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Research Article

Catalytic and Non-Catalytic Roles of DNA Polymerase κ in the Protection of Human Cells Against Genotoxic Stresses

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DNA polymerase κ (Pol κ) is a specialized DNA polymerase involved in translesion DNA synthesis. Although its bypass activities across lesions are well characterized in biochemistry, its cellular protective roles against genotoxic insults are still elusive. To better understand the in vivo protective roles, we have established a human cell line deficient in the expression of Pol κ (KO) and another expressing catalytically dead Pol κ (CD), to examine the cytotoxic sensitivity to 11 genotoxins including ultraviolet C light (UV). These cell lines were established in a genetic background of Nalm-6-MSH+, a human lymphoblastic cell line that has high efficiency for gene targeting, and functional p53 and mismatch repair activities. We classified the genotoxins into four groups. Group 1 includes benzo[*a*]pyrene diolepoxide, mitomycin C, and bleomycin, where the sensitivity was equally higher in KO and CD than in the cell line expressing wild-type Pol κ (WT). Group 2 includes hydrogen peroxide and menadione, where hypersensitivity was observed only in KO. Group 3 includes methyl methanesulfonate and ethyl methanesulfonate, where hypersensitivity was observed only in CD. Group 4 includes UV and three chemicals, where the chemicals exhibited similar cytotoxicity to all three cell lines. The results suggest that Pol κ not only protects cells from genotoxic DNA lesions via DNA polymerase activities, but also contributes to genome integrity by acting as a non-catalytic protein against oxidative damage caused by hydrogen peroxide and menadione. The non-catalytic roles of Pol κ in protection against oxidative damage by hydrogen peroxide are discussed. Environ. Mol. Mutagen. 56:650–662, 2015. © 2015 Wiley Periodicals, Inc.

Key words: translesion DNA synthesis; Nalm-6-MSH+; catalytically dead mutants

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INTRODUCTION

Genomic DNA is continuously exposed to a variety of endogenous and exogenous genotoxic agents. Living organisms employ multiple repair mechanisms to maintain genomic integrity against DNA damage. Nevertheless, unrepaired lesions frequently arrest progress of DNA replication and induce genotoxicity. To alleviate genotoxic effects, cells possess specialized DNA polymerases (Pols) that can bypass DNA lesions, at or before which replicative Pols, e.g., Pol δ and Pol ϵ, stall [Friedberg et al., 2002; Schmitt et al., 2009; Lange et al., 2011]. After successful lesion bypass, the replicative Pols take over the primer DNA from the specialized Pols and continue the chromosome replication [Friedberg et al., 2005]. Therefore, this process, i.e., translesion DNA synthesis (TLS), contributes to the completion of the whole chromosome replication and enhances cell viability [Prakash et al., 2005; Nohmi, 2006]. Although the specialized Pols have an ability to replicate across DNA lesions, they replicate undamaged DNA with lower fidelity compared to the replicative Pols [McCulloch and Kunkel, 2008]. In addition, the bypass across the lesions in DNA does not repair or remove the damaged bases. Thus, TLS is regarded as a mechanism of DNA damage tolerance, instead of DNA repair, which may cause mutations in compensation for cell survival [Sale et al., 2012].

Currently, it is known that humans possess more than 15 Pols per cell and that more than half are involved in TLS across some types of lesions [Lange et al., 2011]. Pol κ is a member of the Y-family Pols [Ohmori et al., 2001], the most predominant class of the specialized Pols, and conserved in all domains of life, i.e., Eukarya, Archaea, and bacteria [Gerlach et al., 1999; Ogi et al., 1999; Wagner et al., 1999; Gruz et al., 2001]. Unlike Pol η, another member of the Y-family Pols, which bypasses relatively specific types of DNA lesions, e.g., ultraviolet light (UV)-induced thymine–thymine dimer and cisplatin-induced intra-strand crosslinks effectively [Johnson et al., 1999; Masutani et al., 1999, 2000], Pol κ seems to be involved in TLS across a variety of DNA lesions that are structurally unrelated. Pol κ appears to bypass N^2-guanyl DNA adducts induced by benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) [Ogi et al., 2002; Suzuki et al., 2002; Huang et al., 2003; Avkin et al., 2004; Niimi et al., 2009; Sassa et al., 2011, 2014], acetylaminofluorene [Zhang et al., 2000], methylglyoxal [Yuan et al., 2011], monoalkylating agents [Choi et al., 2006], a C8-guanyl adduct by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) [Fukuda et al., 2009], 8-oxoguanine [Zhang et al., 2000; Haracska et al., 2002; Jaloszynski et al., 2005], thymine glycol [Fischhaber et al., 2002; Yoon et al., 2010], and inter-strand crosslinks [Minko et al., 2008; Takeiri et al., 2014]. In addition, Pol κ is reported to be involved in nucleotide excision repair [Ogi et al., 2010], microsatellite stability [Baptiste and Eckert, 2012], replication checkpoint [Betous et al., 2013], and repair of DNA strand breaks [Zhang et al., 2013]. However, the characterization of Pol κ has been carried out mostly in biochemistry, and thus it remains largely uncertain what roles Pol κ plays in vivo (at cellular levels). In fact, in some cases, the in vitro biochemical specificity of Pol κ conflicts with the cellular sensitivity of the Pol κ-deficient cells [Ogi and Lehmann, 2006], which emphasizes the importance of characterization of the protective roles of Pol κ at cellular levels.

