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Effects of senescent lens epithelial cells on the severity of age-related cortical cataract in humans

A case–control study

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Abstract

The aging of lens progenitor cell has been repeatedly proposed to play a key role in age-related cataracts (ARCs), but the mechanism is far from being understood. The present study aims to investigate the relationship between aging of lens progenitor/epithelial cells and the 4 subtypes of ARCs in humans.

Lens capsules, which were collected from ARC patients during surgery, were divided into 3 groups according to the age of patients (50–60, 60–80, and >80 years). The expressions of lens progenitor cell-related markers Sox2, Abcg2, and Ki67 were first examined in human lens epithelial cells (HLECs) in situ. Then, the percentage of senescent and SA-β-gal$^+$ HLECs isolated from lens capsules were quantified. Finally, the potential relationships between the percentage of senescent (and SA-β-gal$^+$) HLECs and the severity of ARCs were analyzed.

Ki67$^+$, Sox2$^+$, and Abcg2$^+$ HLECs in lens capsules were clearly more abundant in young people than in patients older than 50 years, and they were almost absent in patients older than 60 years. The percentage of primary HLECs with aging morphology increased with age, consistent with the results of SA-β-gal$^+$ primary HLECs. Only cortical cataract classification was found to be strongly related to the percentage of SA-β-gal$^+$ and senescent HLECs.

Our study gave the initial evidence on the dynamical change of lens stem/progenitor cells in human lens capsule with age and suggested that lens progenitor/epithelial cell aging is important in the severity of cortical cataracts.

Abbreviations: ARC = age-related cataract, LEC = lens epithelial cell, LSC = lens stem/progenitor cell.

Keywords: age-related cataract, lens epithelial cells, lens stem/progenitor cells, senescent cells

1. Introduction

Cataract, which is the opacification of the lens, is the leading cause of blindness worldwide. Among many risk factors such as ultraviolet light exposure, environmental factors, and diabetes, among others, aging is the main cause and is responsible for more than half of the cases of cataracts (referred to as age-related cataracts [ARCs]).[1–3] Although many efforts have been made to understand the process of cataract, its pathological mechanism remains elusive, and thus effective treatments and medicine in curing the disease aside from surgery are lacking.[1,2] Cell aging, especially lens stem/progenitor cell (LSC) aging, has been repeatedly proposed to play a key role in ARCs, but its mechanism remains to be understood.[4]

To maintain the physiological functions of the lens, the LSCs, which are a small subpopulation within lens epithelial cells (LECs), continuously proliferate and/or differentiate into lens fiber cells to sustain the lens transparency.[5–7] The dysfunction of the LSCs may result in blurry lens. Although the LSCs have been identified, their locations in the lens capsules remain elusive. Yamamoto et al[6] demonstrated that LSCs exist at or are near the germinative zone, consistent with the findings from another study that a side population (considered as stem cells in most tissues) from LECs is mainly located around the germinative zone.[5–6] However, Zhou et al[7] suggested that the LSCs, the number of which decrease with age, are localized not only in the germinative zone but also at the center of the lens in mice by undergoing tritiated thymidine and bromodeoxyuridine labeling; this finding indicates that LSC aging could be involved in cataractogenesis. However, studies focusing on human LSCs are rare.

Cellular senescence is defined as the stable arrest of the cell cycle coupled to morphological changes.[8–10] The cell-cycling activity of adult stem cells declines in older tissues and ceases at a certain point; this process is called stem cell exhaustion that

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results in the attenuation of the regenerative potential of tissues.[10] A similar functional attrition of stem cells has been found in many tissues, including mouse forebrain, bone, and muscle fibers, among others.[11–13] A few studies have shown that less LSCs are found in older samples, but studies on the relationship between stem cell exhaustion and ARC remain elusive.[7]

In the present study, lens capsules with human lens epithelial cells (HLECs) were collected from ARC patients during surgery. LSCs were first analyzed in situ in samples from different ages, and they were found to be almost absent in older samples. More senescence cells were found in primary HLECs from older people. Interestingly, our results also suggest that the severity of cortical cataracts is influenced by the cellular senescence of HLECs.

