Versatile and Efficient Genome Editing in Human Cells by Combining Zinc-Finger Nucleases With Adeno-Associated Viral Vectors

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Abstract

Zinc-finger nucleases (ZFNs) have become a valuable tool for targeted genome engineering. Based on the enzyme's ability to create a site-specific DNA double-strand break, ZFNs promote genome editing by activating the cellular DNA damage response, including homology-directed repair (HDR) and nonhomologous end-joining. The goal of this study was (i) to demonstrate the versatility of combining the ZFN technology with a vector platform based on adeno-associated virus (AAV), and (ii) to assess the toxicity evoked by this platform. To this end, human cell lines that harbor enhanced green fluorescence protein (EGFP) reporters were generated to easily quantify the frequencies of gene deletion, gene disruption, and gene correction. We demonstrated that ZFN-encoding AAV expression vectors can be employed to induce large chromosomal deletions or to disrupt genes in up to 32% of transduced cells. In combination with AAV vectors that served as HDR donors, the AAV-ZFN platform was utilized to correct a mutation in EGFP in up to 6% of cells. Genome editing on the DNA level was confirmed by genotyping. Although cell cycle profiling revealed a modest G2/M arrest at high AAV-ZFN vector doses, platform-induced apoptosis could not be detected. In conclusion, the combined AAV-ZFN vector technology is a useful tool to edit the human genome with high efficiency. Because AAV vectors can transduce many cell types relevant for gene therapy, the *ex vivo* and *in vivo* delivery of ZFNs via AAV vectors will be of great interest for the treatment of inherited disorders.

Introduction

IN THE LAST TWO DECADES various platforms have been developed that allow for efficient and precise modification of the human genome. A very promising approach is based on zinc-finger nuclease (ZFN) technology (Urnov *et al.*, 2010; Rahman *et al.*, 2011). ZFNs are designer nucleases that consist of two functional domains: a customized zinc-finger array that specifies DNA binding and the endonuclease domain of the restriction enzyme *FokI* that contains the catalytic activity (Smith *et al.*, 2000). Each zinc-finger array in a ZFN subunit confers binding to the respective target half-site, and upon dimerization of the two subunits in correct spacing and orientation, the nuclease domain introduces a DNA double-strand break (DSB) within the spacer sequence that separates

the two target half-sites (Smith *et al.*, 2000). ZFNs have been shown to cleave target DNA with high specificity and efficiency in a variety of human cell lines and primary cells, including T cells (Perez *et al.*, 2008), hematopoietic stem cells (Lombardo *et al.*, 2007; Holt *et al.*, 2010), mesenchymal stromal cells (Benabdallah *et al.*, 2010), embryonic stem cells (Lombardo *et al.*, 2007; Hockemeyer *et al.*, 2009; Zou *et al.*, 2009), and induced pluripotent stem cells (Hockemeyer *et al.*, 2009; Zou *et al.*, 2009).

A major issue for therapeutic applications of designer nucleases is the balance between ZFN activity and nuclease-associated toxicity (Händel and Cathomen, 2011). ZFNs have undergone several critical improvements in design in the last decade, including the development of novel platforms to generate specific zinc-finger arrays (Maeder *et al.*, 2008;

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Urnov *et al.*, 2010; Kim *et al.*, 2011; Sander *et al.*, 2011), improved architecture of the dimer interface to prevent homodimerization of ZFN subunits (Miller *et al.*, 2007; Szczepek *et al.*, 2007; Söllü *et al.*, 2010; Doyon *et al.*, 2011; Ramalingam *et al.*, 2011), and customized interdomain linkers that connect the DNA-binding domain with the nuclease domain (Bibikova *et al.*, 2001; Händel *et al.*, 2009). After ZFN-mediated DNA cleavage of the target site, the resulting DSB leads to activation of the cellular DNA damage response, involving nonhomologous end-joining (NHEJ) and homology-directed repair (HDR) (Shrivastav *et al.*, 2008). NHEJ is an error-prone repair pathway that can lead to small insertions or deletions (indels) at the junction site. If the DSB occurs within coding sequences, indels can result in frame-shift or nonsense mutations. HDR, on the other hand, is based on homologous recombination and is the basis of ZFN-induced gene targeting. Although NHEJ is the more prominent repair pathway in mammalian cells, when high numbers of donor DNA are delivered to the nucleus, the HDR pathway becomes more prominent for DSB repair (Lombardo *et al.*, 2007; Gellhaus *et al.*, 2010).