To gain insight into the in vivo protective roles of Pol κ against genotoxins in human cells, we have previously constructed human Pol κ knockout (KO) cells [Sassa et al., 2014]. In this study, we constructed Pol κ catalytically dead (CD) mutants, recovered the MSH2 expression of the KO and CD cells, and compared the cytotoxic sensitivity with the Pol κ wild-type (WT) cells against 11 genotoxins including UV light. To generate the mutants, we took advantage of human pre-B acute lymphoblastic leukemia cell line Nalm-6, which possesses a high efficiency for gene targeting by homologous recombination (HR) and has proficient p53 function [Adachi et al., 2006]. We recovered the MSH2 expression by introduction of a synthetic cDNA corresponding to exon 9 to exon 16 of MSH2 as we have done to generate Nalm-6-MSH+ [Suzuki et al., 2013]. The CD cells express catalytically inactive Pol κ that has amino acid substitutions of Asp198 and Glu199 to Ala and Ala (D198A/E199A). The Asp198 and Glu199 are critical to the catalytic activity because they interact with two magnesium ions, the catalysts of polymerase activity, at the active site [Lone et al., 2007]. We engineered not only KO but also CD cells because Pol κ interacts with other proteins, such as REV1, PCNA, and MSH2 [Ohashi et al., 2009; Lv et al., 2013; Yoon et al., 2014]. REV1 is a scaffold of multiple TLS Pols, e.g., Pol η, Pol ι, Pol κ, and REV7, a subunit of Pol ζ, [Wojtaszek et al., 2012a, 2012b] and PCNA interacts with multiple proteins involved in DNA transactions [Ulrich and Takahashi, 2013]. MSH2 forms a complex with MSH6, which plays an essential role in mismatch repair [Zlatanou et al., 2011]. Therefore, a simple KO or knockdown of Pol κ might modulate the functions of other proteins, thereby obscuring the catalytic roles of Pol κ in the protection of human cells against genotoxic stresses. In this study, we found that Pol κ KO cells but not CD cells exhibited hypersensitivity to oxidizing agents such as hydrogen peroxide and menadione. The possible non-catalytic roles of Pol κ in tolerance to oxidative stress are discussed.

MATERIALS AND METHODS

Cell Culture, PCR, and DNA Primers

The human pre-B cell line Nalm-6, Nalm-6-MSH+, and the derivatives, i.e., CD and KO cells, were maintained in RPMI1640 (Nacalai

Tesque, Japan) supplemented with 10% calf serum (Thermo Fisher Scientific), 50 μg/mL kanamycin, and 50 μM 2-mercaptoethanol in 5% CO_2 in an incubator at 37°C. All PCR reactions were performed with KOD FX (Toyobo, Japan) or ExTaq DNA Pol (Takara Bio, Japan), and the primer sets used for PCR and DNA sequence analysis are listed in Supporting Information Table I.

Construction of Targeting Vectors for the *POLK* Gene

Targeting vectors for CD cells (CD vectors) were constructed by the simple vector construction method of MultiSite Gateway® Technology (Life Technologies) as described [Iiizumi et al., 2006]. The 5′-arm of the vectors was designed to introduce a mutation, i.e., 5′-GATGAA-3′ to 5′-GCTGCA-3′, where underlined bases were changed into exon 6 to inactivate Pol κ (Fig. 1A). This mutation directs amino acid changes of D198A/E199A. The sequence change was introduced into the vectors by replacing the genomic sequence containing 5′-GATGAA-3′ with an oligonucleotide containing the 5′-GCTGCA-3′ (see more detail in Supporting Information Fig. 1). The sequence change resulted in the introduction of the restriction enzyme site of *Pst*I. The 5′-arm was amplified with primers CD 5′-arm Fw and CD 5′-arm Rv with the DNA fragment (2.9 kb) containing the *Pst*I site as the template, and the 3′-arm was generated with primers CD, KO 3′-arm Fw and 3′-arm CD, KO Rv with Nalm-6 genomic DNA as the template. The drug resistance gene, i.e., the puromycin-resistance gene (Pur^r) or the hygromycin-resistance gene (Hyg^r), and 5′- and 3′-targeting arms of each targeting vector were assembled into single plasmids in one step according to the supplier's protocol (Life Technologies).

Establishment of *POLK* KO and CD Derivatives of Nalm-6-MSH+ Cells

DNA transfection for gene targeting was performed as described previously [Sassa et al., 2014]. Briefly, the targeting vectors were linearized with *Ahd*I, and the linearized DNA containing floxed Hyg^r (2 μg) was transfected into 2×10^6 cells of Nalm-6 cells that were suspended in 0.1 mL of KitT solution by Nucleofector™ (Lonza) according to the manufacturer's instructions. After incubation for 20–24 h, the cells were re-plated at a density of about 10^6 per 90-mm dish with agarose medium containing 400 μg/mL of hygromycin (Wako). Alternatively, the cells were seeded in 96-well plates at a density of 2,000 cells/well in the medium containing hygromycin. After 2–3 weeks incubation, the resulting drug resistant clones were obtained and they were subjected to PCR analysis with primers 5′-loxP TG and CD-r to confirm that exon 6 of *POLK* was successfully replaced by the targeting vector. Subsequently, the targeting vector containing floxed Pur^r was transfected into the *POLK* heterozygous mutant cells. The transfected cells were selected by culture with the medium containing 0.5 μg/mL of puromycin (Wako), and the homozygous mutants were screened from among the selected cells by PCR analysis with primers 3′-loxP TG and CD-r. The targeted gene replacement was verified by Southern blot analysis, and the introduction of the mutation (GAT GAA to GCT GCA) was verified by PCR of cDNA of *POLK*, followed by *Pst*I digestion, and by DNA sequencing of the cDNA (see below). The drug resistance genes were excised by transient expression of Cre recombinase by electroporation of the Cre expression vector. Finally, MSH2 expression was restored by introduction of the synthetic cDNA sequence corresponding to exon 9 to exon 16 of the *MSH2* gene downstream of exon 8 of *MSH2* as described [Suzuki et al., 2013]. We also restored the *MSH2* expression of KO cells. The resulting cells, i.e., CD and KO cells, along with WT cells, i.e., Nalm-6-MSH+ cells [Suzuki et al., 2013], were used in this study.

Southern Blot and DNA Sequence Analyses

Southern blot analyses of the WT cells and the heterozygous and homozygous CD mutants were conducted as described [Sassa et al., 2014]. The ^{32}P-labeled probe was obtained by PCR amplification from Nalm-6 genomic DNA with the primers South CD F and South CD R. Sequence changes in the genomic DNA of the exon 6 of the CD cells were confirmed by the DNA sequence analysis with a 3130 Avant genetic analyzer (Applied Biosystems). Namely, exon 6 of *POLK* was amplified using genotyping primers, i.e., POLK GT Fw and POLK GT Rv, and sequenced with POLK GT Fw primer using BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies) according to the manufacturer's instructions. For cDNA analysis, total RNA was extracted from the CD cells with RNeasy kit (Qiagen), and transcribed into cDNA with ExScript® RT-PCR Kit (Takara Bio, Japan) with primers RT-PCR Fw and RT-PCR Rv. The cDNA corresponding to the region from exon 5 to exon 7 of the *POLK* gene was analyzed by DNA sequencing.

