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Deregulated expression of DNA polymerase β is involved in the progression of genomic instability

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Abstract

Deregulated expression of DNA polymerase beta (pol β) has been implicated in genomic instability that leads to tumorigenesis, yet the mechanisms underlying the pol β -mediated genetic instability remain elusive. In this study, we investigated the roles of deregulated expression of pol β in spontaneous and xenobiotic-induced genetic instability using mouse embryonic fibroblasts (MEFs) that express distinct pol β levels (wild-type, null and over-expression) as a model system. Three genetic instability endpoints, DNA strand breaks, chromosome breakage and gene mutation, were examined under various expression levels of pol β by comet assay, micronuclei test and *hprt* mutation assay. Our results demonstrate that neither pol β deficiency nor pol β over-expression is sufficient for accumulation of spontaneous DNA damage that promotes a hyper-proliferation phenotype. However, pol β null cells exhibit increased sensitivity to exogenous DNA damaging agents with increased genomic instability compared with pol β wild-type and over-expression cells. This finding suggests that a pol β deficiency may underlie genomic instability induced by exogenous DNA damaging agents. Interestingly, pol β over-expression cells exhibit less chromosomal or DNA damage, but display a higher *hprt* mutation frequency upon methyl methanesulfonate exposure compared with the other two cell types. Our results therefore indicate that an excessive amount of pol β may promote genomic instability, presumably through an error-prone repair response, although it enhances overall BER capacity for induced DNA damage.

Keywords

Base excision repair; DNA polymerase beta; genomic instability; DNA damage and repair

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The authors declare no potential conflict of interest relevant to this article.

Author contributions:

Qingying Luo performed the majority of the experiments; Mei Wu conducted the Western blot; Shukun Liu helped to prepare the figures; Qingying Luo and Yanhao Lai analyzed the data and drafted the original version of the manuscript; Zunzhen Zhang designed the experiments. Yuan Liu conducted the reconstruction of the main theme and ideas of the manuscript, performed the editing for the manuscript and constructed the revised manuscript.

1. Introduction

DNA damage induced genomic instability plays an essential role in tumorigenesis. DNA base lesions, such as oxidized and alkylated bases, and single-strand DNA breaks induced by endogenous and exogenous DNA damaging agents, are the most common forms of DNA lesions. Base excision repair (BER) has been identified as the major cellular defense system for eliminating such damage, thereby preventing development of human diseases such as cancer. Decreased DNA repair capacity by nucleotide polymorphisms of some BER enzymes and proteins has been found to be associated with an elevated risk of lung cancer, prostate cancer and squamous cell carcinoma [Roberts et al., 2011]. Among them, non-synonymous substitutions in 8-oxo-guanine DNA glycosylase (OGG1) and X-ray repair cross-complementing protein 1 (XRCC1) are examples [Roberts et al., 2011]. These findings indicate that BER protein deregulation may lead to deficient or excessive BER that could have a substantial impact on the development or progression of human cancer, and this could be accomplished by modulating genomic stability and integrity [Prasad et al., 2011; Sterpone et al., 2010; Raffel et al., 2010].

Among the various BER proteins, DNA polymerase beta ($\text{pol } \beta$) has received considerable attention due to its role in efficient DNA damage repair, yet its relatively low fidelity. $\text{pol } \beta$ is a central component of BER that fills in a single-nucleotide gap and excises the 5'-terminal deoxyribose phosphate resulting from 5'-incision of an apurinic/apyrimidinic (AP) site by AP endonuclease 1 (APE1) during single-nucleotide (SN)-BER [Liu and Wilson, 2012; Yamtich and Sweasy, 2010; Beard and Wilson, 2006]. In addition, $\text{pol } \beta$ is involved in long patch (LP)-BER in which it coordinates with flap endonuclease 1 (FEN1) through the "Hit and Run" mechanism [Liu et al., 2005] or via strand-displacement synthesis [Wilson, 1998; Asagoshi et al., 2010]. The idea that $\text{pol } \beta$ down-regulation results in a cellular BER deficiency, and thus increased tumorigenic potential, is widely accepted and supported by a recent study [Poltoratsky et al., 2007]. Reduced $\text{pol } \beta$ expression has also been detected in one-fifth of tumors, such as breast and colon cancer [Bhattacharyya et al., 2002], suggesting that $\text{pol } \beta$ deficiency is involved in cancer development and progression.

Over-expression of $\text{pol } \beta$ in cancer appears to be complex and more prevalent than its loss. For example, $\text{pol } \beta$ mRNA levels are significantly increased in approximately one-third of tumors, such as uterine, ovarian, prostate and stomach tumors, compared with normal tissue. In addition, increased $\text{pol } \beta$ protein level has been observed in colon adenocarcinoma cell lines, in prostate and ovarian tumors, as well as in breast adenocarcinoma samples, where $\text{pol } \beta$ expression was 286-fold elevated in comparison to the adjacent normal tissues [Albertella et al., 2005; Bhattacharyya et al., 2002]. Several *in vitro* studies have demonstrated that over-expression of wild-type $\text{pol } \beta$ is directly involved in cancer-associated phenotypic alterations such as apoptosis, spontaneous mutations and chromosomal aberrations [Fréchet et al., 2001; Bergoglio et al., 2002]. However, Sweasy's group showed that over-expression of wild-type $\text{pol } \beta$ failed to produce any transformation phenotype in mouse cells. Instead, over-expression of various mutant forms of $\text{pol } \beta$, i.e., E295K, K289M and I260M, which have been identified in gastric cancer, colon cancer and prostate cancer, led to cellular transformation phenotypes, including anchorage-independent growth and focus formation [Lang et al., 2007]. The results from these studies suggest that $\text{pol } \beta$ over-expression plays a complex and significant role in tumorigenesis.

