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Induction of Homologous Recombination Following *in utero* Exposure to DNA-Damaging Agents

Bijal Karia^{1,2}, Jo Ann Martinez¹, and Alexander J. R. Bishop^{1,2,3}

¹Greehey Children's Cancer Research Institute, The University of Texas Health Science Center at San Antonio, 8403 Floyd Curl Drive, San Antonio, Texas 78229, USA

²Department of Cellular and Structural Biology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78229, USA

³Cancer Therapy and Research Center, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229, USA

Abstract

Much of our understanding of homologous recombination, as well as the development of the working models for these processes, has been derived from extensive work in model organisms, such as yeast and fruit flies, and mammalian systems by studying the repair of induced double strand breaks or repair following exposure to genotoxic agents *in vitro*. We therefore set out to expand this *in vitro* work to ask whether DNA-damaging agents with varying modes of action could induce somatic change in an *in vivo* mouse model of homologous recombination. We exposed pregnant dams to DNA-damaging agents, conferring a variety of lesions at a specific time in embryo development. To monitor homologous recombination frequency, we used the well-established retinal pigment epithelium pink-eyed unstable assay. Homologous recombination resulting in the deletion of a duplicated 70 kb fragment in the coding region of the Oca2 gene renders this gene functional and can be visualized as a pigmented eyespot in the retinal pigment epithelium. We observed an increased frequency of pigmented eyespots in resultant litters following exposure to cisplatin, methyl methanesulfonate, ethyl methanesulfonate, 3-aminobenzamide, bleomycin, and etoposide with a contrasting decrease in the frequency of detectable reversion events following camptothecin and hydroxyurea exposure. The somatic genomic rearrangements that result from such a wide variety of differently acting damaging agents implies long-term potential effects from even short-term *in utero* exposures.

Keywords

Homologous recombination; DNA damaging agents; mouse; pink-eyed unstable; *in utero* exposure; *in vivo*

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Correspondence should be addressed to A.J.R.B., bishopa@uthscsa.edu, phone: (210) 562-9000.

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1. Introduction

Environmental exposures include many potential genotoxic agents, many of which are known carcinogens. Considering the variety of these agents, they are generally classified by their mode of action [1]. Irrespective of how they react with DNA, a DNA lesion that impacts DNA replication, inhibiting its normal progression, might potentially instigate genomic instability via aberrant repair processes, including homologous recombination (HR). HR is usually considered to be a high-fidelity DNA-repair process, because it uses a homologous template of DNA to repair damaged DNA. Therefore, it is not surprising that HR is most prevalent during or just after DNA synthesis, when the sister chromatid is present to act as the template and facilitate this type of repair and restoration of normal DNA replication [2]. However, there is also the potential that an HR event will be mediated by an alternate homologous sequence at some ectopic site, for example between simple repeats present in the genome (SINES or LINEs, segmental duplications, copy number variants, or even genes that share significant homology) [3]. Considering that copy number variations are now understood to constitute approximately 12% [4] of the mammalian genome, increasing the frequency of HR by exposure to DNA-damaging agents might be expected to significantly impact genome stability in a proliferating somatic cell. For instance, unlike genetic diseases (e.g., sickle-cell anemia or Tay-Sachs disease) which are often associated with mutation in a single gene and prevalent in certain populations, recombination-based genomic disorders (e.g. DiGeorge syndrome or Prader-Willi syndrome) result from frequent rearrangements of the genome in certain disease loci [5]. To better understand the impact of such recurrent change, we asked what the consequence would be on genomic stability following exposure to several differently acting DNA-damaging agents when many cells are actively dividing during development. To assess genome rearrangement we used a well-established segmental duplication/deletion pigmentation assay, the pink-eyed unstable mouse model, with *in utero* exposures to several different agents.

The pink-eyed unstable (p^{um}) mutation in the mouse is a head-to-tail duplication of a 70 kb region of DNA—effectively a segmental duplication that disrupts the function of the p gene (also known as *Oca2*). The p gene encodes for an integral membrane protein that is required for the proper assembly of melanin in melanosomes and confers dark brown-black pigmentation [6]. The p^{um} mutation causes a dilution of this color in 2 pigmented cells of the mouse: melanocytes confer coat color to fur and to the retinal pigment epithelium (RPE) of the eye. Spontaneous reversion of the 70 kb duplication via deletion of one copy of the duplicated sequence renders a functional p gene, thus allowing for proper melanin packing in cells. These reversion events can be scored as black spots on an otherwise dilute fur coat or as pigmented cells in the RPE of the mouse eye [7]. Proliferation of these tissues occurs mainly during embryonic development; thus, we were able to expose pregnant dams to various agents during development and determine the impact on somatic HR using this simple pigment-based assay system [8, 9, 10, 11]. For this study we examined the effect of 8 different agents that can be categorized into 6 different classes of action; cisplatin, methyl methanesulfonate, ethyl methanesulfonate, bleomycin, 3-aminobenzamide, etoposide, camptothecin, and hydroxyurea.

