Human Adult Retinal Pigment Epithelial Stem Cell-Derived RPE Monolayers Exhibit Key Physiological Characteristics of Native Tissue

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PURPOSE. We tested what native features have been preserved with a new culture protocol for adult human RPE.

METHODS. We cultured RPE from adult human eyes. Standard protocols for immunohistochemistry, electron microscopy, electrophysiology, fluid transport, and ELISA were used.

RESULTS. Confluent monolayers of adult human RPE cultures exhibit characteristics of native RPE. Immunohistochemistry demonstrated polarized expression of RPE markers. Electron microscopy illustrated characteristics of native RPE. The mean transepithelial potential (TEP) was 1.19 ± 0.24 mV (mean $\pm$ SEM, $n = 31$), apical positive, and the mean transepithelial resistance (R_T) was $178.7 \pm 9.9 \Omega \cdot \text{cm}^2$ (mean $\pm$ SEM, $n = 31$). Application of $100 \mu\text{M}$ adenosine triphosphate (ATP) apically increased net fluid absorption (J_v) by $6.11 \pm 0.53 \mu\text{L} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$ (mean $\pm$ SEM, $n = 6$) and TEP by 0.33 ± 0.048 mV (mean $\pm$ SEM, $n = 25$). Gene expression of cultured RPE was comparable to native adult RPE ($n = 5$); however, native RPE RNA was harvested between 24 and 40 hours after death and, therefore, may not accurately reflect healthy native RPE. Vascular endothelial growth factor secreted preferentially basally $2582 \pm 146 \text{ pg/mL/d}$, compared to an apical secretion of $1548 \pm 162 \text{ pg/mL/d}$ ($n = 14$, $P < 0.01$), while PEDF preferentially secreted apically $1487 \pm 280 \text{ ng/mL/d}$ compared to a basolateral secretion of $864 \pm 132 \text{ ng/mL/d}$ ($n = 14$, $P < 0.01$).

CONCLUSIONS. The new culture model preserves native RPE morphology, electrophysiology, and gene and protein expression patterns, and may be a useful model to study RPE physiology, disease, and transplantation.

Keywords: human, retinal pigment epithelium, cell culture, fluid transport

The RPE is a monolayer of cells between the neural retina and choroidal blood supply, and is critically important for photoreceptor function.^{1–3} For example, in vivo light/dark transitions trigger RPE physiological changes that homeostatically maintain the chemical composition and volume of the extracellular spaces separating the apical membrane/photoreceptor and basolateral/Bruch membrane interfaces.^{4–6} Damage to the RPE with aging and disease can lead to vision loss, as seen in age-related macular degeneration (AMD), Best's disease, retinitis pigmentosa, and other retinal degenerative diseases.^{7–11}

In vitro, primary cultures of human RPE represent an attractive model for the study of RPE-related diseases, because they allow the independent pharmacologic manipulation of the retinal and choroidal sides of the monolayer while monitoring the polarized physiological responses to specific disease-producing agents.^{12–16} Retinal pigment epithelium cell lines, such as ARPE-19, a spontaneously immortalized line,¹⁷ have proven useful for in vitro studies, but are limited in their ability to mimic molecular and physiological functions of primary

RPE.^{18–24} Primary cultures of fetal human RPE (fhrPE) are a highly valuable model, since they exhibit many of the known functions of mammalian and native human RPE (nhRPE). Several protocols have been developed to improve culture reproducibility and allow assessment of a wide variety of RPE functions.^{15,24–36} However, cultures from adult human RPE (ahrPE) may be physiologically more representative of native mature tissue.

Prior pioneering work has demonstrated that ahrPE can proliferate successfully in culture. However, these cultures lose their native physiology, tending to undergo an epithelial-mesenchymal transition and form fibroblast-like progeny more readily than fhrPE.^{37–41} Recently, we have shown that the adult RPE layer includes a subset of cells that can be activated in vitro to self-renew and produce large numbers of new RPE cells.⁴² We have developed a method to culture ahrPE, which, based on the preliminary examinations so far, suggest an improvement of the preservation of morphology.⁴³ These RPE stem cell (RPESC)-derived RPE cultures can be isolated from donor eyes, cultured, and expanded to produce confluent monolayers of

cobblestone RPE. The goal of this study was to determine whether this culture method is a significant advance in preserving native physiology over other attempts to culture adult human RPE. We examined in depth the physiology of ahRPE cultures and found they share many features with fhRPE and nhRPE. These studies suggested the use of ahRPE as an in vitro model of RPE physiology and disease. Since the ahRPE cultures can be generated successfully from aged human donors, even nonagenarians, they offer the opportunity to compare RPE characteristics during aging and between non-affected and diseased donors. These “disease in a dish” models can be useful for therapeutic compound screening. In addition, our studies indicated ahRPESC-derived RPE represent a viable cell source for replacement of atrophic cells in patients suffering from RPE degenerative disease.

