



Cellular debris and ROS in age-related cortical cataract are caused by inappropriate involution of the surface epithelial cells into the lens cortex

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Purpose: To quantify changes in the lens epithelial cells and underlying lens cortex responsible for age-related cortical cataract (ARCC) in the rat.

Methods: Freshly isolated lenses were stained vitally for DNA with Hoechst 33342. Reactive oxygen species (ROS) and mitochondria were visualized and quantified by dihydrorhodamine 123 (DHR). The fluorescence was quantified using Laser Scanning Confocal Microscopy (LSCM) of vitally stained lenses. Cortical DNA was verified as such by DNase I digestion. Cataract reflections were determined from digitalized images of light reflections taken with a low magnification light microscope, or with the LSCM.

Results: The anterior surface epithelia of old rat lenses were full of gaps and ragged in appearance with a decrease of over 50% in lens epithelial cell (LEC) density. The surface LECs were frequently seen to have involuted into the cortex at inappropriate sites, forming deposits full of DNA, nuclear and mitochondrial debris, and abundant ROS. These involutions frequently originated near open gaps in the surface epithelia, where they appear to have detached from the capsular membrane. Cortical cataracts in the rat lenses were seen to co-localize with these LEC involutions, as had been seen previously in mice with ARCC.

Conclusions: ARCC in rats co-localized with inappropriate accumulations of nuclei, mitochondria, DNA, and expression of ROS in debris filled foci. These were the result of both involution of surface LECs into areas of cortical ARCC, and by an extension of the normal bow region deep into the anterior and posterior of cataractous lenses. These results were in complete agreement with our previous studies on ARCC in mice.

Age-related cortical cataract (ARCC) develops in almost all mammalian and avian species so far examined, and is the most common cause of human blindness world-wide [1-5]. In humans, the presence and degree of advancement of cataracts (especially cataracts in the outer cortical part of the lens, here designated ARCC) are inversely related to the mean lifespan of the study subjects, with or without the presence of diabetes [6-9]. We and others have previously shown that a wide variety of normal mice and rats develop ARCC, and that ARCC is delayed in three rodent models with extended life spans: calorie-restricted mice and rats, growth hormone knock-out (GHRKO) mice, and transgenic antioxidant-protected mice, indicating that its incidence is related to the rate of aging in a wide variety of species [10,11].

Several changes in aging lenses have been reported to be associated with ARCC. These include alterations in lens crystallins and damage by reactive oxygen species (ROS) in humans [12-14], and in mice and rats [15-19]. Damage to the lens surface epithelial cells (LECs) has also been reported to occur with ARCC [20,21]. However, the detailed mechanism of age-related cataract in any species remains to be fully defined [22-24].

In normal young animal lenses, only the anterior lens surface is covered by nucleated LECs. LECs on the mid anterior surface (central region) are relatively non-dividing in adults. Between the central zone and equator of the lens, the LECs divide, forming a proliferative zone. Equatorial to this proliferative zone, LECs withdraw from the cell cycle, elongate, enter the lens interior, and begin a program of differentiation [25-28]. As LECs begin to form lens fiber cells at the equator, they are buried deeper in the cortex by newly arriving LECs forming the “bow region”. LECs in the bow region undergo a process whereby their intracellular organelles are degraded by an apoptotic-like process forming clear lens fiber cells. This results in the formation of an “organelle free zone” (OFZ) consisting of the lens nucleus and inner cortex. In the normal adult lens, nuclei are only seen on the anterior surface and in the bow region where they are normally degraded. A residual layer of cortical mitochondria persists in the outer rim of the lens cortex even in the polar regions of the adult lens [28,29]. Maintenance of this OFZ is necessary for normal function and clarity of the adult lens. However, our previous studies demonstrated that in old cataractous mouse lenses, surface LECs have migrated inwards into the normally clear cortex forming deep channels filled with nuclear and mitochondrial debris, DNA, and ROS. These involutions of surface cellular material co-localize with areas of cortical cataract [30]. In the work reported here, we have extended these studies to include young (4-5 months) and old (25-31 months) Brown-Norway rat

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lenses, and report a nearly identical phenomenon. We propose that this condition may be the underlying cause of age-related cortical cataract in various mammalian species.

METHODS

Vital dyes: All dyes were obtained from Molecular Probes, Inc. (Eugene, OR). Hoechst 33342 was kept as a 10 mM stock in water at 4 °C. Dihydrorhodamine 123 (DHR) was kept as a 5 mM stock in DMSO at -20 °C. Propidium iodide (PI) was kept as a 2 mM stock in water at 4 °C.

Rats: Both lenses from each of 13 young (4-5 months) and 13 old (25-31 months) Brown-Norway rats were used in the various studies. The rats were obtained from the National Institutes of Aging colony and were maintained under specific pathogen-free conditions. The rats were sacrificed by carbon dioxide inhalation. At all times we utilized protocols for animal research of the University of Washington Institutional Animal Care Committee, and the American Association for Laboratory Animal Science (AALAS).

Lens preparation and microscopy: Whole eyes were placed corneal side down on sterile gauze and held in place by forceps. An incision was then made across the surface where the optic nerve enters the eye, and the sclera was pulled back to expose the lens. Debris from the ciliary body that remained attached to the equatorial plane of the lens was gently teased away with forceps before staining. As described separately below, 3 different microscopes were utilized, a low-power light microscope for whole lens pictures and cataract assessment, a BioRad Laser Scanning Confocal Microscope (LSCM) used to analyze PI stained parformaldehyde-fixed lenses before and after DNase, and a Zeis two-photon LSCM used to analyze lenses vitally stained with Hoechst 33342 and DHR.

DNase sensitivity of Propidium Iodide and Hoechst positive staining material in cortical inclusions: Heavily cataractous old rat lenses (both posterior and anterior subcapsular cataracts extending deep into the cortex) from old (27 months) rats and young (5 months) noncataractous lenses were fixed by graded alcohol fixation, dried, and post-fixed with 0.5%