Cisplatin works by aquating a chloride ligand, which results in the formation of a DNA adduct and, usually produces intrastrand DNA cross-links, which in turn impedes replication and transcription [12]. In contrast, alkylating agents such as methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS) are not used as frequently in clinical settings as chemotherapeutic agents, but they are considered cytotoxic, mutagenic and carcinogenic, and prototypical of other clinically relevant or environmentally present alkylating agents [13]. Recent findings have shed light on the powerful potential of poly (ADP-ribose) polymerase (PARP) inhibitors to act as anticancer drugs. A first generation PARP inhibitor, 3-aminobenzamide (3AB), acts by attacking the catalytic site of the PARP family of enzymes, thereby competing with NAD^+ and resulting in the inhibition of the activity of PARP [14]. Bleomycin has been classified as an “antitumor antibiotic” by forming complexes with iron, which reduces molecular oxygen to superoxide and hydroxyl radicals, thereby causing single- and double-strand breaks in DNA. Etoposide, sometimes more appropriately termed a topoisomerase poison, acts by disrupting the normal functionality of the ubiquitous topoisomerase II (Top2) enzyme, which plays a role in cutting DNA strands to relieve tangles and supercoiling. Rather than binding or intercalating with DNA directly, etoposide stabilizes a ternary complex of Top2 that is covalently linked to DNA at a strand break [15]. The target of camptothecin is topoisomerase I (TOP I), an enzyme that relaxes DNA supercoiling. Camptothecin is a cell-cycle-specific agent that causes DNA replication-fork collapse in dividing cells, resulting in cell death [16]. Hydroxyurea (HU) works by inactivating the ribonucleotide reductase enzyme, thus reducing the availability of free nucleotides. As a consequence, HU inhibits the entire replicase complex, blocking DNA synthesis and resulting in the arrest of cells in the S phase of the cell cycle [17].

In this paper we sought to determine if *in utero* exposure to different classes of DNA-damaging agents caused the type of lesions that could increase the frequency of HR in our pink-eyed unstable mouse model. We observed a robust induction of HR following cisplatin and alkylating-agent exposure as well as a significant induction of HR following exposure to etoposide, bleomycin, and a PARP inhibitor used in our study, 3AB. In contrast, a marked decrease in the frequency of detectable reversion events was observed for the ribonucleotide reductase inhibitor (hydroxyurea) and Top1 inhibitor (camptothecin) measured in our study. Aside from the alkylating agents and camptothecin, to our knowledge the remaining DNA damaging agents have not been assessed previously using an *in vivo model* such as the p^{un} system.

2. Materials and Methods

2.1 Mouse cohort and breeding

C57BL/6J $p^{un/un}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Experimental cohorts were maintained by breeding homozygous $p^{un/un}$ mice to generate sufficient numbers of animals for exposure experiments. All animal studies were conducted in accordance with University and Institute IACUC policies, as outlined in protocol 07005-34-02-A,B1,C.

2.2 Timing of pregnancy and exposure to agents

Mice homozygous for $p^{un/un}$ were bred with successful copulation, which was indicated by a vaginal plug and timed as 0.5 days *post coitum*. Pregnant dams were then exposed to various DNA-damaging agents (Table A.1) on embryonic day 12.5.

The DNA-damaging agents were prepared in 0.9% normal saline (also used as a control) and injected intraperitoneally to pregnant dams. The dose of each agent was calculated based on the weight of the pregnant dam (per 30 g) and prepared in a 0.2 mL solution.

2.3 Eye dissection and p^{un} reversion (homologous recombination) assay

Eyes from 30-day-old pups derived from exposed pregnant dams were harvested and dissected as previously described [8]. RPE whole mounts were prepared and imaged using a Zeiss Lumar version 12 stereomicroscope, Zeiss AxioVision MRm camera, and Zeiss AxioVision 4.6 software (Thornwood, NY). For each RPE, the total number of pigmented eyespots was scored along with the number of cells that comprised each eyespot as detailed in Claybon *et al.* [18]. The position of each pigmented eyespot was also recorded to confirm the correlation between the time of exposure and the location of any induced reversion events, as previously described [9]. The criterion for scoring a p^{un} reversion event as well as the analysis of its position on the RPE was also previously outlined in Bishop *et al.* [8]. Briefly, an eyespot was scored as one or more reverted cell (indicated by black pigmentation in an otherwise transparent cell layer), separated by no more than one unpigmented cell.

2.4 Statistics

Statistical analysis was performed using GraphPad Prism 6.0 (La Jolla, CA) software. We first performed a normality test and determined that our data did not follow a normal Gaussian distribution. We therefore chose nonparametric tests that compare distributions of 2 unpaired groups. A Mann-Whitney test was used to analyze p^{un} reversion frequency between DNA-damaging agents and control. We used a Kolmogorov-Smirnov test to analyze distribution differences in eyespot position data between control and differing DNA-damaging agents.

