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Radiation-induced DNA Damage and Repair in Lens Epithelial Cells of both *Ptch*1(+/–) and *Ercc*2(+/–) Mutated Mice

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Epidemiological studies suggest an increased incidence and risk of cataract after low-dose (<2 Gy) ionizing radiation exposures. However, the biological mechanism(s) of this process are not fully understood. DNA damage and repair are thought to have a contributing role in radiation-induced cataractogenesis. Recently we have reported an inverse dose-rate effect, as well as the low-dose response, of DNA damage and repair in lens epithelial cells (LECs). Here, we present further initial findings from two mutated strains (*Ercc*2$^{+/-}$ and *Ptch*1$^{+/-}$) of mice, both reportedly susceptible to radiation-induced cataract, and their DNA damage and repair response to low-dose and low-dose-rate gamma rays. Our results support the hypothesis that the lens epithelium responds differently to radiation than other tissues, with reported radiation susceptibility to DNA damage not necessarily translating to the LECs. Genetic predisposition and strain(s) of mice have a significant role in radiation-induced cataract susceptibility.

INTRODUCTION

In a number of epidemiological studies, ionizing radiation has been reported to induce cataract when the eye is exposed to low doses (*1*). More recently, the biological mechanism(s) underpinning radiation cataractogenesis have been further investigated with a number of potential pathways identified (*2, 3*). One such pathway reported to have a role in radiation cataractogenesis was that of DNA damage and repair. DNA damage in the lens epithelial cells (LECs) from natural and environmental insult results in the formation of DNA double-strand breaks (DSB) (*4–7*), which require repair to limit any loss of normal cellular function including progression of LECs to mature lens fiber cells (*2*). As part of the LDLensRad project, researchers have previously reported an inverse dose-rate effect of ionizing radiation on DNA damage and repair within *in vivo* LECs exposed to 0–2 Gy gamma rays. These doses were delivered at dose rates ranging from 0.014 to 0.3 Gy/min, with postirradiation repair periods of 4 and 24 h (*5*). A significantly greater number of mean 53BP1 foci was observed as dose rate reduced, contradicting the dose-rate response from *in vivo* lymphocytes from the same animals (*5*), and the reported (and expected) effect or dose rate (*8*). While the exact role of radiation-induced DNA damage during cataractogenesis remains unclear, the repair pathway(s) have been hypothesized in recent years as a likely contributing factor, interacting with other mechanisms and pathways (*2–4, 6*). Similarly, the dose-rate dependency regarding radiation-induced cataract has recently been under scrutiny with more attention being given to low-dose exposures in the literature (*9*). In 2018, an increased risk of cataract (based on self-reported surgery) at a mean estimated cumulative absorbed dose (over 5 years) of 55.7 mGy (interquartile range 23.6–69.0 mGy) was observed in an epidemiological study of radiologic technologists (*10*), with reported increased excess hazard ratio of cataract when low-radiation doses were protracted. These studies, alongside the recently reported inverse dose rate (*5*) confirm the need for further investigation into the influence of dose rate and the role of DNA repair in the lens.

Age-related cataract has been associated with defective DNA repair in the LECs (*11*), with several DNA repair genes identified as potential contributors to cataract pathogenesis. To investigate the DNA damage and repair response in LECs, two mutant strains of mice, *Ptch*1 and *Ercc*2 heterozygotes [both having been previously reported to have increased incidence of radiation-induced cataract

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(*12, 13*)] received 0.5–2 Gy γ-ray irradiation at two dose rates.

MATERIALS AND METHODS

The reported inverse dose-rate effect in DNA repair was attributed to the different damage repair of LECs from that seen in other tissues (*5*); therefore, the study presented here further investigates this response by exploring the radiation response of two DNA repair-deficient mouse models, as described below.

