Functional Principal Component Analysis Reveals Discriminating Categories of Retinal Pigment Epithelial Morphology in Mice

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PURPOSE. To determine whether multivariate, functional principal component analysis of the size and shape of retinal pigment epithelial (RPE) cell morphology allows discrimination of mouse RPE genotypes and age.

METHODS. Flatmounts of RPE sheets obtained from C57BL/6J ($n = 50$) and rd10 ($n = 61$) mice at postnatal days 30 to 720 were stained for zonula occludens-1 (ZO-1) and imaged with confocal microscopy. A total of 111 flatmounts were prepared. Twenty-one morphometric measurements were made on tiled, composite images of complete flatmounts, including cell location, area, and eccentricity, using automated image analysis software for quantitatively measuring cell phenotypes.

RESULTS. In young (≤ 61 -day-old) C57BL/6J mice, the RPE morphology resembled a regular hexagonal array of cells of uniform size throughout the retina, except near the ciliary body, where the shapes of RPE resembled a soft network. Old (≥ 180 -day-old) C57BL/6J eyes had a subpopulation of large cells. A clear disruption of the regular cell size and shape appeared in rd10 mutants. Aspect ratio and cell area gave rise to principal components that predictively classified mouse age and genotype.

CONCLUSIONS. Quantitative differences in the RPE sheet morphology allowed discrimination of rd10 from C57BL/6J strains despite the confounding effect of aging. This has implications for RPE sheet morphology as a potential early biomarker for diagnosis of eye disease and prognosis of the eye at early stages when disease is subtle. We conclude that an RPE cell's area and aspect ratio are very early quantitative indicators that predict progression to advanced RPE disease as manifested in rd10.

Keywords: RPE, image analysis, retinal degeneration

Retinal pigment epithelium (RPE), a multifunctional cell monolayer that separates the retinal photoreceptors from the choroid, is crucial in maintaining photoreceptor cells. RPE regulates the flow of nutrients and oxygen into the retina, removes waste byproducts from the retina, phagocytizes shed discs from photoreceptors, absorbs stray light that passes through the retina, and regulates the osmolarity of the eye. The RPE sheet functions as a barrier to maintain gradients and protect photoreceptor cells and inner neurons from the immune system and from external assaults from infectious agents.¹ To preserve the integrity of the barrier, when an epithelial cell dies, the apoptotic cell is extruded² from an intact sheet through a p115RhoGEF-mediated process³ that maintains the barrier. However, disruptions in RPE occur with age and in several ocular disorders, including age-related macular degeneration and retinitis pigmentosa.^{4–7}

The biomechanical forces that organize RPE and other epithelial cell-cell contacts include adhesion, tension, and contraction.⁸ An actin-myosin cytoskeleton contributes contractile forces that lead to regular polygonal (mostly hexagonal) shapes.^{8–10} One of many molecules that provides a high-quality outline of the RPE cell border is zonula

occludens-1 (ZO-1), a tight junction protein.¹¹ Trafficking and movement of proteins that are part of the adhesion, tension, and contraction mechanisms lead to changes in the force balance. Additionally, the death of RPE cells, because of retinal disease progression, bystander effects, and mechanical stress from the presence of drusen-like substances, flecks, or the like, must contribute to the changes in force balance.⁸ Consequently, we hypothesize that remodeling of RPE cell shape causes alterations in cell packing and a rearrangement of the patterns and tiling of the RPE sheet. We hypothesize that the RPE morphology changes differently with age and disease progression.¹² This study established the relationship between RPE morphology and the phenotypic degeneration of the retina specified by rd10.¹³

We studied the morphometric properties of RPE sheets from C57BL/6J and rd10 mice on a C57BL/6J background. Here, C57BL/6J mice are considered wild-type (WT) mice and were used to study the effect of aging in the RPE cell morphology, distinct from disease. rd10 mice were used to assess effects of photoreceptor loss and aging on RPE cell morphology. This strain has a missense mutation in *PDE6B*, the gene encoding the beta subunit of rod phosphodiester-

