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Enhanced gene disruption by programmable nucleases delivered by a minicircle vector

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Targeted genetic modification using programmable nucleases such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) is of great value in biomedical research, medicine and biotechnology. Minicircle vectors, which lack extraneous bacterial sequences, have several advantages over conventional plasmids for transgene delivery. Here, for the first time, we delivered programmable nucleases into human cells using transient transfection of a minicircle vector and compared the results with those obtained using a conventional plasmid. Surrogate reporter assays and T7 endonuclease analyses revealed that cells in the minicircle vector group displayed significantly higher mutation frequencies at the target sites than those in the conventional plasmid group. Quantitative PCR and reverse transcription-PCR showed higher vector copy number and programmable nuclease transcript levels, respectively, in 293T cells after minicircle versus conventional plasmid vector transfection. In addition, tryphan blue staining and flow cytometry after annexin V and propidium iodide staining showed that cell viability was also significantly higher in the minicircle group than in the conventional plasmid group. Taken together, our results show that gene disruption using minicircle vector-mediated delivery of ZFNs and TALENs is a more efficient, safer and less toxic method than using a conventional plasmid, and indicate that the minicircle vector could serve as an advanced delivery method for programmable nucleases.

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INTRODUCTION

Targeted genetic modifications are of great value in biomedical research, medicine and biotechnology.[1] Currently, engineered nucleases such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) are widely used as a promising tool for targeted genome engineering. ZFNs and TALENs have a modular structure and are composed of DNA-binding domain linked to a nuclease domain derived from FokI restriction enzyme[2,3] The DNA-binding domains are zinc finger proteins and transcription activator-like effector in ZFNs and TALENs, respectively, and can be custom-designed to bind to specific genomic sequences, enabling targeted genetic modification at those sites. When introduced into cells, ZFNs and TALENs make double-strand breaks at their target sites, which can be repaired either through error-prone non-homologous-end-joining[4,5] or homologous recombination in the presence of donor DNA.[6–8] Non-homologous-end-joining can result in gene knockout and homologous recombination can lead to insertion of transgenes or correction of abnormal sequences at the target site. The efficiency of homologous recombination is usually more than 10 000 times higher in the presence of engineered nuclease activity. ZFN has been successfully used for targeted genome editing in many systems, such as *Drosophila*,[7,9] nematode, fish,[10] frog oocytes,[11] rodents,[12] plants[8,13–15] and human cells.[16–19] TALEN-mediated gene targeting has also been successfully demonstrated in several systems including plants,[20,21] zebrafish,[22] yellow catfish,[23] *Caenorhabditis elegans*,[24] rats,[25] mice[26] and human cells.[3]

To date, conventional plasmids,[3,20,27–32] integrase-defective lentiviral vectors,[18,33] adenoviral vectors,[34,35] adeno-associated viral vectors,[36] direct microinjection into embryos,[12,37–39] and recombinant proteins[40,41] have been used to deliver engineered nucleases. Among these approaches, plasmid-mediated delivery has been predominant because it is easy to generate the necessary components and vector integration into the host genome is relatively rare. However, the overall efficiency of obtaining gene-modified cells with this plasmid-based approach is often low.[29,42] A minicircle vector is a new generation of DNA vectors that lack extraneous bacterial sequences encoding antibiotic resistance genes and a bacterial origin of replication.[43,44] Several lines of evidence suggest that the minicircle possesses merits such as robust transgene expression,[43,45] high efficiency in gene transfer[46–48] and biosafety,[49] all of which indicate that the minicircle could be an efficient and safe delivery system for programmable nucleases.

Because of these advantages, we postulated that ZFN and TALEN delivery via the minicircle vector might facilitate targeted genetic modification. In this study, for the first time, we cloned ZFN and TALEN sequences into a minicircle vector and a conventional plasmid vector and compared the resulting nuclease activities and toxicities in cells. Our studies revealed that minicircle delivery of programmable nucleases resulted in significantly higher levels of target gene modification and reduced cytotoxicity compared with delivery by conventional plasmid vectors, supporting the use of the minicircle vector as an advanced nonviral delivery method for programmable nucleases.

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RESULTS

Preparation of minicircle DNA encoding ZFNs or TALENs

To compare the activities of programmable nucleases expressed by minicircles versus conventional plasmids, we used pcDNA 3.0 (Figure 1a) instead of the minicircle parental plasmid (Figure 1b) as the control vector because pcDNA 3.0 has been the primary vector used for programmable nuclease expression;[50–52] furthermore, the minicircle parental plasmid has not been used as an expression vector for programmable nucleases. Electrophoresis of the isolated minicircle vector showed that a major band of minicircle and a minor band of parental plasmid was present (Figure 1c). Given that the molecular weight of the minicircle vector is lower than that of the parental plasmid, the copy number of the parental plasmid would be much lower than that of the minicircle vector, suggesting high purity of the isolated minicircle vector.

Comparison of nuclease activities from minicircle-ZFN/TALEN and plasmid-ZFN/TALEN using surrogate reporters

To compare the nuclease activities of ZFNs and TALENs expressed by the minicircle vector (referred to as M-ZFN and M-TALEN, respectively) with those expressed by a conventional plasmid vector (referred to as P-ZFN or P-TALEN), we first performed surrogate reporter assays.[51–56] The reporter consists of the *mRFP* gene, followed by the nuclease's target sequence and the enhanced green fluorescent protein (*eGFP*) gene, which is out of frame with the *mRFP* gene (Figure 2a). If ZFNs or TALENs make a double-strand break at the target sequence of the reporter, frame shifts occur because of the generation of indels in the target sequence through the error-prone non-homologous-end-joining repair process, resulting in functional eGFP expression. The level of eGFP expression correlates with the activity of the programmable nucleases.[51–54] Flow cytometry was performed at 72 h after the cotransfection of the reporter plasmid and M-ZFN or P-ZFN into 293T cells. The frequency of $eGFP^+$ cells relative to transfected cells ($eGFP^+$/total $mRFP^+$ cells) was significantly higher, by 1.71-fold, in the M-ZFN-transfected cell population than in the P-ZFN group (Figures 2b and c), suggesting that the ZFN activity is higher when the enzyme is delivered by a minicircle vector versus a conventional plasmid. The total number of $mRFP^+$ cells was higher in the M-ZFN group than in the P-ZFN group, suggesting that the overall transfection efficiency is higher in cells transfected with M-ZFN versus P-ZFN.

