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**Adenoviral vector delivery of RNA-guided
CRISPR/Cas9 nuclease complexes induces
targeted mutagenesis in a diverse array of
human cells**



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ABSTRACT

CRISPR/Cas9-derived RNA-guided nucleases (RGNs) are DNA targeting systems, which are rapidly being harnessed for gene regulation and gene editing purposes in model organisms and cell lines. As *bona fide* gene delivery vehicles, viral vectors may be particularly fit to broaden the applicability of RGNs to other cell types including dividing and quiescent primary cells. Here, the suitability of adenoviral vectors (AdVs) for delivering RGN components into various cell types is investigated. We demonstrate that AdVs namely, second-generation fiber-modified AdVs encoding Cas9 or single guide RNA (gRNA) molecules addressing the Cas9 nuclease to the *AAVS1* “safe harbor” locus or to a recombinant model allele can be produced to high-titers (up to 20×10^{10} transducing units/ml). Importantly, AdV-mediated transduction of gRNA:Cas9 ribonucleoprotein complexes into transformed and non-transformed cells yields rates of targeted mutagenesis similar to or approaching those achieved by isogenic AdVs encoding TALENs targeting the same *AAVS1* chromosomal region. RGN-induced gene disruption frequencies ranged from 18% to 31% in a cell type-dependent manner. We conclude that AdVs constitute a valuable platform for introducing RGNs into human somatic cells regardless of their transformation status. This approach should aid investigating the potential and limitations of RGNs in numerous experimental settings.

Key words: CRISPR/Cas9; RNA-guided nucleases; Adenoviral vectors; Primary cells; Targeted gene knockout; Genome editing

INTRODUCTION

Genome engineering strategies based on designer nucleases can be harnessed to edit and to interrogate the function of genomic sequences in cells of higher eukaryotes. In common, these approaches entail the activation of cellular DNA repair pathways in target cells upon the induction of double-stranded DNA breaks (DSBs) at predefined chromosomal positions^{1,2}. The engagement of non-homologous end-joining (NHEJ) and homologous recombination (HR) machineries at these artificially created DSBs can result in targeted gene knockout and addition, respectively. The potential of genome engineering is considerable for fundamental and applied research. For instance, in the form of new molecular medicine modalities such “genome surgery” approaches might constitute a watershed departure from canonical gene therapy in which chromosomal insertion of the therapeutic DNA is unpredictable³.

Programmable nuclease technologies are developing at a rapid pace^{1,2}. These include zinc-finger nucleases (ZFNs), engineered homing endonucleases, transcription activator-like effector nucleases (TALENs) and, more recently, RNA-guided nucleases (RGNs). RGNs are RNA-dependent DNA nucleases built on components from type II clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) systems. These systems have been evolving in bacteria and archaea as adaptive immune mechanisms against invading nucleic acids of foreign agents such as plasmids and phages^{4,5}. CRISPR loci contain an array of short palindromic repeats separated by protospacer DNA snippets of around 20 bp that have been acquired from invading agents. Following expression, the short palindromic repeats in precursor CRISPR transcripts (pre-crRNA) hybridize to a portion of a trans-activating CRISPR RNA (tracrRNA). The bound tracrRNA, in concert with RNase III and Cas9, lead to the processing of the pre-crRNA into small CRISPR RNAs (crRNAs) each of which encompasses a sequence complementary to a specific protospacer. Finally, crRNAs base-paired to tracrRNAs loaded with Cas9 nucleases direct site-specific DNA cleavage following crRNA:target DNA hybridization. Of note, the fact that foreign DNA harbors a so-called protospacer adjacent motif (PAM) next to the protospacer sequence whereas crRNAs lack this motif, ensures that crRNA:tracrRNA:Cas9 complexes recognize exclusively the incoming exogenous DNA as opposed to the chromosomal CRISPR-incorporated protospacers^{4,5}.

The above-described fundamental insights combined with enticing work done *in vitro* and in bacteria^{6,7} led the type II CRISPR/Cas9 system from *Streptococcus pyogenes* to be rapidly adapted as a gene editing toolbox for mammalian cells⁸⁻¹⁰. These studies comprised the transfection of cell lines with plasmids expressing from RNA polymerase II (Pol-II) and Pol-III promoters, respectively, Cas9 proteins and chimeric single guide RNAs (gRNAs) engineered by fusing sequence-tailored crRNAs to tracrRNA scaffolds. Thus, gRNAs are bifunctional molecules⁷ consisting of crRNA targeting and tracrRNA scaffolding moieties. Owing to the fact that RGN target site specificity is governed by RNA-DNA hybridizations, as opposed

to protein-DNA interactions, make CRISPR/Cas-based systems unique among customizable nucleases. Importantly, this feature allows for facile nuclease retargeting and multiplexing by simply replacing the RNA component of each ribonucleoprotein complex^{1,11}. However, independent studies carried out in cell lines indicate that RGNs display high levels of off-target DNA cleaving activities especially when compared to protein-based platforms such as TALENs with a tradeoff existing between activity and specificity¹²⁻¹⁶. The off-target cleaving profile of programmable nucleases is, clearly, a parameter to take into consideration in the context of both therapeutic and basic-research protocols.

To expand the range of experimental systems in which RGNs can be tested and optimized, it is crucial to introduce these rather large macromolecular complexes not only into established cell lines but also into non-transformed diploid cell types (i.e. Cas9: 1,368 aa; 158 kDa; *Cas9* ORF: 4.1 kb). In this regard, adenoviral vectors (AdVs) constitute interesting RGN delivery candidates especially owing to their episomal nature, large cloning capacity, high-titers and ability to transduce dividing as well as non-dividing cells¹⁷.

In the present work, we sought to investigate the capacity of AdVs for packaging and delivering functional *gRNA* and human codon-optimized *Cas9* transcriptional units into human cells. To this end, we selected previously validated *gRNA* molecules whose sequences target Cas9 to the so-called *AAVS1* “safe harbor” locus located on the long arm of the human chromosome 19 (19q13.42-qter) or to a recombinant model allele consisting of a chromosomally integrated *eGFP* expression unit¹⁰. In parallel, to serve as a reference, we took along in these transduction experiments isogenic AdVs encoding TALENs, which have also been previously validated and target the same *AAVS1* region¹⁸. By deploying targeted mutagenesis assays as well as live-cell readouts based on fluorescence microscopy and flow cytometry, we demonstrate that AdVs can efficiently transfer functional RGN components into human cells regardless of their transformation status.

