UV-induced mutations in epidermal cells of mice defective in DNA polymerase η

and/or ι

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Abstract

Xeroderma pigmentosum variant (XP-V) is a human rare inherited recessive disease, predisposed to sunlight-induced skin cancer, which is caused by deficiency in DNA polymerase η (Pol η). Pol η catalyzes accurate translesion synthesis (TLS) past pyrimidine dimers, the most prominent UV-induced lesions. DNA polymerase ι (Pol ι) is a paralog of Pol η that has been suggested to participate in TLS past UV-induced lesions, but its function *in vivo* remains uncertain. We have previously reported that Pol η -deficient and Pol η /Pol ι double-deficient mice showed increased susceptibility to UV-induced carcinogenesis. Here, we investigated UV-induced mutation frequencies and spectra in the epidermal cells of Pol η - and/or Pol ι -deficient mice. While Pol η -deficient mice showed significantly higher UV-induced mutation frequencies than wild-type mice, Pol ι deficiency did not influence the frequencies in the presence of Pol η . Interestingly, the frequencies in Pol η /Pol ι double-deficient mice were statistically lower than those in Pol η -deficient mice, although they were still higher than those of wild-type mice. Sequence analysis revealed that most of the UV-induced mutations in Pol η -deficient and Pol η /Pol ι double-deficient mice were base substitutions at dipyrimidine sites. An increase in UV-induced mutations at both G:C and A:T pairs associated with Pol η deficiency suggests that Pol η contributes to accurate TLS past

both thymine- and cytosine-containing dimers *in vivo*. A significant decrease in G:C to A:T transition in Pol η /Pol ι double-deficient mice when compared with Pol η -deficient mice suggests that Pol ι is involved in error-prone TLS past cytosine-containing dimers when Pol η is inactivated.

Key Words: DNA polymerase η / DNA polymerase ι / translesion synthesis/ UV-induced mutagenesis/ xeroderma pigmentosum variant (XP-V)

1. Introduction

Xeroderma pigmentosum (XP) is a genetic disorder characterized by ultraviolet light (UV) sensitivity and increased incidence of skin cancers. XP has been classified into eight genetic complementation groups, XP-A~G and XP-V (1). Cells derived from XP-A through XP-G patients are deficient in nucleotide excision repair (NER), which repairs a variety of DNA lesions including UV-induced cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts. The variant form (XP-V) is characterized by proficiency in NER but deficiency in translesion synthesis (TLS). TLS is a mechanism that prevents replication blockage at a DNA lesion by using specialized DNA polymerases that incorporate nucleotides opposite the lesion and continue DNA synthesis past the site of damage (1). We have identified human DNA polymerase η (Pol η) as the product of the gene associated with XP-V, *POLH*. Pol η catalyzes accurate translesion synthesis (TLS) past *cis-syn* thymine-thymine dimers (2-5), an important mechanism to prevent UV-induced skin cancers in human cells.

Mammalian cells are now known to have fifteen DNA template-dependent DNA polymerases, and these are classified into five families, A, B, X, Y, and AEP (archaeo-eukaryotic primase superfamily) according to similarities in their primary structures (6-9). The Y-family, which was shown to carry out TLS past some lesions,

consists of Pol η , Pol ι , Pol κ , and REV1 (10). Pol ι , encoded by the *POL1* gene, incorporates one or two nucleotides opposite (6-4) photoproducts *in vitro* but cannot bypass these lesions by itself (11,12), although limited Pol ι dependent bypass of CPD lesions has been observed depending upon the local sequence context and metal ion used as a cofactor (11, 13-15). Whereas Pol ι is a paralog of Pol η , no human disease related to Pol ι -deficiency has been identified so far, and its physiological relevance has not been clarified. Pol κ is the eukaryotic homologue of the *E. coli* DinB protein (DNA polymerase IV) and has been shown to be involved in accurate TLS past *N*²-adducts of dG and also in both spontaneous and induced mutagenesis (16,17). Although Pol κ was suggested to be involved in NER of UV-induced lesions (18,19), it seems unlikely that Pol κ plays an important role in TLS past UV lesions since the enzyme showed no activity to bypass past CPD and (6-4) photoproduct *in vitro* (20). REV1 has a dCMP transferase activity, but the protein appears to exert its role in UV-induced responses, as a scaffold protein interacting with Pol ζ , Pol η , Pol ι , and Pol κ (10). Pol ζ is a B-family polymerase containing REV3 and REV7 subunits and is believed to function as an “extender” enzyme after a TLS DNA polymerase (for example, Pol ι) inserting a nucleotide opposite a given DNA lesion (10).