TABLE. Sample Group Characteristics for WT and rd10 Young and Old Mice

Characteristic	Group			
	WT Young	WT Old	rd10 Young	rd10 Old
Age range	≤P61	≥P180	≤P61	≥P100
No. of mice	27	23	27	34
Average age ± SD	44.6 ± 13.0	464.7 ± 256.1	49.6 ± 10.2	243.3 ± 214.5
Average cell area ± SD	247.5 ± 12.1	268.4 ± 10.9	247.4 ± 9.5	264.6 ± 16.0
Mode cell area ± SD	195.9 ± 7.1	193.7 ± 5.2	201.6 ± 8.4	190.7 ± 8.4
Average aspect ratio ± SD	0.74 ± 0.01	0.73 ± 0.01	0.70 ± 0.02	0.62 ± 0.03
Mode aspect ratio ± SD	0.78 ± 0.01	0.79 ± 0.01	0.74 ± 0.02	0.63 ± 0.05
Highest peak (x,y,z)	(198,0.78,0.015)	(198,0.78,0.012)	(210,0.73,0.014)	(180,0.64,0.009)
X-Y area including 95% of cells	162.9	211.0	207.6	294.5
Area at $z = 4 \times 10^{-4}$	204.0	248.4	253.7	333.5
Age breakdown per major group; independent mice	P30 $n = 10$	P180 $n = 8$	P30 $n = 3$	P100 $n = 13$
	P45 $n = 8$	P330 $n = 4$	P45 $n = 13$	P180 $n = 13$
	P60 $n = 4$	P720 $n = 6$	P60 $n = 6$	P330 $n = 3$
	P61 $n = 5$	P722 $n = 5$	P61 $n = 5$	P723 $n = 2$
Collapsed data from Figures 2 and 4; ages P60.5 and P722	P60.5; $n = 9$	P721; $n = 11$	P60.5; $n = 11$	P727.5 $n = 5$
	(summed P60 and P61)	(summed P720 and P722)	(summed P60 and P61)	(summed P723 and P732)

Area at $z = 4 \times 10^{-4}$, the area of the contour where $z = 4 \times 10^{-4}$ from the plots given in Figures 6E–H; highest peak, x, y, and z coordinates from Figure 6 of the mode for each major group; mode, the value at the most abundantly represented cell area or aspect ratio; SD, standard deviation; X-Y area including 95% of the cells, area of the contour that includes 95% of the cells.

These statistics suggest that WT young cells were normal in size and shape; WT old were normal in shape but abnormal in size; rd10 young were abnormal in shape but normal in size; and rd10 old were abnormal in size and shape. The larger X-Y areas indicate increased size and shape variability of RPE cells in rd10 and old mice compared to WT young mice.

ase, which normally hydrolyzes cGMP in the phototransduction cascade.¹⁴ Mutations in the *PDE6B* gene result in autosomal recessive retinitis pigmentosa (ARRP) in humans.¹⁵ Similarly, rd10 mice exhibit degeneration of first rod and then cone photoreceptor cells, eventually leading to loss of retinal function.¹⁶ Thus, the rd10 mouse is considered a model of ARRP.¹⁶ rd10 exhibits several properties akin to retinitis pigmentosa.^{12,17–19} rd10 has not been investigated for morphological changes in the RPE, despite evidence of white spots of various sizes in fundus images.¹⁴ Indeed, while much work has been done in the neural retina to understand rd10 retinal degeneration,^{16,20,21} little work has been done on collateral damage to the RPE sheet in common mouse models.

Although qualitative analyses of RPE flatmount images have been used to study disease status and progression, RPE morphometric analyses have been limited to showing RPE images for visual inspection,^{22–27} averaged measurements of cell areas,^{28,29} and a few other measurements, such as number of neighbors and form factor for each cell.¹¹ Herein we report our first efforts in establishing baseline morphological measurements in WT mouse RPE and quantifying differences between morphology due to aging and that of the rd10 mutation, focusing on cell size and cell shape. We demonstrate that novel statistical analyses using multivariate, functional principal component analysis (FPCA)³⁰ and tools used for classification of size and shape of RPE morphology allow discrimination of RPE genotypes and age.

