This project has received funding from the Euratom research and training programme 2014-2018 under grant agreement No 662287.

EJP-CONCERT

European Joint Programme for the Integration of Radiation Protection Research

H2020 – 662287

D9.43 – Mouse proliferation/morphology studies

Lead Author: DU and PHE

With contributions from: All project partners (DH-PHE, HMGU, ENEA, DU, OBU) and Advisory Board members

Reviewer(s): CONCERT coordination team

Work package / Task	**WP 9 T9.2**	**ST 9.2.4**
Deliverable nature:	**Report**	
Dissemination level: (Confidentiality)	**Public**	
Contractual delivery date:	**M49**	
Actual delivery date:	**M49**	
Version:	**1**	
Total number of pages:	**6**	
Keywords:	**Ionising Radiation; Lens; Cataract; Mouse Models; Lens epithelium; cellular organisation**	
Approved by the coordinator:	**M49**	
Submitted to EC by the coordinator:	**M49**	

Abstract

The lens of the eye is more radiosensitive than previously thought, but the mechanism(s) that lead to low dose radiation cataract have yet to be fully elucidated. This is now a critically important knowledge gap for radiation protection because of the recent substantial reduction in occupational dose limits as a result of epidemiological information and reanalyses. The public health issues concern medical radiation workers, many of whom will likely need to amend their working practices as our understanding of the underlying process and ultimate effects of chronic, low dose, ionising radiation exposure becomes more complete.

The LDLensRad project brings together experts from across Europe to answer a number of key research questions on this topic, including: how does low dose radiation cause cataracts; is there a dose rate effect, and how does genetic background influence cataract development after radiation exposure. CONCERT Deliverable D 9.43 of the project, describes methods currently being employed to assess how ionising radiation affects the proliferation rate and the cellular organisation in the lens epithelium. The data are currently being collated and will be analysed for submission for publication.

Progress summary

1 Introduction

In 2012, the International Commission on Radiological Protection (ICRP) reduced its recommended ionising radiation (IR) threshold of 2 Gy for acute exposure and 5 Gy for chronic exposure to a threshold of 0.5 Gy independent of the IR dose rate, to prevent vision impairing cataracts (1). One of the most radiosensitive tissues in the human body is the eye lens, and cataract development has been associated with IR exposure. However, the ICRP acknowledged that our understanding of the biological processes causing cataracts at low-dose IR levels is yet to be fully elucidated.

The eye lens is an avascular, elastic, transparent tissue, which along with the cornea, refracts the incoming light onto the retina. A single layer of lens epithelial cells (LECs) attached to the lens capsule covers the anterior hemisphere of the eye lens. The lens epithelium is divided into central (CZ), germinative (GZ) and transition (TZ) zone. In the central zone, the cells are largely quiescent, whilst proliferation of the LECs peaks in the germinative zone (2). Differentiation of LECs into lens fibre cells (LFCs), which form the bulk of the lens, occurs in the transition zone where the cells eventually align to form the characteristic meridional rows (MR) before being internalized into the body of the lens (figure 1a and b, adapted from Uwineza *et al.* 2019 (3)). The development and maintenance of optical transparency, refraction and elasticity is vastly dependent on these processes. Disruption of these leads to cataractogenesis.

The GZ and TZ at the lens equator are considered the most radiosensitive regions in the lens epithelium (4). Multiple studies on the influence of IR on the lens epithelium showed that exposure to levels >2 Gy cause increased proliferation, aberrant LEC differentiation and organisation in the germinative and transition zone. Only recently have the effects of doses <0.5 Gy been investigated and initial *in vitro* and *in vivo* radiation experiments show changes in proliferation and cell density at these low doses in the germinative and transition zone. We aim to expand our comprehension of the consequences of low dose IR-exposure by studying cell density through 3D-imaging and proliferation studies. For comparison of this advanced imaging technique, traditional flat mounting (5, 6) with proliferation marker Ki67 (7, 8) is performed.