An important aspect in ZFN-based genome engineering is vectorization of the nuclease and the donor. Ideally, ZFNs as well as the donor DNA are present only transiently at high concentrations. Episomal DNA-based ZFN expression systems, such as plasmid DNA or viral vectors, usually harbor strong promoters to ensure high ZFN levels. Thus, the duration of ZFN expression is limited through rapid dilution during cell division in mitotic cells. In addition to plasmid DNA, transient nuclease expression in human cells has been reported from integrase-deficient lentiviral vectors (Cornu and Cathomen, 2007; Lombardo *et al.*, 2007), adenoviral vectors (Perez *et al.*, 2008), and vectors based on adeno-associated virus (AAV) (Porteus *et al.*, 2003; Gellhaus *et al.*, 2010). The use of AAV vectors is of particular interest because these vectors function efficiently as a substrate for HDR, even if the target locus contains only a DNA single-strand nick (Metzger *et al.*, 2011) or no DNA damage at all (Hendrie and Russell, 2005). In proof-of-concept studies using AAV as a donor for HDR and an I-SceI-induced DSB in the target locus to stimulate gene targeting, we and others showed that gene targeting at a chromosomally integrated marker gene can be achieved in up to 65% of cultured human cells (Miller *et al.*, 2003; Porteus *et al.*, 2003; Gellhaus *et al.*, 2010; Hirsch *et al.*, 2010). Moreover, in a recent study ZFN-stimulated *in vivo* gene targeting was reported after AAV-based gene transfer in a mouse model for factor IX deficiency (Li *et al.*, 2011).

The goals of this study were to demonstrate the versatility of combining AAV vectors with the ZFN platform for rational editing of the human genome and to assay platform-associated toxicity. We show that ZFN encoding AAV expression vectors can be employed (i) to delete a lentiviral provirus, (ii) to disrupt an expression cassette, or (iii) to correct a mutational insertion.

Materials and Methods

Plasmids

Plasmids were assembled by polymerase chain reaction (PCR)-directed cloning and standard molecular biology procedures. Relevant parts of the plasmids and the resulting

vectors are shown in Figs. 1 and 2. ZFN pairs EB1/BA1 (here termed EB/BA) (Cornu *et al.*, 2008), E292 and E502 (Maeder *et al.*, 2008), and their respective recognition sites have been described before. For this study, the corresponding zinc-finger arrays were first transferred into a pRK5 plasmid backbone (Alwin *et al.*, 2005) that contained either of the obligate heterodimeric *FokI* variants KV or EA under control of a cytomegalovirus (CMV) promoter (Szczepek *et al.*, 2007; Söllü *et al.*, 2010), and then subcloned into the AAV backbone plasmids pFB-GFP (Urabe *et al.*, 2002) or pTR-UF (gift of Nicholas Muzyczka, Gainesville, FL). Plasmid pAV.LHA-Sce1Δ encodes a CMV-driven, C-terminally truncated version of I-SceI that served as a negative control. The AAV donor ΔGFPiNwpre and the lentiviral vector encoding the mGFPiNwpre target locus (Fig. 2) have been described before (Gellhaus *et al.*, 2010; Söllü *et al.*, 2010). The lentiviral vector encoding target locus dsEGFP (Fig. 1) was generated by adding two PEST domains at the C-terminus of EGFP of vector pLV-CMV.EGFPiNwpre (Cornu and Cathomen, 2007) and inserting the EB/BA target site in the ΔU3 region of the 3' long terminal repeat (LTR). Maps and sequences of all plasmids are available upon request.