3. Results

3.1 Survival analysis of pregnant dams' postexposure to varying doses of DNA-damaging agents

Dosing for each agent was empirically derived based on a combination of reported LD₅₀ (lethal dose 50%) levels, published fetotoxicity data, and previous work done with the p^{un} fur-spot assay [10]. We performed a survival analysis study to determine which doses maximized dam survival, litter size, and p^{un} fur-spot induction (Table 1). The fur-spot assay was used merely as an indicator to confirm that the chosen dose for each agent was capable of inducing HR by our assay, but it generally requires many more mice to establish statistical significance compared to the eyespot assay. For cisplatin and MMS, a dose of 2.5 mg/kg and 0.2 mg/kg, respectively, resulted in robust litter sizes and p^{un} fur-spot induction. The 100 mg/kg dose for EMS did not result in spotted pups; however, given that the eyespot assay is more sensitive (one-cell resolution) compared with the clonal expansion of revertant

cells needed to visualize a fur-spot, we went forward with this dose for our study. Bleomycin proved to be quite toxic to the pregnant dams; therefore, we selected a dose of 5 mg/kg, which gave larger litter sizes and an 18% fur-spot frequency. In contrast, we selected the highest dose tested for 3AB because it did not appear to harm the dams. The topoisomerase inhibitors, etoposide and camptothecin, resulted in very few litters at high doses; however, lower doses of these agents still elicited a robust 36% fur-spot frequency. A midrange dose of the replication inhibitor, HU, resulted in a 27% fur-spot frequency and 100% survival (Table 1).

3.2 Exposure to DNA-damaging agents with different modes of action induces homologous recombination

Either naturally or by design, chemotherapeutic agents with differing modes of action lead to a variety of lesions, such as strand breaks, DNA adducts, and DNA cross-links, among others. These lesions are detected by surveillance machinery that operates by triggering a robust DNA-damage signal cascade, thereby prompting appropriate DNA-repair pathways. HR is one such pathway; however, much of our understanding of this pathway stems from either lower organisms or tissue-culture studies on the repair of induced double-strand breaks. Therefore, we set out to ask whether differing types of DNA lesions could induce somatic HR *in vivo*. We exposed pregnant dams at a specific time in embryo development (12.5 dpc) to DNA-damaging agents that have differing modes of action. The simplest assessment of these agents is to determine the frequency of reversion events in each RPE for each agent exposure compared to the saline control. Upon examining the frequency of eyespots (p^{um} reversions) per RPE, we found that all agents, except camptothecin and HU, significantly induced HR compared to the control (Fig. 1a and Table 2). The cross-linking agent, cisplatin, and both alkylating agents, MMS and EMS, showed a twofold induction of eyespots per RPE ($P < 0.0001$; Mann-Whitney test). Though not as robust, PARP inhibition ($P < .001$; Mann-Whitney test), bleomycin, and the Top2 poison, etoposide ($P < .01$; Mann-Whitney test), also significantly induced HR in comparison to the control agent (Fig. 1a and Table 2). Whereas most of the agents resulted in a concomitant increase in the number of revertant cells (Table 2), camptothecin and HU showed a highly statistically significant decrease in the number of eyespots ($P < .0001$; Mann-Whitney test) and an overall fewer number of cells per RPE, possibly due to the dose selected for this study (Fig. 1a and Table 2). It is interesting that these latter data contrast with the clear induction observed using the fur-spot assay, which indicated that the selected doses should induce p^{um} reversion, albeit with possible cytotoxic effects in the RPE (Table 1). The resultant litters from pregnant dams exposed to EMS, showed a clear induction of p^{um} reversion in the RPE (compared with no induction in the fur), which indicates an increased sensitivity of the eyespot assay to detect damage-induced HR events. These results suggest that many forms of damage—not just strand breaks—are capable of inducing recombinogenic lesions.

3.3 Induction of single-cell and multicell reversion events differ depending on the mode of action of DNA-damaging agents

In addition to directly providing HR frequency information in a developing mouse RPE, the p^{um} RPE-based reversion assay allows for the further subdivision of eyespot frequency into those eyespots consisting of one cell (single-cell eyespots) and those with more than one cell

(multicell eyespots). The idea is that events resulting in multicell eyespots are more likely to have been tied to replication, such that reversion in one cell that is actively replicating its DNA clonally expands into a group of daughter cells. Thus, according to our criteria, we would score a clonally expanded multicell eyespot as a single reversion event. In contrast, a single-cell eyespot might have been derived from an event that was not necessarily tied to replication or in a cell that was in its terminal division and did not continue to divide once the revertant *p* gene segregated into one daughter cell. However, the p^{um} reversions that result in single-cell eyespots may result from cells that are in G1 through a single strand annealing type event. Since we observe that the induced events occur in specific regions of the RPE (and not in regions that are post mitotic at the time of exposure) suggests that whether single-cell or multicell, p^{um} reversions arise in cells that are actively dividing at the time of exposure.