To investigate the physiological roles of Pol η and Pol ι , we have previously

generated mice mutated in *Polh* and/or *Poli* genes (21). Pol η -deficient mice were generated by inserting a G418 resistance gene cassette into exon 8 of the *Polh* gene, resulting in production of a truncated, nonfunctional Pol η protein. Using the 129 mouse derived embryonic stem (ES) cell line, which carries a spontaneous Pol ι nonsense mutation (22), we obtained Pol η /Pol ι double-deficient mice as well as Pol ι -deficient mice. We found that the incidence of skin tumors was greatly increased in the Pol η - and Pol η /Pol ι double-deficient mice after UV irradiation, that Pol η /Pol ι double-deficient mice started to develop skin tumors earlier than Pol η -deficient mice, and that the average number of skin tumors was higher in the double-deficient mice than in the Pol η -deficient mice. In addition, we found that epithelial and mesenchymal tumors were formed in Pol η - and Pol ι -deficient mice, respectively (21). These results suggest that, in addition to Pol η , Pol ι also participates in suppressing skin carcinogenesis. Other groups had also shown the importance of Pol η and Pol ι to suppress UV-induced skin tumors. Pol η -deficient mice, generated by disruption of exon 4 of the *Polh* gene, are highly susceptible to UV-induced skin tumors (23). Pol η /Pol ι double-deficient mice, in turn, develop UV-induced skin tumors earlier than Pol η -deficient mice (24).

To investigate the functions of Pol η and Pol ι in suppressing UV-induced carcinogenesis, we examined the mutation frequencies and spectra in UV-irradiated and

unirradiated epidermis in Polη-deficient, Polι-deficient, and Polη/Polι double-deficient mice, using the *rpsL* transgene as a mutation reporter sequence. Our results show that Polη plays crucial roles in suppressing any types of UV-induced base substitution at dipyrimidine sites *in vivo* and that Polι participates in error-prone TLS past UV lesions in the absence of Polη.

2. Materials and Methods

2.1. Generation of transgenic mice

The *rpsL* transgenic mouse line (ssw2-14p) used in this study was derived from C57BL/6J mice, but carries approximately 100 hemizygous copies of the pSSW plasmid; it has been described previously (25). The pSSW plasmid carries a reporter gene, *rpsL* from *E. coli*, with a dominant streptomycin-sensitive (Sm^s) phenotype in bacterial cells, and a kanamycin-resistant (Km^r) gene. *Polh*^{-/-} and/or *Poli*^{-/-} mice were established as previously described (21). *Polh*^{+/-}, *Poli*^{+/-} mice were mated with the *rpsL* transgenic mice to generate *rpsL*^{Tg/+} *Polh*^{+/-} *Poli*^{+/-} mice. By mating the *rpsL*^{Tg/+} *Polh*^{+/-} *Poli*^{+/-} mice with *Polh*^{+/-} *Poli*^{+/-} mice, *rpsL*^{Tg/+} *Polh*^{-/-} *Poli*^{+/+}, *rpsL*^{Tg/+} *Polh*^{+/-} *Poli*^{-/-}, and *rpsL*^{Tg/+} *Polh*^{-/-} *Poli*^{-/-} mice were generated. The handling and sacrifice of all animals were carried out in accordance with nationally prescribed guidelines, and ethical approval for the studies was granted by the Committee for Animal Experiments, Graduate School of Frontier Biosciences of Osaka University.

2.2. Preparation of UVB irradiated and unirradiated epidermal genomes

At 8 weeks of age, after shaving of dorsal hair, mice from each respective genotype were irradiated with UVB (FL20SE-E; Toshiba, Tokyo, Japan) at 400 J/m².

One week after irradiation, these mice were sacrificed, and the irradiated dorsal and unirradiated ventral skins were collected. These skin samples were treated with 20 mM EDTA in PBS at 37°C, and the epidermis was peeled from the dermis using a spatula. The isolated epidermal samples were frozen in liquid nitrogen and stored at -80°C. To prepare genomic DNA, the epidermal samples were first incubated in lysis buffer (10 mM Tris-HCl (pH 8.0), 100 mM EDTA, 200 mM NaCl, 0.5% SDS, 0.1 mg/ml RNaseA) at 37°C for one hour, followed by 1 mg/ml Proteinase K for 3 h. After phenol/chloroform extraction, genomic DNA was obtained by ethanol precipitation.

2.3. *rpsL* mutagenesis assay

Mutation analyses were performed as described (25) with minor modifications. Briefly, 10 µg of isolated genomic DNA was digested with 35 U of *Ban*II (TaKaRa, Shiga, Japan) at 37°C for 3 h, which incises once the pSSW plasmid, in order to excise the integrated shuttle vector at unit size. The *Ban*II digested DNA was then treated with 350 U of T4 DNA ligase (TaKaRa, Shiga, Japan) for 2 h at 16°C for self-circularization. One microgram of the resulting DNA was introduced into *E.coli* DH10B cells by electroporation (1.8 kV/mm). Then, a portion of the cells were plated onto LB plates containing kanamycin (50 µg/ml), and the remainders were plated onto plates

