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**Involvement of specialized DNA polymerases in mutagenesis
by 8-hydroxy-dGTP in human cells**

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Abstract

The mutagenicity of an oxidized form of dGTP, 8-hydroxy-2'-deoxyguanosine 5'-triphosphate (8-OH-dGTP), was examined using human 293T cells. Shuttle plasmid DNA containing the *supF* gene was first transfected into the cells, and then 8-OH-dGTP was introduced by means of osmotic pressure. The DNAs replicated in the cells were recovered and then transfected into *Escherichia coli*. 8-OH-dGTP induced A:T→C:G substitution mutations in the cells. The knock-downs of DNA polymerases η and ζ , and REV1 by siRNAs reduced the A:T→C:G substitution mutations, suggesting that these DNA polymerases are involved in the misincorporation of 8-OH-dGTP opposite A in human cells. In contrast, the knock-down of DNA polymerase ι did not affect the 8-OH-dGTP-induced mutations. The decrease in the induced mutation frequency was more evident by double knock-downs of DNA pols η plus ζ and REV1 plus DNA pol ζ (but not by that of DNA pol η plus REV1), suggesting that REV1-DNA pol η and DNA pol ζ work in different steps. These results indicate that specialized DNA polymerases are involved in the mutagenesis induced by the oxidized dGTP.

1. Introduction

Nucleic acids are subjected to thousands of oxidative hits per day [1]. The resulting nucleic acid oxidation represents a potentially important source of mutations, and is one of the causative factors of carcinogenesis, neurodegeneration, and aging [2-4]. 8-Hydroxyguanine (8-OH-Gua, also known as 8-oxoguanine) is one of the major oxidized bases produced by reactive oxygen species (ROS) [5,6]. This damaged base has been used as a marker of (DNA) oxidation, due to its sensitive detection by an HPLC-electrochemical detector system [5]. Site-directed mutagenesis experiments using *Escherichia coli* and mammalian cells indicated that 8-OH-Gua in DNA has high mutagenic potential [7-15].

ROS also oxidize DNA precursors (deoxyribonucleotides). 8-Hydroxy-2'-deoxyguanosine 5'-triphosphate (8-OH-dGTP, also known as 8-oxo-dGTP) is the major oxidation product of dGTP in *in vitro* oxidation reactions [16]. An increasing body of evidence indicates that the oxidation of dGTP in the nucleotide pool is another important endogenous source of mutagenesis. Nunoshiba *et al.* reported that one of the mutations found most frequently in an *E. coli* strain lacking superoxide dismutases and a repressor for iron-uptake systems was an A:T→C:G transversion, and concluded that the mutation was caused by 8-OH-dGTP, on the basis of various experiments [17]. Deficiencies in the *E. coli* MutT and mouse MTH1 proteins that catalyze the hydrolysis of 8-OH-dGTP caused

increased mutation rates and tumor formation, respectively [18-23]. In terms of the accumulation of 8-OH-Gua in DNA, the contributions of 8-OH-dGTP from the nucleotide pool and the direct oxidation of G bases in DNA are reportedly almost equal [19]. The presence of 8-OH-dGTP in the mitochondrial nucleotide pool was recently shown [24]. The concentration of this oxidized dGTP was 1–10% of that of unmodified dGTP. Although the amounts of 8-OH-dGTP in the nucleus and cytosol are not presently known, it seems reasonable to hypothesize that 8-OH-dGTP is present in the nucleus.

Recently, we showed that 8-OH-dGTP was mutagenic and specifically induced A:T→C:G transversions in live simian cells [25]. The A:T→C:G mutations would occur due to the incorporation of 8-OH-dGTP opposite A in template DNA by DNA polymerase(s) (pol(s)) [26]. Mammalian cells contain specialized DNA pols that are involved in TLS [27,28], and some TLS DNA pols misincorporate the oxidized dGTP *in vitro* [29,30]. To assess the involvement of the specialized DNA pols in the mutagenesis induced by 8-OH-dGTP, their expression was knocked-down, and then 8-OH-dGTP was introduced into the knocked-down cells. In this study, we used human cells for which the genomic sequence is available, and examined the effects of the knock-downs of specialized DNA pols by siRNAs. The knock-downs of DNA pols η and ζ , and REV1 reduced the

A:T→C:G substitution mutations, suggesting their roles in the mutagenesis by 8-OH-dGTP.

2. Materials and methods

2.1. Materials

dGTP (FPLC-grade) was purchased from GE Healthcare Bio-Sciences (Piscataway, New Jersey, USA). 8-OH-dGTP was obtained from TriLink BioTechnologies (San Diego, California, USA). Purified oligonucleotides were from Hokkaido System Science (Sapporo, Japan) and Sigma Genosys Japan (Ishikari, Japan). siRNAs (“stealth RNAi”, Invitrogen, Carlsbad, California, USA) were synthesized according to the BLOCK-iT RNAi Designer software, on the supplier’s website. The siRNAs against DNA pols η and ι were based on those reported in the literature [31,32]. The other siRNAs were designed by the software. The following siRNAs were used: pol η sense, 5'-GAGGAAUAAACCUUGUGCAGUUGUA; pol η antisense, 5'-UACAACUGCACAAGGUUUAUCCUC; pol ι sense, 5'-AACAAACAGUCUUAUUACCUGAAAG; pol ι antisense, 5'-CUUUCAGGUAAUAAGACUGUUUGU, REV1 sense, 5'-UAAGUUUGGUCUCUGCAAGGAUUUC; REV1 antisense, 5'-GAAAUCCUUGCAGAGACCAAACUUA; REV3 sense,

