# TALENs-mediated homozygous *CCR5Δ32* mutations endow CD4+ U87 cells with resistance against HIV-1 infection

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**Abstract.** Since evidence suggests that transplantation of bone marrow stem cells with the C-C chemokine receptor type 5 (*CCR5*)*Δ32/Δ32* genotype may cure patients infected with human immunodeficiency virus (HIV)-1, the present study aimed to reproduce the *CCR5Δ32* mutation in cluster of differentiation (CD)4+ U87 cells using genome engineering methods. A modified transcription activator-like effector nucleases (TALENs) technique, combined with homologous recombination for site-specific, size-controlled and homozygous DNA deletions, was used to reproduce the homozygous *CCR5Δ32* mutation in CD4+ U87 cells. The results indicated that the frequency of the TALENs-targeted mutation reached 50.4% without any selection, whereas homologous recombination from *CCR5* to *CCR5Δ32* occurred in 8.8% of targeted cells. Notably, a HIV-1 challenge test demonstrated that *CCR5Δ32/Δ32* CD4+ U87 cells were resistant to HIV infection. In conclusion, engineered *CCR5Δ32/Δ32* mutations endowed CD4+ U87 cells with resistance against HIV-1 infection; this site-specific, size-controlled and homozygous DNA deletion technique was able to induce precise genomic editing, i.e., the deletion or insertion of a predetermined length of DNA sequence at a specific locus throughout the genome.

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*Abbreviations:* TALENs, transcription activator-like effector nucleases; CCR5, C-C chemokine receptor type 5; ZFNs, zinc finger nucleases; HSPCs, hematopoietic stem and progenitor cells; SDSA, synthesis-dependent strand annealing; T7E1, T7 endonuclease 1

*Key words:* CCR5Δ32, homologous recombination, genome editing, TALENs

## Introduction

C-C chemokine receptor type 5 (CCR5) is a co-receptor for human immunodeficiency virus (HIV)-1 present on the surface of target cells, including cluster of differentiation (CD)4+ T lymphocytes (1). A small fraction of Europeans (1%) are homozygous for a 32-bp deletion within the coding region of both *CCR5* alleles (*CCR5Δ32/Δ32* genotype), which produces a polymorphic form of CCR5 that is not present on the cell surface, and thus confers strong protection against HIV-1 infection (2). CCR5 antagonists can block HIV-1 entry into target cells; at present, one small molecule CCR5 antagonist has been approved for clinical use (3).

In 2007, an HIV-1-infected patient with acute myeloid leukemia received transplantation of bone marrow stem cells from a donor with the *CCR5Δ32/Δ32* genotype, and the viral load in this patient has since been undetectable (4,5). Therefore, replacement of host CD4+ T lymphocytes with engineered *CCR5Δ32/Δ32* genotype cells is believed to represent a method by which HIV-1 infection may be cured. Various gene-targeting techniques could be used to produce genetically engineered cells, including zinc finger nucleases (ZFNs) (6,7), transcription activator-like effector nucleases (TALENs) (8-10) and the RNA-guided CRISPR/Cas9 nuclease system (11,12), which can be used to induce random mutations (deletion and/or insertions) or insert a specific gene at specific loci.

Various techniques have been used to disrupt *CCR5* in hematopoietic stem and progenitor cells (HSPCs), CD4+ T lymphocytes and induced pluripotent stem cells (iPSCs) (7,13-17). Disruption of *CCR5* by ZFNs can efficiently inhibit HIV-1 infection of CD4+ T cells (7). In addition, ZFN modification of *CCR5* in primary human CD4+ T cells protects cells from infection with CCR5- and CXCR4-trophic HIV-1 strains (6). TALENs recognize only one nucleotide, instead of the three required for ZFNs (9), and can target sites in the *CCR5* loci with less cytotoxicity than ZFNs (8). This technique

has been reported to protect *CCR5*-expressing T cells from R5-tropic HIV (10). In addition, Wang *et al* (11) recently silenced *CCR5* via Cas9 and *CCR5*-specific single-guide RNA in CEM cells, whereas Hou *et al* (12) extended this to *CXCR4* in primary CD4$^+$ T cells.

Although bi-allelic disruption of the *CCR5* gene can prevent infection of target cells, including CD4$^+$ T lymphocytes, concerns have been raised suggesting that cells with non-functional *CCR5* may lose some important immune functions (18); however, individuals with the *CCR5Δ32/Δ32* genotype do not experience any discernable deleterious clinical effects (19,20). Recently, Ye *et al* (21) homozygously reproduced the naturally existing *CCR5Δ32* mutation in iPSCs by combining the TALENs or CRISPR/Cas9 technique with the PiggyBac technique, as a 'TTAA' tetranucleotide sequence happens to be located close to the to-be-deleted 32 bp region. The established *CCR5Δ32/Δ32* iPSC clones maintained pluripotency and resistance to HIV-1 infection, further indicating that the *CCR5Δ32/Δ32* genotype is safe for cells.