METHODS

Human Adult RPESC Culture

Human globes from donors aged between 67 and 93 years were obtained from the National Disease Research Interchange (Philadelphia, PA, USA), the Eye-Bank for Sight Restoration, Inc. (New York, NY, USA), and the Lions Eye Bank (Albany, NY, USA). A detailed protocol of the eye dissection has been published previously⁴³ and the major steps are illustrated schematically in Figure 1. Briefly, globes were obtained within 40 hours of death. They were immersed in Betadine solution for 5 minutes and washed three times in PBS. Then, the anterior portions were removed after a circular cut 2.5 mm posterior to the ora serrata. The vitreous and retina were removed, and the posterior eye-cup three-fourths-filled with dispase-1 solution (2U/mL, cat. no. 1284908; Roche Diagnostics, Indianapolis, IN, USA) containing 12.5 µg/mL DNase (Sigma-Aldrich Corp., St. Louis, MO, USA) and incubated at 37°C for 1 hour. The solution was gently removed and replaced with Dulbecco's modified Eagle's medium (DMEM)/F12 media containing 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, USA). Using a double bevel spoon blade (3.0 mm), the RPE were removed gently from Bruch's membrane as sheets. The media containing the RPE sheets then was collected and gently layered on top of a 10% sucrose solution. After 15 minutes, the sheets sank to the bottom, while the single cells remained in the top fraction. The bottom fraction containing the RPE sheets then was collected and plated on tissue culture plates coated with 10 µg/mL placental ECM (Becton Dickinson, Franklin Lakes, NJ, USA) in RPE media supplemented with 10% FBS, which was changed 3 times a week. After the first week, FBS was reduced to 2%. After 1 month in culture, the cells typically became confluent and exhibited cobblestone morphology. Cells then were removed by adding 0.25% trypsin for 10 to 15 minutes and plated into Transwell inserts (Corning, Inc., Corning, NY, USA) at a density of 1×10^5 cells per well. Cells were cultured for approximately 2 months until they reached a total tissue resistance of $>200 \Omega \cdot \text{cm}^2$, measured within 5 minutes of removal from the incubator using an EVOM2 (World Precision Instruments, Inc., Sarasota, FL, USA) Epithelial Volt-ohmmeter.

Solutions

Retinal pigment epithelium media was formulated by mixing 50% DMEM/F12 (CellGro; Mediatech, Manassas, VA, USA) and 50% α MEM (Sigma-Aldrich Corp.) supplemented with 1% penicillin/streptomycin (Life Technologies), 0.5% N1 supplement (Sigma-Aldrich Corp.), 2% FBS (Life Technologies), nonessential amino acids (1003 solution; Life Technologies), 1% Glutamax I (Life Technologies), 0.25 mg/mL taurine (Sigma-

Aldrich Corp.), 0.02 µg/mL hydrocortisone (Sigma-Aldrich Corp.), and 0.013 ng/mL tri-iodothyronine (Sigma-Aldrich Corp.).

Control Ringer's solution contained 120 mM NaCl, 5 mM KCl, 23 mM NaHCO₃, 2 mM MgCl₂, 10 mM glucose, 2 mM taurine, and 2.4 mM CaCl₂. This solution was bubbled continuously with 5% CO₂, 10% O₂, and 85% N₂ to a stable pH of approximately 7.4 and an osmolarity of 295 ± 6 mOsm.

Electrophysiology

Calomel electrodes in series with Ringer's solutions and agar bridges were used to measure the transepithelial potential (TEP), and the microelectrode signals were referenced to either the apical or basal bath to measure the membrane potentials, (V_A and V_B) where $TEP = V_B - V_A$. Conventional intracellular microelectrodes were made from borosilicate glass tubing (0.5-mm inner diameter and 1-mm outer diameter) with a filament (Sutter Instrument Co., Novato, CA, USA). Prior to use, microelectrodes were back filled with 150mM KCl, and had resistances of 80 to 200 M Ω .

The total transepithelial resistance (R_T), was obtained by passing 4 µA current pulses across the tissue and measuring the resultant changes in TEP, V_A , and V_B . Current pulses were bipolar, with a period of 3 seconds. Total transepithelial resistance (R_T) is the resultant change in TEP divided by 4 µA, and R_A/R_B is the absolute value of the change in V_A divided by the change in V_B ($R_A/R_B = \Delta V_A / \Delta V_B$). The current-induced TEP voltage deflections were digitally subtracted from the records for clarity and continuity of the trace. In the electrophysiology experiments (see Figs. 4–9), the black bars indicate a solution change at the manifold outside of the recording chamber. The response onset was delayed due to the time the replacement solution takes to travel from the manifold to the recording chamber.

Fluid Transport

Monolayers of ahRPE cultured on Transwell inserts were mounted in a modified Ussing chamber, and the rate of transepithelial water flow (steady-state fluid absorption rate [J_v]) was measured using a modified capacitance probe technique described previously.^{26,44} The TEP and R_T of the ahRPE monolayer were measured by injecting bipolar current via Ag/AgCl pellet electrodes that were connected to the solution baths with agar bridges (4% wt/vol). All fluid transport experiments were performed in a Steri-Cult CO₂ incubator (Thermo Fisher Scientific, Halethorp, MD, USA) at 37°C and 5% CO₂. After a 15- to 30-minute incubation in control Ringer (5% CO₂), steady-state J_v , TEP, and R_T were reached and recorded.

Electron Microscopy

The ahRPE cells on Transwell inserts were fixed in 2.5% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (EMS) at room temperature for 2 hours and rinsed three times for 5 minutes in PBS. Then, inserts were treated with 1% ice-cold osmium tetroxide in PBS solution for 1 hour. After osmication, inserts were rinsed in PBS and processed through a battery of ethanol dehydration steps (50%, 70%, 85%, and 100% ethanol) for plastic embedding. A routine transmission electron microscopy protocol was used to obtain the RPE micrographs.²⁶

Immunohistochemistry

Adult human RPE on Transwell inserts were fixed with 4% paraformaldehyde in PBS for 10 minutes, then rinsed three times in PBS. Cells were permeabilized with 0.01% saponin and