5'-UUAAUAGCUCGUUCCAAGGUCUCUC; REV3 antisense, 5'-GAGAGACCUUGGAACGAGCUAUUAA. The control siRNA was that for low GC content (Invitrogen). The pZ189-StuI plasmid was constructed by replacing the linker sequence between the two EcoRI sites in pMY189 [33] by a linker oligodeoxyribonucleotide containing an StuI site. The *E. coli* strain KS40 (*lacZ(am)*, *CA7070*, *lacY1*, *hsdR*, *hsdM*, Δ (*araABC-leu*)7679, *galU*, *galK*, *rpsL*, *thi*, *gyrA*)/pOF105 [34] was provided by Dr. Tatsuo Nunoshiba, of Tohoku University, and was used as an indicator strain of the *supF* mutants.

2.2. Mutagenesis experiments

293T cells (5×10^4 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 hr. The pZ189-StuI plasmid (29 fmol, 100 ng) and the siRNA (7.2 pmol each) were mixed with Lipofectamine (Invitrogen) and introduced into the cultured 293T cells according to the supplier's recommendations. After 24 hr, 8-OH-dGTP (2.4 pmol) was introduced by osmotic pressure, using a buffer solution (30 mM KCl, 10 mM HEPES-NaOH, pH 7.4) according to the literature [35]. After 24 hr of culture, the plasmid amplified in the cells was recovered by the method of Stary and Sarasin [36]. The recovered DNA was treated with DpnI, to digest the unreplicated plasmids. After removing the protein by

passage through a Micropure EZ device (Millipore, Billerica, Massachusetts, USA), the DNA was isolated by ethanol precipitation.

The DNAs recovered from the 293T cells were transfected into *E. coli* KS40/pOF105 cells by electroporation, using a Gene Pulser II Transfection Apparatus with a Pulse Controller II (Bio-Rad, Hercules, California, USA). The *supF* mutant frequency was calculated according to the numbers of white and pale blue colonies on Luria–Bertani agar plates containing nalidixic acid (50 $\mu\text{g/ml}$), streptomycin (100 $\mu\text{g/ml}$), ampicillin (150 $\mu\text{g/ml}$), chloramphenicol (30 $\mu\text{g/ml}$), 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (80 $\mu\text{g/ml}$), and isopropyl- β -D-thiogalactopyranoside (23.8 $\mu\text{g/ml}$), and the numbers of colonies on agar plates containing ampicillin and chloramphenicol, as described in Ref. [34].

The nucleotide sequences of the *supF* gene were analyzed by sequencing, as described previously [37], using an ABI PRISM Big Dye Terminator Cycle Sequencing Kit and ABI 377 and 3130 DNA sequencers (Applied Biosystems, Foster City, California, USA).

2.3 Analysis of 8-OH-Gua in DNA

293T cells (5×10^5 cells) were plated into a 60-mm dish 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 hr. The pZ189-StuI

plasmid (1 μg) was introduced into the cultured 293T cells with Lipofectamine. After 24 hr, 8-OH-dGTP (24 pmol) was introduced by osmotic pressure. After 16 hr, the treated cells were trypsinized and washed twice with phosphate-buffered saline. The cellular DNA was isolated by the sodium iodide method, using a DNA Extraction WB Kit (Wako Pure Chemical Industries, Osaka, Japan). Desferal (deferroxamine mesylate, 1 mM) was added to the solution used for cell lysis. The total DNA isolated from the 293T cells was treated with nuclease P1 and calf intestine alkaline phosphatase. The amount of 8-OH-Gua was determined by HPLC, essentially as described previously [38]. Namely, the nucleoside solution was filtered through an Ultrafree-Probind filter (Millipore, Bedford, Massachusetts, USA). The filtrate was then injected onto an HPLC column (Capcellpak C18 MG, 4.6 mm X 250 mm, 5 μm , Shiseido Fine Chemicals, Tokyo, Japan) equipped with UV (UV-8020, Tosoh, Tokyo, Japan) and electrochemical (ECD-300, Eicom, Kyoto, Japan) detectors, with isocratic elution with a solution containing 8% methanol, 10 mM NaH_2PO_4 , and 50 mg/l EDTA•2Na, at a flow rate of 1 ml/min. The effluent was monitored by UV absorption at 260 nm for 2'-deoxyguanosine and by the electrochemical response (applied voltage, 550 mV) for 8-hydroxy-2'-deoxyguanosine. The 8-OH-Gua level in the DNA sample was calculated from HPLC runs of standard samples of 8-hydroxy-2'-deoxyguanosine and 2'-deoxyguanosine.

2.4 Quantitative 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) as described by the manufacture's instructions. Each of the mRNA transcripts was measured by quantitative PCR method with an ABI 7500 Real Time PCR System and SYBR-Green chemistry (Applied Biosystems) using the following primer sets:

5'-dCTGATTGAATGCCTTGTCTTAAGTG;	Pol	ι	upper,
5'-dCAGCATCTGCCTGTTAGTTTGG,	Pol	ι	lower,
5'-dTGTTGCCAAGCACTTACATTGAA;	Pol	η	upper,
5'-dTGTTTTTCGCATCCCCTCTTT,	Pol	η	lower,
5'-dCTCTGTCCGTGATGTCTTCCAA;	REV1		upper,
5'-dACAGCAGCCCGAAATACTTCTT,	REV1		lower,
5'-dCCAGCTCCTCGCGAAGTG;	REV3		upper,
5'-dACAGGACAGTGTAAGGTAGTAAAATATTGT.	REV3		lower,

Data were expressed as a ratio to the GAPDH mRNA which was determined using the following primers: GAPDH upper, 5'-dAACTTTGGCATTGTGGAAGG; GAPDH lower, 5'-dGTCTTCTGGGTGGCAGTGAT.