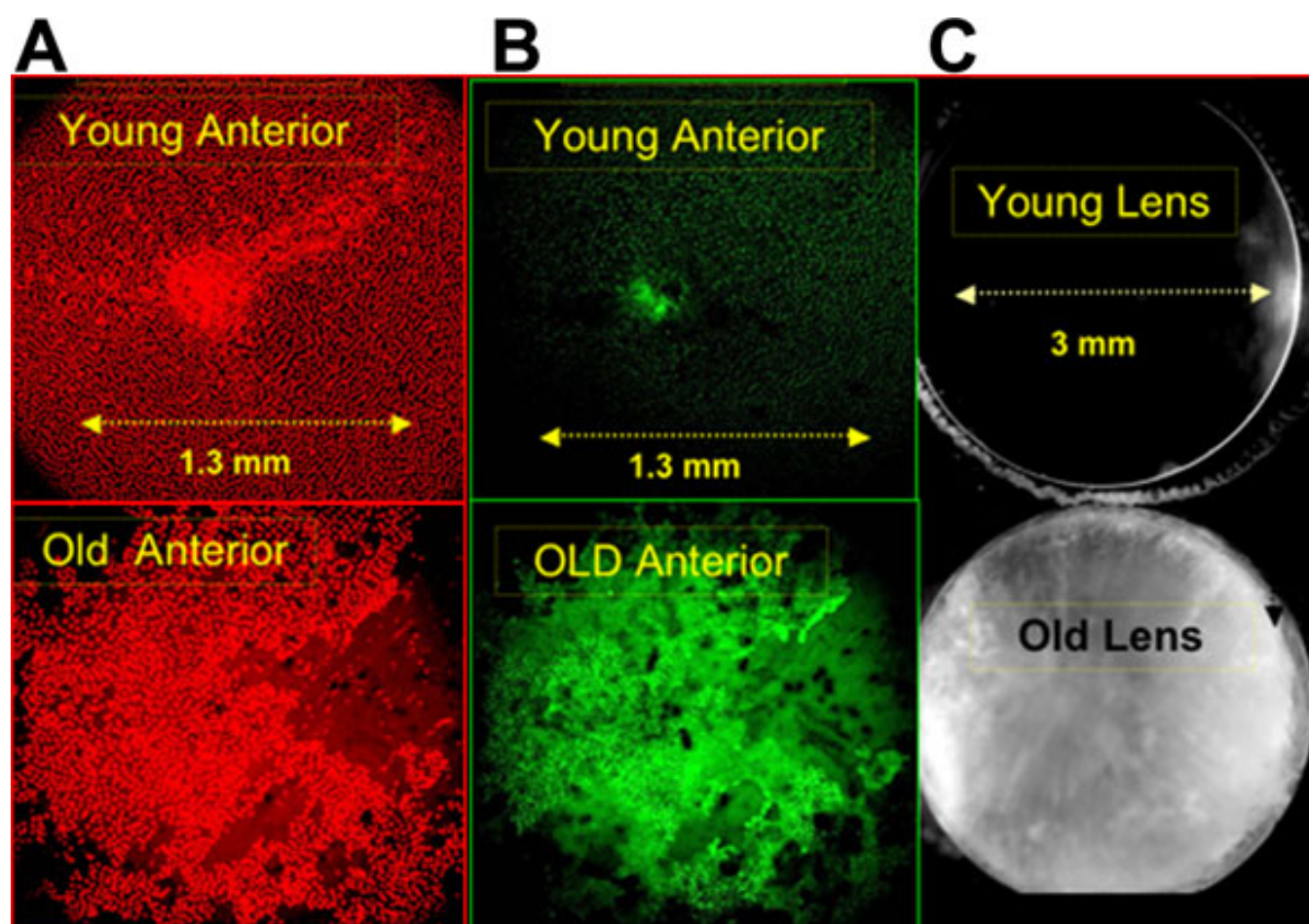


Figure 1. A comparison of lens anterior surfaces of young and old rat lenses vitally stained for DNA and ROS. **A:** Comparison of DNA staining with Hoechst 33342 (computer coded **red**) of the anterior surface of young and old lenses. **B:** Comparison of vital staining for ROS fluorescence (with DHR shown in **green**) of the same surface areas. **C:** Comparison of low magnification photos taken with a reflected light microscope of the anterior sides of the same young and old lenses. Each LSCM image is a projection of the average of 25 separate LSCM frames scanning from the mid anterior surface inwards 100 μ m. The confocal settings are identical for young and old pairs so that relative comparisons of DNA fluorescence and cataract reflectance can be made.

paraformaldehyde in PBS for 5 min to stabilize DNA during the succeeding incubations. Longer fixation (15 min or more) blocked DNase digestion. The post-fixed lenses were rinsed in Dulbeccos Modified Eagles Medium (DMEM) with 25 mM HEPES and without bicarbonate (Invitrogen, Carlsbad, CA), dehydrated with graded alcohols and stored at -20 °C. Prior to digestion, the dried lenses were marked with inkblots to determine orientation following digestion when little DNA remains. Lenses were then stained with 2 µg/ml Propidium Iodide (PI) in DNase buffer (20 mM Tris pH 8.5, 2 mM MgCl₂) for 15 min at 37 °C. The DNA in and on the stained lenses was analyzed prior to DNase using the Biorad LSCM and the images stored, and then the lenses were treated with 0.4 ml of 200 mg/ml RNase free DNase I (Sigma-Aldrich, St. Louis, MO) dissolved in DNase buffer for 30 min at 37 °C to digest DNA. The lenses were then reanalyzed with the BioRad LSCM for any residual DNA. The position of the previously analyzed areas was determined from the orientation of inkblots. More than 90% of the PI fluorescence was removed by the DNase treatment.

Staining of freshly isolated lenses with Hoechst 33342 and DHR: The rat lenses were viably stained with DHR and Hoechst 33342 as previously described for mouse lenses [30]. DHR (5 mM) was added to the lenses presently in 10 ml of lens medium (DMEM containing 25 mM HEPES with 7% fetal bovine serum) chilled to 0 °C for 45 min with mild agitation. Staining of the lenses with DHR was performed at 0 °C (but not frozen) to optimize differential staining of the abnormal ROS present in cataractous lenses with that produced by normal respiration of surface LECs at 37 °C. DHR staining of the rat lens inclusions at 0 °C was just as intense as when done at 37 °C. Following DHR treatment, the lenses were rinsed briefly in lens medium, to remove free DHR, and stained with 10 µM Hoechst 33342 in lens medium at 37 °C for 15 min as previously described [30]. Freshly isolated metabolizing lenses were placed in chambered slides (Nalge Nunc International, Naperville, IL) in 2.0 ml of lens medium. The lenses were then photographed with a low power light microscope and chilled to 0 °C to stabilize the DHR and the DNA fluorescence during analysis with the LSCM. The doubly stained

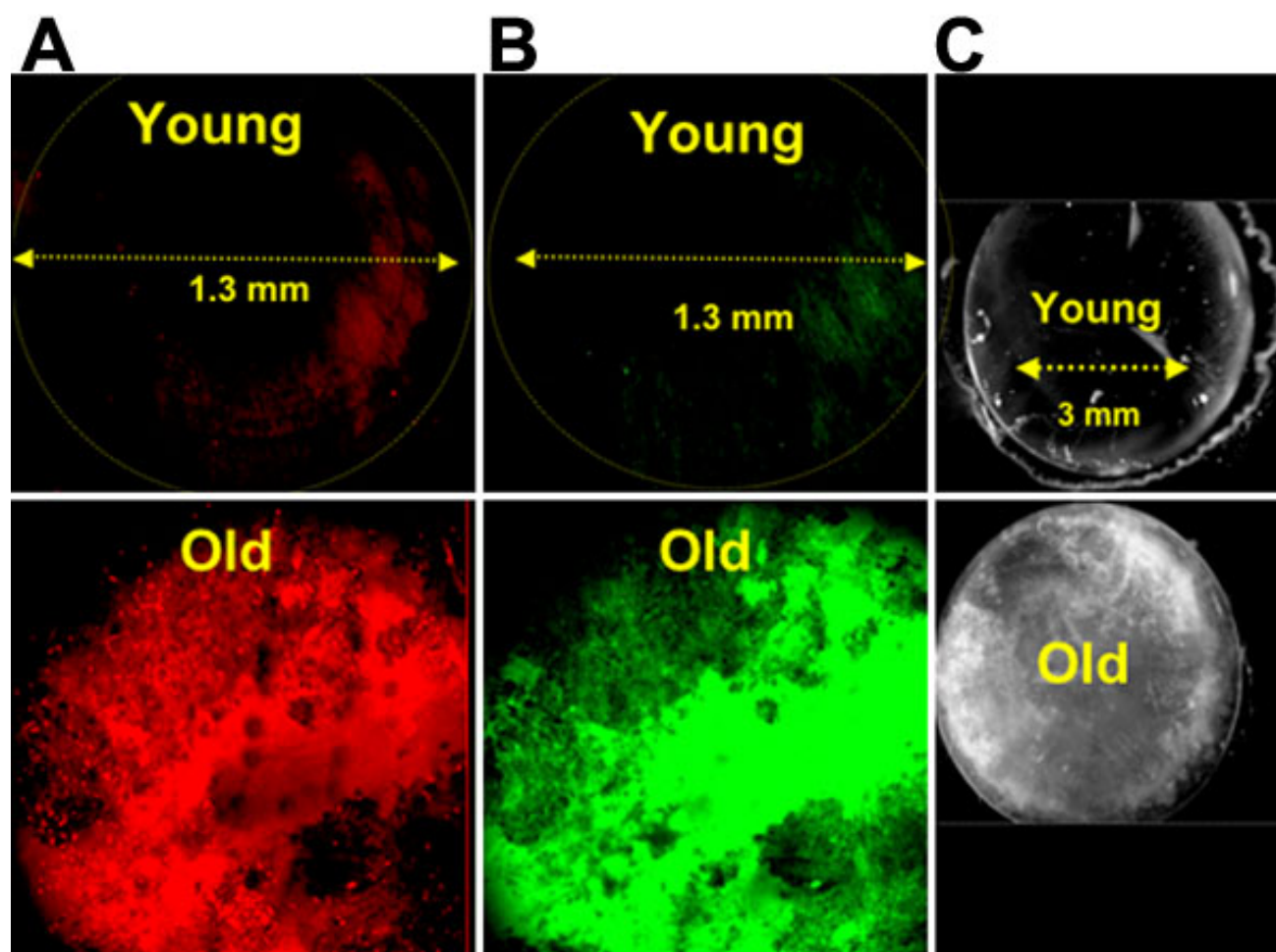


Figure 2. LSCM projections of typical posterior surfaces of young and old rat lenses stained for DNA and ROS. **A:** Vital staining of posterior cortical DNA with Hoechst 33342 (fluorescence computer coded as red) of a young clear (5 months) compared to an old (31 months) cataractous rat lens. **B:** Vital staining of the same lens posteriors for ROS with DHR (computer coded green). **C:** Low power microscope photos of posterior cataract reflectance from the same lenses.

lenses were then kept on ice until analyzed with the LSCM. The fluorescence of the stained lenses was stable for at least 3 h on ice.