METHODS

Mice

B6.CXB1-Pde6b rd10/J (rd10) mice and C57BL/6J (WT) mice were obtained from Jackson Laboratories, Inc. (Bar Harbor, ME). The original rd10 mouse lesion was discovered in a C57BL/6J mouse, and, thus, rd10 is fully congenic with C57BL/6J. Mice were housed in Emory University Division of Animal Resources managed facilities according to Association for Research in Vision and Ophthalmology (ARVO) guidelines and as approved by the Emory University IACUC. Mice were maintained on a 12-hour light:12-hour dark cycle at an approximate light level of 50 to 100 lux in the cages during daytime. Mice were offered rodent chow (product 5001; Purina, St. Louis, MO) and water ad libitum. The mice were examined at ages ranging from postnatal day (P)30 until approximately 2 years of age.

RPE Flatmount Technique

Mice were euthanized with CO₂ in accordance with Emory IACUC guidelines and ARVO guidelines for treatment of animals. One eye from each mouse was marked on the superior side with a fine point, permanent ink pen (Sharpie, Oak Brook, IL), enucleated, fixed for 10 min in 10% neutral buffered formalin (Fisher; Pittsburgh, PA), and washed three times with PBS (catalog no. 46-013-CM; Cellgro, Manassas, VA). Excess tissue was removed from the outside of the globe. No

FIGURE 1. RPE flatmount images. Cell boundaries were stained *green* with anti-ZO-1, and cell nuclei were stained *red* with propidium iodide. (A) Merged image of whole-RPE flatmount of an rd10 mouse at P100 is shown. *Scale bars* and sizes are indicated on the images. Highly compressed, elongated, and tortuous cells in the midperiphery are indicated by the four *white arrowheads*. (B) Region of RPE flatmount of a WT mouse at P45 is shown. A patch of approximately 795 cells is illustrated. Most cells were regular in shape and consistent in size. (C) RPE is shown in the mid-peripheral region of an rd10 mouse eye at P100. A transition zone that separated compressed cells from large and variably sized cells posterior to the

transition ran horizontally through the image (*arrow*). This reflects underlying pathology of the RPE layer in the rd10 mice, which is fatter and thicker in the compressed area. Demonstration of the compression is a key point in presenting this image.

incision was made in the cornea before or during fixation. The incision was made only after the initial fixative was removed and replaced with PBS. Flatmounting was done by making four radial cuts from the center of the cornea back toward the optic nerve. A drop of Dulbecco calcium- and magnesium-free PBS was placed on the eye to keep it moist. The flaps were peeled away from the lens, and the lens was removed. The iris and retina were removed using forceps. Tension from the sclera was relieved by making cuts halfway through each flap at the ciliary body/cornea margin and small cuts through the ciliary body.

ZO-1 Staining

The RPE flatmounts were blocked with Hanks' balanced salt solution HBSS (SH30588.01; Hyclone, Logan, UT) containing 0.1% (v/v) Triton X-100 and 1% (w/v) bovine serum albumin (antibody buffer) for 30 minutes. Immunostaining with a 1:100 dilution of rabbit anti-ZO-1 antibody (catalog no. 61-7300; Invitrogen, Carlsbad, CA) in antibody buffer was conducted for approximately 16 hours at room temperature. The flatmounts were washed 5 times with HBSS plus 0.1% (v/v) Triton X-100 (wash buffer) for 2 minutes and then stained for 1 hour with Oregon Green-conjugated goat anti-rabbit immunoglobulin G secondary antibody (catalog no. O11038; Invitrogen) in antibody buffer and then washed three times with wash

buffer. The flatmounts were mounted with Vectashield hardset (catalog no. H-1400; Vector Laboratories, Burlingame, CA) and allowed to harden overnight.

Imaging

Imaging of the flatmounts was performed with a confocal imaging system (model C1; Nikon) using argon laser excitation at 488 nm. Confocal images were digitally merged (Adobe Photoshop CS2; Adobe, San Jose, CA). Selected representative regions of equal size (181×266 pixels; $225 \times 331 \mu\text{m}$) were cropped from the merged flatmount image from areas devoid of dissection artifacts. As many selected representative regions as possible were taken from each image (45–60 selected representative regions per image). Twenty-one morphometric measurements for every cell identified in the selected representative region, including cell location, cell area, perimeter, eccentricity, aspect ratio, form factor, and number of neighbors, were calculated using CellProfiler.³¹