Our data showed a significant increase in frequency of single-cell reversion events for cisplatin, EMS, and MMS ($P < .0001$; Mann-Whitney) with only a small increase in multicell events for MMS ($P < .05$; Mann-Whitney; Figs. 1b and 1c). 3AB and bleomycin also showed an increase in single-cell events ($P < .05$ and $P < .01$, respectively; Mann-Whitney) with no induction of multicell events (Figs. 1b and 1c). In contrast, HU exposure resulted in a significant decrease in single-cell events ($P < .01$; Mann-Whitney); in addition, both HU and camptothecin appeared to have a significant decrease in the frequency of multicell eyespots compared with the control agent ($P < .001$ and $P < .0001$, respectively; Mann-Whitney; Figs. 1b and 1c). An interesting finding was that, of the 17 RPE analyzed following camptothecin exposure, only 5 of 31 eyespots were scored as multicell reversion events, compared with 50% in the control group. Further, we observed an induction of reversion events following camptothecin exposure using the p^{um} fur-spot assay (similar to that reported by others; *eg*, [19]), but a decrease in the p^{um} eyespot assay for the same animals. This suggests a tissue-specific difference in response, perhaps with RPE cells being more sensitive to camptothecin, resulting in greater cell death, permanent cell cycle arrest, or repair by other pathways without induction of HR events.

3.4 Position frequency of reversion events following exposure to DNA-damaging agents

In addition to examining the frequency of events, another aspect of the p^{um} eyespot assay is that the position of events found in the adult RPE can be related to the specific time during development when they occurred [8]. The RPE development results from an edge-biased pattern of proliferating cells that orient outward radially [20]. Previously, Bishop et al. demonstrated that the time of exposure to a DNA-damaging agent during development correlates strongly with the location of induced revertant events in the adult RPE [8]. Therefore, those eyespots that are near to the centrally located optic nerve occurred early in development, whereas those positioned toward the edge of the RPE (distal to the optic nerve head) most likely occurred later in embryo development. Considering that all of the exposures were conducted at embryo day 12.5, we expect this to correlate to an induction of p^{um} reversion events at approximately one third of the distance from the optic nerve head to the edge of the RPE (*ie* “position 0.3”).

To determine whether there was a positional effect of the observed increase in eyespots following exposure to DNA-damaging agents, we analyzed the distribution of p^{um} reversion events using a nonparametric Kolmogorov-Smirnov test to evaluate pattern shifts of eyespots between the control RPE and those exposed to DNA-damaging agents (Fig. 2a). Cisplatin ($P < .01$; Kolmogorov-Smirnov), MMS and EMS ($P < .05$; Kolmogorov-Smirnov) showed a significant shift in distribution of total reversion events compared to control (Fig. 2a). We further compared and contrasted the pattern of eyespot distribution for single- and multicell events separately. An examination of RPE for single-cell eyespots (Fig. 2b) revealed an induction pattern similar to that observed for total eyespots but positioned more toward the proximal region of the RPE for these agents ($P < .01$, $P < .05$, $P < .01$, respectively; Kolmogorov-Smirnov), with no significant distribution difference in multicell reversion events compared to the control agent (Fig. 2c).

Bleomycin did not have a significant pattern change in total eyespots but there was a significance in single-cell eyespots ($P < .05$; Kolmogorov-Smirnov) with an apparent spike of reversion events in regions following induction suggesting a quick mode of action for this agent compared to the control agent (Figs. 2a and 2b). PARP inhibition and etoposide exposure also resulted in a robust distribution change in total eyespots ($P < .01$; Kolmogorov-Smirnov) and a shift to more proximal regions for single-cell eyespots ($P < .05$, $P < .01$, respectively; Kolmogorov-Smirnov), which was more substantial following 3AB induction compared to the control agent (Fig. 2b). Again no significance was indicated in multicell reversion events (Fig. 2c). The differences in position and pattern shifting between single and multicell events have been reported for various genetic backgrounds [21] and following DNA-damage exposure [8, 9].

It is interesting that the significant decrease seen in the total frequency of eyespots ($P < .05$, $P < .01$, respectively; Kolmogorov-Smirnov) was also recapitulated in the distribution pattern of these eyespots following camptothecin and HU exposure (Fig. 2a). For HU, the distribution difference is significant amongst single-cell reversion events ($P < .05$; Kolmogorov-Smirnov), with an apparent lack of eyespots directly following exposure, possibly due to cell death (Fig. 2b). Although not significant, the patterning of eyespots following camptothecin exposure indicates a loss in multicell eyespots in the more distal regions of the RPE, suggesting cytotoxicity in proliferating cells or an alternative pathway to repair the damage (Fig. 2c).