Lens opacity measurements with low-power reflecting light microscope: The intensity of light reflected from lens opacities was quantified by 2 methods; either by image analysis (Photoshop version 7.01, Adobe Systems, San Jose, CA) of digital photographs of the lenses taken with a low magnification reflected light microscope, or by using a separate reflected light channel on the LSCM. The anterior side was determined from prior visualization of the Hoechst 33342 stained anterior epithelium under a fluorescent microscope. The lenses were then centered in the field of a low power light microscope (ZST; Unitron, Bohemia, NY), equipped with microscope adapter (MM3XS; Martin Microscope, Easley, SC), and photographed with a digital camera using 16x magnification (Coolpix 5400; Nikon, Tokyo, Japan). Back lighting was provided by a tungsten light source (T-Q/FOI-1; Techni-Quip Hollywood, CA) with dual light guides positioned for side

lighting. The lenses were then turned upside down, to photograph the posterior sides. The conditions were identical for young and old lenses photographed on each day.

LSCM scanning of vital staining with Hoechst 33342 and DHR: A Zeiss 2-photon LSCM (model 510 NLO, Carl Zeiss MicroImaging, Inc., Thornwood, NY) was used for analysis of whole lenses vitally stained with the DNA fluorochrome Hoechst 33342 (10 mM) and 5 mM DHR. For Hoechst 33342, the two-photon laser (Mira 900 IR femtosecond pulse laser 50 mW Titanium Sapphire; Coherent Inc., Santa Clara, CA) was tuned to 750 nm. Emitted light was passed through a primary dichroic mirror passing light only below 650 nm, a secondary optic passing light only below 490 nm, and finally a bandpass filter near the Hoechst 33342 optima between 435 and 485 nm. The DHR (oxidized by ROS to rhodamine 123 inside the lens fibers) was analyzed using the 488 nm line of an argon laser (5 mW, run at 60% of full power; Lasertechnik GmbH, Berlin, Germany). Emitted light was selected with a primary dichroic that blocked the 488 nm exciting light, a secondary

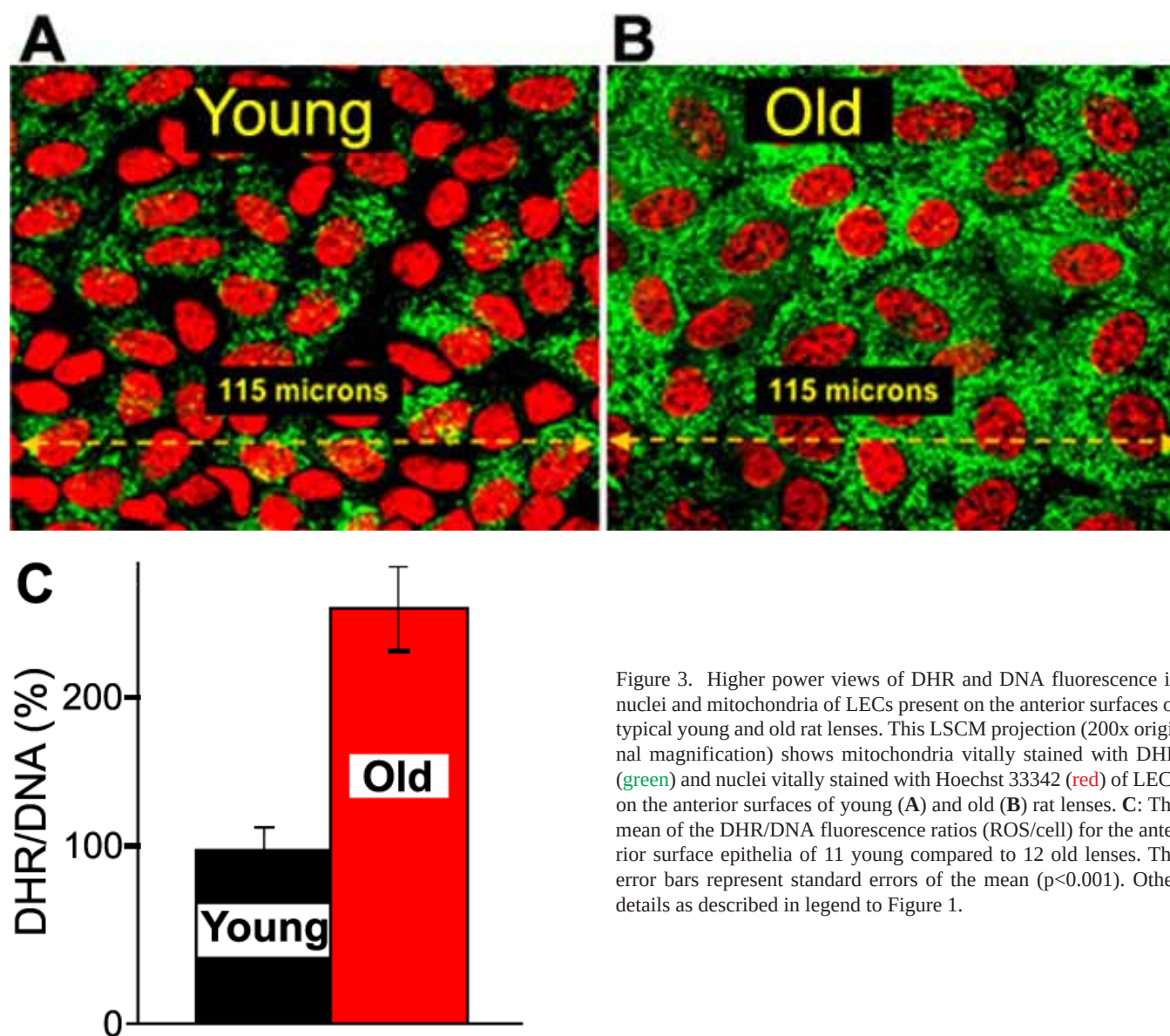


Figure 3. Higher power views of DHR and DNA fluorescence in nuclei and mitochondria of LECs present on the anterior surfaces of typical young and old rat lenses. This LSCM projection (200x original magnification) shows mitochondria vitally stained with DHR (green) and nuclei vitally stained with Hoechst 33342 (red) of LECs on the anterior surfaces of young (A) and old (B) rat lenses. C: The mean of the DHR/DNA fluorescence ratios (ROS/cell) for the anterior surface epithelia of 11 young compared to 12 old lenses. The error bars represent standard errors of the mean ($p < 0.001$). Other details as described in legend to Figure 1.

optic that transmits light waves shorter than 635 nm, a tertiary optic that transmits light waves longer than 490 nm, and a band-pass filter near the rhodamine 123 optima between the 500 and 550 nm wavelengths. In some cases, a separate reflection channel was set up that measured 543 nm light reflected from opacities. This was performed with the 543 nm excitation line from the Helium-Neon laser at 10% power, and detecting reflected light passing through a 500-550 nm band-pass filter. Only one fluorochrome was excited at a time per frame scan to assure that only the desired probe was visualized. Unless otherwise specified, analyses were performed at both the anterior and posterior poles of the lens using a 10x objective and scanning 26 frames at 4 mm intervals from an area of the lens 1.3 mm in diameter beginning at the surface and ending 100 mm deep beneath the surface. Freshly isolated viable lenses were used for all vital staining.