Site-specific, size-controlled and homozygous DNA deletion remains a major challenge in mammalian genome engineering. The present study established an efficient method to homozygously reproduce the natural *CCRΔ32* mutation in CD4$^+$ U87 cells using a TALENs-mediated homologous recombination technique. Engineered CD4$^+$ U87 cells with the *CCR5Δ32/Δ32* genotype exhibited significant resistance to HIV-1 infection.

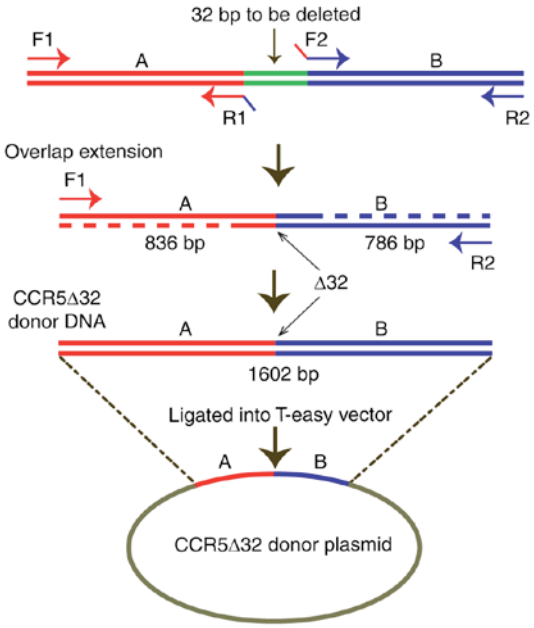


Figure 1. Schematic diagram of *CCR5Δ32* donor DNA construction. Homologous arms A and B were first amplified separately with F1 and R1, and F2 and R2, respectively. Their products, 836 and 786 bp in length, were then mixed and annealed with each other, followed by the next round of polymerase chain reaction with F1 and R2. The final product (A+B, 1,602 bp in length) was finally ligated into the *Eco*RI/*Bam*HI digested T-easy vectors. CCR5, C-C chemokine receptor type 5.

## Materials and methods

*Cell culture*. CD4$^+$ U87 cells were acquired from American Type Culture Collection (Manassas, VA, USA). CD4$^+$ U87 cells were originally derived from glioma cells expressing *CCR5* and *CXCR4*, and were stably transfected with a CD4 receptor gene to mimic CD4$^+$ T lymphocytes, and a puromycin gene resistance for selection. CD4$^+$ U87 cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 1% penicillin and streptomycin, 1%, amphotericin B, 1% sodium pyruvate, 1% L-glutamine and 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in a $CO_2$ incubator at 37˚C.

*Construction of CCR5Δ32 donor DNA plasmids*. *CCR5Δ32* DNA plasmids were constructed by overlap extension PCR. To mimic the naturally occurring *CCR5Δ32*, mutation, the 32 bp DNA fragment (3,299-3,330 bp) was deleted from the wild-type *CCR5* (Gene ID:1234, https://www.ncbi.nlm.nih.gov/gene/1234). Two sets of primers, F1 (5'-CACAAGATTTTATTTGGTGAGA-3') and R1 (5'-CTATCTTTAATGTATGGAAAATGAGAGCTG-3'), and F2 (5'-TTTCCATACATTAAAGATAGTCATCTTGGG-3') and R2 (5'-ATACATAAGGAACTTTCGGAGT-3'), were designed for both sides of the 32 bp DNA fragment, as indicated in Fig. 1. The two homologous arms, 836 and 786 bp in lengths, were separately amplified by PCR with the primers F1/R1 and F2/R2, respectively, and were then used as DNA templates for the next round of PCR with the primers F1 and R2. The products (1,602 bp in length) were confirmed to contain the correct *CCR5Δ32* sequence by gene sequencing (data not shown), and were finally ligated into *Eco*RI/*Bam*HI digested T-easy vectors (Promega Corporation, Madison, WI, USA).