Similar results were obtained with TALENs. Flow cytometry after cotransfection of the reporter and a *DGAT1*-targeting TALEN plasmid into 293T cells showed that the ratio of $eGFP^+$/total $mRFP^+$ cells was 1.54-fold higher in M-TALEN-transfected cells than in P-TALEN-transfected cells (Figures 3a and b), implying that TALEN activity also increased when the enzyme was delivered using a minicircle vector. Furthermore, the total number of $mRFP^+$ cells was 2.1-fold higher in the M-TALEN group than in the P-TALEN group, indicating that the overall transfection efficiency is higher in M-TALEN-transfected cells, which is compatible with the result using ZFNs.

Comparison of the endogenous gene mutation frequency from M-ZFN/TALEN and P-ZFN/TALEN using the T7E1 assay

We next tested the nuclease activity on the endogenous target sequence in human cells by analyzing genomic DNA isolated from M-ZFN/TALEN- or P-ZFN/TALEN-transfected cells using a T7 endonuclease I (T7E1) DNA cleavage assay. To analyze activity at the Z-224 target site, we designed PCR primers to obtain a 780 bp PCR amplicon with the target site at the center (at position 387). After T7E1 treatment, heteroduplexed DNA generated by the activity of Z-224 gives rise to two DNA bands of similar sizes (387 and 389 bp), which appear as a single band after gel electrophoresis. The T7E1 assay showed a 1.83-fold higher frequency of mutations in the M-ZFN-transfected cells (average 43%) than in the P-ZFN group (25%) (Figures 4a and b).

Next, we performed similar experiments using a pair of TALENs targeting the *DGAT1* gene. In this case, we designed primers to obtain a 590-bp PCR amplicon, with the target site at position 320. After T7E1 treatment, heteroduplexed DNA cleaved by the activity of the *DGAT1*-targeting TALEN gave rise to two DNA bands of unequal sizes (320 and 270 bp). The assay showed 1.76-fold higher frequency of mutations in M-TALEN- versus P-TALEN-transfected cells (Figures 5a and b). Thus, together our data suggest that minicircle vector-based delivery of ZFNs and TALENs is more effective than conventional plasmid-based delivery, leading to a higher frequency of endogenous gene mutations driven by programmable nucleases.

The mechanisms underlying higher gene editing efficiency after minicircle-mediated ZFN delivery

To determine why gene editing is more efficient after minicircle-mediated ZFN delivery, we evaluated the transcript expression level and vector copy numbers in cells transfected with equivalent amounts of P-ZFN and M-ZFN. Quantitative reverse transcription-PCR showed that the ZFN transcript level in cells transfected with M-ZFN was 4.8-fold significantly higher than in those transfected with P-ZFN (Figure 6a), indicating that the more efficient gene editing was attributable to higher ZFN expression. This finding is in line with previous results showing that ZFN protein levels correlate with ZFN activity.[51,53,57] Furthermore, quantitative PCR revealed that the vector copy number in cells transfected with M-ZFN was 1.8-fold significantly higher than in those transfected with P-ZFN (Figure 6b), suggesting that the M-ZFN transfection was higher than P-ZFN. To determine if the higher ZFN transcript expression in the M-ZFN group is solely due to the higher transfection, we calculated the transcript copy number normalized to the vector copy number, and found that it was 2.6-fold significantly higher in the M-ZFN versus the P-ZFN group (Figure 6c). This result suggests that the transcription activity from each copy of M-ZFN is higher than that from each copy of P-ZFN. Taken together, these results indicate that the higher gene editing efficiency after minicircle-mediated ZFN delivery is attributable to higher ZFN expression from M-ZFN versus P-ZFN, which is mediated by both higher transfection and higher transcription activity from each transfected copy of M-ZFN.

Comparison of cell viability after transfection of M-ZFN and P-ZFN

The gene editing by programmable nucleases, including ZFNs and TALENs, is often associated with reduced cell viability.[30,58,59] Although several methods have been developed to reduce ZFN-associated cytotoxicity,[59–62] the effects of changing the delivery method on cytotoxicity have not been tested. To address this issue, we compared the cytotoxicity associated with transfection of M-ZFN and P-ZFN using two different viability assays. A tryphan blue exclusion assay revealed that the viability of M-ZFN-transfected cells was significantly higher than that of P-ZFN-transfected cells (Figure 7a). Flow cytometry after annexin V and propodium iodide (PI) staining also showed that the number of PI^- annexin V^- cells (live cells) and PI^+annexin V^+ cells (dead cells) was significantly higher and lower, respectively, in the M-ZFN versus the P-ZFN group (Figures 7b–d), corroborating that the cytotoxicity of M-ZFN is lower than that of P-ZFN.

Lack of transgene integration after transfection with M-ZFN

To determine if the minicircle transgene undergoes genomic integration, we performed PCR using genomic DNA as template. Electrophoresis of the PCR amplicons showed that the minicircle

