locus was used in concert with recombinant plasmids encoding the Rep 78-III protein, or the left and right domains of the AAVS1-specific ZFNSh, or the Cas9 nuclease. The donor plasmid, referred to as pAAVS1-p5IEE-SA-2A-puro-pA, bears a 148 bp p5IEE sequence element derived from the AAV2 p5 promoter,¹⁰ a SA sequence, and a puromycin resistance gene and a pA sequence, flanked by homology arms corresponding to the AAVS1 locus⁸ (Fig. 2A, middle part). To investigate HDR events, HEK 293 cells were co-transfected with the pAAVS1-p5IEE-SA-2A-puro-pA donor plasmid, and a plasmid(s) encoding a nuclease/nickase, including the Rep 78-III nickase (Fig. 2B, top left part), the AAVS1-specific ZFNSh (consisting of the ZFNSh-L and ZFNSh-R subunits;

Figure 1. Analysis of indel formation. **(A)** Outline of the trs/Rep binding site (RBS) sequences and the sequences recognized by the AAVS1-specific ZFNSh⁸ and the T2 single-guide RNA (sgRNA).²⁰ The **black rectangles** refer to exons 1, 2, and 3 of the *PPP1R12C* gene. The trs and RBS sequences and the ZFNSh and T2 sgRNA target sequences are shown in bold. **(B)** Surveyor analysis. Sequences spanning the Rep 78 trs/RBS site 12 reference 12 and the AAVS1 ZFNSh cleavage site⁸ were polymerase chain reaction (PCR) amplified using genomic DNA from HEK 293 cells transfected using plasmid constructs encoding the nucleases and nickases listed. To amplify the sequence spanning the trs/RBS sequence, the TRS-RBS B-Fwd and TRS-RBS B-Rev primers were used (*horizontal arrows*). The CEL-1 primer set (*horizontal dashed arrows*)⁸ was used to amplify the sequence spanning the AAVS1 ZFNSh and Cas9 cleavage sites and the Cas9D10A nickase site. The PCR products shown in *lanes 1, 2, 5, and 7* were generated using the CEL-1 primers set, and the products shown for *lanes 3, 4, and 6* using the TRS-RBS B primer set. *Lanes 1 and 6:* Surveyor analysis of PCR products generated using mock-transfected HEK 293 cell DNA; *Lane 2:* Surveyor analysis of PCR products using genomic DNA from HEK 293 DNA co-transfected using the ZFNSh-L plus ZFNSh-R nuclease encoding plasmids; *Lanes 3 and 4:* Surveyor analysis of PCR products using genomic DNA from HEK 293 DNA transfected using the Rep 78 WT or the Rep 78-III-encoding plasmids, respectively; *Lanes 5 and 7:* Surveyor analysis of PCR products using genomic DNA from HEK 293 DNA transfected using the Cas9 and Cas9D10A-encoding plasmids, respectively. Samples were analyzed using a 10% TBE/polyacrylamide gel. M: 100 bp DNA ladder (New England Biolabs, Ipswich, MA).

Fig. 2B, middle left part), or an AAVS1-directed Cas9 nuclease²⁰ (Fig. 2B bottom left part). Control transfections included the donor plasmid alone. Transfected cells were subjected to puromycin selection for a total of 15 days, starting 3 days after initiating the transfection. Single-cell clones were randomly picked, the cells were expanded, and genomic DNA was prepared and characterized by PCR using the AAVS1-F and Puro-R primer set (Fig. 2A, bottom part). Three independent clones each were analyzed. The genomic DNA from one of the clones obtained using the donor plasmid alone served as a negative control.

The PCR products from HEK 293 cell clones obtained using the pAAVS1-p5IEE-SA-2A-puro-pA donor plasmid and either the Rep 78-III plasmid, the ZFNSh-L plus ZFNSh-R plasmids, or the Cas9 and sgRNA T2-encoding plasmids are displayed in Fig. 2B (top-right, middle-right, and bottom-right panels, respectively). A PCR product of the expected size (1,190 bp) was observed, with all the genomic DNAs from cells transfected with the donor plasmid plus a nuclease/nickase plasmid. Cells transfected with the donor plasmid alone did not reveal a specific PCR product, indicating that the presence of the nuclease promoted the site-specific