*Designation and selection of TALENs*. CCR5-TALENs were designed with the right arm mostly overlapping the to-be-deleted 32 bp region and the left arm 14-18 bp upstream (Fig. 2A). It is critical to ensure arms are mostly within the specific regions to prevent the donor DNA and the mutated *CCR5Δ32* from further targeting by CCR5-TALENs. In addition, the CCR5Δ1-TALENs were designed with both arms overlapping the to-be-deleted 31 bp region (Fig. 2B). The TALENs plasmids were constructed by one-step ligation using the Fast TALE™ TALEN Assembly kit (Sidansai Biotechnology Co., Ltd., Shanghai, China). Altogether, six pairs of TALENs plasmids were designed and constructed to target both *CCR5* and *CCR5Δ1*. After a preliminary transfection, two pairs of TALENs plasmids, L4309/R4324 for *CCR5*, and L4336/R4352 for *CCR5Δ1*, with the highest targeting efficiencies were selected for use in the subsequent experiments. The plasmids were designated CCR5-TALENs and CCR5Δ1-TALENs accordingly.

*Transfection*. CD4$^+$ U87 cells (~1x10$^6$) were mixed with 8 $\mu$g paired CCR5-TALENs plasmids (each 4 $\mu$g) and 2 $\mu$g *CCR5Δ32* donor DNA in a cuvette (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with 100 $\mu$l Opti-minimal essential medium (Gibco; Thermo Fisher Scientific, Inc.). Electroporation was conducted at 150 V using a transfection system (NEPA21; Nepa Gene Co., Ltd., Chiba, Japan). Transfected CD4$^+$ U87 cells were transferred into one well of a 6-well plate and cultured at 37˚C for 3 days, after which the cells underwent a second and third round of transfection using the same conditions. After three

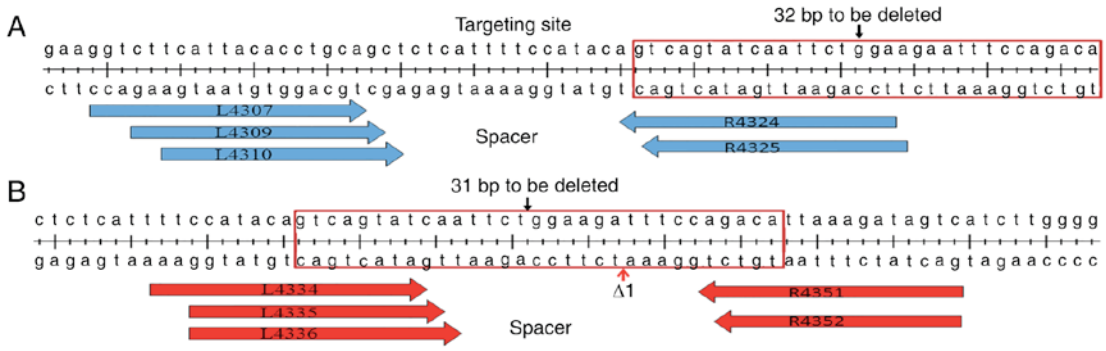


Figure 2. CCR5-TALENs and CCR5Δ1-TALENs. (A) CCR5-TALENs, three left (L4307, L4309 and L4310) and two right arms (R4324 and R4325) were designed. The binding domains of the three left arms were located mostly within the to-be-deleted 32 bp region. The spacer lengths ranged between 14 and 18 bp. (B) CCR5Δ1-TALENs, three left (L4334, L4335 and L4336) and two right arms (R4351 and R4352) were designed. All binding domains of the five arms more or less overlapped the to-be-deleted 31 bp region. The spacer lengths ranged between 15 and 18 bp. CCR5, C-C chemokine receptor type 5.

rounds of transfection, DNA was extracted using the Blood Genomic DNA Extraction Mini kit (Tiangen Biotech Co., Ltd., Beijing, China) for T7 endonuclease 1 (T7E1) enzyme analysis, and ≥50 transfected cells were cultured individually. A single clone with the *CCR5Δ32* mutation was screened by monoculture and sequencing. In order to generate homozygous *CCR5Δ32* mutations, transfection of the $CD4^+$ U87 cells with *CCR5Δ32/Δ1* was performed under the same conditions using the CCR5Δ1-TALENs plasmids for a further two rounds in place of the CCR5-TALENs plasmids, and no donor DNA was added.

*T7E1 enzyme analyses*. The genomic region, encompassing the TALENs targeting site, was amplified by polymerase chain reaction (PCR), and PCR products were denatured and annealed. Some heteroduplex DNA was formed as a result of random mutations or homologous recombination. The annealed DNA was then digested with 5 units of T7E1 enzyme (Beijing Viewsolid Biotech Co., Ltd., Beijing, China) at 37˚C for 15 min. The heteroduplex DNA fragments were cut at the mismatch point and 1% agarose gel electrophoresis was performed to separate the DNA fragments. The TALENs targeting rate was calculated according to the DNA band intensities measured by grayscale technique (Photoshop CS6; Adobe Systems, Inc., San Jose, CA, USA).

