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TALE nickase mediates high efficient targeted transgene integration at the human multi-copy ribosomal DNA locus

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ABSTRACT

Although targeted gene addition could be stimulated strikingly by a DNA double strand break (DSB) created by either zinc finger nucleases (ZFNs) or TALE nucleases (TALENs), the DSBs are really mutagenic and toxic to human cells. As a compromised solution, DNA single-strand break (SSB) or nick has been reported to mediate high efficient gene addition but with marked reduction of random mutagenesis. We previously demonstrated effective targeted gene addition at the human multicopy ribosomal DNA (rDNA) locus, a genomic safe harbor for the transgene with therapeutic potential. To improve the transgene integration efficiency by using TALENs while lowering the cytotoxicity of DSBs, we created both TALENs and TALE nickases (TALENickases) targeting this multicopy locus. A targeting vector which could integrate a GFP cassette at the rDNA locus was constructed and co-transfected with TALENs or TALENickases. Although the fraction of GFP positive cells using TALENs was greater than that using TALENickases during the first few days after transfection, it reduced to a level less than that using TALENickases after continuous culture. Our findings showed that the TALENickases were more effective than their TALEN counterparts at the multi-copy rDNA locus, though earlier studies using ZFNs and ZFNickases targeting the single-copy loci showed the reverse. Besides, TALENickases mediated the targeted integration of a 5.4 kb fragment at a frequency of up to 0.62% in HT1080 cells after drug selection, suggesting their potential application in targeted gene modification not being limited at the rDNA locus.

1. Introduction

Gene addition has a wide range of applications in gene therapy and basic research. It is usually achieved by a non-viral or a viral vector mediated transgene integration. Non-viral vectors are totally less efficient than viral vectors. Owing to the randomness of integration, it might be mutagenic if a physiological gene is disrupted or the transgene might be silenced if the integration takes place in a non-active chromatin area. Despite their high transfection efficiency, viral vectors are not ideal due to their immunogenicity and insertional mutagenesis, especially in gene therapy application. Lethal liver inflammation after administration of large dose of adenoviruses had been reported. The immune system will destroy the infected cells, making sustained gene expression impossible [1]. Another mostly used virus, retrovirus had been reported to induce deregulated premalignant cell proliferation through activating the proto-oncogene *LMO2* [2]. A following study revealed this type of retrovirus integrated into the genome in a semi-random manner, which is very mutagenic because it tends to integrate into transcription start regions [3].

As a result, numerous researchers are striving to develop more reliable transgenic methods via non-viral vectors into a defined safe locus meanwhile keep a relatively considerable efficiency. The site-specific integration is acknowledged to be mediated by a homologous recombination (HR) directed DNA repair pathway. The HR efficiency differs from site to site in the human genome as reviewed in some gene targeting study [4]. We have reported a successful targeted gene addition at the 45S ribosomal DNA (rDNA) locus in human embryonic stem cells [5]. The rDNA locus consists of hundreds of copies of 45S pre-RNA (rRNA) gene clustered on the short arms of the ten acrocentric chromosomes in diploid human cell. Active recombinational property of this area was reported during both meiosis and mitosis [6], which is consistent with the high gene targeting efficiency we achieved. In the general population, loss or gain of some copies of the *rRNA* gene is very

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common without any phenotypic effects and could be inherited stably. Research has shown that carriers with balanced translocation involving the short arms of the acrocentric chromosomes are usually clinically normal though have an increased risk of unbalanced offspring [7]. We therefore proposed that the rDNA locus is a suitable harbor for transgene and more likely to be targeted efficiently.

The emerging designer endonucleases, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), have become powerful tools in targeted genome manipulation. Moehle et al. showed that ZFNs stimulated targeted integration of a 1.5 kb fragment at a frequency of up to 5% without selection [8]. However, design of ZFN is very sophisticated and laborious. Even with optimized platforms such as 'Oligomerized Pool Engineering', it is difficult to produce effective ZFNs targeting a given site [9]. TALENs consist of a non-specific endonuclease domain of restriction enzyme FokI and TALE repeats initially derived from plant pathogens of the *Xanthomonas* genus [10]. The natural repeat units share a one-to-one correspondence with the four types of DNA bases. More than 80% of modularly ligated TALENs were proved to be efficient and it is possible to design more than one pair of TALENs targeting any base among random sequence [11,12].

