Cleavage of Target DNA Promotes Sequence Conversion with a Tailed Duplex

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Much attention has been paid to targeted sequence alteration, since it would provide a powerful tool for dissecting gene functions, creating animal models with mutations in genes of interest, and treating patients with diseases caused by genetic alterations. Sequence alterations have been accomplished by using nucleases, nucleic acids, and their combinations. In particular, sequence conversion using artificial nucleases is receiving considerable attention.1–5) Donor nucleic acids, co-particular, sequence conversion using artificial nucleases is using nucleases, nucleic acids, and their combinations. In this study, we examined the effects of target DNA cleavage on sequence conversion with a TD were examined. Plasmid DNAs with and without cleavage near the target position were each introduced into HeLa cells, together with the TD. The cleavage promoted the sequence alteration efficiency by ca. 7-fold. These results suggested that the sequence conversion efficiency with the TD fragment is increased when an artificial nuclease introduces cleavage near the target site.

Key words sequence conversion; tailed duplex; genome editing

MATERIALS AND METHODS

Oligodeoxyribonucleotides (ODNs) ODNs were obtained from Fasmac (Atsugi, Japan) and Eurofins Genomics (Tokyo, Japan) in purified forms.

Construction of Phage and Plasmid DNAs The pBluescript II SK(+) plasmid (Agilent Technologies, Santa Clara, CA, U.S.A.) was digested with SspI and PvuII and ligated with a linker ODN containing KpnI and SalI sites, to obtain the pBS-BamKpnSal plasmid. The 6th AAT codon in the lacZα gene in the M13mp18 DNA (TaKaRa, Otsu, Japan) was converted to the synonymous AAC codon, to eliminate the unique EcoRI site. Two novel EcoRI sites were introduced into the upstream and downstream positions of the gene, to yield the M13EcoZEco phage DNA. The 783-bp EcoRI–EcoRI fragment containing the “wild-type” lacZα gene was inserted into the pBS-BamKpnSal plasmid cleaved by KpnI and SalI, using a GeneArt Seamless Cloning and Assembly kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.), to obtain the pLacZα plasmid (Fig. 2A). The 17th TCG codon (Ser) in the gene was converted to the TAG sequence, to introduce a termination codon. The plasmid DNA bearing the mutared lacZα gene was named pLacZα(C295A) (Fig. 2B).

Preparation of the Target Plasmid and the TD DNA Fragment The pLacZα(C295A) plasmid was purified with a GenElute HP Plasmid Miniprep kit (Sigma-Aldrich, St. Louis, MO, U.S.A.) and digested with either PstI or XbaI, for cleavage near the target position.

The 783-base ss fragment was prepared from the M13EcoZEco phage DNA, by annealing with its respective scaffold ODNs (5′-dGGT TTT TTA ATT CAC CAG TG-3′ and 5′-dAAAGTTAGAATTCTGTTAA-3′) followed by EcoRI digestion, and was purified by low-melting point agarose gel electrophoresis and gel filtration chromatography. The TD fragment was prepared by annealing the ssDNA fragment.
and a 35mer ODN (5'-dCTG TTA AAA TTC GCA TTA AAT TTT TGT TAA ATC AG-3'), as described.10)

**Transfection and Determination of Sequence Conversion Efficiency** The TD fragment (5 pmol) and the target plasmid (pLacZα(C295A)) (12.5 fmol) were transfected into HeLa cells (RCB0007, provided by the RIKEN BRC through the National BioResource Project of the MEXT, Japan) using the Lipofectamine Reagent (Life Technologies, Carlsbad, CA, U.S.A.), as described previously.12) After 48 h, the plasmid DNA was recovered and electroporated into Escherichia coli DH10B cells, as described previously.9,12) The bacteria were seeded onto Luria–Bertani (LB) agar plates containing 30 µg/mL of ampicillin and 80 µg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Sequence conversion of codon 17 from TAG to TCG restores the β-galactosidase activity, and the efficiencies were calculated by dividing the number of blue colonies by the total number of colonies on the plates.

**RESULTS AND DISCUSSION**

**Increased Sequence Conversion Efficiency by Cleavage of Target DNAs** We previously reported sequence conversions with 5'-TD DNA fragments, using the genes encoding hygromycin-resistance and enhanced green fluorescent protein and ribosomal protein S12 as model target genes.10–13) In this study, we used the lacZα gene from a cloning vector (M13mp18), since sequence conversion from the mutated (TAG) sequence to the “wild-type” (TCG) could be easily judged by the blue-white selection and the PstI and XbaI sites are located near the target position (Fig. 2B). The pLacZα(C295A) plasmid was cleaved by either restriction enzyme. The undigested plasmid was also used for comparison.

