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Roles of specialized DNA polymerases in mutagenesis by 8-hydroxyguanine in human cells

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Abstract

The formation of 8-hydroxyguanine (8-OH-Gua, 7,8-dihydro-8-oxoguanine) in DNA and in the nucleotide pool results in G:C→T:A and A:T→C:G substitution mutations, respectively, due to the ability of 8-OH-Gua to pair with both C and A. In this study, shuttle plasmid DNAs containing 8-OH-Gua paired with C and A in the supF gene were transfected into human 293T cells, in which specialized DNA polymerases were knocked-down. The DNAs replicated in the cells were recovered and then introduced into an indicator strain of *Escherichia coli*. Mutation analysis indicated that the knock-downs of DNA polymerases η and ζ by siRNAs enhanced the G:C→T:A mutations caused by 8-OH-Gua:C. The 8-OH-Gua:C-induced mutation frequency was not further increased by double knock-downs of DNA polymerases η and ζ, suggesting that the two DNA polymerases work in the same pathway. In addition, the reduction of DNA polymerase η slightly decreased the A:T→C:G substitutions caused by 8-OH-Gua:A. These results suggest that DNA polymerases η and ζ are involved in the bypass of 8-OH-Gua in human cells.

Keywords: 8-Hydroxyguanine; specialized DNA polymerases; oxidative mutagenesis
Abbreviations: 8-OH-dGTP, 8-hydroxy-2'-deoxyguanosine 5' triphosphate; 8-OH-Gua, 8-hydroxyguanine; MBL; methylene blue plus light; pol, polymerase; ROS, reactive oxygen species; RT, reverse transcription; TLS, translesion synthesis.
1. Introduction

DNA is continuously oxidized by reactive oxygen species (ROS) that are produced endogenously by normal oxygen metabolism as well as by many environmental mutagens and carcinogens. 8-Hydroxyguanine (8-OH-Gua, 7,8-dihydro-8-oxoguanine) is one of the major oxidized nucleobases produced by ROS, and 100–500 residues are generated per cell per day [1-3]. This oxidized G base is miscoding, due to its ability to form base pairs with A in addition to C [4-7]. Mutagenesis experiments with DNA containing 8-OH-Gua at a predetermined site indicated that 8-OH-Gua is highly mutagenic and induces G:C→T:A transversion mutations in *Escherichia coli* and mammalian cells [8-16]. ROS oxidize DNA precursors (2'-deoxyribonucleotides) as well as DNA. 8-Hydroxy-2'-deoxyguanosine 5'-triphosphate (8-OH-dGTP, 7,8-dihydro-8-oxo-dGTP) is the major oxidation product of dGTP in *in vitro* oxidation reactions [17]. Pursell et al. demonstrated that the concentration of 8-OH-dGTP was 1–10% of that of unmodified dGTP in the mitochondrial nucleotide pool [18]. 8-OH-dGTP is mutagenic and specifically induced A:T→C:G transversions in living bacterial and mammalian cells [19-21]. The A:T→C:G mutations seem to be triggered by the incorporation of 8-OH-dGTP opposite A in template DNA, resulting in the formation of 8-OH-Gua:A base pairs, and the subsequent incorporation of dCTP opposite 8-OH-Gua during the next round of replication would cause the transversion [22].

Mammalian cells contain specialized DNA polymerases (pols) that play important roles in translesion synthesis (TLS) [23,24]. It has been shown that TLS DNA pols bypassed 8-OH-Gua *in vitro* [25-34]. Thus, to understand the mutagenesis and carcinogenesis processes by 8-OH-Gua, it is important to examine the contributions of the polymerases to the induction and suppression of the G:C→T:A mutations caused by this lesion in living mammalian cells. Previously, Lee and Pfeifer
transfected plasmid DNA after a treatment with methylene blue plus light (MBL), which produces predominantly 8-OH-Gua in DNA, into human cells in which DNA pol η was knocked-down and mutated [35]. However, the MBL treatment could potentially produces other types of DNA damages [36]. Therefore, we surmised that the transfection of plasmid DNA containing 8-OH-Gua at a specific position would provide more direct evidence. In addition, we attempted to examine the involvement of other TLS DNA pols in the 8-OH-Gua-induced mutagenesis.