2. Methods

2.1. Anterior lens capsule collection from ARC patients

Lens capsule collection in this study was approved by the ethics committee of Zhejiang University. Our research adhered to the tenets of the Declaration of Helsinki. A total of 190 anterior lens capsules were collected from ARC patients between 50 and 102-year old and free from other ocular diseases, during cataract surgeries at the Eye Center of the 2nd Affiliated Hospital, Medical College of Zhejiang University. Cataract patients caused by reasons other than aging were excluded. All ARC patients were graded for cataract severity by the Lens Opacities Classification System III before surgery. Samples were preserved in an HLEC culture medium and kept on ice immediately after anterior continuous curvilinear capsulorhexis. Another 10 lens capsules were collected from young people (0–10 years) who were suffered from diseases during which lens were not affected during surgery from the same hospital. Informed consent was obtained from the subjects (or the parents of the young subjects [0–10 years]) after the nature and the possible consequences of the study were explained to them.

2.2. Primary HLEC culture

Primary HLECs were isolated and cultured as previously described.[14] The anterior lens capsules were cut into 1 mm × 1 mm pieces and placed on 35 mm dishes, with the epithelium facing down in Dulbecco modified Eagle medium (DMEM) (Gibco, Invitrogen, NY) containing 10% fetal bovine serum (FBS) (Gibco, Invitrogen, NY), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco, NY). HLECs migrated from the explants and proliferated while the capsules were still attached to the bottom of the dish for 7 days. Then, they were passaged or prepared for further experiments after the capsules were discarded.

2.3. SA-β-gal staining

SA-β-gal staining was performed as described by Dimri.[15] In brief, primary HLECs were fixed in 4% paraformaldehyde (in PBS) for 5 minutes, washed and incubated with fresh senescence-associated β-Gal (SA-β-Gal) stain solution, and then kept in an incubator at 37 °C without CO_2 supply. SA-β-Gal stain solution contains 1 mg of 5-bromo-4-chloro-3-indolyl β->d-galactoside (X-Gal) (Sigma–Aldrich, MO) per mL, 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM potassium ferrocyanide, 5 mM NaCl, and 2 mM $MgCl_2$. Cells were incubated for 4 hours for optimized staining. Their images were captured using an Olympus CKX41 microscope (Olympus) equipped with ToupView software.

2.4. Immunofluorescence examination of HLECs in situ

The anterior lens capsules were fixed in 4% paraformaldehyde (in PBS) for 15 minutes immediately after being collected from surgery. After permeabilization with 0.4% Triton (Sigma–-Aldrich) in PBS, the samples were incubated with the primary antibodies: rabbit anti-sox2 antibody (1/250) (Millipore, CA), mouse anti-BCRP antibody (Abcg2) (1/100) (Millipore, CA), and rabbit anti-ki67 (1/100) antibody (Thermo Scientific, IL) at room temperature overnight. Before labeling the nuclei with 4′,6-diamidino-2-phenylindole (DAPI) (0.5 μg/mL; Sigma–Aldrich), the cells were incubated for 1.5 hours with AlexaFluor 555/488-labeled secondary antibody (1/1000; Invitrogen, OR). Finally, the lens capsules were mounted on Vectashield (Vector Laboratories, CA) using carrier slices. Images were captured using an Olympus IX71 microscope (Olympus) equipped with DP2-BSW software (Olympus) and prepared with Image J software and Microsoft PowerPoint (2007).

2.5. Statistical analysis

SPSS software (Version 17.0, SPSS, Inc., Chicago, IL) was used in the present study. One-way ANOVA, followed by the least significant difference (LSD) post hoc multiple comparison, was performed on all experiments when more than 2 groups required comparison. Statistical significance was defined as $^{*}P < 0.05$, $^{**}P < 0.01$.