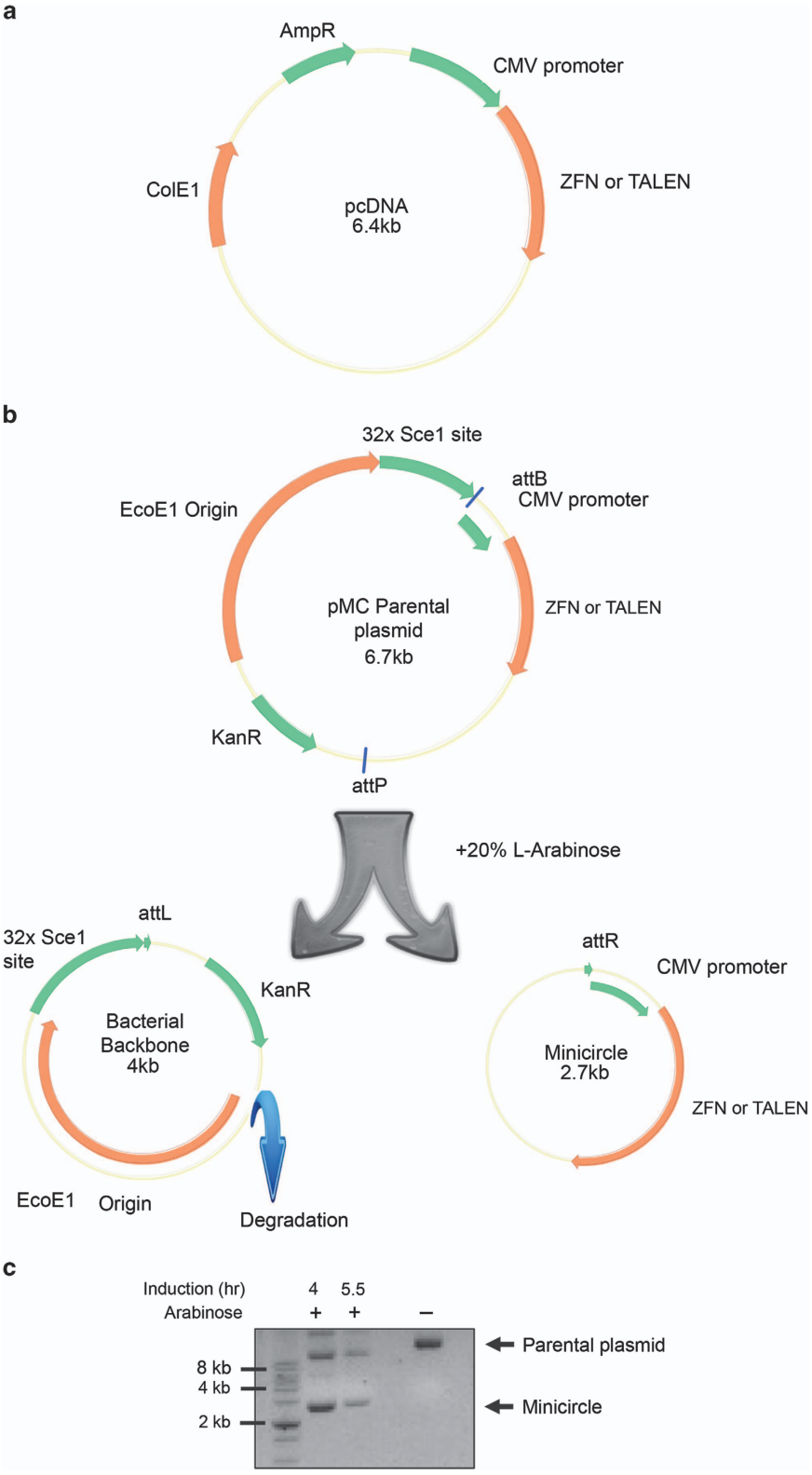
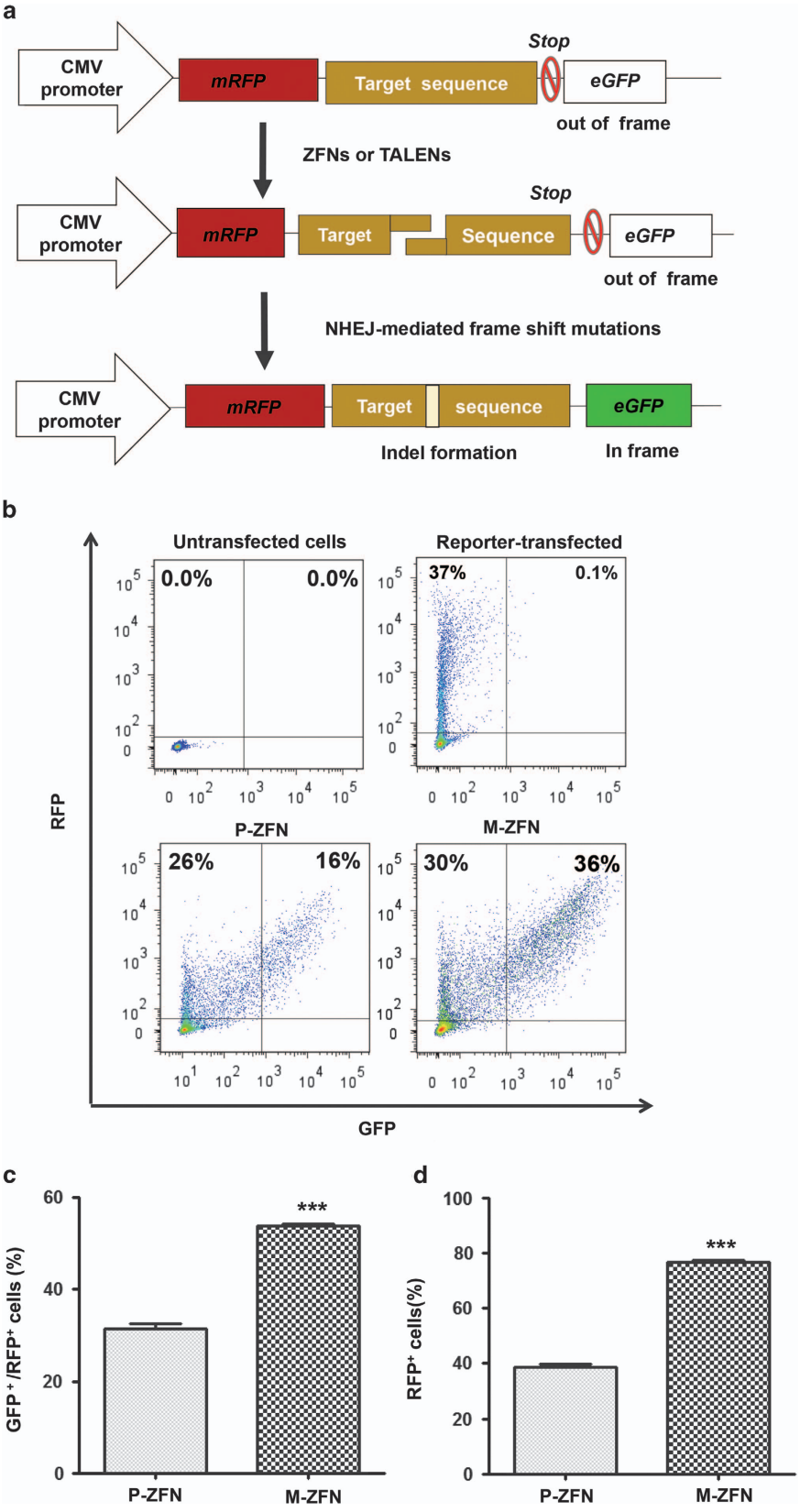