Although previous studies have suggested that BER dysfunction resulting from $\text{pol } \beta$ deregulation (deficiency or over-expression) is closely associated with genetic instability that can lead to tumorigenic processes, many questions about the roles of $\text{pol } \beta$ deregulation in genetic instability remain to be answered. For example, is $\text{pol } \beta$ deficiency or over-expression sufficient to cause spontaneous genomic instability? What may be the role $\text{pol } \beta$

plays in modulating the genomic instability induced by exogenous mutagens? Furthermore, what are the molecular mechanisms underlying xenobiotic-induced genetic instability in situations of deregulated pol β expression? Based on the fact that pol β plays a pivotal role in repairing DNA base lesions, while it is error-prone, we hypothesized that under exogenous stress, pol β deficiency and the associated reduced BER capacity would lead to progression of genomic instability. We postulated that pol β over-expression would enhance BER efficiency and improve cellular viability, yet would also increase error-prone DNA synthesis during BER, thereby leading to a mutator phenotype and genetic instability. To test the above hypotheses, we used mouse embryo fibroblasts (MEFs) that express various levels of pol β , wild-type (pol $\beta^{+/+}$), pol β null (pol $\beta^{-/-}$) and pol β over-expression (pol β^{oe}) cells, as models to evaluate the effects of deregulated pol β expression on the progression of genetic instability at the chromosome, DNA and gene level in the presence and absence of a xenobiotic challenge.

2. Materials and methods

2.1 Cell lines and culture

SV40-transformed pol $\beta^{+/+}$ wide-type cells (termed 16.3), pol $\beta^{-/-}$ (termed 19.4 knock-out) and pol β^{oe} (termed 19 HBS over-expressed) mouse embryonic fibroblasts (MEFs) were a kind gifts from Dr. Samuel H. Wilson (National Institute of Environmental Health Sciences (NIEHS)/National Institutes of Health (NIH), Research Triangle Park, NC, USA) [Sobol et al., 1996]. The three cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St. Louis MO, USA) with 10% fetal calf serum (FBS) and hygromycin (80 μ g/ml; Gibco BRL, Grand Island, N.Y., USA) at 37 °C in a 5% CO₂ incubator. G418 (600 μ g/ml; Gibco BRL, Grand Island, N.Y., USA) was added in the medium for culturing pol β^{oe} cells to maintain the stable expression of plasmid.

2.2 Western blotting

Total cell lysates were prepared in a sodium dodecyl sulfate buffer. Proteins in the same amount were separated by 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. After incubation with the primary antibody specific against pol β (kindly provided by Dr. Samuel H. Wilson at NIEHS), the PVDF membrane was incubated with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase and visualized with enhanced chemiluminescence.

2.3 Cell growth kinetics

Cells were seeded in 12-well plates at a density of 1×10^4 cells per well. After 24 h, cells were collected and counted on days 1–8. The cell number was obtained from three parallel experiments and was plotted against culture time.

2.4 Cell population doubling time

Cells were plated into 24-well dishes at 2×10^4 cell per well (three wells for each type of cells). After 48 h, cells were trypsinized and counted microscopically. The cell population doubling time was calculated based on the formula: Cell population doubling time = $(T - T_0) \lg 2 / (\lg N_t - \lg N_0)$. Here, T_0 and T represent starting time and ending time of cell culture, respectively. N_0 and N_t stand for the cell number at the start and the end of each culture time interval.

2.5 Single cell gel electrophoresis -Comet assay

Alkaline comet assay was used to detect DNA single-strand and double-strand breaks and alkali-labile sites. In this study, cells were seeded in 24-well plates at a density of 10^5 per well. After 24 h, cells were treated with different concentrations of potassium dichromate ($K_2Cr_2O_7$) (0, 0.05, 0.1, 0.5 mM) at 37 °C for 2 h. Cells were then harvested by trypsinization. Trypan blue exclusion method was employed to check cell viability. The protocol of slide preparation, lysis and electrophoresis were conducted as described previously [Wu et al., 2008]. Duplicates of slides were made for cells treated at each concentration of $K_2Cr_2O_7$. Comet rate and olive tail moment (OTM) were used as the qualitative and quantitative measurement of DNA damage. 200 cells were randomly selected and scored from each slide for comet rate, while 30 randomly selected comet cells were analyzed for OTMs. Comet rate (%) = (total number of cells with tails/total number of counted cells)×100%. OTMs were obtained using comet assay score software (CASP).

2.6 Chromosomal breakage/aberrations measured by micronucleus test

The micronucleus test is utilized to measure micronuclei, which mainly originate from chromosome breakage or a chromosome that is detached from the mitotic spindle during cell division. Cells were seeded in a 6-well plate at a density of 1×10^6 cells per well. After cell attachment, various concentrations of mitomycin (MMC) (0, 0.05, 0.5 mg/ml) were added into each individual well for 24 h allowing the capture of micronuclei that were produced during cell division. Cells were then harvested. Trypan blue exclusion was used to examine cell viability as described previously [Wu et al., 2008]. 1000 cells were randomly selected from each slide and examined under fluorescent microscope for measuring micronucleus rate (400× magnifications).