We initially intended to further promote the gene targeting efficiency of rDNA locus using TALENs. As some researchers have addressed, although human rDNA sites could serve as a safe genomic target for transgene integration in gene therapy, tailored endonuclease targeting the multicopy *rRNA* genes may not be an appropriate solution to increase the integration efficiency, as the enzyme would fragment the rDNA units by multiple double-stranded DNA cleavage that is very likely to be fatal to the cell [13,14]. Three teams reported that ZFNs can be engineered to induce a site-specific DNA single-strand break (SSB) or nick, which could stimulate gene addition using a homologous donor template but failed to induce significant levels of the small insertions and deletions (indels) [15–17]. As TALENs cut the target DNA based on principles similar to those of ZFNs, both upon dimerization of two FokI domains in correct spacing and orientation, TALENickases are very likely to be designed and applied in targeted gene manipulation as ZFNickases did.

Here, we designed TALENs and TALENickases targeting the human rDNA locus to test the enzyme efficiency in targeted gene addition with or without drug selection and found that TALENickases are even more efficient than their correspondent TALENs at the multicopy rDNA locus.

2. Materials and methods

2.1. Cell culture

HT1080 and HEK293T cells were maintained in Dulbecco's modified Eagles medium (hyclone) supplemented with 10% fetal bovine serum (Gibco) at 37 °C humidified 5% CO_2 atmosphere.

2.2. Plasmids

The plasmids encoding TALENs were constructed using the Joung Lab REAL Assembly TALEN Kit (addgene, #1000000017) according to the previous publication [11]. We designed one left TALEN (TALEN-L) and three right TALENs (TALEN-R) targeting the 5468 site of the human rDNA repeat unit. Mutagenesis of TALEN-Rs was performed by PCR amplification of the FokI coding sequence by JSD-Muta-F (5′GTCAAAAAGGGTCTGCCTCA3′) and JSD-D450A-R (5′CCGCTATAAGCTTTAGTATCCACGATCACACCGTAATCAATAGGAGATCCGACAGTATAAATTGCTCCGGCCGGTTTCCTTGATCCACC3′) or JSD-D450N-R (5′CCGCTA TAAGCTTTAGTATCCACGATCACACCGTAATCAATAGGAGATCCGACAGTATAAATTGCTCCGTTCGGTTTCCTTGATCCACC3′) or JSD-D467A-R (5′CCGCTATAAGCTTTAGTAGCCACGATCACACCGTAAT3′), respectively. Then the PCR products were digested by HindIII and BamhI and substituted to the TALEN coding plasmids. The targeting vector pHr-IRES-GFP was constructed by ligation of an IRES-GFP fragment digested from phr1-IRES2-ACGFP1 using SmaI and NheI with the backbone fragment digested from F9-pro using AfeI and NheI. Our group previously constructed the plasmids phr1-IRES2-ACGFP1 and F9-pro, and a human rDNA targeting vector pHrnF9 [5].

2.3. Targeted integration of GFP under the Pol I promoter

For gene targeting, 700,000 HEK293t cells were plated per well on 6-well plate, and transfection were performed 24 h later using lipofectamine 2000 following the manufacturer's instructions. For each gene-targeting assay, 750 ng of pHr-IRES-GFP and 500 ng of left and 500 ng of right TALENs or TALENickases were used. GFP positive cells were determined by flow cytometry (FACSCalibur; BD Biosciences) from three days after transfection.

2.4. Assessment of apoptosis

HEK293T cells were seeded on 6-well plate at the density of 700,000 cells per well. Transfection of TALENs or TALENickases using lipofectmine 2000 was performed 24 h later. Three days after transfection, the percentage of apoptotic cells was determined using the Annexin-FITC apoptosis detection kit (sigma) according to the manufacturer's instructions. Briefly, 5 μl AnnexinV FITC conjugate and 10 μl of Propidium iodide (PI) solution were added to every 0.5×10^6 cells. Cells treated with 1 μg/ml staurosporine were used as a positive control. After incubation the tubes at room temperature for exactly 10 min, the fluorescence was determined by flow cytometry (FACSCalibur; BD Biosciences).