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. Induction of mutations by 8-OH-dGTP in 293T cells

Previously, we observed that A:T→C:G transversions were specifically induced by 8-OH-dGTP in simian COS-7 cells [25]. To knock-down various DNA pols, human cells for which the genomic sequence is available are a better choice. In this study, we first examined the mutagenic potential of 8-OH-dGTP in living human 293T cells. The pZ189-StuI plasmid, containing the *supF* gene, the SV40 large T antigen gene, and the SV40 origin of replication, was used in this study. The large T antigen is produced from the gene on the plasmid in addition to the gene on the chromosome in 293T cells, and the use of this plasmid enhanced 8-OH-dGTP-induced mutations in comparison with the pSVKAM189/Zeo(+) plasmid used in the previous study [25] (data not shown), possibly due to the increased amount of the large T antigen necessary for replication initiation of the plasmid. 8-OH-dGTP was introduced into the 293T cells carrying the pZ189-StuI plasmid by the

osmotic pressure method [35], rather than with the cationic lipids used previously [25]. The replicated DNA was recovered from the treated 293T cells and then was transfected again into the indicator *E. coli* strain, KS40/pOF105 [34]. Solutions containing dGTP or no deoxyribonucleotide (none) instead of that containing 8-OH-dGTP were used in the control experiments.

As shown in Fig. 1, the introduction of 8-OH-dGTP into 293T cells enhanced the total mutant frequency. This result was in contrast to the previous result that 8-OH-dGTP did not increase the total mutation frequency in COS-7 cells [25]. This discrepancy could be due to the differences in the 8-OH-dGTP introduction methods, the cell line, and the plasmid DNA used. In contrast, the observed *supF* mutant frequency did not increase when the unmodified dGTP was introduced (Fig. 1).

We measured the 8-OH-Gua content in DNA to examine the introduced 8-OH-dGTP was actually incorporated into DNA. The introduction of 8-OH-dGTP was carried out on a 10-fold larger scale than that of the mutagenesis experiment described above, keeping the same concentration of 8-OH-dGTP, and total (genomic plus plasmid) DNA, not plasmid DNA alone, was analyzed, due to the amount of DNA required. 8-OH-Gua was quantitated by the highly sensitive HPLC-electrochemical detector method [5]. In the dGTP (control) experiment, the 8-OH-Gua level in DNA was $1.52 (\pm 0.20)/10^6$ Gua, at 16 hr after the treatment (mean \pm

standard deviation of three separate experiments). This 8-OH-Gua level can be attributed to oxidation that could occur during the experimental procedure as well as to DNA oxidation plus the incorporation of endogenous 8-OH-dGTP in the cells. The treatment with 8-OH-dGTP increased the 8-OH-Gua level to $3.54 (\pm 0.16)/10^6$ Gua at the same time point ($P < 0.0002$, statistically significant). Thus, the 8-OH-Gua level of $2.02/10^6$ Gua over the background was induced by the 8-OH-dGTP treatment, suggesting that the added oxidized nucleotide actually entered the live human cells and was incorporated by DNA pol(s).

3.2. Mutation spectrum of 8-OH-dGTP

We analyzed the sequences of the *supF* genes in 197, 126, and 60 colonies, obtained in the none (no deoxyribonucleotide), dGTP, and 8-OH-dGTP experiments, respectively (Table 1). The most frequent mutation in the control experiments was the deletion/insertion of ≥ 3 bases (49 and 53%). The ratio of this type of mutation was reduced in the 8-OH-dGTP experiment (17%). The ratio of single-base substitution mutations in the 8-OH-dGTP experiment (82%) was much higher than that in the control experiments. Among the substitutions, 8-OH-dGTP induced A:T→C:G transversions specifically. Nearly 90% of the single-base substitution mutations were A:T→C:G mutations when 8-OH-dGTP was introduced. In contrast, A:T→C:G mutations were infrequently found in the control

experiments. This result is in agreement with the previously reported A:T→C:G induction by 8-OH-dGTP in *E. coli* and simian cells [25,39].

The distributions of the mutations in the *supF* gene found in this study are shown in Supplementary Figs. 1 and 2. Positions 95–97 correspond to those of the anticodon in the encoded tRNA, and many mutations were found at the 96th and 97th positions, in the case of the 8-OH-dGTP-treated group.

3.3. Knock-downs of specialized DNA polymerases affect the induced mutations

We next examined the effects of the knock-downs of specialized DNA pols by siRNAs. In this study, DNA pols η , ι , and ζ , and REV1 were selected as the knock-down targets. These knock-downs were measured by quantitative RT (reverse transcription)-PCR (Table 2) and checked by western blotting in the cases of pols η and ι (data not shown). The treatment of 293T cells with siRNA reduced mRNAs for these DNA pols. Since we did not observe the effective knock-down of DNA pol κ , this pol was excluded from the analysis. Total of $>3.8 \times 10^6$ transformed *E. coli* cells were obtained by the electroporation when we determined the mutant frequencies.