3. Results

3.1. Proportion of human LSCs (HLSCs) within HLECs gradually decreases with age

A total of 190 anterior lens capsules from ARC patients were divided into 3 groups with age: group 1 (50–60 years), group 2 (60–80 years), and group 3 (>80 years). Details are presented in Table 1. One of the hypotheses for ARC is senescent and exhaustion of LSCs in lens capsules.[10] Therefore, the expressions of lens stem cell markers Sox2, Ki67, and Abcg2 in situ were first examined through immunofluorescence. A large amount of $Ki67^{+}$ cells were found in the lens capsules from children aged 0 to 10 years, while they were almost absent in the lens capsules from patients older than 50 years. This finding suggests that LECs gradually lose their proliferation ability with age (Fig. 1A–C, J). Sox2 and Abcg2 were found to express in many kinds of stem cells, such as embryonic stem cells and neural stem cells, among others.[16,17] In our study, the number of $Sox2^{+}$ and $Abcg2^{+}$ gradually decreased, and they were not observed within HLECs from patients older than 60 years. This finding indicates the exhaustion of stem cell pool within HLECs (Fig. 1D–L).

Table 1

Number of age-related cataract (ARC) patients during different groups divided according to age.

Age gender	50—60 years	60—80 years	Older than 80 years
Male	3	32	23
Female	34	81	17
Total	37	113	40

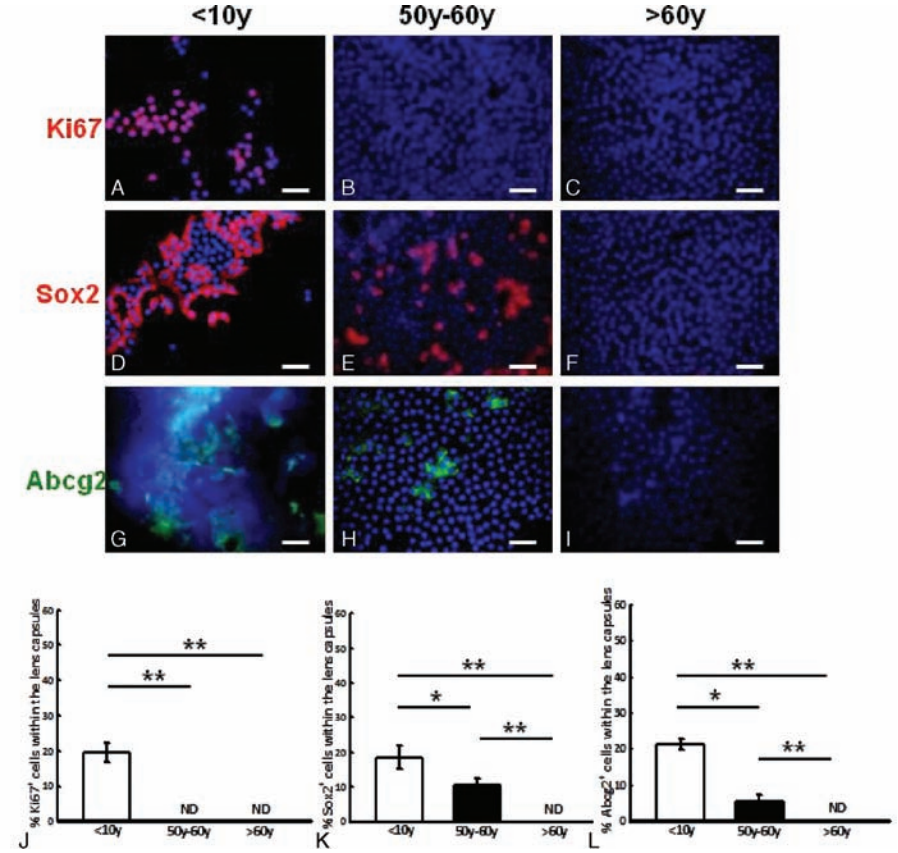

Figure 1. Expressions of Ki67, Sox2, and Abcg2 in lens capsules from patients. Ki67$^+$, Sox2$^+$, and Abcg2$^+$ cells were observed in the lens capsules from young people (A, D, G), but they were reduced in the lens capsules from older patients. Eventually, Ki67 (red) signaling could not be detected in the samples from patients older than 50 years (B, C), and Sox2 (red) and Abcg2 (green) signaling was absent in the samples from patients older than 60 years (E, F, H, I). J–L show the quantified data which are presented as mean ± SEM (group <10 years, n = 5; group 50–60 years, n = 5; and group >60 years, n = 5). Scale bar, 40 μm. $^*P < 0.05$ and $^{**}P < 0.01$ versus proportion of Ki67$^+$, Sox2$^+$, or Abcg2$^+$ HLECs from <10 years. HLEC = human lens epithelial cell, ND = not detected, SEM = standard error of mean.