Western Blot Analysis

Total cell lysates were prepared from exponentially growing cells of KO, CD, and WT cells with PRO-PREP™ protein extract solution (iNtRON Biotechnology, Korea). The protein extracts were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to an immobilon-P membrane (Millipore). The membrane was soaked with blocking buffer including 5% non-fat skim milk, and then incubated with either rabbit polyclonal antibody against Pol κ [Sassa et al., 2014] or mouse anti-β-actin monoclonal antibody (Sigma-Aldrich), followed by incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody, respectively (GE Healthcare). The proteins were visualized by chemiluminescence with the ECL system (GE Healthcare). MSH2 was detected with a mouse anti-MSH2 monoclonal antibody (Santa Cruz Biotechnology) [Suzuki et al., 2013]. The expression levels were quantified by CS analyzer (ATTO, Japan).

Cell Viability Assay

The growth inhibition potential of the various genotoxic agents and UV was evaluated with Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Japan), where water-soluble tetrazolium salt is reduced to a yellow-color formazan by the activities of dehydrogenases of living cells. Briefly, 5×10^5 cells were seeded in 96-well plates and treated with different amounts of each genotoxic agent for 24 hr at 37°C. After the treatment, the cells were incubated with 10 μL of CCK-8 solution for 4 hr at 37°C. Then the soluble formazan product was quantified at 450 nm by Multiskan GO™ Microplate Spectrophotometer (Thermo Scientific). Cell viability was calculated as a percentage of the amount of formazan formed in the treated cells versus that of formazan in vehicle-treated cells. The experiments were repeated at least three times. The names of chemicals, CAS registry numbers, and suppliers used in this assay are listed in Table I. The UV radiation was achieved by a germicidal lamp (Toshiba GL10, Japan) emitting predominantly 254 nm. The lamp was placed 50 cm above the cells and the exposure time was increased to elevate the doses of UV up to 8 J/m^2.

Colony Formation Assay

To confirm the results of cell viability assay described above, we conducted colony formation assay with hydrogen peroxide. Briefly, 5×10^6 cells in 6-well plates were treated with increasing doses of hydrogen peroxide for 1 or 3 hr at 37°C. The cells were then washed twice and seeded in duplicate 96-well plates at a density of 2.5 cells/well. After 18–21 day culture, colonies were counted. The relative survival was calculated from plating efficiency according to the method of the mouse lymphoma assay [Clements, 2000].