containing both kanamycin and streptomycin (200 µg/ml). These plates were incubated at 28°C for 48 h. Total numbers of kanamycin-resistant colonies were calculated from the number of colonies formed and the amount of DNA used. Mutation frequency was calculated as the ratio of total colony numbers on the plate containing kanamycin and streptomycin to those on the plate containing kanamycin only. For mutation spectrum analysis, DNA fragments containing the *rpsL* gene were amplified by PCR from the kanamycin- and streptomycin-resistant colonies. The primers F (5'-CACCTGATTGCCCGACATTA-3') and R (5'-CAGGGTCGGAACAGGAGAGC-3') were used for the PCR. The amplified PCR products were directly sequenced with the forward primer (5'-GACGAATTCCCGGTTTGACTGGTC-3') and the reverse primer (5'-GGATTGTCCAAACTCTACGAG-3'). Mutations found in the *rpsL* coding region and surrounding sequences containing the promoter and ribosomal recognition regions (from position -120 to position 375) were exhibited in the mutation spectrum.

2.4. Statistical analyses

Statistical analyses for mutation frequency included the Student's *t*-test and Fisher's exact probability test. The CLUSTERM program

(<http://www.itb.cnr.it/webmutation/>) was employed to predict mutation hotspots (26).

3. Results

3.1. Polh but not Poli suppresses UV-induced mutations in epidermal cells

Polh^{+/+} *Poli*^{+/+}, *Polh*^{-/-} *Poli*^{+/+}, *Polh*^{+/+} *Poli*^{-/-} and *Polh*^{-/-} *Poli*^{-/-} mice carrying the *rpsL* transgene hemizygotously were obtained by crossing of *Polh*^{+/+} *Poli*^{+/+} mice with *rpsL* transgenic mice. Those mice had its back shaved and irradiated with 400 J/m² UVB once. One week after irradiation, the mice were sacrificed, and irradiated dorsal skin samples and unirradiated ventral skin samples were collected and separated into epidermis and dermis. Genomic DNAs were extracted from the epidermis and subjected to mutation analysis as described in Materials and Methods. Six mice from each genotype were examined. The number of screened and mutated colonies, together with mutation frequencies of each individual mouse, is shown in Table 1. The mean mutation frequencies for six mice are depicted in Figure 1. There was no significant difference in the mutation frequency of the unirradiated epidermal cells among the four genotype. The increases in mutation frequency following UV-irradiation were statistically significant in all genotypes. UV-induced mutation frequencies for *Polh*^{-/-} *Poli*^{+/+} (4.07×10^{-4}) and *Polh*^{-/-} *Poli*^{-/-} mice (3.36×10^{-4}) were approximately 13- and 11-fold higher, respectively, than that of *Polh*^{+/+} *Poli*^{+/+} mice (3.07×10^{-5}), indicating that Polh exerts a crucial role for suppressing mutations by UV-irradiation. On the other hand, the

UV-induced mutation frequency in *Polh*^{+/+} *Poli*^{-/-} mice (3.22×10^{-5}) were not significantly different from that in wild-type mice ($P=0.82$), suggesting that PolI does not influence UV-induced mutagenesis in the epidermis in the presence of Polη. Interestingly, however, the UV-induced mutation frequency in the genome from *Polh*^{-/-} *Poli*^{-/-} mice was significantly lower than that of *Polh*^{-/-} *Poli*^{+/+} mice ($P=0.020$), suggesting that PolI contributes to increasing mutations in the absence of Polη.

3.2. Sequence analysis of mutations

To identify the types and locations of mutations, we sequenced the *rpsL* coding region and its upstream region containing the promoter and the ribosomal recognition sequences (from position -120 to position 375) in the mutants. The identified mutations are shown in Figure 2A-D. As summarized in Table 2, base substitutions were among the most common mutations induced by UV-irradiation in all mouse genotypes. Statistical analysis revealed that *Polh*^{-/-} *Poli*^{+/+} and *Polh*^{-/-} *Poli*^{-/-} mice had significantly higher base substitutions, namely, 4.03×10^{-4} and 3.22×10^{-4} , respectively, than *Polh*^{+/+} *Poli*^{+/+} (2.51×10^{-5}) and *Polh*^{+/+} *Poli*^{-/-} (2.78×10^{-5}) mice ($P<0.0001$) (Table 3). On the other hand, there were no statistically significant differences in the frequencies of UV-induced frameshift mutations between the four genotypes. An important point to

note is that most of the UV-induced mutations were found at dipyrimidine sites (Figure 2A-D, Table 3). The frequencies of base substitutions at dipyrimidine sites in *Polh*^{-/-} *Poli*^{+/+} and *Polh*^{-/-} *Poli*^{-/-} mice were significantly higher than those in *Polh*^{+/+} *Poli*^{+/+} and *Polh*^{+/+} *Poli*^{-/-} mice ($P < 0.0001$). These results indicate that Polη is predominantly responsible for suppressing mutations upon UV-irradiation, by correctly bypassing UV-induced pyrimidine dimers.

