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Lens Epithelial Cell Proliferation in Response to Ionizing Radiation

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Lens epithelial cell proliferation and differentiation are naturally well regulated and controlled, a characteristic essential for lens structure, symmetry and function. The effect of ionizing radiation on lens epithelial cell proliferation has been demonstrated in previous studies at high acute doses, but the effect of dose and dose rate on proliferation has not yet been considered. In this work, mice received single acute doses of 0.5, 1 and 2 Gy of radiation, at dose rates of 0.063 and 0.3 Gy/min. Eye lenses were isolated postirradiation at 30 min up until 14 days and flat-mounted. Then, cell proliferation rates were determined using biomarker Ki67. As expected, radiation increased cell proliferation 2 and 24 h postirradiation transiently (undetectable 14 days postirradiation) and was dose dependent (changes were very significant at 2 Gy; $P = 0.008$). A dose-rate effect did not reach significance in this study ($P = 0.054$). However, dose rate and lens epithelial cell region showed significant interactions ($P < 0.001$). These observations further our mechanistic understanding of how the lens responds to radiation.

INTRODUCTION

The lens is one of the most radiosensitive tissues in the body (*1*). The consequence of lens exposure to ionizing radiation is cataract, but this is considered a deterministic effect, or tissue reaction, which occurs only above a threshold of approximately 0.5 Gy. Previously published studies have suggested that below this threshold there was no significant effect during low-LET exposures (*2*). There are three main types of cataract, depending on where in the lens they form: nuclear, cortical or posterior subcapsular (PSC) (*3*). Of these, the most prevalent type of radiation-induced cataract is the latter (*4–8*), with increased incidence of cortical cataract also reported (*9, 10*). PSC are not unique to radiation exposure, but are also induced by aging (*11*), steroid use and environmental and health-related risk factors (*11, 12*). An increased incidence of cataract has been reported in Chernobyl clean-up workers (*10*). A dose of 1 Gy has been associated with the loss of visual acuity and cataract (*13*), but less data exist for doses <0.5 Gy. In 2012, the advised threshold for cataract induction was lowered to 0.5 Gy absorbed dose for radiation workers (*2, 14*), resulting in new occupational exposure recommendations (*15*) and dose limits for the lens (*1, 16*) of 20 mSv per year (averaged over 5 years, with no single year exceeding 50 mSv) within the EU Basic Safety Standards (*17*). These regulatory changes provided additional scientific impetus to establish the mechanistic link between radiation exposure and cataract formation (*14, 18*). As recent human epidemiological studies have increasingly reported the induction of clinically relevant cataract from low radiation doses (<2 Gy), the ICRP in their recommendation of a lower deterministic threshold did not rule out the possibility of a no-threshold model (*2*). Occupational exposure groups such as industrial and medical workers represent the largest subject cohorts potentially at risk of radiation-induced health effects. These groups are most likely to be affected by the reduction in lens dose limits (*14*). Indeed, one of the largest cohort studies of radiology technologists in the U.S. reported an excess risk of cataract from low dose (<100 mGy cumulative lens exposure) and low-dose-rate exposures (*19, 20*) strongly supporting further investigations. Other groups may also exceed 20 mSv dose to the lens annually (*14*). Investigations of radiation dose and threshold must also be considered in terms of latency for cataract formation; a recent hypothesis of cataractogenic load suggests exposure to radiation adds to the ''load'' already placed upon the lens to develop cataract, with radiation simply decreasing that latency period (*21*).

There have been few animal studies investigating early lens changes after low-dose irradiation (*18, 22–25*) to support these epidemiological findings. Mechanistic pathways involved in radiation-induced cataract are being investigated (*1*), but it is too early at this stage to draw definitive conclusions, especially with respect to a stochas-

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tic or deterministic narrative for radiation protection purposes. Indeed, these studies suggest that favoring either interpretation at this stage without further scientific investigation is premature.