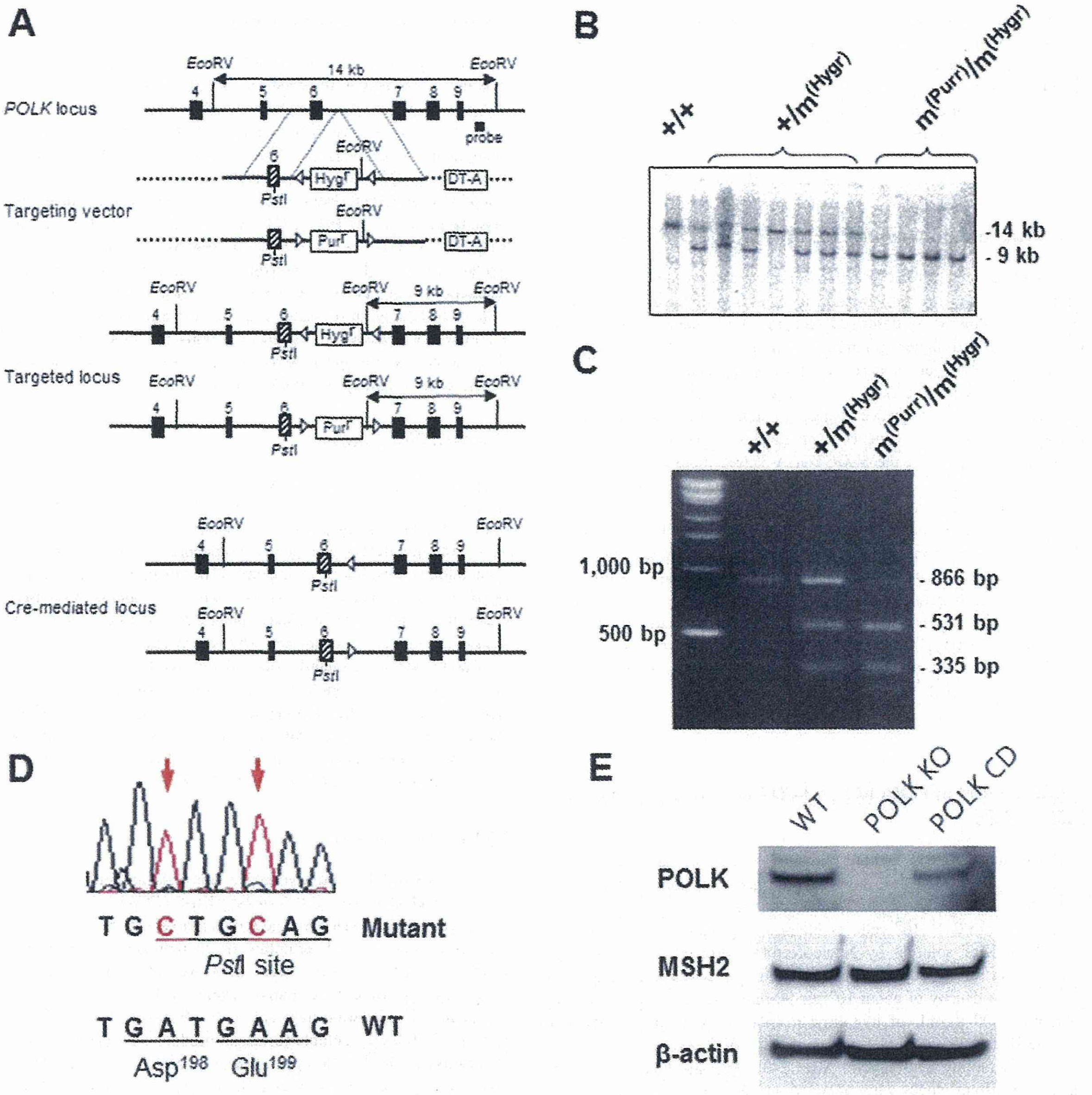

Fig. 1. A: Targeting strategy for generation of POLK CD mutant cells. Schematic representation of knocking-in a catalytically dead mutation into the *POLK* gene. The *POLK* locus, the targeting constructs, the targeted locus, and the Cre-mediated locus are shown here. The black boxes and triangles represent exons and loxP sequences, respectively. D198 and E199 are coded in exon 6. The mutated exon 6 having PstI site is shown as a hatched box. DT-A represents the gene encoding diphtheria toxin A, which is used to prevent random integration of the targeting vectors. The black box with probe represents the position of DNA probe for Southern blot analysis. **B**: Southern blot analyses of replacement of exon 6 with the introduced targeting CD vectors. *Eco*RV-digested genomic DNA from wild-type (WT), heterozygous (+/m(Hygr)), and homozygous POLK cells (m(Purr)/m(Hygr)) was loaded onto each lane. The probe used for the hybridization is indicated in Fig. 1A. The wild-type allele (14 kb) and targeted alleles (9 kb) are indicated at right. One mutant lost the band of 9 kb (lane 5 from the most left). **C**: Verification of introduction of *Pst*I site in exon 6 of the POLK gene. Total RNA from wild-type and mutant POLK cells, i.e., heterozygous (+/m(Hygr)) and homozygous POLK mutant cells (m(Purr)/m(Hygr)), was extracted and subjected to RT-PCR. The resulting cDNAs were digested with *Pst*I and separated by agarose gel electrophoresis. Molecular sizes of the wild-type allele (866 bp) and the targeted allele (separated to 531 bp and 335 bp) are indicated at right. The most left lane represents molecular weight markers. **D**: The result of DNA sequencing of the amplified cDNA fragment from the homozygous mutant POLK cells (m(Purr)/m(Hygr)). The sequences around codon 198 and 199 are shown. **E**: Western blot analysis for POLK and MSH2 protein. Whole cell extracts from WT, KO, and CD cells were loaded onto a 10% of SDS-polyacrylamide gel. β-actin served as a loading control.

TABLE I. Genotoxins for Cell Viability Assay

Genotoxin	CAS number	Supplier
Benzo[*a*]pyrene diol epoxide (BPDE)	68366-05-2	Midwest Research Institute
Mitomycin C (MMC)	50-07-7	Nacalai Tesque
Etoposide	33419-42-0	Sigma-Aldrich
Bleomycin hydrochloride (Bleomycin)	67763-87-5	Wako
Hydroxyurea	127-07-1	Sigma-Aldrich
Methyl methanesulfonate (MMS)	66-27-3	Nacalai Tesque
Ethyl methanesulfonate (EMS)	62-50-0	Nacalai Tesque
Hydrogen peroxide	7722-84-1	Wako
Potassium bromated	7758-01-2	Wako
Menadione	58-27-5	Wako
Ultraviolet C light (UV)	—	—

Complementation Assay

For the complementation assay, a full-length cDNA of the *POLK* gene was amplified from total RNA of WT cells by RT-PCR with primers of pEF-polk2-F and pEF-polk-R carrying restriction sites of *Bam*HI and *Pme*I, respectively. The RT-PCR product was digested with the restriction enzymes, and the resulting fragment was inserted into pEF6/Myc-His B vector (Life Technologies) digested with *Bam*HI and *Pme*I. The resulting plasmid was named pEF6-POLK. The catalytically inactive POLK expression vector, pEF6-POLK-CD, which carries a sequence change directing the amino acid substitutions of D198A/E199A, was generated from pEF6-POLK by using QuickChange^{TM} Site-Directed Mutagenesis Kit (Agilent Technologies) with primer POLK ex6mut-Fw and POLK ex6mut-Rv. The sequences of the *POLK* gene in pEF6-POLK and pEF6-POLK-CD vectors were confirmed by DNA sequencing with primers K4, R7, K5, R3, R1, F1, F3 and R8. The vectors were linearized by *Nsb*I prior to transfection. Then, KO cells were transfected with 5 μg of linearized vector DNA of pEF6-POLK or pEF6-POLK-CD. The transfection procedure was described above. Forty-eight hr after transfection, the cells were transferred into medium containing 5 μg/mL of blasticidin S (Life Technologies) and cultured for 2 weeks. The drug registrant clones were examined for the protein expression by Western blotting as described above. For KO cells transfected with linearized pEF6-POLK-CD, the sequence change was confirmed by DNA sequencing of cDNA generated by RT-PCR with primers POLK cDNA Fw and POLK cDNA Rv. Several clones were obtained for KO cells transfected with linearized pEF6-POLK or pEF6-POLK-CD, and used for the colony formation assay with hydrogen peroxide at 30 μM and bleomycin at 50 μg/mL.

Statistical Analysis

Data are presented as mean ± standard deviation (SD) unless otherwise indicated. All statistical analyses were performed using JMP ver. 9.0 software (SAS Institute). For the cell viability assay, the concentration at which 50% growth inhibition occurred (IC50) was determined by non-linear regression analysis for each of the genotoxic agents. Statistical comparison of IC50 values between WT cells and either KO or CD cells was carried out by Dunnett's test after ANOVA. The levels of $P < 0.05$ were considered significant.

RESULTS

Establishment of Pol K KO and CD Cells

The *POLK* gene includes 15 exons on chromosome 5 and the catalytic residues of D198 and E199 reside on exon 6. To investigate the roles of human Pol κ in DNA damage tolerance, we generated Pol κ KO and CD cells. Because we have generated KO cells in a background of Nalm-6 in previous work [Sassa et al., 2014], we established CD cells in the same background and restored the MSH2 expression in both cell lines, thereby establishing KO and CD cells in a background of Nalm-6-MSH+.

