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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.

Table 1
Sequences and the structural characteristics of nonspecific oligonucleotides used for targeted gene repair

Sequence (5′–3′)		Length
100-1:	CATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCA CCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAG	100nt
100-2:	CAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCG CACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGA	100nt
100-3:	AGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGAC TTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAA	100nt
50-1:	CCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAG	50nt
50-2:	CACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGA	50nt
50-3:	AGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGAC	50nt
25-1:	CAGCACGACTTCTTCAAGTCCGCCA	25nt
25-2:	TGCCCGAAGGCTACGTCCAGGAGCG	25nt
25-3:	CACCATCTTCTTCAAGGACGACGGC	25nt
25-4:	AACTACAAGACCCGCGCCGAGGTGA	25nt
12-1:	CAGCACGACTTC	12nt
12-2:	TGCCCGAAGGCT	12nt
12-3:	CACCATCTTCTT	12nt
12-4:	AACTACAAGACC	12nt

Different single-stranded DNA nonspecific oligonucleotides (12nt, 25nt, 50nt, 100nt) were designed for the SSO-mediated targeted gene repair in mammalian cell reporter system (F5 cells). The sequences of these oligonucleotides were randomly selected, and the significant similarity with the sequence of SSO was avoided. Also, no modifications were added to these nonspecific oligonucleotides.

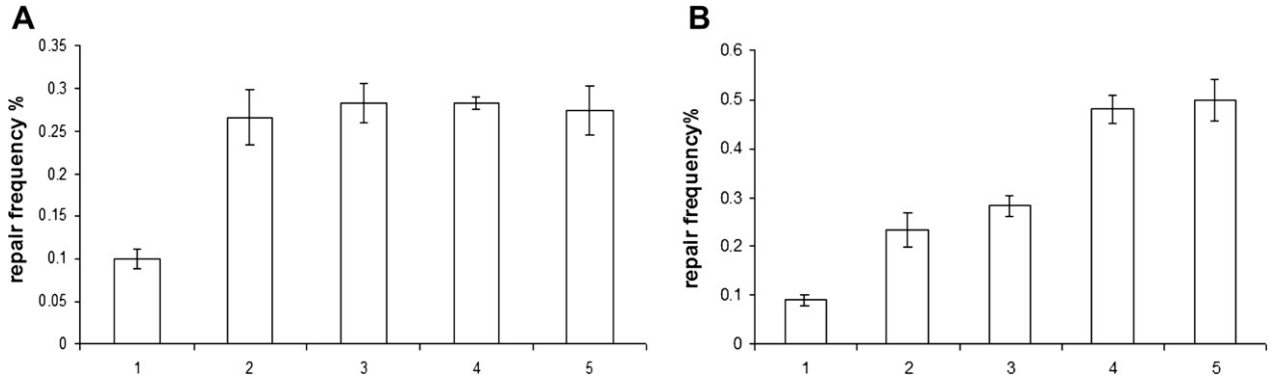

Fig. 1. (A) Evaluation of E6(SSO)-mediated repair frequency with nonspecific oligonucleotides in different lengths (100nt, 50nt, 25nt, 12nt). Nonspecific oligonucleotide (3 μg) and E6(3 μg) were used for each group, 1. Control: F5 cells were treated with E6 only; 2–5: F5 cells were treated with 100nt long nonspecific oligos/E6, 50nt long nonspecific oligos/E6, 25nt long nonspecific oligos/E6, 12nt long nonspecific oligos, respectively. (B) Repair efficiencies of E6(SSO) in the conditions with different doses of 25nt nonspecific oligonucleotides (1, 3, 6, 9 μg). The amount of E6 was 3 μg for each group. 1. Control: F5 cells were treated with E6 only; 2–5: F5 cells were treated with 1 μg nonspecific oligos/E6, 3 μg nonspecific oligos/E6, 6 μg nonspecific oligos/E6, 9 μg nonspecific oligos/E6, respectively.