Figure 1. pcDNA and minicircle vector encoding zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). (**a**) Vector map of the pcDNA plasmid for expression of a ZFN or TALEN with the CMV promoter. (**b**) Generation of a minicircle vector for expression of a ZFN or TALEN with the CMV promoter. Addition of L-arabinose leads to the expression of ΦC31 integrase and SceI homing endonuclease in the host *E. coli* strain (ZYCY10P3S2T). ΦC31 integrase drives site-specific recombination between the attB and attP sites, leading to the generation of (i) a minicircle vector containing the CMV promoter, ZFN or TALEN, and hybrid sequence (attR) and (ii) a bacterial backbone plasmid containing the bacterial origin of replication (EcoE1 Origin), the antibiotic resistance gene (KanR) and the left hybrid sequence (attL). This backbone plasmid is subsequently linearized by SceI endonuclease and removed by bacterial exonucleases. (**c**) The quality of the minicircle preparation was analyzed by agarose gel electrophoresis. After arabinose induction for 4 or 5.5 h, the parental vector (6.7 kb) was degraded, giving rise to the minicircle vector (2.7 kb).

transgene is not present in genomic DNA (Figure 8), which is compatible with previous results reporting a lack of minicircle vector integration.[49]

DISCUSSION

Genome editing using ZFNs and TALENs is of great value in many different applications. Transient transfection using conventional



plasmids has been the predominant way to deliver these programmable nucleases into cells.[3,20,27–32] In this study, we report that minicircle vector-mediated delivery of programmable nucleases leads to higher frequencies of target gene modification and lower cytotoxicity than delivery with a conventional plasmid, indicating that the minicircle vector is an attractive delivery method.

The nuclease delivery method is critical to the success rate and activity of ZFNs and TALENs.[1] Minicircles contain very short minimal bacterial sequences, an important advantage over conventional plasmids in the field of gene therapy.[43,63,64] Thus, we used minicircles to deliver ZFNs and TALENs to achieve efficient and safe nuclease delivery for genome editing.

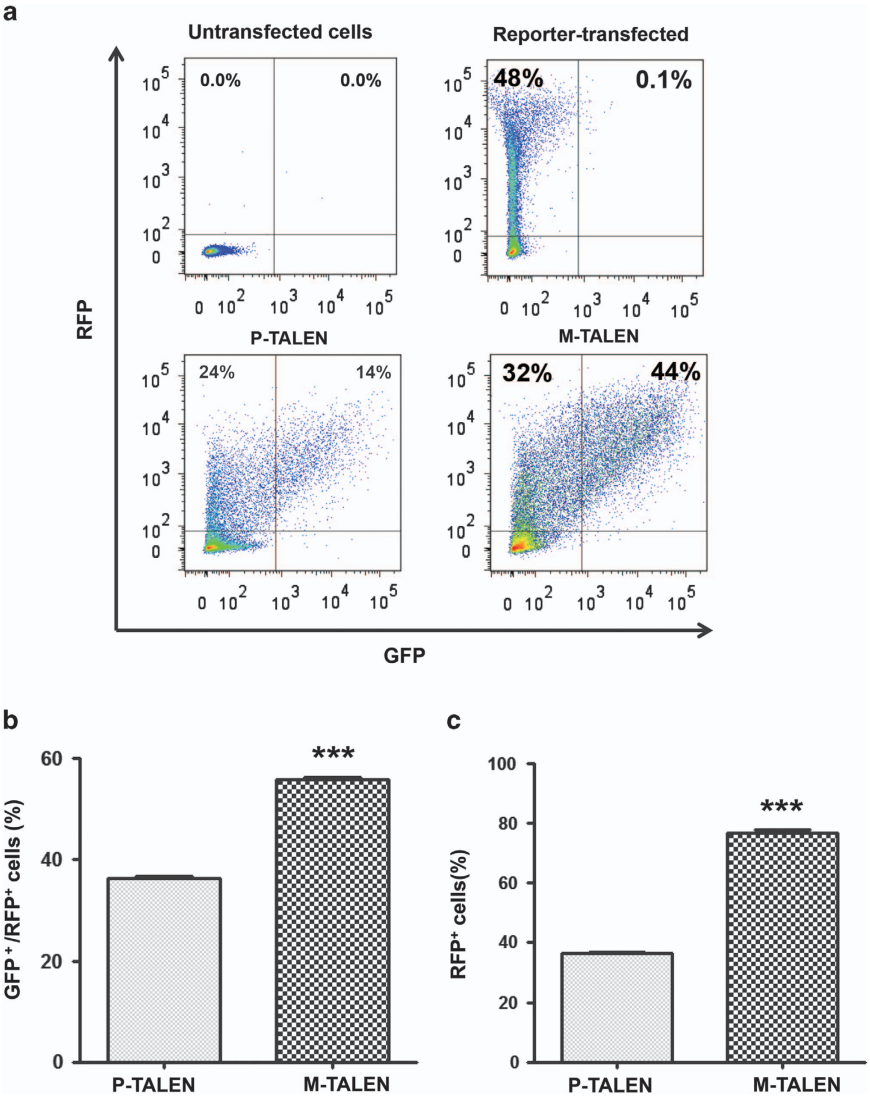


Figure 3. Surrogate reporter assay comparing TALEN activity. Flow cytometry was performed at 4 days after the transfection of 293T cells with either minicircle (M) or pcDNA (P) vector encoding the *DGAT1*-targeting TALEN together with reporter plasmid. (**a**) Representative flow cytometry. Untransfected cells and cells transfected with only reporter were used as analysis controls. (**b**) The percentage of GFP^+ cells among transfected cells (total RFP^+ cells). (**c**) The percentage of RFP^+ cells, which reflects the overall transfection efficiency. Error bar represents standard error. $n = 15$, $^{***}P < 0.001$.

Figure 2. Surrogate reporter assay comparing ZFN activity. (**a**) Working mechanism of the surrogate reporter. The reporter comprises the *mRFP* gene, the programmable nuclease's target sequence and the *eGFP* gene. *mRFP* is constitutively expressed from the CMV promoter, whereas eGFP is not expressed because its sequence is out of frame in the absence of programmable nuclease activity. When a double-strand break is introduced into the target sequence by ZFNs or TALENs, indels are generated in the target sequence as the break is repaired by nonhomologous end-joining (NHEJ), which often causes frameshift mutations. Such mutations can make *eGFP* in frame, leading to the expression of the mRFP-eGFP fusion protein. (**b**, **c**) Surrogate reporter assay using flow cytometry. Flow cytometry was performed at 72 h after the cotransfection of 293T cells with either minicircle (M) or pcDNA (P) vector encoding the *CCR5*-targeting ZFN together with reporter plasmid. (**b**) Representative flow cytometry. Untransfected cells and cells transfected with only the reporter were used as analysis controls. (**c**) The percentage of GFP^+ cells among transfected cells (total RFP^+ cells). (**d**) The percentage of RFP^+ cells, which reflects the overall transfection efficiency. Error bar represents standard error. $n = 18$, $^{***}P < 0.001$.