To establish the mutant cells, the CD vectors were constructed to introduce the mutation into exon 6, which directs the amino acid substitutions of D198A/E199A (Fig. 1A). The expressed Pol κ D198A/E199A lost the Pol activity completely in vitro (Supporting Information Fig. 2). The vectors have Hyg^r for the first targeting or Pur^r for the second targeting. The drug resistance genes are flanked by the *loxP* sites and the vectors contain the diphtherotoxin A (DT-A) gene to eliminate random integrants. As a result of the first targeting, we obtained 23 heterozygous clones out of 168 hygromycin-resistant clones. For the second targeting, the vectors having Pur^r were transfected into the $POLK^{+/m(Hygr)}$ clone. We obtained 9 homozygous mutated clones out of 72 puromycin-resistant clones. The targeted gene replacement was verified by Southern blot analysis with *Eco*RV-digested genomic DNA with an external 3′-probe (Figs. 1A and 1B). Successful introduction of the mutation (GAT GAA to GCT GCA) into exon 6 was verified by PCR of cDNA of *POLK*, followed by *Pst*I digestion (Fig. 1C) and DNA sequencing of the cDNA (Fig. 1D). The drug-resistance genes were removed by transient expression of Cre recombinase. Then, the synthetic cDNA corresponding to exon 9 to 16 of *MSH2* was introduced into KO and the cured cells, and restored the expression of MSH2, which was verified by Western blotting (Fig. 1E and Supporting Information Fig. 3). The expression of Pol κ and the inactive derivative (D198A/E199A) was examined by Western blot analysis with anti-Pol κ antibody (Fig. 1E, Supporting Information Fig. 3), and the results indicated that the proteins were expressed in WT cells and CD cells but not in KO cells. The expression level of Pol κ in CD cells was 84% of that of Pol κ in

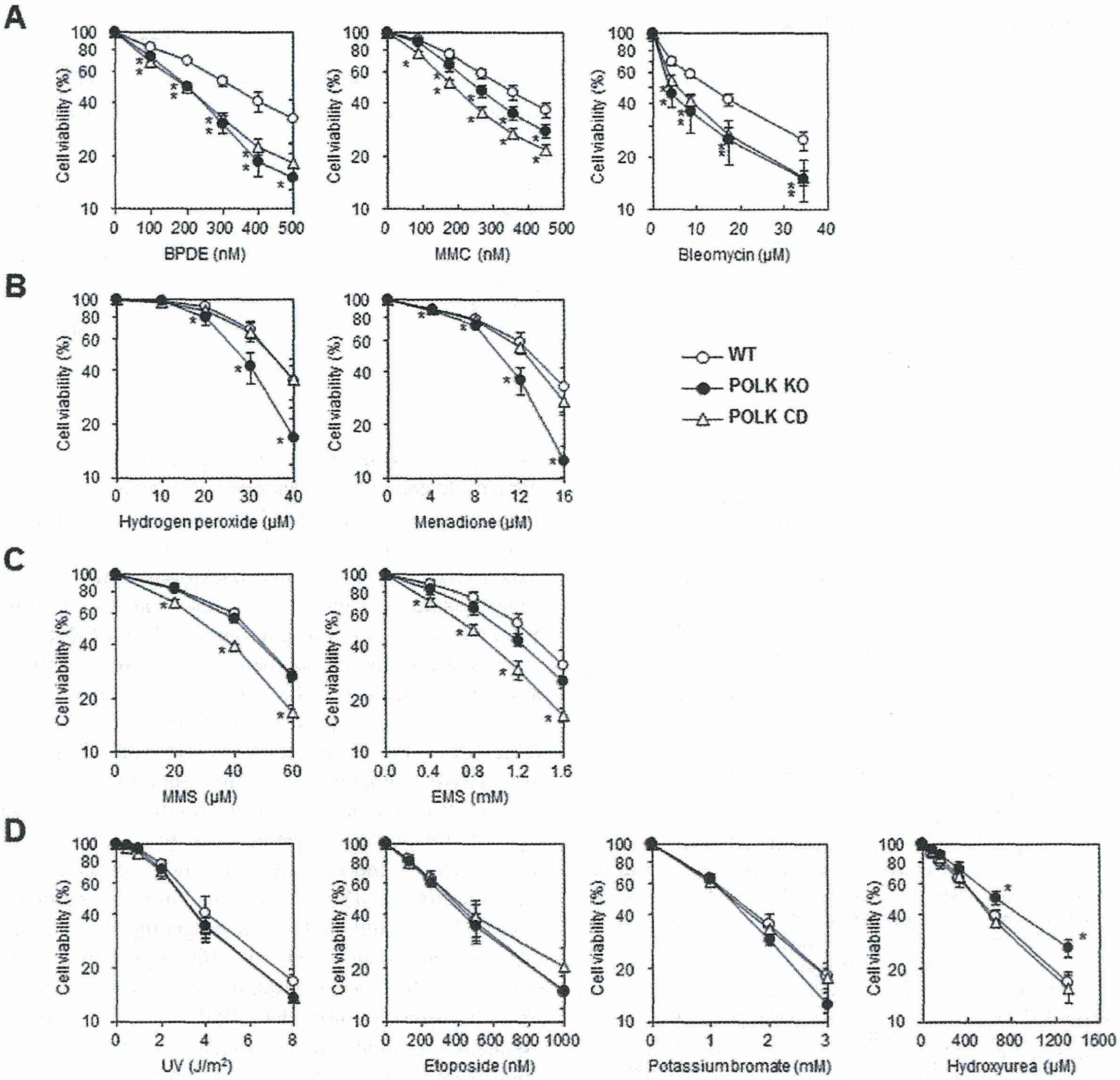

Fig. 2. Cell viability assay of 11 genotoxins with WT, KO, and CD cells of POLK. Cell viability, which was evaluated with Cell Counting Kit-8, was plotted against the dose of chemicals or UV. **A**: Results of Group 1, which includes BPDE, MMC, and bleomycin. **B**: Results of Group 2, which includes hydrogen peroxide and menadione. C: Results of Group 3, which includes MMS and EMS. D: Results of Group 4, which includes UV, etoposide, hydroxyurea, and potassium bromate. *Represents the statistical significance of $P < 0.05$ of the difference of cell viability of CD or KO cells compared to that of WT cells. The experiments were repeated at least three times with all three cell lines in parallel and the mean ± S.D. are presented.