The anterior surface of the lens is covered in a single cell monolayer of lens epithelial cells (LEC). These are responsible for the growth, symmetry and function of the lens as they give rise to the lens fiber cells (LFC) that comprise the lens mass and deliver its optical function (*26–28*). The LEC population is subdivided into those resident either in the central or peripheral region of the epithelium. The central region is coincident with the optical axis, and at the center is the anterior pole of the lens epithelium, which is defined geometrically. The peripheral region is positioned around the equator of the lens, which is at right angle to the anterior pole. There is a high rate of cell proliferation in the lens periphery, and the most proximal LEC relative to the central pole differentiate into the LFC and leave the epithelium to increase the mass of the lens throughput life (*29, 30*). Changes in cell proliferation rates and regional differences around the equatorial circumference alter lens morphology (*31, 32*). Increases in proliferation and growth factor FGF2 have been observed in human LEC exposed to heavy ions, along with changes in transcription factor cyclin dependent kinase inhibitor 1A (CDKN1A), which has a known role in cell proliferation (*33, 34*). Radiation induces oxidative stress in the lens, which results in altered proliferation, cell migration and differentiation (*35, 36*).

Proliferation is easier to study *in vitro*, but this does not replicate fully the *in vivo* situation due to the carefully regulated nature of cell division and differentiation needed to support the growth and geometry in the lens (*1, 35, 36*). The proliferating LEC in the lens periphery are thought to be radiosensitive (*22*), but it is technically challenging to replicate this *in vitro*. Radiation-induced stimulation of LEC proliferation has been observed *in vitro* (*37, 38*) but the interpretation is complicated by the collective inhibition of cell growth suggestive of two separate cell subsets in the HLEC1 cell line, illustrating the importance of further animal studies. These are critical to understanding how to model this using *in vitro* alternative techniques.

Within this study, inbred female C57BL/6 mice were selected; this is a documented radioresistant strain (*39*) including the observed levels of radiation-induced DNA damage (*40, 41*). The strain survives well after >1 Gy doses (*42*) and is the strain of choice for radiation-induced cataract studies (*22, 43*). Strain-based radiosensitivity is an important consideration for studies such as this; radioresistant characteristics associated with C57BL/6 mice may or may not extend to the lens.

MATERIALS AND METHODS

Irradiations

Mice received whole-body irradiation (WBI) with a ^{60}Co source at approximately 10 weeks of age (*44*), a point slightly after establishment of emmetropia and adult growth characteristics (*45*). Irradiation, 0.5, 1 or 2 Gy, was delivered at dose rates of 0.3 and/or 0.063 Gy/min. Alongside the WBI mice, sham-irradiated controls were transported to and from the irradiation facility. The irradiation system is calibrated and traceable to national standards. All doses were delivered to within 5% accuracy. Most of this error comes from the difference in the size of the mice compared to the standard build-up cap that is used (18.6-mm diameter). The ^{60}Co air-kerma correction factor (from the calibration certificate) and then air kerma to tissue correction factor of 1.113 [the same correction factor as used by Dalke *et al*. (*24*)] were used. All irradiations took place at room temperature and were whole-body *in vivo* exposures. A combination of two or four ^{60}Co sources was used to achieve dose rates of 0.063 and 0.3 Gy/min, respectively. Access to the gamma-ray irradiation facility at MRC Harwell (UK) was granted as part of the LDLensRad project. Source decay factors were calculated according to the date of irradiation.

Mice

Inbred female C57BL/6 mice (C57BL/6Jola/Hsd; EngivoRMS, UK Ltd., Blackthorn, Bicester, UK) were used in this study ($n = 5$ per treatment group). Groups of up to five mice were housed together. Mice were housed per cage in accordance with minimum space requirements as outlined by the Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes under the Animals (Scientific Procedures) Act 1986 (ASPA). A minimum 200 cm^2 is required for groups of five mice. Food [RM3(E), LBS Biotechnology, Hookwood, UK] and water were available *ad libitum*. Health status of the mice was checked daily. All procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 via approved project licensing granted by the UK Home Office (PPL no. PA66E1512). Additional approval from the local Animal Welfare and Ethical Review Body (AWERB) at Public Health England was granted for the duration of the LDLensRad project.