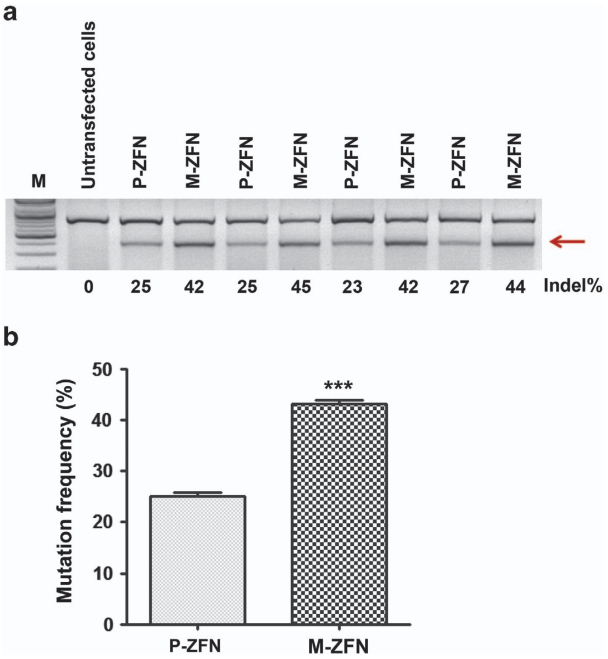


Figure 4. T7E1 assay after delivery of ZFN using the minicircle vector or pcDNA plasmid. Genomic DNA was isolated from 293T cells at 72 h after transfection with either minicircle (M) or pcDNA (P) vector encoding the *CCR5*-targeting ZFN and subjected to the T7E1 assay. (**a**) T7E1 assay. The numbers at the bottom of the gel denote mutation percentages calculated by band intensities. (**b**) Bar graph representation of the T7E1 assay analysis result. Error bar represents standard error. $n = 8$, $^{***}P < 0.001$.

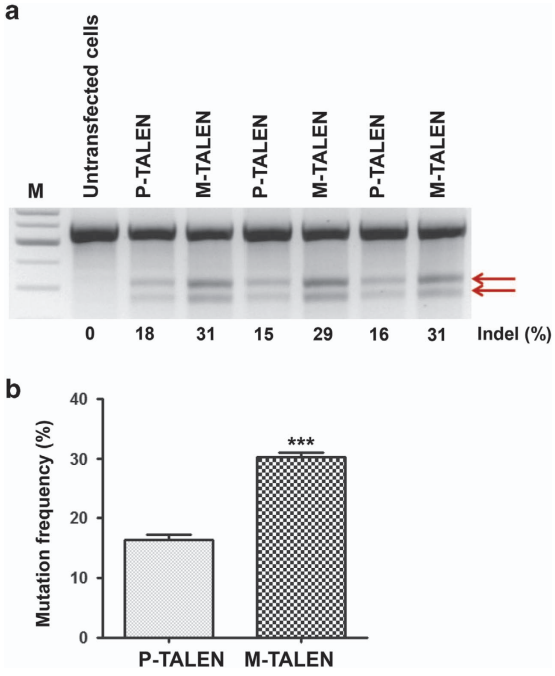


Figure 5. T7E1 assay after delivery of TALEN using the minicircle vector or pcDNA plasmid. Genomic DNA was isolated from 293T cells at 4 days after transfection with either minicircle (M) or pcDNA (P) vector encoding the *DGAT1*-targeting TALEN and subjected to the T7E1 assay. (**a**) T7E1 assay. The numbers at the bottom of the gel denote mutation percentages calculated by band intensities. (**b**) Bar graph representation of the T7E1 assay analysis result. Error bar represents standard error. $n = 8$, $^{***}P < 0.001$.

The higher level of target gene modifications in the minicircle group may be the result of higher expression of programmable nucleases in such vectors as compared with conventional plasmids; the ZFN protein level correlates with ZFN activity.[51,53,57] This idea is compatible with previous reports showing that minicircle vectors lead to higher transgene expression *in vitro*[65] and *in vivo*[43,66,67] as compared with conventional plasmids. Several factors have been proposed as possible mechanisms of robust transgene expression by minicircle vectors: higher transfection efficiency due to small size,[65] higher entry rate to the nucleus from the cytoplasm[65] and lower heterochromatin-associated histone modifications of the transfected minicircle vector.[67]

Given that higher prolonged expression of ZFNs often leads to enhanced cytotoxicity at the expense of higher levels of target gene modification,[59] our data showing that M-ZFN cytotoxicity was significantly lower than P-ZFN is of great value. This finding is compatible with a very recent report showing that minicircle vector-based transfection is associated with higher cell viability than that associated with conventional plasmids.[65] The bacterial sequences in the conventional plasmid have been reported to elicit immune responses because the unmethylated CpG motifs of bacterial sequences bind to Toll-like receptor 9,[68] which leads to the activation of a MyD88-mediated signal cascade. This cascade in turn results in the activation of innate and adaptive immune responses, leading to toxicity.[69] The finding that CpG-free plasmids are associated with reduced inflammation and robust, sustained gene expression as compared with conventional plasmids[70] indicates that the CpG sequence in the conventional plasmid might have a role in this toxicity.

In the surrogate reporter assay, the overall transfection efficiency in the minicircle group was higher than in the pcDNA group. This difference would be at least partly attributable to the toxicity of pcDNA transfection as shown in Figure 7. If pcDNA-transfected cells are selectively damaged, the consequent transfection efficiency in viable cells would be higher in the minicircle group.

Given that programmable nucleases are and will be actively investigated to develop therapeutic modalities, the biosafety of their delivery is an important issue. Because there are safety issues associated with viral vectors,[71] nonviral delivery methods such as plasmid DNA are considered for transgene expression. However, bacterial sequences can cause unwanted inflammatory responses[68] and, if the antibiotic resistance genes are taken up by pathogenic bacteria, can lead to serious problems such as infection with antibiotic-resistant bacteria. Thus, minicircle vector-mediated delivery of programmable nucleases would be a safer method than delivery with conventional plasmids.