FIGURE 1. Schematic illustrating the method to culture ahRPE. Human globes are received in sterile moist chambers on ice. After rinsing, a circumferential incision is made posterior to the ora serrata and the vitreous and retina are removed. The eye cup is rinsed, then Dispase I is put into the cup and incubated. The RPE layer is scraped gently from the Bruch's membrane using an angled, double-beveled spoon blade to minimize damage. The RPE are collected and run on a sucrose gradient to separate RPE sheets from single RPE cells. The RPE sheets are plated on ECM-coated plates and typically attach within 48 hours. Confluence is reached after 2 months, with routine media changes.

blocked with normal goat serum (5%) in 1% BSA in PBS for 1 hour, then incubated with primary antibody as shown in Table 1 in the same solution overnight at 4°C. Cells were washed with PBS three times, then incubated with the corresponding Alexa Fluor conjugated secondary antibodies (1:1000, Alexa Fluor 647, goat anti-mouse IgG (H+L, Cat# A-21237; Life Technologies), Alexa Fluor 647 goat anti-rabbit IgG (H+L; Cat# A-21244), at room temperature for 45 minutes. Cells on Transwell inserts then were mounted on glass slides with antifade reagent Prolong gold (Life Technologies) and imaged using a confocal microscope (Leica Biosystems, Nussloch, Germany).

Immunoblots

Samples were prepared, as described previously.¹⁵ Briefly, the cultured ahRPE and fhRPE were solubilized on ice in 200 μ L 25 mM Tris buffer, pH 8.0 containing 2% SDS and 10 μ L/ml Protease Inhibitor Cocktail (Sigma-Aldrich Corp.). Melanin granules were removed by centrifugation. To prevent detergent-resistant multimers of claudin from forming, EDTA was added to 5 mM along with 50 μ L 5 $\times$ gel loading buffer. The samples were incubated for 10 minutes at 37°C and then for 5 minutes in a boiling water bath. Protein concentration was determined using the Micro BCA protein assay kit (Pierce,

TABLE 1. Antibody Information

Vendor	Antibody	Species	Dilution	Catalog #
Abcam, Cambridge, MA, USA	Bestrophin	Ms monoclonal	1:100	ab2182
R&D Systems, Minneapolis, MN, USA	Carbonic anhydrase	Ms monoclonal IgG2A	1:33	MAB21881
R&D Systems	Claudin-19	Ms monoclonal IgG1	1:300	MAB6970
Abcam	CRALBP	Ms polyclonal IgG	1:100	ab15051
Sigma-Aldrich Corp.	MCT1	Rb polyclonal IgG	1:100	HPA003324
Sigma-Aldrich Corp.	MCT3	Rb polyclonal IgG	1:100	AV43935
Abcam	NaKATPase	Ms monoclonal	1:100	ab7671
Invitrogen, Grand Island, NY, USA	Phalloidin 555	Dir conj	1:100	A34055
Invitrogen	ZO-1	Rb polyclonal	1:100	716300
Cell Signaling, Beverly, MA, USA	Ezrin	Rb monoclonal IgG1	1:100	3149
Sigma-Aldrich Corp.	Cytokeratin 8	Ms monoclonal IgG1	1:100	C5301
Michael Redmond, Bethesda, MD, USA	RPE-65	Rb polyclonal	1:50	Private stock

Rockford, IL, USA). Equal amounts of protein were resolved by SDS–polyacrylamide gel electrophoresis and immunoblotted.

Gene Expression

48-Well Assay Protocol. For nhRPE, RNA was isolated within 24 to 40 hours of death. For cultured RPE, RNA was isolated from cells at passage one, 4 to 6 months after initial seeding, after treatment with RNAProtect (Qiagen, Venlo, The Netherlands) to preserve RNA integrity. RNA was isolated using the RNeasy Plus kit (Qiagen), using Nanodrop. RNA samples were shown to have a ratio of absorbance at 260 and 280 nm above 1.9, with the maximum 260/280 ratio observed being 2.09. Messenger RNA (mRNA) was retrotranscribed using the Superscript III kit (Life Technologies) and cDNA used at a concentration equivalent to 2 ng starting RNA per well. A 20 μ l amount of PCR Reaction Mix was used per well of the custom RPE-gene expression array (custom reference no. CA-PH10651A; SABioscience/Qiagen, Valencia, CA, USA). To benchmark RNA quality further, two donors were used exclusively for assaying RNA quality using the above methods, 24 and 32 hours after death using Agilent 2100 bioanalyzer. Their RNA quality index was measured to be RIN: 6.5 and 7.5, respectively, which are of acceptable quality for RNAseq.⁴⁵ To analyze the expression of genes for gap, adherens, and tight junctions, mRNA expression was estimated using custom qRT²PCR arrays prepared by BioRad Laboratories, Inc. (Hercules, CA, USA). Each primer set was matched to have an amplification efficiency of 100% under the conditions used; ACTB, GAPDH, and B2M were used to normalize the data. Expression was expressed relative to CLDN19 using the delta-delta C(t) method.⁴⁶

Enzyme-Linked Immunosorbent Assay (ELISA). Vascular endothelial growth factor and pigment epithelium-derived factor (PEDF) protein were measured in media samples collected from the apical and basal sides of the same Transwell insert 24 hours after complete media change. Human VEGF ELISA kit (Life Technologies) uses polyclonal antibodies specific for human VEGF coated onto the wells of microtiter strips. The VEGF was immobilized with incubation buffer, then conjugated to biotin, treated with streptavidin-horseradish peroxidase (HRP) and colored with stabilized chromogen (tetramethylbenzidine). Optical densities were obtained within 2 hours of test using a microplate reader (1420 multi-label counter; Perkin Elmer, Waltham, MA, USA) at 450-nm wavelength, and data analyzed using Microsoft Excel software (Microsoft, Redmond, WA, USA). The PEDF ELISA was performed according to kit instructions (ChemiKine PEDF Sandwich ELISA; EMD Millipore, Billerica, MA, USA). Briefly, samples were treated with 8 M urea for 1 hour on ice, loaded on a mouse monoclonal antibody

precoated plate after addition of 1:100 PBS diluent, and incubated for 1 hour at 37°C. Biotinylated mouse anti-human PEDF then was added for 1 hour. Detection of color product was similar to the VEGF ELISA described above. Cytokine concentrations were corrected for volume differences between the apical and basal media chambers.