2.5. Gene targeting of HT1080 cells with drug selection

HT1080 cells at confluence of eighty percent were used. After detached using trypsin, 250,000 cells were resuspended in Cell Line Nucleofector® Kit T (Lonza). After adding of 2 μg of linearized pHrnF9 and 750 ng of each TALEN or TALENickase, cells were nucleofected at Nucleofector II (Lonza) using program L005. Then the cells were seeded at the density of 10,000 or 25,000 per 10 cm dish. Three days later, 400 μg/ml G418 was added to the medium. About 12 days after nucleofection, resistant clones were picked up for expansion or stained for counting.

Genomic DNA was isolated from the clones using phenol/chloroform extraction. HT1080 resistant clones were firstly PCR screened using the primers 5′GGGTGGGGCAGGACAGCAAGGGGGAGGAT3′ and 5′ GGCGATTGATCGGCAAGCGACGCTCAGACAG 3′, and a 1.4 kb product would be amplified from homologous recombinants. The PCR positive samples were than confirmed by Southern blotting. A F9 probe was labeled using the PCR DIG Probe synthesis Kit (Roche) and the primers 5'GCTCCATGCCCTAAAGAGAA 3′ and 5′ TCCATCAACATACTGCTTCCA3′. If the transgene was integrated into the rDNA locus via homologous recombination, a 10.3 kb restriction fragment will be detected after digested using pvuII, besides a 4.3 kb band from the endogenous *F9* gene on chromosome X. For RT-PCR of *F9* transgene expression, total RNA was extracted using Trizol reagent (Sigma) and reverse transcribed using Promega's transcription system. A 141 bp fragment will be amplified using primers 5′ATGCAGCGCGTGAACATGA 3′ and 5′ TACCTCTTTGGCCGATTCAGA 3′. For testing of F9 protein secretion, 24-h-old supernatants were collected from six well plates. Paired Antibodies for Factor IX (Cedarlane Laboratories) was used.

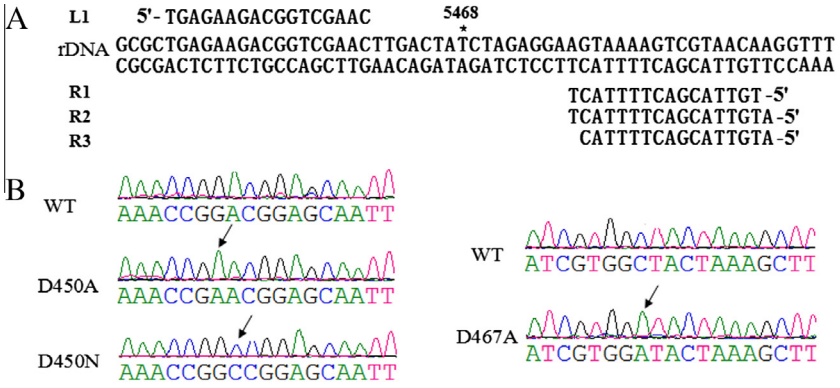

Fig. 1. Design of TALENs and sequencing of TALENickases. (A) Design and generation of one left TALEN and three right TALENs targeting the 5468 site of the multicopy human rDNA locus. (B) Three types of mutagenesis (D450A, D450N and D467A) were introduced to each of the three right TALENs to make TALENickases. Substituted bases were indicated by the arrows. All TALENickases were confirmed by Sanger sequencing.