Ptch*1*$^{+/-}$ *Mice*

One model used in this study was comprised of mice heterozygous for the Patched 1 (*Ptch*1$^{+/-}$) gene (bred on CD1 background), a gene involved in sonic hedgehog cell signaling pathway (Shh) which has a role in cell proliferation and differentiation in LEC (*13–15*), as well as cell fate and epithelial-to-mesenchymal transition. In humans, germinal *Ptch*1 heterozygous mutation results in Gorlin Syndrome (skin cancer susceptibility) in 60% of patients (*16*). Fibroblasts of Gorlin syndrome patients show radiosensitivity. Patients often become hyper-susceptible to secondary cancer after radiation therapy (*16*). In mice, *Ptch*1 heterozygosity increases spontaneous and radiation-induced cataract incidence (*15*) and radiation-induced cancers (*17*).

Ercc*2*$^{+/-}$ *Mice*

XPD/*Ercc*2 mutant mice, a gene involved in DNA repair by nucleotide excision repair (NER) (*18, 19*), were also used during this study (*12, 20*). This strain was bred on a B6C3F1 background (F1 hybrids of C57BL/6JG and C3HeB/FeJ; B6C3F1). In mice, a heterozygous point mutation in the *Ercc*2 gene has shown a sensitivity to radiation (*12*). Xpd/Ercc2 mutation effects the repair response to oxidative stress (*21*). *Ercc*2$^{+/-}$ mice develop nuclear and cortex cataract, with primary and secondary lens fiber cells hypothesized to be affected most (*12*). *Ercc*2$^{+/-}$ mice have a DNA repair deficiency and would therefore be expected to be radiosensitive (*22*), recently demonstrated using γ-H2AX foci detection in lymphocytes (*12*).

Both *Ptch*1$^{+/-}$ and *Ercc*2$^{+/-}$ mice, alongside wild-type (WT) counterparts, received whole-body ^{60}Co γ-ray irradiation at dose rates of 0.3 and/or 0.063 Gy/min at approximately 10 weeks of age. Both strains were irradiated at respective institutions of ENEA (Rome, Italy) and HMGU (Neuherberg, Germany). At HMGU, mice were kept under SPF conditions in accordance with the German Law of Animal Protection, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the tenets of the Declaration of Helsinki. Treatment of the mice was approved by the Government of Upper Bavaria (ROB-55.2-2532.Vet_02-16-167). At ENEA, experiments were performed according to the European Community Council Directive 2010/63/EU, approved by the local Ethical Committee for Animal Experiments of the ENEA, and authorized by the Italian Ministry of Health (no. 1233/2015-PR). At 4 and/or 24 h postirradiation, eyes were extracted and formaldehyde fixed (n = 402 lenses), lens epithelia isolated using microdissection followed by immunofluorescent staining for 53BP1 foci as described extensively elsewhere (*5–7*). The use and significance of 53BP1 foci as a marker for DSB repair have also been discussed elsewhere (*5–7, 23*). Using Minitab® 18, general linear model analysis of variance (ANOVA) was applied to all data including dose, dose rate, lens epithelial region and time after irradiation. Both central and peripheral LECs were analyzed separately. Due to project constraints and issues with fixation of some lenses, it was not possible to irradiate at both dose rates and all doses at each institute; therefore, a full complement of exposure data for each strain was not possible.

RESULTS AND DISCUSSION

*Ercc*2$^{+/-}$ mice and wild-type (WT) counterparts, as well as inbred C57BL/6J mice from a previously published LDLensRad study (*5*), received 0, 0.5, 1 and 2 Gy γ-ray irradiation at 0.3 Gy/min. DNA DSB repair was quantified using 53BP1 (tumor suppressor p53-binding protein 1) foci detected at 4 and 24 h postirradiation. There was a clear dose response for all strains at 4 and 24 h. The lowest mean foci per cell, observed at both 4 and 24 h postirradiation and in either LEC region, were those of the *Ercc*2$^{+/-}$ mice. Compared to the WT B6C3F1 and inbred C57BL/6J mice, this observation was most clear at 24 h postirradiation for all doses. At 2 Gy and 4 h postirradiation, approximately one half the mean number of 53BP1 foci were observed in *Ercc*2$^{+/-}$ mice. Mean 53BP1 foci/cell frequencies reduced significantly from 4 to 24 h, with y-axis adjusted accordingly in Fig. 1.