AAV-mediated genome engineering

All experiments were performed with recombinant AAV type 2 vectors. With the exception of AAV.ZFN_{EB} and AAV.ZFN_{BA}, all AAV vectors were produced and purified as previously described (Gellhaus *et al.*, 2010). Vectors AAV.ZFN_{EB} and AAV.ZFN_{BA} were generated using the baculovirus system, as described in detail elsewhere (Smith *et al.*, 2009). AAV vector titers were determined by quantitative real-time PCR (LightCycler, Roche) using SYBR Green (DyNamo™ Capillary SYBR Green, Finnzymes) and appropriate primers, as previously described (Gellhaus *et al.*, 2010). For AAV-mediated genome editing, 5×10^4 target cells were infected with vector doses ranging from 10^3 to 10^5 genome copies (gc)/cell in 500–2000 μL of standard medium. To analyze AAV-mediated EGFP expression kinetics, 10^5 U2OS cells were transduced with either 50 or 500 gc/cell of AAV2.EGFP (Schwartz *et al.*, 2007). At the indicated time points, cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences). U2OS-based cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (Invitrogen). The polyclonal target cells were generated by lentiviral transduction (Cornu *et al.*, 2008), followed by selection with 0.4 mg/mL geneticin (Händel *et al.*, 2009).

Genotyping

Genomic DNA from target cells was extracted with the Blood MiniKit (Qiagen). To amplify part of the proviral DNA containing the EGFP locus, 100 ng of genomic DNA was used as a template, along with 10 μM of each primer (#1270 5'-tacctcaatggcgctggata and #598 5'-gaactccagcaggaccatgt), 10 mM dNTP, and 0.125 U of Phusion High-Fidelity DNA Polymerase (Finnzymes) in 1× reaction buffer for 25 cycles. A 150-bp amplicon of the PTBP2 allele (primers #1274 5'-tctcattccctatgttcacgc and #1275 5'-gttcccgcagaatggtgaggtg) served as a control. T7 endonuclease I (T7E1) assays were carried out as described previously (Hoher *et al.*, 2011; Mussolino *et al.*, 2011). In brief, 100 ng of the purified 544-bp

A

target

product

% EGFP-negative cells

gc/cell ($\times 10^4$)

EB BA EB&BA

B

EGFP

PTBP2

1 2 3 4 5 6 7 8 NT U H₂O

C

target

product

% EGFP-negative cells

gc/cell ($\times 10^4$)

vector E292 E502

D

ZFN

T7E1

544

446

E

5'-junction

3'-junction

E502L

E502R

F

ZFN NT H₂O

5'-junction 3'-junction

~800

Western blot analysis to detect ZFN expression was performed as previously (Shimizu *et al.*, 2011). To assess ZFN expression at the RNA level, 10^5 U2OS cells were transduced with 10^5 gc/cell of AAV.ZFN_{EB} and AAV.ZFN_{BA} each, and harvested in TRIzol reagent (Invitrogen) at indicated time points. The cDNA was generated using QuantiTect reverse transcription kit (Qiagen) and used as a template to amplify a 162-bp fragment of the *FokI* nuclease using primers #1436 5'-agtcaagagcgagctggaag and #1437 5'-cggtagccgtacaccttcac. The PCR products were separated on a 2% agarose gel and quantified from nonsaturated gel images with Quantity One

V.4.6.9 (BioRad) software (Hoher *et al.*, 2011; Mussolino *et al.*, 2011).

