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Improvement of SSO-mediated gene repair efficiency by nonspecific oligonucleotides

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ABSTRACT

Targeted gene repair mediated by single-stranded DNA oligonucleotides (SSOs) is a promising method to correct the mutant gene precisely in prokaryotic and eukaryotic systems. We used a HeLa cell line, which was stably integrated with mutant enhanced green fluorescence protein gene (mEGFP) in the genome, to test the efficiency of SSO-mediated gene repair. We found that the mEGFP gene was successfully repaired by specific SSOs, but the efficiency was only ~0.1%. Then we synthesized a series of nonspecific oligonucleotides, which were single-stranded DNA with different lengths and no significant similarity with the SSOs. We found the efficiency of SSO-mediated gene repair was increased by 6-fold in nonspecific oligonucleotides-treated cells. And this improvement in repair frequency correlated with the doses of the nonspecific oligonucleotides, instead of the lengths. Our evidence suggested that this increased repair efficiency was achieved by the transient alterations of the cellular proteome. We also found the obvious strand bias that antisense SSOs were much more effective than sense SSOs in the repair experiments with nonspecific oligonucleotides. These results provide a fresh clue into the mechanism of SSO-mediated targeted gene repair in mammalian cells.

Targeted gene repair mediated by oligonucleotides has been an important technique in functional genomics and gene therapy for the past few years [1]. This approach can create deletions, short insertions, and point mutations with the specific alternation of short stretches DNA. Because the DNA mutations are corrected at their endogenous loci, the repaired gene will still be regulated by the natural elements [2,3]. Therefore, it is an ideal method for the treatment of genetic diseases, which are caused by small DNA alternations [4].

Both RNA/DNA chimeric oligonucleotides (RDOs) and SSOs can complete the targeted gene repair in bacteria, fungi, and mammalian cells [5–8]. In RDO-mediated repair, DNA moiety directs the gene correction event, and the RNA moiety is responsible for stabilizing structure [9]. But the unstable efficiency and poor reproducibility have been observed in RDO-mediated gene repair [10]. Compared with RDOs, the repair efficiency of SSOs is more stable and reproducible. Also, SSOs are easy for synthesis, purification and modification. And SSOs have shown higher efficiency than RDOs, especially in the *in vivo* repair systems [11].

The basic structure of SSOs is the single-stranded DNA oligonucleotide with modifications at terminus, and the mismatch-forming nucleotide is centrally located. And the optimal structure of SSOs must be tested in different reporter systems. For example, the optimal SSO in our system (HeLa cells) is 25nt long, terminal stabilized by six phosphorothioate linkages [12]. In *Saccharomyces cerevisiae*, the efficient SSO is 70–80nt long and has three terminal phosphorothioate modifications [13]. Further, even the unmodified SSO are able to complete the targeted gene repair in the HEK-293 cell line [14].

Interestingly, many laboratories have found that two complementary SSOs usually show different repair efficiencies for the same locus. This phenomenon is called as strand bias [6,11,15–17]. And the SSOs, which are complementary with the nontranscriptional strand of the targeted gene, are named as antisense SSOs. The complementary sequence of the antisense SSOs are called as sense SSOs. The antisense SSOs have shown higher repair efficiency than sense SSOs in most reporter systems [12]. However, opposite strand bias for correcting two individual mutations in a single targeted gene has been found in an episomal β-galactosidase gene reporter system [15]. And DNA replication, transcription, mismatch repair, as well as local chromosomal environment have been found to influence strand bias in SSO-mediated gene repair [16,17].

The SSO-mediated gene repair is a complicated event, and the exact mechanism is still unclear. Recently, a two-step mechanism

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has been proposed, which involves the following steps: (i) DNA pairing/annealing, followed by (ii) DNA repair/recombination [18–20]. First, the SSO anneals with its complementary strand in the double-stranded DNA to form a D-loop structure. This process is mediated by cellular protein factors, such as Rad51 [6,21,22]. Since a structural perturbation is induced by the mismatched base pair in the D-loop structure, endogenous protein machinery might be activated to initiate the second step (site-specific DNA repair/recombination). The annealed SSO will direct the site-specific base conversion in the targeted gene [23]. Some evidences suggested that annealed SSO may be dissociated from its targeted sequence at the end of the repair process, but it appears inconsistent with the observation that SSO will be incorporated into the genome eventually [24].

It is very important to establish a sensitive and reliable reporter system to investigate the mechanism of SSO-mediated gene repair in mammalian cells. In the previous study, we introduced two modifications to the EGFP gene in plasmid pEGFP-C1: an A to T point mutation that inactivates the initiation codon of EGFP gene, and a replacement of the restriction site (from AgeI to EcoRI). The plasmid with these two modifications was named as pmEGFP-C1. The EGFP expression was abolished for the mutant initiation codon, and the introduced EcoRI restriction site provided an important maker to distinguish the repaired gene from the wild type plasmid contamination. The SSO was designed to repair the mutated initiation codon, and restore the EGFP expression [12].