Previously, minicircle vectors required a somewhat complicated preparation process as compared with conventional plasmids, but minicircle DNA production and induction conditions have now been optimized and simplified.[72] Furthermore, these vectors are also commercially available from various companies.

In general, the gene editing efficiency of ZFNs is higher than that of TALENs,[1] although in this study, the specific ZFNs we used showed higher activity than the TALENs. In both systems, enhanced delivery of nucleases using a minicircle vector leads to improved gene editing efficiency. This enhanced gene editing by improved delivery is in line with enrichment of gene edited cells by selection of transfected cells.[73–75]

In conclusion, we have shown that minicircle vector-mediated delivery of programmable nucleases such as ZFNs and TALENs into human cells leads to higher levels of target gene modification with reduced cellular toxicity as compared with delivery by a conventional plasmid. Thus, we propose a minicircle vector as an efficient, safe and attractive method to deliver programmable nucleases, facilitating their use.

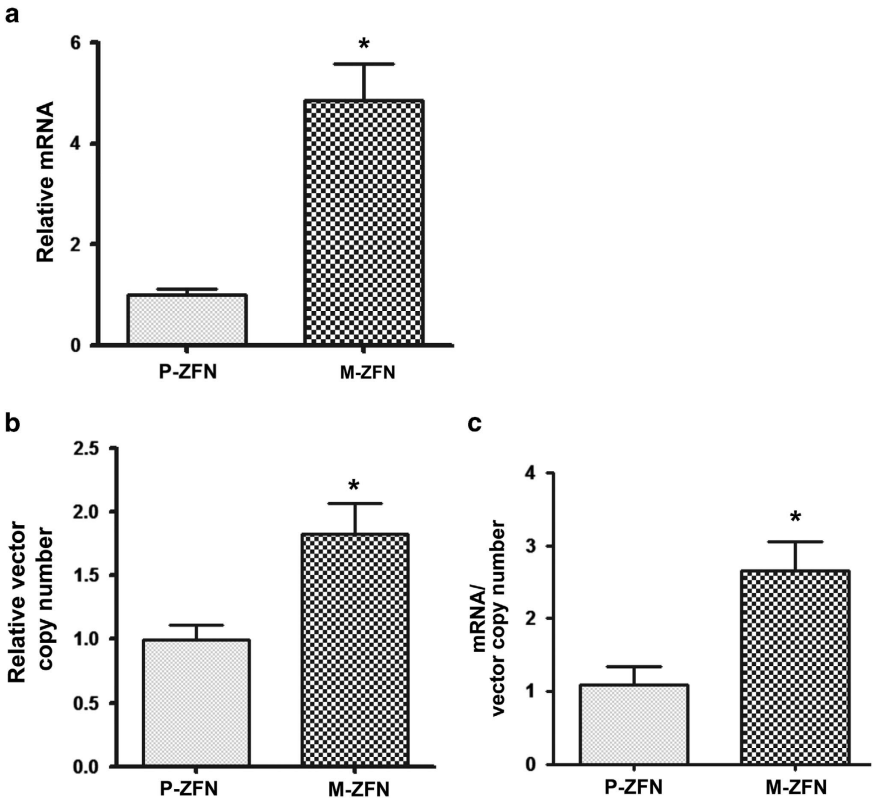


Figure 6. Transcript and vector copy numbers in cells after minicircle- or conventional plasmid-mediated ZFN delivery. 293T cells were transfected with equal copy numbers of either minicircle (M) or conventional plasmid (P, pcDNA 3.0) vector encoding the *CCR5*-targeting ZFN (Z-224). At 24 h after transfection, total RNA isolated from the cells was subjected to quantitative reverse transcription-PCR for evaluation of transcript copy number (**a**) and total cell lysates were subjected to PCR for determining vector copy number (**b**). The transcript copy number normalized to the vector copy number was calculated (**c**). The transcript copy number, vector copy number and the transcript copy number normalized to vector copy number were all significantly higher in the minicircle group than in the conventional plasmid group. $n = 4$, $^*P < 0.05$.

MATERIALS AND METHODS

Plasmids encoding ZFNs and TALENs

We subcloned sequences encoding a pair of *CCR5*-targeting ZFNs (Z-224[19,50,53]) and a pair of *DGAT1*-targeting TALENs into a minicircle parental plasmid and the pcDNA 3.0 plasmid; in both cases, expression of the programmable nuclease is driven by the cytomegalovirus (CMV) promoter. Detailed information about ZFN-224, which targets *CCR5*, has been previously described.[19,50,53] The target sequence of the *DGAT1*-targeting TALENs is 5′-TGCCATCGCCTGCAGGATTC-3′(Left)-tttattcagctct(spacer)-5′-GACAGTGGCTTCAGCAACTA-3′(right). The plasmids encoding *DGAT1*-targeting TALENs were prepared as recently described[76] and purchased from Toolgen (Seoul, South Korea; Order number H157465, http://www.talenlibrary.net/). All plasmid vectors were amplified using the DH5α strain of *Escherichia coli*.

Minicircle vector

Recombinant minicircles were prepared from the parental plasmid using a specifically engineered strain of *E. coli* (*ZYCY10P3S2T*) that expresses ΦC31 integrase and SceI endonuclease in the presence of arabinose. After arabinose induction, ΦC31 integrase splits the full-size parental plasmid into (i) a minicircle DNA encoding a ZFN or TALEN and (ii) the extraneous bacterial backbone (Figure 1b). The extraneous bacterial sequence, which contains multiple SceI restriction sites, undergoes Sce1-mediated destruction, whereas the minicircle DNA, which lacks SceI restriction sites, remains intact and can be isolated. To construct a minicircle parental plasmid with the CMV promoter, the EF1 alpha promoter was removed from the parental minicircle plasmid (MN502-1; System Biosciences, Mountain View, CA, USA) using SpeI and XbaI and replaced with a CMV promoter amplified from the pMIRNA1 vector (System Biosciences) using PCR. The DNA fragment encoding ZFN-224 and the *DGAT1*-targeting TALENs were then cloned into the minicircle parental plasmid vector containing the CMV promoter after PCR amplification. The minicircle plasmid was obtained from the parental plasmid as previously described.[77] Briefly, we inoculated cells from one transformed colony into 2 ml of Terrific Broth (pH 7.0) with kanamycin (50 μg ml^{-1}) and incubated the cells at 37 °C with shaking at 250 r.p.m. We later combined 10 μl of culture with 50 ml Terrific Broth containing kanamycin (50 μg ml^{-1}) and continued incubation for 16 to 18 h. At this time, the A_{600} of the culture was 3.5–4.2 with a pH of ~6.5. We then prepared a minicircle induction mixture that comprised of 50 ml fresh Luria-Bertani Broth, 2 ml 1N sodium hydroxide, and 0.5 ml 20% l-arabinose, combined it with a 50 ml overnight culture, and incubated the culture at 32 °C with shaking at 250 r.p.m. for an additional 5–5.5 h. Minicircle DNA was isolated from 4 ml of bacterial lysate using a miniprep plasmid purification kit (Bioneer, Daejun, South Korea) according to the manufacturer's instructions and used for transfection. The purity of isolated minicircle vector was evaluated using 0.8% agarose gel electrophoresis.