Second, we explored if the dose of the nonspecific oligos could affect the gene repair efficiencies. We analyzed F5 cells by treatment with the following groups: 25-1/E6, 25-2/E6, 25-3/E6, 25-4/E6, or E6, respectively. The dose gradient of the nonspecific oligonucleotide was set up as 1, 3, 6, and 9 μg. Gene repair efficiencies were detected by flow cytometry. We found that about 0.3–0.5% of the cells treated with nonspecific oligonucleotides were repaired successfully, while 0.1%of the repaired cells were detected in the control group only treated with E6. And the gene correction efficiency of SSO(E6) was positively correlated with the dose of nonspecific oligonucleotides, since the repair efficiencies were increased up to 0.5% in the 6 μg and 9 μg nonspecific oligos treated groups, while 0.3% of the repaired cells were detected in the groups treated with 1 and 3 μg nonspecific oligos (Fig. 1B). And a modest difference in gene repair frequency was observed when increasing the amount of nonspecific oligonucleotides to 9 μg. As far as the cytotoxicity was concerned, we chose 6 μg of nonspecific oligonucleotides as the standard amount for the further experiments.

Third, to further confirm that the increased repair efficiencies were independent on the sequence of nonspecific oligos, we treated F5 cells with the followings: C25-1, C25-2, C25-3, C25-4, which were the complementary oligonucleotides of 25-1, 25-2, 25-3, and 25-4, respectively. After 48 h of transfection, we also found that about 0.5% of the cells treated with those nonspecific oligonucleotides were corrected successfully, while the repaired cells in the control group (only treated with E6) were 0.1% (see Supplemental material 2). This result confirmed that the increased repair efficiency was due to the treatment of nonspecific oligonucleotides, instead of the specific sequences.

Then we analyzed the mechanism of the increased repair efficiency by the nonspecific oligos treatment. One possibility is that nonspecific oligos may integrate into the chromosome. To test this likelihood, we treated F5 cells with thymidine and nonspecific oligos together. According to the replication fork leakage hypothesis, thymidine, which can inhibit the formation of dCTP (one of the essential precursors for cellular DNA synthesis), will slow down or retard the progression of the replication fork. So the period of the chromosomal DNA existing in single-stranded form will be lengthened, which will increase the possibility of nonspecific oligos integration [25]. If this possibility exits, the beneficial effect on the oligos integration would cause a more significant increase in the repair efficiency. To test it, we incubated F5 cells with nonspecific oligonucleotides and thymidine, and the repair efficiencies were measured after 48 h of transfection. As shown in Fig. 2A, the gene repair efficiencies increased up to 1% with the treatment of thymidine, but no further improvement in the repair efficiencies was observed in the