2.7 hprt gene mutation assay

Hprt gene mutation assay was performed to investigate genotoxicity of methyl methanesulphonate (MMS) at gene level in MEFs with distinct pol β expression as described previously [Liu et al., 2010]. Approximately, 2×10^5 cells were seeded in 100 ml flasks overnight. Cells were then exposed to MMS (final concentration: 0, 5, 10, 50 μ M) in serum-free medium for 24 h. Subsequently, the medium was removed and washed by PBS, and cells were incubated at 37 °C for additional 1 week to provide sufficient time to express mutation in treated cells. At the end of incubation, the majority of the cells was re-seeded at a density of 10^6 cells per flask in new flasks (three flasks for each concentration) and cultured with 6-Thioguanine (6-TG, final concentration was 2×10^{-7} mM) selective medium that inhibited the growth of the normal cells leaving mutant cells to proliferate. The rest of cells were plated at a density of 200 cells per well (three wells for each concentration) in a 24-well plate in serum-free DMEM without 6-TG. After incubation for 14 days, these plates and flasks were stained by 10% Giemsa, and colonies of 50 cells were counted. Cloning efficiency (CE) and mutation frequency (MF) were calculated with following equations: CE (%) = (number of treated cell/number of negative well)/200×100%. MF = (average number of mutation forming of each type of cell/CE)× 10^{-6} .

2.8 Cell survival studies

A colorimetric assay was used to measure the number of viable cells under MMS environment. Briefly, cells were seeded at a density of 10^4 cells/well in 96-well dishes. The following day, cells were exposed to increasing dosages of MMS in growth medium for 24 h and were subsequently subject to MTT assay with 5 mg/ml of MTT solution at 37 °C incubation for 4 h. Soluble purple color formazan crystals that formed were dissolved in dimethyl sulfoxide (DMSO). Absorbance was read at 570 nm using an ELISA reader (Bio-Rad, Hercules CA, USA). Cell viability was calculated using the formula: (%) = (A_{570} of

test group/ A_{570} of control group) $\times 100\%$, while 50% inhibitory concentration (IC_{50}) was obtained by probit analysis. Each concentration was tested in 8 parallel wells.

2.9 Colony forming assay

Colony forming assay was a commonly used method in evaluating cytotoxicity. This assay was used to test the effects of imbalanced pol β expression on cell proliferation when cells were under MMS treatment. Cells were plated into 24-well plates at 200 cells and then exposed to 0, 0.01, 0.05, 0.1, 0.25, 0.5 mM MMS in growth medium for 24 h. After treatment, cells were washed with PBS and re-incubated for consecutive 10 days to allow colony formation. The colonies that contain 50 or more cells were scored under anatomical microscopy. For each type of cell line, colonies were obtained from 3 wells. The rate of colony formation (%) = (average number of colony forming of each experimental group/inoculation cell number) $\times 100\%$.

2.10 Statistical methods

All the experiments were conducted in triplicates. Statistical analysis was performed by SPSS 13.0 software. In growth kinetics, cell population doubling time experiment, comet assay, MTT assay and colony forming test, the data were expressed as mean $\pm$ standard error. One-way analysis of variance (ANOVA) was used to evaluate significant difference among various experimental groups and least-significant difference (LSD) was applied for multi-group comparison. Statistical comparisons of the 50% inhibitory concentration (IC_{50}) among the three types of cell lines were analyzed by Student's t-test. Poisson distribution was employed to analyze the significant difference among the three types of cells in micronucleus test and *hprt* gene mutation assay. $P < 0.05$ was considered to be statistically significant.

3. Results and Discussion

3.1 Cell growth characteristics under different pol β expression levels

A cell growth advantage is one of the major features associated with the initiation and progression of cancer, yet the effect of deregulated pol β expression on cellular growth has been debated. Excessive expression of wild-type pol β was reported to enhance cellular microsatellite instability, and thus accelerate cellular malignant proliferation [Yamada and Farber, 2002]. These findings support the notion that pol β over-expression may lead to development of cancer by promoting cell growth and proliferation. However, not all the studies on pol β over-expression support this notion. For example, NIH3T3 cells that over-express wild-type pol β failed to exhibit a growth advantage over the parental cell line [Du et al., 2006]. Furthermore, Sweasy's group reported that over-expression of wild-type pol β did not result in focus formation or anchorage-independent growth, indicating that increased expression of wild-type pol β was not sufficient to cause a cell growth advantage [Lang et al., 2007]. The different results from these studies may stem from different experimental conditions or systems. To further explore if and how aberrant pol β expression may alter cellular growth characteristics, we studied cellular morphology, growth kinetics, cell proliferation and colony-forming capacity under different pol β expression levels.

To verify pol β expression level in pol β null, pol β wild-type and pol β over-expression cells (pol β^{oe}), we measured pol β protein using Western blot. Pol β protein was not detected in pol β null cells. Wild-type cells expressed a moderate level of pol β protein, whereas pol β^{oe} cells expressed about 2-fold higher protein level than pol β wild-type cells (Fig. 1). Our studies found that there are no significant differences in cellular morphology, growth kinetics, cell proliferation and colony-forming capacity among the pol $\beta^{-/-}$, pol $\beta^{+/+}$ and pol β^{oe} cells (Fig. 2, Tab. 1, Tab. 2). These findings are consistent with the results from

previous studies of pol β deficiency as well as pol β over-expression [Lang et al 2007]. This indicates that over-expression of wild-type pol β is not sufficient to confer MEFs with a growth advantage, and thus does not produce a spontaneous hyper-proliferation phenotype. Since pol β is a housekeeping gene that is constitutively expressed independent of cell cycle in mammalian cells, expression of this protein might not be expected to affect cell cycle regulation or cell proliferation.