Image analysis of LSCM data: The LSCM data from the two LCSMs (Carl Zeiss Microimaging, Inc. and Bio-Rad) were downloaded to a computer (Macintosh; Apple Computer,

Cupertino, CA) and analyzed with the NIH program Image J developed by Wayne Rasband (National Institutes of Health, Bethesda, MD) as previously described [30].

Normalization of DNA, DHR, and sight reflectance readings to young control subjects: Measurements were made on two to four old lenses and two to four young lenses at a time (per LSCM session). To control for possible changes in staining or instrument sensitivities for the 3 months over which the measurements were made, the values of DNA, DHR, and reflectance for each lens was normalized to (divided by) that of the two young lens controls measured on each day. All results were presented as a percentage of the young control animals unless otherwise stated.

Statistics: Regression coefficients between cataract scores and DNA and DHR were made using the linear regression program on computer (SPSS version 11 for Macintosh; SPSS Inc., Chicago, IL). A 2 tailed Mann-Whitney nonparametric (also from SPSS) was used for comparing young and old groups of mice.

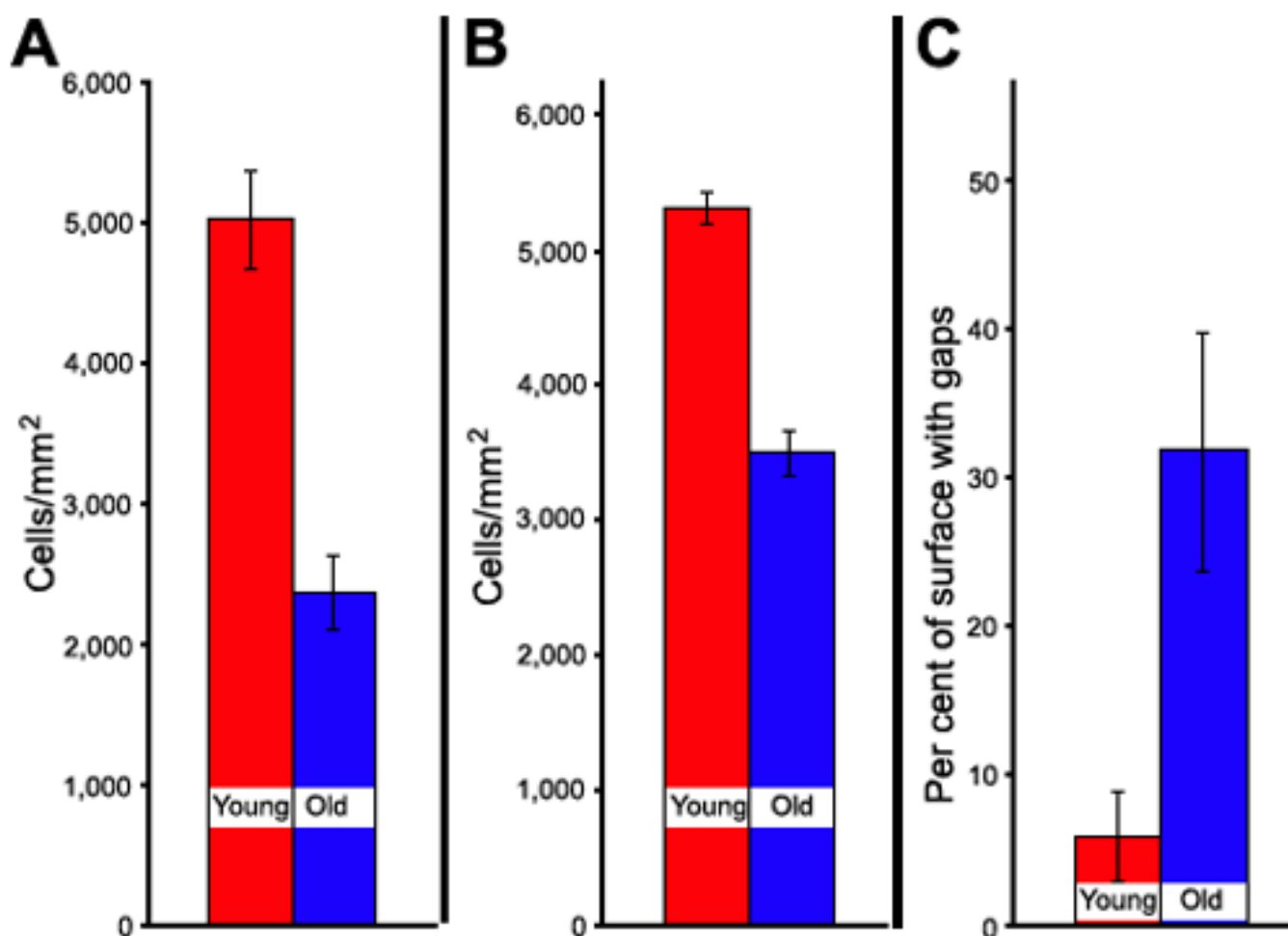


Figure 4. Age-related loss of surface LECs from old rat lenses. The relative nuclear density (nuclei/mm²) is shown for the mid central surface epithelia of 11 pairs of lenses from each of 11 young and 12 old rat lenses analyzed. Images show the nuclear densities including epithelial gaps (A), nuclear densities excluding epithelial gaps (B), and the fraction of cell surface that was gapped (C). The error bars represent standard errors of the mean ($p < 0.001$). Other details as described in legend to Figure 3.

RESULTS

Most, but not all of the old rat lenses were cataractous, while none of the young rat lenses were. Importantly, confocal virtual slices of the old cataractous lenses displayed a reduction in epithelial coverage of the front half of the lens, often accompanied by large non-epithelialized gaps. In lenses with anterior cortical cataracts, strands of epithelial cells, originating from the surface, were always seen descending into the lens interior, and these inclusions encompassed light-reflecting cataractous regions. These were not present in cataract free clear lenses. These inclusions contained broken nuclear elements, free DNA, and mitochondria containing localized ROS. Figure 1 shows typical examples of vital staining of the anterior surfaces of a clear young lens and old cataractous rat lens (initial magnification, 100x) for DNA (red) and ROS (green) with a comparison to a low power light microscope photo showing cataract reflections from the same lenses (the colors displayed are computer generated). Note that young surfaces have a relatively uniform covering of LECs (small red dots) whereas the old lenses have a lower density of surface LECs with frequent gaps, and with DNA-positive diffuse material (cloudy red areas) visible in the cortical areas be-

neath the gaps along with the nuclear debris (Figure 1A). The young lenses are relatively free of ROS expression both on the surface and in the underlying cortex, whereas, ROS is heavily expressed in old cataractous lenses both on the surface and in the underlying cortex (Figure 1B). This is quite similar to that we have reported previously for ARCC in mouse lenses [30].

The posterior surfaces of vertebrate lenses lack an epithelium. Nevertheless, as shown in Figure 2, the posterior cortical regions of cataractous old lenses were also filled with nuclear and mitochondrial debris and free DNA, while the same regions of young rat lenses (as well as those old rat lenses lacking cataracts) were relatively free of both DNA and ROS. In some cataractous lenses, the posterior debris could be seen to originate from the bow region (not shown) in other cases it may originate from migration posteriorly of anterior involutions.

Figure 3A,B are typical higher power (20x objective) views of the continuous (gap-free) surface epithelia of young (Figure 3A) and old (Figure 3B) rat lenses. The figure displays the typical heavier DHR staining of the old rat LECs compared to young LECs even in areas free of surface gaps.