RESULTS

A major aim of this study was to analyze ahRPE cultures derived from adult human RPESCs⁴² to determine if they exhibit the physiological characteristics typical of native RPE. The nhRPE isolation steps are illustrated in Figure 1. RPE cells in small groups or sheets attach within 24 hours of plating and begin proliferating. Over the first month in culture, the cells continue to proliferate and then differentiate, making a transition from a flat cobblestone layer to acquire a columnar and hexagonal morphology. After 2 months they form flat, confluent monolayers of cobblestone ahRPE. When the cells divide, the melanin concentration decreases; they are largely unpigmented at 1 month, but repigment over the second month, still to a lower level than typical nhRPE.

Immunohistochemistry

To determine the polarization of ahRPE monolayer cultures, immunostaining of tight junctions, visual cycle proteins, and membrane-specific and cytoskeletal markers was performed, and images were captured using confocal imaging (Fig. 2). In these monolayers, Claudin-19 was present at the apical end of the lateral membrane^{15,47} next to the tight junction complex protein ZO-1⁴⁸ consistent with the presence of tight junctions. Phalloidin, which binds to F-Actin,⁴⁹ was apically localized, illustrating the polarized epithelial nature of the cultures. Ezrin, a membrane-associated protein involved in cytoskeletal organization and found preferentially in nhRPE microvilli,⁵⁰ was located appropriately in the cultures. Cytoplasmic proteins cellular retinaldehyde binding protein (CRALBP) and RPE65, which are involved in the retinal visual cycle,^{51,52} also were present, as was the secretory epithelium-specific marker Cytokeratin 8.⁵³ Bestrophin, an RPE-specific calcium-activated chloride channel,^{54,55} showed perinuclear and basal expression in ahRPE cultures. Monocarboxylate transporters 1 and 3 (MCT1 and 3) were present apically and basally, respectively,⁵⁶ while carbonic anhydrase IX (CA IX) was located apically and basally¹⁴ as seen in native tissue. Na⁺K⁺ATPase also was observed apically and basally, as reported previously in native RPE^{57,58} and other adult human RPE cultures.⁴⁰ Immunoblotting revealed the presence of claudin-3 and claudin-19 in amounts comparable to fhRPE (Supplementary Fig. S1A).

FIGURE 2. Adult human RPE cultures express markers typical of native RPE. Nuclei are labeled with 4',6-diamidino-2-phenylindole (DAPI; cyan), whereas all other immunofluorescence is red or yellow. Claudin 19, Phalloidin (F-actin), Ezrin, ZO1, and MCT1 are preferentially located on the apical side, as observed by looking at the Z-plane located to the right of the 2D image. RPE65, Cytokeratin 8, CRALBP are cytoplasmic. Bestrophin is found perinuclear and on the basal side. CA IX and Na⁺K⁺-ATPase are located on the apical and basolateral membranes. MCT3 is observed basally. Scale bars: 10 μ M.

Unlike fhRPE, Claudin-1, Claudin-2, and Claudin-10 could not be detected (data not shown). In summary, the cultured ahRPE monolayers exhibited polarized distribution of proteins similar to nhRPE.

Electron Microscopy

Electron microscopy was used to evaluate the morphology of cultured ahRPE grown in Transwells, which have a semiporous polyester membrane. Scanning electron micrographs of the surface of the RPE monolayer (Fig. 3A) demonstrate cobblestone morphology along with highly dense apical microvilli.⁵⁹ After 4 months in culture, ahRPE showed many features of nhRPE, including tight junctions, microvilli, polarized pigmentation, and basally localized nuclei. Figure 3B shows the ahRPE had secreted a substantial basement matrix, seen as electron-dense material between the basal side of the cell and the polyester Transwell. A time-course of the culture shows that within the first 2 weeks, the cells are relatively flat, with no obvious tight junctions formed and no microvilli (Fig. 3Ci). By 2 months, small microvilli covered the apical surface, tight junctions were forming, pigmentation was polarized to the apical membrane, and the nucleus was moving basolaterally (Fig. 3Cii); that is, the monolayer was developing into a more native-like morphology.

Electrophysiology

Membrane transport systems and receptors that regulate ion and fluid transport across RPE have been analyzed extensively.^{13,14,44,60–69} While native transport properties have been

demonstrated in cultured adult RPE of other mammalian species,^{5,26,61,68,70} those of cultured ahRPE have not yet been examined to our knowledge. We surveyed a selection of physiological properties to assess functionality of the ahRPE cultures.