*Nested PCR*. In order to avoid contamination of undegenerated *CCR5Δ32* donor DNA, two pairs of primers were designed to amplify the targeting sites. The first round of PCR was performed with primers F3 (5'-TTCATCATCCTCCTGACA ATCG-3') and R3a (5'-CTCAAGAATCAGCAATTCTC-3'), and product length was 1,048 bp. Since R3a was located 18 bp downstream from the primer R2 used to amplify donor DNA, there was no chance for R3a to anneal to donor DNA when contaminated. The products from the first round of PCR were purified by gel extraction, and the second round of PCR was performed with primers F3 and R3b (5'-TGGTCCAACCTG TTAGAGCTAC-3') to amplify the targeting sites; product length, 479 bp. The PCR product was ligated into T-easy vectors and then transformed into DH5a competent cells, and successfully transformed clones were gene sequenced using Sanger sequencing.

*Determination of the p24 antigen*. Cells were challenged with BaL-HIV-1 obtained from State Key Laboratory for Infectious Disease Prevention and Control (Beijing, China), a CCR5-trophic virus isolate, at a multiplicity of infection of 0.06, for 4 h at 37˚C with 8 $\mu$g/ml Polybrene. The challenged cells were rinsed three times to remove the free virus and were cultured as above for 12 days. The culture supernatants were collected every 48 h and replaced with fresh medium. p24 content in the culture supernatants was assessed in triplicate by ELISA (632200; Clontech Laboratories, Inc., Mountainview, CA, USA) according to the manufacturer's protocol (22,23). This experiment was performed in a P-3 laboratory situated in the Chinese Center for Disease Control and Prevention (Beijing, China) strictly according to the guidelines.

## Results

*TALENs-mediated homozygous CCR5Δ32 mutation*. CCR5-TALENs and CCR5Δ1-TALENs plasmids were used to induce homozygous CCR5Δ32 mutation (Fig. 3). $CD4^+$ U87 cells were initially transfected with CCR5-TALENs plasmids and the *CCR5Δ32* donor DNA fragments carried in T-easy vectors. After three rounds of transfection without any antibiotic selection, the *CCR5* gene in ≤50% of $CD4^+$ U87 cells was targeted by T7E1 enzyme analysis (Fig. 3A). Two of the 29 (6.9%) single-cell cultured clones were revealed to carry bi-allelic mutations, and one of these contained a 1 bp deletion (Δ1) on one allele and a 32 bp deletion (Δ32) on the other (representing 1.7% of the transfected alleles). The latter was confirmed to carry the natural *CCR5Δ32* mutation by gene sequencing (Fig. 3C). The bi-allelic mutated $CD4^+$ U87 cells with the *CCR5Δ32/Δ1* genotype underwent a further two rounds of transfection with CCR5Δ1-TALENs, without any donor DNA. It was assumed that the mutated *CCR5Δ32* alleles themselves could be used as donor DNA for the predicted homologous recombination.

As expected, PCR revealed that the genomic DNA of cells with the *CCR5Δ32/Δ1* genotype contained similar levels of *CCR5Δ32* and *CCR5Δ1* nucleic acids. However, after two rounds of transfection, the *CCR5Δ32* alleles were gradually enriched and became the major alleles with increasing homologous recombination. The intensity of the *CCR5Δ32* band was