Cell cycle profiles and apoptosis

U2OS target cells were synchronized by serum starvation for 1 day. Then, 10^5 cells were transduced with 10^5 gc/cell each of both AAV-ZFN_{subunit} expression vectors or treated with 100 nM vinblastine for 4 hr. After 4 days, cells were fixed overnight in ethanol and stained with propidium iodide (PI) staining solution (3.8 mM sodium citrate, 25 μ g/mL of PI, 0.2 mg/mL of RNase A in phosphate-buffered saline). Before analysis by flow cytometry (FACSCalibur), low molecular weight DNA was released by incubating cells in DNA extraction buffer (200 mM Na₂HPO₄, 100 mM citric acid, pH 7.8). The fractions of cells in G1, S, and G2/M phases were determined by plotting FL3-W versus FL3-A. The number of cells containing a fragmented genome due to

apoptosis was quantified by determining the percentage of subG1 events by plotting the whole cell population as FL2-H versus cell counts.

Statistical analysis

All experiments were performed at least three times, with the exception of data point E502 in Fig. 4. Error bars represent standard deviation. Statistical significance was determined using a one-sided Student's *t*-test with unequal variance.

Results

AAV-ZFN-mediated gene disruption and provirus excision

As a paradigm for inactivating and/or deleting a retroviral sequence in the human genome, we generated a reporter line based on human osteosarcoma U2OS cells that expresses a destabilized EGFP (U2OS.693) by lentiviral transduction. Quantitative PCR established that U2OS.693 cells contain between three and seven copies of the provirus (data not shown). The lentiviral LTRs of the lentivector used contain a target site for the previously characterized ZFN pair EB/BA (Cornu *et al.*, 2008). Upon expression of both ZFN subunits the entire provirus should be excised, leaving just a single LTR signature in the genome (Fig. 1A). To this end, U2OS.693 cells were infected with 3×10^3 or 1×10^5 gc of AAV particles per cell, either individually with a single ZFN subunit or in combination, and the percentage of EGFP-expressing cells was determined 5 days later. As expected, successful excision of the provirus was dependent on the expression of both ZFN subunits and on the vector dose. After co-transduction with 10^5 gc/cell of AAV vectors

FIG. 2. AAV-mediated genome editing by homology-directed repair (HDR). **(A)** Schematic of gene correction in U2OS.893 cells. The target locus consists of a mutated EGFP (mGFP) gene under control of a cytomegalovirus (CMV) promoter, followed by an ires-Neo^R-wpre cassette. The mutation in mGFP is based on a 43-bp insertion that includes three in-frame stop codons and a recognition site for ZFN pair EB/BA (fat arrow). The AAV donor vector contains a 5'-truncated EGFP gene (ΔGFP) followed by the ires-Neo^R-wpre cassette. HDR (indicated by two crosses) between target locus and AAV donor leads to expression of a functional EGFP gene (product). **(B)** AAV-ZFN mediated gene targeting. U2OS.893 cells were transduced with the indicated vector dose of AAV donor and ZFN expression vectors, and assessed by flow cytometry 6 days post-transduction. The graph displays the average percentage of EGFP-positive cells; * and ** indicate statistically significant increase in EGFP-positive cells as compared to mock infected cells (*p* < 0.01 and *p* < 0.002, respectively). **(C)** AAV-mediated ZFN expression. U2OS cells were transduced separately with AAV-ZFN_{EB}, AAV-ZFN_{BA}, or a control vector at 10⁴ or 10⁵ gc/cell, harvested after 72 hr, and ZFN expression levels detected using an HA tag specific antibody. **(D)** Genotyping. Genomic DNA of U2OS.696 cells infected with 10⁴ gc/cell of AAV donor or AAV-ZFN, as indicated, was isolated 30 days post-transduction. The DNA was subjected to nested PCR analysis (primer positions shown in panel A) to detect the corrected target locus; 10⁴ and 10⁵ copies of an EGFP plasmid served as positive controls (cto1, cto2).

encoding the EB and BA subunits, 11% of U2OS.693 cells turned EGFP-negative. To verify ZFN-mediated excision of the provirus on the genome level, single clones were expanded and the genomic DNA of EGFP-negative cells isolated. PCR-based amplification of a region spanning most of the EGFP gene and part of the CMV promoter revealed that in the majority of clones all proviral genomes were excised (Fig. 1B). In three out of the eight clones at least one provirus copy remained untouched. Since all clones were EGFP negative, the respective expression cassettes must have been silenced before or after AAV transduction.