We recently found that the knock-downs of DNA pols η and ζ, and REV1 reduced the frequency of A:T trans (C:G mutations induced by exogenous 8-OH-dGTP [21], suggesting that these proteins are involved in the mutagenesis induced by 8-OH-dGTP. This result raised the question of whether these DNA pols contribute to the incorporation of 8-OH-dGTP and/or to the insertion of dCTP opposite 8-OH-Gua. The discrimination would be possible if we introduced plasmid DNA containing an 8-OH-Gua:A pair, an intermediate in the mutagenic process of 8-OH-dGTP [22], into cells with knocked-down TLS DNA pols.

In this study, double-stranded plasmid DNA containing an 8-OH-Gua:C pair was transfected into human cells in which DNA pols η, ι, and ζ, and REV1 were knocked-down. Moreover, plasmid DNA containing an 8-OH-Gua:A pair was also introduced into the knocked-down cells, as the intermediate in the mutagenic process of 8-OH-dGTP. We found that the knock-downs of DNA pols η and ζ enhanced the G:C trans (T:A transversion mutation induced by the 8-OH-Gua:C pair. Moreover, the knock-down of DNA pol η reduced the A:T trans (C:G substitution mutation by the 8-OH-Gua:A pair. These results suggest that some TLS DNA pols indeed play important roles in the mutagenesis processes of 8-OH-Gua.
2. Materials and methods

2.1. Materials

Oligodeoxyribonucleotides containing 8-OH-Gua and their control oligodeoxyribonucleotides were purchased from Japan Bio Services (Asaka, Japan) and purified by reverse phase and anion exchange HPLC, as described previously [37]. Other oligodeoxyribonucleotides were obtained from Hokkaido System Science (Sapporo, Japan) and Sigma Genosys Japan (Ishikari, Japan) in purified forms. The siRNAs (“stealth RNAi”, Invitrogen, Carlsbad, California, USA) were the same ones used in the previous studies [21]. The control siRNA was that for low GC content (Invitrogen). The E. coli strain KS40/pOF105 [38] was provided by Dr. Tatsuo Nunoshiba, of International Christian University, and was used as an indicator strain of the supF mutants.

2.2. Plasmid DNAs containing 8-OH-Gua

The oligodeoxyribonucleotides indicated below were synthesized and chemically phosphorylated at their 5'-end on the support during synthesis.

T-96, 5'-dGCAGACTCTAAATCTGCCGTCAT;

ohG-96, 5'-dGCAGACTCG*AAATCTGCCGTCAT;

G-122, 5'-dCGACTTCGAAGGTTCCAATCC;

ohG-122, 5'-dCGACTTCGAAGG*TTCGAATC; where G* represents 8-OH-Gua.
Double-stranded plasmid DNAs containing 8-OH-Gua were constructed as described [39] from the single-stranded forms of pZ189-StuI [21] and the oligodeoxyribonucleotides listed above, by annealing and treatments with T4 DNA pol and T4 DNA ligase, and with Dam methylase.

2.3. Mutagenesis experiments

For the experiments using the G:C and 8-OH-Gua:C plasmid DNAs, 293T cells (3 X 10⁴ cells) were plated into 24-well dishes and were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum, at 37°C under a 5% CO₂ atmosphere for 24 h. The siRNA (7.2 pmol each) and “carrier DNA”, the pZeo plasmid (100 ng), which was constructed by ligation of the EM7-Zeo and pUC ori fragments of pCMV/Zeo (Invitrogen), were mixed with Lipofectamine (Invitrogen) and introduced into the cultured 293T cells according to the supplier’s recommendations. Note that the pZeo plasmid lacks the ampicillin resistance gene and thus does not affect the mutagenesis assay. After 24 h, the plasmids containing G:C and 8-OH-Gua:C (100 ng, 29 fmol) was transfected with Lipofectamine. After 48 h of culture, the plasmid amplified in the cells was recovered by the method of Stary and Sarasin [40]. The recovered DNA was treated with DpnI, to digest the unreplicated plasmids, and was introduced into E. coli KS40/pOF105 cells by electroporation. The supF mutant frequency was determined as described previously [21,38]. For the T:A and 8-OH-Gua:A plasmids, the mutagenesis experiments were performed similarly, using 293T cells (6 X 10⁴ cells) in 12-well dishes.