3.2 Deregulation of pol β expression was not sufficient to increase spontaneous DNA damage and genomic instability

Spontaneous DNA damages occur at a rate of 10,000 lesions per cell per day, and most of these damages are repaired by the BER pathway. Thus, it is possible that pol β deficiency could lead to the accumulation of spontaneous DNA damage, leading to genomic instability, whereas pol β over-expression could remove spontaneous DNA damage effectively, thereby preventing genomic instability. However, the roles of pol β deregulation, especially pol β deficiency in repairing spontaneous DNA damage and genomic stability, remain somewhat unclear [Frechet et al., 2001; Bergoglio et al., 2002; Lang et al., 2007]. To address this issue, we examined spontaneous DNA strand breaks, chromosomal breakage and gene mutation of all three cell lines using the comet assay, micronucleus test and *hprt* gene mutation assay. The results showed that no hyper-mutagenic phenotype was observed under pol β deficiency or over-expression in the absence of exogenous chemical challenges (Fig. 3, Fig. 4 and Fig. 5). This result could be explained by several possible mechanisms. First, BER is a complex system in which multiple proteins with overlapping functions cooperate with each other, yet work in an independent manner. DNA polymerase λ (pol λ), DNA polymerase gamma (pol γ) and DNA polymerase iota (pol ι) all have been proposed to possess 5'-dRP lyase activity and could partially substitute for the function of pol β during BER [Luke et al., 2010; Braithwaite et al., 2010]. Therefore, spontaneous DNA lesions could be effectively removed by these alternative polymerases in pol β null cells. Second, in the absence of xenobiotics, the level of spontaneous DNA damage is relatively low. The wild-type pol β level is sufficient to repair this low level of DNA lesions, and thus, excessive pol β expression may be redundant for removing spontaneous DNA damage. Taken together, our data support the notion that deregulated pol β expression neither increases DNA damage nor confers a cell growth advantage in the absence of exogenous DNA damaging agents. In this scenario, deregulation of pol β expression might not affect cellular genomic stability and integrity significantly.

3.3 Deregulation of pol β expression on genotoxicity and genomic instability induced by exogenous DNA damaging agents

We next measured three major genetic instability endpoints of pol $\beta^{+/+}$, pol $\beta^{-/-}$, and pol β^{oe} cell lines following challenge with an exogenous DNA damaging agent. Since accumulation of unrepaired DNA single- and double-strand breaks are potential substrates for DNA homologous recombination repair and play a crucial role in genomic stability, we measured DNA strand breaks using the comet assay, a relatively sensitive and fast technique for monitoring DNA strand breaks at a single cell level. DNA strand breaks induced by a well-known oxidative DNA damaging agent, potassium dichromate ($K_2Cr_2O_7$), was measured in pol $\beta^{+/+}$, pol $\beta^{-/-}$ and pol β^{oe} cells. As illustrated in Fig. 3, the comet rate and OTM in all three cell lines significantly increased in a dose-dependent manner after cells were treated with $K_2Cr_2O_7$ for 2 h, during which time repair could take place. Pol $\beta^{-/-}$ cells exhibited the highest comet rate and OTM, whereas pol β over-expression cells showed the lowest comet rate and OTM, indicating a high efficiency of coping with DNA strand breaks (Fig. 3A and 3B). For example, when pol $\beta^{-/-}$ and pol β^{oe} cells were treated with 0.1 mM $K_2Cr_2O_7$, the comet rate of pol $\beta^{-/-}$ cells was 100%, whereas that of pol β^{oe} cells was less than 50% (Fig. 3A). On the other hand, the OTM of pol $\beta^{-/-}$ cells was 2-fold higher than that of pol $\beta^{+/+}$

cells and nearly 4-fold higher than that of pol β^{oe} cells (Fig. 3B). These results indicate that pol β deficiency leads to an accumulation of DNA strand breaks that result from the exogenous oxidative stressor. This suggests that the accumulated DNA strand breaks could result in chromosome breakage and gene mutations.

Employing a micronucleus test, we examined chromosomal breakages induced by mitomycin (MMC), a commonly used chromosome clastogen also known to induce oxidative stress, in pol $\beta^{+/+}$, pol $\beta^{-/-}$ and pol β^{oe} cells (Fig. 4). A significant difference in the rate of micronucleated cells was detected among the three cell types following high dose MMC (0.5 mg/ml) treatment (Fig. 4). The micronucleus rate of pol $\beta^{-/-}$ cells was 8% ($P<0.05$), and that of pol $\beta^{+/+}$ cells was 6%, whereas the micronucleus rate of pol β^{oe} cells was lower than 5% ($P<0.05$) (Fig. 4). The above data indicate that pol β deficiency results in accumulation of DNA strand breaks that subsequently exacerbate cellular chromosomal breakages, and that pol β over-expression effectively removes DNA strand breaks, thereby preventing chromosomal breakages.