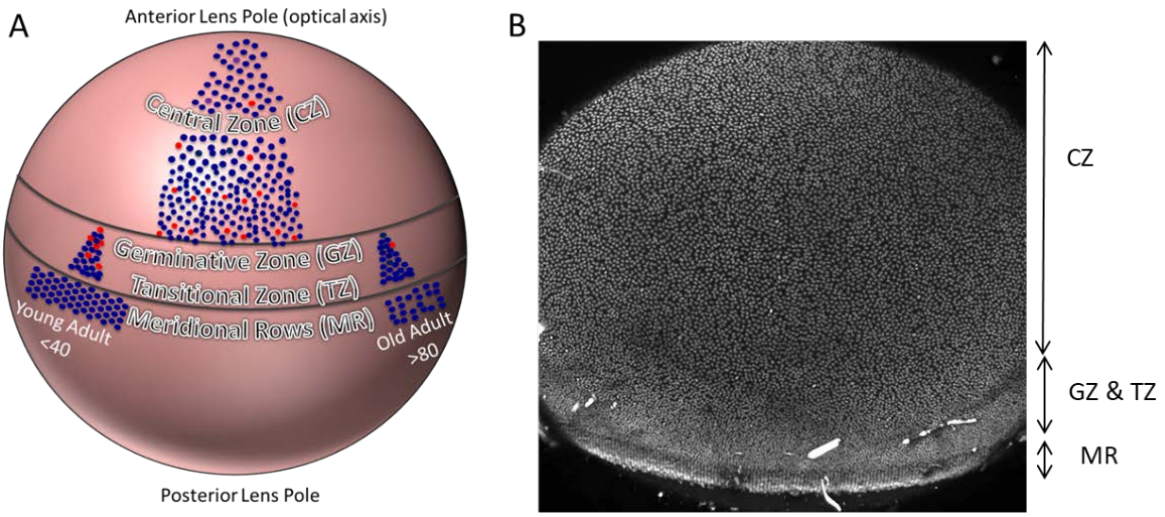

***Figure 1**: (A) Schematic of the epithelium that covers the anterior hemisphere of the eye lens. (B) Example of a mouse lens stained with DAPI to identify cell nuclei in the lens epithelium. The lens is orientated in the same way as the schematic. Notice the change in cell density between the different zones and the organization in the meridional rows.*

2 Methods

To understand how LECs manage IR induced stress, nominally radio-sensitive CD1 and radio-resistant C57BL/6J (Ptch1+/- and wild type (WT) mice) were exposed to 0.5, 1 and 2 Gy using 60Co gamma-rays (broad beam, whole body) at 0.3 and 0.063 Gy/ min dose rates. Moreover, C57BL/6J x C3HeB/FeJ (WT and Ercc2+/-) and 129 Sv were also exposed to 0.5, 1 and 2 Gy using 60Co gamma-rays (broad beam, whole body) at only 0.3 Gy/ min dose rate. Subsequently, the animals were returned to their cages and received standard care. Mice were sacrificed 24 hours, 4 months and 12 moths post irradiation. The eyes were removed and eye lenses collected using a dissection microscope. Lenses were then fixed in paraformaldehyde and stained with Hoechst to stain nuclei (Figure 1b). Images were collected using a Confocal Laser Scanning Microscope in conjunction with software analytical tools such as LAS-X, Image J. An in-house written cell density calculating MatLab program was used to analyse the cell density in the captured images. Alongside 3D whole mount imaging, isolated and flat mounted epitheliums were stained with proliferation marker Ki67, DNA double stranded breaks marker 53BP1 and nuclear counterstain DAPI (figure 2, as part of deliverable 9.42 DNA DAMAGE).