2.4. Quantitative reverse transcription (RT)-PCR analysis of mRNA
Total RNA was extracted from 293T cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) combined with RNase-free DNase I (Takara, Otsu, Japan) for the degradation of genomic DNA in total RNA samples. First-strand cDNA synthesis was performed on 500 ng of total RNA using an oligo dT primer and an RNA PCR Kit (AMV) (Takara), according to the manufacturer’s instructions. Each of the mRNA transcripts was measured by the quantitative PCR method with an ABI 7500 Real Time PCR System and SYBR-Green chemistry (Applied Biosystems, Foster City, CA), as described previously [21]. Data are expressed as the ratio to the GAPDH mRNA.

2.5. **Statistical analysis**

Statistical significance was examined by the Student’s *t*-test. Levels of *P*<0.05 were considered to be significant.

3. **Results**

3.1. **Design of plasmid DNAs involving 8-OH-Gua**

We constructed plasmid DNAs containing 8-OH-Gua:C and 8-OH-Gua:A base pairs (Fig. 1). The parental plasmid, pZ189-StuI, contains the *supF* gene, the SV40 large T antigen gene, and the SV40 origin of replication [21]. 8-OH-Gua was introduced into positions 122 and 96 of the sense strand of the *supF* gene for 8-OH-Gua:C and 8-OH-Gua:A, respectively. Position 96 was one of the
A:T→C:G hot spots in the *supF* gene when 8-OH-dGTP was introduced [20,21]. Plasmid DNAs containing G:C and T:A at positions 122 and 96, respectively, were used as control plasmids.

3.2. **Knock-downs of DNA polymerases η and ζ affect the induced mutation frequency of the 8-OH-Gua:C pair**

We first examined the effects of the knock-downs of specialized DNA pols by siRNAs on mutations induced by 8-OH-Gua:C. DNA pols η, ι, and ζ (REV3), and REV1 were selected as the knock-down targets, as in the previous study [21]. These knock-downs were measured by quantitative RT-PCR (Table 1). The treatment of 293T cells with siRNA reduced the amounts of the mRNAs encoding these DNA pols. Since we did not observe the effective knock-down of DNA pol κ, this pol was excluded from the analysis.

The treatment with siRNAs against DNA pols η, ι, and ζ, and REV1 resulted in *supF* mutant frequencies similar to the case for the control siRNA in the experiments using the G:C plasmid (∼1 X 10⁻³, Fig. 2, open columns), indicating that the reduction in these DNA pols did not affect the background mutant frequency in this experimental system. When the plasmid with the 8-OH-Gua:C pair at position 122 was introduced into 293T cells treated with the control siRNA, the *supF* mutant frequency was increased to ∼4 X 10⁻³ (Fig. 2), indicating the mutagenicity of 8-OH-Gua in DNA. The *supF* mutant frequencies were enhanced to ∼6.5 X 10⁻³ in the cells where DNA pols η and ζ were knocked-down (Fig. 2). This result suggests that the two specialized DNA pols are involved in “high-fidelity” bypass of the 8-OH-Gua lesion.
We next examined the effects of the double knock-downs of DNA pols $\eta$ and $\zeta$. These knock-downs could further increase the $supF$ mutant frequency when the two DNA pols act independently. However, we did not observe the expected additive effect upon the double knock-downs (Fig. 3). This result may suggest that DNA pols $\eta$ and $\zeta$ work in the same pathway in the mutation process of 8-OH-Gua, although the knock-down efficiencies for these DNA pols were lower than those in the single knock-down experiments at 24 h, at the time point of transfection of the $supF$ plasmid (Table 1).