WT cells (Supporting Information Fig. 4). The introduction of the mutation was confirmed again by DNA sequence analyses of the genomic and cDNA after the recovery of MSH2 expression (data not shown). The growth of KO cells (doubling time: 20.8 ± 0.6 hr) was slightly faster than WT cells (22.0 ± 0.6 hr), but the difference was not statistically significant. CD cells (22.6 ± 0.3 hr) grew at a similar rate to WT cells. The doubling time of the three cell lines was not substantially affected by the recovery of MSH2.

Cell Viability Assay

We examined viability of KO and CD cells after treatments with various genotoxic agents (Fig. 2) and calculated IC50 values for each tested agent with each-type of cells (Table II). According to the difference of sensitivities, we classified the genotoxic agents into 4 groups.

Group 1 includes BPDE, MMC, and bleomycin. Both KO and CD cells exhibited significantly higher sensitivity to these agents than WT cells (Fig. 2A). In the case of

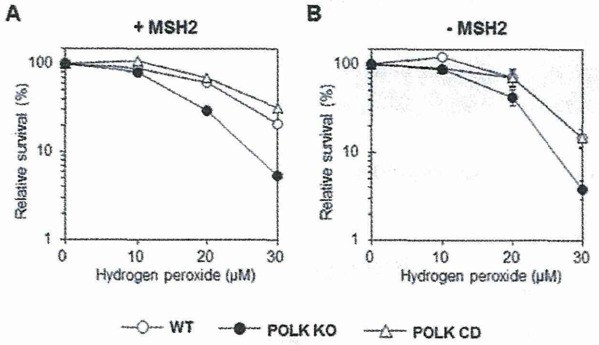

Fig. 3. Colony formation assay of hydrogen peroxide with WT, KO, and CD cells in the presence or the absence of MSH2. The cells were treated with hydrogen peroxide for 3 h (+MSH2 cells) or 1 h (−MSH2 cells) and after washing, the cells were seeded in 96-well plates at a density of 2.5 cells/well. After 18–21 day culture, colonies were counted and the relative survival was calculated from the plating efficiency [Clements, 2000]. **A**: Results of colony formation assay in the presence of MSH2, i.e. Nalm-6-MSH+ background. The experiments were conducted twice and the mean values are presented. **B**: Results of colony formation in the absence of MSH2, i.e. Nalm-6 background. The cells were the same as CD and KO cells but without restoration of MSH2. The experiments were conducted more than three times and the mean ± S.E. are presented.

BPDE, more than 40% decrease in IC50 was observed in KO and CD cells compared to WT cells. Although KO and CD cells displayed similar high sensitivity to BPDE and bleomycin, CD cells displayed slightly higher sensitivity than KO cells against MMC. Group 2 includes two oxidizing agents, i.e., hydrogen peroxide and menadione (Fig. 2B). KO cells exhibited higher sensitivity to these oxidative damaging agents than the WT and CD cells. WT and CD cells exhibited similar sensitivity to these agents. For hydrogen peroxide, there was ~22% decrease in IC50 in KO compared to WT, but IC50 was unaltered in CD cells. Group 3 includes MMS and EMS (Fig. 2C). CD cells exhibited higher sensitivity to these agents than the WT and KO cells. KO and WT cells exhibited similar sensitivity to the chemicals. More than 30% decrease was observed in IC50 in CD cells compared to WT cells, but there was no significant reduction in KO cells. Group 4 includes UV, etoposide, hydroxyurea, and potassium bromate (Fig. 2D). Neither KO nor CD cells exhibited higher sensitivity than WT cells for these genotoxins.

To confirm the results of the cell viability assay with hydrogen peroxide, we conducted colony formation assays. The results indicated that KO cells were more sensitive to the killing effects of hydrogen peroxide than CD and WT cells, and also that CD and WT cells exhibited similar sensitivity (Fig. 3A). When we examined the sensitivity of WT, CD, and KO cells in a background of Nalm-6 cells, which are deficient in mismatch repair functions, KO cells were more sensitive to the killing effects of hydrogen peroxide than CD and WT cells (Fig. 3B).

Complementation Assay

To further confirm the results that inactive Pol κ having D198A/E199A protects cells from the toxic effects of hydrogen peroxide, we expressed Pol κ or the inactive Pol κ in KO cells, and examined the sensitivity to the killing effects of hydrogen peroxide at 30 µM or bleomycin at 50 µg/mL. Although one clone expressing Pol κ (KO+pEF-POLK 2–5) did not exhibit substantially enhanced cell survival compared to KO cells when treated with hydrogen peroxide, two other clones expressing Pol κ (KO+pEF-POLK 2–10, 2–12) exhibited similar survival to WT cells (Fig. 4A). Similarly, the hypersensitivity to hydrogen peroxide in KO cells was restored in one clone expressing the inactive Pol κ (KO+pEF-POLK-CD 1). The relative survival was similar to that of WT cells. When cells were treated with bleomycin, the clone expressing Pol κ (c KO+pEF-POLK 2–5) exhibited enhanced cell survival compared to KO cells (Fig. 4B). The relative survival was more than that of WT cells. In contrast, the clone expressing the inactive Pol κ (KO+pEF-POLK-CD 1) exhibited very similar survival to KO and CD cells.

DISCUSSION

In this study, we have engineered two human cell lines, one lacking the expression of Pol κ (KO cells) and the other expressing Pol κ D198A/E199A (CD cells) (Fig. 1) in a Nalm-6-MSH+ background, and examined their sensitivity to various genotoxins including UV (Table I). We included CD cells along with KO cells for the study because we expected that Pol κ might have a non-catalytic role in the protection of cells from genotoxins. This assumption is based on the fact that Pol κ interacts with other proteins [Ohashi et al., 2004; Lv et al., 2013; Yoon et al., 2014]. As a result, we classified the genotoxins into four groups based on their cytotoxic pattern to the three cell lines, i.e., KO, CD, and WT cells (Table II).