RESULTS

Adenoviral vectors encoding RGN components can be produced at high-titers

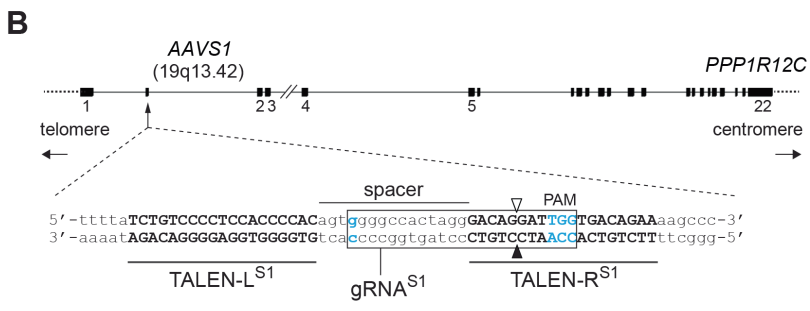
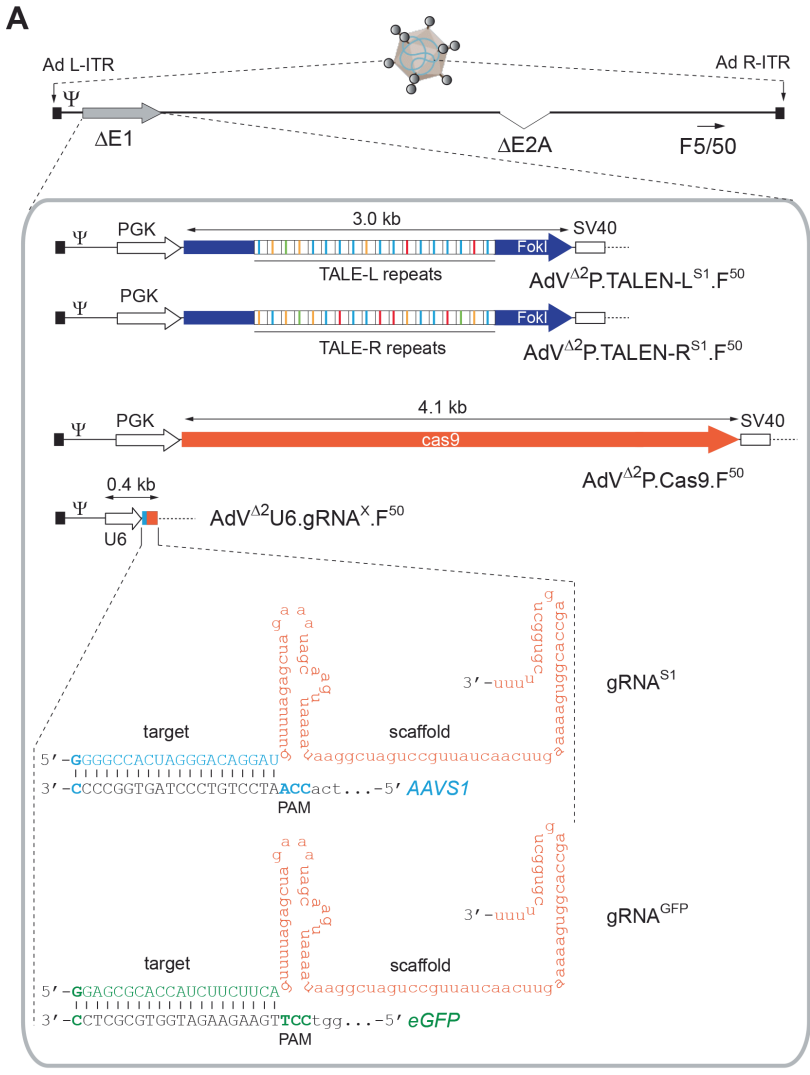
We started by investigating the feasibility of producing AdVs containing *Cas9* and *gRNA* transcriptional units. For this purpose, we selected *E1*- and *E2A*-deleted AdVs displaying fiber motifs recognizing the ubiquitous cellular receptor CD46 (Fig. 1A). This retargeted second-generation AdV system offers several advantages when compared to its conventional *E1*-deleted AdV counterpart including higher genetic capacity, lower levels of “leaky” viral gene expression and efficient transduction of cells lacking, or with low amounts of, the coxsackievirus-adenovirus receptor CAR on their surface. Among these are human cell types often utilized in experimental and applied research settings such as hematopoietic cells,

skeletal muscle progenitor cells (hereinafter referred to as myoblasts), and bone marrow-derived mesenchymal stromal cells (hMSCs)¹⁹⁻²².

In the current work, we selected the previously validated gRNA_AAVS1-T2 unit¹⁰, herein named gRNA^{S1}, to edit endogenous chromosomal sequences via AdV delivery of CRISPR/Cas9-derived RGNs. The nucleotide sequence of this gRNA targets the Cas9 nuclease to the human *AAVS1* locus (Fig. 1B). To serve as a reference, we took along the optimized designer nuclease pair TALEN-L^{S1}/TALEN-R^{S1} specific for the same *AAVS1* region¹⁸ (Fig. 1B) and placed their ORFs under the control of the same transcriptional elements as those regulating *Cas9* expression. To this end, AdV shuttle plasmids expressing *Cas9*, *TALEN-L^{S1}* or *TALEN-R^{S1}* from the Pol-II *PGK* promoter and short gRNA^{S1} molecules from the Pol-III *U6* promoter were constructed. The choice for the former promoter was motivated by its ubiquitous expression in a diverse array of human cell types and, owing to its human origin, higher resistance to silencing phenomena than that offered by commonly-used viral transcriptional elements.

Transient transfection experiments using as readout a surrogate DSB formation assay dependent on the NHEJ DNA repair pathway, showed that co-transfecting test cells with gRNA^{S1}- and Cas9-encoding AdV shuttle plasmids resulted in *AAVS1* targeted mutagenesis (Fig. 2). The functionality of the AdV shuttle plasmids encoding the *AAVS1*-specific TALENs was also confirmed through similar transient transfection experiments (not shown). Next, we used these validated reagents to assemble full-length AdV molecular clones for rescuing and propagating the *E1*- and *E2A*-deleted (AdV^{Δ2}) fiber-modified (F⁵⁰) vectors AdV^{Δ2}P.Cas9.F⁵⁰, AdV^{Δ2}U6.gRNA^{S1}.F⁵⁰, AdV^{Δ2}P.TALEN-L^{S1}.F⁵⁰ and AdV^{Δ2}P.TALEN-R^{S1}.F⁵⁰. Following the rescue, propagation and purification of AdV particles, we determined that the AdV functional titers in the resulting preparations were within the typical range expected for these research-scale production rounds (i.e. 23.7×10^{10} , 13.3×10^{10} , 2.4×10^{10} and 4.2×10^{10} TU/ml for AdV^{Δ2}P.Cas9.F⁵⁰, AdV^{Δ2}U6.gRNA^{S1}.F⁵⁰, AdV^{Δ2}P.TALEN-L^{S1}.F⁵⁰ and AdV^{Δ2}P.TALEN-R^{S1}.F⁵⁰, respectively). In addition, as previously shown for AdVs harboring *TALEN* expression units¹⁸, structural analysis of packaged AdV genomes isolated from purified particles of AdV^{Δ2}P.Cas9.F⁵⁰ and AdV^{Δ2}U6.gRNA^{S1}.F⁵⁰ revealed restriction patterns diagnostic for AdV backbones containing intact transgenes (Fig. 3).

Figure 1. Schematic representation of the genome-modifying AdVs deployed in this study and corresponding target sequences. (A) The AdVs encode the Cas9 nuclease or TALEN proteins specific for the human “safe harbor” locus *AAVS1* within *PPP1R12C* or gRNA molecules addressing Cas9 to *AAVS1* or to *eGFP* sequences. All vectors were assembled on the basis of a second-generation (i.e. *E1*- plus *E2A*-deleted) AdV backbone (AdV^{Δ2}) coding for chimeric fibers composed of basal domains from prototypic human adenovirus serotype 5 fused to the apical shaft and knob motifs from human adenovirus serotype 50 (F5/50). The *Cas9* and *TALEN* ORFs are under the transcriptional control of the human *PGK-1* gene promoter (PGK) and the simian virus 40 (SV40) polyadenylation signal whilst the *gRNAs* are regulated by a RNA Pol-III promoter (*U6*) and terminator sequences. Ψ, human adenovirus serotype 5 packaging signal; Ad L-ITR and Ad R-ITR, “left” and “right” adenoviral inverted terminal repeat, respectively. The structure of the single guide RNA molecules gRNA^{S1} and gRNA^{GFP}, composed of Cas9 targeting and scaffolding portions, are depicted base-paired to their respective *AAVS1* and *eGFP* target sequences. TALE-L and TALE-R, *AAVS1*-binding transcription activator-like effector repeats of designer nucleases TALEN-L and TALEN-R, respectively (TALE portions coding for repeat variable di-residues [RVDs] recognizing A, T, C and G are color-coded); FokI, nuclease domain of the type IIS restriction enzyme FokI; PAM, protospacer adjacent motif. **(B)** Location of the *AAVS1* target sites for the RNA- and TALE-guided nuclease complexes gRNA^{S1}:Cas9 and TALEN-L^{S1}:TALEN-R^{S1}, respectively, in relation to the whole *PPP1R12C* gene. The target sequences for the TALEN-L^{S1} and TALEN-R^{S1} proteins are in upper case, whereas that for the gRNA^{S1} is boxed (i.e. G[N]₁₉NGG). PAM, protospacer adjacent motif. Solid and open vertical arrowheads indicate the positions at which DNA nicking takes place catalyzed by the two nuclease domains of Cas9. ►