Lens Extraction and Fixation

Lenses were dissected and epithelia isolated, flat mounted and prepared for analysis as per published protocols (*18, 22, 46*). A brief analysis of early time response was conducted using a single radiation dose of 0.5 Gy (delivered at 0.3 Gy/min). Time points of 30 min, 4, 24 and 48 h, and 3, 7, 10 and 14 days were investigated.

Ki67

Cell proliferation marker Ki67 was detected by immunofluorescence microscopy using a conjugated fluorescently labeled monoclonal antibody (SolA15) with FITC (Thermo Fisher Scientific, UK) (Fig. 1) together with 200 nm/ml 4′, 6-diamidino-2-phenylindole (DAPI; Roche Diagnostics, Burgess Hill, UK), each diluted 1:500 in 1% (w/v) BSA and incubated on the epithelium for 45 min. Samples were then washed with phosphate buffered saline (PBS). Using fine watchmaker forceps, epithelia were transferred to microscope slides and air dried in the dark. Once dried slides were mounted in ProLong gold antifade (Invitrogen, Paisley, UK), the coverslip was sealed using nail varnish.

Scoring and Statistical Analysis

Once lens epithelia were prepared, they were imaged at 20× magnification using a Nikon® Optiphot 2 fluorescence inverted microscope and Nikon NIS-Elements Advanced Research software package (Tokyo, Japan). LEC scoring was performed, as described elsewhere (*18, 22, 25*). Briefly, the recommended scoring of Ki67 was followed; the percentage of positively stained cells of the total cell number was recorded. The method applied to LEC analysis in this study incorporated the collection of digital images followed by manual scoring of Ki67-positive cells within fields of view and recording the total cell number. The proliferative index was then calculated. No

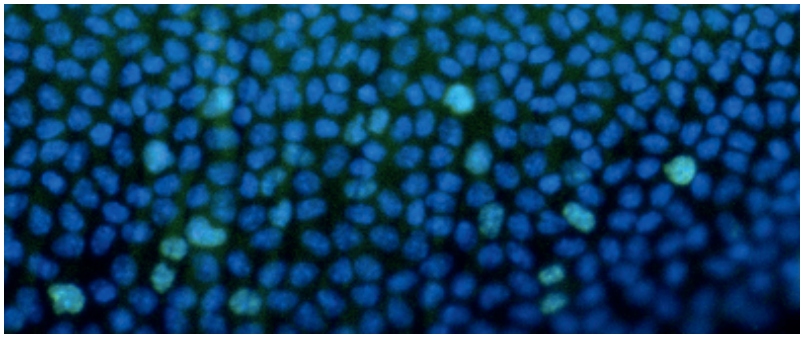

FIG. 1. Immunofluorescent staining of mouse lens epithelium using proliferation marker Ki67 (green) and and nuclear stain DAPI (blue).

previous data of Ki67-positive cell frequency scored within the LEC were available, therefore power calculations were performed once initial data were collected. Two-sample *t* test sample size power calculations were performed based on a power of 0.8, with results indicating that in most cases the sample number was likely to detect any significant effect for factors investigated (dose, dose rate, region and time). Two-way general linear analysis of variance (ANOVA) was then used to analyze Ki67 proliferation in response to the factors of time, dose, dose rate and LEC region. Furthermore, Tukey's pairwise comparisons were also performed.

RESULTS

Early Time Response of Ki67

Time points postirradiation included 30 min, then 4 and 24 h alongside sham-irradiated animals. The number of Ki67-positive cells were counted and analyzed, with mean percentages shown in Fig. 2. Both the central- and peripheral-region LEC are plotted together highlighting the differential proliferative response of each. ANOVA revealed a significantly higher percentage of Ki67 positivity within the peripheral-region LEC compared to those centrally located ($P = 0.009$). The percentages of positive Ki67 cells at the different time points were not significant from the control value, suggesting that at 0.5 Gy (delivered at 0.3 Gy/min), radiation did not induce an effect in cell proliferation at up to 24 h postirradiation. A fluctuation was observed in the peripheral region whereby the percentage of Ki67 positivity declined from control up to 4 h, returning to a value similar to control at 24 h postirradiation. The percentage of Ki67-positive LEC within the central region did not appear to change across all time points and demonstrated smaller standard error.