The mutant frequencies upon the treatment with siRNAs against DNA pols η , ι , and ζ , and REV1 (and control siRNA) were similar in the

control experiments ($(1.0-1.5) \times 10^{-4}$, data not shown), indicating that the decrease in these DNA pols did not affect the background mutant frequency in this experimental system.

We then introduced 8-OH-dGTP into the knocked-down 293T cells. The *supF* mutant frequencies were affected by the knock-downs of three of the four specialized DNA pols examined in this study. As shown in Fig. 2, the induced mutant frequencies were significantly decreased in the cells in which DNA pols η and ζ , and REV1 were reduced. In contrast, the knock-down of DNA pol ι did not affect the mutant frequency.

The mutation spectra in the knocked-down cells are shown in Table 3. In all cells treated with siRNAs, 8-OH-dGTP specifically induced A:T \rightarrow C:G transversions. The mutation frequency for the induced A:T \rightarrow C:G substitution was calculated by multiplying the mutant frequency by the proportion of mutants containing A:T \rightarrow C:G substitutions (Table 4). The knock-downs of DNA pols η and ζ , and REV1 reduced the frequency of the A:T \rightarrow C:G mutations, suggesting that these DNA pols, but not DNA pol ι , are involved in the mutagenic event induced by 8-OH-dGTP.

The overall distributions of the mutations were unchanged by the knock-downs, as shown in Supplementary Figs. 3-5.

3.4. Effects of double knock-downs of specialized DNA polymerases

We finally examined the effects of the double knock-downs of specialized DNA pols. DNA pols η and ζ (REV3), and REV1 were selected as the knock-down targets, based on the results shown in Fig. 2. These knock-downs were measured by quantitative RT-PCR (Table 5). The double knock-down of these DNA pols did not affect the background mutant frequency in this experimental system (data not shown).

The knock-down of both DNA pol η and REV1 decreased the mutant frequency induced by 8-OH-dGTP (Fig. 3). However, the effect of the double knock-down was similar to those of the single knock-downs of DNA pol η and REV1. In contrast, the induced mutant frequencies were decreased by ~50% in the cells in which DNA pol η plus REV3 (DNA pol ζ) and REV1 plus REV3 were knocked-down. These results may suggest that REV1-DNA pol η and DNA pol ζ work in different steps in the mutation process of 8-OH-dGTP.

4. Discussion

A:T→C:G transversion mutations were induced by 8-OH-dGTP in human 293T cells (Table 1). The same type of mutation is induced by 8-OH-dGTP in *E. coli* and simian cells [25,39]. These findings suggest that 8-OH-dGTP was incorporated opposite A, and dCTP was then inserted opposite the

incorporated 8-OH-Gua residue during the next round of replication [26]. It was previously shown that mammalian DNA pols misinserted this oxidized dGTP opposite A *in vitro* [29,30,40-44]. In particular, DNA pol η , a Y-family DNA pol, erroneously incorporated 8-OH-dGTP opposite A quite frequently [29,30].

This study examined the involvement of specialized DNA pols in the mutagenesis by 8-OH-dGTP. DNA pols η and ι , and REV1, which are members of the Y-family DNA pols, and DNA pol ζ , a B-family DNA pol, were knocked-down, and 8-OH-dGTP was introduced into the knocked-down cells. As shown in Fig. 2 and Table 4, the decrease in DNA pol η significantly reduced the 8-OH-dGTP-induced mutations. In contrast, the knock-down of DNA pol ι , another member of the Y-family DNA pols, did not affect the mutagenicity of 8-OH-dGTP. Recently, the incorporation of 8-OH-dGTP by DNA pols η and ι was analyzed *in vitro* [30]. The kinetic parameters indicated that DNA pol η incorporates 8-OH-dGTP opposite A with ~60% efficiency relative to TTP incorporation opposite A. On the other hand, the 8-OH-dGTP incorporation opposite A by DNA pol ι is quite inefficient, with only 3% efficiency of the Watson–Crick pairing. The results shown in Fig. 2 are in line with the *in vitro* observations. Thus, DNA pol η , but not pol ι , may incorporate 8-OH-dGTP in an error-prone manner during replication in 293T cells. The recent finding that DNA pol η

induced A→C mutations during *in vitro* gap-filling DNA synthesis in the presence of 8-OH-dGTP supports this interpretation [45].

In addition, the knock-down of REV3 (the catalytic subunit of DNA pol ζ) reduced the mutagenicity of 8-OH-dGTP (Fig. 2 and Table 4). DNA pol ζ is a highly specialized pol, specifically adapted for extending primer termini opposite from a diverse array of DNA lesions [28]. This unique characteristic of pol ζ led us to speculate that pol ζ may extend a nascent strand from the 8-OH-Gua residue that was incorporated by pol η. However, DNA pol η extends a primer with 8-OH-Gua paired with template A *in vitro* [30]. We cannot disregard the possibility of the incorporation of 8-OH-dGTP by DNA pol ζ at this time, since no report about it is available.