Cell culture and transfection

HEK 293T cells were cultured in Dulbecco's Modified Eagle's medium (Gibco-BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (Gibco-BRL) and penicillin (100 U ml^{-1})/streptomycin (100 mg ml^{-1}) and were transfected using Fugene HD (Roche, Branford, CT, USA). To perform surrogate reporter assays, the weight ratio of the DNA encoding one member of a ZFN or TALEN pair: DNA encoding the other member of the pair: reporter was 1:1:1; 0.5 μg of each plasmid (total 1.5 μg DNA) was transfected into cells (at 80% confluency) in a well of 12-well plate. At 72 h after transfection of ZFN-encoding plasmids, the cells were harvested and subjected to flow cytometry. In the case of TALENs, cells were subjected to transient cold shock to enhance nuclease stability and activity.[57] The cells were cultured at 37 °C for 24 h after transfection and then cultured at 30 °C for 3 days until flow cytometry. For the T7E1 assay, 0.5 μg of DNA encoding

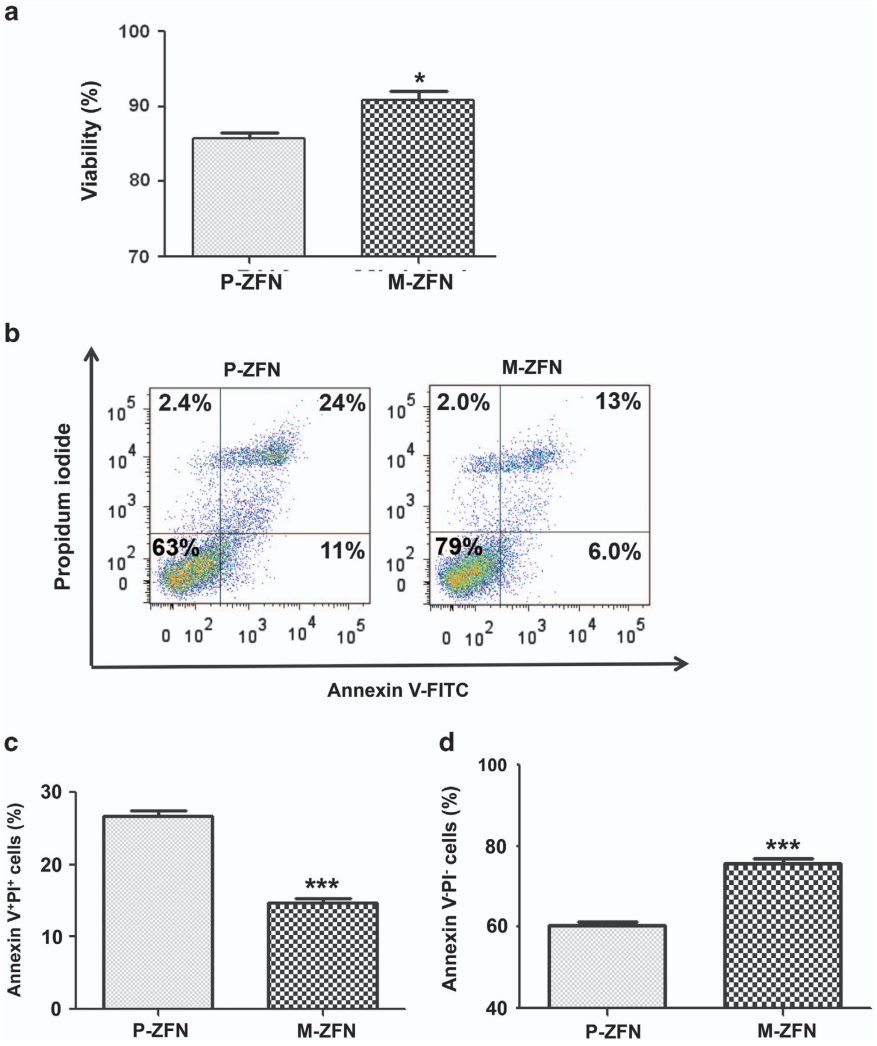


Figure 7. Viability of cells after minicircle- or conventional plasmid-mediated delivery of ZFNs. The viability of 293T cells was determined using tryphan blue staining (**a**) or annexin V and propodium iodide (PI) staining (**b**, **c** and **d**) at 72 h after transfection with either minicircle (M) or conventional plasmid (P, pcDNA 3.0) vector encoding the *CCR5*-targeting ZFN (Z-224). (**a**) Tryphan blue assay. Error bar represents standard error. $n = 4$, $^{*}P < 0.05$. (**b**) Representative flow cytometry after annexin V and PI staining. (**c**) Bar graph representation of annexin V- and PI-positive cells (dead cells). $n = 5$, $^{***}P < 0.001$. (**d**) Bar graph representation of Annexin V- and PI-negative cells (live cells). Error bar represents standard error. $n = 5$, $^{***}P < 0.001$.

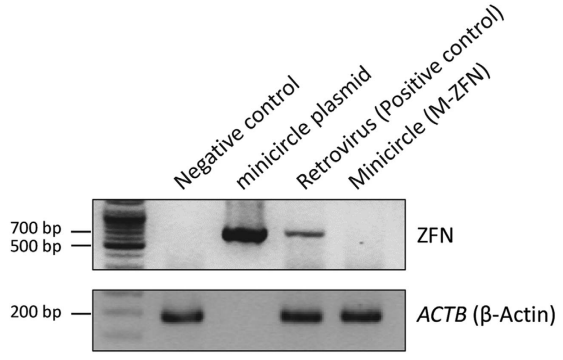


Figure 8. Lack of transgene integration after transfection with minicircles. After M-ZFN transfection into 293T cells, the genomic DNA that is not contaminated with episomal plasmid was isolated and subjected to PCR to detect minicircle vectors integrated in the host genome. Genomic DNA isolated from untransfected cells and cells transduced with ZFN-expressing retrovirus were used as negative and positive controls, respectively. Minicircle plasmid was also used as control for the PCR.

one member of a ZFN or TALEN pair and 0.5 μg of DNA encoding the other member of the pair were co-transfected into cells (at 80% confluency) in a well of 12-well plate. To evaluate transcript levels and vector copy numbers, 1 μg of DNA encoding one member of a ZFN pair and 1 μg of DNA encoding the other member of the pair were co-transfected into 293T cells (at 80% confluency) in a well of six-well plate.