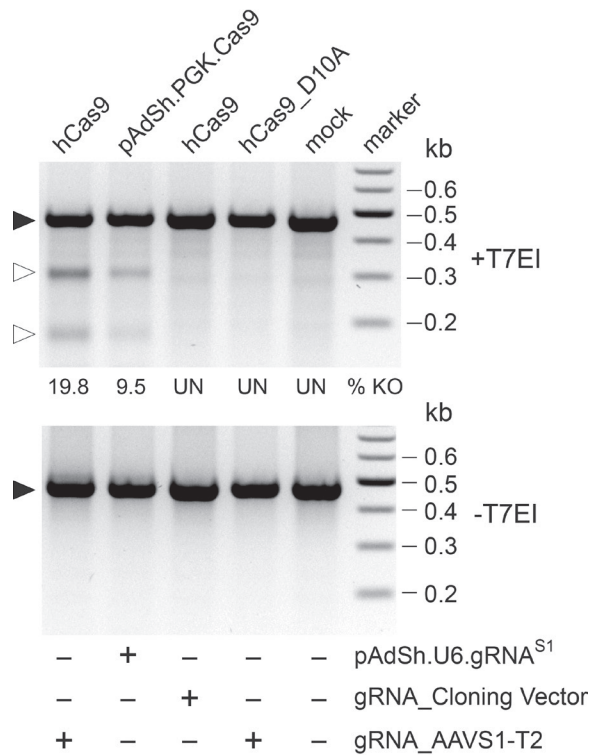


Figure 2. Transient transfection experiments for validating AdV shuttle plasmids pAdSh.PGK.Cas9 and pAdSh.U6.gRNA^{S1}. AAVS1-specific PCR products amplified from E1-transformed PER.C6 cells co-transfected with hCas9 (9.6 kb) and gRNA_AAVS1-T2 (4 kb), pAdSh.PGK.Cas9 (11.6 kb) and pAdSh.U6.gRNA^{S1} (7.3 kb), hCas9 and “empty gRNA” construct gRNA_Cloning Vector (3.9 kb), hCas9_D10A (9.6 kb) and gRNA_AAVS1-T2 or mock-transfected. Marker, Gene Ruler DNA Ladder (Fermentas). Plasmids hCas9 and hCas9_D10A express Cas9 nucleases which induce a DSB and a nick at AAVS1, respectively. After amplicon denaturation and reannealing, the presence of mismatches derived from NHEJ-mediated repair of site-specific DSBs *in cellula* was probed by T7 endonuclease I (T7EI) digestions (upper panel). Negative controls were provided by amplicons not exposed to T7EI (-T7EI) as well as T7EI-treated amplicons (+T7EI) corresponding to mock-transfected cells, to cells co-transfected with hCas9 and gRNA_Cloning Vector and to cells co-transfected with hCas9_D10A and gRNA_AAVS1-T2 encoding the “nickase” mutant version of Cas9 (i.e. Cas9^{D10A}) and gRNA^{S1}, respectively. Solid and open arrowheads indicate the positions of, respectively, undigested and T7EI-digested DNA fragments whose sizes are consistent with DSB formation at the AAVS1 target site. % KO and UN, knockout frequency and undetected, respectively.

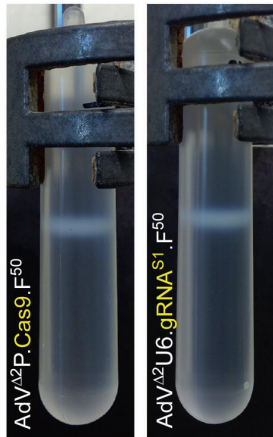
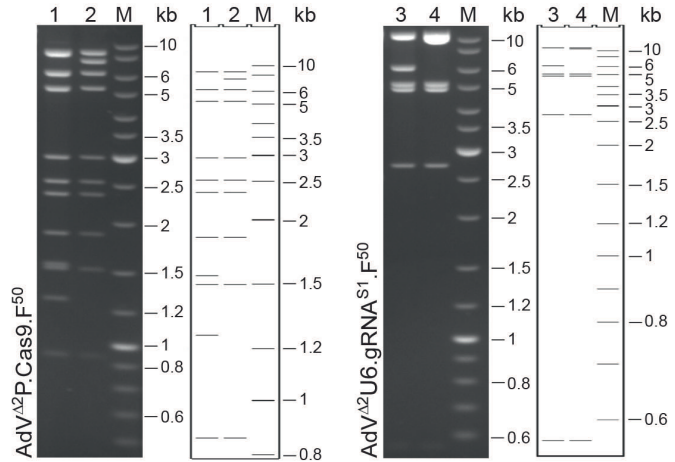
A**B**

Figure 3. Structural analysis of AdV genomes. (A) Genome-containing AdV particles after ultracentrifugation banding through continuous CsCl density gradients **(B)** Characterization by restriction fragment length analysis of DNA isolated from CsCl-purified AdV particles. Left panels, in-gel and *in silico* restriction patterns of AdV^{Δ2P}.Cas9.F⁵⁰ genomes (lanes 1) and respective pAdV^{Δ2P}PGK.Cas9.F⁵⁰ parental plasmid (lanes 2) treated with BglII. Right panel, in-gel and *in silico* restriction patterns of AdV^{Δ2U6}.gRNA^{S1}.F⁵⁰ DNA (lanes 3) and corresponding pAdV^{Δ2U6}.sgRNA^{S1}.F⁵⁰ parental plasmid (lanes 4) digested with Bsp1407I. Lanes M, GeneRuler DNA Ladder marker.

5

AdVs deliver functional RGN complexes into human cells inducing robust site-specific mutagenesis at native and recombinant target chromosomal loci

Subsequently, we sought to test via transduction experiments followed by gene knockout assays, the functionality of the gRNA^{S1}- and Cas9-encoding AdVs in a panel of human cell types. These target cells comprised cervix carcinoma HeLa cells, osteosarcoma U2OS cells, hMSCs and myoblasts. In addition, we took advantage of the fine-tuned adenoviral infection mechanism to introduce into target cell nuclei a range of well-defined dosages of DNA templates encoding each RGN component. Related to this, it is noteworthy mentioning that, as of yet, bipartite CRISPR/Cas nuclease complexes have been introduced into target cells by transfection-based protocols mostly deploying fixed amounts of the two RGN components. In this regard it is relevant pointing out that in contrast to ZFNs and TALENs, in which the deployment of equimolar amounts of similarly sized monomers directly follows from their *modus operandi* (i.e assembly of catalytically active dimers at the target site), RGN activities might improve by optimizing total and relative quantities of *gRNA* and *Cas* templates. Therefore, for the experiments deploying AdV^{Δ2P}.Cas9.F⁵⁰ and AdV^{Δ2U6}.gRNA^{S1}.F⁵⁰ we set-up “co-transduction grids” to determine gene disruption levels resulting from different combined dosages as well as ratios of the two RGN elements. Transduction experiments in the HeLa and U2OS cell lines led to dose-dependent AAVS1 disruption following AdV-mediated gene transfer as revealed by the detection of NHEJ-derived small insertions/deletions (indels) at

the target site (Fig. 4, Fig. 5A and Fig. 5B). Importantly, control transduction experiments in which only one of the two vectors was used did not result in detectable indel formation (Fig. 4). Moreover, co-transduction experiments in U2OS cells deploying side-by-side vector pairs $AdV^{\Delta 2}P.Cas9.F^{50}/AdV^{\Delta 2}U6.gRNA^{S1}.F^{50}$ and $AdV^{\Delta 2}P.TALEN-L^{S1}.F^{50}/AdV^{\Delta 2}P.TALEN-R^{S1}.F^{50}$ showed that, in addition to a clear AdV dose dependency, the gene knockout frequencies achieved by $gRNA^{S1}:Cas9$ and $TALEN-L^{S1}:TALEN-R^{S1}$ complexes were similar (compare Fig. 5B with the lower panel in Fig. 5C).