Interestingly, in *Polh*^{-/-} *Poli*^{-/-} mice, the frequency of UV-induced total base substitutions was significantly lower than in *Polh*^{-/-} *Poli*^{+/+} mice ($P = 0.029$, see Table 3). Similarly, the frequency of base substitutions at dipyrimidine sites in *Polh*^{-/-} *Poli*^{-/-} mice was also significantly lower than in *Polh*^{-/-} *Poli*^{+/+} mice ($P = 0.025$, see Table 3). In addition to this, several mutational hotspots were observed at dipyrimidine sites in the UV-irradiated epidermal genomes of *Polh*^{-/-} *Poli*^{+/+} mice, including -104C, -103T, -104/-103CT, -101G, -79A, -11G, -10G, -11/-10GG, 3G, 16C, 28A, 40C, 73G, 83C, 82/83CC, 88A, 94G, 106C, 127-130A, 170T, 184G, 194C, 208G, 226G, 233C, 262A, 263A, and 272C (Figure 2B). Among these, -104C, 40C, 82/83CC, 94G, 106C, 170T, 184G, 194C, 226G, and 262A were no longer mutational hotspots in the UV-irradiated *Polh*^{-/-} *Poli*^{-/-} mice (Figure 2D). These results are consistent with our interpretation that Polη participates in error-prone TLS past UV-induced lesions in an error-prone manner,

especially when bypassing C within pyrimidine dimers under the Pol η -deficient background.

The frequencies for each type of base substitution are depicted in Figure 3. The G:C to A:T transition was the most prominent UV-induced mutation in all genotypes. In the genomes of *Polh*^{-/-} *Poli*^{+/+} and *Polh*^{-/-} *Poli*^{-/-} mice, the UV-induced mutational frequencies of all types of base substitutions were higher than in *Polh*^{+/+} *Poli*^{+/+} and *Polh*^{+/+} *Poli*^{-/-} mice, although G:C to C:G and A:T to C:G transversions in *Polh*^{-/-} *Poli*^{+/+} mice were not statistically significant compared with those in *Polh*^{+/+} *Poli*^{+/+} mice. These observations strongly suggest that Pol η suppresses UV-induced mutations at both C and T residues in the epidermis *in vivo*. Interestingly, in *Polh*^{-/-} *Poli*^{-/-} mice, the frequency of G:C to A:T transitions was statistically lower than that in *Polh*^{-/-} *Poli*^{+/+} mice ($P=0.0013$), suggesting that Pol ι contributes to UV-induced G:C to A:T transitions in the absence of Pol η , probably by inserting A opposite cytosine within pyrimidine dimers.

4. Discussion

4.1. Roles of Pol η in suppressing UV-induced mutagenesis

In the present study, we analyzed the UVB-induced mutations occurring in the *rpsL* transgene in epidermal cells of wild type, Pol η -deficient, Pol ι -deficient, and Pol η /Pol ι double-deficient mice. Although several studies have investigated the effects of Pol η and/or Pol ι deficiencies on UV-induced mutagenesis (24,27-33), these studies were done using cultured cells *in vitro*. To the best of our knowledge, this is the first report addressing the roles of Pol η and Pol ι in UV-induced mutagenesis *in vivo*. When irradiated with UVB, Pol η -deficient mice showed significantly higher mutation frequencies than control mice. Most of the UV-induced mutations in the genomes of Pol η -deficient mice were base substitutions at dipyrimidine sites (both C and T residues). These findings clearly indicate that Pol η is crucial for accurate TLS past UV-induced pyrimidine dimers in the epidermal cells and in the absence of Pol η , other TLS polymerases carries out mutagenic bypass past the lesions. These results account for previous findings that Pol η -deficient mice suffer from high incidences of UV-induced skin tumors (21,23,24).

The most prominent UV-induced mutations were G:C to A:T transitions in all genotypes. Hydrolytic deamination of the cytosine in CPDs, which produces

uracil-containing CPDs, is thought to play a key role in the UV-induced mutations at G:C base pairs (34-36). Polη preferentially incorporates dAMP opposite uracil of T-U CPDs (37), resulting in a G:C to A:T transition, if the CPD is repaired after TLS. This may account for the frequent G:C to A:T transitions observed in Polη-proficient mice. However, the large increase in the frequency of UV-induced G:C to A:T transitions in Polη-deficient mice indicates that Polη rather functions to suppress such transitions. Similar results showing that the most prominent UV-induced mutations caused by Polη deficiency are G:C to A:T transitions were observed in two previous experiments. In the first of these, the *supF* gene was introduced into XP-V cells and human kidney 293T cells in which Polη expression was suppressed by siRNA. In the second, the *LacZ'* gene was introduced into Burkitt's lymphoma BL2 cells in which the *POLH* gene was inactivated by gene targeting (28,29,33). Abundant G:C to A:T transitions were also observed following UV-induced mutation analyses of the *HPRT* locus in XP-V cells and primary fibroblasts obtained from Polη-deficient mice, although equivalent levels of G:C to T:A transversions were also observed in these studies (24,27,31). These observations suggest that Polη is critical for suppressing at C (some of them might be deaminated) within UV-induced pyrimidine dimers. Although we do not know which kind of nucleotides Polη incorporates opposite the cytosine in CPDs, its preference for