Statistical analysis using general linear ANOVA revealed that the region of the lens epithelium ($P < 0.001$) and genotype ($P = 0.002$) were statistically significant. Interaction between strain and region was highly statistically significant ($P < 0.001$). No significant effect of sex, dose or time was observed. Further Tukey pairwise comparison of strain revealed that the response observed in *Ercc*2$^{+/-}$ mutants was statistically significantly different from both inbred C57BL/6J and WT B6C3F1 mice.

The DNA DSB repair response in the LECs of *Ptch*1$^{+/-}$ mice compared to WT counterparts (CD1 background) was also investigated. The most striking observation was in the 4-h postirradiation *Ptch*1$^{+/-}$ mice, where greater numbers of mean 53BP1 foci/cell were present in both central and peripheral LECs irradiated at 2 Gy at the lower 0.063 Gy/min dose rate (see Fig. 2). This effect was less noticeable in the WT mice at 4 h postirradiation. At 24 h postirradiation, detectable 53BP1 foci were very low, with less than 0.1 foci/cell observed within all variables. In *Ptch*1$^{+/-}$ mice, time postirradiation (4 and 24 h), dose (0 and 2 Gy) and dose rate (0.3 and 0.063 Gy/min) of irradiation were all statistically significant ($P < 0.001$). The LEC regions, whether central or peripheral, also showed significantly different responses ($P = 0.031$). *Ptch*1$^{+/-}$ and their counterpart WT mice were not statistically significant from each other, neither was sex of mice. After 2 Gy irradiation, B6C3F1 LECs showed the largest reduction factor in foci from 4 to 24 h, 7.58 and 8.53 for the central and peripheral regions, respectively (Table 1). After 0.5 and 1 Gy irradiation, *Ercc*2$^{+/-}$ LECs showed the largest reduction factors.

It must be noted that these data have a significant lack of fit ($P < 0.001$), despite using the most scientifically appropriate model. Levene's test for equality of variance indicates significant variation in variance between groups. However, ANOVA was still judged to be the most appropriate method in this case, as the power is likely to be higher than a non-parametric test.