As a proof of principle that demonstrates AAV-ZFN mediated inactivation of functional gene expression, ZFN pairs targeting the EGFP marker gene at positions 292 (E292) and 502 (E502), respectively (Maeder *et al.*, 2008), were employed. U2OS.693 cells were infected with 10^3 , 10^4 , or 10^5 gc/cell of each ZFN expression vector and evaluated after 5 days. The percentage of EGFP-negative cells was dependent on both the vector dose and the ZFN pair used (Fig. 1C). Whereas 9% of cells lost functional EGFP expression upon infection with 10^5 gc/cell of the AAV vectors encoding the left and right subunits of the E292 ZFN pair, 32% of cells transduced with 10^5 gc/cell of the E502 encoding AAV vectors turned EGFP-negative. Molecular characterization of genomic DNA from pooled cells using the mismatch-sensitive T7E1 confirmed the presence of indels at the E502 site after expression of the respective ZFNs (Fig. 1D). To assess whether the AAV-ZFN expression vectors can integrate into a ZFN-induced DSB, a PCR strategy to detect vector-host genome junctions at the E502 site was applied (Fig. 1E). Both PCR amplifications produced the expected ~800-bp fragments (Fig. 1F), confirming AAV integration into nuclease-induced DSBs at low frequencies as previously shown (Gellhaus *et al.*, 2010).

Together these experiments demonstrated that AAV vectors encoding ZFNs can be efficiently employed to modify the human genome by harnessing the cellular NHEJ pathway for DNA repair. The AAV-ZFN platform was successfully used to either disrupt a gene or to delete an entire expression cassette, as illustrated by the excision of a lentivirus vector genome.

AAV-ZFN-mediated gene correction

To demonstrate AAV-mediated gene correction by HR, we established an U2OS-based cell line harboring a promoter-proximal 43-bp insertion that contains the recognition site for the ZFN pair EB/BA at position 327 of the EGFP marker gene (U2OS.893). Because promoter-free donors are advantageous, such a target configuration requires the generation of AAV donors with asymmetric homology arms. The AAV donor contained a 5'-truncated EGFP (δ GFP) and a homologous 3'-sequence stretch (ires-Neo^R-wpre). Of note, the 5'-homology arm between the donor and target was only 268 bp long (Fig. 2A).

U2OS.893 cells were co-infected with different amounts of AAV donor vector, AAV-ZFN_{EB}, and AAV-ZFN_{BA}, and the percentage of EGFP-positive cells was determined 10 days post-transduction (Fig. 2B). Co-infection of target cells with 10^4 gc/cell of donor vector and 10^5 gc/cell of both AAV-ZFN_{EB} and AAV-ZFN_{BA} led to correction of 6% of transduced cells. The percentage of EGFP-positive cells dropped significantly to 0.9% when the amount of donor was in-

creased to 10^5 gc/cell, suggesting that the ratio between donor and ZFN expression vector is crucial. Immunoblot analysis confirmed equal expression of both ZFN subunits upon transduction (Fig. 2C).

To verify that EGFP-positive cells were the result of a genuine HR event between the target locus and the AAV donor vector, genomic DNA from U2OS.893 cells was extracted 30 days post-transduction. The amplification products of an allele-specific PCR, in which primers that specifically recognize the corrected target locus but neither the AAV donor DNA nor the mutated target locus were employed (Gellhaus *et al.*, 2010), confirmed that efficient gene targeting was dependent on the presence of all three vectors (Fig. 2D).