To further explore genomic stability under deregulation of pol β expression at the gene level, we measured the mutation frequency at the *hprt* locus in pol $\beta^{+/+}$, pol $\beta^{-/-}$ and pol β^{oe} cells treated with methyl methanesulfonate (MMS). The X-linked *hprt* gene is the most commonly used mammalian locus for detecting mutagenic effects of xenobiotics. The results indicate that pol $\beta^{-/-}$ cells and pol β^{oe} cells all exhibit a higher mutation frequency (MF) in the *hprt* locus than pol $\beta^{+/+}$ cells under the same dosage of MMS treatment (Fig. 5). At 50 μM , pol $\beta^{-/-}$ cells exhibit a 3-fold higher mutation frequency than pol $\beta^{+/+}$ cells, and pol β^{oe} cells exhibit a similar mutation frequency rate as pol $\beta^{-/-}$ cells (Fig. 5), indicating that both pol β deficiency and over-expression promote MMS-induced mutations. Our results demonstrate that exogenous DNA damaging agents induce genomic instability phenotypes in pol β null cells. The increase in DNA strand breaks, micronucleus formation and *hprt* gene mutations suggest that accumulation of unrepaired DNA damage in pol β null cells leads to chromosomal abnormalities and gene mutation. This further indicates that pol β is required for efficient BER because of its dRP lyase and gap-filling synthesis activities. This is consistent with previous reports [Luke et al., 2010; Horton et al., 2003; Sobol et al., 2002].

Interestingly, pol β over-expression cells exhibit decreased DNA damage and micronucleus rate (Fig. 3, Fig. 4), but elevated MMS-induced *hprt* gene mutations (Fig. 5). This indicates that an excessive amount of pol β enhances BER capacity, yet also facilitates xenobiotic-induced mutations that lead to genomic instability. Several possible mechanisms may underlie this finding. First, pol β lacks a proofreading activity, compromising its ability to discriminate correct nucleotides from incorrect nucleotides during gap-filling synthesis [Beard and Wilson, 2006; Wilson, 1998]. Thus, pol β over-expression may increase the likelihood that the enzyme incorporates an incorrect nucleotide or mutagenic nucleotide analog into DNA. Consistently, it has been found that an excessive amount of pol β facilitates frameshift mutations during one-nucleotide gap filling synthesis [Chan et al., 2007]. Second, BER enzymes and cofactors interact and coordinate with each other forming a series of protein complexes in which substrates and products can be passed along in a highly orchestrated manner. This has been proposed as the “passing the baton” mechanism by Wilson and Kunkel [Wilson and Kunkel, 2000]. It is conceivable that over-expression in one of the BER enzymes or proteins may lead to imbalanced interactions among the other repair proteins, thereby disrupting the coordinated steps during the “passing the baton” process.

3.4 Cell survival response to MMS under deregulated pol β expression

To further explore the consequences of deregulated pol β expression on cell survival, we examined the effects of pol β deficiency and over-expression on cell viability and proliferative capacity following MMS treatment using the MTT and colony forming assays. The results show that pol $\beta^{-/-}$ cells, by and large, exhibit the lowest cell viability and colony formation rate over an increasing MMS dosage, with the pol β^{oe} cells exhibiting the greatest survival (Fig. 6A and 6B). The 50% inhibitory concentration (IC₅₀) of MMS for viability of pol $\beta^{+/+}$, pol $\beta^{-/-}$ and pol β^{oe} cells was (1.85±0.43) mM, (1.26±0.35) mM and (2.67±0.51) mM, respectively. The differences in IC₅₀ among the three cell lines were statistically significant ($P<0.05$). Our results indicate that pol β deficiency significantly reduces the cellular survival response to DNA base lesions, whereas excessive amount of pol β increases cell survival by removing DNA damages effectively.

It should be noted that both pol $\beta^{-/-}$ and pol β^{oe} cells were found to bear a homozygous null mutation of pol ι [Sobol, 2007], a member of the Y-family of DNA polymerases. Since pol ι contains weak dRP lyase activity and DNA lesion bypass synthesis activity [Bebenek et al., 2001], deficiency of this polymerase could compromise BER capacity of pol $\beta^{-/-}$ and pol β^{oe} cells to some extent. However, it is unlikely that pol ι deficiency would significantly affect our results based on the fact that pol β is the major contributor of cellular dRP lyase activity and gap-filling synthesis during BER. In addition, it was found that both pol ι and pol λ , another DNA polymerase that harbors dRP lyase activity, play limited roles in backing up pol β function [Braithwaite et al., 2010]. Poltoratsky et al. also reported that pol ι deficiency has negligible effect on cellular sensitivity to alkylation DNA damage under pol β deficiency [Poltoratsky et al., 2008], indicating that pol ι does not play a major role in BER. Since both pol $\beta^{-/-}$ and pol β^{oe} cells were derived from the same embryonic fibroblasts containing a null mutation in the pol ι gene, these cells share identical genetic background except for the pol β expression level. Thus, the significant differences in genomic instability and cell viability between pol $\beta^{-/-}$ and pol β^{oe} cells likely reflect the variation in pol β expression.