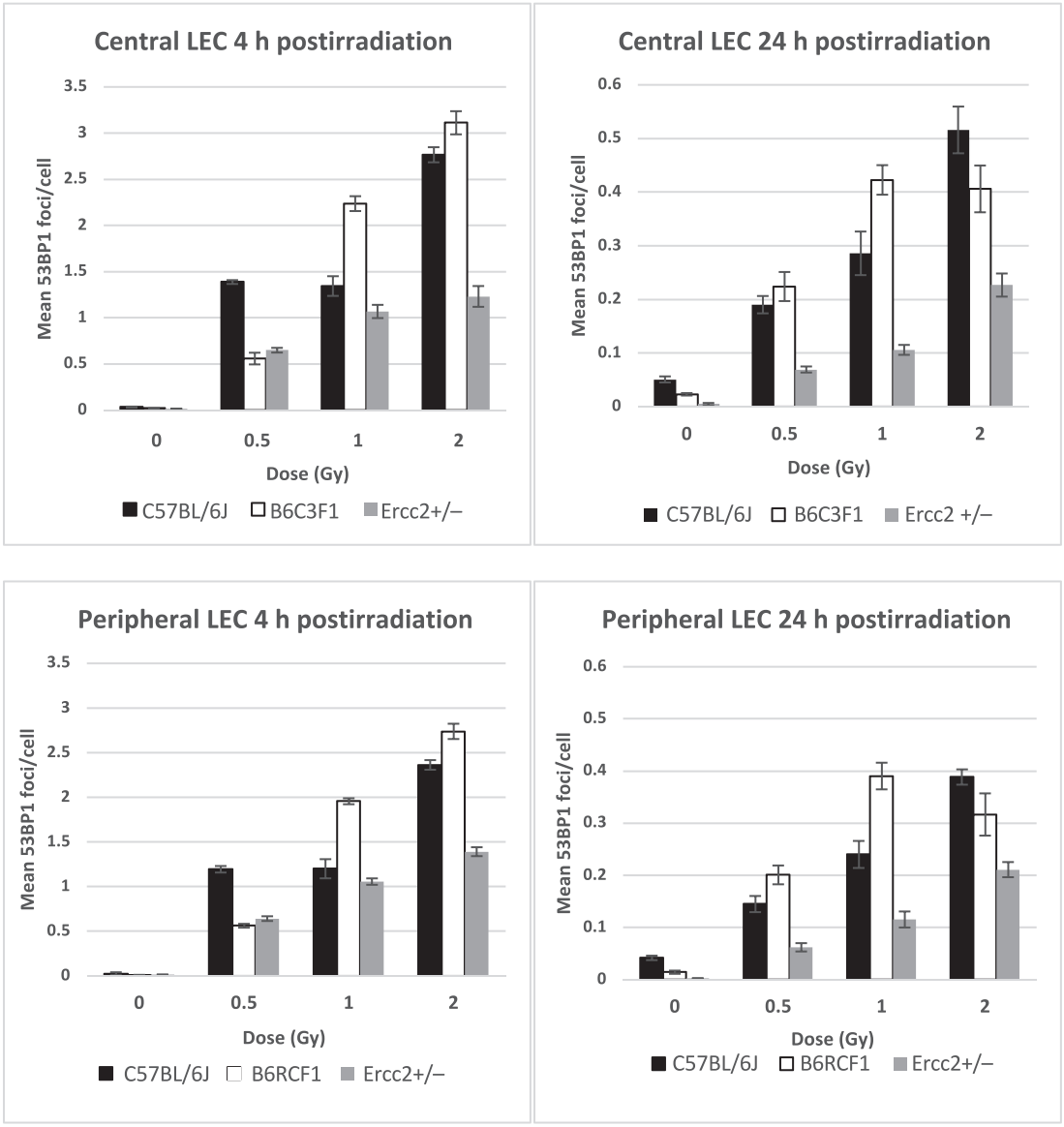

FIG. 1. Mean 53BP1 foci/cell observed in central and peripheral region LECs of *Ercc2*$^{+/-}$ (n = 67) mutants, their wild-type counterparts (B6C3F1, n = 66) and inbred C57BL/6J (n = 78) mice. Mice received 0, 0.5, 1 and 2 Gy ^{60}Co irradiation at a dose-rate of 0.3 Gy/min, and were sacrificed at 4 and 24 h postirradiation. Data are presented with standard error bars. Note y-axis scales are different for 4 and 24 h (total n = 211 mice).

Both mutated strains demonstrated less detectable mean 53BP1 foci/cell than classically radioresistant C57BL/6 inbred mice in both central and peripheral LECs after 2 Gy irradiation (at 0.3 Gy/min) (Fig. 3).

Little was known about the effect of mouse genotype on *in vivo* irradiated lens studies (*2*). Strong strain dependency of DNA damage repair within inbred strains after low-dose irradiation across multiple inbred strains of mice has been reported (*6*). Inbred strains likely underrepresent human populations, with all their complexities (*2, 24, 25*). The effect of murine strain in radiation-induced cataractogenesis research studies has been previously discussed elsewhere (*2, 26*). When comparing Atm$^{+/-}$ and Atm$^{-/-}$ mice, a greater incidence of radiation-induced cataract was reported in the heterozygote mice at doses of 0.5–2 Gy (*27–29*). At high-dose (8 Gy) irradiation, there was no significant difference between heterozygous and WT mice. Rad51 and Atm deficiencies, both with a role in DNA repair, have also been used to demonstrate an increased radiation-induced cataract incidence in mice (*27, 30, 31*). Genetic effect(s) are expected to be greater in repair-deficient mutant and knock-out mouse models at low doses (*2*). In humans, gene polymorphisms in base excision repair results in an increased risk of senile cataract (*32*). Elevated DNA single-strand breaks (SSB) have been reported in the LECs of patients with cataract (*33*).