Expression kinetics

Transient expression of ZFNs is crucial for many applications because prolonged expression of the designer nucleases may be toxic to the cell. To characterize expression kinetics of AAV-based transgene expression both at the protein and RNA levels, we transduced U2OS cells either with an AAV2-based EGFP expression vector or the AAV-ZFN vectors. U2OS cells transduced with 50 or 500 gc/cell of AAV-EGFP, respectively, were assessed by flow cytometry at 2, 5, and 8 days post-transduction (Fig. 3A). EGFP fluorescence dropped more than threefold between days 5 and 8, suggesting that the vector is rapidly diluted out in these

FIG. 3. AAV expression kinetics. **(A)** Protein expression. U2OS cells were transduced with an AAV-EGFP vector at 50 or 500 gc/cell, respectively, and subjected to flow cytometric analysis at the indicated time points. The graph displays mean fluorescence intensity measured in channel FL-1. **(B)** RNA expression. U2OS cells were transduced with AAV-ZFN_{EB} and AAV-ZFN_{BA} at 10^5 gc/cell. RT-PCR amplifying a fragment contained in the ZFN *FokI* domain was performed on RNA extracted from transduced cells at indicated time points. The position of the expected 162-bp band is indicated. Numbers below the gel indicate signal intensities relative to day 2. NT, nontransduced cells; AAV, cells transduced with an AAV control vector.

FIG. 4. Platform-associated toxicity. U2OS.693 cells were infected with 10^5 gc/cell of AAV-ZFN vectors, as indicated, fixed in ethanol 4 days after infection, stained with propidium iodide (PI) and analyzed by flow cytometry. Cells treated with 100 nM vinblastine (Vin) were assayed after 1 day and served as a control. **(A)** Cell cycle profile. The plots show the respective cell populations in G1 (gray), S (white), and G2/M phase (black). The average percentage of cells in G2/M phase is indicated. **(B)** Apoptosis. The graph displays the average fraction of cells in the subG1 population at day 4 post-transduction.

cultured cells. Total RNA was isolated from cells transduced with 10^5 of AAV-ZFN_{EB} and AAV-ZFN_{BA} each at days 2, 5, and 8 post-transduction. Reverse-transcription PCR confirmed rapid loss of ZFN expression over the timeframe of 1 week (Fig. 3B). Together these results confirmed the transient, short-lived nature of ZFN expression in mitotic cells.

AAV-ZFN-associated toxicity

In the present study we applied three ZFN pairs that were generated by selection-based approaches (Hurt *et al.*, 2003; Maeder *et al.*, 2008). To assess cytotoxicity associated with AAV-based expression of these ZFNs, U2OS cells were co-infected with 10^5 gc/cell of each vector encoding either ZFN subunit. After 2 and 4 days cells were fixed and stained with PI to perform a cell cycle analysis and determine the percentage of apoptotic cells by quantification of the subG1 fraction. The cell cycle profiles at day 2 (not shown) and day 4 post-transduction (Fig. 4A) revealed a modest increase in the G2/M populations for cells expressing the E292 and E502 ZFN pairs. In agreement with this modest effect on the cell

cycle, the percentage of the subG1 fraction, representing apoptotic cells, did not increase above background for all samples analyzed (Fig. 4B). Also Annexin V staining of these cells did not reveal any significant signs of apoptosis (data not shown). Together, these results suggest that AAV-mediated expression of ZFNs can induce a mild G2/M arrest but does not induce measurable levels of apoptosis.