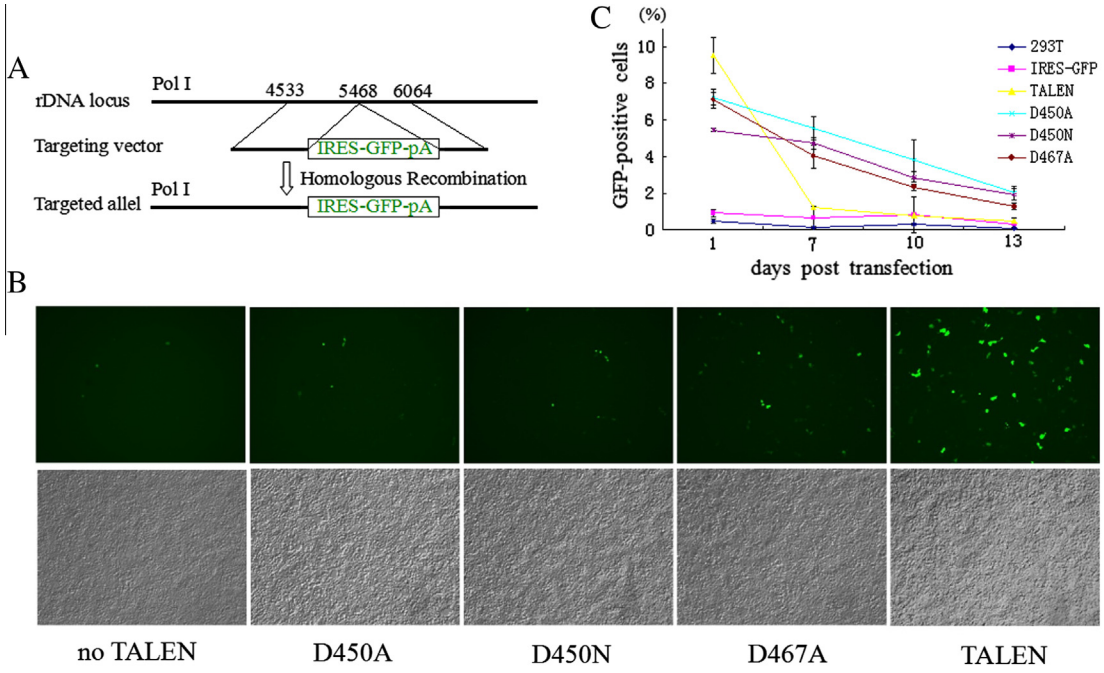

Fig. 2. Targeted knock-in of a promoterless GFP at the rDNA locus with TALENs or TALENickases. (A) Schematic representation of the rDNA unit, donor vector, and targeted allele after HR. After HR, the GFP expresses under control of the upstream Pol I promoter with the help of the encephalomyocarditis virus internal ribosome entry site (EMCV-IRES) element. (B) The targeting vector was co-transfected into HEK293T cells with or without TALENs or TALENickases as indicated. Four days after transfection, the cells were examined under a fluorescence microscope. Cells were microphotographed with a magnification of 100. (C) At day 3, 7, 10 and 13 post transfection, the percentage of GFP-positive cells of all the groups was determined by flow cytometry.

Reference curves were constructed using serial dilutions of normal pooled plasma, with correlation coefficient (R^2) of at least 0.990 using a 5-parameter logistic curve fit algorithm as previously reported [5].

3. Results

3.1. Generating of TALENs and TALENickases

In previous studies, ZFNickases were generated by substitution of one FokI in the ZFN pair with a catalytic inactive mutant. An Asp-450 to Ala (D450A) or an Asp-467 to Ala (D467A) single amino acid substitutions have been reported to eliminate the FokI catalytic activity [18]. An Asp-450 to Asn (D450N) mutant was reported structurally more similar to the wild type sequence with the similar inactivation effect [15]. We designed one left TALEN and three right TALENs targeting the 5468 site of human rDNA repeating unit (Fig. 1A). We performed all the three mutagenesis on the catalytic domain of the type IIS restriction enzyme FokI and applied them to the three right TALENs. All of the resulting TALENickases with mutation were confirmed by Sanger sequencing (Fig. 1B).

3.2. Targeted GFP knock-in under control of the Pol I promoter

In order to test the efficiencies of TALENs and TALENickases in stimulating targeted gene addition, we constructed a pHr-IRES-GFP vector which could integrate a 1.7 kb promoter-less EMCV-IRES-GFP-polyA cassette into the 5468 site of rDNA locus. The targeting vector was co-transfected with TALENs or TALENickases, fluorescent cells will be detected if the cassette was either targeted integrated under control of the endogenous RNA polymerase I (Pol I) promoter or randomly integrated under other active promoters

(Fig. 2A). Up to 9.54% ± 0.98% of 293T cells were GFP positive three days after co-transfection with pHr-IRES-GFP and TALENs. The stimulated frequencies of TALENickases were slightly lower than those of TALENs, up to 7.24% ± 0.41%, 5.45% ± 0.09% and 7.09% ± 0.42% for D450A, D450N and D467A mutant, respectively (Fig. 2B and C, Table S1). Similar results have been reported in previous research using ZFNs and ZFNickases, which demonstrated the DSB-inducing ZFNs were more effective than ZFNickases in stimulating homology-directed repair (HDR) [15]. However, the stimulated frequency of TALENs dropped sharply to 1.2% ± 0.08% 4 days later, while the groups using TALENickases maintained the average frequencies from 4.00% to 5.56% at the same time point (Fig. 2C, Table S1). Even 13 days after transfection, 2.01% ± 0.36% of the cells co-transfected with D450A mutant were GFP positive, while those with TALENs dropped to only 0.48% ± 0.17% (Fig. 2B, Table S1). Our results revealed that TALENickases are obviously effective in promotion of targeted gene addition at the multicopy rDNA locus. Then we tested the cytotoxicity of TALENs and TALENickases on HEK293T cells. After transfection of the TALENs or TALENickases, the percentage of apoptotic cells was determined using the Annexin-FITC apoptosis detection kit with a FACSCalibur flow cytometer (BD Biosciences) (Fig. 3). Our result revealed TALENs are more cytotoxic than any type of the three TALENickases significantly.