Figure 2. Insertion of transgene sequences at the AAVS1 site in HEK 293 cells mediated by the Rep 78-III protein or using a zinc finger nuclease (ZFNSh) or the CRISPR/Cas 9 components. **(A)** Outline of transgene insertion approach. *Top part:* Schematic of the AAVS1 locus. *Middle part:* Schematic of plasmid donor construct containing the left and right homology arms, a puromycin resistance gene encoding puromycin N-acetyltransferase, a splice acceptor (SA) sequence, the p5IEE element, and a polyadenylation (pA) signal. *Bottom part:* Schematic of p5IEE-SA-puromycin-pA sequence inserted within the first intron of the *PPP1R12C* gene. The AAVS1-F and Puro-R primers used are indicated by horizontal arrows. **(B)** The nuclease-encoding plasmids used are shown on the left. The right panels show the PCR products obtained using genomic DNA from HEK 293 cells transfected using the donor plasmid plus the nuclease plasmids listed. M: GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific, Carlsbad, CA). The arrows refer to the major PCR products observed.

insertion of donor sequences at the predicted genomic site.

Design and testing of lentiviral vectors encoding AAV2 Rep 78 proteins

The next goal was to construct lentiviral vectors bearing the Rep 78-III coding region and to test the impact on vector titers. To do this, the pCDH-CMV-MCS-EF1-copGFP-based lentiviral vector containing the constitutive CMV-IE promoter was used (Fig. 3A). VSV-G-pseudotyped lentiviral vectors were prepared and titrated, as described.^{24,25} The results presented in Fig. 3B show that the titers of lentiviral vectors encoding the Rep 78 WT and the Rep 78-III proteins were in the order of 2×10^5 TU/mL determined by flow cytometry, while the titers of a control vector (MCS) lacking a transgene sequence were in the order of 6×10^6 TU/mL (Fig. 3B), indicating that the presence of the Rep 78

WT or Rep 78-III transgene sequences and/or proteins reduced vector titers.

To determine whether a lack of Rep 78-III expression during vector packaging would increase titers, the doxycycline (Dox) inducible SLIK-Venus lentiviral vector²² system was used (Fig. 3C). Figure 3D shows that the titers were higher (up to 1.5×10^6 TU/mL) in the absence of Dox (–Dox), and only slightly lower (up to 1×10^6 TU/mL) in the presence of Dox (+Dox). This indicates that the vector platform used impacted vector titers and that the presence of the Rep 78 protein during vector packaging reduced vector titers only slightly. The Western blot analysis presented in Fig. 3E shows that the expected Rep 78-III protein was produced in the presence but not in the absence of Dox.

Taken together, these results show that the presence of the wild type or the engineered Rep 78-III protein during lentiviral vector production

Figure 3. Production of lentiviral vectors encoding the AAV2 Rep protein. **(A)** Expression constructs. The Rep 78 WT and the Rep 78-III proteins were expressed from the pCDH-CMV-MCS-EF1-copGFP lentiviral vector bearing the constitutive CMV-IE promoter. MCS refers to a control vector lacking a Rep 78 sequence. **(B)** Titers observed for the various vectors in transduced HEK 293 cells. **(C)** Rep 78-III expression using the Dox-inducible SLIK-Venus vector.²² **(D)** Vector titers in HEK 293 cells observed using the Dox-inducible vector. Dox (1 μ g/ml) was either absent during vector production (-Dox), or present (+Dox). Vector titers were determined by flow cytometry 3 days post transduction. **(E)** Western blot analysis. Extracts were derived from HeLa cells transduced using the SLIK-Venus-based Rep 78-III vector. HeLa cells were transduced at a MOI of 2 and treated with or without Dox (1 μ g/ml) for 72 h. Bars represent means $\pm$ standard deviation (SD) of three independent experiments.

decreased vector titers in the context of the CDH-CMV-MCS-EF1-copGFP vector platform and that the titers involving the Dox-inducible SLIK-Venus vector were higher.

Directed insertion of transgene sequences mediated by IDLV at the AAVS1 locus in HEK 293 cells and in human iPSCs

Next, the specificity and efficiency of the Rep78-III-based integration system were tested in the context of HEK 293 cells and human iPSCs transduced with IDLVs. To do this, a series of IDLVs encoding the AAV2 Rep 78-III protein bearing the CMV-IE or CAG promoters was constructed.^{21,35} These vectors are referred to as NL(CMV)Rep78-III/CMV/WPRE Δ U3 and NL(CMV)Rep78-III/CAG/WPRE Δ U3, respectively (Fig. 4A, top part). In addition, two separate IDLVs encoding the left (ZFNSH-L) and right (ZFNSH-R) subunits of the

ZFNSH nuclease were constructed (referred to as NL(CMV)AAVS1 ZFNSH-L/CMV/WPRE Δ U3 and NL(CMV)AAVS1 ZFNSH-R/CMV/WPRE Δ U3, respectively; Fig. 4A, middle part). An IDLV donor vector referred to as NL8/AAVS1-2A-SA-P5IEE-Puro Δ U3 was also designed (Fig. 4A, bottom part).