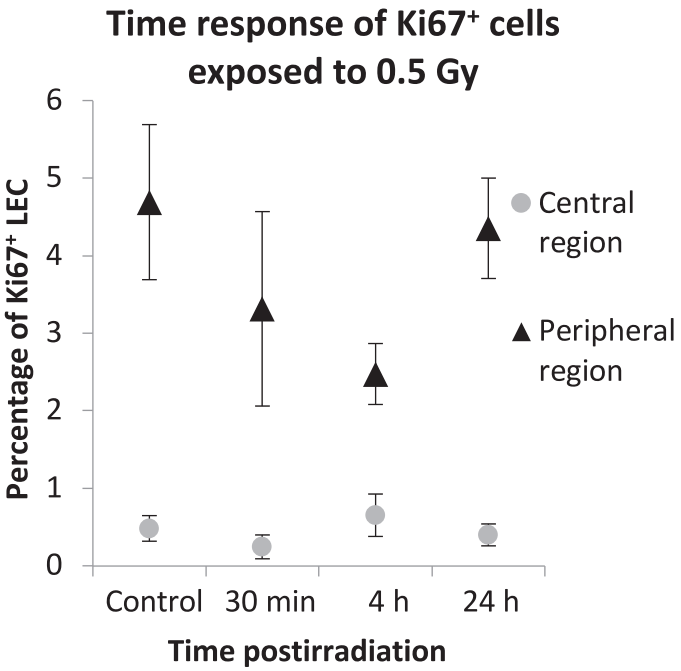

FIG. 2. Time response of Ki67-positive cells (%) in both the centrally and peripherally located LEC after 0.5 Gy irradiation (0.3 Gy/min). Error bars represent standard error. Region of LEC statistically significant ($P = 0.009$).

Later Time Response of Ki67

Postirradiation times from 4 h to 14 days were chosen to detect the radiation effect on cell proliferation during the first two weeks (*47*). Ki67-positive LEC were counted in the central and peripheral regions and plotted as a percentage of total cells (Fig. 3).

No statistically significant difference was observed using Ki67 as the marker for cell proliferation for any time point between the control and irradiated lenses; 2 Gy irradiation had no significant effect on proliferation up to 14 days postirradiation. The peripheral region showed greater fluctuation in Ki67 positivity; at days 3 and 14 there was an observable increase in Ki67-positive LEC in the irradiated lenses, alongside larger standard errors (SE). This fluctuation was not statistically significantly different from the sham-irradiated control LEC. Less fluctuation was seen in the central-region LEC.

Dose-Rate Effects

During the investigation of dose and dose-rate influence, 74 female inbred C57BL/6 mice received 0.5, 1 and 2 Gy WBI (including time-matched sham-irradiated controls) and were sacrificed at 4 and 24 h postirradiation. ANOVA revealed LEC region to be highly significant ($P < 0.001$),