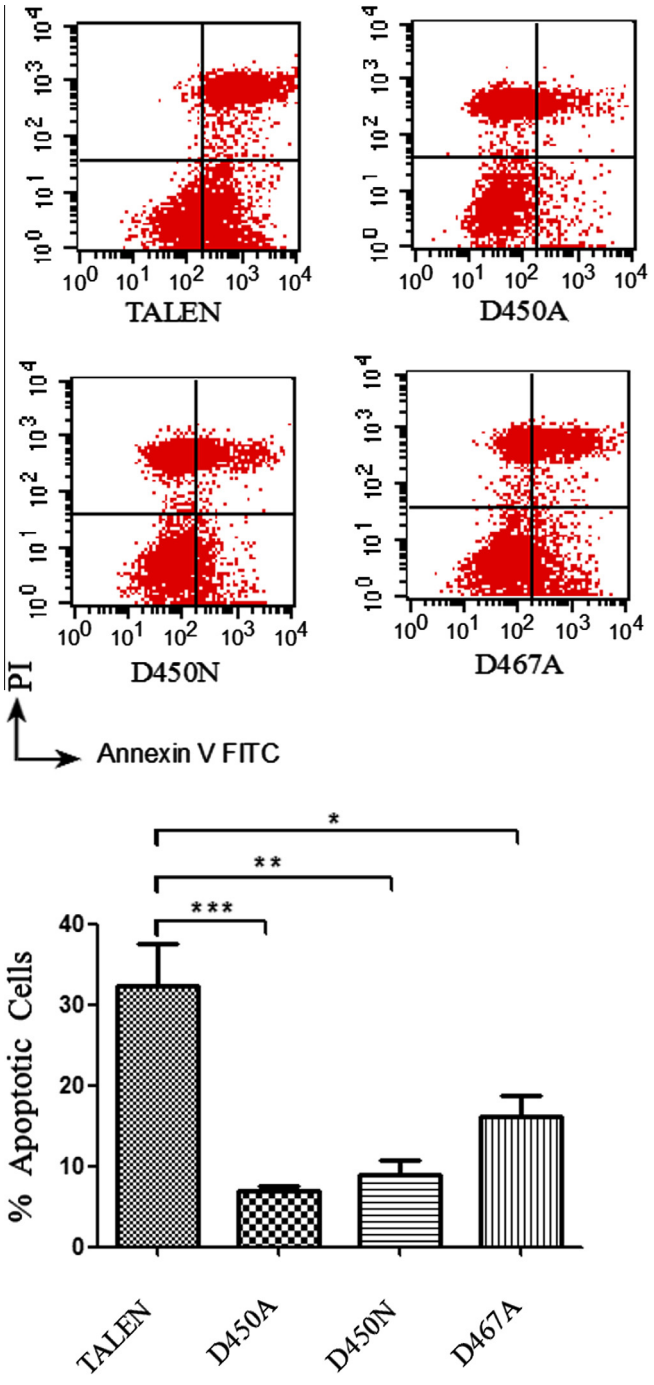

Fig. 3. Detection of apoptosis after transfected with TALENs or TALENickases. TALENs were more cytotoxic to cells than any of the three types of TALENickases. Data are mean ± SD ($n = 3$).