incorporating dAMP opposite uracil suggests that Pol η needs to cooperate with other TLS polymerases or unknown factors to promote accurate bypass of the lesion. Pol ι is a possible candidate for this role. It is capable of incorporating dGMP opposite uracil in CPDs with half the efficiency of dAMP (14). Pol η also interacts with Pol ι and UV-induced nuclear foci formation by Pol ι is largely dependent on Pol η (38). Pol η interacts with REV1 (39). Supporting this, we have shown that the accumulation of endogenous REV1 in UV-damaged sites in nuclei depends on Pol η , and that Pol η -REV1 interaction is required to suppress spontaneous mutations in human cells (40). These observations suggest that coordinated TLS by multiple DNA polymerases may take place in the presence of Pol η , contributing to greater suppression of base substitutions at dipyrimidine sites than by Pol η alone.

UVB exposure in mice may produce reactive oxygen species that generate oxidative base damage, such as 8-oxoG (41). Mammalian Pol η is efficient at incorporating dC opposite 8-oxoG to suppress G:C to T:A transversion *in vitro* (42,43). Furthermore, Lee *et al.* reported that G:C to T:A transversions were predominant in XP-V and Pol η -knockdown human cells (44). Hence, it is reasonable to suggest that some, if not all, G:C to T:A transversions in UV-exposed Pol η -deficient mice are caused by UV-induced oxidative base damage. On the other hand, there were no

significant differences in the spontaneous mutation frequencies in several tissues between one-year-old Pol η -deficient and -proficient mice (32). It remains uncertain whether or not oxidative base damage is involved in the enhancement of mutagenesis in UV-exposed Pol η -deficient mice.

4.2. Roles of Pol ι in UV-induced mutagenesis

UV-induced mutation frequencies in epidermal genomes from Pol ι -deficient mice were not significantly different from those of wild-type mice, but the mutation frequency in Pol η /Pol ι double-deficient mice was statistically lower than in Pol η -deficient mice. These results imply that Pol ι would not cause mutations in the UV-irradiated epidermal genome when Pol η is present, but would cause UV-induced mutations in its absence. This is especially true for Pol ι and G:C to A:T transitions, in which the difference is statistically significant in Pol η -deficient mice. This is consistent with a previous report that Pol ι preferentially incorporates dAMP opposite T-U CPDs *in vitro* (14), which would give rise to G:C to A:T transitions. Pol ι also preferentially incorporates dTMP opposite TT-CPD *in vitro* (11), which would generate A:T to T:A transversion. In the experiments described in this report, however, we have not yet observed any statistical difference in the frequency of transversion between

Pol η -deficient and Pol η /Pol ι double-deficient mice.

Gueranger *et al.* (33) reported that Pol ι deficiency did not affect UV-induced mutations in a shuttle vector in the presence of Pol η but did reduce some mutational hotspots observed in Pol η -targeted BL2 cells. Wang *et al.* (31) also reported that suppression of Pol ι expression decreased UV-induced mutation frequencies in Pol η -deficient XP-V cells. These observations are consistent with our findings that Pol ι acts as a mutator in the absence of Pol η . However, Dumstorf *et al.* (24), investigating primary fibroblasts isolated from Pol η - and/or Pol ι -deficient mice, have reported that Pol ι deficiency causes a decrease in UV-induced base substitutions not only in the absence of Pol η but also in its presence. We cannot explain the discrepancies between these observations, but it may be possible that the difference depends on the type of cells employed, i.e. that there is an inherent difference between dermal fibroblasts and epithelial cells. Dumstorf *et al.* also reported biases for UV-induced base substitutions between nontranscribed and transcribed strands of the *Hprt* locus in wild-type and Pol η -deficient cells which were lower in Pol η /Pol ι double-deficient cells (24). We did not, however, observe this strand bias. This is probably due to the different transcription status of the *Hprt* gene and the *rpsL* reporter gene, i.e., the *Hprt* gene is an actively transcribed housekeeping gene but the *rpsL* reporter gene has no eukaryotic promotor.

So, UV-induced photoproducts could be selectively removed from the transcribed strand of the *Hprt* gene (45) but not of the *rpsL* gene.