The uncleaved and cleaved pLacZα(C295A) plasmid DNAs were separately introduced into human HeLa cells using cationic lipids, together with a 400-fold molar excess amount of the TD fragment (Fig. 2C). After recovery from the transfected cells 48 h later, the plasmid DNAs were electroporated into E. coli DH10B (recA, lacZΔM15) cells. The transformed DH10B cells were seeded onto agar plates containing X-gal. The ratio of blue colonies was defined as the sequence conversion efficiency.

The sequence conversion efficiencies when the TD frag-
ment was co-introduced with the undigested and digested plasmid DNAs are shown in Fig. 3. The co-introduction with the undigested plasmid resulted in ca. 1% sequence conversion efficiency (none, Fig. 3). The efficiency was increased to 7.2% when the plasmid DNA was digested with PstI. This value was ca. 7-fold higher than that of the undigested DNA, indicating that the cleavage of the target DNA dramatically promoted the sequence conversion. Likewise, the efficiency (7.1%) was increased when the target DNA was cleaved by XbaI. These results indicated that the cleavage of the target DNA enhanced the sequence conversion efficiency and that comparable efficiencies were obtained independently of the cleavage positions.

**Sequence Analysis of Plasmids Recovered from Transfected Cells** To examine whether the expected sequence conversion was induced, we sequenced the EcoRI–EcoRI region (corresponding to the TD fragment) of the plasmid DNAs isolated from the blue colonies. In total 25, 18, and 19 colonies were analyzed for the undigested, PstI-digested, and XbaI-digested DNAs, respectively. The sequence alterations observed in the analyzed plasmids are shown in Table 1. In the undigested DNA experiment, 24 among the 25 colonies analyzed contained the on-target A-to-C change without mutations in other positions. One clone was a false-positive, since no sequence change was detected (no mutation, Table 1). Likewise, the correct sequence alteration was confirmed in almost all of the plasmids in the PstI and XbaI experiments. Large (ca. 300bp) deletion mutations were found in one colony in both experiments. Thus, the sequence conversion efficiencies calculated as the ratios of blue colonies essentially reflected the actual efficiencies. The results obtained in this study indicated that the cleavage of the target DNA promotes the sequence conversion efficiency with a TD fragment.

It remains unclear whether the large deletions observed in the PstI and XbaI experiments were attributable to the double-strand break and/or the TD fragment. Additional studies are necessary to elucidate the involvement of TD in the unexpected mutagenic events in cells.

**Implications** Although the molecular mechanisms of gene correction by the ss and TD DNA fragments have not been elucidated, homologous recombination (HR) proteins are considered to be involved. First, the order of the sequence conversion efficiencies for the base-substitution mutations (5′-TD>3′-TD>ssDNA fragment) is identical to that of efficiencies in the in vitro DNA strand exchange reactions catalyzed by the RAD51 protein, an important enzyme in HR. Second, the target plasmid DNA became radioactively labeled when radiolabeled ss and TD DNA fragments were introduced into mammalian cells, suggesting the integration of the fragments (ref. 15 and Tsuchiya et al., unpublished results). In the case of the experiment with undigested DNA, the TD fragment could be recognized as damaged DNA strand(s) by proteins involved in double-strand break repair, such as HR. In contrast, the cleaved target DNA and/or the TD fragment might be recognized as damaged DNA by these proteins, in the experiments with digested DNAs. The effects of the cleavage on conversion efficiency could be due to enhanced recognition of DNA(s). Alternatively, protein(s) involved in the sequence conversion process could be changed at least in part by the cleavage. Further improvement of sequence conversion could be achieved when the cellular proteins involved in the process are identified.

To utilize the TD fragment in combination with cleavage of target sequence in genomic DNA, efficient delivery of the TD plus an artificial nuclease (as protein, mRNA, or DNA) into cells is required. In particular, highly effective and selective delivery systems are necessary when this technology is applied to gene therapy, together with decrease in off-target effects (cleavage at untargeted positions) by the nuclease.

In conclusion, cleavage of the target DNA dramatically promoted the sequence conversion efficiency in human cells. The results obtained in this study suggest that the TD fragment is a useful donor nucleic acid in combination with artificial nucleases.

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**Conflict of Interest** The authors declare no conflict of interest.

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