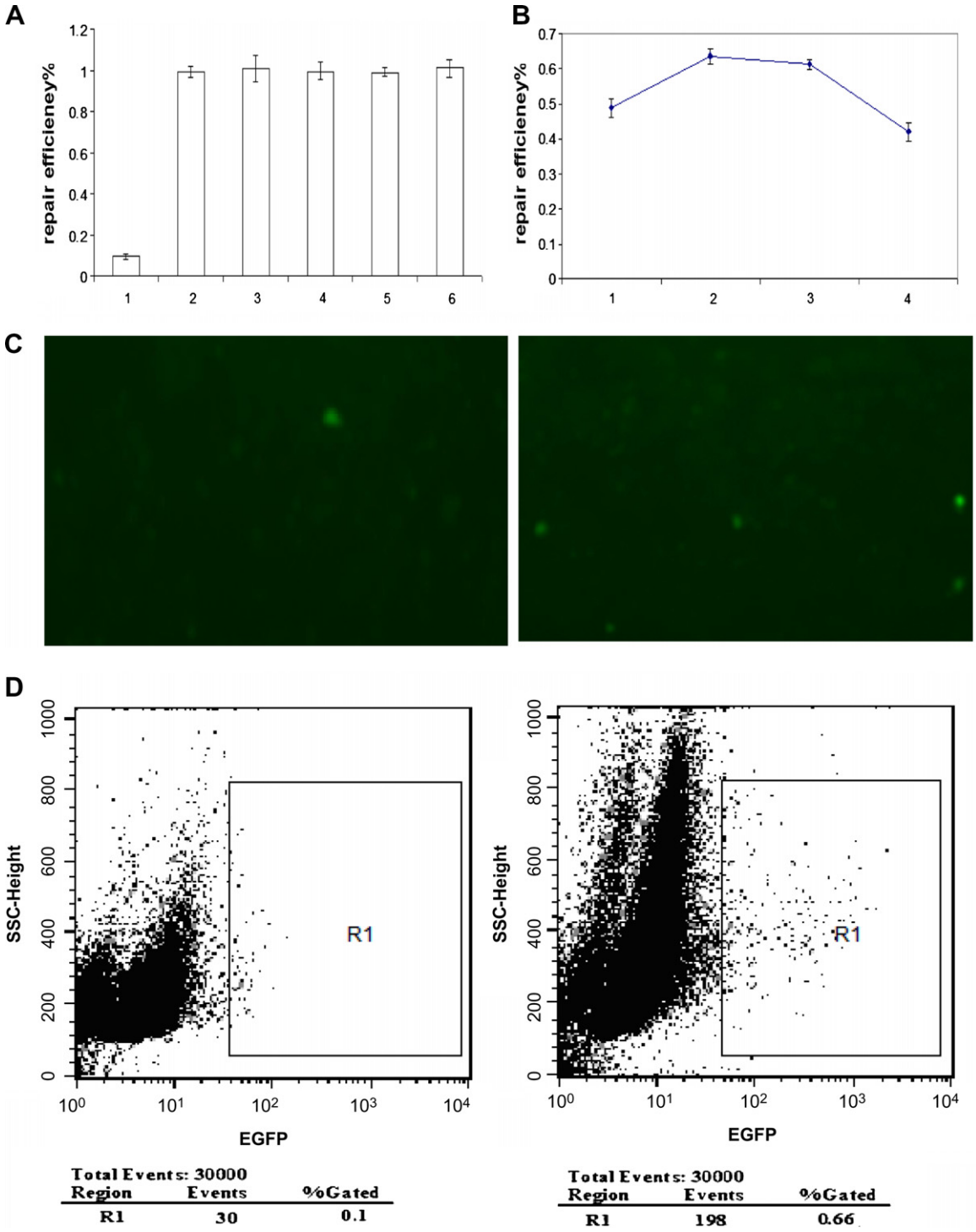

Fig. 2. (A) Repair efficiency of E6 with the treatment of nonspecific oligonucleotide and thymidine. 1. Control: F5 cells were treated with E6(3 μg)only; 2: F5 cells were treated with thymidine (2 mM) and E6; 3–6: F5 cells were treated with 25-1/E6/thymidine, 25-2/E6/thymidine, 25-3/E6/thymidine, and 25-4/E6/thymidine, respectively. (B) Repair efficiency of E6 with the preincubation of nonspecific oligonucleotides. For each group, F5 cells were treated with 25nt nonspecific oligonucleotides (6 μg), as well as E6(3 μg). 1. cotransafection of E6 and nonspecific oligonucleotides; 2–4: preincubation of nonspecific oligonucleotides 0.5, 3, 6 h before the transfection of E6. (C) Repaired cells detected under UV light. (*Left*) without nonspecific oligonucleotides, there was only one green cell in several eye fields. (*Right*) with the 0.5 h preincubation of nonspecific oligonucleotides, there were five green cells in one eye field. (D) FACS results comparing frequencies of SSO-mediated repair 48 h after transfection by E6 without (1) or with (2) nonspecific oligonucleotides preincubation (0.5 h).

coincubation of thymidine and nonspecific oligonucleotides. These results suggested that the increased repair efficiency by nonspecific oligonucleotides was not achieved by integrating into the chromosome.