The lower UV-induced mutation frequency in Pol η /Pol ι double-deficient mice than in Pol η -deficient mice was unexpected, as it is inconsistent with previous observations that Pol η /Pol ι double-deficient mice start to develop skin tumors earlier, and go on to develop higher numbers of skin tumors than Pol η -deficient mice (21,24). It should be noted that UV-irradiation procedures used for tumor formation are different from those used for mutagenesis analyses, i.e. mice were irradiated every day, with 2 kJ/m² of UVB, for 20 weeks, in tumor formation assays, but were only irradiated once, with 0.4 kJ/m² of UVB, in mutagenesis assays. In addition, we have reported that Pol η and Pol ι deficiencies induce epithelial and mesenchymal tumors, respectively, suggesting that the contributions of Pol η and Pol ι to mutation avoidance in the epidermis are different from those in the dermis. Petta *et al.* demonstrated that Pol ι is involved in oxidative base damage tolerance independently of Pol η (46). Our present study, however, did not show a greater number of mutations in UV-irradiated epidermal cells from Pol ι -deficient mice. These are issues that we hope to solve in the near future.

4.3. UV-induced mutagenesis in the absence of Pol η

Small numbers of UV-induced G:C to C:G and A:T to C:G transversions may suggest that cells do not have DNA polymerases that efficiently incorporate dCMP opposite cytosine, uracil, or thymine dimers. However, a clear increase in UV-induced transitions in the Pol η -deficient mice indicates that an error-prone TLS mechanism exists in the epidermis *in vivo*. We showed that Pol ι partially causes mutations in the UV-irradiated genome in the absence of Pol η ; however, the majority of these mutations could be produced by other TLS polymerases. Pol ζ and REV1 are likely candidates for error-prone enzymes. Previous studies using both mouse and human cells have shown that reduced expression or disruption of *REV3* or *REV7* genes, which encode Pol ζ , results in a decrease in UV-induced mutation frequencies (33,39,47,48). In human cells, reduced *REV1* gene expression has been reported to decrease the frequency of UV-induced mutation (39,47,49). Moreover, a targeted deletion of the BRCT domain in mouse cells decreased UV-induced mutation frequency (50). In addition, Polk-deficient and *Polk*-knockdown cells exhibit UV sensitivity (51-53), indicating that this polymerase is also involved in UV-damage tolerance mechanisms. Polk participates in nucleotide excision repair (NER) (18,19). However, it also appears to play a role in TLS past UV-induced lesions, as shown in *Xenopus* egg extracts and NER-deficient XP-A cells (53-55). Finally, it should be pointed out that replicative DNA polymerase(s) may

add one or more nucleotides (mostly one dAMP) to the primer terminus before dissociating from it, Polζ extending further (56). If A is inserted opposite to C in pyrimidine dimers in such a way by a replicative DNA polymerase and extended by Polζ, it should result in a G:C-to-A:T transition. Although disruption of the *Rev3* gene results in embryonic lethality in mice (47), *Rev1*, *Rev7* and *Polκ* knockout and *Rev3* conditional knockout animals have been successfully generated (57-61). To further understand the mechanisms that protect against and produce mutations and tumors after UV-irradiation, it will be necessary to perform *in vivo* UV-induced mutagenesis experiments with mice defective in multiple TLS polymerases.

4.4. Conclusions

In this study, we show that Polη is important for suppressing any types of UV-induced base substitution at dipyrimidine sites, and that Polι acts as a mutator at UV lesions in the absence of Polη in epidermal cells.

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Figure Legends

Figure 1

Mutation frequencies of the *rpsL* transgene in UVB-irradiated and unirradiated epidermis from *Polh*^{+/+} *Poli*^{+/+}, *Polh*^{-/-} *Poli*^{+/+}, *Polh*^{+/+} *Poli*^{-/-}, and *Polh*^{-/-} *Poli*^{-/-} mice. Mean mutation frequencies of six mice are plotted with standard deviations. Open and filled bars indicate data for unirradiated and irradiated epidermis, respectively. All differences in mean mutation frequencies between UV-irradiated and unirradiated mice are statistically significant ($P=0.0006$ for *Polh*^{+/+} *Poli*^{+/+} mice, and $P<0.0001$ for *Polh*^{-/-} *Poli*^{+/+}, *Polh*^{+/+} *Poli*^{-/-} and *Polh*^{-/-} *Poli*^{-/-} mice). Differences between irradiated *Polh*^{-/-} *Poli*^{+/+} mice and irradiated *Polh*^{+/+} *Poli*^{+/+} mice, and between irradiated *Polh*^{-/-} *Poli*^{-/-} mice and irradiated *Polh*^{+/+} *Poli*^{+/+} mice are both statistically significant ($P<0.0001$). The difference between irradiated *Polh*^{-/-} *Poli*^{-/-} mice and irradiated *Polh*^{-/-} *Poli*^{+/+} mice is statistically significant ($P=0.021$).