Discussion

An important safety measurement when developing platforms for therapeutic ZFN expression in human cells is to make sure that nuclease expression quickly reaches a high peak value after transfection/transduction but that expression lasts only transiently. The latter point is of utmost importance because long-lasting high ZFN expression may increase ZFN-associated off-target activity and hence cytotoxicity (Alwin *et al.*, 2005; Doyon *et al.*, 2008; Meng *et al.*, 2008; Foley *et al.*, 2009; Pruett-Miller *et al.*, 2009). Nucleofection of plasmid DNA and transduction with adenoviral or integrase-deficient lentiviral vectors (IDLVs), respectively, have been successfully used to achieve ZFN-mediated gene editing in primary human cells (Lombardo *et al.*, 2007; Perez *et al.*, 2008; Holt *et al.*, 2010). Moreover, microinjection of ZFN-encoding mRNA into one-cell zebrafish and rat embryos was shown to allow for efficient gene disruption (Doyon *et al.*, 2008; Meng *et al.*, 2008; Geurts *et al.*, 2009). In this study we present AAV vectors as a potentially valuable vector platform for ZFN-mediated genome engineering based on NHEJ and HDR. We demonstrated that combining the two platforms allows for efficient genome editing in human cells, such as the targeted deletion of an entire expression cassette, the targeted disruption of an expressed gene, and the correction of mutations in an open reading frame.

The concentration and configuration of the donor DNA is a crucial parameter in HDR-based genome editing (Hirata and Russell, 2000; Moehle *et al.*, 2007; Gellhaus *et al.*, 2010; Orlando *et al.*, 2010). It has been reported previously that AAV vectors serve as efficient templates for HDR in human cells, both in the absence (Chamberlain *et al.*, 2004; Khan *et al.*, 2010) and the presence (Miller *et al.*, 2003; Porteus *et al.*, 2003; Gellhaus *et al.*, 2010; Hirsch *et al.*, 2010) of a DSB. For the experimental systems used in this and in our previous studies, however, we were not able to detect AAV-based gene targeting in the absence of a DSB-inducing enzyme. The reason for this discrepancy likely resides in the donor architecture, which in our case was optimized to work efficiently for DSB-stimulated gene targeting.

Co-applying the AAV donors with AAV-ZFN expression vectors resulted in functional gene correction in up to 6% of cells. Although this number is therapeutically relevant, it is about four times smaller as compared with a similar setting in which U2OS target cells were co-infected with the same donor and an AAV I-SceI expression vector (25% EGFP-positive cells) (Gellhaus *et al.*, 2010). Moreover, when the same cells were infected with a single AAV vector that combined donor function and I-SceI expression, 65% of cells revealed a corrected target locus (Gellhaus *et al.*, 2010). Hence, the lower gene targeting activity cannot be solely reduced to differences in nuclease activities. This is in agreement with previous comparisons between I-SceI and

ZFNs pairs, in which we did not detect more than twofold differences in the ability of these nucleases to stimulate gene targeting when plasmid transfection was used (Cornu *et al.*, 2008; Szczepek *et al.*, 2007). We therefore speculate that the drop in promoting gene targeting is a vector-associated phenomenon, and reducing the number of different AAV vector constructs from three to two may be crucial to further increase the gene targeting frequency. Future experiments will consequently focus on generating AAV vectors that harbor a so-called two-in-one ZFN construct; i.e., a combined expression cassette in which the two ZFN subunits are separated by a 2A autoprotease (Söllü *et al.*, 2010).

High peak expression of ZFN is a prerequisite to reach the critical nuclear concentration of the enzyme necessary to induce a targeted DSB. As shown previously for IDLVs and adenoviral vectors, sufficient ZFN expression can only be achieved with relatively high vector doses (Lombardo *et al.*, 2007; Perez *et al.*, 2008). In many experiments we observed efficient NHEJ and HDR-based genome editing only when applying 10^5 AAV-ZFN gc/cell. Although this is a large number, 10^5 copies of an expression cassette per cell correspond to about 0.5 μ g of a 4-kb plasmid vector per 10^6 cells, which is typically used for ZFN expression vectors in nucleofection protocols. On the other hand, we sometimes noticed inhibitory effects on gene targeting when using very high AAV doses. Whether this is related to saturation of the cellular transport machinery that shuttles the capsids from the cell surface to the nucleus or saturation of the DNA repair pathways that have to deal with high numbers of incoming single-stranded DNA vectors, needs to be addressed in future experiments.