To compare the gene targeting efficiency at the AAVS1 locus mediated by the Rep78-III protein with that of the AAVS1-specific ZFNSH, 10⁵ of HEK 293 cells were co-transduced using the Rep 78-III-encoding NL(CMV)Rep78-III/CMV/WPRE Δ U3 IDLV vector at a MOI of 10 plus the IDLV donor vector NL8/AAVS1-2A-SA-P5IEE-Puro Δ U3 at MOIs ranging from 1 to 10 (black bars). Alternatively, the donor vector alone (gray bars) was used (Fig. 4B). The results presented in Fig. 4B show that the frequency of puromycin-resistant colonies was increased at higher MOIs of the donor vector (up to 360 colonies per 10⁵

Figure 4. Delivery of transgene sequences to the AAVS1 site in HEK 293 cells and in human induced pluripotent stem cells (iPSCs) mediated by the Rep 78-III protein or by a zinc finger nuclease (ZFNSh) encoded by integrase defective lentiviral vectors. **(A)** *Top and middle parts:* Outline of the NL(CMV)Rep78-III/CMV/WPREΔU3, the NL(CMV)Rep78-III/CAG/WPREΔU3, the NL(CMV)ZFNSh-L/CMV/WPREΔU3, and NL(CMV)ZFNSh-R/CMV/WPREΔU3 IDLV vector constructs. *Bottom part:* Schematic of the NL8/AAVS1-SA-2A-pSIEE-PuroΔU3 IDLV donor vector. **(B)** Number of puromycin-resistant colonies obtained using HEK 293 cells transduced with the NL8/AAVS1-SA-2A-pSIEE-PuroΔU3 IDLV donor vector alone (gray bars) or the donor vector plus the Rep 78-III-encoding NL(CMV)Rep78-III/CMV/WPREΔU3 IDLV vector at MOIs of 1, 5, and 10, respectively (black bars). Puromycin-resistant cell clones obtained were stained with crystal violet and counted. The results shown represent means $\pm$ SD obtained from three different experiments. **(C)** PCR analysis of insertion events at the AAVS1 locus. The PCR analysis of the puromycin-resistant colonies obtained after transduction of HEK 293 cells using the IDLV donor vector alone (referred to as Donor alone), the IDLV donor vector plus the ZFNSh-encoding IDLV vectors (referred to as Donor plus ZFNSh), and the IDLV donor vector plus the Rep 78-III-encoding IDLV vector (referred to as Donor plus Rep 78-III) are shown in the *top-left*, *top-right*, and *bottom-left* panels, respectively. The *upper panels* refer to the PCRs involving the AAVS1-F/Puro-R primer set, and the *lower panel* to the P5/P6 primer set that is specific for the puromycin resistance gene. The (+) control refers to genomic DNA from transduced HEK 293 cells bearing a donor cassette at the AAVS1 locus. **(D)** Targeted integration mediated by Rep78-III in human iPSCs. ND2.0 human iPSCs were transduced with the IDLV NL8/AAVS1-SA-2A-P5IEE-PuroΔU3 donor vector with or without the Rep 78-III-encoding NL-Rep78-III/CAG/WPREΔU3 IDLV. Puromycin-resistant cell clones were randomly picked for characterization by PCR using primers AAVS1-F and Puro-R. *Upper panel:* PCR product amplified with primer set AAVS1-F and Puro-R. *Lower panel:* PCR amplified with primers P5 and P6.

cells used for transduction). Also, the number of colonies obtained using the donor vector plus the Rep 78-III-encoding IDLV vector was up to 10-fold higher compared to the number of colonies obtained using the donor IDLV alone (Fig. 4B).

A number of puromycin-resistant HEK 293 cell colonies were characterized by PCR using the AAVS1-F and Puro-R primer set (see Fig. 2A). For cells transduced with the donor IDLV vector in concert with the ZFNSh-encoding IDLVs, 100% of

the colonies tested revealed a PCR fragment consistent with targeted insertion of the donor cassette at the AAVS1 locus (Fig. 4C, top-right part). For cells transduced with the donor IDLV vector in concert with the Rep78-III-encoding IDLV, 84% of the colonies tested revealed a PCR fragment consistent with targeted insertion of the donor cassette at the AAVS1 locus (Fig. 4C, bottom-left part). None of the clones from cells transduced with the donor IDLV donor vector alone revealed a specific PCR product using the AAVS1-F/Puro-R primer set (Fig. 4C, top-left part). All the clones tested, including clones that did not reveal a PCR product using the AAVS1-F/Puro-R primer set, revealed a PCR fragment using the P5 and P6 primers that are specific for puromycin resistance gene sequences.