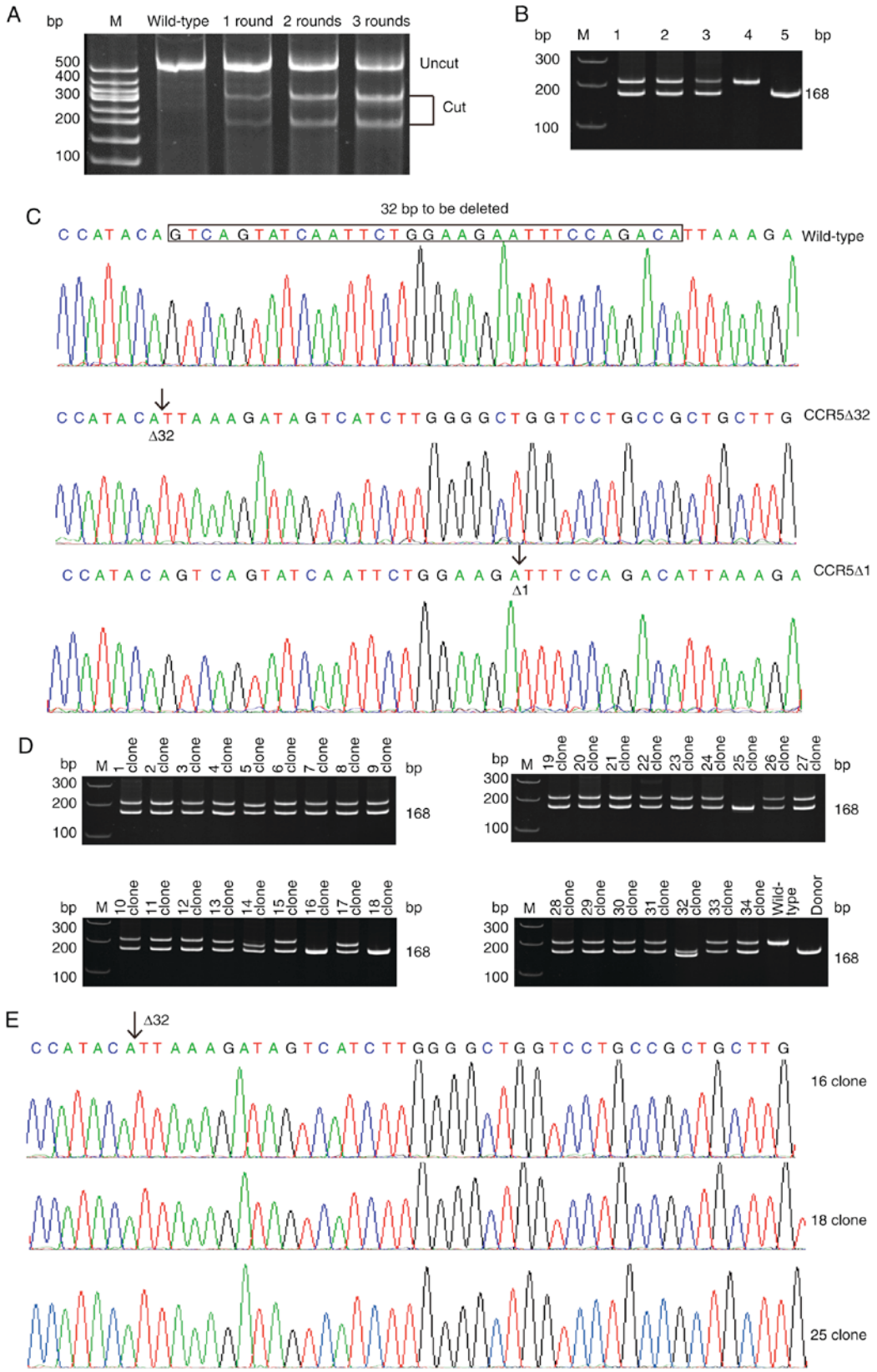


Figure 3. TALENs-mediated homozygous recombination. (A) T7E1 enzyme analysis of transfected CD4$^+$ U87 cells after each transfection with CCR5-TALENs. The targeting efficiencies after first, second and third rounds of transfection were 14.80, 38.20 and 50.04%, respectively. M, 100 bp marker; wild-type, wild-type cells; lanes 1-3, transfected cells after the first, second and third rounds of transfections. (B) PCR of *CCR5Δ32/Δ1* genotype cells. M, 100 bp marker; lane 1, prior to transfection; lane 2, after CCR5-TALENs transfection; lane 3, after CCR5Δ1-TALENs transfection; the *CCR5Δ32* band (168 bp) was 2.87-times brighter than the *CCR5Δ1* band; lane 4, wild-type cells (negative control); lane 5, *CCR5Δ32* donor DNA (positive control). (C) Sequencing of wild-type CD4$^+$ U87 cells and those with *CCR5Δ32/Δ1* mutations. (D) PCR of 34 single-cell cultured clones of CD4$^+$ U87 cells with CCR5Δ32/Δ1 mutations post-transfection with CCR5Δ1-TALENs. The *CCR5Δ32* band was observed in all clones; however, as a single band, it was only seen in clones 16, 18 and 25 post-transfection with CCR5Δ1-TALENs. M, 100 bp marker; lanes 1-34, single-cell cultured cells post-transfection with CCR5Δ1-TALENs; wild-type, wild-type cells (negative control); donor, *CCR5Δ32* donor DNA (positive control). (E) Gene sequencing of clones 16, 18 and 25. CCR5, C-C chemokine receptor type 5; CD4, cluster of differentiation 4; PCR, polymerase chain reaction; T7E1, T7 endonuclease 1; TALENs, transcription activator-like effector nucleases.