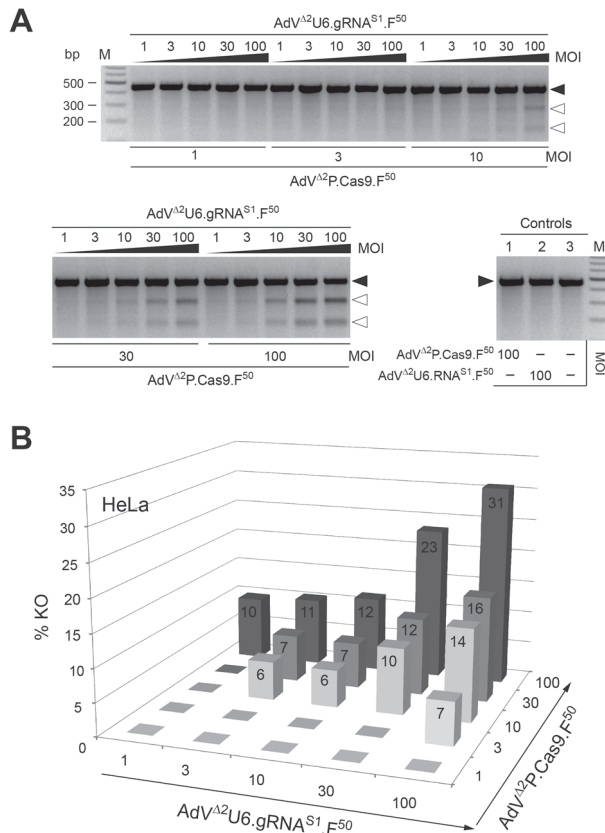


Figure 4. Transduction experiments in HeLa cells for the functional validation of $AdV^{\Delta 2}P.Cas9.F^{50}$ and $AdV^{\Delta 2}U6.gRNA^{S1}.F^{50}$. (A) Genotyping assays based on the detection of indels generated by NHEJ-mediated DSB repair at the chromosomal target site. The *AAVS1*-specific amplicons derived from genomic DNA of HeLa cells co-transduced with $AdV^{\Delta 2}P.Cas9.F^{50}$ and $AdV^{\Delta 2}U6.gRNA^{S1}.F^{50}$ at the indicated MOIs (in TU/cell) were denatured and re-annealed, treated with T7EI and resolved through agarose gel electrophoresis. As negative controls, it was used genomic DNA isolated from HeLa cells exposed to 100 TU/cell of $AdV^{\Delta 2}P.Cas9.F^{50}$ (lane 1), 100 TU/cell of $AdV^{\Delta 2}U6.gRNA^{S1}.F^{50}$ (lane 2) or to no vector (lane 3). Solid and open arrowheads indicate the positions of undigested and T7EI-digested DNA molecules, respectively. Lane M, Gene Ruler DNA Ladder marker. (B) Target gene knockout frequencies in HeLa cells co-transduced with $AdV^{\Delta 2}P.Cas9.F^{50}$ and $AdV^{\Delta 2}U6.gRNA^{S1}.F^{50}$ as measured by densitometry of the DNA species depicted in Fig. 4A.

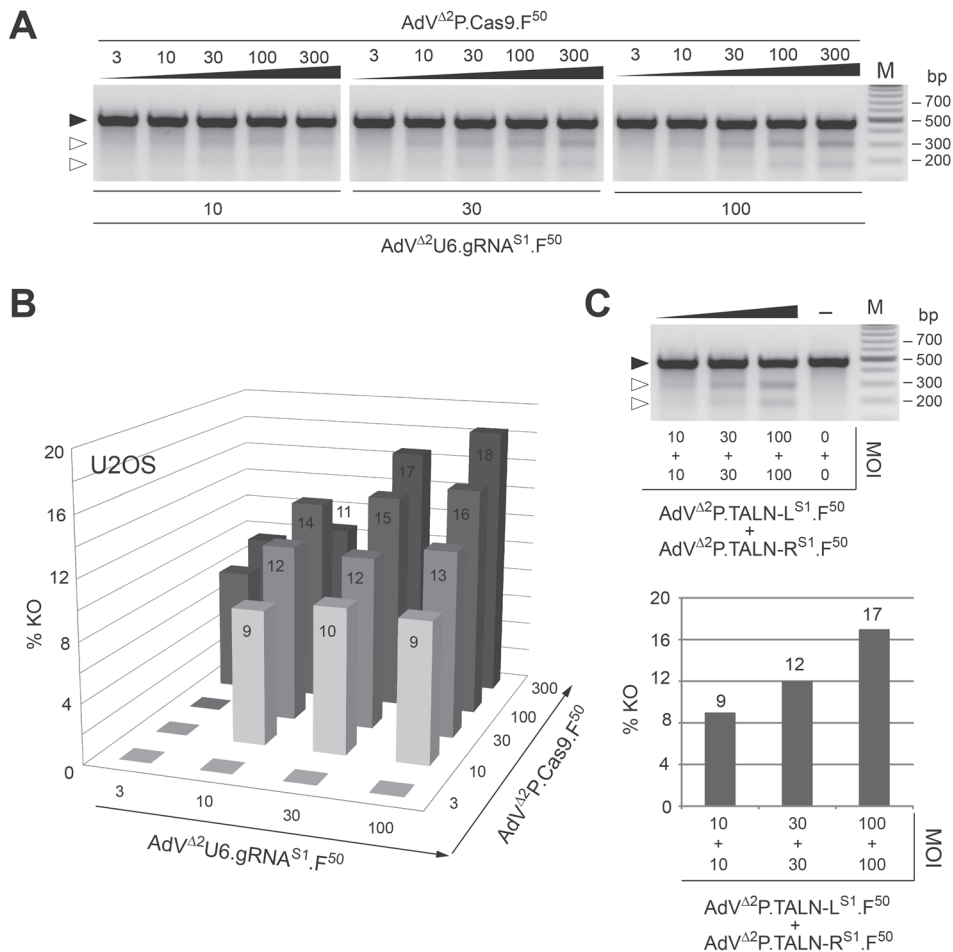


Figure 5. Analysis of site-specific DSB formation in U2OS cells following AdV-mediated delivery of transgenes encoding RGN and TALEN complexes. (A) Targeted mutagenesis experiments with AdV^{Δ2}P.Cas9.F⁵⁰ and AdV^{Δ2}.U6.gRNA^{S1}.F⁵⁰. Agarose gel electrophoresis of AAVS1-specific PCR products amplified from chromosomal DNA of U2OS cells co-transduced with the indicated MOIs of AdV^{Δ2}P.Cas9.F⁵⁰ and AdV^{Δ2}.U6.gRNA^{S1}.F⁵⁰ (in TU/cell). Prior to loading the PCR products were denatured, re-annealed and treated with the mismatch-sensitive enzyme T7E1. Solid and open arrowheads point to the positions of undigested and T7E1-digested DNA molecules, respectively. Lane M, Gene Ruler DNA Ladder marker. **(B)** Target gene knockout frequencies in U2OS cells co-transduced with AdV^{Δ2}P.Cas9.F⁵⁰ and AdV^{Δ2}.U6.gRNA^{S1}.F⁵⁰ as measured by densitometry of the DNA species shown in Fig. 5A. **(C)** Targeted mutagenesis experiments with AdV^{Δ2}P.TALEN-L^{S1}.F⁵⁰ and AdV^{Δ2}P.TALEN-R^{S1}.F⁵⁰. Upper panel, T7E1-based assays on AAVS1-specific amplicons corresponding to genomic DNA of mock-transduced U2OS cells (-) or of U2OS cells co-transduced with the specified MOIs of AdV^{Δ2}P.TALEN-L^{S1}.F⁵⁰ and AdV^{Δ2}P.TALEN-R^{S1}.F⁵⁰ (in TU/cell). Solid and open arrowheads indicate the positions of undigested and T7E1-digested DNA molecules, respectively. Lanes M, Gene Ruler DNA Ladder marker. Lower panel, target gene knockout frequencies in U2OS cells co-transduced with AdV^{Δ2}P.TALEN-L^{S1}.F⁵⁰ and AdV^{Δ2}P.TALEN-R^{S1}.F⁵⁰ as determined by densitometry of the DNA fragments presented in the upper panel.

Follow-up transduction experiments in myoblasts generally yielded results similar to those obtained in HeLa and U2OS cells. Crucially, at matched MOIs, the levels of RGN-induced targeted mutagenesis were higher in myoblasts than in the two cancer-derived cell lines (Fig. 6A and Fig. 6B). For instance, 1:1 mixtures of AdV^{Δ2}P.Cas9.F⁵⁰ and AdV^{Δ2}U6.gRNA^{S1}.F⁵⁰ corresponding to a combined MOI of 60 TU/cell led to gene disruption frequencies in HeLa, U2OS and myoblasts of 12%, 12% and 27%, respectively (compare Fig. 4B and Fig. 5B with Fig. 6B). Importantly, myoblasts exposed to only one of the two RGN or TALEN pair members did not contain detectable indels at *AAVS1*. Finally, it is also noteworthy pointing out that at the high-end of the MOI scale tested in myoblasts (i.e. double dose of 30 TU/cell) the AdV^{Δ2}P.TALEN-L^{S1}.F⁵⁰/AdV^{Δ2}P.TALEN-R^{S1}.F⁵⁰ set yielded roughly a 2-fold higher gene knockout activity when compared to equivalent MOIs of the AdV^{Δ2}P.Cas9.F⁵⁰/AdV^{Δ2}U6.gRNA^{S1}.F⁵⁰ pair (compare Fig. 6B with the lower panel in Fig. 6C). To establish the AdV transduction levels achieved in the CRISPR/Cas9-mediated targeted mutagenesis experiments corresponding to Figs. 4, 5 and 6, the various target cell types were exposed to particles of AdV^{Δ2}U6.gRNA^{S1}.F⁵⁰ and reporter vector AdV^{Δ2}P.eGFP.F⁵⁰ under the same conditions as those used in transduction experiments with AdV^{Δ2}U6.gRNA^{S1}.F⁵⁰ and AdV^{Δ2}P.Cas9.F⁵⁰. Of note, the transgene regulatory elements in AdV^{Δ2}P.eGFP.F⁵⁰ are identical to those in AdV^{Δ2}P.Cas9.F⁵⁰. Flow cytometric analyses clearly showed that the panel of MOIs selected for transducing each of the target cell types led to a wide range of gene delivery activities. Among these were MOIs yielding transduction levels of 100% (Supplementary Fig. 1).