The use of self-complementary AAV (scAAV) vectors (McCarty *et al.*, 2003; Wang *et al.*, 2003) may effectively reduce the required ZFN vector dose. scAAVs harbor a double-stranded DNA genome and are therefore not dependent on conversion into duplex forms, either via second strand synthesis or re-association kinetics, leading to faster and usually higher transgene expression. In a proof-of-concept approach Hirsch *et al.* (2010) showed that scAAV-based I-SceI expression vectors performed better in a gene targeting assay than single-stranded AAV counterparts. On the downside, the limited vector genome size of scAAV will not permit the packaging of a two-in-one ZFN expression cassette or a combination of donor and ZFN. The high AAV doses required to see biological effects in our studies suggest that high vector genome concentrations in the nucleus may allow for pairing of complementary genomes of (+) and (−) polarity and as a result bypass the requirement of DNA second strand synthesis.

ZFN and vector-associated toxicity is of utmost importance when transferring the AAV-ZFN platform to the clinic. It is known that episomal DNA vectors can integrate into naturally occurring DSBs (Miller *et al.*, 2004), and we and others have also shown that nuclease-induced DSB serve as a prominent integration site for viral vectors, including IDLVs and AAV (Cornu and Cathomen, 2007; Gellhaus *et al.*, 2010; Petek *et al.*, 2010). Also in this study, we detected integration of AAV vectors into the ZFN-induced DSB. However, although AAV vectors can integrate into nuclease-induced DSBs at low frequency, the majority of integrations seem to occur outside the target locus (Gellhaus *et al.*, 2010), most likely in naturally occurring DSBs or nuclease off-target sites (Petek *et al.*, 2010; Gabriel *et al.*, 2011). Moreover, we also

reported that a targeted DSB shifts the balance from illegitimate recombination to gene targeting (Gellhaus *et al.*, 2010), which strongly supports the use of designer nucleases to promote HDR-based genome engineering.

Quantitative assessment of ZFN-associated toxicity has been challenging, especially since the platforms to generate ZFNs improved in terms of producing designer nucleases with higher activity and lower toxicity (Händel and Cathomen, 2011). In this study we applied two assays to quantify AAV-ZFN-induced apoptosis but did not detect signs of toxicity. Cell cycle analysis, on the other hand, demonstrated a mild arrest of cells expressing ZFN pairs E292 and E502 in the G2/M phase. A G2/M phase arrest is indicative of DNA damage, likely caused by the combined on- and off-target activities of the respective ZFNs. Previous studies have further shown that transduction of cells with wild-type AAV2 or UV-inactivated AAV2 led to cell cycle arrest in the G2/M phase (Saudan *et al.*, 2000; Raj *et al.*, 2001). Under the experimental conditions used in our study, we only observed minimal effects of AAV transduction on the cell cycle. The absence of both Rep expression and UV irradiation of our AAV vectors could explain the different experimental outcomes. In summary, this study emphasizes the importance of a comprehensive and thorough analysis of the activities and toxicities triggered by the designer nucleases and/or the vector system.

In conclusion, this study validates AAV vectors as a valuable platform to deliver designer nucleases to target cells. Given the safety record of AAV vectors in clinical and preclinical applications, one can even envisage *in vivo* gene editing therapies, as recently demonstrated in the liver of neonatal mice (Li *et al.*, 2011). AAV vectors have also been used to efficiently transduce human iPS cells or mesenchymal stem cells *ex vivo*, opening the door for a wide range of applications in regenerative medicine by combining cell therapy with targeted genome engineering.

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Author Disclosure Statement

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