Statistical Analysis

The RPE images and the quantitative data output from CellProfiler analysis were examined, and cell area and cell shape (measured by aspect ratio) were chosen as two key features to discriminate between the genotypes and age

FIGURE 2. Analysis of RPE cell area. (A) Boxplots of median cell area (μm^2) as a function of age for WT eyes are shown. Boxplots show 5-number summaries of cell areas: smallest cell area, lower quartile (25%), median area, upper quartile (75%), and largest observed cell area. (B) Density distributions are shown for each WT eye (young, black; old, red). The mode is at approximately $200 \mu\text{m}^2$ for young and old mice. Shoulders at approximately $380 \mu\text{m}^2$ and tails differ between young and old WT mice. The black curves (young, $\leq P61$) have a taller and narrower peak at a smaller cell area, whereas red curves (old, $\geq P180$) have broader and lower peaks and thicker tails, representing cells with large cell areas. (C) Boxplots of median cell areas as a function of age for rd10 eyes are shown. (D) Density distributions are shown for each rd10 eye. The mode is at approximately $200 \mu\text{m}^2$ for young and old mice. Black lines and symbols are for young eyes, and red represents old eyes. The older (red) curves showed broadening and lower density near the mode, a larger shoulder at approximately $380 \mu\text{m}^2$, and more cells with areas $>400 \mu\text{m}^2$ than in young rd10 mice. Numbers of mice per age group and genotype are given in the Table.

FIGURE 3. Principal component score plots of cell area are shown for all 4 genotype and age groups. *Black squares* are WT eyes, and *red triangles* are rd10 eyes. The *x*-axis on each plot (A–C) is a different first principal component because the dataset from which it was derived was different in each plot; the same applies to each *y*-axis; each second PC score axis is different in the three plots. (A) The plot of the first two principal components for WT eyes is shown. This panel shows the clear separation WT young from WT old based only on cell area. (B) The rd10 mice are resolved, old mice from young, but not as well as in the WT mice. (C) Data are derived from all 4 groups; old are resolved from young but do not clearly resolve the four distinct groups (WT young, WT old, rd10 young, rd10 old) based solely on cell area. Panel (C) illustrates the central point of this experiment, that old can be separated from young, but not WT young from rd10 young.

groups. The classification rule was constructed using the following four steps:

1. Density functions were generated for the area and shape data, respectively, from each eye, using a penalized likelihood method. A joint density function of the area and the aspect ratio was estimated by the kernel method for each eye based on its area and shape data.
2. A newly proposed FPCA method was applied to the collection of joint density functions for all eyes to perform dimension reduction.³⁰ The first four principal components were chosen, and the corresponding principal component scores were calculated for each eye. Therefore, we obtained a 111×4 matrix of PC scores.
3. Three popular classification methods were selected and applied to the matrix of PC scores obtained in step 2:

linear determinant analysis (LDA), quadratic determinant analysis (QDA), and support vector machine (SVM).

4. The leave-one-out cross-validation method was used to determine the accuracy of the classification results: the PC scores of one eye image were selected as the test data, and the PC scores of the remaining eye images were used as training data to construct the classification rule applied to the test data. This process was repeated such that each RPE image was used once as the test data.

RESULTS

We assessed a total of 111 flatmounts, including 50 from WT and 61 from rd10 mice. For each genotype, we sampled a wide age span, from P30, P45, P60, P100, P180, and P330 to over P720. The Table lists the sample sizes and average ages of the mice in each of the four major groups.

Figure 1 sample images show the differences between the RPE sheet morphology of WT mouse eyes and that of rd10 mouse eyes. Figure 1A is a merged image of a whole RPE flatmount from an rd10 mouse at P100; it illustrates the flatmounting of the RPE sheet. This panel gives an overview of the entire sheet and shows the important areas of the RPE sheet that need to be analyzed and the overall characteristics of the different types of alterations to the RPE sheet depending on location on the sheet. The green borders of RPE cells stained with anti-ZO-1 can be recognized. The entire back of the eye was a uniform sheet with the exception of artifacts such as knife marks or scratches of the RPE monolayer. Brightly stained corneal endothelium was highlighted in bright orange. We routinely removed the superior tab of the cornea to mark that quadrant and to orient the flatmounts.