Suppression of REV1 expression also decreased the 8-OH-dGTP-induced mutations (Fig. 2 and Table 4). REV1 is a member of the Y-family DNA pols, but it acts as deoxycytidyl transferase opposite a range of damaged bases [27,28]. More importantly, REV1 binds with other specialized DNA pols, including DNA pols η and ζ [46-51]. Human REV1 was suggested to have a central role in TLS, as a scaffold that recruits the DNA pols involved in TLS to the replication fork. In addition, REV1 functions together with pol ζ in promoting translesion DNA synthesis. Thus, the decrease in REV1 might cause the inefficient recruitment of

DNA pols η and ζ to the replication fork, resulting in reduced 8-OH-dGTP incorporation and/or extension from the primer terminus containing 8-OH-Gua. The results of double knock-downs (Fig. 3) may suggest involvement of REV1-DNA pol η in a step and DNA pol ζ in another step in the 8-OH-dGTP-induced mutation process. However, verification of this hypothesis requires further experiments from various viewpoints.

Another possibility is that DNA pols η and ζ , and REV1 participated in the bypass of 8-OH-Gua in the template DNA and/or the extension from the 3'-terminal deoxyribonucleotide incorporated opposite 8-OH-Gua during the second round of replication (after 8-OH-dGTP was incorporated into DNA). In particular, the insertion of dCTP opposite 8-OH-Gua and/or the extension from a C:8-OH-Gua pair would promote the occurrence of A:T \rightarrow C:G transversion mutations. Avkin and Livneh reported that the efficiency of bypass across 8-OH-Gua was similar in DNA pol η -proficient and -deficient human cells (80% and 71%, respectively), when a gapped plasmid carrying 8-OH-Gua in the ss DNA region was introduced [52]. In addition, the ratio of dCTP insertion was also nearly identical in both cell lines (~80%). Thus, the effect of the knock-down of DNA pol η could at least be interpreted as the efficient incorporation of 8-OH-dGTP by this Y-family DNA pol, but not the error-free bypass across 8-OH-Gua. However, further studies are necessary with regard to this possibility.

As shown in Table 2, the knock-down of the specialized DNA pols was not very efficient. In particular, only 30% of reduction was observed for REV3. However, the knock-downs of DNA pols η and ζ , and REV1 reduced the 8-OH-dGTP-induced mutation (Fig. 2). Thus, these decreased levels of mRNA are seemed sufficient to affect the induced mutagenesis.

This study focused on specialized DNA pols in the 8-OH-dGTP-induced mutagenesis. It is accepted that most incorporation of deoxyribonucleotides into nascent DNA chains is conducted by replicative DNA pols. Contribution of the replicative DNA pols to 8-OH-dGTP incorporation is unknown and could be larger than that of the specialized DNA pols examined in this study. Efficiency and fidelity of the 8-OH-dGTP incorporation by the replicative and specialized DNA pols would determine their contributions to the mutations by the oxidized dGTP.

We introduced the replicated DNA recovered from the treated 293T cells into *E. coli* cells by electroporation. Judging by the numbers of colonies on the titer plates, little, if any, toxicity of 8-OH-dGTP was observed in 293T cells (data not shown). In addition, the toxicity of 8-OH-dGTP in the 293T cells with knocked-down DNA pol seemed negligible, although the 8-OH-dGTP treatment appeared to yield slightly reduced bacterial colonies in the case of DNA pol η . However, the numbers of *E. coli* colonies are affected by efficiencies of the

electroporation and further studies will be required to conclude the toxicity of 8-OH-dGTP.

When 8-OH-dGTP was introduced into an *E. coli* strain lacking a Y-family DNA pol, pol IV, its deficiency did not affect the mutations induced by 8-OH-dGTP [53]. On the other hand, the deficiency in DNA pol V, another *E. coli* Y-family pol, significantly facilitated the mutagenicity of 8-OH-dGTP in a pol-activity independent manner. Thus, the *E. coli* Y-family DNA pols did not seem to be involved in the incorporation of 8-OH-dGTP, at least under our experimental conditions, in contrast to some human Y-family DNA pols.

In conclusion, the knock-downs of DNA pols η and ζ , and REV1 reduced the mutagenicity of 8-OH-dGTP in human 293T cells. This result suggests that these DNA pols are involved in the incorporation of oxidized dGTP. The defense mechanisms against this damaged deoxyribonucleotide produced by ROS in mammalian cells are of great interest. Experiments along this line are currently in progress.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure legends

Fig. 1 Frequencies of *supF* mutants after replication in 293T cells treated with 8-OH-dGTP. The deoxyribonucleotide and the plasmid containing the *supF* gene were introduced into the cells, as described in the Materials and Methods. Mutant frequencies were calculated according to the numbers of *supF* mutant colonies on agar plates containing nalidixic acid, streptomycin, ampicillin, and chloramphenicol, and the numbers of colonies on agar plates containing ampicillin and chloramphenicol. Data are expressed as means \pm standard deviation (n=4–12) (***) P <0.001).

Fig. 2 Effects of knock-downs of specialized DNA pols in 293T cells on 8-OH-dGTP-induced mutant frequencies. Data are expressed as means \pm standard deviation (n=4–12) (***) P <0.001 vs control).

Fig. 3 Effects of double knock-downs of specialized DNA pols in 293T cells on 8-OH-dGTP-induced mutant frequencies. Data are expressed as means \pm standard deviation (n=3) (**) P <0.005 vs control; (***) P <0.001 vs control).