3.2. Proportion of senescent cells within primary HLECs increases with age

In some tissues, adult stem cells, which spend most of their time in quiescent state, are activated by stimulations such as injury and start to express different markers.[18] In the present study, whether the LSCs from older patients temporarily lost the expression of stem cell markers and would regain their properties in certain circumstances, such as after cataract surgery, was examined. The expressions of Sox2, Ki67, and Abcg2 were examined in primary HLECs from individuals older than 60 years as soon as the HLECs migrated from the explants. Unsurprizingly, no signal was detected for Sox2, Ki67, and Abcg2 in cultured primary HLECs (Fig. 2), thus suggesting that properties of LSCs were genuinely lost and could not be retrieved after stimulation.

Interestingly, some HLECs from older people were observed to become larger, flatter, and irregular with massive vacuoles in the cytoplasm (referred to as senescent HLECs) during culturing, whereas others showed uniform rectangle morphologies, as in the samples from children. The representative images are shown in Fig. 3A–C. Moreover, the senescent HLECs stopped proliferating and gradually died afterwards. After being cultured for 3 weeks, the proportion of senescent cells were 10.67% ± 3.62% cells within the HELCs in group 1, and then it increased and reached

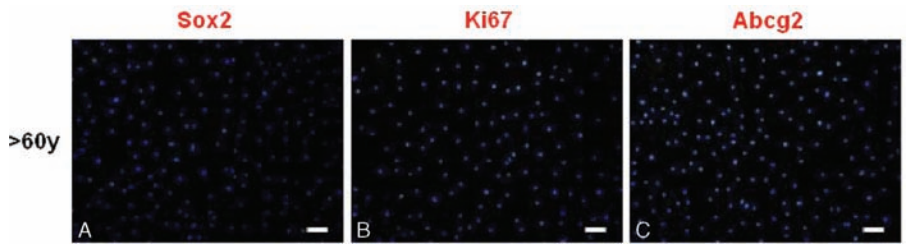

Figure 2. Expressions of Ki67, Sox2, and Abcg2 in the cultured primary human lens epithelial cells (HLECs) from patients older than 60 years. Ki67$^+$, Sox2$^+$, and Abcg2$^+$ cells were not observed in the cultured primary HLECs from patients older than 60 years, representative pictures showed as A–B. Scale bar, 40 μm.