In order to gather more information on target gene disruption activities attainable with the *AAVS1*-specific TALENs and RGN complexes within specific cell types and, simultaneously, probe for possible cell type-dependent effects on these activities following AdV-mediated gene transfer, we carried out parallel co-transduction experiments with both AdV sets on myoblast and hMSC cultures. In addition, to evaluate the impact of different relative concentrations of gRNA^{S1} and Cas9 molecules on gene knockout levels, we exposed these target cells to a constant total vector dose of 80 TU/cell spanning different ratios between AdV^{Δ2}P.Cas9.F⁵⁰ and AdV^{Δ2}U6.gRNA^{S1}.F⁵⁰.

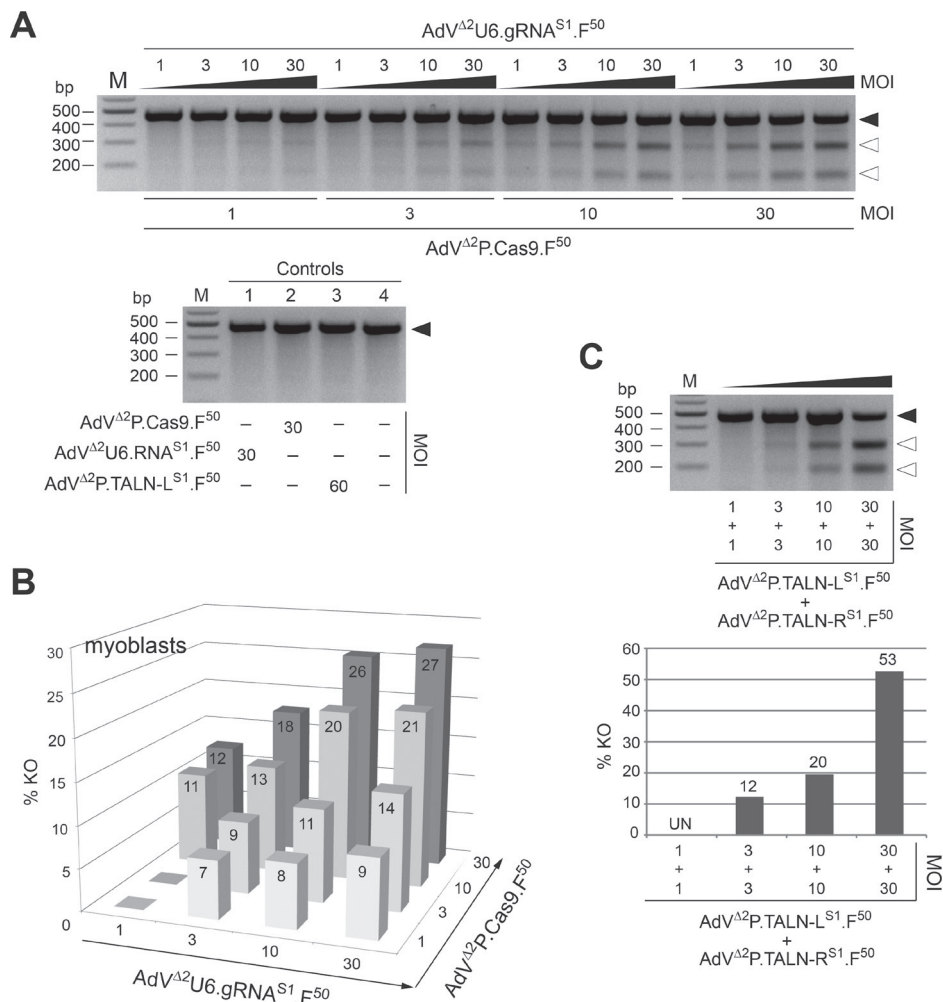


Figure 6. Analysis of targeted mutagenesis in human myoblasts following AdV-mediated transfer of DNA coding for RGN and TALEN complexes. (A) Targeted mutagenesis experiments with AdV^{Δ2}P.Cas9.F⁵⁰ and AdV^{Δ2}U6.gRNA^{S1}.F⁵⁰. Upper panel, AAVS1-specific PCR products corresponding to genomic DNA of myoblasts co-transduced with the indicated MOIs of AdV^{Δ2}P.Cas9.F⁵⁰ and AdV^{Δ2}U6.gRNA^{S1}.F⁵⁰ (in TU/cell) subjected to T7EI-based assays. Lower panel, negative controls corresponding to human myoblasts that were singly transduced with AdV^{Δ2}P.Cas9.F⁵⁰ (lane 1), AdV^{Δ2}U6.gRNA^{S1}.F⁵⁰ (lane 2) and AdV^{Δ2}P.TALEN-L^{S1}.F⁵⁰ (lane 3) or that were mock-transduced (lane 4). (B) Target gene knockout frequencies in human myoblasts co-transduced with AdV^{Δ2}P.Cas9.F⁵⁰ and AdV^{Δ2}U6.gRNA^{S1}.F⁵⁰ as measured by densitometry of the DNA species depicted in Fig. 6A. (C) Targeted mutagenesis experiments with AdV^{Δ2}P.TALEN-L^{S1}.F⁵⁰ and AdV^{Δ2}P.TALEN-R^{S1}.F⁵⁰. Upper panel, T7EI-based assays on AAVS1-specific amplicons obtained from genomic DNA of myoblasts co-transduced with the indicated MOIs of AdV^{Δ2}P.TALEN-L^{S1}.F⁵⁰ and AdV^{Δ2}P.TALEN-R^{S1}.F⁵⁰ (in TU/cell). Solid and open arrowheads indicate the positions of undigested and T7EI-digested DNA molecules, respectively. Lanes M, Gene Ruler DNA Ladder marker. Lower panel, target gene disruption frequencies in myoblasts co-transduced with AdV^{Δ2}P.TALEN-L^{S1}.F⁵⁰ and AdV^{Δ2}P.TALEN-R^{S1}.F⁵⁰ as determined by densitometry of the DNA fragments shown in the upper panel.

The results depicted in Fig. 7A and in Fig. 7B corroborated the data on the higher TALEN pair activity in myoblasts presented in Fig. 6C and extended it to hMSCs. Indeed, gene knockout frequencies in myoblasts and hMSCs exposed to vector mixtures consisting of different ratios between $\text{AdV}^{\Delta 2\text{P.Cas9.F}50}$ and $\text{AdV}^{\Delta 2\text{U6.gRNA}^{S1.F}50}$ were consistently lower than those resulting from co-transductions with 1:1 mixtures of $\text{AdV}^{\Delta 2\text{P.TALEN-L}^{S1.F}50}$ and $\text{AdV}^{\Delta 2\text{P.TALEN-R}^{S1.F}50}$. Of note, T7EI-digested DNA species corresponding to genomic DNA from cells transduced with the two nuclease systems displayed different migration profiles upon electrophoresis (Fig. 7A). These molecular weight differences are consistent with the predicted target site cleaving positions for the TALEN and RGN complexes i.e. within the spacer and three nucleotides “upstream” of the PAM, respectively (Fig. 1B). AdV transduction levels corresponding to these targeted mutagenesis experiments in myoblasts and hMSCs were determined by applying $\text{AdV}^{\Delta 2\text{U6.gRNA}^{S1.F}50}$ and $\text{AdV}^{\Delta 2\text{P.eGFP.F}50}$ (Fig. 7C) under the same experimental conditions as those used with $\text{AdV}^{\Delta 2\text{U6.gRNA}^{S1.F}50}$ and $\text{AdV}^{\Delta 2\text{P.Cas9.F}50}$ (Fig. 7B). Collectively, these experiments also demonstrated that, at equivalent MOIs, AdV-mediated delivery of the *AAVS1*-specific RGNs resulted in higher gene disruption levels in myoblasts than in hMSCs regardless of the proportions of each vector type in the co-transducing $\text{AdV}^{\Delta 2\text{P.Cas9.F}50}/\text{AdV}^{\Delta 2\text{U6.gRNA}^{S1.F}50}$ mixtures (Fig. 7D).