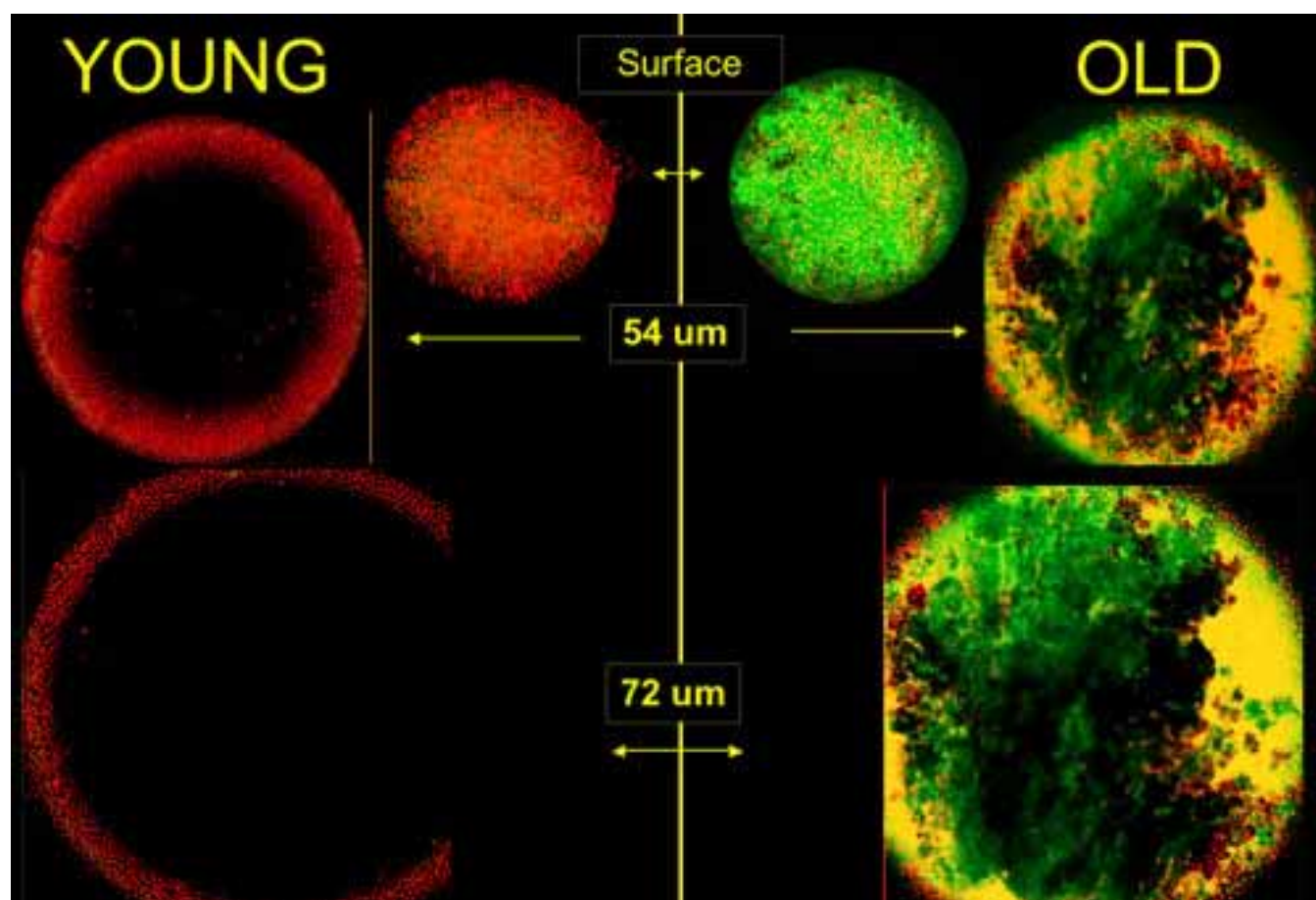


Figure 5. LSCM scans through the anterior cortex of young (5 months) and old (31 months) rat lenses showing vital staining for DNA and DHR at three different focus levels. The top row of images is of DNA (Hoechst 33342, red) and ROS (DHR, green) fluorescence at the anterior surface, the second row is focused 53 μm below the anterior surface, and the bottom row is focused 72 μm below anterior surface.

Also visible is the typical lower density of LECs on the surface of old rat lenses (compare Figure 3A,B). DHR is oxidized to free rhodamine 123, a lipophilic anionic dye, which is concentrated in mitochondria by the negative mitochondrial membrane potential of the mitochondria [31,32]. Mitochondrial localization is plainly visible in lenses of both age groups. Figure 3C displays the average value of DHR/DNA content in areas of continuous surface epithelia from young compared to old rat lenses, showing that ROS (DHR staining) per unit of DNA was increased by 250% in old lenses. No significant increase in DNA/cell was detected for one group compared to the other, so this can be interpreted as a significant increase in ROS/cell in the old rat surface LECs. In addition to the increased DHR per cell, the density of LECs on the surface of old rat lenses (nuclei/mm²) was reduced by 51% relative to young rat lenses (Figure 4A; $p < 0.001$). Much of this was due to the presence of epithelial gaps devoid of LECs in the old rat lens, which average 30% of the anterior surface of old lenses (Figure 4C; $p < 0.001$). However, even in the continuous areas of the old rat epithelia, the cell densities were significantly reduced compared to young rat lenses (Figure 4B; $p < 0.001$).

Age-related changes were even more dramatic in the cortical areas beneath the anterior surface, where diffuse DNA

and ROS were seen to penetrate deep into the cortical areas, accompanying cellular involutions from the surface. Figure 5 shows typical DNA and DHR fluorescence in inclusions at different depths beneath the anterior surface of young and old ARCC rat lenses. The young lenses are relatively debris free and the interior has little ROS. Old cataractous rat lenses have high levels of DNA, nuclear debris, and ROS. Analysis of confocal frames progressively deeper beneath the surface showed that the cortical DNA staining was due to undegraded or partially degraded nuclei, and free cytoplasmic DNA from LECs that had detached from the lens membrane and migrated down into the cortex, as we have previously reported for mouse ARCC [30]. In Figure 6, the DNA fluorescence is shown separately (Figure 6A) to more clearly reveal the DNA (red) connections to the surface LECs. This was very similar to that seen in ARCC in old cataractous mouse lenses [30].

To demonstrate that the Hoechst 33342 stained material was indeed DNA, we fixed some old cataractous lenses with alcohol, stained them with PI and treated with DNase I. PI was used instead of Hoechst 33342 for DNase sensitivity since Hoechst 33342 is not retained in fixed material. As shown in Figure 7, DNase I was able to degrade the DNA in both the surface nuclei and in cataractous cortical inclusions just be-