Previous work by Bishop *et al.* correlated an induction of damage-induced HR (using benzo[a]pyrene) to a particular time in fetal development [8]. Based on these calculations, we performed our inductions at embryo day 12.5 to maximize exposure to proliferating cells. However, the lack of significance in multicell reversion events following most of the DNA-damaging agents suggests that we might have missed the optimal “signal-to-noise” ratio for the agents used. Still, the results reported here suggest a significant difference in the repair of different types of lesions resulting from exposure to different types of agents.

4. Discussion

Exposure to environmental genotoxins or endogenous byproducts of normal cellular processes represents a peril to genomic integrity. Given this, many species have adapted a robust set of DNA-repair processes that can remove DNA lesions and hopefully maintain the integrity of the genetic material. Each repair process has a preferred substrate, although the significant redundancy that exists between these processes creates overlap in the substrates. For example, it has been shown that base excision repair, nucleotide excision repair, and recombination can all repair the alkylation damage associated with MMS exposure [22]. Actually, HR is a process that should be capable of repairing any genotoxic DNA lesion given the appropriate homologous template. However, the fact that HR must be kept in delicate balance to maintain genomic integrity and curb tumorigenesis is evidenced by mouse models deficient in key HR proteins. For instance, previous work using the p^{un} eyespot assay with mice deficient in BRCA1 (breast cancer, 1 early onset) resulted in a decreased frequency of HR compared to wild-type mice. In contrast, a hyperrecombination HR phenotype was seen in mice lacking RecQ helicase, BLM, the gene associated with Bloom syndrome [23]. BRCA1 and BLM deficiency yield a polarized HR phenotype, yet both lead to a higher incidence of cancer, most likely because of increased chromosomal instability due to an imbalance of HR repair. In this study, we sought to determine whether previously established DNA-damaging agents, either naturally occurring or manufactured, were able to elicit deletion events at a particular locus by using the *in vivo* p^{un} mouse model. By determining the frequency of pigmented cell spots in the RPE, we demonstrated that *in utero* exposure to these agents at an established time in embryo development resulted in an increased frequency of reversion events in our system compared to a saline control. This increased frequency of HR in the RPE following exposure to DNA-damaging agents is likely to be representative of the somatic mutation and genetic alterations that may be induced in other highly proliferative cells during embryo development. Any such somatic change in development has the potential to be clonally expanded, altering the genetic makeup of subsets of cells in the adult body with long-term consequences on the genetic integrity of cells.

The cytoplasm of a wild-type RPE cell is packed with melanosomes (specialized organelles filled with melanin granules), which give these light-sensitive cells their dark guise. The murine pink-eyed dilution gene, p (also called the *Oca2* gene) encodes for the P protein, which is involved in maintaining the pH balance necessary for melanin production [24]. When the p gene is nonfunctional (as in the $p^{un/un}$ genotype), melanin production is compromised, resulting in a dilute color compared to the normal, robust, black-brown pigmentation in the RPE and other pigmented tissues, such as the fur. In the event of an HR reversion of the disrupting 70 kb duplication segment in the p^{un} mutation, a pigmented eyespot can be visualized among a clear background. This readout allows us to determine baseline frequencies of HR, which we suggest is normal maintenance to ensure genomic integrity as well as any deviations—either hypo- or hyperrecombination—as evidenced by the aforementioned work done with mouse models deficient in HR proteins [23], as well as others [18, 25].

Studies that offer information about the developmental timing in which drugs might act can provide insight into consequences of clonal expansion of these somatic mutations. Due to its modes of action (adduction and cross-linking of DNA), cisplatin results in DNA damage. Once this damage is detected, DNA-repair machinery (such as HR) is initiated, or else the damage elicits an apoptotic response. Exposure to cisplatin has been shown to increase sister chromatid exchanges (SCEs) [26], a crossover event between 2 sister chromatids that is most likely a result of HR [27]. In agreement with the findings of this *in vitro* study, cisplatin was also a strong inducer of HR in our assay for the dose that was used. We saw a robust increase in total and single-cell eyespots and a nonsignificant increase in multicell eyespots in the RPE of embryos harvested from pregnant dams exposed to a single dose of 2.5 mg/kg of cisplatin. This dose would be considered relatively low by clinic standards, which are often higher and given in multiple regimens; nonetheless, the dose was able to elicit a response in our system, suggesting that HR is instigated in response to lesions involving DNA adducts and cross-linking damage.