Just inner to the corneal endothelium, the remnants of the ciliary body in bright green were identified. Just inside the ciliary epithelium, a ring of 8 to 10 cells of a loosely knit group of RPE cells in a transitional zone at the far periphery of the RPE sheet was seen. Inside the transitional zone, the cells were packed in regular hexagonal and pentagonal patterns, with RPE cells a little smaller than in the transitional zone. The optic nerve head was present in the center of the preparation. The retina was detached from this point, and frequently there was a small dissection artifact from which the RPE cells were missing. Underlying the RPE was the choroid, which could be readily observed if the RPE cells were stretched thinly, or if there was an artifact or scratch to the RPE sheet that exposed the choroid. Figure 1B (from normal WT RPE at P45) shows a patch of 795 RPE cells with 1176 nuclei (Fig. 1B shows 48% of the RPE cells had two or more nuclei and the remainder were mononucleate). These counts excluded any cells on the edge or incomplete cells. Most of the RPE cells were regular, with four or six sides, and the sides were all of approximately the same length. Thus, the cells were isotropic, and few if any carried internal vertices with angles greater than 145° (i.e., most of the angles were close to 108° in the pentagons and 120° in hexagons). In the case of changes to the RPE caused by bystander or collateral damage from the rd10 mutation, expressed in the rod photoreceptor cell, many irregularities in the shape of RPE cells began to develop (Figs. 1A, 1C). Cells lost their regular hexagonal shape, became elongated, and the size of the cells became more variable in comparison to WT RPE cells of the same age. Figure 1C (same magnification as Fig. 1B) shows 844 complete cells; nuclei were not counted due to variations in the plane of focus that did not allow counting nuclei. This is unavoidable, as this reflects underlying pathology of the RPE layer in the rd10 mice, which is fatter and thicker in the bulge. The demonstration of the bulge is one of the purposes of presenting this image. There appeared to be

FIGURE 4. Analysis of RPE cell aspect ratio. (A) Boxplots of median cell aspect ratio (dimensionless) is shown as a function of age for WT eyes. Boxplots display the five-number summaries of the aspect ratio: smallest aspect ratio, lower quartile (25%), median, upper quartile (75%), and largest observed aspect ratio. There was no significant change in aspect ratio relative to age, indicating that baseline cell shape change in age was very small in WT mice. (B) Density plots are shown for each WT eye. The mode is at an aspect ratio of approximately 0.8 for both young and old WT. (C) Boxplots of median cell aspect ratios are shown as a function of age for rd10 eyes. (D) Densities were plotted for each rd10 eye. The mode is at an aspect ratio of approximately 0.8 for young rd10 and approximately 0.6 for old rd10. Black lines and symbols are for eyes $\leq$ P61 and red for $\geq$ P100 (rd10) or P180 (WT) eyes. Numbers of mice per age group and genotype are given in the Table.

a distinct bulging ring or bull's-eye pattern of highly compressed or elongated cells at the midperiphery in the sheet, which was noticeable to the human observer (Fig. 1A). Generally, parts of the RPE sheet more peripheral to the ring did not change at this time point in the rd10 mice. Large cells with more than six sides were found posterior to the ring of compressed cells in the rd10 sheets (Figs. 1A, 1C).

Figure 2 summarizes metrics and statistics of changes in the RPE cell during the aging of both WT and rd10 mice. Approximately 10,000 cells per mouse eye (of the approximately 55,000 RPE cells total per eye) and sample sizes of approximately six to eight mice per age group (Table) were analyzed. The median cell area, en face (Fig. 2A), the general trend was that with age, the cell size gradually increased by approximately 10% to 15% in WT mice over the approximately 2-year life span of the mouse. The greatest area change occurred between 60 and 180 days. In the rd10 mice (Fig. 2C), cell areas trended upward with increasing age as well. However, there were no obvious differences in median cell area between WT and rd10 eyes at the same ages. Because the median may not explain the most variability in the data, we plotted the density distributions (which contain all data) and extracted the first four principal components by FPCA. We grouped the density distributions according to age as either young ($\leq$ P61) or old ($\geq$ P180 for WT or $\geq$ P100 for rd10) based on the aforementioned greatest cell area change, the generally accepted notions of juvenile versus adult mouse ages, and arbitrary partitioning of two age groups. After the cell area density distributions were plotted (Fig. 2B, WT; Fig. 2D, rd10), qualitative properties of younger versus older mice ($\leq$ P61 versus $\geq$ P180) could be seen. The density distributions were not normal (Shapiro-Wilk test, data not shown): a peak