To determine the stability of these monolayers, TEP and R_T were measured for a minimum of 60 minutes. Figure 4 shows a simultaneous recording of TEP and R_T conducted approximately 15 minutes after mounting the Transwell insert in the experimental chamber. All measurements are reported as mean $\pm$ SEM. Figure 4A shows that TEP and R_T were stable for over 60 minutes ($n = 5$) and the mean data from 31 experiments is summarized in Figure 4B. The average TEP and R_T was 1.2 ± 0.24 mV and $178 \pm 8.9 \Omega\text{-cm}^2$, respectively. These measurements indicated that ahRPE cultures maintain apical and basolateral membrane resting potentials, constant over time, thus, maintaining a transport potential across the monolayers. Moreover, a mean R_T of $178 \Omega\text{-cm}^2$ is consistent with the resistance of native RPE monolayers and suggests that the tight junctions are intact.^{5,64,70–72}

In vivo, the subretinal space (SRS) separates the photoreceptors outer segments and the RPE apical membrane. The chemical composition and volume of the SRS changes following transitions from dark to light and these changes alter RPE ion and fluid transport. In vitro, switching the apical bath K⁺ concentration from 5 to 1 mM mimics the potassium ([K⁺]_o) changes that occur in the SRS following the transition from dark to light. Therefore the electrophysiological responses to this perturbation is an informative test for the presence of the appropriate in vivo channels and cell membrane trans-

FIGURE 3. In electron microscopy, ahRPE cultures exhibit multiple features of native tissue. (A) Scanning electron microscopy (SEM) images of the surface of an ahRPE culture at multiple magnifications. (B) A 6-month culture of ahRPE. (C) Time course of ahRPE polarization: 1 week (*Cit*), 2 weeks (*Cit*), and 2 months (*Cit*). Tight junctions (arrow 1), apical microvilli (arrow 2), polarized apical pigmentation (arrow 3), basally localized nucleus (arrow 4), basement membrane (arrow 5) are identified.

porters, including apical membrane K channels, the apical $\text{Na}^+\text{K}^+\text{-ATPase}$, and Na/K/2Cl cotransporters.

Figure 5A summarizes the data from five experiments in which we recorded the electrophysiological responses to a decrease of $[\text{K}^+]_o$ in the apical bath.⁷³ The decrease in apical $[\text{K}^+]_o$ hyperpolarized the apical (V_A) and basolateral (V_B) membranes by 14 ± 1.2 mV. Following the decrease in apical $[\text{K}^+]_o$, the TEP increased by 0.46 ± 0.52 mV ($n = 21$). These experiments showed that the apical membrane hyperpolarized at a greater rate than the basolateral membrane and that the response is reversible following the increase in $[\text{K}^+]_o$ from 1 to 5 mM, which mimics the light to dark transition in vivo. Concomitantly, the ratio of the apical to basolateral membrane resistance (R_A/R_B) decreased by 0.01 ± 0.0021 ($n = 5$) in the

intracellular recording experiments. In all 21 experiments (5 intracellular and 16 TEP/ R_T), total tissue resistance (R_T) increased by $10.0 \pm 1.81 \Omega\cdot\text{cm}^2$. The box plots in Figure 5B show the variation between Transwells measurements of TEP and R_T . The ahRPE TEP- and R_T -induced changes are statistically significant ($P < 0.001$) and Figure 5A shows that the transport potential and tissue resistance changes following the $[\text{K}^+]_o$ changes are reversible in this culture model of adult human RPE.

Table 2 compares the data plotted in Figure 5B to published data of nhrPE⁷⁴ and data collected from primary cultures of fhrPE. A number of features stand out. Native human RPE, ahRPE, and fhrPE all share similar baseline measurements of V_A and R_A/R_B , and their TEPs are not appreciably different. The

FIGURE 4. Steady-state and TER of ahRPE primary cultures. Retinal pigment epithelium cultivated on polyester membrane Transwells maintain stable TEP and TER for over 1 hour. (A) TEP (solid line) and R_T (dotted line) plotted as a function of time. (B) Box plots of the average baseline TEP (mV) and TER ($\Omega\cdot\text{cm}^2$) of 31 experiments.

FIGURE 5. Effect of reducing apical bath $[K^+]_o$ from 5 to 1 mM on V_A , V_B , TER, and TEP in ahRPE cultures. (A) Reduction of apical K^+ from 5 to 1 mM (solid bar) depolarized V_A (dotted orange line), V_B (solid green line) each from -48.4 to -66.7 , while TEP increased (solid line) by 0.1 mV and R_T increased by $7 \Omega \cdot \text{cm}^2$ (dotted line). (B) Box plots of the average change in TEP and TER upon application of 1 mM K^+ apically in 16 experiments. * $P < 0.001$.

ratio of apical-to-basolateral membrane resistance (R_A/R_B) is more closely aligned in nhrPE and ahRPE cultures compared to fhrPE. The total tissue resistance (R_T) is significantly different among the three cell models. Although this variation may be largely due to differences in damage expressed within the circumferential mechanical seal around the tissues, it also could include resistance differences in the cellular or paracellular pathways. The comparison of primary cultures (adult versus fetal human) shows more robust membrane voltage and resistance responses to a step change in apical $[K^+]_o$ from the fetal tissue (ΔV_A , ΔV_B and ΔTEP , ΔR_T and R_A/R_B). Part of the ΔR_T and perhaps part of the ΔTEP difference may be due to edge damage that is not physiological. These culture models also may differ in apical or basolateral membrane K^+ channel density and/or conductance or paracellular properties. An examination comparing these models in terms of permeability, selectivity, and total conductance of the cellular and paracellular pathways would help distinguish between these possibilities.⁷⁵

As a first step in testing for the presence of K^+ channels on the basolateral membrane, we increased the basal bath solution isoosmotically from 5 to 25 mM $[K]_o$ and measured the TEP and R_T responses. The data in Figure 6 show that the resting TEP was 1.29 ± 0.33 mV, while the resting R_T was $216 \pm 7.1 \Omega \cdot \text{cm}^2$ ($n = 16$). After elevating $[K]_o$, TEP increased by 0.39 ± 0.05 mV, while R_T decreased by $30.6 \pm 4.2 \Omega \cdot \text{cm}^2$ —consistent with the presence of ahRPE K^+ channels at the basolateral membrane. The ahRPE TEP- and R_T -induced changes are statistically significant ($P < 0.001$).