Alkylating chemotherapy drugs, such as temozolomide, ifosfamide, and cyclophosphamide, have their effect in every phase of the cell cycle and are thus desirable for use on a wide range of cancers. They have been shown to be very effective in the treatment of slow-growing cancers, solid tumors, and leukemia but are also used to treat lung cancer, ovarian cancer, breast cancer, and others. Both MMS and EMS are S(N)2 agents involved in base N-methylation that can lead to the formation of apurinic sites that block replication [28]. Point mutations are a common and typical consequence of alkylation damage due to guanine alkylation, as are DNA strand breaks and DNA fragmentation. That these agents induce HR has been indicated by an increase in SCEs in various murine tissues (including bone marrow, liver, and kidney tissues) following intraperitoneal injection of MMS [29]. The frequency of HR was also examined using recombination between 2 tandemly arranged neomycin gene fragments in the ovary cells of Chinese hamsters (CHO:5) and was found to be increased by MMS treatment [30]. Using the *in vivo* p^{um} fur-spot assay, other studies have demonstrated that HR is induced by either EMS or MMS [10], which we now recapitulate in this paper using the more sensitive RPE-based p^{um} mouse model. We observed a significant increase in total and single-cell eyespots and a less robust increase in eyespots consisting of more than one cell later in development (distal regions of RPE) following MMS exposure. The latter results contrast our initial finding with the p^{um} fur-spot assay, which is reliant on clonal expansion of p^{um} reversion events to produce observable fur spots, indicating that we were able to detect multicell events in this tissue. We interpret this result to mean that the induction of multicell events was too weak for the dose used to observe an increase above the background frequency of spontaneous events in the RPE. Alternatively, if we had performed our exposures at an earlier time in fetal development (for example at E10.5 similar to previous studies with other agents [9]), then perhaps we could have observed a clearer induction as the background spontaneous frequency of multicell events is considerably less in the proximal region of the RPE. This may explain the general lack of significant induction of multicell events for all our exposures in this study.

Seminal work showing that PARP1 is involved in DNA repair [31] and activated by ionizing radiation and alkylation damage paved the way for PARP inhibitors to be used in a

“synthetic lethality” approach with chemotherapeutic agents [32]. Much work has been done in the form of mouse models of PARP and in *in vitro* work that reveals its role as an early responder to DNA damage and facilitator of HR [33, 34]. PARP inhibition by 3AB is a potent inducer of SCEs in CHO cells [35, 36]. A hyperrecombination phenotype was also observed in work done by Claybon *et al.* using Parp1 nullizygous mice [18]. In the present study, we also show that pharmacological inhibition of PARP1 activity can induce HR in the *in vivo* p^{um} eyespot assay. We saw a significant increase in total and single-cell eyespots, and although not significant, a spike in multicell events closely following 3AB exposure. In the Parp1 nullizygous study, the greatest increase was in multicell events, which is to be expected considering that PARP1 preferentially binds to single-strand breaks, which is a critical step in base-excision repair. In the absence or inhibition of PARP1, a situation occurs where single-strand breaks are not repaired correctly, resulting in a collapsed replication fork due to recombinogenic-associated double-strand break lesions in replicating cells [37, 38]. The weaker response in multicell events observed in the present study might reflect either the transient nature of the inhibitor exposure or the aforementioned issue with high background in spontaneous multicell events for our selected time of exposure.

Several studies involving radiation influence have reported an increased occurrence of cancer following *in utero* exposure [39, 40]. Bleomycin is generally considered to be radiomimetic and a potent source of oxidative stress [41]. Bleomycin and the other enediyne antibiotics achieve site-specific free radical attacks on sugar moieties in both strands of DNA that can result in double-strand breaks [41]. These breaks have been shown to induce HR following bleomycin exposure, as measured by SCEs in CHO cells [42]. A similar phenomenon of robust induction of HR reversion events in more central regions of the RPE, and specifically in single-cell eyespots as seen in this study, was also reported in a paper by Bishop *et al.* using the p^{um} eyespot assay with X-ray damage [9]. Further, the single-cell reversion events appear to have occurred quickly and for a limited duration, following bleomycin exposure, similar to the inductions observed with ionizing radiation reported previously [43].

Etoposide belongs to one of 2 classes of agents involved in enzyme-mediated anticancer properties through the stabilization of TOP2-DNA complexes and halting processes, such as transcription or replication. Although effective in clinical therapy, etoposide often results in secondary malignancies; indeed, it is thought that chemotherapy drugs targeting the TOP2 β isoform may be the source. Etoposide has been shown to induce recombination in several studies. For instance, etoposide induces SCEs in both CHO cells [44] and cultured human lymphocytes [45]. Furthermore, this potent TOP2 inhibitor has been shown to induce somatic intrachromosomal recombination in a whole mouse transgenic (pKZI) mutagenesis model in which a lacZ transgene is only expressed after a DNA inversion [46, 47]. In our study we were able to directly demonstrate that etoposide is capable of strongly inducing HR, specifically resulting in an increase of single-cell eyespots.