3.3. Targeted integration of a complete gene cassette with drug selection

We next use the targeting vector pHrnF9 that contains a 5.4 kb fragment consisting of a promoter-less IRES-Neo and an EF1α-FIX cassette to test if either TALENs or TALENickases could stimulate large gene fragment integration under the circumstance of drug selection. The donor vector was co-transfected with TALENs or TALENickases into HT1080 cells. After drug selection, clones were picked up for expansion or stained for counting (Fig. 4A). The resistant clones were firstly screened by PCR then verified by southern blotting (Fig. 4B and C, Fig. S1). In a set of experiments, the targeting frequency of 9.1×10^{-4} was achieved via co-transfection with TALENs, comparing with 1.2×10^{-4} without using TALENs (Table S2). When TALENickases were used, 30.8×10^{-4}, 26.4×10^{-4} and 62.4×10^{-4} were achieved using D450A, D450N and D467A mutant, respectively (Table S2). These data are in keeping with those in the transfected 293T cells after continuous culture (Table S1). Our results demonstrate that TALENickases are highly efficient to stimulate large gene cassette addition at the rDNA locus, even more efficient than their TALEN counterparts.

Then we try to test if the transgene targeted at the rDNA locus with the help of TALENickases could be expressed. Reverse transcription PCR (RT-PCR) analysis revealed that the *F9* gene in the targeted cells was transcribed, while the transcript of the endogenous *F9* in HT1080 cells were not detected (Fig. S2A). After that the cell culture supernatants were analyzed using enzyme linked immunosorbent assay (ELISA). The targeted clones secreted F9 protein up to more than 200 ng/10^6 cells/24 h, but the F9 secretion was undetectable in HT1080 supernatant (Fig. S2B). These data indicated that the targeted clones stimulated by TALENickases could transcribe the transgene *F9* and secrete the synthesized protein robustly.

4. Discussion

Stable insertion of functional transgenes is of great value in both basic and applied biomedical research. The environment of the integration site can largely affect the credibility of the research findings. If the transgene is integrated randomly, its expression will be unpredictable because of position effects. Conversely, the random or semi-random integration of the transgene may alter the expression of the surrounding genes, leading to the change of the cell's fate or transformation of the cells [5,19]. For these reasons, it is necessary to target the transgene to a predetermined genomic site. Owing to its special properties, the repeating human rDNA locus might serve as an ideal harbor for transgenes. Targeting one or a few copies of the *rRNA* gene might be tolerable to cells because there are hundreds copies of this non-coding gene clustered at the rDNA locus. As the rDNA locus does not contain protein coding sequences, manipulation of these sites will be less mutagenic compared with other sites [13]. Furthermore, integrated transgenes