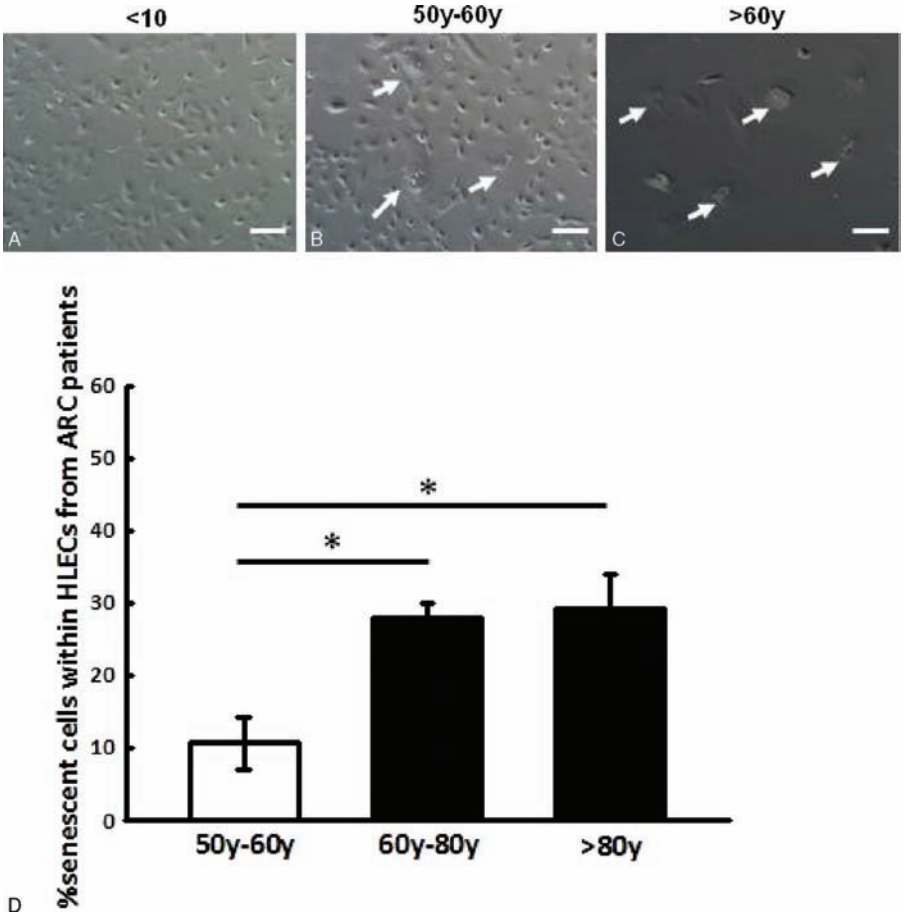

Figure 3. Proportion of senescent cells within primary HLECs from different ages. Representative pictures of primary HLECs from different ages are denoted by A, B, and C. Senescent HLECs were not observed in young samples (A) (<10 years), but they were observed and increased with age in B (50–60 years) (arrow) and C (>60 years) (arrow). D shows the quantified data which are presented as mean ± SEM (group 50–60 years, n = 12; group 60–80 years, n = 39; and group >80 years, n = 14). Scale bar, 40 μm. $^{*}P < 0.05$ versus proportion of senescent HLECs from 50 to 60 years. HLEC=human lens epithelial cell, SEM=standard error of mean.

27.96% ± 2.05% in group 2 and 29.21% ± 4.86% in group 3 (Fig. 3D). These results indicate that the functions of HLECs in older individuals decline probably because senescent HLECs cannot be replenished due to the exhaustion of stem cell pools.

Aging is defined as the time-dependent functional decline occurring in all living organs, and it is the primary risk factor for degenerative diseases such as AD disease and ARC.[10] To further investigate senescent cells in each group, SA-β-gal staining was performed in primary HLECs. The results showed that the SA-β-gal^{+} HLECs showed senescent HLECs morphology and were observed in the samples from patients older than 50 years and that the number of SA-β-gal^{+} cells increased with age. Non-SA-β-gal^{+} HLECs were observed from the samples from the children (0–10 years). Representative pictures are shown in Fig. 4A–C. After quantification, 24.27% ± 4.66% in group 1, 39.24% ± 2.98% in group 2, and 44.34% ± 3.47% in group 3 were found to be SA-β-gal^{+} within primary HLECs (Fig. 4D). These results were in accordance with what was found for senescent HLECs above (Fig. 3).

3.3. Loss of functional HLECs may influence the severity of cortical subcapsular cataracts

Many mechanisms for ARC, including the aging of HLECs, have been investigated, but the relationship between the aging of HLECs and cataract grades remains unknown.[2] Therefore, the correlation between the severity of different ARC subtypes and the proportion of senescent cells within HLECs was analyzed. All samples were divided into groups according to cataract severity based on the Lens Opacities Classification System III in terms of 4 features: cortical cataracts (C), nuclear opalescence (N), posterior subcapsular cataracts (P), and nuclear color (NC) (Table 2).[19] The results showed that proportion of senescent cells within HLECs increased with the grade of cortical cataracts. The proportion of senescent cells within HLECs was found to be 19.27% ± 4.83% in grade I, increased to 32.31% ± 4.99% in grade II and 32.67% ± 2.81% in grade III, and finally reached 38.86% ± 5.91% in grade IV and 38.57% ± 3.90% in grade V

Table 2

Number of age-related cataract (ARC) patients during different subtype cataracts groups.