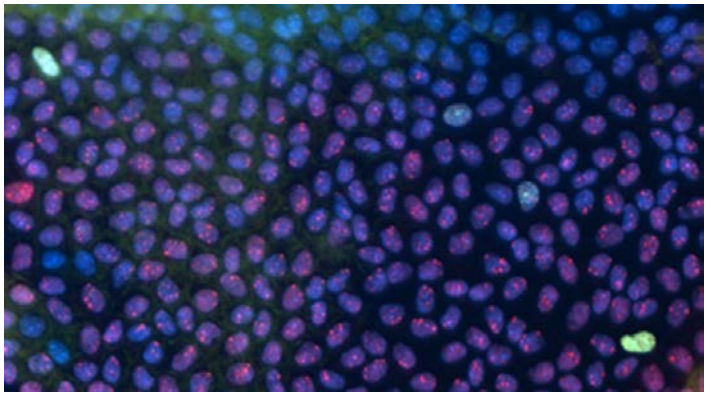

Figure 2: *Dual immunofluorescent staining of 53BP1 (DNA DSB marker) and proliferation marker Ki67 in flat mounted lens epithelium*

The results are currently being collated and prepared for submission for publication.

3 Discussion

3D-imaging is a methodological breakthrough for studying the lens epithelium. Marckiewicz and colleagues (5, 6) used flat mounts to study IR-induced changes in the LECs. The latter technique is very challenging, requiring highly trained individuals, and is extremely laborious (due to the need for overnight data accumulation). With our whole mount approach, the technical challenges and data collection times are significantly reduced enabling us to more easily study the changes in cell density across the different zones in the epithelium in larger animal cohorts. Flat mounts are still vital as this approach ensures complete accessibility of the epithelium by exposing these cells directly. The LECs are still covered by the lens capsule when deploying whole-mount staining, which can affect some staining protocols. Alongside investigating the effects of IR on LEC proliferation and cell organisation, comparisons between the two observational techniques discussed above can be drawn.

Epidemiological data show that there can be a very long latency period between low dose IR-exposure and cataract development (1). This accentuates the importance of longitudinal studies to understand IR-induced cataract. Our experimental design allows us to study short and long-term effects, and therefore to identify new tissue-defined characteristics that occur early after IR-exposure associated with cataract development later in life.

Bibliography

1. Stewart FA, Akleyev AV, Hauer-Jensen M, Hendry JH, Kleiman NJ, Macvittie TJ, et al. ICRP publication 118: ICRP statement on tissue reactions and early and late effects of radiation in normal tissues and organs--threshold doses for tissue reactions in a radiation protection context. Annals of the ICRP. 2012;41(1-2):1-322.
2. Song SH, Landsbury A, Dahm R, Liu YZ, Zhang QJ, Quinlan RA. Functions of the intermediate filament cytoskeleton in the eye lens, J Clin Invest. 2009;119(7):1837-48.
3. Uwineza A, Kalligeraki AA, Hamada N, Jarrin M, Quinlan RA. Cataractogenic load - A concept to study the contribution of ionizing radiation to accelerated aging in the eye lens. Mutation research. 2019;779:68-81.
4. Markiewicz E, Barnard S, Haines J, Coster M, van Geel O, Wu W, et al. Nonlinear ionizing radiation-induced changes in eye lens cell proliferation, cyclin D1 expression and lens shape. 2015;5(4):150011.
5. Barnard SGR, Moquet J, Lloyd S, Ellender M, Ainsbury EA, Quinlan RA. Dotting the eyes: mouse strain dependency of the lens epithelium to low dose radiation-induced DNA damage. Int J Radiat Biol. 2018:1-9.
6. Markiewicz E, Barnard S, Haines J, Coster M, van Geel O, Wu W, et al. Nonlinear ionizing radiation-induced changes in eye lens cell proliferation, cyclin D1 expression and lens shape. Open biology. 2015;5(4):150011.
7. Denkert C, Budczies J, von Minckwitz G, Wienert S, Loibl S, Klauschen F. Strategies for developing Ki67 as a useful biomarker in breast cancer. Breast (Edinburgh, Scotland). 2015;24 Suppl 2:S67-72.
8. Jurikova M, Danihel L, Polak S, Varga I. Ki67, PCNA, and MCM proteins: Markers of proliferation in the diagnosis of breast cancer. Acta histochemica. 2016;118(5):544-52.