Assuming that pol ι has a role in repairing DNA damage by inserting correct nucleotides, lack of pol ι would result in increased genetic instability, such as elevated *hprt* gene mutations, in pol $\beta^{-/-}$ and pol β^{oe} cells. Thus, the *hprt* gene mutation rate of these cells could be overestimated in our study. However, given the fact that pol ι plays a minor role in BER [Braithwaite et al., 2010; Poltoratsky et al., 2008], it is unlikely that our gene mutation rate results were significantly overestimated. Moreover, as a lesion bypass DNA polymerase, pol ι could potentially increase cellular gene mutations leading to an overestimation of the gene mutation rate. Thus, cells with a pol ι knockout, such as in the pol $\beta^{-/-}$ or pol β^{oe} cells, may very well be advantageous for studying the mutagenic effects of deregulation of pol β expression. We conclude that the genomic instability observed herein mainly results from deregulation of pol β expression, especially pol β over-expression. The overlapping roles between pol β deregulation and pol ι in genetic instability during BER is an interesting question that needs to be elucidated in the future.

In summary, our study reveals several important aspects about the roles of pol β expression in genomic instability. First, a wild-type level of pol β , as would be expected, does not lead to genomic instability, and thus does not promote a spontaneous hyper-proliferation phenotype. Second, when cells are under challenge of xenobiotics, a wild-type level of pol β is crucial in maintaining genomic stability through the efficient removal of induced DNA damage, whereas a deficiency in pol β results in genomic instability. Third, over-expression of pol β , although it can protect cells from genotoxic stress by effectively removing DNA damage, results in increased gene mutation and genomic instability, presumably via decreased fidelity during repair synthesis.

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The abbreviations used are

BER	base excision repair
pol β	DNA polymerase beta
dRP	deoxyribose phosphate
Comet assay	single cell gel electrophoresis
OTM	Olive tail moment
MMC	mitomycin
MMS	methyl methanesulphonate
hprt	hypoxanthine-guanine phosphoribosyl transferase
CE	cloning efficiency
MF	mutation frequency
pol ι	DNA polymerase iota

References

- Albertella MR, Lau A, O'Connor MJ. The overexpression of specialized DNA polymerases in cancer. *DNA Repair (Amst)*. 2005; 4:583–593. [PubMed: 15811630]
- Asagoshi K, Liu Y, Masaoka A, Lan L, Prasad R, Horton JK, Brown AR, Wang XH, Bdour HM, Sobol RW, Taylor JS, Yasui A, Wilson SH. DNA polymerase beta-dependent long patch base excision repair in living cells. *DNA Repair (Amst)*. 2010; 9:109–119. [PubMed: 20006562]
- Beard WA, Wilson SH. Structure and mechanism of DNA polymerase Beta. *Chem Rev*. 2006; 106:361–382. [PubMed: 16464010]
- Bebenek K, Tissier A, Frank EG, McDonald JP, Prasad R, Wilson SH, Woodgate R, Kunkel TA. 5'-Deoxyribose phosphate lyase activity of human DNA polymerase iota in vitro. *Science*. 2001; 291:2156–2159. [PubMed: 11251121]
- Bergoglio V, Pillaire MJ, Lacroix-Triki M, Raynaud-Messina B, Canitrot Y, Bieth A, Garès M, Wright M, Delsol G, Loeb LA, Cazaux C, Hoffmann JS. Deregulated DNA polymerase beta induces chromosome instability and tumorigenesis. *Cancer Res*. 2002; 62:3511–3514. [PubMed: 12067997]
- Bhattacharyya N, Chen HC, Wang L, Banerjee S. Heterogeneity in expression of DNA polymerase beta and DNA repair activity in human tumor cell lines. *Gene Expr*. 2002; 10:115–123. [PubMed: 12064574]
- Braithwaite EK, Kedar PS, Stumpo DJ, Bertocci B, Freedman JH, Samson LD, Wilson SH. DNA polymerases beta and lambda mediate overlapping and independent roles in base excision repair in mouse embryonic fibroblasts. *PLoS One*. 2010; 18:e12229. [PubMed: 20805875]
- Chan K, Houlbrook S, Zhang QM, Harrison M, Hickson ID, Dianov GL. Overexpression of DNA polymerase beta results in an increased rate of frameshift mutations during base excision repair. *Mutagenesis*. 2007; 22:183–188. [PubMed: 17267816]