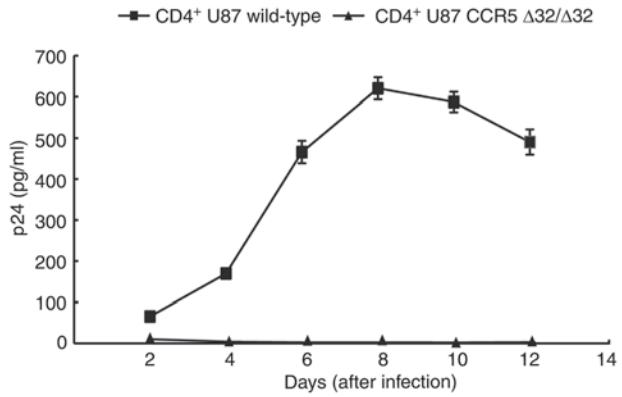


Figure 4. HIV-1 challenge. The mean concentration of p24 in the supernatant at 2, 4, 6, 8, 10 and 12 days after challenge was 58.47±2.35, 162.23±4.78, 458.78±27.34, 613.35±26.78, 580.35±24.73 and 483.34±30.85 in wild-type CD$^+$ U87 cells, whereas almost no p24 was detected in *CCR5Δ32/Δ32* genotype cultures. CCR5, C-C chemokine receptor type 5; CD4, cluster of differentiation 4; HIV-1, human immunodeficiency virus-1.

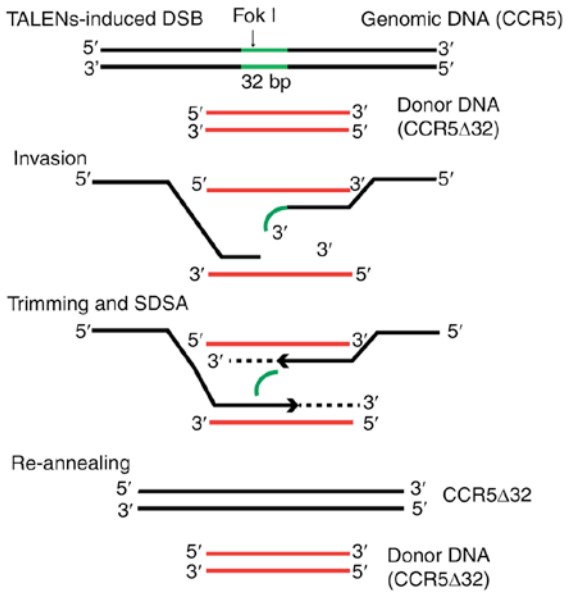


Figure 5. Mechanism underlying TALENs-mediated homologous recombination. DSBs were induced by *Fok*I connected with TALENs. The two 5' to 3' ends invaded into the opened double strands of donor DNA templates and annealed with the corresponding regions with at least one of them bearing a non-homologous 'floating' tail. These non-homologous tails were excised by some unidentified DNA polymerase, followed by SDSA on the donor DNA templates. The two newly synthesized DNA single strands leave the donor DNA templates, re-anneal to each other and may continue to carry on SDSA with each other as templates if necessary until the DSB is completely repaired. CCR5, C-C chemokine receptor type 5; DSB, double-strand break; SDSA, synthesis-dependent strand annealing; TALENs, transcription activator-like effector nucleases.

eventually close to 3-fold (2.87-fold, as determined by gray scale measurement) that of the *CCR5Δ1* band (Fig. 3B).

At this point, a total of 50 single cells were randomly selected for single cell culture, and DNA was separately extracted from 34 successfully cultured clones. Subsequent PCR analysis using primers F4 (5'-CTCCCAGGAATCATC TTTACC-3') and R4 (5'-TCATTTCGACACCGAAGCAG-3'), with a short product length of 200 bp, indicated that clones 16, 18 and 25 were homozygous for the *CCR5Δ32* mutation, whereas in clones 5, 13, 14, 17 and 32 *CCR5Δ1* appeared to be randomly mutated (Fig. 3D). Subsequent gene sequencing confirmed that clones 16, 18 and 25 (representing 8.8% of the transfected alleles, and 37.5% of the targeted alleles) were homozygous for the *CCR5Δ32* mutation (Fig. 3E).

