Supplementary Material

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The Project MACULA retinal pigment epithelium grading system for histology and optical coherence tomography in age-related macular degeneration

Methods

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Methods

Tissue Preparation

We evaluated eyes recovered from non-diabetic white donors for research by the Alabama Eye Bank (1995-2012). Median death-to-preservation time was 3:49 hours (range, 0:40-11:40 hours). Eyes were preserved by immersion in 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer following anterior segment excision. When accessioned, eyes underwent ex vivo color photography. When processed, eyes underwent ex vivo infrared reflectance imaging, 488 nm and 787 nm autofluorescence, and SDOCT volume scans (Spectralis, Heidelberg Engineering).

Donor eyes with gross macular appearance consistent with AMD and lacking other chorioretinal macular pathology were processed. An 8-mm diameter full eye-wall punch centered on the fovea was trephined, post-fixed by osmium tannic acid paraphenylenediamine to accentuate extracellular lipid and embedded in epoxy resin.
(PolyBed 812, Polysciences, Warrington PA). Sub-micrometer-thick sections\textsuperscript{43, 48} were stained with toluidine blue, scanned with a microscope (BX51, Olympus, Center Valley, PA) equipped with a 40x 0.95 NA total internal reflection fluorescence objective and image-stitching software (CellSens; Olympus, Center Valley, PA). Digital sections (visible at http://projectmacula) were used for annotating morphology and thicknesses using custom ImageJ plug-ins (http://rsbweb.nih.gov/ij/). Sections were also photodocumented with a 60X oil-immersion objective (numerical aperture = 1.4) and digital camera (XC10, Olympus) and reviewed at 1900X on a monitor. Sections were initially annotated 2012-2013 by author CAC and re-annotated by author ECZ in 2014 to incorporate newly defined RPE grades.

Annotation and layer thickness measurements

The Superior section was the longest possible near the ring of high rod density (3-5 mm superior to the foveal center).\textsuperscript{49} The Central section contained fovea, parafovea, and perifovea, and the Superior section contained only perifovea.\textsuperscript{50} The nominal sampling scheme contained 13 locations in the Superior section and 25 in Central, from 3500 µm nasal to 3500 µm temporal to the fovea. The Central section contained more locations to capture features dependent on steep gradients of foveal neuronal cell density. The number of sampling locations varied among eyes, because 1/13 GA eyes and 3/39 CNV eyes had only a Central section, retinal detachment sometimes precluded use of section ends, and Superior sections, being chords of the circular tissue punch, were shorter than Central sections, which were diameters. Locations in detached neurosensory retina, which shrunk more than RPE-choroid-sclera, were digitally adjusted to match.

Definitions of RPE cells, RPE-derived cells, and the RPE layer are given in the main text. If BLamD was not present, then there was no RPE layer, and attributing scars to sub-retinal or sub-RPE spaces required examination of surrounding tissue. We did not determine whether
vessels in fibrovascular scars originated in choroid or retinal circulation.

The procedure for measuring the thickness of epithelial RPE is given in the main text. The non-epithelial component of Shedding was not measured. The non-epithelial components of Sloughed and Intraretinal (i.e., individual desquamated cells) were measured only if they contacted the epithelial component. Dissociated RPE was not measured. We measured non-epithelial Entombed RPE when cells formed a continuous layer inside the scar, and where layered with other cells and fluid, only those cells closest to BLamD were measured. We did not measure a hyalinized envelope, if present.

Early BLamD is palisade-like, located close to BrM, has noticeable lipoprotein-derived debris within it, and appears in all BLamD. Late BLamD is scalloped (convex toward the RPE), located close to the RPE, has little lipoprotein-derived debris within it, and appears only in thick BLamD. BLamD was measured perpendicular to its outer extent if detached from BrM by sub-RPE scar or basal linear deposits. The latter are diffuse, gray-staining, lipid-rich soft drusen material between the RPE basal lamina and the inner collagenous layer of BrM; these were not measured.
Supplementary Table 1: Demographics of study eyes*