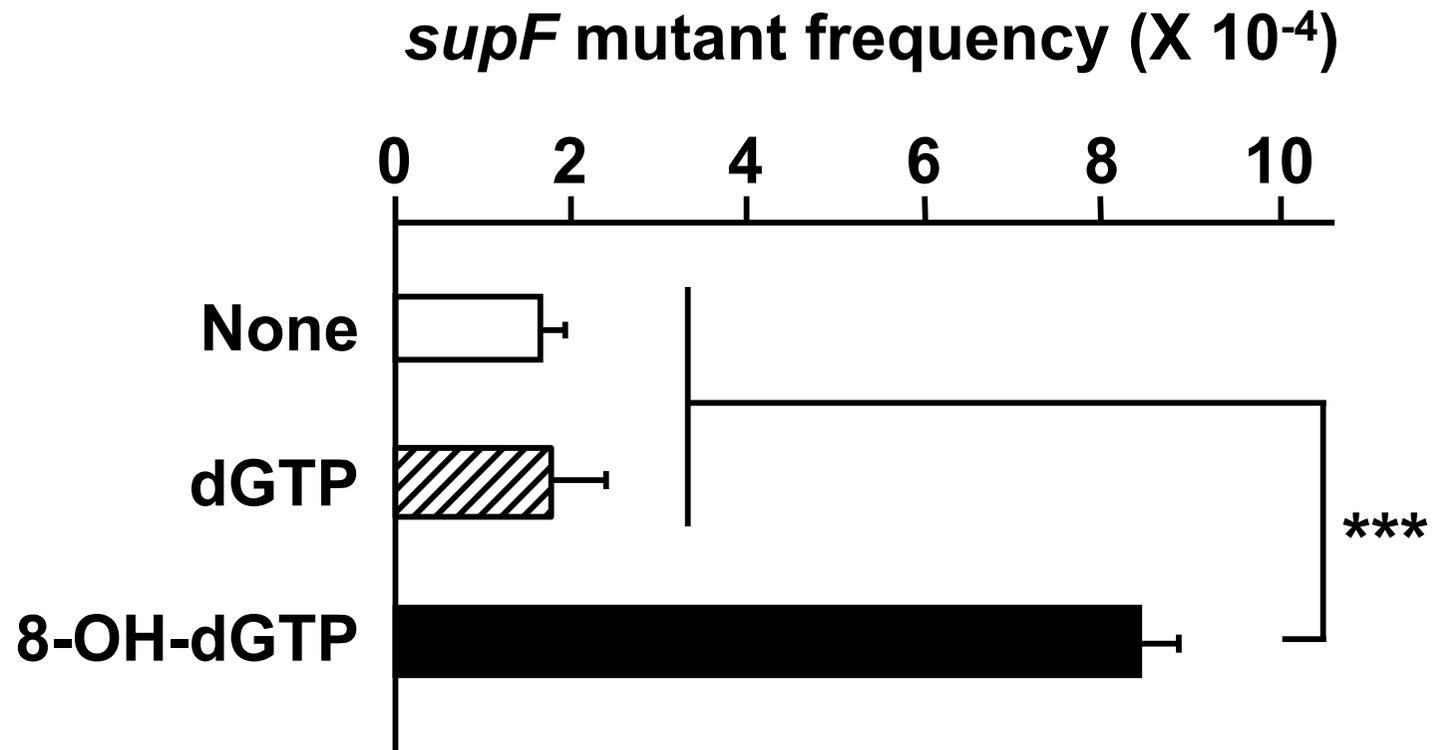


Fig. 1

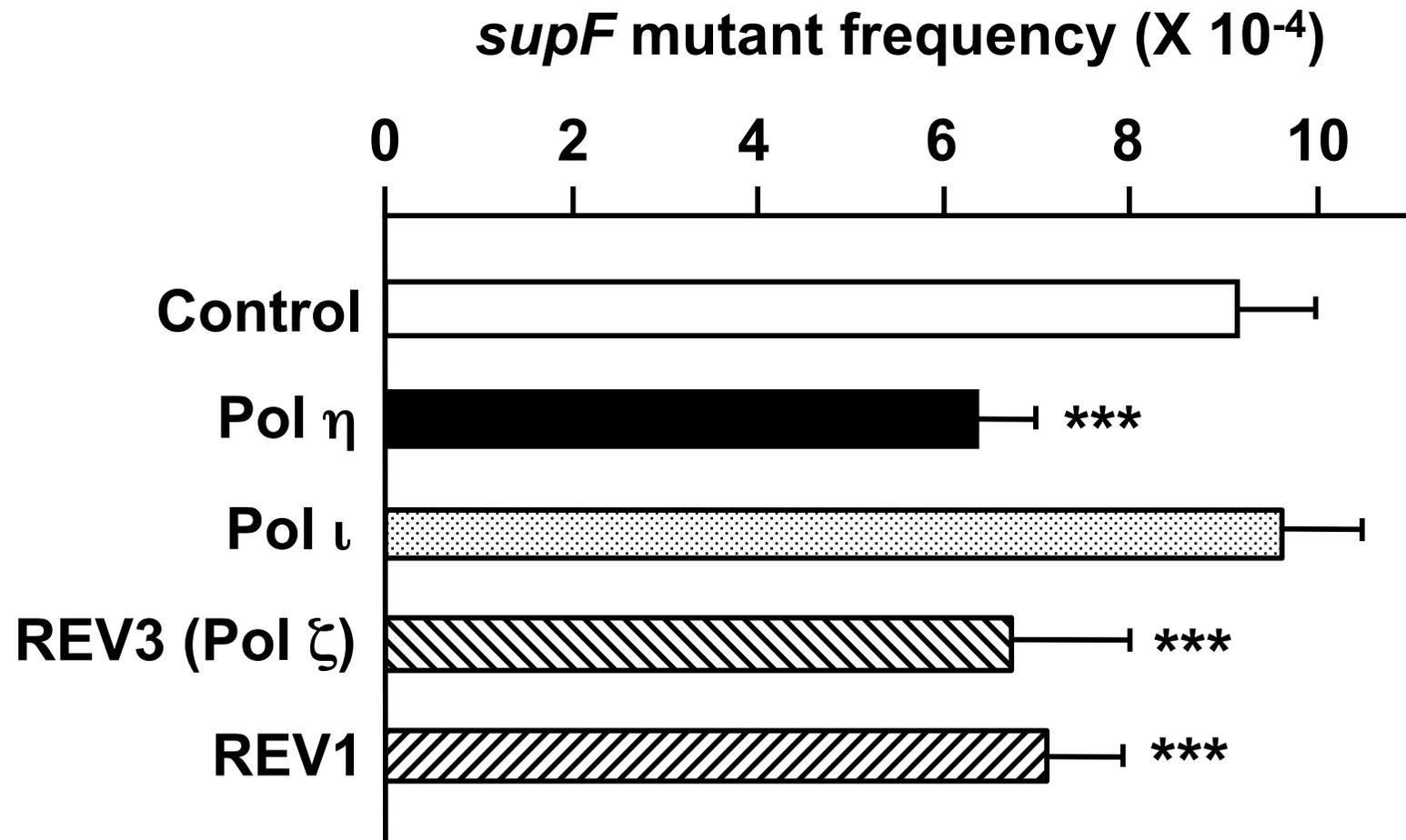


Fig. 2

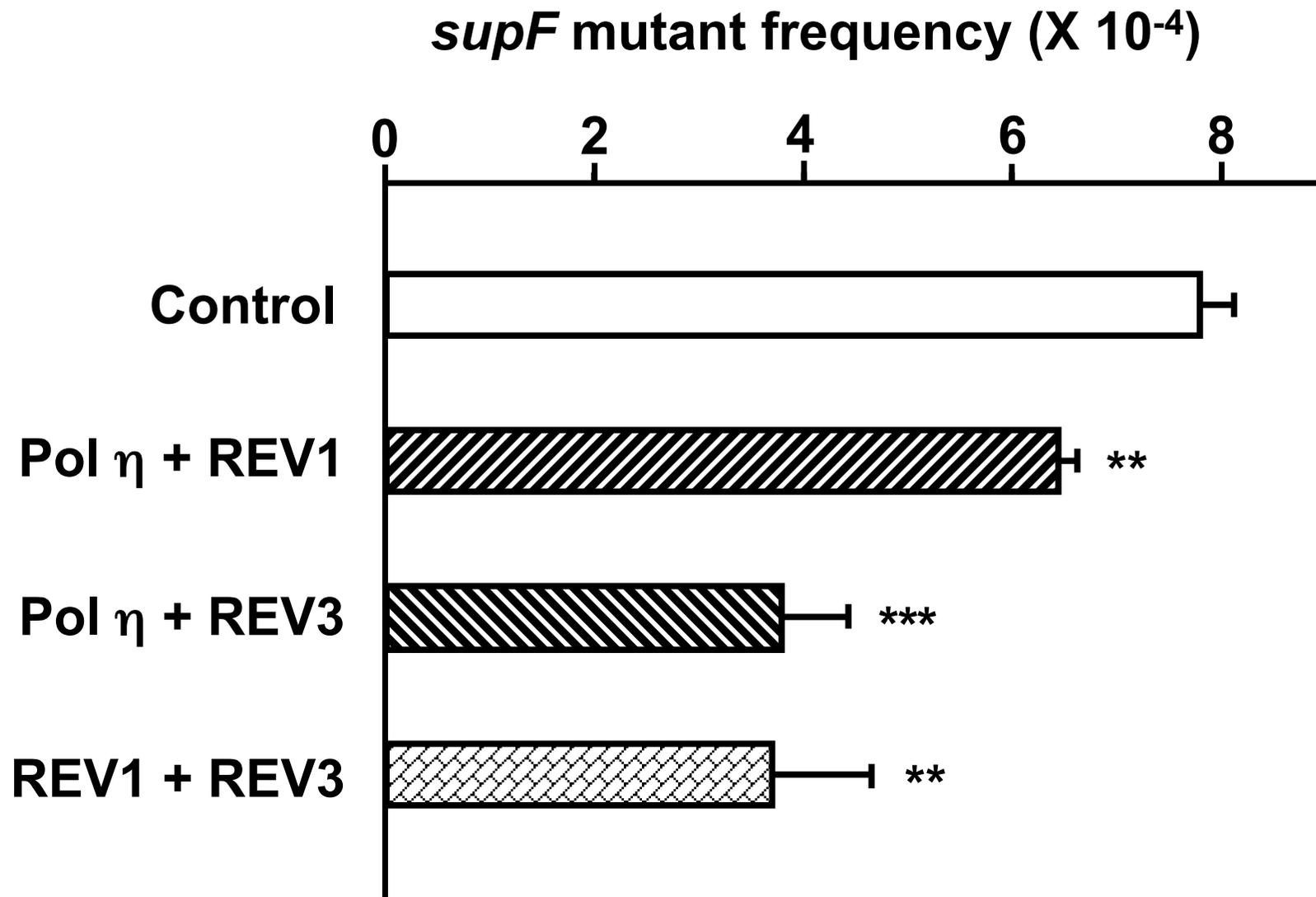


Fig. 3

Table 1. Mutations induced by 8-OH-dGTP in 293T cells

	None	dGTP	8-OH-dGTP
Single base substitution			
Transition			
A:T-->G:C	23 (12)	16 (13)	2 (3)
G:C-->A:T	19 (10)	15 (12)	0 (0)
Transversion			
A:T-->T:A	3 (2)	2 (2)	0 (0)
A:T-->C:G	2 (1)	2 (2)	45 (75)
G:C-->T:A	27 (14)	11 (9)	0 (0)
G:C-->C:G	6 (3)	4 (3)	2 (3)
Deletions/Insertions			
1-2 bases	14 (7)	4 (3)	1 (2)
≥3 bases	96 (49)	67 (53)	10 (17)
Complex	0 (0)	1 (1)	0 (0)
Others	2 (1) ^a	4 (3) ^b	0 (0)
No mutation	25 (13)	4 (3)	0 (0)
Total	217	130	60
Total colonies analyzed	197 (100)	126 (100)	60 (100)

All data are represented as cases found (%).

^a CC --> TA (at positions 71 and 72) and GA --> CC (at positions 91 and 92) mutations.

^b GT --> TC (at positions 62 and 63), GA --> AT (at positions 73 and 74), CC --> AT (at positions 130 and 131), and CC --> AA (at positions 135 and 136) mutations.

Table 2. Amounts of mRNAs in 293T cells with knocked-down TLS DNA pols

DNA Pol	24 hr		48 hr	
Pol η	0.44	\pm 0.13	0.44	\pm 0.18
Pol ι	0.29	\pm 0.15	0.23	\pm 0.08