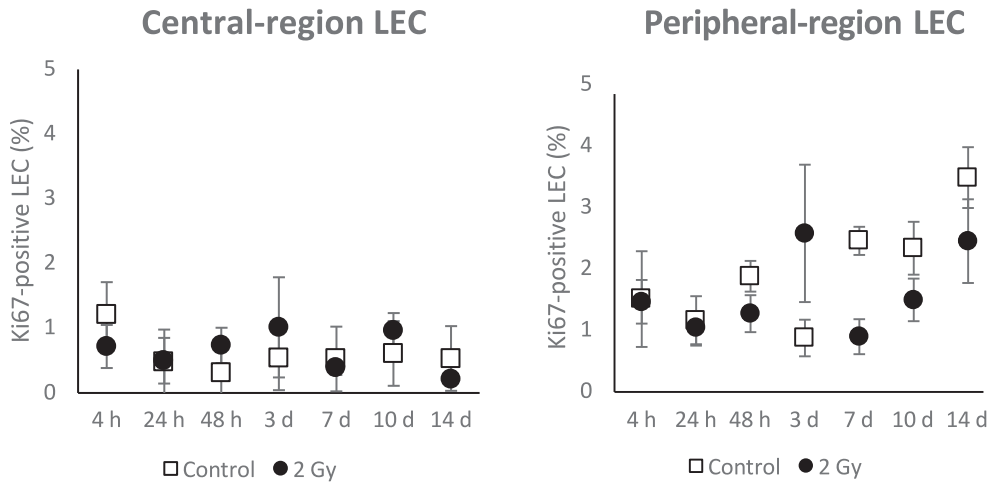

FIG. 3. Percentage of Ki67-positive LEC in the central and peripheral region 4 h to 14 days after 2 Gy X-ray irradiation. SE bars are plotted. Time postirradiation not statistically significant.

with the central LEC expressing significantly less Ki67 positivity than the peripheral region. Dose was highly significant ($P = 0.003$); further post hoc Tukey's pairwise analysis confirmed that LEC of control and 2 Gy WBI mice were significantly different from each other ($P = 0.008$). The 2 Gy irradiated LEC was also significantly different from 0.5 Gy ($P = 0.034$). Figure 4 shows this data, with central and peripheral LEC plotted separately, and for each time point. Radiation doses used in this study were delivered at 0.063 and 0.3 Gy/min.

Statistical analysis of these data showed a significant decrease in Ki67-positive cell percentage in the lens epithelium after 2 Gy irradiation. The significance of LEC region was revealed by ANOVA analysis ($P < 0.001$), suggesting that further interaction analysis with other variables be performed. Of these, only the dose rate and LEC region variables were found to have a very close to significant interaction ($P = 0.054$). Post hoc Tukey's comparison revealed the breakdown of these interactions (Table 1). There were clear significant P values occurring within the various combinations of interactions. For example, each dose rate had a significantly different effect on the central compared to the peripheral region of LEC ($P < 0.001$).

DISCUSSION

Early published studies using high-dose exposures in frogs have reported radiation-induced changes in LEC proliferation rates within 14 days postirradiation (*47*); therefore, the goal of the current work was to determine if significant changes to LEC proliferation could still be observed at lower doses. To investigate the effect of dose rate, radiation was delivered at either 0.063 or 0.3 Gy/min. These dose rates are both considered acute, and were agreed upon as part of the LDLensRad project for calibration and replication at three different institutes.

Ionizing radiation has been demonstrated to affect the lens epithelium when observing proliferation markers 5-ethynyl-2′-deoxyuridine (EdU) and cyclin-D1 in slightly younger (6-week-old) mice (*22*), although the response after doses at different dose rates had not been previously investigated. Lens radiosensitivity is attributed not to radiation-induced cell killing, but to excessive LEC proliferation resulting in abnormal differentiation, impaired DNA repair and non-targeted effects, to name a few effects (*48*). Radiation-induced effects on LEC proliferation in the mouse lens are observable within 24 h after exposure (*22*).

The identification and scoring of both central- and peripheral-region LEC was undertaken because proliferation rates of each differ (*28*), suggesting the radiation response may show regional differences. The results from these inbred C57BL/6 mice support the known organization of the epithelium (*28*); centrally located LEC have a lower proliferating rate, with percentage of Ki67-positive cells being significantly greater in the peripheral region for the lenses that had reached a mature growth pattern. While peripheral-region LEC are known to cycle at an increased rate, this is relatively low (3–5% of LEC; Fig. 4).