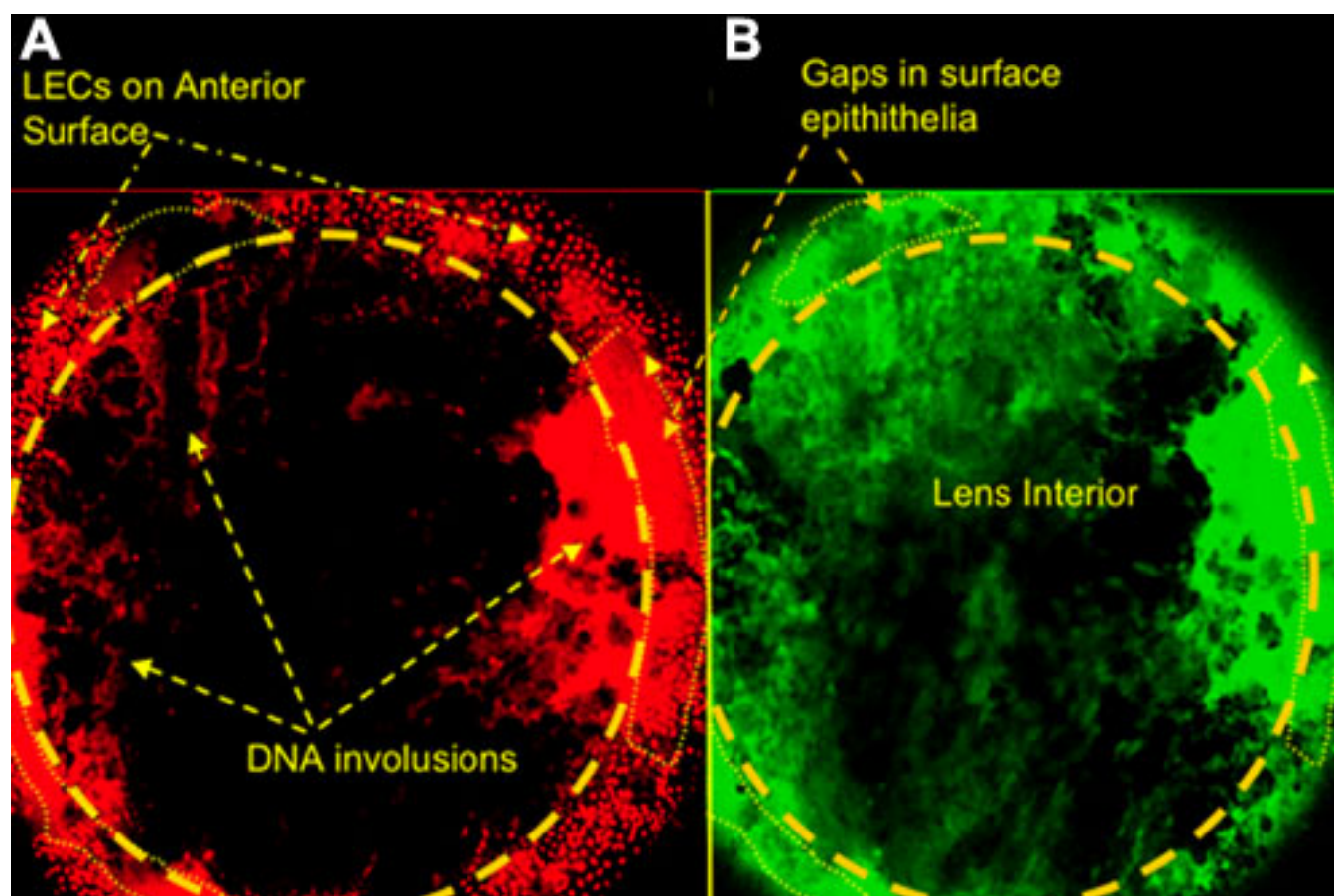


Figure 6. Involution of surface LECs in cataractous old lens. **A:** DNA (only) view of LSCM projection section 72 μm below the anterior surface of the old cataractous rat lens shown in Figure 5 to better illustrate the LEC origin of DNA involutions (DNA, red). **B:** ROS expression (DHR, green) is shown for same LSCM frame. The dotted lines surround gaps in surface epithelium.

neath the surface (compare Figure 7A,B). DNA in fixed lenses incubated without DNase I was not degraded (Figure 7C,D). Inkblots were used to mark the anterior sides of fixed lenses, so that the same general areas could be imaged before and after DNase I.

In Figure 8A-C the relative fluorescent intensities of cortical DNA and ROS were measured for each young and old rat lens, and then plotted against the cataract reflection intensity of each lens. The correlations between cataract reflectance and cortical debris or cortical ROS were very high. The r^2 for DNA versus cataract reflectance was 0.74 (Figure 8A); and the r^2 between ROS and cataract was 0.59 (Figure 8B). As would be expected, cortical ROS also correlated well with cortical DNA ($r^2=0.70$; Figure 8C). The old rat lenses also had much higher mean values for cortical DNA and DHR, and cortical cataract (Figure 8D-F) than did young rat lenses. The comparisons shown are for the lens as a whole (both the anterior and posterior regions), but the anterior and posterior regions also individually demonstrated significant age-related increases in DNA and cataract reflectance ($p<0.01$, data not

shown). These correlations are also in good agreement with those we recently reported for young and old mouse lenses [30].

Visual inspection indicated that the positions of individual cortical cataracts matched the positions of the most intense cortical DHR and DNA fluorescence within the lens. To document this, we set up a separate reflection channel on the LSCM and compared cataract reflections to DNA and ROS fluorescence at the same focal planes in two sets of young and old lenses. The results of this are shown in Figure 9A,B. Confocal sections through both the anterior (Figure 9A) and posterior (Figure 9B) of heavily cataractous old rat lenses produced cataract reflections that co-localized well with the DHR and DNA fluorescence at the same position. Young lenses had little reflectance or cortical fluorescence, as was expected.

DISCUSSION

Using DNA and ROS specific vital fluorescent dyes and laser scanning confocal microscopy we have shown that the development of cortical cataracts in old Brown-Norway rats

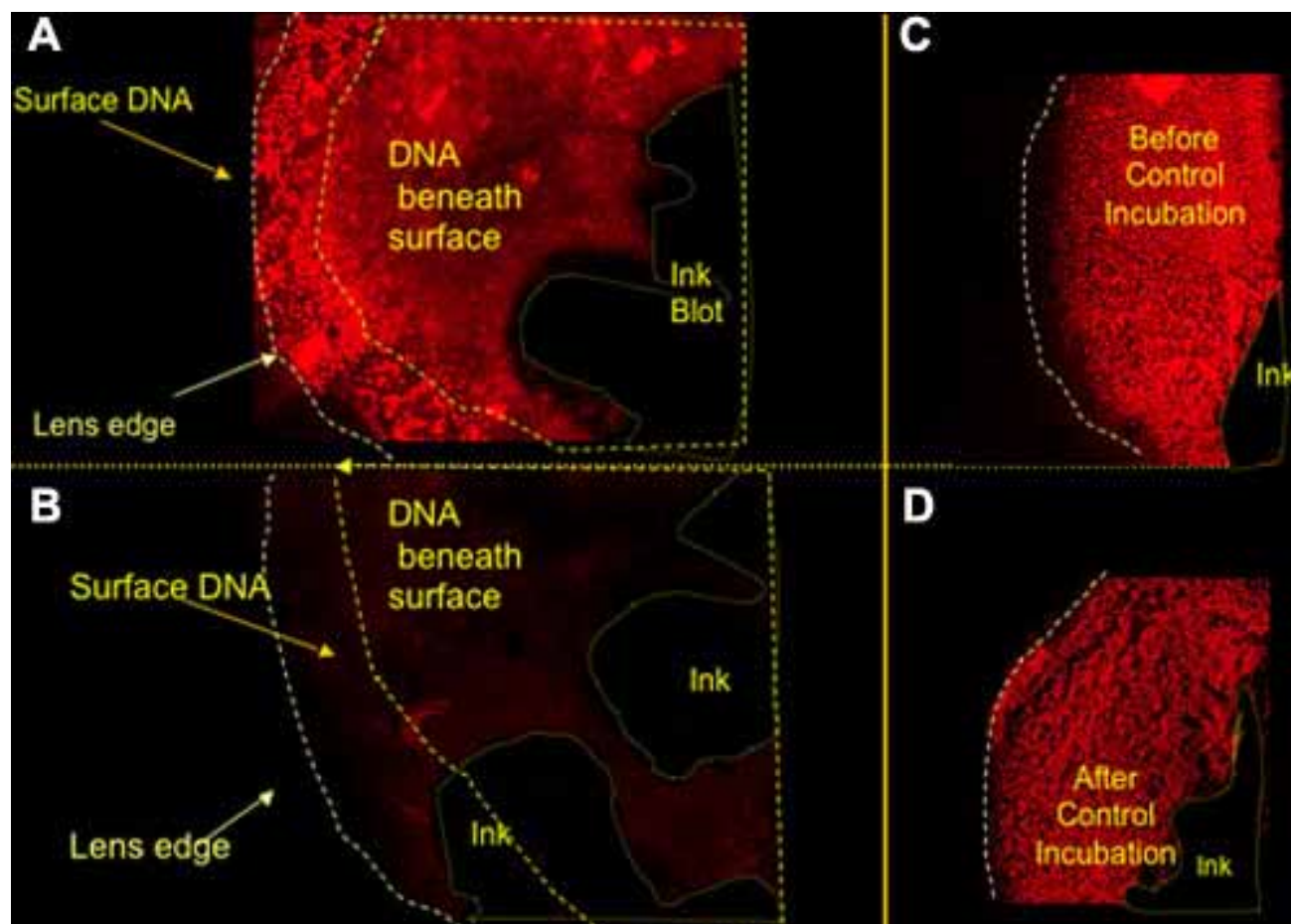


Figure 7. DNase I susceptibility of cortical DNA fluorescence in old cataractous lenses. **A:** Pre-DNase images of the anterior of an old rat lenses fixed and stained for DNA with PI. The frame has been focused 100 μ m below the anterior surface so that both surface and cortical (necrotic inclusions) DNA can be seen (DNA below surface is marked by dashed lines). **B:** The same lens is shown after DNase I digestion for 30 min. **C,D:** The anterior view of a control young lens before (**C**) and after (**D**) control incubation in buffer without DNase I. The inkblots were used to locate the same areas on the surface of the lens before and following DNase digestion.

co-localize with areas of cortical DNA, mitochondria, and ROS. These cortical accumulations arise from invasion of anterior surface LECs, or from extension of the bow region followed by partial degradation of organelles in the invading cells and expression of ROS. This results in strand-like inclusions stretching from the anterior surface into the subcapsular space and cortex. These involutions spread from their sites of entry both anteriorly and posteriorly and can be seen to join with similar debris arising from an extended bow region. Cor-

tical cataracts in all parts of the lens were seen to co-localize with these involutions, and usually in the most debris-laden part of the involution. These processes were identical to those found previously for ARCC in old mouse lenses [30]. Importantly, DNA debris and ROS staining are much lower or absent completely from young and old lenses without cataract. This indicates that the debris and ROS are correlated to the cataract pathology.