("mode") at approximately $200 \mu\text{m}^2$ was observed in both WT and rd10 mice. Distinguishing the older from the younger mice, the major peak at approximately 200 decreased in magnitude with age, and a tail of larger sized cells was appreciated in both WT and old rd10 mice. However, the distribution of older cells was different in comparing rd10 and WT mice. The WT mice had fewer cells over $400 \mu\text{m}^2$ (Fig. 2B) with a distinct shoulder at approximately $380 \mu\text{m}^2$. The rd10 older mice lacked the shoulder at $380 \mu\text{m}^2$ and had a larger and longer tail with more cells evident at $600 \mu\text{m}^2$ (Fig. 2D) than the WT mice.

When principal components were extracted from the area density distributions, there was a clear resolution of the young (black squares) from old (red triangles) RPE cells in both WT (Fig. 3A) and rd10 (Fig. 3B). Figure 3C combines the joint data for cell area from genotypes (both WT and rd10) and age (young versus old) from the density distributions. Figure 3C shows the first two PC scores plotted on the x - and y -axes. The separation between the solid symbols and the open symbols was clear, indicating that the young ($\leq$ 61 days, solid symbols) and the old (open symbols) could be clearly discriminated. However, the two colors, black and red, were more mixed, indicating that the discrimination between the two genotypes (black for WT, red for rd10) was not distinct, indicating that the two genotypes were not well discriminated solely by cell area.

In Figure 4, the aspect ratio of the RPE cell was evaluated, much as the RPE cell area in Figures 2 and 3. The aspect ratio is defined as the ratio of the minor axis length to the major axis length, calculated as the lengths of the major (minor) axis of an ellipse that has the same normalized second central moments as the cell region. The major and minor axes lengths were the output of CellProfiler.³¹ When the aspect ratio is equal to 1, the

FIGURE 5. The principal component score plots of cell aspect ratio are shown for genotypes and age groups. *Black squares* are for WT eyes, and *red triangles* are for rd10 eyes. (A) A plot of the first two principal components for WT eyes is shown. This plot partially resolves WT old from WT young based on aspect ratio. (B) A plot of the first two principal components for rd10 eyes is shown, illustrating ample resolution of rd10 young from rd10 old based on aspect ratio. (C) Based solely on aspect ratio, we could not fully discriminate among the four classes (WT young, WT old, rd10 young, rd10 old).

cell shape is a circle. The smaller the aspect ratio, the more elongated the cell. We found RPE cell aspect ratios in WT had values of approximately 0.74, with no significant change with age (Fig. 4A), suggesting that cell shape change was minimal in WT eyes over time. Figure 4B illustrates the close similarity in aspect ratio of young and old WT mice in the density distributions. In the rd10 mouse (Fig. 4C), the median aspect ratio at P30 started at the same value, 0.74, but it decreased with age to 0.60 from P180 to P330 and slightly increased to approximately 0.65 at 2 years of age, suggesting an elongation during disease progression in the rd10 mouse. Note that this median aspect ratio over the whole eye did not take into account the obvious spatial/regional inhomogeneity of distortion in rd10 mouse, as is evident in Figures 1A and 1C. The density curves showed a more substantial difference, with a significant shift to the smaller values for old rd10 mice. When FPCA was conducted using aspect ratio, there was a limited distinction of young from old WT (Fig. 5A) but a clear distinction of young from old rd10 (Fig. 5B) mice. Figure 5C

combines data from aspect ratios of both genotypes and ages. Again, symbols correspond to the ages (Fig. 5B, solid symbols for the young and open symbols for the old), and colors correspond to genotypes (Fig. 5, black for WT and red for rd10). But we were unable to completely separate the four groups (WT young, WT old, rd10 young, rd10 old) based solely on aspect ratio (Fig. 5C). However, we did separate WT young from rd10 young, which is a goal of this work.