We analyzed the sequences of the $supF$ genes in colonies on the selection agar plates. As expected, the G:C$\rightarrow$T:A transversion was the mutations found most frequently in the colonies obtained from the control siRNA-treated and knocked-down cells (Table 2). The total $supF$ mutant frequencies were multiplied by the percentages of the targeted G:C$\rightarrow$T:A transversion, and the targeted G:C$\rightarrow$T:A frequencies were calculated to be 2.6, 5.0, and $4.8 \times 10^{-3}$ in the control, pol $\eta$, and pol $\zeta$ experiments, respectively. Thus, we concluded that the G:C$\rightarrow$T:A mutation was increased by the knock-down of the two TLS DNA pols, and that they play suppressive roles in the mutagenesis caused by 8-OH-Gua:C. In contrast, no G:C$\rightarrow$T:A mutation at position 122 was observed for the transfection of the control G:C plasmid (Supplementary Table 1).

Interestingly, the G:C$\rightarrow$A:T transition mutation was found in five of the 57 colonies obtained by transfection of the 8-OH-Gua:C plasmid into the cells where pol $\iota$ was knocked-down (Table 2). The G:C$\rightarrow$C:G mutation was detected in all cases, except for the DNA pol $\eta$ experiment.
3.3. Effects of knock-downs of specialized DNA polymerases on the induced mutation frequency of the 8-OH-Gua:A pair

Recently, we found that the knock-downs of DNA pols η and ζ, and REV1 decreased the A:T→C:G mutations induced by exogenous 8-OH-dGTP [21]. In the 8-OH-dGTP experiment, knock-down of a certain DNA pol could affect (i) incorporation of 8-OH-dGTP opposite A during the first round of replication and (ii) that of dCTP opposite 8-OH-Gua after 8-OH-dGTP was incorporated into DNA. We next examined the effects of the knock-downs of specialized DNA pols on the mutations induced by 8-OH-Gua:A, to assess their contributions to the insertion of dCTP opposite 8-OH-Gua. The plasmid DNA containing an 8-OH-Gua:A pair at position 96 of the supF gene was prepared. This position was one of the hot spots of the A:T→C:G mutations induced by 8-OH-dGTP [20,21].

The treatment with siRNAs against the TLS DNA pols did not affect the supF mutant frequencies in the experiments using the T:A plasmid (~1 X 10^{-3}, Fig. 4A), although a slightly higher frequency was observed for the knock-down of DNA pol ζ (P=0.08). When the plasmid with the 8-OH-Gua:A pair at position 96 was introduced into 293T cells treated with the control siRNA, the supF mutant frequency was 88 X 10^{-2} (88%), which is quite high (Fig. 4B). This high mutant frequency was due to the MUTYH protein, which removes A opposite 8-OH-Gua [41]. The supF mutant frequencies were decreased to 81 X 10^{-2} (81%) in the cells where DNA pol η was knocked-down (P<0.05). This result suggests that this DNA pol is involved in the incorporation of dCTP opposite the 8-OH-Gua base, although the effect of the knock-down was minimal.
We analyzed the sequences of the supF genes in colonies on the selection agar plates (Table 3 and Supplementary Table 2). As expected, the A:T→C:G transversion was found in most cases for the 8-OH-Gua:A plasmid. In the cases of the control and pol η siRNAs, all colonies examined contained the A:T→C:G mutation. Thus, the knock-down of DNA pol η reduced the occurrence of the A:T→C:G mutation induced by 8-OH-Gua:A by 8%, while the knock-down decreased the A:T→C:G mutations caused by exogenous 8-OH-dGTP by 32% [21]. Mutations other than A:T→C:G at position 96 were found in three and one colonies for the knock-downs of DNA pols ι and ζ, respectively (Table 3).