REV3	0.68	\pm 0.15	0.61	\pm 0.38
REV1	0.30	\pm 0.07	0.19	\pm 0.03

The amount of mRNA was measured by quantitative RT-PCR at 24 hr and 48 hr after siRNA introduction. The amount of mRNA was normalized relative to that of the human GAPDH mRNA contained in each sample. Relative values to that in 293T cells treated with the control siRNA are shown. Data are expressed as means \pm standard deviation (n=3-4).

Table 3. Mutations induced by 8-OH-dGTP in 293T cells with knocked-down TLS DNA pols

Mutations	Control	Pol η	Pol ι	REV3 (Pol ζ)	REV1
Single base substitution					
Transition					
A:T-->G:C	2 (1)	2 (2)	1 (2)	0 (0)	0 (0)
G:C-->A:T	2 (1)	0 (0)	2 (3)	0 (0)	1 (1)
Transversion					
A:T-->T:A	2 (1)	1 (1)	0 (0)	2 (2)	1 (1)
A:T-->C:G	150 (84)	74 (82)	53 (88)	75 (83)	70 (78)
G:C-->T:A	4 (2)	1 (1)	1 (2)	3 (3)	3 (3)
G:C-->C:G	0 (0)	1 (1)	0 (0)	2 (2)	0 (0)
Deletions/Insertions					
1-2 bases	1 (1)	1 (1)	0 (0)	1 (1)	1 (1)
≥ 3 bases	12 (7)	9 (10)	1 (2)	7 (8)	11 (12)
Others	0 (0)	0 (0)	1 (2) ^a	0 (0)	1 (1) ^b
No mutation	6 (3)	2 (2)	2 (3)	2 (2)	3 (3)
Total	179	91	61	92	91
Total colonies analyzed	179 (100)	90 (100)	60 (100)	90 (100)	90 (100)