By combining cell area and aspect ratio, we cleanly separated each of the four classes with the first two principal components, (Figs. 6, 7). Three dimensional plots of area and aspect ratio as the independent variables (x - and y -axes, respectively) and the density as the dependent variable (z -axis) in Figure 6 supported the resolution of each of the four experimental groups from one another. Figures 6A to 6D show results from WT young (Fig. 6A), WT old (Fig. 6B), rd10 young (Fig. 6C), and rd10 old (Fig. 6D). The main peak was sharpest and highest in the WT young mice (Fig. 6A), with less pronounced changes in aspect ratio and a more pronounced shoulder at slightly higher cell sizes in old WT mice (Fig. 6B). However, RPE cells from the rd10 mouse lost their regularity of size and aspect ratio to a greater extent with age (Fig. 6D) and showed generally greater variability, leading to the appearance of a reduced maximum peak height.

The contour plots of areas and aspect ratio densities (Figs. 6E–H) corroborated this finding. Tightly bunched isometric density lines, indicated a narrow distribution of size and aspect ratio in young WT mice (Fig. 6E), with little change in the location of the maximum on aging (Fig. 6F). Even in young rd10 mice, the RPE maximum was flatter and wider (Fig. 6G), meaning that there was increased variability in size and shape. By older ages, the rd10 mice (Fig. 6H) showed a decreased modal aspect ratio, increased variability in aspect ratio and cell area, and much more spreading of the peak. The Table lists the peak locations and the areas enclosed by the same contour lines.

We calculated the first 4 PC scores for both genotypes using two-dimensional, joint area and shape measurements. These four PC scores from 111 eyes form a matrix of size 111×4 , which we used to construct classification rules. We applied each of the three classification methods, LDA, QDA, and SVM, to the matrix to discriminate the genotype and age group. We then used the leave-one-out cross-validation method to assess the predictive accuracy. The prediction accuracies were 96.4% (i.e., 107 were correctly classified among 111 total eyes), 96.4%, and 97.3%, respectively. These high accuracies showed that, using cell area and shape, FPCA readily resolves all four groups (WT young, WT old, rd10 young, and rd10 old). Results with the first two PC scores are illustrated graphically in Figure 7, where each of the four classes were resolved from one another.

DISCUSSION

In this study, we showed that novel statistical analyses using multivariate FPCA and tools for the classification of size and shape of RPE morphology allowed discrimination of RPE genotypes and age. The use of the joint density distributions from roughly 10,000 RPE cells per mouse and FPCA instead of a simple median were key to deriving discriminatory power. We draw two conclusions: (1) there are distinctive bystander effects on the RPE cells of the rd10 mutant that are obvious in flatmount preparations; and (2) the effects include characteristic patterns and changes in tiling that can be readily recognized at early stages; these changes can be quantified quite precisely, and we can distinguish these quantitative pathological changes in rd10 eyes from normal aging changes.

FIGURE 6. Joint cell area and shape density distributions: WT young eyes (**A**), WT old eyes (**B**), rd10 young eyes (**C**), and rd10 old eyes (**D**). The height of the peaks indicates the density of cells. The *colors* show that WT young eyes had the sharpest and most concentrated peak of cell size aspect ratio distribution. The three other mountain plots (**B–D**) were blunted in height, had shoulders, and were broadly dispersed in combinations of size and shape. (**E–H**) Contours through the mountains illustrate the wider distribution and variation in combined cell size and shape (e.g., [**E**] WT young; [**F**] WT old; [**G**] rd10 young; and [**H**] rd10 old) of the effects of increasing age and affected genotype.

FIGURE 7. First two PC scores of the joint areas and aspect ratios in all four sample groups. The plot illustrates the discrimination of each group from the three others. Color, age, young (red), old (black). Triangles, WT mice; squares, rd10 mice.

It remains to be determined whether this morphometrics approach can be generalized: could this approach distinguish rd10 from rd1, rd8, or other animal retinal degenerations, or AMD from Stargardt or other human retinal disease at young ages in a masked study? The present study provides a proof-of-principle and a set of tools to answer these questions in the future.