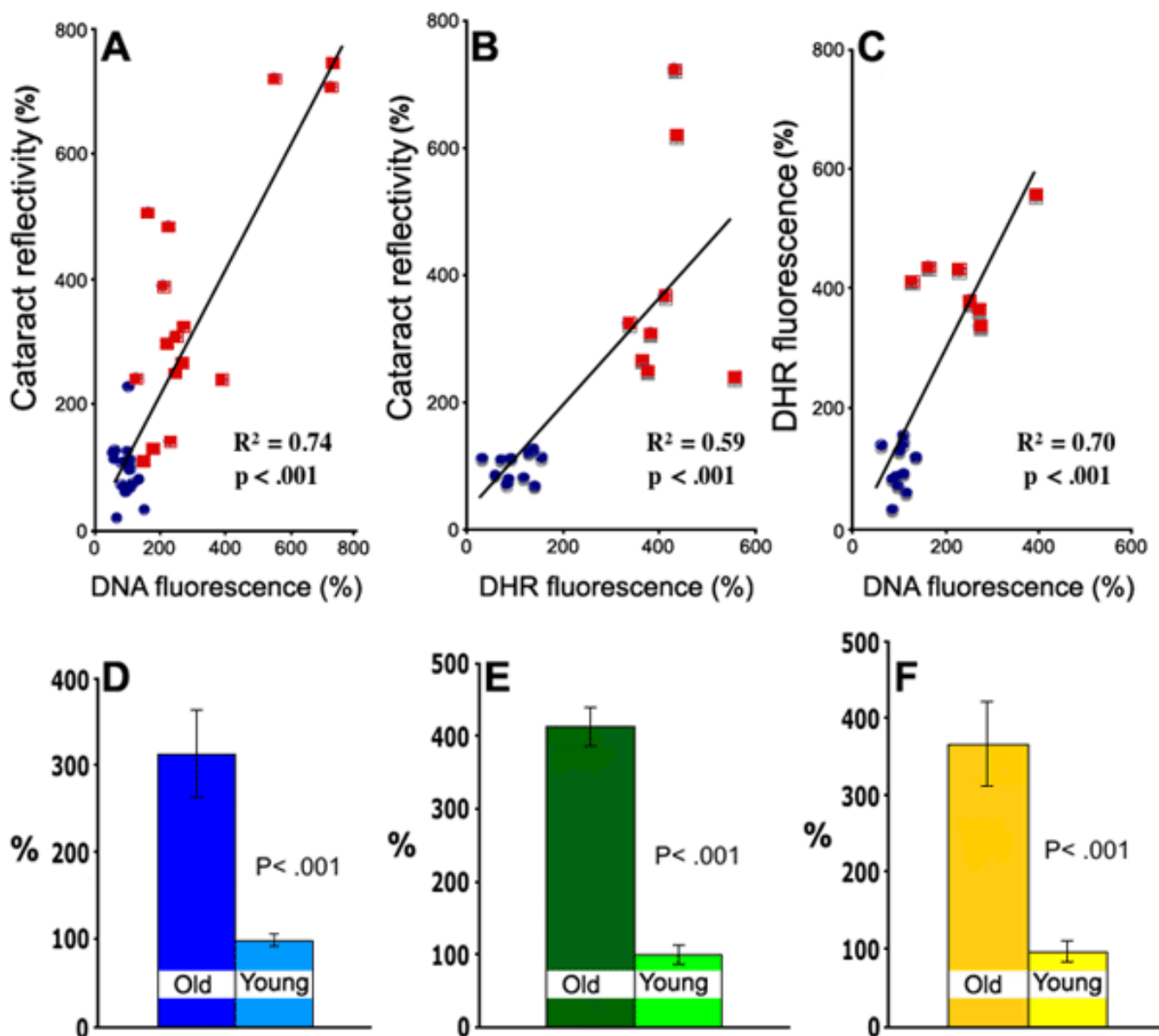


Figure 8. Quantitation of cataract reflection intensities, cortical DNA fluorescence, and cortical DHR fluorescence in young and old lenses. **A:** The correlation between cataract reflectivity (determined from the intensity of reflected light in digitalized low power light microscope photos) and cortical DNA fluorescence (mean Hoechst33342 fluorescence) averaged for both the posterior and anterior sides of young and old rat lenses. The relative fluorescence for each old rat lenses (25-31 months) is shown as red squares, and each young rat lens (4-5 months) is shown as blue circles. **B:** The correlation between cataract reflectivity and cortical DHR fluorescence of young and old lenses (note that fewer lenses were stained with DHR). **C:** The correlation between cortical DNA and cortical DHR in lenses stained with both stains. The p values (**A-C**) are from the 2 tailed Pearson correlation test. **D-F:** Bar graphs comparing the means of the cortical DNA, DHR, and cataract reflectivity for all old and young lenses in each age group; error bars represent standard errors of the mean.

ROS was detected by the fluorescence of oxidized DHR, which has been widely used as a fluorescent probe for ROS [31-33]. The probe is sensitive to a wide variety of ROS, but especially to H_2O_2 [32]. Since ROS oxidizes non-fluorescent DHR to red fluorescent rhodamine that becomes concentrated in mitochondria due to their negative mitochondrial membrane potential, it is especially sensitive to mitochondrial ROS [31]. However, it may be oxidized in the cytoplasm of the cell prior to being concentrated in the mitochondria. We, therefore, cannot specify whether the ROS is produced by damaged mitochondria, or from some other source such as NADPH oxidase [32]. The ROS detected by DHR fluorescence appears to arise from a pre-existing stable pool of ROS because DHR fluorescence did not increase after 15 min (needed for dye penetra-

tion) for up to 2 h of labeling with DHR (unpublished observations). Other probes for ROS also stained the LEC inclusions including dihydroethidium (HE), and dichlorofluorescein diacetate (DCF-DA; unpublished observations). DHR was preferred because it did not require esterase that is needed for DCF-DA staining, and might be limiting in lenses [28]; and because HE has too narrow a specificity for types of ROS, staining mainly superoxide [32].