The frequency of mean 53BP1 foci/cell in LECs were compared from our previously published inbred C57BL/6J

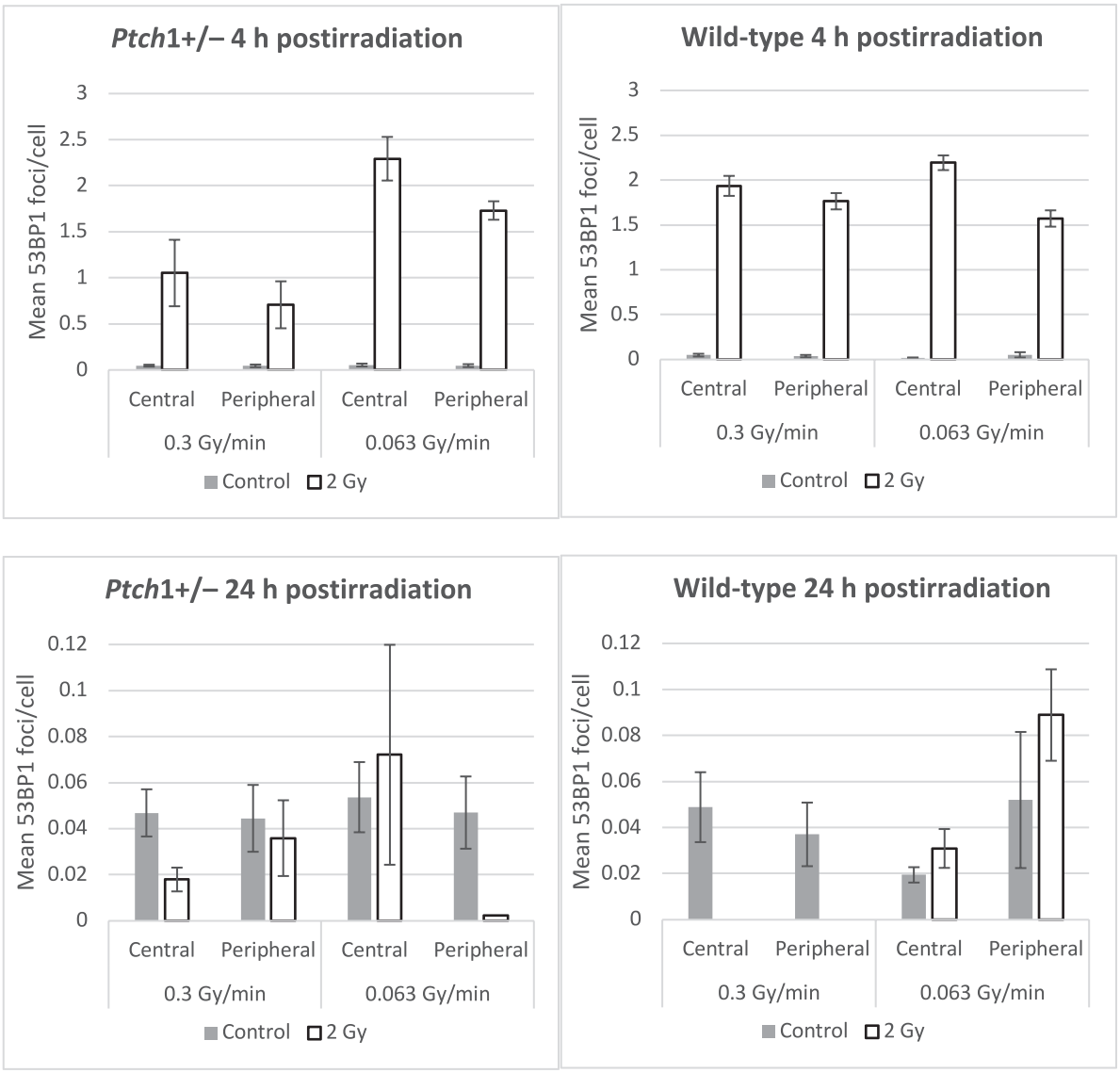

FIG. 2. *Ptch*1$^{+/-}$ and CD1 wild-type mice received 2 Gy ^{60}Co irradiation delivered at 0.3 and 0.063 Gy/min and sham-irradiated mice served as control. Mean 53BP1 foci per cell were recorded in both central and peripheral region LEC from mice sacrificed at 4 and 24 h postirradiation (n = 68 mice).