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th># eyes</th>
<th># donors</th>
<th>Gender (F:M)</th>
<th>Mean age, years (± SD)</th>
<th>Ex vivo imaging and histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geographic atrophy</td>
<td>13</td>
<td>12</td>
<td>8:4</td>
<td>85.6 ± 4.9</td>
<td>3 unilobular; 10 multilobular - 7 foveal affected; 6 foveal spared (Two fellow eyes had almost identical fovea-sparing lesions.)</td>
</tr>
<tr>
<td>Choroidal neovascularization</td>
<td>39</td>
<td>39</td>
<td>25:14</td>
<td>85.4 ± 7.2</td>
<td>37 CNV in foveola: 25 sub-retinal+sub-RPE scar; 4 sub-retinal scar, 5 sub-RPE scar; 1 sub-retinal scar+sub-RPE NV; 2 sub-RPE NV; 2 CNV extra-foveola: sub-RPE scar</td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
<td>51</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* For 22 donors with clinical histories, the median time between the last clinic visit and death was 22.6 months (range, 2.1-106.1 months). No chorioretinal pathology except AMD was present. In all eyes a foveal depression could be detected even if the disease involved the fovea. The foveola was defined as 500 µm in diameter.  
CNV= choroidal neovascularization; NV= neovascularization; RPE= retinal pigment epithelium.
**Supplementary Figure 1. RPE morphology and granules.**

**A.** Transmission electron microscopy distinguishes definitively between lipofuscin (L), melanolipofuscin (MLF), and melanosomes (M) in desquamated RPE of an 85-year-old woman with early AMD. **B.** Sub-micrometer epoxy resin section, toluidine blue stain in an 88-year-old woman with early AMD. Essentially an electron microscopy tissue preparation viewed by light microscopy, this preparation shows RPE granules clearly and is recommended for histology-OCT imaging comparisons. Sloughed RPE consists of desquamated spherical cells internal to an epithelial layer, which in turn overlies basal linear deposit (BLinD). Desquamated cells with spindle-shaped melanosomes and numerous lipofuscin granules resemble the epithelial cells in granule types, sizes, packing density, and staining properties.
**Supplementary Figure 2: Cytoskeleton of Bilaminar RPE.**

A, B: RPE cells are delineated by their cytoskeleton. In each panel, two different RPE cytoskeleton layers are visible. Each individual layer is organized as a monolayer of multiple RPE cells. The arrowheads highlight the superimposed cytoskeleton of two RPE cells of slightly different sizes and orientations (blue and white arrowheads), which are lying upon each other. The RPE mosaic around Bilaminar was intact. Images were captured as part of a larger survey of RPE autofluorescence and cytoskeletal changes in aging and AMD. Donor: 86 year-old female, neovascular AMD. Alexa647-phalloidin label. Scale bar: 50 µm.
Appendix: Key to Figures at http://projectmacula

Figure 2 RPE Grading System

A) Non-uniform RPE (RPE1): Section 2007012R-85F-4025, Eccentricity 2210 (perifovea).

B) Very non-uniform RPE (RPE2): Section 2005025R-88F-3950, Eccentricity -2590 (perifovea).

C) Non-epithelioid Dissociated RPE (RPE7): Section 2007003R-94-F-3550 Neovascular AMD, Eccentricity 1500 (perifovea).


E) Intraretinal RPE (RPE3): Section 2006001R-79M-3875, Eccentricity -800 (parafovea).


G) Sloughed RPE (RPE2A): Section 2011013R-95M-3975, Eccentricity 700 (parafovea).


K) RPE Atrophy without BLamD (RPE5): Section 2011017R-83F-4000, Eccentricity -2720 (perifovea-peripapillary).

Figure 3 Dissociated RPE

A) Section 2008003L-96F-4100, Eccentricity 1790 (perifovea).

B) Section 2011017R-83F-4000, Eccentricity 260 (parafovea).

C) Section 2000020R-85F-3975, Eccentricity -600 (parafovea).

D) Section 2011013R-95M-2000, Eccentricity -2800 (perifovea).

Figure 4 Entombed RPE
A) Section 0099028L-87F-4075, Eccentricity -1850 (perifovea)
B) Section 2003037R-77M-3975, Eccentricity 850 (parafovea)
C) Section 2000003R-93F-2000, Eccentricity 2000 (perifovea)
D) Section 0099076L-77F-2000, Eccentricity -1500 (perifovea)

**Figure 7 Clinicopathologic correlation**

Not currently online.