	Cortical (C)	Nuclear (N)	Posterior subcapsular (P)	Nuclear color (NC)
0	–	5	6	–
I	6	30	16	26
II	23	24	26	33
III	33	37	46	29
IV	26	–	5	5
V	10	–	–	1

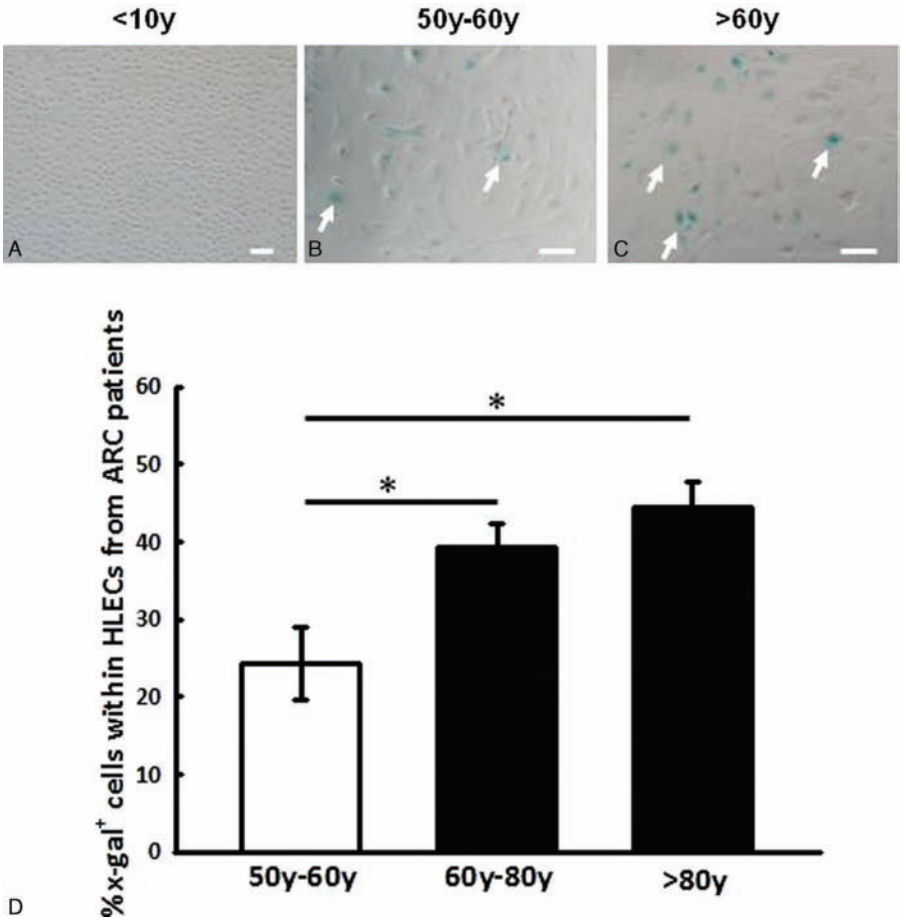

Figure 4. Proportion of SA-β-gal$^+$ cells within primary HLECs from different ages. Representative pictures of primary HLECs from different ages are denoted by A, B, and C. SA-β-gal$^+$ HLECs (arrows) were not observed in young samples (A) (<10 years), but they were observed and increased with age in B (50–60 years) (arrow) and C (>60 years) (arrow). D shows the quantified data which are presented as mean ± SEM (group 50–60 years, n = 12; group 60–80 years, n = 33; and group >80 years, n = 12). Scale bars, (A) 50 μm; (B, C) 40 μm. $^*P < 0.05$ versus proportion of SA-μ-gal$^+$ HLECs from group 50 to 60 years. HLEC=human lens epithelial cell, SEM=standard error of mean.

(Fig. 5). No relevance was found between senescent HLECs and nuclear opalescence (N), nuclear color (NC), or posterior subcapsular cataracts (P) (Fig. 5).