Retinal catecholamines are released following light/dark transitions and their receptors are located on the RPE apical membrane.^{76,77} These paracrine signals, for example, dopamine and epinephrine, can modulate RPE membrane potential and conductance as well as transepithelial fluid transport.^{63,64,78} To determine whether adrenergic receptors were present apically in ahRPE cultures, we applied apical epinephrine and measured the TEP and R_T responses. In four experiments the mean baseline TEP was 1.81 ± 0.41 mV and mean R_T was $186.7 \pm 19.2 \Omega \cdot \text{cm}^2$, TEP and R_T increased by 0.31 ± 0.035 mV and $19.9 \pm 4.8 \Omega \cdot \text{cm}^2$, respectively, consistent with the presence of apical membrane adrenergic receptors (Fig. 7). The ahRPE TEP- and R_T -induced changes are statistically significant ($P < 0.001$). Side-by-side comparison of nhrPE, ahRPE, and fhrPE changes in TEP and R_T in response to epinephrine show that ahRPE respond similarly to nhrPE and fhrPE (Supplementary Table S1).

Retinal pigment epithelial cells have been shown to express P2Y₂ purinoceptors on the apical membrane.^{5,26,79} Previous studies have described three phases of the ATP response in RPE. The first phase is due to a Ca^{2+} activated increase in Cl^-

TABLE 2. Native Human RPE (nhrPE) Was Compared to ahRPE, and Primary Cultures of fhrPE at Steady-State (5 mM $[K^+]_o$ in Apical and Basal Baths) and Following Reduction of Apical $[K^+]_o$ to 1 mM K^+

$\Delta[K]_o$ -Induced Changes in RPE Electrophysiology						
	Steady-State ($[K^+]_o = 5$ mM K)				1 mM K^+	
	nhrPE	ahRPE Cultures	fhrPE Cultures $n = 5$		ahRPE Cultures $n = 5$	fhrPE Cultures $n = 5$
V_A , mV	-49 ± 3.5 ($n = 4$)*	-48.6 ± 2.0 ($n = 5$)	-54.6 ± 1.0	ΔV_A , mV	-14 ± 1.2	-24 ± 3.4
V_B , mV	NA	-48.1 ± 2.1 ($n = 5$)	-52.3 ± 1.1	ΔV_B , mV	-14 ± 1.2	-23 ± 3.3
TEP, mV	1.9 ± 0.3 ($n = 4$)*	1.2 ± 0.24 ($n = 31$)	2.2 ± 0.4	ΔTEP , mV	0.46 ± 0.05	2.2 ± 0.2
R_T , $\Omega \cdot \text{cm}^2$	79 ± 24 ($n = 4$)*	178 ± 8.9 ($n = 31$)	566 ± 42	ΔR_T , $\Omega \cdot \text{cm}^2$	10 ± 1.8	-51 ± 11
R_A/R_B	0.7 ± 0.25 ($n = 15$)*	0.79 ± 0.02 ($n = 5$)	0.34 ± 0.06	$\Delta R_A/R_B$	0 ± 0.01	-0.05 ± 0.08

* Data from Quinn and Miller.⁷⁴ Measurements reported are mean $\pm$ SEM. NA, not available.

FIGURE 6. Effect of a 5-fold increase in basal side $[K^+]_o$ on TEP and R_T in ahRPE. (A) Changing the basal bath $[K^+]_o$ from 5 to 25 mM (solid bar) reversibly increased TEP by 0.91 (solid line) and decreased R_T by 39 $\Omega \cdot \text{cm}^2$ (dotted line). (B) Box plots of TEP and R_T changes ($n = 16$). Ba, Basal. * $P < 0.001$.

channel conductance at the basolateral membrane that increases TEP. During the second phase of this response, TEP is reversed due to a decrease in conductance of apical membrane K^+ channels. The electrochemical basis of phase three is less well understood. We tested for the presence of purinoceptors in cultured ahRPE with the apical application of 100 μM ATP (Figure 8). This elicited a typical triphasic response, with an initial rise in TEP of 0.53 ± 0.048 mV and a R_T decrease of 18.5 ± 3.5 $\Omega \cdot \text{cm}^2$ ($n = 25$) consistent with the activation of basolateral membrane Cl^- channels. The ahRPE TEP- and R_T -induced changes are statistically significant ($P < 0.01$).

An essential property of RPE is the net steady-state absorption of fluid from its apical to basolateral side.

Therefore, we measured transepithelial fluid transport concomitant with TEP and R_T as shown in Figure 9. Adult human RPE cultures transported fluid from the apical to basolateral side at a steady-state rate of 4.3 ± 1.3 $\mu\text{L} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$ (J_v), while TEP was 1.2 ± 0.3 mV and R_T was 138 ± 24.4 $\Omega \cdot \text{cm}^2$ ($n = 6$). Apical application of ATP increased steady-state J_v to 10.4 ± 1.6 $\mu\text{L} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$, while TEP increased during phase I to 1.7 ± 0.5 mV and R_T decreased to 130 ± 27.0 $\Omega \cdot \text{cm}^2$. After washout, the cultures returned to a baseline rate of 3.06 ± 0.51 $\mu\text{L} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$, while TEP was 1.2 ± 0.3 mV, and R_T was 137 ± 26 $\Omega \cdot \text{cm}^2$ ($n = 6$; $P < 0.03$). These transport rates were similar to those found in native bovine and fhRPE measurements, indicating a close similarity between native tissues and these cultures.^{5,12,13,26,79} Fluid transport across the RPE is driven by

FIGURE 7. Epinephrine-induced electrical responses (TEP, R_T) in ahRPE. (A) Transepithelial potential (solid line) and R_T (dotted line) plotted as a function of time. Apical epinephrine (10 nM, solid bar) increased TEP by 0.41 mV and R_T by 14 $\Omega \cdot \text{cm}^2$. (B) Box plots of the change in amplitude of TEP and R_T upon 10 nM epinephrine administration to apical bath ($n = 5$). * $P < 0.001$.