*Off-targeting analysis of CCR5-TALENs and CCR5Δ1-TALENs.* A Basic Local Alignment Search Tool (BLAST) search of the National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed *CCR2* to have the most similar genomic DNA sequence to *CCR5*; therefore, the off-target effects of the two TALENs on this gene were investigated. An analysis investigating the homology between the potential targeting region of *CCR2* (4,312-4,711 bp) and that of *CCR5* (4,023-4,422 bp), which was the targeting region of the CCR5-TALENs and the CCR5Δ1-TALENs, revealed a score of 73%, analyses of all other potential targeting regions revealed scores <50%. The present study amplified the potential targeting region of *CCR2* in CD4$^+$ U87 cells with *CCR5Δ32/Δ32* mutations by PCR, followed by T7E1 analysis and gene sequencing (data not shown). The present study confirmed that no off-target *CCR2* mutations were generated.

*HIV-1 challenge test.* Wild-type CD4$^+$ U87 cells and the *CCR5Δ32/Δ32* genotype clones were challenged with BaL-HIV-1 for 4 h to assess resistance to infection. The level of p24 in the culture supernatants was assessed every 48 h for the following 12 days by ELISA. In wild-type cultures, p24 was detected after 48 h and peaked after 8 days. Conversely, p24 was not detected in the supernatants of *CCR5Δ32/Δ32* genotype cells at any time point (Fig. 4).

## Discussion

The present study successfully reproduced the naturally existing *CCR5Δ32/Δ32* genotype in CD4$^+$ U87 cells by combining TALENs with a traditional homologous recombination technique. This study reported a novel technique capable of producing site-specific, size-controlled and homozygous DNA deletion within the mammalian genome. Introduction of the *CCR5Δ32/Δ32* mutation, rather than entirely knocking out *CCR5*, may ease concerns regarding the potentially unfavorable clinical effects of *CCR5* knockout (18). TALENs-mediated homologous recombination achieved a significantly higher frequency of recombination, without application of selection, compared with traditional homologous recombination techniques without off-target DNA integration into the genome (24).

In order to ensure that the donor *CCR5Δ32* genes are saved from further targeting by TALENs, the binding domain of one arm of the TALENs must be located mostly within the to-be-deleted 32 bp region of *CCR5* (Fig. 2A and B). Once *CCR5* is randomly mutated with insertions or deletions, including *CCR5Δ1*, new TALENs may be needed to target the randomly mutated *CCR5*, even though the insertions or deletions did not occur within the binding domains of the original TALENs arms. After repeated transfection with CCR5-TALENs, gene sequencing indicated that the *CCR5Δ32/Δ1* genotype remained unchanged. These results suggested that a new pair of TALENs may be required even though mutation did not occur within the binding domains of

CCR5-TALENs. Six pairs of CCR5Δ1-TALENs were therefore designed; one of which was revealed to work well. These results indicated that these TALENs are not only specific for the binding domains, but may also be specific for the 3-dimensional structures surrounding the binding domains. This property increases safety of these TALENs, and suggests that off-target effects will be rare. In the present study, the originally designed CCR5-TALENs were no longer functional when a 1 bp deletion had occurred 3 bp downstream of the right arm of the CCR5-TALENs (Fig. 2).

To the best of our knowledge, homozygous deletion of a predetermined length of genomic DNA using ZFNs, TALENs or CRISPR/Cas9 has not been previously reported. The mechanism involved, therefore, requires discussion. Theoretically, homology-directed repair occurs in a manner most consistent with the synthesis-dependent strand annealing (SDSA) model of double strand break (DSB) repair (25,26). However, since constructed *CCR5Δ32* donor DNA templates were used, when DSB occurred, one out of two or both 5' to 3' broken ends of each DSB, bearing the to-be-deleted sequence, will not find the homologous sequence on the donor DNA templates to anneal with; therefore, SDSA would not be able to start. However, the present study suggested that this non-homologous end could be trimmed off by some unidentified DNA polymerase. Therefore, the DSB repair process with the predetermined 32 bp deletion used in this study may involve the five steps as illustrated in Fig. 5: i) DSB, double strand DNA was broken apart in the targeting region by *Fok*I connected with TALENs; ii) invasion, both 5' to 3' ends invaded into the opened double strands of donor DNA and annealed with the corresponding homologous sequences, at least one bearing a non-homologous 'floating' tail; iii) trimming, the non-homologous tail is excised by an unidentified DNA polymerase; iv) SDSA, SDSA starts with donor DNA sequences as templates; and v) re-annealing, the two newly synthesized DNA single strands leave the donor DNA templates, re-anneal, and may continue SDSA with each other as templates, if necessary, until the DSB is completely repaired.