To further investigate the relationship between the aging of HLECs and cataract subtypes, the proportion of SA-β-gal$^+$ cells in each subtype and grade were analyzed. The proportion of SA-β-gal$^+$ cells were 13.17% ± 2.00% in grade I, increased to 35.18% ± 4.67% in grade II and 39.87% ± 4.81% in grade III, and finally reached 43.09% ± 4.65% in grade IV and 36.23% ± 2.99% in grade V of cortical cataracts (Fig. 6). These results were consistent with those of senescent HLECs, thus further confirming the influence of the aging of HLECs in the severity of cortical cataracts. The number of SA-β-gal$^+$ cells in each grade of posterior subcapsular cataract was also analyzed. Surprizingly, the severity of posterior subcapsular cataracts could also be influenced by SA-β-gal$^+$ HLECs. The proportion of SA-β-gal$^+$ cells within HLECs were 27.28% ± 7.95% in grade 0, increased to 35.33% ± 3.88% in grade I, and reached the highest at 45.16% ± 6.01% in grade II of posterior subcapsular cataracts (Fig. 6). However, no significant difference was observed among the groups. Similar results to those of senescent HLECs were found for nuclear opalescence (N) or nuclear color (NC) (Fig. 6).

In sum, our results reveal that the severity of cortical cataracts may be influenced by the senescent cells within HLECs, the number of which increased in older people probably because of the aging and exhaustion of human lens stem cells.

4. Discussion and conclusions

In this study, we showed that the number of functional HLSCs within HLECs declined with age and could be exhausted in samples from a certain old age. The results of our study demonstrated that a higher percentage of senescent cells within HLECs could result in severe cortical cataracts.

Several studies have provided evidence for the existence of LSCs, which continue to divide and differentiate into lens fiber cells throughout the lifetime of animals.[5–7] Although the germinative zone has been repeatedly proposed as a stem cell niche in the lens, Zhou et al[7] suggested that stem cells could also be located in the central zone of mice lens.[7] However, whether this is also the case in human lens is still unknown. Our study provided the initial evidence that LSCs genuinely exist in the central zone of human lens, and this evidence is supported by the expression of stem cell markers Ki67, Sox2, and Abcg2. Moreover, the dividing activities of these cells, which could be recruited under certain circumstances, decreased and even disappeared in older people. This finding suggests that LSCs, which are exhausted in older people, may be involved in the formation of ARCs.

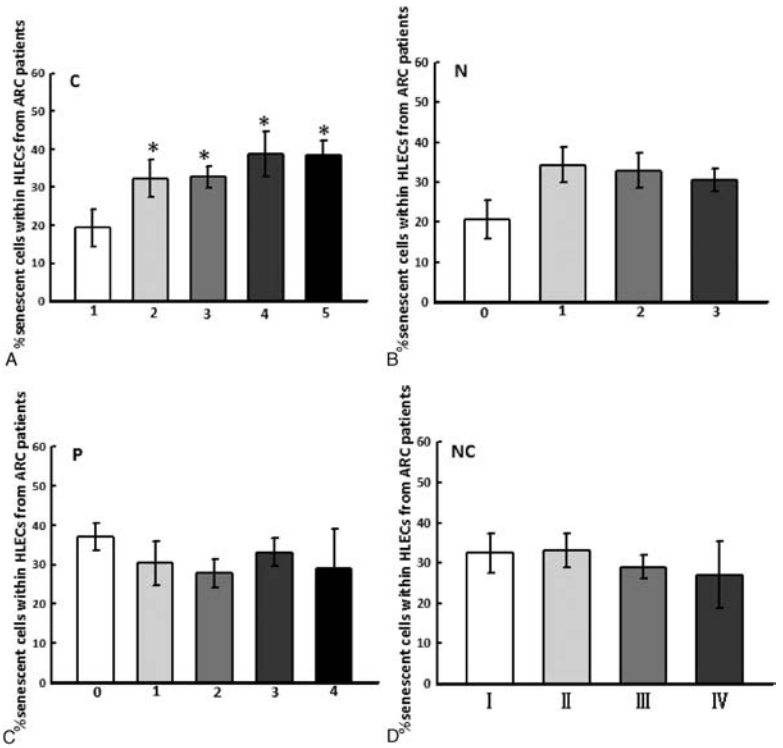

Figure 5. Proportion of senescent HLECs from different subtypes of cataracts. C is short for cortical cataracts, N for nuclear opalescence, P for posterior subcapsular cataract, and NC for nuclear color. Different bars show the different grades of each cataract subtype. Data are presented as mean ± SEM. $^{*}P < 0.05$ versus proportion of senescent HLECs from the group of cortical cataracts grade 1. HLEC=human lens epithelial cell, SEM=standard error of mean.