- Du LT, Xu L, Yang XF, He Y, Wei Q, Zhuang ZX. Effects of overexpression of human pol-beta on cellular response to DNA damage. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi*. 2006; 24:88–91. [PubMed: 16600112]
- Fréchet M, Canitrot Y, Cazaux C, Hoffmann JS. DNA polymerase beta imbalance increases apoptosis and mutagenesis induced by oxidative stress. *FEBS Lett*. 2001; 505:229–232. [PubMed: 11566181]
- Horton JK, Joyce-Gray DF, Pachkowski BF, Swenberg JA, Wilson SH. Hypersensitivity of DNA polymerase beta null mouse fibroblasts reflects accumulation of cytotoxic repair intermediates from site-specific alkyl DNA lesions. *DNA Repair (Amst)*. 2003; 4:27–48. [PubMed: 12509266]
- Lang T, Dalal S, Chikova A, DiMaio D, Sweasy JB. The E295K DNA polymerase beta gastric cancer-associated variant interferes with base excision repair and induces cellular transformation. *Mol Cell Biol*. 2007; 27:5587–5596. [PubMed: 17526740]
- Liu S, Wu M, Zhang Z. Involvement of DNA polymerase beta in repairing oxidative damages induced by antitumor drug adriamycin. *Toxicol Appl Pharmacol*. 2010; 246:163–170.
- Liu Y, Beard WA, Shock DD, Prasad R, Hou EW, Wilson SH. DNA polymerase beta and flap endonuclease 1 enzymatic specificities sustain DNA synthesis for long patch base excision repair. *J Biol Chem*. 2005; 280:3665–3674. [PubMed: 15561706]
- Liu Y, Wilson SH. DNA base excision repair: a mechanism of trinucleotide repeat expansion. *Trends Biochem Sci*. 2012 [Epub ahead of print]. 10.1016/j.tibs.2011.12.002
- Luke AM, Chastain PD, Pachkowski BF, Afonin V, Takeda S, Kaufman DG, Swenberg JA, Nakamura J. Accumulation of true single strand breaks and AP sites in base excision repair deficient cells. *Mutat Res*. 2010; 694:65–71. [PubMed: 20851134]
- Poltoratsky V, Prasad R, Horton JK, Wilson SH. Down-regulation of DNA polymerase beta accompanies somatic hypermutation in human BL2 cell lines. *DNA Repair*. 2007; 6:244–253. [PubMed: 17127106]
- Poltoratsky V, Horton JK, Prasad R, Beard WA, Woodgate R, Wilson SH. Negligible impact of pol iota expression on the alkylation sensitivity of pol beta-deficient mouse fibroblast cells. *DNA Repair (Amst)*. 2008; 7:830–833. [PubMed: 18434259]
- Prasad R, Beard WA, Batra VK, Liu Y, Shock DD, Wilson SH. A review of recent experiments on step-to-step “hand-off” of the DNA intermediates in mammalian base excision repair pathways. *Mol Biol (Mosk)*. 2011:45586–45600.
- Raffel A, Eisenberger CF, Cupisti K, Schott M, Baldus SE, Hoffmann I, Aydin F, Knoefel WT, Stoecklein NH. Increased EpCAM expression in malignant insulinoma: potential clinical implications. *Eur J Endocrinol*. 2010; 162:391–398. [PubMed: 20097833]
- Roberts MR, Shields PG, Ambrosone CB, Nie J, Marian C, Krishnan SS, Goerlitz DS, Modali R, Seddon M, Lehman T, Amend KL, Trevisan M, Edge SB, Freudenheim JL. Single-nucleotide polymorphisms in DNA repair genes and association with breast cancer risk in the web study. *Carcinogenesis*. 2011; 32:1223–1230. [PubMed: 21622940]
- Sobol RW, Horton JK, Kühn R, Gu H, Singhal RK, Prasad R, Rajewsky K, Wilson SH. Requirement of mammalian DNA polymerase-beta in base-excision repair. *Nature*. 1996; 379:183–186. [PubMed: 8538772]
- Sobol RW, Watson DE, Nakamura J, Yakes FM, Hou E, Horton JK, Ladapo J, Van Houten B, Swenberg JA, Tindall KR, Samson LD, Wilson SH. Mutations associated with base excision repair deficiency and methylation-induced genotoxic stress. *Proc Natl Acad Sci USA*. 2002; 99:6860–6865.
- Sobol RW. DNA polymerase beta null mouse embryonic fibroblasts harbor a homozygous null mutation in DNA polymerase iota. *DNA repair (Amst)*. 2007; 6:3–7. [PubMed: 16979388]
- Sterpone S, Mastellone V, Padua L, Novelli F, Patrono C, Cornetta T, Giammarino D, Donato V, Testa A, Cozzi R. Single-nucleotide polymorphisms in BER and HRR genes, XRCC1 haplotypes and breast cancer risk in Caucasian women. *J Cancer Res Clin Oncol*. 2010; 136:631–636. [PubMed: 20140625]
- Wilson SH. Mammalian base excision repair and DNA polymerase beta. *Mutat Res*. 1998; 407:203–215. [PubMed: 9653447]

- Wilson SH, Kunkel TA. Passing the baton in base excision repair. *Nat Struct Biol.* 2000; 7:176–178. [PubMed: 10700268]
- Wu M, Zhang Z, Che W. Suppression of a DNA base excision repair gene, hOGG1, increases bleomycin sensitivity of human lung cancer cell line. *Toxicol Appl Pharmacol.* 2008; 228:395–402. [PubMed: 18234257]
- Yamada NA, Farber RA. Induction of a low level of microsatellite instability by overexpression of DNA polymerase beta. *Cancer Res.* 2002; 62:6061–6064. [PubMed: 12414629]
- Yamtich J, Sweasy JB. DNA polymerase family X: function, structure, and cellular roles. *Biochim Biophys Acta.* 2010; 1804:1136–1150. [PubMed: 19631767]

Fig. 1. Western blot for determining pol β protein levels of pol β^{-/-}, pol β^{+/+} and pol β^{oe} cells
 Total cell lysate was prepared in sodium dodecyl sulfate (SDS) buffer. Same amount of total proteins isolated from pol β^{-/-}, pol β^{+/+} and pol β^{oe} cells were separated by 6% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane. After incubation with the primary polyclonal antibody against pol β, the PVDF membrane was incubated with a goat anti-rabbit secondary antibody conjugated with horseradish peroxidase. Pol β protein was visualized with enhanced chemiluminescence. Western blot of actin protein was used as a loading control.