To further test the AdV system as a delivery platform for CRISPR/Cas9-derived RGNs, $\text{AdV}^{\Delta 2\text{U6.gRNA}^{\text{GFP.F}50}}$ was generated. This vector has the genetic make-up of $\text{AdV}^{\Delta 2\text{U6.gRNA}^{S1.F}50}$ except that it encodes the *eGFP*-specific gRNA^{GFP} in place of gRNA^{S1} (Fig. 1A). As with the other genome-modifying AdVs, $\text{AdV}^{\Delta 2\text{U6.gRNA}^{\text{GFP.F}50}}$ could also be produced to high titers of functional vector particles (i.e. 10.4×10^{10} TU/ml). Next, $\text{AdV}^{\Delta 2\text{U6.gRNA}^{\text{GFP.F}50}}$ was deployed together with $\text{AdV}^{\Delta 2\text{P.Cas9.F}50}$ in transduction experiments in indicator H27 cells23. These cells contain a single-copy constitutionally active *eGFP* transcriptional unit providing, as a result, a convenient model system to evaluate target gene knockout strategies by live-cell tracing and quantification of gene-modified populations. Thus, as in experiments using $\text{AdV}^{\Delta 2\text{P.Cas9.F}50}$ and $\text{AdV}^{\Delta 2\text{U6.gRNA}^{S1.F}50}$, we set-up a “co-transduction grid” to determine targeted mutagenesis frequencies resulting from different combined dosages of $\text{AdV}^{\Delta 2\text{P.Cas9.F}50}$ and $\text{AdV}^{\Delta 2\text{U6.gRNA}^{\text{GFP.F}50}}$. Direct fluorescence microscopy (Fig. 8A) as well as flow cytometry (Figs. 8B and 8C), revealed a clear dose-dependent increase in *eGFP* disruption levels varying from a minimum of 0.8% to a maximum of 65%. Controls consisting of H27 cells transduced either with $\text{AdV}^{\Delta 2\text{P.Cas9.F}50}$ or with $\text{AdV}^{\Delta 2\text{U6.gRNA}^{\text{GFP.F}50}}$, each applied at an MOI of 100 TU/cell, did not yield frequencies of *eGFP*-negative cells above background levels (Fig. 8B).

Taken our data together, we conclude that AdVs encoding RGN components can be produced to high titers and achieve robust targeted mutagenesis in a diverse array of human cell types.

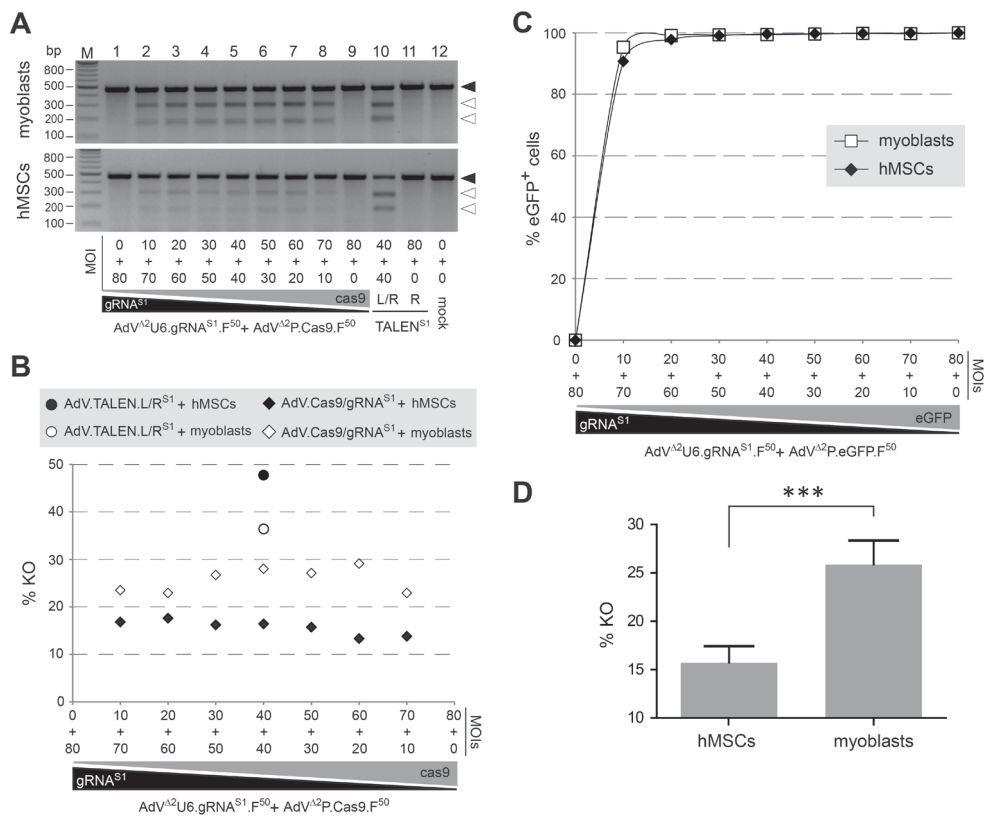
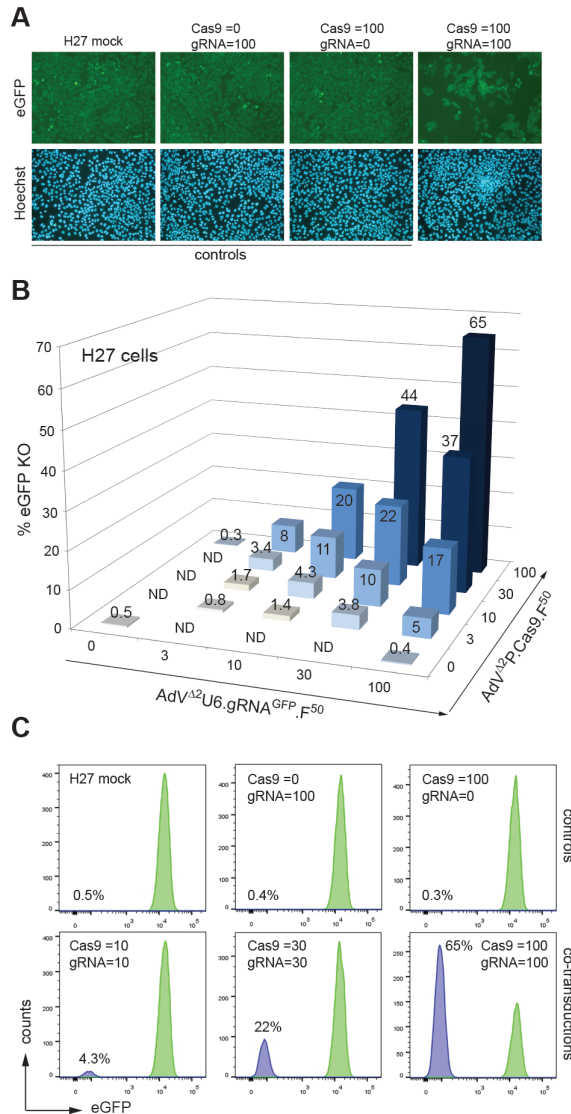