Here, we found that, qualitatively, RPE cells in a WT mouse eye (Fig. 1B) had rather homogeneous sizes and nearly hexagonal packing. The cells appeared isotropic, with no obvious elongation or orientation bias. In contrast, RPE cells in the rd10 eyes looked more irregular, and variability increased with age (Fig. 1).^{11,32,33} Thus, qualitatively, as RPE in rd10 mice were greatly affected compared to those in WT mice, we sought to develop quantitative discriminators among the four groups (WT young, WT old, rd10 young, and rd10 old). Three tests were conducted to find accurate quantitative discriminators (cell area alone versus shape alone versus area-shape jointly).

First, in Figures 2 and 3, the cell area density curves and their first and second PC scores showed distinct features that resolved young from old WT RPE cells. To a lesser extent, young and old rd10 cells were resolved, but the four groups (WT young, WT old, rd10 young, and rd10 old) were not well resolved based only on RPE cell area.

Second, to test a measure of RPE cell shape, we chose aspect ratio. The smaller the aspect ratio, the more elongated the cell. Beyond P30, at each age tested, WT always had a greater median aspect ratio than rd10 (Figs. 4A, 4C). The density distributions and FPCA of the aspect ratio (Figs. 4, 5) showed the clear discrimination between two genotypes despite the confounding factor of age, although we felt that further refinement of our analysis would achieve better discrimination among the four groups.

Third, to discriminate among four genotype-age groups (WT young, WT old, rd10 young, rd10 old), it was necessary to use a joint area-shape dataset. Three-dimensional density plots (Figs. 6A–D) and their corresponding contour plots (Figs. 6E–H) showed (1) a tight and narrow size-shape density distribution of the WT young RPE cells; (2) the WT old RPE cells developed a shoulder extending to larger cell areas but remained confined in aspect ratio; (3) the young rd10 had area ranges similar to those of young WT eyes but greater variability and ranges in aspect ratio; (4) the old rd10, on the other hand, varied widely in both aspect ratio and area. Thus, qualitatively, these combined size-shape plots suggested good discrimination of each group from the other three. This was borne out by FPCA analyses with the first two PC scores as shown in Figure 7 and a clear visualization of the separation among the four sample

groups. This led to a clear quantitative discrimination based on the first four PC scores for each group of the joint area-shape measurements. This reduced the morphometric data from each flat mount to four PC scores. Each classification method had an accuracy of >96%, that is, given a single RPE sheet for which the area and aspect ratio density distributions are known, the odds are <4% that we would misclassify its age and genotype.

This method (using joint density distributions, FPCA, and classification) might be generally applicable to RPE morphometric data, such that different categories of RPE could be distinguished despite the age factor. Discrimination between normal subjects and patients with mutations that affect the RPE at an early age would be important diagnostic and prognostic goals, if the approach described here could be applied to highly novel noninvasive human RPE sheet imaging techniques (Rossi E, et al. *IOVS* 2013;54:ARVO E-Abstract 6282).

Here, we focused on the rd10 model. The RPE cells are bystanders to the rd10 degeneration that begins within photoreceptors.^{14,16,34} Here we showed that collateral damage to the RPE sheet occurred after the initial genetic insult to the photoreceptors. It is not yet apparent how the collateral changes occurred, but there ought to be severe oxidative stress in the RPE cells during the degeneration of photoreceptors and the remodeling of the neural retina.³⁵ We found alterations in RPE cell shape and size, which resembled known stimulation of migration and mechanical stress following the death of neighboring cells. We previously found regional differences in the rd10 RPE sheet as the mice aged and the disease progressed.^{11,33} Also we found differences in morphology, comparing the posterior RPE with far periphery regardless of age, genotype, or species. Analyzing by zone, region, quadrant, or ring, we might expect even more robust differences that may discriminate better than the whole-sheet analyses (without considering cell location) as we did here. We plan to test this hypothesis in the future.

In summary, we are now able to discriminate between age ($\leq P61$ versus $\geq P180$) and genotype (WT versus rd10) of the mouse by RPE morphometry in the present proof-of-principle study. This result is exciting because in the future we might be able to consider RPE morphometry as a diagnostic and prognostic tool. Validation on human samples is an essential next step. Non-invasive imaging of the living human eye may soon provide comparable morphometric data for the human RPE (Rossi E, et al. *IOVS* 2013;54:ARVO E-Abstract 6282). In conclusion, we compared RPE characteristics of young and old rd10 and WT mice, and we found that principal component analysis distinguished between age and pathology.

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