Group 1 includes BPDE, MMC, and bleomycin (Table II and Fig. 2A). Both KO and CD cells exhibited higher sensitivity to the chemicals than did the WT cells. The results with BPDE and MMC are consistent with in vitro results that Pol κ bypasses N^2-guanyl adducts induced by BPDE and also that it bypasses crosslinks in DNA induced by MMC [Suzuki et al., 2002; Minko et al., 2008; Sassa et al., 2011]. The results for MMC are consistent with the report that Pol κ knock-in mice, in which inactive Pol κ is expressed, exhibited hypersensitivity to MMC-induced mutagenicity and clastogenicity in the bone marrow relative to wild-type mice [Takeiri et al., 2014]. Interestingly, this group includes bleomycin, which induces single- and double-strand DNA breaks (SSBs and DSBs) in DNA. Furthermore, the hypersensitivity of KO and CD cells to bleomycin was also confirmed in colony formation assay with

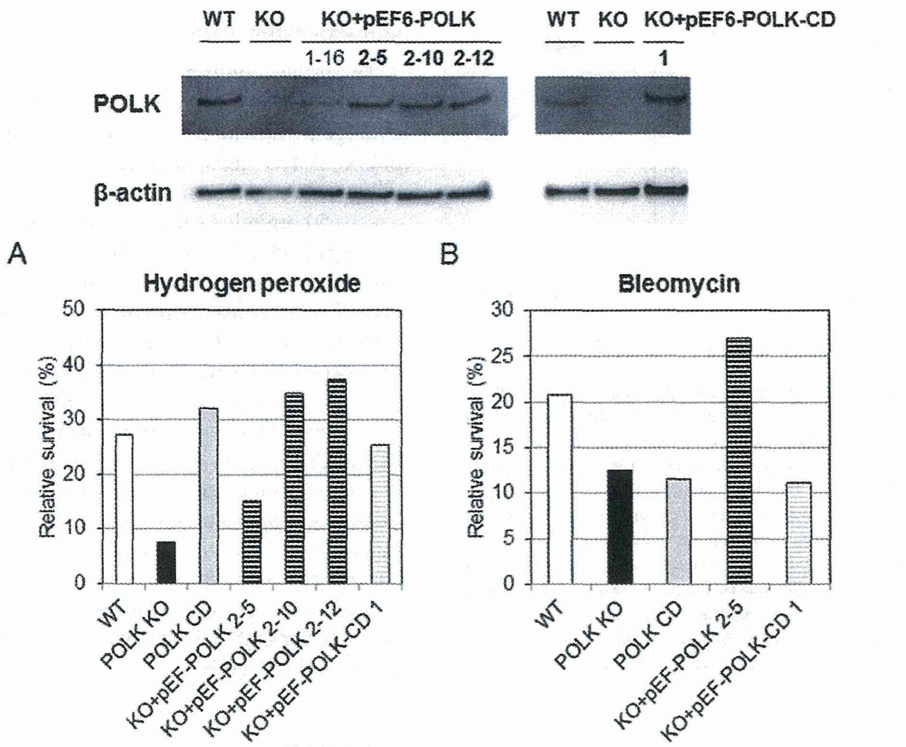

Fig. 4. Complementation assay of KO cells treated with hydrogen peroxide or bleomycin by introduction of linearized DNAs that allow stable expression of active or inactive Pol κ. **A**: WT, KO, CD cells, and KO cells expressing Pol κ (pEF-POLK 2–5, 2–10, and 2–12) or inactive Pol κ (pEF-POLK-CD 1) by stable transfection were treated with hydrogen peroxide at 30 μM for 3 h, and the cell viability was evaluated by colony formation assay. The relative survival was calculated with the cell survival of each untreated cell line as 100%. KO cells transfected with linearized pEF-POLK2–5, pEF-POLK2–10 or pEF-POLK2–12 expressed wild-type Pol κ as shown in the upper part of the graph. The cells transfected with linearized pEF-POLK-CD 1 expressed Pol κ variant of D198A/E199A. The Western blot was conducted with Pol κ antibody and β-actin served as a loading control. The cells transfected with linearized plasmid pEF-POLK1-16 were not used for the complementation assay because of the low expression level of Pol κ. **B**: WT, KO, CD cells, and KO cells transfected with linearized pEF-POLK 2–5 or pEF-POLK-CD 1 were treated with bleomycin at 50 μg/mL for 3 h, and the cell survival was evaluated as described in (A). The experiments were conducted once.

the Pol κ-complemented cells (Fig. 4B). Bleomycin is a therapeutic agent that induces the strand breaks in DNA through the production of free radicals in the vicinity of DNA [Chen and Stubbe, 2005]. Although SSBs and DSBs in DNA are induced, the latter is postulated as a major cause for the cytotoxicity [Povirk, 1996]. In the protozoa *Trypanosoma cruzi*, overexpression of the protozoan Pol κ increases resistance to zeocin, an analogue of bleomycin, and the Pol κ extends the primer DNA from a recombination intermediate with D-loop in vitro, raising the possibility that Pol κ is involved in HR [Rajao et al., 2009]. In contrast, etoposide, which induces topoisomearse II-associated direct DSBs, did not exhibit any sensitivity to KO and CD cells (Group 4, Fig. 2D). DSBs induced by etoposide are predominantly repaired by non-homologous end-joining in human cells [Adachi et al., 2004]. Taken together, we suggest that the increased sensitivity of KO cells and CD cells to bleomycin, but not etoposide, implicates a catalytic role of Pol κ in DSB repair via HR.