The homologous recombination frequency after the first three rounds of transfection was 1.7% when the TALENs targeting rate was ≤50.4%. However, after the last two rounds of transfections it reached 8.8%, when the TALENs targeting rate was only 23.5% (Fig. 3). However, dividing these two homologous recombination frequencies by the TALENs targeting rate, indicates that the homologous recombination frequency after the first three rounds of transfection was 3.4%, and after the last two rounds of transfection was 37.5%. To better determine the frequencies of TALENs targeting and homologous recombination, three types of frequency were discussed: i) Frequency of targeting (T7E1 analysis), which reflects how many cells with mutated CCR5 were generated; ii) frequency of homologous recombination, which reflects the number of clones containing the *CCR5Δ32* allele (formula: $F_2$=the number of *CCR5Δ32* alleles/2x the number of sequenced clones); iii) frequency of homologous recombination in targeted *CCR5*, which reflects the accuracy of DNA autonomous repair (formula: $F_3$=the number of *CCR5Δ32* alleles/the number of targeted alleles including *CCR5Δ32* alleles). This homologous recombination difference may be attributed to homologous recombination, as longer homologous arms will have higher homologous recombination frequencies. The total length of the homologous arms for the first three rounds of transfection was only 1,602 bp, whereas for the last two rounds of transfections the homologous arms were the whole chromatids. This significant difference may explain the 10-fold difference in homologous recombination frequency between the last two rounds and first three rounds of transfection. The homologous recombination frequencies could be further improved if the homologous arm length was increased. Notably, since 3 of the 8 targeted mutations edited by CCR5Δ1-TALENs that occurred during DSB repair processes in the last two rounds of transfection were caused by homologous recombination, the DSB induced by TALENs may have a tendency to be repaired, restoring the 'original' sequences.

A major disadvantage of the present study is choosing the $CD4^+$-U87 cell line to generate homozygous *CCR5Δ32* mutations instead of directly using T lymphocytes. T lymphocytes could be used directly once the technique is ready for clinical application. The present study chose the $CD4^+$-U87 cell line to preliminarily establish the technique simply because these cells grow fast and are easy to manipulate. Furthermore, they mimic T lymphocytes very well in terms of expressing CD4, *CCR5* and *CXCR4*, which is required for the HIV challenge test following gene editing. Since this modified TALENs technique has been well established in editing the U87 cell line, our further studies aim to optimize the technique, and make it easier to alter the *CCR5* gene in T lymphocyte cells.

To the best of our knowledge, off-target occurrence should be avoided when using genome editing for therapeutic applications. To minimize off-target modification, the following strategies were employed in the present study: i) Potential off-target sites prediction, after obtaining homozygous *CCR5Δ32* mutations in three clones, potential off-target sites were analyzed in the targeting regions of CCR5-TALENs and CCR5Δ1-TALENs. Homology (73%) was detected between *CCR5* (4,023-4,422 bp) and *CCR2* (4,312-4,711 bp). The *CCR2* gene was revealed to possess the highest homology to the targeting region of *CCR5*, whereas other genes exhibited lower homology (≤50%) when aligned with *CCR5* (4,023-4,422 bp), as determined using a BLAST search in NCBI. ii) Detecting off-target modification by T7E1 analysis and gene sequencing; since the *CCR2* gene exhibited the highest homology to the targeting region of *CCR5*, a primer was designed to specifically amplify *CCR2* (4,312-4,711 bp) with corresponding template DNA extracted from wild-type cells, *CCR5Δ32/Δ1* cells, and clones 16, 18 and 25. Amplified 400 bp CCR2 fragments were analyzed by T7E1 analysis, and the results demonstrated that none were cleaved by T7E1, indicating that no potential off-target effects occurred in the *CCR2* homologous region (data not shown). In addition, 400 bp PCR products were ligated into T-easy vector and sequenced (50 successfully transformed clones for each PCR product were sent for gene sequencing) and the sequencing results confirmed that no potential off-target effects occurred in the *CCR2* homologous region (data not shown). Since no off-target effects were detected in the *CCR2* gene, it is very unlikely that other off-target effects will occur in genes with low homology to *CCR5*. Furthermore, TALENs, instead of CRISPR/Cas9, were used in the present study to edit the target gene due to the following reasons: i) TALENs exhibit an

efficient editing efficacy, although it is lower compared with CRISPR/Cas9 (21); ii) notably, TALENs exhibit much lower off-target modification than CRISPR/Cas9 (27).

In conclusion, to the best of our knowledge, the present study reproduced the *CCR5Δ32/Δ32* genotype without selection for the first time in $CD4^+$ U87 cells. This mutation conferred resistance against HIV-1 infection. Our future studies aim to adapt this technique in HSPCs or $CD4^+$ T lymphocytes, producing clinically useful cells for therapeutic use in HIV-positive patients.

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