Fig. 2. Growth kinetics of pol $\beta^{-/-}$, pol $\beta^{+/+}$ and pol β^{oe} cells

Cells were seeded in a 12-well plate at a density of 1×10^4 cells per well. After 24 h, cells were collected and counted on day 1 to day 8, respectively. Cell number was obtained from triplicates. Then growth rate of cells was calculated. No significant difference was observed among the three cell lines ($P > 0.05$).

Fig. 3A **Fig. 3B**

Fig. 3. DNA damage in pol β^{+/+}, pol β^{-/-} and pol β^{oe} cell treated with K₂Cr₂O₇
 Comet assay was used to detect cellular K₂Cr₂O₇-induced DNA strand breaks under various expression levels of pol β. (A) The comet rate (%) resulting from pol β^{-/-}, pol β^{+/+} and pol β^{oe} cells was calculated according to the equation provided in Section 2 and was plotted against various concentrations of K₂Cr₂O₇. 200 cells were scored per slide. (B) The OTM values were expressed as mean ± S.D. and obtained from 30 comet images for each slide. For both of comet rate and OTM, One-way analysis of variance (ANOVA) including least-significant difference (LSD) multiple comparison test was used to identify statistically

significant difference. “a” denotes a significant difference ($P<0.05$) between $K_2Cr_2O_7$ -treated cells and untreated cell control, “b” indicates a significant difference ($P<0.05$) between $pol\ \beta^{-/-}$ or $pol\ \beta^{oe}$ cells and $pol\ \beta^{+/+}$ at the same concentration of $K_2Cr_2O_7$.

Fig. 4. Effect of MMC on the micronucleus rate in pol $\beta^{+/+}$, pol $\beta^{-/-}$ and pol β^{oe} cells
 The cells were treated by 0, 0.05, 0.5 mg/ml MMC for 24 h. Micronucleus rate (%) was calculated as the ratio of micronucleated cells in total cells examined. The data were analyzed by Poisson distribution. “a” denotes a significant difference ($P < 0.05$) between MMC treated cells and untreated control. “b” indicates a significant difference ($P < 0.05$) between pol $\beta^{-/-}$ or pol β^{oe} cells and pol $\beta^{+/+}$ cells.

Fig. 5. MMS-induced mutations at *hprt* gene in pol β^{+/+}, pol β^{-/-} and pol β^{oe} cells

The cells were treated by increasing dosages of MMS for 24 h. The *hprt* gene mutation frequency (%) was calculated by the equation described in Section 2 and was plotted against the concentration of MMS. Poisson distribution was used to determine significant differences. “a” denotes a significant difference ($P < 0.05$) between treated groups and untreated control, whereas “b” indicates a significant difference ($P < 0.05$) between pol β^{-/-} or pol β^{oe} cells and pol β^{+/+} cells.

Fig. 6. Cell survival responses to MMS of $\text{pol}\beta^{+/+}$, $\text{pol}\beta^{-/-}$ and $\text{pol}\beta^{\text{oe}}$ cells

Cells survival responses to MMS at various dosages were examined. (A) Cell viability was measured using MTT assay that was performed according to the description of Section 2 and was plotted against the concentration of MMS. (B) Cells proliferative capacity was detected by colony forming assay. Cells were exposed to 0, 0.01, 0.05, 0.1, 0.25, 0.5 mM MMS. The rate of colony forming (%) was calculated according to the equation provided in Section 2. For both assays, data were illustrated as mean $\pm$ standard error derived from three independent assays. One-way analysis of variance (ANOVA) including least-significant difference (LSD) multiple comparison test was employed to determine statistical differences, $P < 0.05$ was considered to be statistically significant.

Table 1Cell population doubling time of pol $\beta^{+/+}$, pol $\beta^{-/-}$ and pol β^{oe} Cells

Cell line	cell number at the start ($\times 10^4$)	Cell number after 48 h($\times 10^4$)	Cell population doubling time (h)
pol $\beta^{+/+}$	1.0	7.03 $\pm$ 0.55	26.61 $\pm$ 1.68
pol $\beta^{-/-}$	1.0	6.96 $\pm$ 0.48	26.82 $\pm$ 1.55
pol β^{oe}	1.0	7.28 $\pm$ 0.65	26.12 $\pm$ 1.56

Proliferation capacity was determined by population doubling time assay. Population doubling time was calculated according to the formula which described in Section 2. The data were obtained from three independent experiments and presented as mean $\pm$ standard error. No significant difference was observed among the three cell lines ($P>0.05$).

Table 2Colony forming ability of pol β $^{+/+}$, pol β $^{-/-}$ and pol β oe Cells

Cell line	Number of clone formation	Rate of clone formation (%)
pol β $^{+/+}$	43.33 $\pm$ 7.41	0.22 $\pm$ 0.04
pol β $^{-/-}$	44.33 $\pm$ 9.88	0.22 $\pm$ 0.05
pol β oe	41.00 $\pm$ 6.98	0.21 $\pm$ 0.03

Pol β $^{+/+}$, pol β $^{-/-}$ and pol β oe cells were plated in 24-well plates and cultured for 14 d. Rate of colony formation (%) = (average number of colony/total number of the cells plated) $\times$ 100 %. The data were obtained from three independent assays and were expressed as mean $\pm$ S.D.. One-way analysis of variance (ANOVA) including least-significant difference (LSD) multiple comparison test was used to determine statistically significant differences. No significant difference was observed among the three cell lines ($P>0.05$).