Camptothecin binds the TOP1-DNA complex selectively and efficiently, making it a potent chemotherapeutic. This agent has been shown to be involved in recombination in many tissue culture studies involving the induction of SCEs in CHO and human lymphoid cell lines [45, 48, 49, 50, 51, 52]. Enhanced frequency of reversion was seen in an *Hprt*-gene-

based reversion-mutation assay in a CHO V79 plasmid system measuring intrachromosomal recombination following camptothecin exposure [53]. Surprisingly, we did not have a robust induction of HR in our p^{um} eyespot assay, although our preliminary fur-spot results indicated an induction recapitulating an earlier report with the p^{um} fur-spot assay using this agent [19]. Again, we observed a clear difference between our RPE and the fur-spot assay, suggesting tissuespecific differences. A developmental requirement for functional TOPI has also been evidenced by the death of *Top1* knockout mice early in embryogenesis [54].

Nucleotide pool disequilibrium is now a strong possibility in explaining many phenomena, such as replication stress associated with aberrant activation of the RB-E2F pathway in early stages of cancer [55] and slowed replication speed in Bloom syndrome [56]. HU inhibits ribonucleotide reductase, an enzyme crucial in the conversion of ribonucleotides into deoxyribonucleotides essential for DNA synthesis. A tight balance of intracellular deoxyribonucleoside triphosphate (dNTP) pools must be kept to maintain genomic integrity in yeast and at sites of damage in mammalian cells. In fact, many studies have shown that alterations in dNTP pools are coupled with genomic instability, mutagenesis, and tumorigenesis [57]. Insufficient nucleotides will cause DNA replication stress and perhaps replication fork collapse, situations known to induce HR. In this respect HU treatment has been shown to be a positive inducer of SCEs in CHO cells [58, 59]. However, we did not observe an induction of HR in the eyespot assay following HU exposure, but did observe a significant decrease, similar to camptothecin.

Both camptothecin and HU exposure produced a surprising result in that, for the doses chosen, there was an apparent induction of fur-spots but a very significant decrease in eyespots. It is possible that, at the time during development that we performed our exposures, E12.5—melanocyte precursors (dormant migrating melanoblasts) that pigment the fur—are more robust or refractory to DNA damage, while the rapidly dividing RPE cells are either more sensitive or utilize an alternative pathway to repair the DNA lesions produced by camptothecin and HU. Cultured human RPE cells appear to be very sensitive to camptothecin [60], and camptothecin cytotoxicity has been shown to be dependent on DNA synthesis, as the DNA polymerase inhibitor aphidicolin completely blocks camptothecin cytotoxicity [61]. Although a tissue specific cytotoxicity in the developing RPE is a plausible explanation, it does not explain why we observed a decrease in HR events in the RPE below that of spontaneous levels. We have only ever observed a decreased frequency of eyespots when there was a genetic deficiency impairing the homologous recombination process, such as BRCA1 or BRCA2 deficiency ([23] and unpublished data). An alternative explanation is that the camptothecin-induced damage in the p^{um} gene is repaired differently in RPE cells compared to melanoblasts. It is interesting to note that the *Oca2* gene within the RPE is expressed at this time of development but not in melanoblasts [62]. Camptothecin will interrupt both RNA and DNA polymerase progression; thus, it is possible that any damage within the p^{um} gene could be repaired by another mechanism, possibly a transcription-coupled repair mechanism, obviating the need for HR repair. Similarly, the lack of recombination following HU exposure might be due to a higher order response that results in a replication restart (thus avoiding frequent double-strand breaks caused by fork collapse) rather than recombination-mediated gene conversions that would be visualized by

the p^{um} assay (see model in [23]). For instance, DNA fiber analysis revealed a defect in replication-fork recovery following HU-mediated imbalance of dNTP pools and a subsequent replication block in BLM-deficient cells [63]. This suggests that BLM may be involved in recovering a stalled fork before a collapse can occur (presumably induced by HU). If a low level of exposure to HU stimulates BLM activity, then converse to the hyperrecombination phenotype in the p^{um} eyespot assay we reported in the absence of BLM [23], activated BLM would be expected to reduce the frequency of p^{um} reversion events and may provide a reasonable explanation for the decreased frequency of eyespots we observed. It is interesting that both camptothecin and HU are known to result in BLM phosphorylation and presumably its activation [64].

According to the American Cancer Society, in 2013 about 580,350 Americans are expected to die of cancer. Cancer is the second most common cause of death in the United States, and cancer in children has an annual incidence of about 150 new cases per 1 million U.S. children. All cancers involve the malfunction of genes and the other contributions may be additive over a lifetime of internal and external risk factors. Given the number of cell divisions during *in utero* development, it is likely that there is a higher sensitivity during this time to carcinogen exposure [66, 67, 68, 69]. In addition, this might be due to less efficient DNA repair or defective apoptotic mechanisms that facilitate DNA damage into subsequent cell divisions [70]. Further, in mice, about one third of mutations arise before birth [71]. Animal and human epidemiologic studies show a causal relationship for *in utero* exposures (*ie*, diagnostic radiographs [72]) and cancer incidence in children [73, 74, 75]. Given this, *in utero* exposure during critical times in development to DNA-damaging agents or other sources of damage may cause a clonal expansion of somatic mutations. The present study showed that a relatively low single exposure to a wide variety of differently acting agents resulted in an increase of genomic rearrangement (a DNA deletion) at one site in the genome. Notably, this one genomic site can be considered to be representative of up to 12% of the genome with similar structures. This would seem to suggest that a significant amount of somatic genomic rearrangements might be occurring as a consequence of environmental fetal exposures, which likely has a significant impact on the genetic diversity present in somatic tissues. This may explain some of the higher incidences of cancer associated with prenatal exposures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