All data are represented as cases found (%). ^a A CC --> T mutation at positions 71 and 72.

^b A TT --> GG mutation at positions 123 and 124.

Table 4. Mutation frequency for A:T→C:G transversions induced by 8-OH-dGTP in 293T cells with knocked-down TLS DNA pols ($\times 10^{-4}$)

Control	Pol η	Pol ι	REV3 (Pol ζ)	REV1
7.7	5.2	8.5	5.6	5.6

The mutation frequency was calculated by multiplying the mutant frequency (shown in Fig. 2) by the proportion of mutants containing A:T→C:G substitutions (shown in Table 3).

Table 5. Amounts of mRNAs in 293T cells with knocked-down TLS DNA pols (double knock-down)

siRNA	DNA Pol	24 hr		48 hr	
Pol η + REV1	Pol η	0.30	\pm 0.04	0.26	\pm 0.06
	REV1	0.30	\pm 0.05	0.21	\pm 0.05
Pol η + REV3	Pol η	0.58	\pm 0.06	0.40	\pm 0.03
	REV3	0.66	\pm 0.16	0.40	\pm 0.13
REV1 + REV3	REV1	0.32	\pm 0.03	0.26	\pm 0.03
	REV3	0.64	\pm 0.28	0.46	\pm 0.06

The amount of mRNA was measured by quantitative RT-PCR at 24 hr and 48 hr after siRNA introduction. The amount of mRNA was normalized relative to that of the human GAPDH mRNA contained in each sample. Relative values to that in 293T cells treated with the control siRNA are shown. Data are expressed as means \pm standard deviation (n=3-4).