Flow cytometry

Single-cell suspensions were analyzed using FACSAria II (BD Biosciences, San Jose, CA, USA) as previously described.[51] For the reporter assay, untransfected cells and cells transfected with reporters alone were used as analysis controls.[51–53] For the viability assay, unstained cells and single-color stained cells were used as analysis controls. In each sample, 20 000 cells were analyzed. FlowJo (version 10.0, Tree Star, Inc., Ashland, OR, USA) was used to analyze the data.

T7E1 assay

The T7E1 assay was performed as previously described.[50,51,53] Briefly, genomic DNA was isolated using a genomic DNA purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The region including the engineered nuclease target sites on the genomic DNA was PCR-amplified using the following primers: Z-224 (Forward primer,

5′-GAGCCAAGCTCTCCATCTAGT-3′; Reverse primer, 5′-CTGTA GG AA TGAGAGCTGC-3′) and *DGAT1*-targeting TALEN (Forward primer 1, 5′-CAAG CTCCATGTAGGTCCAG-3′; Reverse primer 1, 5′-GCTCTCCTGGGATCCAATG-3′; Forward primer 2, 5′-TCTGACCCTGACATGCTCGT-3′; Reverse primer 2, 5′-CCAATGGGAAGCAGCAAGTA-3′). For analysis of the *DGAT1* gene, nested PCR was performed; the first PCR product using primer pair 1 was subjected to a second round of PCR using primer pair 2. The PCR amplicons were denatured by heating and annealed to form heteroduplex DNA, which was treated with 5 U of mismatch-sensitive T7 endonuclease 1 (New England Biolabs, Hitchin, UK) for 20 min at 37 °C and then analyzed using 2% agarose gel electrophoresis. If small indels are generated in the target sequence of the ZFN-224 group, T7E1 treatment gave rise to 387 and 389 bp DNA fragments, which appear as only one band, whereas treatment in the *DGAT1*-targeting TALEN group gave rise to 320 and 270 bp DNA fragments, which were observed as two separate bands after gel electrophoresis.

Cell viability test

Cells were harvested using trypsinization at 72 h after transfection and cell viability was determined using either tryphan blue staining or flow cytometry after annexin V and PI staining. For tryphan blue staining, the harvested cells were stained using 0.4% tryphan blue (Gibco-BRL) and the cell viability was determined using microscopic evaluation. For flow cytometric determination of viability, the cells were stained with the annexin V-FITC kit (BD biosciences) according to the manufacturer's protocol. Briefly, a total of 1×10^6 cells per ml were washed twice with cold PBS, resuspended in 100 µl 1X binding buffer, and incubated for 10 min at room temperature in the dark after addition of 5 µl of fluorescein isothiocyanate-conjugated annexin V (catalog number 51-65874X) and 5 µl of PI (50 µg ml^{-1} stock solution). At the end of the incubation, 200 µl of 1X binding buffer was added and the cells were analyzed within 1 h using flow cytometry.

Real-time reverse transcription-PCR and PCR for quantification of transcript and vector copy number

Using an RNeasy mini kit (Qiagen, Mississauga, ON, USA), total RNA was isolated from 293T cells 24 h after transfection with 1 µg of one member of a M-ZFN or P-ZFN pair and 1 µg of the other member of the pair. Using the isolated RNA as template, complementary DNA synthesis was carried out using the Superscript III enzyme (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR was performed according to the instructions provided by the company (KAPA SYBR FAST qPCR kit; KAPA Biosystems, Boston, MA, USA) using the C1000 Thermal cycler detection system (BIO-RAD, Hercules, CA, USA), 100 ng prepared complementary DNA, and primers designed to amplify a 134 bp sequence within the ZFN FokI domain (forward primer: 5′-TTTGGGTGGATCAAGGAAACCG-3′ and reverse primer: 5′-GTTGCATTTCA TCTGCTTGGCC-3′). The thermocycler conditions were 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, 10 s at 58 °C and 15 s at 72 °C. To determine vector copy number, 20 000 transfected 293T cells were subjected to quantitative PCR as described above.

Determination of transgene integration into the host genome

293T cells transfected with a CCR5-targeting M-ZFN were harvested at 72 h after transfection; genomic DNA was isolated and subjected to PCR for determining transgene integration. The primers designed to amplify a 697 bp sequence that include the junction between the ZFN and FokI domain (forward primer: 5′-AAGAAGCGGAAGGTGGGCAT-3′ and reverse primer: 5′-GCATTTCATCTGCTTGGCCAAT-3′) and a 184 bp sequence within the *ACTB* (β-actin) gene (forward primer: 5′-TCCTCTCCCAAGTCCACACAG-3′ and reverse primer: 5′-AGACCAAAAGCCTTCATACATCTC-3′) were used. As a positive control for genomic integration, we used 293T cells transduced with a retrovirus expressing the CCR5-targeting ZFN. To generate this retrovirus, a murine stem cell virus retroviral vector (kindly provided by Professor Chung Hee Yong, Hanyang University, South Korea) and packaging plasmid pIK6.1MCV.ecopac.UTD (Ecopac; M. Finer Cell Genosys, Redwood City, CA, USA) were co-transfected into 293T cells. Two days after transduction, genomic DNA was isolated from the harvested cells and used as the positive control for PCR. To remove any residual episomal vectors from the genomic DNA, isolated genomic DNA was subjected to gel electrophoresis on a 0.8% agarose gel and purified from the gel at least three times.

Statistical analysis

All data were expressed as mean ± s.e.m and statistical analysis was conducted using PASW Statistics, version 18 (SPSS Inc., Chicago, IL, USA). Student's *t*-test was used for the statistical analysis for continuous variables between two groups. A P-value < 0.05 was considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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