Earlier workers using other methods [34] have also reported loss of surface LECs associated with human ARC [35-37]. Our findings confirm this, and importantly, extend these earlier findings to show that this cellular debris associated with ARCC arises from involuting surface LECs that undergo partial degradation and express ROS, as we have reported previ-

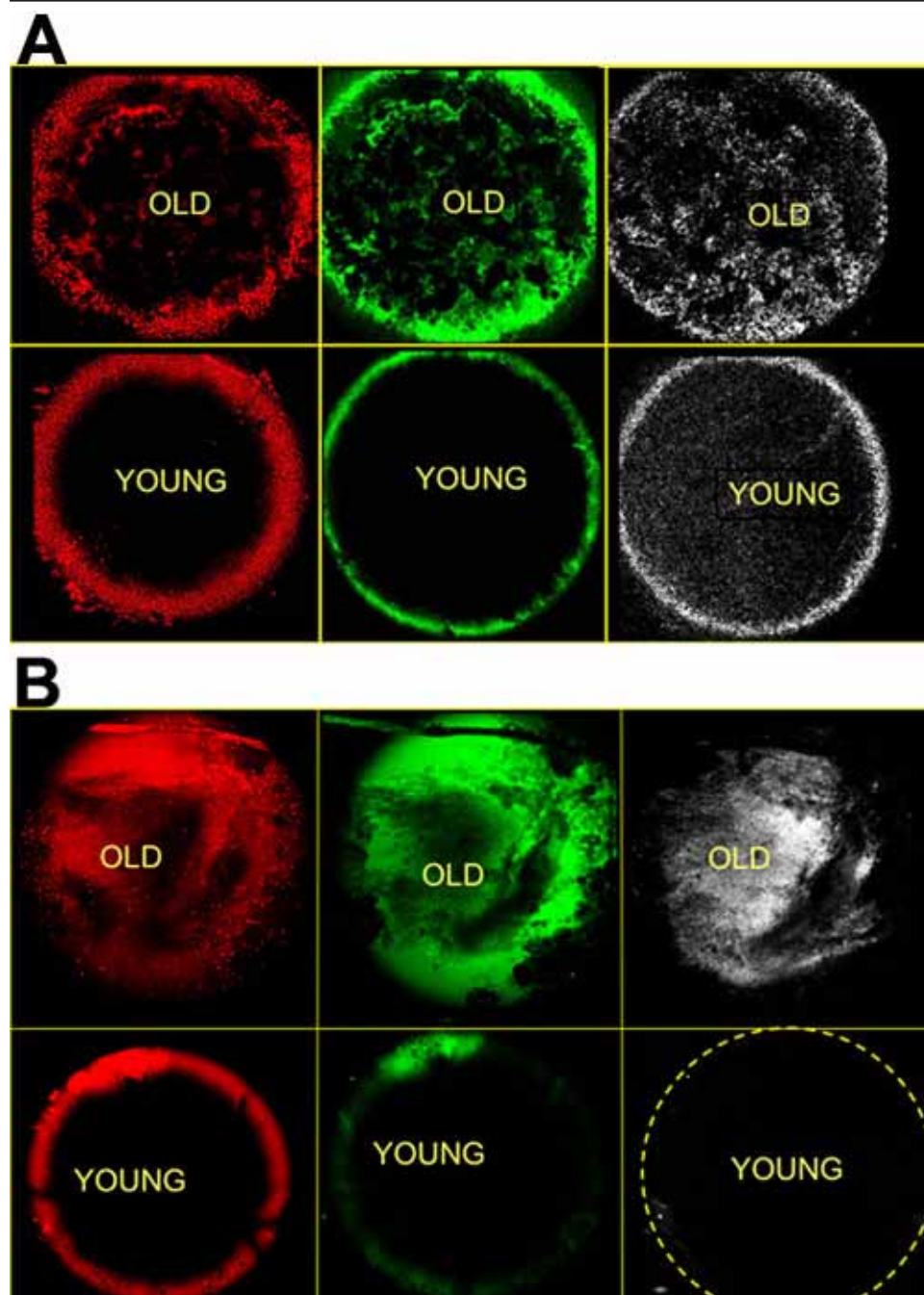


Figure 9. Co-locality of cataract reflections and cortical deposits of DNA and DHR in lens anteriors (A) and in lens posteriors (B). The cataract reflections (white) were measured by creating a separate reflection channel on the Zeiss LSCM focused at the same focal depth as the DNA and DHR fluorescence shown.

ously for mice. We have just begun similar studies on young and old human and dog lenses from recently deceased donors. Only a small number of these samples have been examined so far. However, these displayed loss of surface LECs with involution of LECs with associated debris and ROS filled channels, just as seen in ARCC affected lenses from mice and rats (unpublished data).

Some ARC-related losses of surface cells may be the result of cell death and probably apoptosis, but most of it is directly associated with migration of the cells inward into the cortex. ROS expression was seen at all stages of this involution process. None of the previous models that we have found in the literature reported this full series of events. Some of the reflective properties of cataractous inclusions may arise directly from reflections from LEC debris, but it is likely that the debris and ROS also interferes with proper crystalline-containing fiber formation that is necessary for lens clarity [38]. Interference with degradation of nuclei and DNA has been shown to disrupt lens fibers in lenses from tryptophan deficient mice [39].

Failure to degrade organelles in cortical lens cells has been shown to cause cataracts in several types of recombinant mice. Nishimoto et al. [40] have shown that recombinant mice lacking the DNase II-related enzyme, are unable to digest DNA in the cortical (and nuclear) lens fiber cells. This results in an accumulation of nuclear debris and DNA and produces early cataracts, which worsen with age. Similarly, mice overexpressing BCL-2 [41,42], deficient in p53 but transfected with SV40 large T antigen [41], and overexpressing retinoic acid-binding protein [43] all fail to degrade organelles during lens cell differentiation and all develop cataracts. These models produce both cortical and nuclear cataract, since organelle degradation is blocked early during lens development, whereas the ARC-related events occur late in adult life and only directly affect lens fibers formed at that time.

These studies raise the question of what is responsible for the abnormal involution of surface LECs and extension of the bow into the cortex, accompanied by the expression of ROS in cataractous lenses. We do not believe LEC involutions are the result of accelerated LEC replication or transformation, since the surface cell density is significantly decreased in old lenses, and the involutions frequently can be seen to arise from the edges of denuded surface epithelia. In addition, involutions are initiated over the whole anterior surface including the central region, which undergoes little or no cell replication in adults. Furthermore, we have observed an overall decrease in vivo in LEC turnover in lenses from older rodents [44]. Loss of adhesion factors from the outer lens capsular membrane might account for the gaps and involution of surface LECs, but not the extended bow region. One hypothesis that could explain all these phenomena is that age-related damage to surface mitochondria permits excessive penetration of O_2 into the normally hypoxic lens cortex. Increased pO_2 inside the lens could support involution of LECs and slow the degradation of organelles in the bow region. The pO_2 at the anterior lens surface of the lens is only 2-3% and even lower near the posterior surface [45]. The mitochondria in the

outer 5-10% of the lens normally metabolize 90% of this low level of O_2 diffusing to the lens surface inducing a very hypoxic situation in the inner lens. This is necessary for normal lens homeostasis and clarity [46]. Increased levels of ambient O_2 reaching the lens are reported to block normal differentiation and degradation of lens fiber organelles [47], and this has been shown to lead to cataracts where it occurs, such as following vitrectomy in human patients [12,45,48]. We propose that age-related damage to mitochondrial respiration on or near the lens surface allows unmetabolized O_2 to penetrate deeper into the normally hypoxic lens interior, supporting involution of surface LECs, suppressing organelle degradation, and stimulating ROS production. In support of this we see a 37% ($p<0.01$) decrease in mitochondrial respiration per cell in old mice as determined by respiration induced oxidation of reduced mitotracker red to fluorescent mitotracker red (unpublished observations). Furthermore, this loss of respiratory ability per cell is on top of a 51% decrease in LEC density (Figure 4A). The pO_2 levels in ARCC lenses have not yet been measured.

It is also possible that an age-related rise of the interior lens pO_2 will alter conditions in the lens nucleus, precipitating nuclear cataracts in lenses with a significant loss of mitochondrial respiration. Trusscott [23] and Giblin [18] have proposed that loss of reduced glutathione in the lens interior is a crucial feature in the development of nuclear cataracts. Penetration of O_2 into the lens nucleus would likely compromise internal stores of glutathione, inducing nuclear cataracts [23].

In summary, we present here evidence that involution of surface LECs, and an extension of the bow region accompanied by ROS expression and partial failure to remove DNA-containing debris all occur at the sites of cortical age-related cataract in the rat. These events fully conform to those that we have previously reported in the mouse [30]. At this time, we are unable to prove the initiating cause(s) of the age-related damage, but suggest that age-related damage to LEC mitochondria leads to high levels of intralens O_2 , and initiates these conditions. However, other potential candidates certainly exist such as damage to the factors responsible for the apoptotic-like degradation of organelles, or age-related damage to the capsule membrane.

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