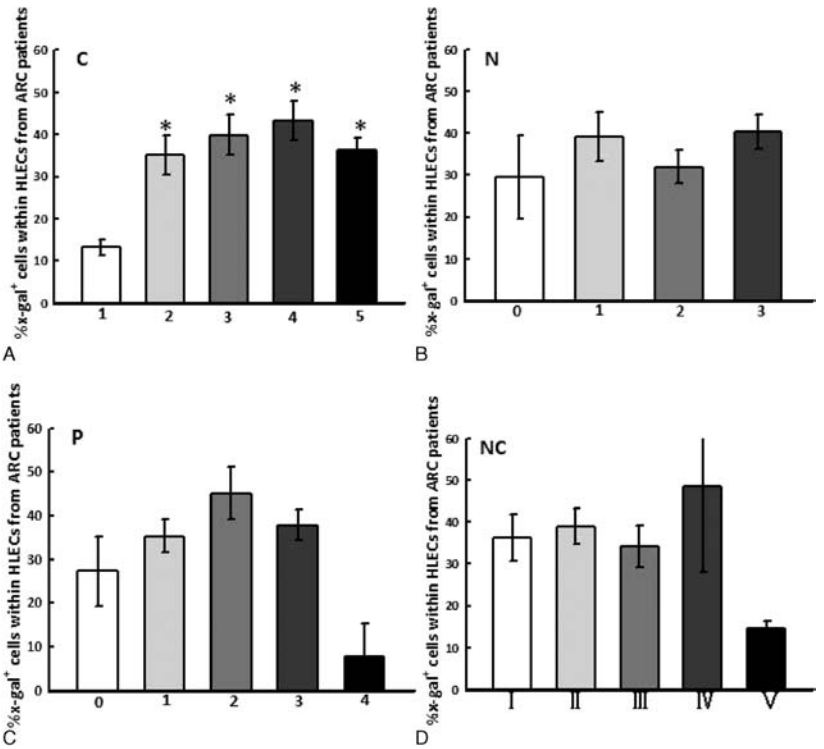

Figure 6. Proportion of SA-β-gal^{+} HLECs from different cataract subtypes. C is short for cortical cataracts, N for nuclear opalescence, P for posterior subcapsular cataract, and NC for nuclear color. Different bars show the different grades of each cataract subtype. Data are presented as mean ± SEM. $^{*}P < 0.05$ versus proportion of SA-β-gal^{+} HLECs from the group of cortical cataracts grade 1. HLEC=human lens epithelial cell, SEM=standard error of mean.

One characteristic of aging in essentially all tissues is the decline in the regenerative capacities and activities of adult stem cells.[10] For example, the decline of hematopoiesis in old tissues may result in a diminished production of adaptive immune cells and in an increased incidence of anemia and myeloid malignancies.[20] In the current study, the increase in senescent HLECs may be caused by the decrease in cell-cycle activity of HLSCs and the increase in senescent progress of differentiated LECs, thus resulting in ARCs. This finding suggests that the anti-aging of LECs may be one of the possibilities of ARC treatment.

Studies have shown age-related cortical cataracts may be caused by the dysfunction of lens surface epithelial cells.[4] However, the mechanisms of the functional attrition of LECs during lifespan remain ambiguous. Many studies on other tissues have shown that the deficient proliferation of stem cells is clearly detrimental to the maintenance of the physiological function of organisms and causes diseases.[10] Their findings indicate that the exhaustion of LSCs may be one reason for the occurrence of ARCs. In the present study, the relationship between the cataract subtypes and the proportion of senescent LECs, parts of which could be caused by the declining proliferative activity of LSCs, was analyzed. Our results provide the initial evidence that the severity degree of age-related cortical cataracts, but not the other subtypes, correlates with senescent LECs. This finding supports our viewpoint in searching for the clinic target of age-related cortical cataracts in LECs.

In summary, our study provides direct and important evidence on the relationship between the severity of age-related cortical cataracts and senescent LECs. Moreover, a new direction in age-related cortical cataract treatment may involve the antiaging or nutrition of LECs.

Acknowledgments

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