FIGURE 8. Electrical responses of ahRPE following addition of 100 μM ATP (solid bar) to the apical bath. (A) Transepithelial potential (solid line) and R_T (dotted line) plotted as a function of time. The ATP elicited a triphasic response indicated as I, II, III, and by vertical dotted lines. Phase I is characterized by a rapid 0.63 mV increase in TEP concomitant with a drop in R_T of 39 $\Omega\cdot\text{cm}^2$. This increase was followed in phase II by a drop in TEP. In phase III, TEP again increased by 0.46 mV at a slower rate. (B) Box plots of the TEP and R_T changes in amplitude following addition of apical ATP (100 μM , $n = 25$). Ap, Apical. * $P < 0.01$.

a cassette of apical and basolateral membrane transporters, receptors, channels and intracellular signaling pathways.^{5,12,13,61,64,80} Two such proteins are the cystic fibrosis transmembrane conductance regulator (CFTR) that mediates the transport of Cl^- out across the basolateral membrane accompanied by an appropriate counter ion and the osmoti-

cally obliged fluid apical membrane Na^+ , K^+ , 2Cl^- cotransporter, which drives salt and water across the apical membrane. The data summarized in Supplementary Figure S2 show that fluid transport is substantially blocked by the addition of CFTR inhibitor-172 (15 μM) to the basal bath. Supplementary Figure S3 shows that fluid transport across

FIGURE 9. Adenosine triphosphate-induced fluid absorption (J_v) by ahRPE. (A) J_v (solid circles) plotted as a function of time (absorption indicated by positive values). (B) Concomitant TEP (solid line) and R_T (dotted line) measurements. Solid bar indicates addition of Ringer's solution containing 100 μM ATP which increased J_v from 2.6 to 5.6 $\mu\text{L}\cdot\text{cm}^2\cdot\text{h}^{-2}$ and decreased R_T by 10.3 $\Omega\cdot\text{cm}^2$; EP increased by 0.95 mV. (C) Summary of six experiments as plotted. * $P < 0.05$ from previous condition.

FIGURE 10. Gene expression comparing ahRPE to nhrRPE and fhrRPE. Complementary DNA from freshly isolated RPE from adult cadaver donors was compared to their genetically-matched cultured RPE counterparts as well as to nongenetically matched cultured fetal RPE by quantitative PCR. Adult cultured RPE was normalized to nhrRPE (A) and fhrRPE (B) data and plotted in LOG_{10} scale. *Significant difference, with a $P < 0.05$, $n = 5$.

ahRPE is blocked by the addition of a specific Na^+ , K^+ , 2Cl^- cotransporter inhibitor bumetanide (100 μM) to the apical bath. A much larger inhibition of J_v was observed by the simultaneous addition of bumetanide and a 3-fold smaller (5 μM) addition of CFTR inhibitor-172 to the apical and basal baths, respectively (Supplementary Fig. S4). Together these experiments suggested ahRPE fluid transport depends on similar mechanisms to nhrRPE and fhrRPE.

Gene Expression

Global expression profiling of native adult and fetal RPE and cultured fetal RPE compared to the Novartis expression data base of 78 tissues²⁴ from throughout the body revealed a unique signature set of 154 genes whose expression levels distinguish RPE from other cell types. This set is defined by genes that are a factor of 10 or more highly expressed in RPE compared to their 78 counterparts in the Novartis set. By this definition, genes that are possibly important for RPE function may go undetected either because they are expressed at relatively low levels (e.g., *CFTR*) and/or are expressed globally at relatively high levels in tissues throughout the body (e.g., regulators of the complement pathway and cholesterol homeostasis). This signature set, thus, is incomplete, but in a preliminary analysis, it has enabled us to compare ahRPE cultures with nhrRPE and fhrRPE over a broad range of RPE functions. Since nhrRPE is processed between 24 to 40 hours after death and has been found to affect RNA quality,⁸¹ the data may not accurately reflect the in vivo human RPE. Further research will be required to evaluate the effects time, method of procurement, and storage have on RPE physiology and, thus, more accurately interpret data from postmortem RPE.

We compared ahRPE cultures with their respective genetically matched, acutely isolated nhrRPE and with nongenetically matched fhrRPE (Fig. 10). Due to the limited sample of nhrRPE RNA, we analyzed a subset genes selected from the 154 gene list, including membrane and channel proteins, RPE development and identity genes, pigmentation, and visual cycle genes. Overall, cultured ahRPE expressed either similar or higher levels of expression of membrane and channel proteins, RPE development and identity genes, pigmentation, and visual cell cycle genes compared to nhrRPE, as shown in Figure 10A. The genes whose expression was significantly higher in ahRPE than nhrRPE were *COL11A1*, *GPNMB*, *RAB27A*, *CSPGS*, *ALDH1A3*, *CLDN19*, *MITF*, *RPE65*, *MYRIP*, and *TRYP1* (Fig. 10A). On the other hand, RPE markers were generally expressed at lower levels in the ahRPE compared to fhrRPE (Fig. 10B). All genes associated with the visual cycle trended to be more highly expressed in ahRPE, but only one gene was significantly higher. Interestingly, *TFEC*, a paralog of *MITF*, was much more highly expressed in fhrRPE and nhrRPE, while *MITF* was much more highly expressed in ahRPE.