HR repairs a variety of DNA lesions as measured by the p^{un} assay

In utero exposure to cisplatin and alkylation damage increased HR frequency

In utero exposure to PARP inhibition and bleomycin increased HR frequency

In utero exposure to etoposide increased HR frequency

In utero exposure to camptothecin and HU decreased HR frequency

Figure 1.

Tukey box and whiskers plot of the frequency of p^m reversion events for (a) total (b) single-cell and (c) multicell eyespots per RPE following saline control and DNA-damaging agents. The dashed line indicates the control frequency for comparison. A nonparametric Mann-Whitney test was used to analyze the frequency data; increased significance is denoted by asterisks (* $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$) and a significant decrease is denoted by diamonds (◆◆ $P < .01$, ◆◆◆ $P < .001$, ◆◆◆◆ $P < .0001$).

Figure 2.

Position analysis of reversion events per RPE. Position intervals are indicated on the x-axis, where the 0.0 position interval corresponds to the central region of the RPE near the optic nerve and the position interval 1.0 represents the edge of the RPE. A Kolmogorov-Smirnov test was used to analyze position distributions with individual significance denoted in each figure.

Table 1

p^{um} Fur-spot assay results following exposure to varying DNA-damaging agents and a saline control

agent	dose (mg/k)	number of dams injected	litters	number of pups						total pups	spotted pups	% spotted
Control	0.9% normal saline	8	8	8	8	8	8	8	8	50	9	18
Cisplatin	2.5	7	4	9	8	5	3			25	8	32
MMS	100.0	6	4	7	6	6	4			23	23	100
	0.2	6	6	9	8	8	7	5		45	10	22
EMS	100.0	4	3	7	6	5				18	0	0
Bleomycin	10.0	5	2	3	1					4	3	75
	5.0	4	3	11	8	3				22	4	18
	2.5	8	5	8	7	6	6	5		32	5	16
	1.0	3	1	3						3	0	0
3AB	400.0	4	3	8	6	6				20	0	0
	300.0	4	3	9	8	7				24	0	0
	200.0	4	3	7	6	4				17	0	0
	100.0	4	3	11	10	6				27	0	0
Etoposide	60.0	3	0									
	40.0	3	0									
	20.0	7	2	6	8					14	3	21
	10.0	7	0									
	5.0	6	1	4						4	3	75
	2.5	3	3	7	5	2				14	5	36
Camptothecin	1.0	3	3	7	5	4				16	3	19
	40.0	4	0									
	20.0	4	0									

agent	dose (mg/k)	number of dams injected	litters	number of pups						total pups	spotted pups	% spotted
Hydroxyurea	10.0	4	0									
	5.0	3	0									
	2.0	9	2	2	4				6	1	17	
	1.0	5	5	8	5	5	4		28	10	36	
	5000.0	4	0									
	3000.0	4	1	8					8	2		
	1000.0	4	0									
	300.0	3	3	8	6	8		22	6	27		
	200.0	2	1	3					3	1	33	
	100.0	2	2	6	2				8	0	0	

Table 2
Summary of p^{um} reversion events and RPE examined following a saline control and various DNA-damaging agents

Agent	Dose (mg/kg)	Total			Average		
		RPE	Eyespots	Cells	Eyespots per RPE	Cells per RPE	Cells per eyespots
Control	Saline	26	104	203	4.0 ± 1.1	7.8 ± 4.0	2.0 ± 1.3
Cisplatin	2.5	16	140	404	8.8 ± 3.2	25.3 ± 22.5	2.9 ± 4.4
MMS	0.2	15	123	290	8.2 ± 3.6	19.3 ± 14.2	2.4 ± 2.9
EMS	100.0	17	133	281	7.8 ± 2.6	16.5 ± 9.6	2.1 ± 2.2
3AB	400.0	17	104	215	6.1 ± 2.6	12.6 ± 9.6	2.1 ± 2.0
Bleomycin	5.0	17	106	274	6.2 ± 3.0	16.1 ± 15.7	2.6 ± 3.4
Etoposide	2.5	16	97	255	6.1 ± 2.4	15.9 ± 10.5	2.6 ± 3.5
Camptothecin	1.0	17	31	45	1.8 ± 1.9	2.6 ± 3.3	1.5 ± 1.7
Hydroxyurea	300.0	17	29	78	1.7 ± 2.4	6.5 ± 14.4	3.0 ± 3.5