Group 2 includes hydrogen peroxide and menadione. Remarkably, only KO cells exhibited higher cytotoxic sensitivity to the chemicals than WT cells (Table II and Fig. 2B). CD cells displayed similar sensitivity to WT cells. To confirm the results of the cell viability assay, we conducted colony formation assay with hydrogen peroxide (Fig. 3A). The results were in good concordance with those of the cell viability assay. Namely, hydrogen peroxide exhibited higher cytotoxicity in KO cells than CD and WT cells. Expression of inactive Pol κ enhanced the cellular survival of KO cells treated with hydrogen peroxide while it did not increase the survival of KO cells treated with bleomycin (Fig. 4). To examine whether MSH2 has any influence on the results [Zlatanou et al., 2011], we examined the sensitivity of KO and CD cells of Nalm-6, which are deficient in MSH2, to hydrogen peroxide. As in the case of Nalm-6-MSH+, hydrogen peroxide exhibited higher cytotoxicity in KO cells than in CD and WT cells in MSH2-deficient background and both CD and

TABLE II. Summary of Cell Viability Assay

		IC50 value		
Genotoxin	Unit	WT	POLK KO	POLK CD
Group 1				
BPDE	nM	329 ± 40 (1.0)	190 ± 3[a] (0.6)	180 ± 4[a] (0.5)
MMC	nM	331 ± 28 (1.0)	257 ± 23[a] (0.8)	189 ± 12[a] (0.6)
Bleomycin	μM	11.7 ± 1.7 (1.0)	4.03 ± 1.92[a] (0.4)	5.59 ± 1.00[a] (0.5)
Group 2				
Hydrogen peroxide	μM	35.1 ± 2.4 (1.0)	27.9 ± 2.3[a] (0.8)	35.1 ± 3.4 (1.0)
Menadione	μM	13.4 ± 1.3 (1.0)	10.5 ± 0.6[a] (0.8)	12.6 ± 0.7 (0.9)
Group 3				
MMS	μM	45.3 ± 2.2 (1.0)	42.6 ± 2.3 (0.9)	30.6 ± 0.9[a] (0.7)
EMS	mM	1.23 ± 0.12 (1.0)	1.03 ± 0.10 (0.8)	0.72 ± 0.10[a] (0.6)
Group 4				
UV	J/m^2	3.54 ± 0.48 (1.0)	3.06 ± 0.32 (0.9)	3.02 ± 0.38 (0.9)
Etoposide	nM	356 ± 79 (1.0)	326 ± 33 (0.9)	371 ± 56 (1.0)
Hydroxyurea	μM	472 ± 65 (1.0)	651 ± 84[a] (1.4)	477 ± 38 (1.0)
Potassium bromate	mM	1.40 ± 0.13 (1.0)	1.31 ± 0.07 (0.9)	1.31 ± 0.07 (0.9)

Values are means ± S.D. ($n \geqq 3$). Genotoxins were classified into four groups based on the cytotoxic response.

Group 1: highly sensitive in KO and CD cells, Group 2: sensitive only in KO, Group 3: sensitive only in CD, Group 4: no significant difference among three cell lines. The number in parenthesis represents the relative values compared to IC50 value of POLK WT as 1.0. Statistical significance was analyzed by Dunnett's test.

[a] Represents the statistical significance of $P < 0.05$ versus WT.

WT cells exhibited very similar sensitivity to the cytotoxicity (Fig. 3B). The results exclude the possibility that MSH2 plays an important role in the protection of cells from hydrogen peroxide. Taken together, we suggested that Pol κ may have a non-catalytic role in protecting human cells from oxidative damage induced by hydrogen peroxide and menadione. It is reported that *Polk*$^{-/-}$ mouse embryonic fibroblast (MEF) cells display higher cytotoxic sensitivity to hydrogen peroxide than *Polk*$^{+/+}$ MEF cells and that DNA repair activity with oligonucleotide having a G:U mismatch is reduced in the cell extracts of the *Polk*$^{-/-}$ MEF cells [Zhang et al., 2013]. However, no catalytically dead cells were used in the study. Therefore, this is the first report to suggest a non-catalytic role for Pol κ in the protection of cells from genotoxicity induced by oxidative damage.

However, what is the non-catalytic role of Pol κ in protecting cells from the oxidative stress? One possibility is that Pol κ interacts with other protein(s) and recruits it (them) to the site of oxidative damage in DNA. Pol κ is recruited to sites of laser-induced DNA damage where strand breaks and oxidative damage in DNA are induced [Zhang et al., 2013]. In addition, Pol κ is known to interact with REV1, which interacts with multiple specialized Pols [Ohashi et al., 2004]. Intriguingly, human MRC-5 cells, which have depressed expression of Pol ι are sensitive to the cytotoxicity of hydrogen peroxide and menadione [Petta et al., 2008]. Pol ι is a member of the Y-family Pol, interacts with REV1, and has an intrinsic 5′-deoxyribose phosphate (dRP) lyase activity, which removes 5′-dRP group and allows the ligase to seal the gap [Bebenek et al., 2001]. This activity plays an important role in base excision repair (BER). Pol λ, an X-family Pol, which has dRP lyase activity, may also be involved in protecting cells from oxidative stress because mouse Pol $\lambda^{-/-}$ cells exhibit sensitivity to the killing effects of hydrogen peroxide [Vermeulen et al., 2007]. It has been reported that the Werner syndrome protein physically interacts with Pol λ and Pol κ [Kanagaraj et al., 2012; Maddukuri et al., in press]. Therefore, we speculate that Pol κ might directly or indirectly assist the recruitment of these Pols to oxidative damaged sites in DNA and enhance the repair activity of the whole cell. Although 8-oxo-guanine is a major oxidative lesion in DNA, Pol κ may not be involved in protection against the cytotoxic effects of this lesion in vivo because both KO and CD cells exhibited similar sensitivity to WT cells against potassium bromate (Group 4, Fig. 2D). Potassium bromate more selectively induces 8-oxo-guanine in DNA than other oxidative stresses [Kawanishi and Murata, 2006]. It is possible, however, that KO and CD cells may exhibit lower mutation frequencies induced by potassium bromate than WT cells because it has been reported that Pol κ is involved in error-prone TLS across 8-oxo-guanine in vivo [Kamiya and Kurokawa, 2012]. Another possibility for the non-catalytic role of Pol κ is that it may participate in repair pathway of oxidized dNTPs [Sekiguchi and Tsuzuki, 2002]. Mouse *Mth1*$^{-/-}$ cells are hypersensitive to the cytotoxicity of hydrogen peroxide [Yoshimura et al., 2003]. Mth1 is a sanitizing enzyme for the oxidized nucleotide pool and hydrolyzes oxidized dGTP and dATP to their corresponding dNMPs. Although