Another possibility is that nonspecific oligos may increase the gene repair efficiency by creating a more favorable intracellular proteomic environment. To test this likelihood, we treated F5 cells with nonspecific oligos for a certain period before transfection with

SSO. If this possibility exits, the beneficial effect on the proteome would cause a more obvious increase in the repair efficiency. To explore it, we incubated the F5 cells with nonspecific oligonucleotides for 0.5, 3, and 6 h before transfection with E6(SSO). And the nonspecific oligonucleotides were maintained in the medium of F5 cells until the gene repair efficiencies were measured. As shown in Fig. 2B–D, after 48 h of transfection, the gene correction efficiencies increased up to 0.66% with the 0.5 and 3 h preincubation of nonspecific oligonucleotides, and then dropped to 0.4% when the preincubation time was extended to 6 h. These results suggested that (i) the increased repair efficiency was achieved by the favorable alterations of the cellular proteome, which was induced by nonspecific oligonucleotides; (ii) this effect was transient, which probably due to the rapid degradation of nonspecific oligonucleotides by the cellular nuclease.

Strand bias in the repair experiments with nonspecific oligonucleotides

Strand bias means that two complementary SSOs usually have different repair efficiencies in the same reporter system [6,11,17,27,28]. And in most repair systems, the antisense SSO, which is complementary with nontranscriptional strand of target gene, has shown higher repair efficiencies than the sense SSO, which is complementary with the transcriptional strand of target gene [12]. But there are different opinions. For example, the repair efficiency of sense SSO was significantly higher than that of antisense SSO in bleomycin treated BHK-21 cells [29]. And the opposite strand bias was also observed when two individual mutations in episomal β-galactosidase gene were repaired by SSO [15]. Now we need to find out the strand bias in our reporter system (F5 cells), which were treated with nonspecific oligonucleotides. We synthesized RE6, which is the complementary sequence of E6, then cotransfected F5 cells with different 25nt nonspecific oligonucleotides and E6 or RE6. After 48 h of transfection, we found that about 0.5% of the cells treated with E6 and nonspecific oligonucleotides were corrected successfully, while almost no repair events were observed in the groups treated with RE6 (Fig. 3). This result is consistent with previous reports that antisense SSO were more efficient than their sense counterparts in the gene correction of chromosomal target.

In summary, we used a sensitive and stable mammalian system to investigate the effect of nonspecific oligonucleotides on the targeted gene repair mediated by SSO. And we found that the nonspecific oligonucleotides could increase the repair efficiency in a dose dependent pattern. The possible mechanism is that the nonspecific oligonucleotides might create a transient and favorable intracellular proteomic environment. Finally, we speculate that this approach has great potential for further application in other gene targeting systems.

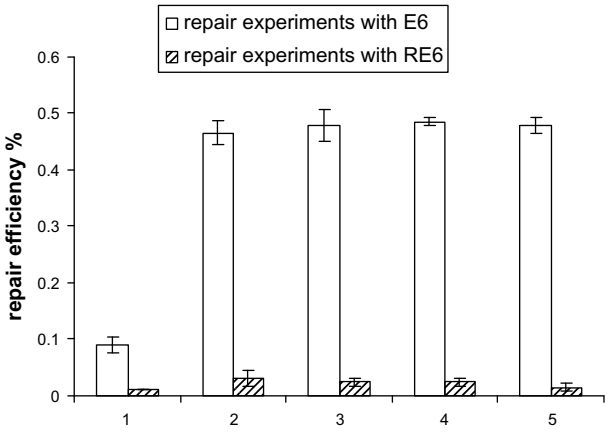

Fig. 3. Stand bias in nonspecific oligonucleotides-treated repair experiments. Nonspecific oligonucleotides (6 μg) and E6(3 μg) or RE6(3 μg) were cotransfected into F5 cells. 1. F5 cells were treated with E6 or RE6 only; 2–5: F5 cells were treated with 25-1/E6 or 25-1/ RE6, 25-2/E6 or 25-2/RE6, 25-3/E6 or 25-3/RE6, 25-4/E6 or 25-4/RE6, respectively.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.08.119.

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