TABLE 1
Reduction Factor of Mean 53BP1 Foci/Cell from 4 to 24 h after 0.5, 1 and 2 Gy (0.3 Gy/min) Irradiation in Both Central and Peripheral LECs of C57BL/6J, B6C3F1 and *Ercc*2$^{+/-}$ Mice

Dose	Strain	Central region LEC	Peripheral region LEC
0.5 Gy	C57BL/6J	7.26 ± 0.19	7.93 ± 0.22
	B6C3F1	2.54 ± 0.3	2.8 ± 0.19
	*Ercc*2$^{+/-}$	10.83 ± 0.17	10.6 ± 0.18
1 Gy	C57BL/6J	4.78 ± 0.38	5 ± 0.36
	B6C3F1	5.5 ± 0.32	5 ± 0.24
	*Ercc*2$^{+/-}$	9.64 ± 0.28	9 ± 0.22
2 Gy	C57BL/6J	5.4 ± 0.35	6.21 ± 0.26
	B6C3F1	7.58 ± 0.41	8.53 ± 0.35
	*Ercc*2$^{+/-}$	5.36 ± 0.36	7.25 ± 0.25

Note. Table includes standard error. n = 211 mice.

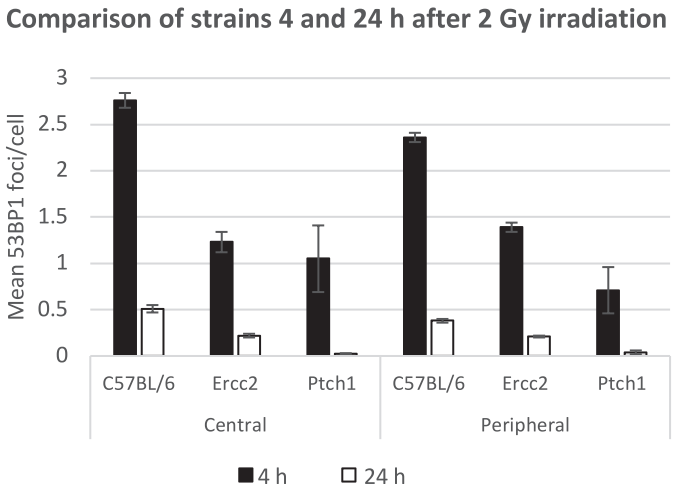

FIG. 3. Mean 53BP1 foci/cell in both the central and peripheral LEC of inbred C57BL/6 mice, compared to mutations *Ercc*2$^{+/-}$ and *Ptch*1$^{+/-}$ at 4 and 24 h after 2 Gy ^{60}Co irradiation (0.3 Gy/min).

(*5*), with *Ercc*2$^{+/-}$ and WT counterpart B6C3F1 mice (Fig. 1). We observed a statistically significant lower number of detectable repair foci in the *Ercc*2$^{+/-}$ mice compared to both B6C3F1 WT and inbred C57BL/6J mice. Furthermore, no statistically significant difference was observed between B6C3F1 WT and inbred C57BL/6J mice.

Here, *Ercc*2$^{+/-}$ mice demonstrate a faster repair of DSBs in LECs, as quantified by a lower mean number of detectable 53BP1 foci compared to WT counterparts. Notably, *Ercc*2$^{+/-}$ mice showed the largest reduction, or faster repair, in mean foci/cell values from 4 to 24 h compared to B6C3F1 and inbred C57BL/6 mice at the lower doses of 0.5 and 1 Gy irradiation. However, after 2 Gy irradiation, the WT mice showed the largest reduction, or faster repair, of DNA damage. The lymphocytes of *Ercc*2$^{+/-}$ mice have previously been shown to be radiosensitive, with slower repair of γ-H2AX foci compared to WT counterparts (*12*). Here we present results from the same *Ercc*2$^{+/-}$ mice, albeit bred on a different background strain (B6C3F1) showing significantly less detectable 53BP1 foci in the LEC of heterozygotes compared to WT B6C3F1 mice. This would support the previously documented hypothesis (*5, 6*) that the DNA repair mechanism(s) in LECs behave differently from those observed in other tissues, thus, a differential radiosensitivity of lymphocytes and LEC. Furthermore, we have previously suggested DNA damage repair is favored over proliferation when radiation is delivered at an acute dose rate (0.3 Gy/min) compared to one that is more protracted (0.063 Gy/min) (*5*).