We introduced the pmEGFP-C1 plasmid into HeLa cells, and selected a stably integrated single-cell clone (F5). Also, we designed the SSO(E6), which was a 25nt long single-strand DNA oligonucleotide with six phosphorothioate linkages at both termini, to repair the inactivated initiation codon of mEGFP. The sequence of E6 was complementary with the nontranscriptional strand of mEGFP gene, expect for the centrally located mismatched nucleotide. We treated F5 cells with E6, and the repair efficiency was measured after 48 h of transfection. The mean repair efficiency of E6 was 0.1%, and it was stable and reproducible. In order to confirm the targeted gene repair in chromosome level, we extracted the genome DNA from the F5 cells treated with E6, and finished the DNA sequencing for the fragment contained the targeted site. The DNA sequencing result showed the repaired ATG initiation codon and an upstream EcoRI site, which also ruled out the contamination of the wild type pEGFP-C1. We used the F5 cells and E6 for the further experiment [25].

Based on the evidence that nonspecific carrier oligonucleotides can increase the targeted repair efficiency in *S. cerevisiae* [26], we investigated the effect of nonspecific oligonucleotides on the efficiency of SSO-mediated gene repair in mammalian cells. We found that the repair efficiency was elevated by ≈6-fold in nonspecific oligonucleotide-treated cells. And we evaluated the influence of length and dose of the nonspecific oligonucleotides on the repair efficiency. Also, we analyzed the possible mechanism for the increased repair efficiency, as well as the strand bias.

Materials and methods

Plasmids and single-strand DNA oligonucleotides. The mutant EGFP plasmid (pmEGFP) was derived from plasmid pEGFP-C1 (Clontech, Palo Alto, CA, USA). A → T alteration (to inactivate the ATG initiation codon of the EGFP gene) and an AgeI (ACCGGT) to EcoRI (GAATTC) restriction site change (nucleotides 600–605) were introduced into plasmid pEGFP-C1 to generate plasmid pmEGFP-C1 by PCR-based mutagenesis strategy (see Supplemental material 1) [12]. The oligonucleotides (SSOs and the nonspecific oligonucleotides) used in this reporter system were synthesized and purified by PAGE at Sangong Company (Shanghai, China).

Cell culture. HeLa cells were cultured in DMEM medium supplemented with 10% heat inactivated fetal bovine serum (FBS, Hyclone, Logan, UT), antibiotics (100 mg/ml streptomycin and 100 U/ml penicillin) at 37 °C and 5% CO_2. The F5 cell clone was obtained from the HeLa cells, which was transfeted with pmEGFP-C1 and cultured in selective medium (supplemented with 600 μg/ml G418).

Transfection. Cells were seeded at 8×10^5 per 60-mm dishes and grown for 24 h to reach 60–80% confluence. SSO (3 μg) and nonspecific oligonucleotides(3–6 μg), as well as Lipofectamine2000 (3 μl, 1 mg/ml, Invitrogen) were mixed, diluted with 100 μl of OPTIMEM (Invitrogen), and incubated at room temperature for 20 min. The mixtures were added to the cells without changing the culture medium (DMEM supplemented with 10% FBS), which was continuously cultured for 48 h.

FACS analysis. Flow cytometry was used to detect repaired cells, which had green fluorescence from EGFP. Cells were harvested after trypsin digestion, washed with phosphate-buffered saline (PBS), and filtered through the 35 μm-pore size nylon mesh to get a single-cell suspension. Thirty thousand cells were analyzed per sample on a FACSCalibur flow cytometer (Becton Dickinson) using a 488-nm argon-ion laser.

Results and discussion

SSO-mediated gene repair with nonspecific oligonucleotides

In our previously studies, we established a sensitive and reliable reporter system (HeLa-mEGFP-F5 cell line) by integrating mEGFP reporter gene into chromosome at low copy numbers. With this reporter system, we have found that the SSO, which is 25nt in length and contains six phosphorothioate linkages at each terminus, exhibits the optimal balance among the size, purity, and stability *in vivo*, for maximum repair activity. And the targeted gene repair events completed by this SSO(E6) were stable and reproducible, which had been confirmed by DNA sequencing analysis [12].

Previously evidence has shown that the nonspecific, carrier oligonucleotides could increase the frequency of targeted nucleotides exchange in the *S. cerevisiae*. These molecules, when added to the reaction, increase the repair frequency up to 25-fold in some cases. The possible mechanism is by providing a molecular trap to bind factors, which may inactivate the specific targeting oligos [26].

With HeLa-mEGFP-F5 line, we investigated that if nonspecific oligonucleotides could increase the SSO-mediated targeted repair efficiency in mammalian cell lines. As shown in Table 1, we synthesized a series of nonspecific single-stranded DNA oligonucleotides with different lengths (12nt, 25nt, 50nt, 100nt). In order to avoid the cellular toxicity, no modifications were added to these oligos. Also, based on the DNA blast result, no significant similarity has been found among the sequence of SSO(E6) and the ones of the nonspecific oligos. First, we investigated that if the length of nonspecific oligos could affect the efficiencies of SSO-mediated gene repair. We treated F5 cells with E6(3 μg), as well as one of these nonspecific oligos(3 μg) in each group, and measured the repair efficiencies after 48 h of transfection. As seen in Fig. 1A, cells treated with nonspecific oligonucleotides showed three times higher repair efficiencies than the control, which was only treated with E6. And this increase had no relationship with the lengths of the oligos, since no significant difference had been observed among the groups treated with nonspecific oligos, which had different lengths. This result suggested that nonspecific oligonucleotides could improve the efficiencies of SSO-mediated gene repair in the length independent pattern. Since the 25nt nonspecific oligos treated groups showed more stable repair efficiencies than others, we used them for the further experiments.