4. Discussion

In this study, we examined the involvement of TLS DNA pols in the mutagenesis by 8-OH-Gua. As shown in Fig. 2, the reduction of DNA pol η, a Y-family DNA pol, significantly increased the mutations induced by 8-OH-Gua paired with C in DNA. This result suggests that DNA pol η plays a suppressive role in the mutagenesis by 8-OH-Gua in human cells. The incorporation of 2′-deoxyribonucleotides opposite 8-OH-Gua by human DNA pol η was analyzed in vitro. Human DNA pol η incorporates dATP and dCTP opposite the lesion with comparable efficiencies, but proliferating cell nuclear antigen (PCNA) and replication protein A (RP-A) enhance the correct 8-OH-Gua bypass [25-30]. The mammalian replicative DNA pols α and δ are error-prone against 8-OH-Gua [27,28,42-47]. Thus, the knock-down of DNA pol η might force the “error-prone” replicative DNA pols to bypass 8-OH-Gua.
In addition, the knock-down of DNA pol ζ, a B-family DNA pol, increased the mutagenesis caused by 8-OH-Gua paired with C (Fig. 2). This result suggests that DNA pol ζ also plays a suppressive role in the 8-OH-Gua-induced mutations in human cells. DNA pol ζ is a highly specialized pol, and it extends primer termini opposite from a diverse array of DNA lesions, including 8-OH-Gua [24,48]. Thus, DNA pol ζ might extend a nascent strand from C (correct 2'-deoxynucleotide) opposite 8-OH-Gua that was incorporated by other DNA pols.

The double knock-downs of DNA pols η and ζ did not further increase the mutant frequency (Fig. 3) and this result may suggest that these DNA pols work in the same pathway. Their properties described above prompted us to speculate that DNA pol η incorporated dCTP opposite 8-OH-Gua and that DNA pol ζ extended the C nucleotide opposite 8-OH-Gua. However, this interpretation should be reviewed carefully, since the knock-down efficiencies for these DNA pols in the double knock-down experiment were lower than those in the single knock-down experiments at 24 h, at the time point of transfection of the supF plasmid (Table 1). Moreover, this result was in contrast to the previous finding that the double knock-downs of DNA pols η and ζ affected additively when mutations induced by 8-OH-dGTP were analyzed [21].

In contrast, the suppression of a Y-family DNA pol, DNA pol ι, did not affect the mutant frequency of the plasmid containing 8-OH-Gua:C (Fig. 2). Although the incorporation of 2'-deoxynucleotides opposite 8-OH-Gua in template DNA by DNA pol ι in vitro was reported [31-33], the result obtained in this study suggests that the contribution of the DNA pol to 8-OH-Gua bypass is minimal, at best, at least in this human cell line. REV1 is a member of the Y-family DNA pols, but it acts as deoxycytidyl transferase opposite a range of damaged bases [23,24].
REV1 inserts dCTP opposite 8-OH-Gua \textit{in vitro} [34]. However, the knock-down of REV1 did not affect the mutation caused by 8-OH-Gua (Fig. 2), suggesting that this error-free function of the protein is not important in the case of 8-OH-Gua:C.

We recently reported the A:T$\rightarrow$C:G mutations induced by 8-OH-dGTP in living simian and human cells [20,21]. In this study, we introduced plasmid DNA containing 8-OH-Gua:A as an intermediate in the mutagenic process of 8-OH-dGTP (Fig. 1). Unexpectedly, the total \textit{supF} mutant frequency was $88 \times 10^{-2}$ in the case of 293T cells treated with the control siRNA (Fig. 4B). Since all colonies among the 50 colonies obtained in the experiment contained the targeted A:T$\rightarrow$C:G mutation (Table 3), the mutation frequency of 8-OH-Gua:A was $88 \times 10^{-2}$ in 293T cells. Le Page et al. reported that the mutation frequencies of 8-OH-Gua:A were 36 and $32 \times 10^{-2}$ in simian COS-7 and human MRC5V1 cells, respectively [49]. This discrepancy would be due to the difference in the MUTYH activity, which removes an A base opposite 8-OH-Gua. The knock-down of MUTYH in 293T cells reduced the mutagenicity of the 8-OH-Gua:A base pair (to $\sim 40 \times 10^{-2}$) [41].