Figure 7. Analysis of target gene knockout frequencies following AdV-mediated introduction of gRNA^{S1}:Cas9 and TALEN-L^{S1}:TALEN-R^{S1} complexes into hMSCs versus human myoblasts. (A) Target site genotyping assays. Upper and lower panels, agarose gel electrophoresis of *AAVS1*-specific PCR products amplified from chromosomal DNA of myoblasts and hMSCs, respectively, co-transduced with the indicated MOIs of AdV^{Δ2}P.Cas9.F⁵⁰ and AdV^{Δ2}.U6.gRNA^{S1}.F⁵⁰ (in TU/cell). Before loading the amplicons were denatured, re-annealed and treated with the mismatch-sensitive enzyme T7E1. The cells were co-transduced at a combined MOI of 80 TU/cell with different ratios of AdV^{Δ2}P.Cas9.F⁵⁰ and AdV^{Δ2}.U6.gRNA^{S1}.F⁵⁰ or with 1:1 mixtures of AdV^{Δ2}P.TALEN-L^{S1}.F⁵⁰ and AdV^{Δ2}P.TALEN-R^{S1}.F⁵⁰. Mock-transduced cells or cells exposed to 80 TU/cell of AdV^{Δ2}P.Cas9.F⁵⁰, AdV^{Δ2}.U6.gRNA^{S1}.F⁵⁰ or AdV^{Δ2}P.TALEN-L^{S1}.F⁵⁰ served as negative controls. Solid and open arrowheads indicate the positions of undigested and T7E1-digested amplicons, respectively. Lanes M, Gene Ruler DNA Ladder marker. **(B)** Target gene knockout activities in hMSCs and myoblasts transduced with AdVs. Target gene disruption frequencies in cells co-transduced with AdV pairs AdV^{Δ2}P.Cas9.F⁵⁰/AdV^{Δ2}U6.gRNA^{S1}.F⁵⁰ and AdV^{Δ2}P.TALEN-L^{S1}.F⁵⁰/AdV^{Δ2}P.TALEN-R^{S1}.F⁵⁰ as determined by densitometry of DNA fragments depicted in Fig. 7A. **(C)** Gene delivery activities in hMSCs and myoblasts transduced with AdVs. Percentages of transduced cells incubated with different mixtures of reporter vector AdV^{Δ2}P.eGFP.F⁵⁰ and AdV^{Δ2}U6.gRNA^{S1}.F⁵⁰, applied at a combined MOI of 80 TU/cell. Cells exposed only to AdV^{Δ2}U6.gRNA^{S1}.F⁵⁰ at an MOI of 80 TU/cell served as negative controls. The frequency of eGFP-positive cells was determined by flow cytometry at three days post-transduction. **(D)** Cumulative data corresponding to the gene knockout frequencies in hMSCs versus human myoblasts following AdV-mediated delivery of gRNA^{S1}:Cas9 complexes into these cells. Plotted data show the mean ± standard deviation (n=7).



DISCUSSION

One can postulate that AdV technologies, in addition to their utility in well-established biomedical research fields such as oncology and vaccinology²⁴⁻²⁶, will start having an increasing role in experimental and applied genome engineering as well. An initial effort in this direction is provided by an AIDS therapeutic strategy based on knocking out the HIV-1 co-receptor gene *CCR5* following AdV-mediated *ZFN* transfer into CD4⁺ T cells cultured *ex vivo*²⁷. Related to this, we have recently shown that AdVs can also serve as delivery vehicles for introducing functional TALENs into human cells¹⁸, whereas Ebina and colleagues have provided a proof-of-concept for using RGNs to disrupt HIV-1 proviral DNA²⁸. In the current work, we have extended the utility of the AdV technology to genome engineering strategies based on RNA-dependent DNA nucleases derived from a type II CRISPR/Cas system.

Assuming that designer nuclease gene expression should ideally occur at high levels^{29,30} and be short-term, AdVs may ultimately be preferable expression platforms when compared to other episomal viral vector systems such as silencing-prone integrase-defective lentiviral vectors (IDLVs)^{30,31} and ssDNA adeno-associated viral vectors (AAVs)³². Moreover, the relatively large size of *Cas* ORFs may, to some extent, compromise the production of viral vectors whose optimal nucleic acid packaging capacities are significantly lower than those offered by AdV backbones and capsids. Still related to this payload capacity issue, our results further suggest that efficient chromosomal DSB formation seems to display a higher dependency on the overall RGN concentrations in target cell nuclei rather than on the use of a specific ratio between the Pol-II and Pol-III expression units. Thus, integrated vector designs in which both *Cas9* and *gRNA* expression units are co-delivered within single vector particles may turn out to be advantageous in certain experimental settings.

By carrying out transduction experiments with AdVs encoding validated *gRNA*^{S1}:*Cas9* complexes and optimized TALEN-L^{S1}:TALEN-R^{S1} heterodimers, we found that the latter sequence-specific nuclease system yielded gene disruption levels that were similar to or higher than those achieved by the former. This outcome is different from that reported recently by Ding and colleagues³³. Through co-transfection experiments in human pluripotent stem cell lines comparing *gRNA*:*Cas9* sets with TALEN pairs based on a specific architecture, these authors showed that, for each targeted locus, the RGNs consistently and substantially outperformed the TALENs. At this stage it is difficult to pinpoint any culprit(s) for these different outcomes since the variables are numerous and include the nature of the scaffolds for each platform, the delivery methods as well as the cell types and chromosomal target sequences tested. Nonetheless, our results do support the view that the TALEN platform is not *per se* inferior to that of RGNs in what site-specific chromosomal cleaving activity is concerned. Finally, we have shown that, at matched doses, AdV-mediated transfer of the *AAVS1*-specific RGNs led to higher gene disruption levels in myoblasts than in hMSCs.

Whether these data are a consequence of different epigenetic signatures at *AAVS1* or some other cell type-specific variable(s) remains to be investigated.

In conclusion, our findings demonstrate that AdVs constitute a valuable delivery option for introducing type II CRISPR/Cas-derived RGN complexes into human cells regardless of their transformation status and open as a result the perspective for RGN deployment and optimization in human primary cells. This research might include, in addition to on-target versus off-target assessments and gene knockout approaches, the testing of genome editing strategies based on donor DNA templates and AdV-mediated delivery of RGNs into target cell nuclei.

METHODS

Cells. The U2OS human osteosarcoma and the HeLa cervix carcinoma cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% and 5% fetal bovine serum (FBS), respectively, at 37°C in a 10% CO₂ atmosphere. The *E1*- and *E2A*-complementing AdV packaging cell line PER.E2A³⁴, was kept in DMEM supplemented with 10% FBS, 10 mM MgCl₂ and 250 µg/ml of Geneticin (Invitrogen). PER.E2A cells were regularly sub-cultured at 39°C and were shifted to 34°C during AdV production for the proper folding of their *E2A*-encoded thermo-sensitive DNA-binding protein. The origin of and culture conditions for the HeLa cell-derived clone H27, constitutively expressing *eGFP*, as well as those of myoblasts and hMSCs have been detailed elsewhere^{23,35,36}. PER.C6 cells³⁷ were cultured at 37°C in DMEM with 10% FBS and 10 mM MgCl₂.

Recombinant DNA. Standard recombinant DNA techniques were applied for the construction of the various AdV shuttle plasmids³⁸. The TALEN-encoding plasmids pAdSh.PGK.TALEN-L⁵¹³⁹ and pAdSh.PGK.TALEN-R⁵¹³⁹ harbor the *TALEN* ORFs derived from 1383.pVAX.AAVS1.TALEN.L-94¹⁸ and 1384.pVAX.AAVS1.TALEN.R-95¹⁸, respectively. These constructs contain as transcription regulatory elements the human house-keeping phosphoglycerate kinase 1 gene (*PGK*) promoter and the simian virus 40 polyadenylation signal (SV40)³⁹. The pAdSh.PGK.Cas9 and pSh.AAVS1.eGFP⁴⁰ constructs have these *PGK* and SV40 regulatory elements controlling the human codon-optimized ORF encoding hCas9¹⁰ (herein referred to as Cas9) and the reporter eGFP, respectively. The *Cas9* ORF was isolated from Addgene plasmid 41815. The expression units gRNA_AAVS1-T2¹⁰ and gRNA_GFP-T2¹⁰ based on the *U6* RNA Pol III promoter (herein dubbed gRNA^{S1} and gRNA^{GFP}, respectively) were retrieved from Addgene plasmid 41818 and 41820 and were inserted into the MCS of pAdSh.MCS.SV40, resulting in constructs pAdSh.U6.gRNA^{S1} and pAdSh.U6.gRNA^{GFP}, respectively.

The *E1*- and *E2A*-deleted (i.e. second-generation) fiber-modified AdV molecular clones pAdV Δ 2P.TALEN-L^{S1}.F⁵⁰, pAdV Δ 2P.TALEN-R^{S1}.F⁵⁰, pAdV Δ 2P.Cas9.F⁵⁰, pAdV Δ 2U6.gRNA^{S1}.F⁵⁰, pAdV Δ 2U6.gRNA^{GFP}.F⁵⁰ and pAdV Δ 2P.eGFP.F⁵⁰ were assembled via homologous recombination after transformation of BJ5183^{pAdEasy-2.50} *E. coli* cells⁴¹ with MspI-treated AdV shuttle plasmids pAdSh.PGK.TALEN-L^{S1}, pAdSh.PGK.TALEN-R^{S1}, pAdSh.PGK.Cas9, pAdSh.U6.gRNA^{S1}, pAdSh.U6.gRNA^{GFP} and pSh.AAVS1.eGFP⁴⁰, respectively.