LEC proliferation is a slow process, with cells dividing once every 17–20 days (*49*). While proliferative activity was significantly lower in central LEC, a small number of Ki67-positive cells were detected. Proliferating cells in most tissues are known to be radiosensitive (*50, 51*). Of the few studies that document the effects of radiation in the lens *in vivo*, low doses were first shown to increase LEC proliferation in rabbits after 15 Gy X-ray irradiation (*52*). An over-proliferation of LEC followed by an adjustment period where proliferation rate returns to ''normal'' was observed. It has been suggested previously that radiation-induced cataract occurs due to a ''pathological morphogenesis'' in peripheral-region LEC after irradiation. This hypothesis suggested that mitotic changes in LEC, demonstrated by abnormal cell densities in this peripheral region,

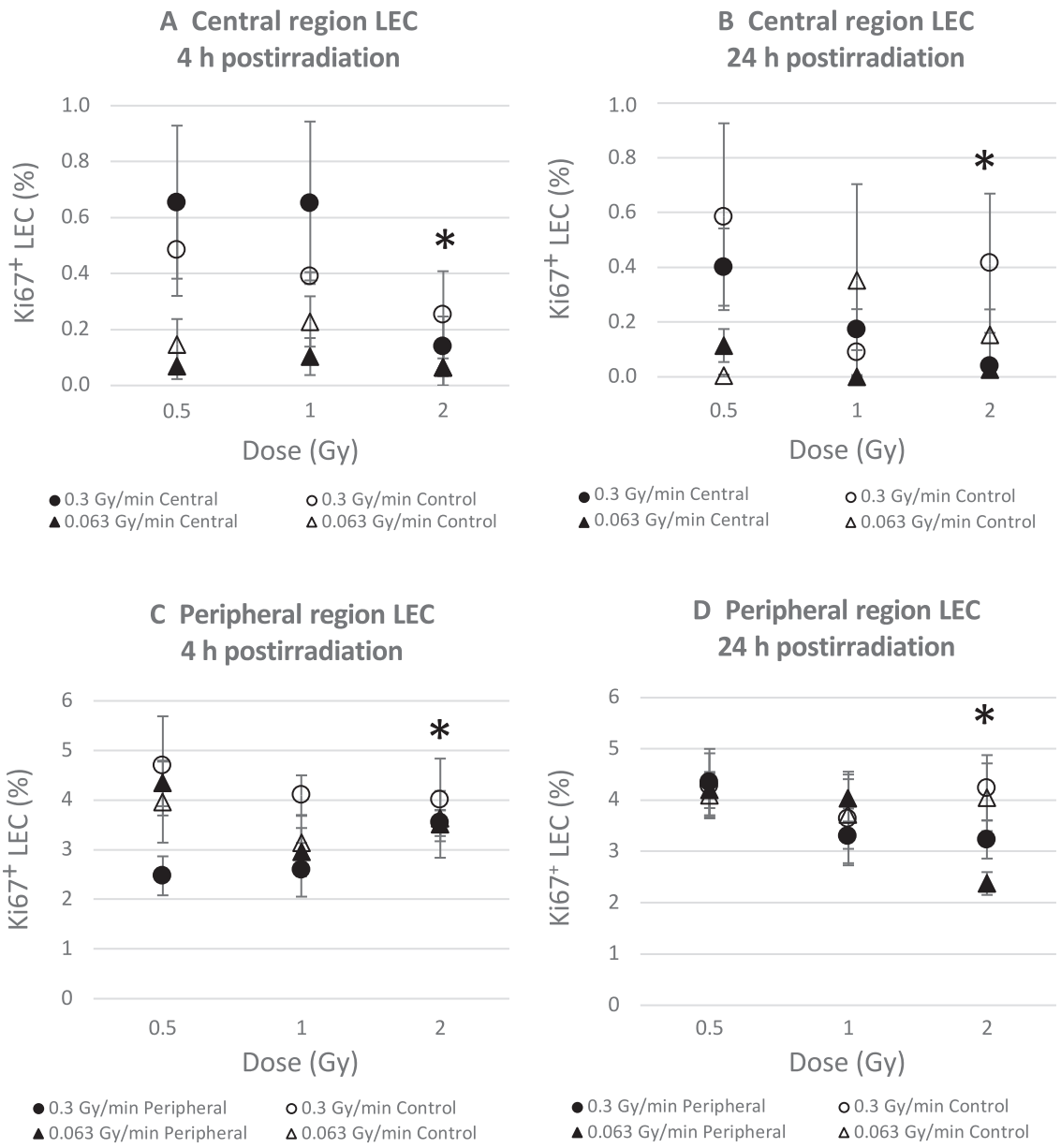

FIG. 4. LEC proliferation response 4 and 24 h after 0–2 Gy (0.063 and 0.3 Gy/min) irradiation. Mean percentage of Ki67-positive cells in the central region of the lens epithelium at 4 h (panel A) and 24 h (panel B), and the peripheral region at 4 h (panel C) and 24 h (panel D) after 0.5, 1 and 2 Gy irradiation at two dose rates with time-matched sham-irradiated controls. Error bars represent standard error. Note the change in y-axis scale from central to peripheral regions. Region of LEC significant ($P < 0.001$). Dose significant ($P = 0.003$). *2 Gy significant from control ($P = 0.008$) and from 0.5 Gy ($P = 0.034$).

TABLE 1
Post Hoc Tukey's Pairwise Comparison Results from Dose Rate (0.063 and 0.3 Gy/min) and Lens Epithelium Region Interactions

Difference of dose-rate*region levels	Difference of means	SE of difference	Simultaneous 95% CI	Adjusted *P* value
(0.063 2)–(0.063 1)	3.551	0.160	(3.140, 3.962)	0.000
(0.3 1)–(0.063 1)	0.243	0.158	(–0.163, 0.650)	0.416
(0.3 2)–(0.063 1)	3.360	0.161	(2.946, 3.774)	0.000
(0.3 1)–(0.063 2)	–3.308	0.159	(–3.717, –2.899)	0.000
(0.3 2)–(0.063 2)	–0.191	0.160	(–0.602, 0.221)	0.633
(0.3 2)–(0.3 1)	3.117	0.158	(2.711, 3.523)	0.000