NER is the main pathway used by mammals to repair DNA lesions (*34*), with *Ercc*2 identified as one of the key genes involved in this pathway and in basal transcription (*35, 36*). Alongside *Ercc*2, the *Ercc6* gene is also involved in NER and was recently demonstrated to show repressed expression after UVB exposure in LECs of age-related nuclear cataract patients (*37*).

*Ptch*1 heterozygous mutant mice have previously been reported radiosensitive to cataract in a nonlinear fashion (*13*) when irradiated at postnatal age 2 days old. During this age, the LECs undergo rapid expansion prior to forming a functioning tissue, with many stem/progenitor cell properties. This could make them particularly hypersensitive to radiation-induced damage, thus the increase in cataract incidence previously observed elsewhere (*13*). As mentioned, *Ptch*1 is a tumor suppressor gene which, when deficient, leads to an aberrant activation of the Shh pathway, a cell signaling pathway required for proper cell differentiation in the LEC, particularly in embryos but also with a role during adulthood, including tissue regeneration and some repair processes (*38*). Shh is essential for activating epithelial-to-mesenchymal transition (*39*). An impaired Shh pathway interferes with almost all DNA repair types in human cancers (*39*). DSBs may have an important role in *Ptch*1 mutant mice as they can cause a loss in heterozygosity (*40, 41*). During this study, adult (10-week-old) mice rather than postnatal 2-day-old mice were irradiated, therefore, we do not observe a dramatic hypersensitivity in *Ptch*1$^{+/-}$ compared to WT mice, although the repair response observed between them is significantly different. *Ptch*1$^{+/-}$ repaired radiation-induced DSBs much faster at 4 h after irradiation at the dose rate of 0.3 Gy/min compared to that of 0.063 Gy/min. This effect is more obvious in the heterozygote mice, but is also observed in the WT mice. These results support the inverse dose-rate effect of radiation in LECs (*5*) irradiated with 2 Gy at an independently calibrated ^{60}Co gamma-ray irradiation facility in Italy. This data supports the hypothesis of an inverse dose-rate response to radiation-induced DNA damage occurring in the lens epithelium, and that this observation is reproducible in a new strain of mice irradiated in another facility.

The involvement of the DNA damage response during radiation-induced cataractogenesis is further suggested based on the findings of this study. Genetically different mouse strains, with effects in DNA repair, therefore, influence the significance of this effect.

Studying the DNA damage response *in vivo* in LECs has limitations. The intricacies of this pathway within LECs needs to be better characterized using *in vitro* culture (although this also has its limitations, as discussed). Future studies need to incorporate both *in vitro* and *in vivo* models, which could best be achieved by isolating primary LECs from the same mice that are being studied (*12*), making DNA damage response observations highly translatable from *in vitro* to *in vivo* findings.

From the very few published studies that have investigated the radiation-induced DNA damage repair response in the lens epithelium, a non-linear, strain-dependent response has been observed (*6, 7*) and more recently, an inverse dose-rate response (*5*). Dose, dose rate and genotype appear to have some effect on the response of LECs to radiation-induced damage, each likely adding to the cataractogenic

load already upon the lens, leading to cataractogenesis and visual impairment (*3*). When observing these effects, it has been hypothesized that the lens may not necessarily be a radiosensitive tissue, but rather responding to and repairing radiation-induced DNA damage differently compared to other tissues of the body. This hypothesis has been discussed in the previously published article as part of the LDLensRad project by Barnard *et al*. (2019) (*5*). The DNA damage response and continued (and uninterrupted) LEC proliferation/differentiation mechanisms interact and balance against each other, with low dose and low dose rate favoring the latter rather than DNA damage repair. Investigations are ongoing to fully understand the role and effect of DNA damage and repair in LECs, and how this pathway might interact with other mechanism(s) such as cellular proliferation and differentiation.

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