Figure 2

Site-distribution of UV-induced mutations observed in (A) *Polh*^{+/+} *Poli*^{+/+}, (B) *Polh*^{-/-} *Poli*^{+/+}, (C) *Polh*^{+/+} *Poli*^{-/-} and (D) *Polh*^{-/-} *Poli*^{-/-} mice. Base substitutions are indicated above the wild-type *rpsL* sequence by the following symbols. A, T, C and G are

indicated by circle, square, triangle and diamond, respectively. Deletions and additions are indicated by an asterisk (*) and plus (+), respectively. Tandem mutations are indicated below the sequence in parentheses. The nucleotide positions, starting from the first position of the initiation codon, for translation are shown at the right side of the sequence. The start codon and the termination codon are surrounded by boxes. The promoter sequence and the ribosomal recognition sequence (Shine-Dalgarno sequence; SD) are underlined.

Figure 3

UV-induced base substitutions observed in *Polh*^{+/+} *Poli*^{+/+}, *Polh*^{-/-} *Poli*^{+/+}, *Polh*^{+/+} *Poli*^{-/-} and *Polh*^{-/-} *Poli*^{-/-} mice. White, dotted, striped, and black bars indicate *Polh*^{+/+} *Poli*^{+/+}, *Polh*^{-/-} *Poli*^{+/+}, *Polh*^{+/+} *Poli*^{-/-} and *Polh*^{-/-} *Poli*^{-/-} mice, respectively. The mean mutation frequencies for the six mice are plotted along with their standard deviations. Note that these mean values differ from the total mutation frequencies listed in Table 3. Values for G:C to A:T, A:T to G:C, G:C to T:A, and A:T to T:A mutations of *Polh*^{-/-} *Poli*^{+/+} mice are significantly different from those of *Polh*^{+/+} *Poli*^{+/+} mice ($P<0.0001$, $P=0.0006$, $P<0.0001$, and $P<0.0001$, respectively). Values for G:C to A:T, A:T to G:C, G:C to T:A, A:T to T:A, and A:T to C:G mutations of *Polh*^{-/-} *Poli*^{-/-} mice are significantly different

from those of *Polh*^{+/+} *Poli*^{+/+} mice ($P<0.0001$, $P=0.0068$, 0.0005 , $P<0.0001$, and $P=0.042$, respectively). The value for the G:C to A:T transition of *Polh*^{-/-} *Poli*^{-/-} mice is significantly different from that of *Polh*^{-/-} *Poli*^{+/+} mice ($P=0.0013$).

Table 1

Mutation frequencies in UVB irradiated and unirradiated epidermal genomes from *Polh*^{+/+} *Poli*^{+/+}, *Polh*^{-/-} *Poli*^{+/+}, *Polh*^{+/+} *Poli*^{-/-}, and *Polh*^{-/-} *Poli*^{-/-} mice.

Genotype	UV irradiation	A ^a (x10 ⁵)	B ^b	Mutation frequency (x10 ⁻⁵)
<i>Polh</i> ^{+/+} <i>Poli</i> ^{+/+}	-	3.44	5	1.45
		4.81	1	0.21
		5.25	3	0.57
		9.22	3	0.33
		7.73	1	0.13
		6.56	2	0.30
	+	5.46	13	2.38
		3.99	15	3.76
		3.80	5	1.32
		7.81	24	3.07
		7.25	23	3.17
		3.37	16	4.75
<i>Polh</i> ^{-/-} <i>Poli</i> ^{+/+}	-	3.94	9	2.28
		4.67	4	0.86
		6.45	3	0.47
		2.76	4	1.45
		3.10	6	1.94
		5.83	2	0.34
	+	3.55	158	44.5
		2.73	102	37.4
		2.96	124	41.9
		3.02	140	46.4
		3.16	127	40.2
		2.55	87	34.1
<i>Polh</i> ^{+/+} <i>Poli</i> ^{-/-}	-	6.75	6	0.89
		3.43	1	0.29
		3.06	1	0.33
		3.15	1	0.32
		4.62	1	0.22
		3.36	4	1.19
	+	3.23	10	3.10
		4.95	20	4.04
		3.11	8	2.57
		3.36	7	2.08
		3.62	11	3.04
		3.57	16	4.48
<i>Polh</i> ^{-/-} <i>Poli</i> ^{-/-}	-	3.65	2	0.55
		5.36	4	0.75
		3.51	2	0.57
		4.59	4	0.87
		3.91	10	2.56
		4.24	10	2.36
	+	2.16	90	41.7
		3.17	109	34.4
		2.85	92	32.3
		3.42	101	29.5
		2.20	75	34.1
		2.69	79	29.4

^aA: Total number of Km^r colonies, as calculated from the number of Km^r colonies counted and the amount of DNA sample used for A and B.

^bB: Total number of Km^r and Sm^r colonies counted.

Table 2

Class-distribution of *rpsL* mutations.