As shown in Fig. 4B, a reduction in the amount of DNA pol $\eta$ significantly decreased the mutation induced by 8-OH-Gua paired with A ($88 \times 10^{-2}$ to $81 \times 10^{-2}$). This $\sim 8\%$ reduction in the mutant frequency was in contrast to the $\sim 32\%$ decrease in mutant frequency by the knock-down of DNA pol $\eta$ in the previous 8-OH-dGTP introduction experiment [21]. Since the knock-down efficiency of the pol $\eta$ mRNA (79\% at 24 h, Table 1) was higher than that in the previous study (56\% at 24 h), the slight effect observed in this study was not due to low knock-down efficiency. As mentioned above, the A:T$\rightarrow$C:G mutation induced by 8-OH-Gua:A was MUTYH-dependent. Based on the quantitatively different effects of the knock-down of DNA pol $\eta$ on the mutations
induced by 8-OH-dGTP and 8-OH-Gua:A, we speculated that DNA pol η did not play the major role in gap-filling after the removal of A opposite 8-OH-Gua by MUTYH. DNA pol λ was suggested to be involved in the bypass of 8-OH-Gua in the gap-filling reactions [28,50]. Taken together with the increased mutation by 8-OH-Gua:C and the decreased mutation by 8-OH-Gua:A in the cells with knocked-down pol η, this DNA pol would bypass 8-OH-Gua during DNA replication, with a higher dCTP/dATP incorporation ratio than those of the replicative DNA pols. Moreover, the decrease in the A:T → C:G mutations by 8-OH-dGTP in the pol η-knocked-down cells, observed in the previous study [21], was suggested to be mainly due to reduced 8-OH-dGTP incorporation into the nascent strand. This speculation is consistent with the highly erroneous incorporation of 8-OH-dGTP opposite A by this DNA pol in vitro [51,52].

In contrast to the previous study [21], no obvious effects were observed when DNA pol ζ and REV1 were knocked-down (Fig. 4B). Thus, DNA pol ζ and REV1 seemed to play important roles in the incorporation of 8-OH-dGTP but not in the insertion of dCTP opposite 8-OH-Gua.

Lee and Pfeifer introduced MBL-treated plasmid DNA into human cells in which DNA pol η was knocked-down and mutated [35]. They observed an increase in the total mutant frequency by the reduction/loss of DNA pol η, as we have shown in this report (Fig. 2). However, the ratios of G:C → T:A transversions in the knocked-down/knock-out cells were similar to (or less than) that in the control cells. Since the MBL treatment produces other damaged bases besides 8-OH-Gua, such as 2,5-diamino-4H-imidazol-4-one [36], their results might also partly reflect the effects of the reduction/loss on mutations caused by several damaged bases.
Avkin and Livneh introduced a gapped plasmid carrying 8-OH-Gua in the single-stranded DNA region into DNA pol η-proficient and -deficient human cells [53]. They reported that the ratio of dATP insertion (corresponding to the G:C→T:A mutation) was nearly identical in both cell lines. Their result was in contrast to our present observation that the knock-down of DNA pol η increased the mutation frequency and the ratio of the G:C→T:A mutation (Fig. 2 and Table 2). This discrepancy is a consequence of the gapped plasmid that Avkin and Livneh used. DNA pol η might not be recruited to the gap, and thus may not participate in the 8-OH-Gua bypass.

In conclusion, the knock-downs of DNA pols η and ζ enhanced the mutagenicity of 8-OH-Gua paired with C in human 293T cells. This result suggests that these DNA pols are involved in the error-free bypass of the oxidized G base. Moreover, the knock-downs of DNA pol η slightly reduced the mutations induced by 8-OH-Gua paired with A. In addition to DNA pols, DNA repair enzymes and nucleotide pool sanitization enzymes would play important roles in the mutagenesis processes of damaged DNA and DNA precursors [54]. Experiments to reveal their roles are currently in progress.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

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References


[22] H. Kamiya, Mutations induced by oxidized DNA precursors and their prevention by nucleotide pool sanitization enzymes, Genes Environ. 29 (2007) 133-140.


Figure legends

**Fig. 1** Structures of plasmid DNAs containing 8-OH-Gua, used in this study. 8-OH-Gua was introduced to either positions 96 (8-OH-Gua:A) or 122 (8-OH-Gua:C) in the *supF* gene. The X’s indicate the positions where 8-OH-Gua was incorporated.

**Fig. 2** Effects of knock-downs of specialized DNA pols in 293T cells on the mutant frequencies induced by 8-OH-Gua:C. Open columns, control plasmid containing G:C at position 122; closed columns, plasmid containing 8-OH-Gua:C at position 122. Data are expressed as means ± SEM (n=4 for control siRNA (G:C and 8-OH-Gua:C), pol η siRNA, (8-OH-Gua:C), and pol ι siRNA (G:C), and n=3 for others). (*P<0.05 vs control).