Validation of Cas9- and gRNA^{S1}-encoding AdV shuttle plasmids. PER.C6 cells were plated at a density of 1.5×10^6 cells per well of 6-well plates (Greiner Bio-One). The next day, a total amount of 6 μ g of DNA corresponding to 1:1 mixtures of hCas9 (Addgene plasmid 41815) and gRNA_AAVS1-T2 (Addgene plasmid 41818), pAdSh.PGK.Cas9 and pAdSh.U6.gRNA^{S1}, hCas9 and gRNA_Cloning Vector (Addgene plasmid 41824) or hCas9_D10A (Addgene plasmid 41816) and gRNA_AAVS1-T2, were transfected by deploying a 1 mg/ml polyethyleneimine (PEI) solution (Polysciences) essentially as described elsewhere⁴¹ except for the use of 6 μ g of DNA and 19.7 μ l of PEI instead of 6.25 μ g of DNA and 18.8 μ l of PEI. At 3 days post-transfection genomic DNA from mock-transfected cells and from co-transfected cells, was isolated according to a previously described method⁴². Targeted gene disruption was assessed by using the T7 endonuclease I (T7EI)-based assay as described below.

Viral vector production, purification and titration. The production of viral vectors AdV Δ 2P.TALEN-L^{S1}.F⁵⁰, AdV Δ 2P.TALEN-R^{S1}.F⁵⁰, AdV Δ 2P.Cas9.F⁵⁰, AdV Δ 2U6.gRNA^{S1}.F⁵⁰, AdV Δ 2U6.gRNA^{GFP}.F⁵⁰ and AdV Δ 2P.eGFP.F⁵⁰, was carried out essentially as described elsewhere⁴¹. In brief, the rescue of AdV particles was initiated by transfecting 1.25×10^6 PER.E2A cells seeded in medium without Geneticin in wells of 6-well plates (Greiner Bio-One). The next day, the cells were transfected by using 18.8 μ l of a 1 mg/ml PEI solution and 6.25 μ g of PacI-linearized plasmids pAdV Δ 2P.TALEN-L^{S1}.F⁵⁰, pAdV Δ 2P.TALEN-R^{S1}.F⁵⁰, pAdV Δ 2P.Cas9.F⁵⁰, pAdV Δ 2U6.gRNA^{S1}.F⁵⁰, pAdV Δ 2U6.gRNA^{GFP}.F⁵⁰ and pAdV Δ 2P.eGFP.F⁵⁰. Following an overnight incubation period at 39°C, the transfection media was replaced and the cells were transferred to the permissive temperature of 34°C. After the emergence of complete cytopathic effect (CPE), the producer cells were harvested and subjected to three cycles of freezing and thawing in liquid N₂ and a 37°C water bath, respectively. Rescued AdVs present in clarified producer cell supernatants, were subsequently amplified through propagation on newly seeded PER.E2A cells. Large-scale AdV stocks were prepared by infecting producer cells at 70-80% confluence in 16 175-cm² cell cultures flasks (Greiner Bio-One). The details on the isopycnic CsCl density-gradient ultracentrifugation and ultrafiltration used to purify and de-salt AdV preparations, respectively, have been described before⁴¹. The titers of purified AdV stocks were determined by TCID₅₀ assays as follows. One hundred microliters of a PER.E2A cell suspension containing 5.0×10^4 cells/ml in DMEM containing 2% FBS and 10 mM MgCl₂, were dispensed in wells of

96-well plates (Greiner Bio-One). After an overnight incubation period at 39°C in a 10% CO₂ atmosphere, the cells were exposed in octuplicate to 100 µl of 10-fold dilutions (range: 10⁻⁵ through 10⁻¹²) of the various viral vector preparations. Next, the cells were incubated for 2.5 weeks at 34°C in a 10% CO₂ atmosphere having 100 µl of DMEM containing 2% FBS and 10 mM MgCl₂ being added at 1 week post-infection. After this incubation period, wells with cultures containing viral plaques or at full CPE were scored to calculate the titers in terms of transducing units per ml (TU/ml) as specified elsewhere⁴¹.

Isolation of viral vector DNA for structural analysis. The isolation of AdV genomes from purified particles was carried out as specified elsewhere⁴¹. The *in silico* restriction patterns were made with the aid of Gene Construction kit (version 2.5) software (Textco BioSoftware, Inc.).

Transduction experiments. Myoblasts, hMSCs and U2OS cells were seeded in their respective media at a density of 1×10⁵ cells per well of 24-well plates (Greiner Bio-One), whereas HeLa cells were plated in the same vessel format at a density of 5×10⁴ cells. Approximately 24 hours after seeding, the cells were exposed in 500-µl volumes to the two different AdV pair combinations (i.e. AdV^{Δ2P}.TALEN-L^{S1}.F⁵⁰/AdV^{Δ2P}.TALEN-R^{S1}.F⁵⁰ or AdV^{Δ2P}.Cas9.F⁵⁰/AdV^{Δ2}U6.gRNA^{S1}.F⁵⁰) at the indicated multiplicities of infection (MOI). Mock-transduced cells or, whenever indicated, cells transduced exclusively with one element of each vector pair, served as negative controls. Three days post-infection, total cellular DNA was isolated by using the DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's recommendations.

Transduction experiments on indicator H27 cells. One day before transduction, 1.0×10⁵ H27 cells were seeded in wells of 24-well plates. After the removal of the culture medium, the H27 cell monolayers were incubated in 500-µl volumes consisting of various total and relative amounts of AdV^{Δ2P}.Cas9.F⁵⁰ and AdV^{Δ2}U6.gRNA^{GFP}.F⁵⁰. Mock-transduced H27 cells and H27 cells exposed exclusively to AdV^{Δ2P}.Cas9.F⁵⁰ or to AdV^{Δ2}U6.gRNA^{GFP}.F⁵⁰ each applied at an MOI of 100 TU/cell, provided for negative controls. Subsequently, after extensive sub-culturing to remove reporter protein from cells with disrupted *eGFP* ORFs, the presence of eGFP-negative cells was assessed by direct fluorescence microscopy and flow cytometry at 15 days post-transduction.

Direct fluorescence microscopy. Targeted *eGFP* knockout in H27 cell cultures was monitored by direct fluorescence microscopy. The H27 cell nuclei were stained by adding Hoechst 33342 (Molecular Probes) at 10 µg/ml for 10 min. Next, the cell cultures were washed twice with PBS, after which cultured medium was added. The eGFP- and Hoechst 33342-specific

signals were detected by using an Olympus IX51 inverse fluorescence microscope (Olympus). Micrographs were captured and archived by deploying a XC30 Peltier CCD camera and the CellF software (both from Olympus).

Flow cytometry. The frequencies of eGFP-positive cells in cultures transduced with AdV^{A2P}. eGFP.F⁵⁰ and of eGFP-negative cells in H27 cell cultures were determined by using a BD LSR II flow cytometer (BD Biosciences). Data were analyzed with the aid of FlowJo 7.2.2 software (Tree Star). Mock-transduced H27 cells served to establish the background fluorescence. Samples of at least 10,000 viable single cells were analyzed per experimental condition.

T7 endonuclease I-based genotyping assays. Target gene disruption levels were assessed by using mismatch-sensitive T7EI endonuclease I. To this end, genomic DNA samples from target cells were subjected to PCR for the amplification of a 469-bp DNA segment encompassing the target sites for the gRNA^{S1}:Cas9 and TALEN-L^{S1}:TALEN-R^{S1} complexes. The primers, PCR reagents and cycling conditions used have been described in detail before¹⁸. Next, the resulting amplicons were denatured and reannealed in a thermocycler by applying the program specified in Supplementary Table S1. One fifth of each PCR mixture was incubated in 15- μ l reactions with 1 \times NEBuffer 2 and 5 U of T7EI (both from New England Biolabs). Control reactions lacked T7EI. After 15 minutes at 37°C, the samples were subjected to electrophoresis through 2% (w/v) agarose in 1 \times Tris-Acetate-EDTA buffer. Finally, ethidium bromide-stained DNA species were measured in a Molecular Imager Gel-DocTM XR+ with the aid of Bio-Rad Image Lab 4.1 software (both from Bio-Rad).

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AUTHOR CONTRIBUTIONS

I.M. and M.H. contributed equally to this work. I.M. and M.H. performed the experiments. J.L., J.M.J. and X.C. produced and characterized reagents. I.M., M.H., J.M.J. and M.A.F.V.G. designed the experiments and analyzed the data. M.A.F.V.G. conceived and initiated the research. M.A.F.V.G. wrote the manuscript with the help from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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