	<i>Polh</i> ^{+/+} <i>Poli</i> ^{+/+}		<i>Polh</i> ^{-/-} <i>Poli</i> ^{+/+}		<i>Polh</i> ^{+/+} <i>Poli</i> ^{-/-}		<i>Polh</i> ^{-/-} <i>Poli</i> ^{-/-}	
UV	-	+	-	+	-	+	-	+
Base substitutions	0.16 (6) ^a	2.62 (83) ^c	0.37 (10) ^c	43.2 (777) ^c	0.12 (3)	3.11 (68)	0.24 (6)	33.2 (547)
Transitions								
G:C→A:T	0.08 (3)	1.58 (50) ^c	0.22 (6)	23.5 (422) ^c	0.12 (3)	2.11 (46) ^c	0.16 (4)	16.4 (271) ^c
A:T→G:C	0.08 (3)	0.28 (9)	0.04 (1) ^c	3.95 (71) ^c	<0.04 (0)	0.32 (7) ^c	<0.04 (0)	4.00 (66) ^c
Transversions								
G:C→T:A	<0.03 (0)	<0.03 (0)	0.04 (1)	5.45 (98) ^c	<0.04 (0)	0.14 (3)	0.04 (1)	3.94 (65) ^c
G:C→C:G	<0.03 (0)	0.03 (1)	0.04 (1)	0.50 (9) ^c	<0.04 (0)	<0.05 (0)	<0.04 (0)	0.43 (7)
A:T→T:A	<0.03 (0)	0.41 (13)	0.04 (1)	5.95 (107) ^c	<0.04 (0)	0.32 (7)	0.04 (1)	4.73 (78) ^c
A:T→C:G	<0.03 (0)	0.13 (4)	<0.04 (0)	0.56 (10) ^c	<0.04 (0)	0.09 (2)	<0.04 (0)	0.73 (12) ^c
Tandems	<0.03 (0)	0.19 (6)	<0.04 (0)	3.34 (60) ^c	<0.04 (0)	0.14 (3)	<0.04 (0)	2.91 (48) ^c
Others	0.24 (9)	0.60 (19) ^c	0.75 (20) ^c	0.61 (11) ^c	0.45 (11)	0.41 (9)	1.03 (26)	1.33 (22)
1bp deletions	0.14 (5)	0.32 (10) ^c	0.49 (13) ^c	0.11 (2)	0.25 (6)	0.18 (4)	0.44 (11)	0.30 (5)
1bp insertions	<0.03 (0)	0.10 (3) ^c	<0.04 (0)	0.28 (5) ^c	0.04 (1)	0.05 (1)	<0.04 (0)	<0.06 (0)
large deletions ^b	0.08 (3)	0.19 (6)	0.26 (7)	0.22 (4) ^c	0.16 (4)	0.14 (3)	0.55 (14)	0.97 (16)
large insertions ^b	0.03 (1)	<0.03 (0)	<0.04 (0)	<0.11 (0)	<0.04 (0)	0.05 (1)	0.04 (1)	0.06 (1)
Total	0.41 (15)	3.22 (102)	1.22 (30)	43.9 (788)	0.58 (14)	3.53 (77)	1.27 (32)	34.5 (569)

Mutation frequencies ($\times 10^{-5}$) were determined by total number of mutations identified from six mice for each of genotypes.

^a Numbers in parentheses are the number of independent mutations.

^b Large deletion and large insertion include deletions and insertions of two or more base pairs.

^c Mutants contained two or three mutations were found.

Table 3

Mutation frequencies ($\times 10^{-5}$) of UV-induced base substitutions at dipyrimidine sites and other sites, and other mutations, including deletions and insertions.

	<i>Polh</i> ^{+/+} <i>Poli</i> ^{+/+}	<i>Polh</i> ^{-/-} <i>Poli</i> ^{+/+}	<i>Polh</i> ^{+/+} <i>Poli</i> ^{-/-}	<i>Polh</i> ^{-/-} <i>Poli</i> ^{-/-}
Base substitutions	2.51±1.06	40.3±4.01 ^a	2.78±1.03	32.2±5.85
at dipyrimidine sites	2.31±0.95	40.1±4.19 ^b	2.62±0.81	31.7±5.73
at other sites	0.20±0.21	0.39±0.28	0.21±0.23	0.67±0.72
Other mutations	0.69±0.33	0.68±0.47	0.44±0.53	1.34±2.57

Mean mutation frequencies of six mice are shown, along with standard deviations.

^aValues are significantly different from those of non-irradiated *Polh*^{+/+} *Poli*^{+/+} and *Polh*^{-/-} *Poli*^{-/-} mice ($P<0.0001$ and $P=0.029$, respectively).

^bValues are significantly different from those of non-irradiated *Polh*^{+/+} *Poli*^{+/+} and *Polh*^{-/-} *Poli*^{-/-} mice ($P<0.0001$ and $P=0.025$, respectively).

Figure 1

Figure 2a

A

Figure 2b

B

Figure 2c

C

Figure 2d

D

Figure 3