**Fig. 3** Effects of double knock-downs of DNA pols η and ζ in 293T cells on the mutant frequencies induced by 8-OH-Gua:C. Open columns, control plasmid containing G:C at position 122; closed columns, plasmid containing 8-OH-Gua:C at position 122. Data are expressed as means ± SEM (n=3). (*P<0.05 vs control).

**Fig. 4** Effects of knock-downs of specialized DNA pols in 293T cells on mutant frequencies induced by 8-OH-Gua:A. (A) Mutant frequencies upon transfection of the control plasmid containing T:A at position 96. (B) Mutant frequencies upon transfection of the plasmid containing
8-OH-Gua:A at position 96. Data are expressed as means ± SEM (n=4 for REV3 siRNA (8-OH-Gua:A), n=6 for control siRNA (8-OH-Gua:A), and n=3 for others). (*P<0.05 vs control).
Fig. 1

Fig. 2
Fig. 3

Fig. 4
Table 1
Amounts of mRNAs in 293T cells with knocked-down specialized DNA polys.

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<th>24 h</th>
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<td>Single knock-down experiment</td>
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<td>REV1</td>
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The amount of mRNA was measured by quantitative RT-PCR at 24, 48, and 72 h after siRNA introduction according to the methods described in Section 2. Relative values to that in 293T cells treated with the control siRNA are shown. Data are expressed as means ($\pm$SEM).

$^a n=3$.

$^b n=2$.

$^c n=5$.

$^d n=4$. 
Table 2. Targeted mutations at position 122 found in progeny plasmid of 8-OH-Gua:C plasmid, replicated in 293T cells with knocked-down DNA pols.

<table>
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<td>Single base substitutions</td>
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</tr>
<tr>
<td>Transition</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>G:C--&gt;A:T</td>
<td>0 (0)</td>
<td>1 (2)</td>
<td>5 (9)</td>
<td>1 (2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Transversion</td>
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<td></td>
</tr>
<tr>
<td>G:C--&gt;T:A</td>
<td>38 (68)</td>
<td>41 (76)</td>
<td>39 (68)</td>
<td>37 (71)</td>
<td>34 (68)</td>
</tr>
<tr>
<td>G:C--&gt;C:G</td>
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<td>0 (0)</td>
<td>1 (2)</td>
<td>1 (2)</td>
<td>1 (2)</td>
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<tr>
<td>Single base deletion</td>
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<td></td>
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</tr>
<tr>
<td>Others</td>
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<td>2 (4)</td>
<td>1 (2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>No mutation</td>
<td>15 (27)</td>
<td>10 (19)</td>
<td>11 (19)</td>
<td>13 (25)</td>
<td>15 (30)</td>
</tr>
<tr>
<td>Total</td>
<td>56 (100)</td>
<td>54 (100)</td>
<td>57 (100)</td>
<td>52 (100)</td>
<td>50 (100)</td>
</tr>
</tbody>
</table>

All data are represented as cases found (%).
Table 3. Targeted mutations at position 96 found in progeny plasmid of 8-OH-Gua:A plasmid, replicated in 293T cells with knocked-down DNA polys.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Control</th>
<th>Pol h</th>
<th>Pol i</th>
<th>REV3 (Pol z)</th>
<th>REV1</th>
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<tbody>
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<tr>
<td>Single base substitutions</td>
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<tr>
<td>Transition</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>A:T→G:C</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Transversion</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A:T→T:A</td>
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<td>0 (0)</td>
<td>1 (2)</td>
<td>1 (2)</td>
<td>0 (0)</td>
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<tr>
<td>A:T→C:G</td>
<td>50 (100)</td>
<td>46 (100)</td>
<td>47 (94)</td>
<td>53 (98)</td>
<td>58 (100)</td>
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<tr>
<td>Single base deletion</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>No mutation</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
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<tr>
<td>Total</td>
<td>50 (100)</td>
<td>46 (100)</td>
<td>50 (100)</td>
<td>54 (100)</td>
<td>58 (100)</td>
</tr>
</tbody>
</table>

All data are represented as cases found (%).

*a8-OH-Gua --> AG.