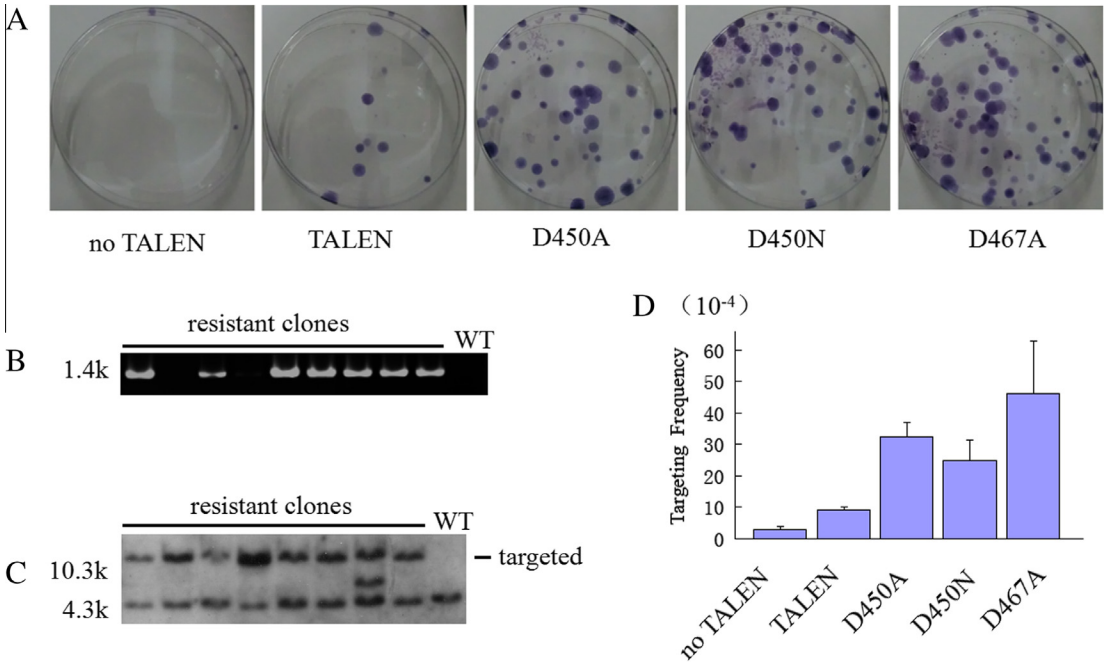

Fig. 4. TALENs or TALENickases induced targeted integration of a 5.4 kb gene fragment at the rDNA locus with drug selection. (A) The linearized donor vector phrnF9 was co-transfected into HT1080 cells with or without TALENs or TALENickases via nucleofection. After drug selection, a part of the resistant clones were stained by Giemsa stain for counting. (B) A portion of the resistant clones from each group were picked up and expanded. A PCR screening was carried out for the picked clones, and a 1.4 kb fragment was amplified if the cells underwent HR. (C) PCR-positive clones were then sent to Southern blot analysis for confirming the HR. A 10.3 kb fragment was detected if the targeted gene addition happened, besides the 4.3 kb fragment corresponding to the endogenous *F9* gene. (D) The gene targeting experiment was carried out in triplicate independently. All the data were summarized as the histogram.

have been proved to be able to express under control of the endogenous RNA Pol I promoter or Pol II promoter integrated with the transgenes at the rDNA locus [5,20]. A recent research achieved 2.7% of rDNA-directed integration using lentivirus vector coding an HIV-1 integrase-I-PpoI fusion protein [21]. However, as the integration did not follow the classic HR manner, it is hard to exclude random integration via conventional molecular biology methods.

ZFNs have been used in targeted gene manipulation for nearly two decades since its first design [22]. The tailored nuclease is really useful as it can create a targeted DSB to stimulate the cell's self-repair process via error-prone non-homologous end joining (NHEJ) or precise HR. As NHEJ usually results in indel mutagenesis, ZFNs were mostly used in targeted gene knock-out. There has been a clinical trial evaluating the ZFNs in AIDS gene therapy, in which the ZFNs were used to disrupt the *CCR5* gene to produce blood cells resistant to HIV-1 infection [23]. In fact, the defective genes should be repaired rather than disrupted in gene therapy strategy for most hereditary diseases. DSBs produced by ZFNs were reported to stimulate HR by several thousand-fold [24], and recently ZFNickases were designed to reduce the unwanted indels markedly while retaining a compromised efficiency of HR induction [15–17].

However, as a powerful tool of molecular biotechnology, ZFNs have not been widely used in conventional biomedical research. The low efficiency and laborious selection process limited their commercialization at Sangamo BioSciences and several professional laboratories [9,25,26]. After the discovery of the simple modular DNA recognition code of the TALE proteins, TALEN was designed immediately for targeted gene mutagenesis [10,27,28]. In the past three years, hundreds of laboratories used TALENs to speed up their research because of its high effectiveness and ease to design [29,30].

In this study, we designed TALENs targeting the human rDNA locus, and introduced inactivating mutations into one of the two FokI domains to make TALENickases. In the GFP knock-in experiment on HEK293T cells, TALENs stimulated the fraction of GFP positive cells up to 9.54% three days after transfection without selection. Although the efficiency achieved by TALENickases was slightly lower than that by TALENs, the cells transfected by TALENs were not stable because of the high cytotoxicity. After seven days of continuous culture, the efficiency by TALENs dropped sharply to a level below that by TALENickases. Even in a gene addition experiment with drug sellection, the targeted insertion of a 5.4 kb fragment was stimulated by TALENickases at a frequency of up to 0.62%, which is much higher than that (0.09%) by TALENs. This result fails to comply with the law that DSBs are more effective than SSBs in stimulating HDR, possibly owing to the unique characteristics of the rDNA locus. There are hundreds of copies of *rRNA* gene per cell serving as potential targets for SSB-induced HDR on the one hand, while on the other hand, the multicopic property might promote the toxicity of the DSBs. And given that there are hundreds of potential targets for a single pair of the designed TALENs or TALENickases, it is hard to detect the resulting indels or nicks. Nevertheless, here we demonstrate that TALENs can be easily mutagenized to generate TALENickases with a similar strategy used for ZFNickases and that TALENickases-induced SSBs can remarkably stimulate targeted gene addition as ZFNickases do, despite that TALENs and ZFNs are not the same in all respects [31]. As a consequence, TALENickases show their potential application in targeted gene modification not being limited at the rDNA locus.

Competing interests

The authors have declared that no competing interests exist.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.099.

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