Notes. Several significant *P* values from interactions are revealed. Individual confidence level = 98.92%. *1 = central region, 2 = peripheral region. SE = standard error.

resulted in cataract. Doses used during the current study can be considered low compared to these seminal studies from a previous century. While no significant effect is observed up to 24 h postirradiation at 0.5 Gy, a dose of 2 Gy resulted in a significant decrease in Ki67-positive cells, most noticeably in the central region (Fig. 4), evidencing that the central-region LEC retain proliferative capacity (*49*). LFC formation requires cell proliferation of LEC in the equatorial peripheral region [specifically the germinative zone (GZ)] of the lens epithelium (*47*). This study suggests that proliferating LEC in both regions are the more radiosensitive, contributing to the mechanism(s) that lead to cataract after irradiation (*1*). Cycling cells are known to be sensitive to radiation-induced damage due to a greater relaxation state of chromatin, and this effect has been linked to the radiosensitivity of equatorial LEC (*29, 53*). A further consideration is that newly differentiating LFC are transcriptionally supercharged, making them susceptible to radiation-induced DNA damage effects. All LFC are terminally differentiated LEC originating in the GZ; any radiation-induced damage which is retained throughout LFC maturation will have significant ramifications in lens homeostasis. Studies of frogs suggest that cataract occurred due to a ''pathological morphogenesis'' in peripheral-region LEC after irradiation (*47*); mitotic changes demonstrated by abnormal cell densities in this region resulted in cataract. However, these changes in peripheral-region LEC density and organization of the meridional rows were seen in frogs with actively dividing LEC (*47*), and also in 6-week-old C57BL/6 mice (*22*). X-ray doses of less than 100 mGy increased LEC proliferation in murine lenses using markers cyclin D1 and EdU labeling, but also the variance increased markedly (*22*).