Overall, the results obtained also indicate that the targeted integration efficiency mediated by Rep 78-III in HEK 293 cells is comparable to that obtained using the AAVS1-specific ZFNSh but that the specificity observed using the Rep 78-III approach was not as high.

To assess the usefulness of the Rep 78-III-mediated targeted transgene integration approach in human iPSCs, human iPSCs were co-transduced with an IDLV donor vector plus an IDLV encoding Rep 78-III under the control of the hybrid CAG promoter³⁵ at a MOI of 100 each. Puromycin-resistant human iPSC clones were picked and characterized by PCR³⁶ using the AAVS1-F/Puro-R primer set. Of the 15 puromycin-resistant human iPSC clones obtained, two revealed transgene sequences that were specifically targeted to the AAVS1 locus (Fig. 4D, top-right panel). In contrast, none of the clones analyzed from cells treated with the donor vector alone revealed a specific PCR fragment using this particular primer set (Fig. 4D, top-left panel). Also, all the clones tested using the P5/P6 primer set were positive for puromycin resistance gene sequences (Fig. 4D, bottom panels). These results show that the Rep 78-III approach can be used to engineer human iPSCs, although the efficiency and specificity were lower compared to those observed with HEK 293 cells.

The HEK 293 and human iPSC cell clones that were PCR-positive using the P5/P6 primers but negative using AAVS1-F/Puro-R primer set likely represent off-targeted integration events. Such off-targeted vector integration events may have been caused by illegitimate integration^{37,38} and/or the p5 promoter sequence present in the p5IEE element,¹⁰ and/or the capacity of the p5IEE element to stimulate gene editing events.³⁹

CONCLUDING REMARKS

This study investigated an alternative approach for targeted integration of transgene sequences using the Rep 78-III nickase. The results obtained using the Surveyor assay and deep sequencing are consistent with earlier findings that Rep 78 WT does not lead to a double-strand break. The same may not apply to other engineered site-specific nickases. For example, recent results reported by Lei *et al.*⁴⁰ revealed that APOBEC3-catalyzed deamination of genomic single-stranded DNA formed during the repair of Cas9 nickase-generated single-strand breaks in human cells were further processed to yield indels.

In this study, the efficiency and specificity of transgene insertion involving both the plasmid approach (Fig. 2) and the IDLV approach (Fig. 4) were assessed based on the formation of puromycin-resistant cell clones and the analysis by PCR of genomic DNA from such cell clones. It remains to be determined whether approaches that are independent of a functional transgene sequence, such as high-throughput sequencing methods, will lead to the same outcomes.

The production of adenoviral vectors encoding the Rep 78/68 polypeptides has been challenging due to low titers.¹⁸ However, adenoviral vectors encoding the reengineered Rep 78-III protein involving synonymous codon pair recoding^{18,34} revealed titers comparable to those obtained with adenoviral vectors lacking the Rep 78 transgene sequence. The results presented in Fig. 3B show that the titers of lentiviral vectors expressing Rep 78 proteins from the constitutive CMV promoter were lower compared to the titer of the control vector lacking a Rep 78 sequence, independent of whether the Rep 78 WT protein or the reengineered Rep 78-III protein were used. For the Dox inducible SLIK-Venus lentiviral vector, titers were slightly reduced in the presence of Dox (Fig. 3D). This indicates that the presence of the Rep 78-III protein may have inhibited vector production, similar to the inhibitory effects of Rep 78 on HIV-1 production observed before.⁴¹

In conclusion, the results indicate that the Rep 78-III nickase can successfully mediate targeted insertion of transgene sequences using IDLV donor vectors in HEK 293 cells with an efficiency similar to that observed using the AAVS1-specific ZFNSh. The study further demonstrated that Rep 78-III can also be used for genome editing in human iPSCs. However, the specificity of transgene insertion involving Rep 78-III in human iPSCs was lower compared to HEK 293 cells, possibly due to toxicity effects exerted by Rep 78⁴² and/or the higher vector MOIs used for human iPSCs.

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AUTHOR DISCLOSURE

No competing financial interests exist.

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