Radiation causes a disorganization of meridional rows in the lens epithelium. This was first observed after high dose X-ray irradiations of frogs, where radiation-induced cataract was almost always preceded by meridional row disorganization (*47*). Inhibiting proliferation by hypophysectomy (removal of the pituitary gland) resulted in no significant effect of radiation on meridional row organization, evidencing how both cell proliferation and also LEC differentiation can contribute mechanistically to cataract. The study concluded that radiation-induced cataractogenesis must be a result of a mitosis-driven formation of abnormal LFC due to damage or changes within the meridional rows LEC (*47*). Acute X-ray exposure (although at a relatively lethal dose) of rabbit lenses resulted in the disarrangement of LFC in the bow of the lens, including the peripheral-region LEC after one week (*54*). Furthermore, at three weeks postirradiation, a decrease in the number of LEC within the peripheral region was observed, suggesting a lack of cell repopulation (*54*). Rabbit lenses exposed to 15 Gy X rays resulted in a complete inhibition of cell proliferation at 30 min postirradiation, with this inhibition continuing for several days (*52*). Proliferation returned thereafter and continued to increase to an overcompensation approximately one week later and lasting for approximately a further week before returning to baseline. This proliferative ''compensation'' acts as a catch-up mechanism to regain lost mitotic divisions and suggests there are both feed-forward and feed-back mechanisms in play to maintain the linear growth of the adult mammalian lens.

While the methods in this study allow for the observation of proliferative activity using a single marker, this was merely a snapshot of what is occurring at the time of sacrifice postirradiation. Ki67 has been reported to decay during G_0/G_1 cell cycle phase, dependent on cell type, and therefore is a graded rather than a binary marker (*55*). During this study, the effects of radiation are perhaps more obvious within the central-region LEC, where long-term quiescent cells are the easiest to identify as they re-enter the cell cycle after exposure. This perhaps explains the observation of a time lag before effects are visible in the peripheral region, while being more immediate in the central region. Choice of proliferation marker is therefore crucial; future studies should incorporate a panel of proliferation markers to further elucidate the role of cell cycle state depending on the region of LEC both at the time of exposure and whether radiation has an effect on cell cycle checkpoints and progression. The current difficulties to follow or track individual LEC *in vivo* make conclusions regarding cell fate difficult. Strain dependency of the lens in response to radiation has been reported in mice (*18*), and it should be noted that inbred populations of mice are not representative of human and epidemiological cohorts, where the genetic pool is much wider (*56*). Observations of LEC proliferation and cell cycle effects after irradiation can be difficult to correlate from one study to another, especially when a different marker(s) have been used and conclusions of radiation-induced proliferation can be biased depending on the choice of marker and what phase(s) of cell cycle are being observed. Future studies would benefit from incorporating a panel of markers spanning all cell cycle stages to ensure that accurate conclusions can be drawn, and further investigations are needed to understand the possible relationship between altered LEC proliferation and the subsequent impact on differentiation. Advancements in whole lens imaging techniques have recently been reported (*45*), and may provide a useful tool to investigate possible aberrant differentiation as a result of altered proliferation. While this study has used different dose rates during irradiations, both are considered to be acute, and therefore a chronic, or even fractionated, exposure would be desirable during future investigations of dose-rate effects.

In conclusion, the radiation effects on LEC proliferation rates are not as pronounced at the low radiation doses used in this study compared to those historical studies reporting on the effects of high-dose acute radiation. However, a significant reduction in proliferation was observed 24 h after 2 Gy irradiation. Although in this study only two dose rates were investigated, no dose-rate effect was identified. However, the close-to-significant interaction between dose

rate and LEC region identified using post hoc analysis suggests further investigation is needed to understand the differential dose-rate responses occurring depending on LEC region, as observed in the DNA damage response (*25*). This highlights the influence of genotype on the radiation response in the lens, and further highlights the continued need for both low- vs. high-dose radiation studies. Remarkably, the lens epithelium relative cell density measurements at 12 months postirradiation in B6C3F1 mice showed that cell density remained constant over time.

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