Cytokine Secretion

Polarized cytokine secretion of VEGF and PEDF in RPE monolayers has been demonstrated previously^{26,27,82–85} and, therefore, can be used as a benchmark of RPE monolayer health and function. Figure 11 illustrates data from 14 donors in which we examined the apical and basolateral secretion of VEGF and PEDF in ahRPE monolayers plated in polyester Transwell inserts. Vascular endothelial growth factor was preferentially secreted to the basal bath at a rate of 2582 ± 146 pg/mL/d compared to apical secretion, which occurred at a rate of 1548 ± 162 pg/mL/d ($n = 14$, $P < 0.01$). Pigment

FIGURE 11. Polarized expression of PEDF and VEGF Adult human RPE was passaged onto Transwell inserts and cultured for at least 2 months. Media then was taken from the top and bottom wells 24 hours after media change, and assayed for concentrations of VEGF and PEDF proteins via ELISA. In the 14 donors assayed, mean VEGF secretion was 1548 pg/mL/d in the apical side and 2582 pg/mL/d in the basal side. Mean PEDF secretion was 1487 ng/mL/d in the apical side and 863.8 ng/mL/d in the basal side. *Significant difference, with a $P < 0.01$, $n = 14$.

epithelium-derived factor was preferentially secreted to the apical bath at a rate of 1487 ± 280 ng/mL/d compared to a basolateral secretion of 864 ± 132 ng/mL/d ($n = 14$, $P < 0.01$). In seven of those experiments, R_T also was measured and found to be $194.9 \pm 8.6 \Omega \cdot \text{cm}^2$, with the cultures having a polarized secretion of PEDF and VEGF indistinguishable from the larger group. The polarized secretion of these neuroprotective/angiogenic factors can be represented as a ratio comparing basal (BA) to apical (AP) $[\text{PEDF/VEGF}]_{\text{AP}} = 959$ and $[\text{PEDF/VEGF}]_{\text{BA}} = 332$, and this comparison is consistent with fhRPE ($[\text{PEDF/VEGF}]_{\text{AP}} = 76$ and $[\text{PEDF/VEGF}]_{\text{BA}} = 19.4$). The secretion of apical PEDF/VEGF likely provides neuroprotective/neurotrophic support for the retina and possible maintenance of nonfenestrated retinal blood vessel fenestration.⁸⁶ The observed decrease in $[\text{PEDF/VEGF}]$ secretion from the basolateral surface of the RPE may reflect a coordinated decrease in PEDF and an increase in VEGF secretion to support choroidal blood vessel fenestration.^{26,87,88}

DISCUSSION

There is considerable interest in developing “disease in a dish” models of human RPE.^{7,8,89–91} Successful maintenance of epithelial polarity and function in vitro has been a long sought goal for studying RPE-related diseases.^{32,92–94} In this study, we showed that ahRPE cells isolated and cultured from nhrRPE⁴³ have functional and morphologic characteristics resembling native tissue. The primary ahRPE cultures exhibit a variety of characteristics fundamental for maintaining the homeostatic environment of the retina/RPE/choroidal complex including polarized protein expression and EM morphologic features, RPE electrophysiological responses and gene signature markers, and polarized cytokine secretions. These data indicated that this culture method reliably and robustly produces ahRPE monolayers with physiological characteristics that are stable for many months in vitro and are potentially valuable for studying RPE function, RPE disease, and RPE transplantation. While no direct tests were conducted to assay phagocytosis and the visual cycle, the gene expression data and protein

expression suggested that ahRPE cultures might be capable of performing these functions as well, which will be examined in depth in the future.

The characteristics of the nhrRPE, fhRPE, and ahRPE models provide a framework for evaluating their optimal use. With regard to cultured fhRPE one feature consistently shown is higher transepithelial resistance (TER) compared to native nhrRPE (Table 2), and from other adult species explants.^{5,64,70–72} How and why this divergence from normal native adult RPE occurs currently is unknown, though culture medium has an important role. When serum is removed from the apical medium chamber, the TER of fhRPE falls more in line with other tissues,¹⁵ and serum-free medium can further the maturation of stem cell-derived RPE.⁹⁵ More generally, the environmental impact of aging, smoking, the accumulation of stress, and the immune responses to stress, can significantly alter RPE gene expression and phenotype. With regard to the ahRPE, these cells were cultured from donors between 67 and 93 years of age and, therefore, the results represent aged RPE. Concerning nhrRPE, long-term changes in gene expression are observed when cells are in hypoxic conditions for less than 24 hours.⁹⁶ The low R_T of nhrRPE may hint that the circumferential mechanical seal around the tissue may be incomplete and not representative of the in vivo state. Moreover, the length of time after death increases protein degradation,⁹⁷ and tissue transplant rejection.⁹⁸ Long-term culture of fetal and adult RPE may reverse some of these changes and contribute to the higher gene expression and electrophysiological measurements observed in fetal and adult cultured RPE compared to native. However, perhaps these effects are still reflected in the behavior of ahRPE cells as they possess more heterogeneity in packing geometry (Fig. 3A) and heterogeneity (in a cell to cell comparison) of some proteins (Fig. 2), both of which are features of aged or less healthy RPE, which could be an advantage or a limitation of the use of these cells, depending on the application of the model.^{99,100}

In many species the Na^+K^+ -ATPase is preferentially found on the apical side of RPE in vivo. However, in our cultures we find Na^+K^+ -ATPase expressed apically and basolaterally, which is consistent with previous reports of cultured adult human RPE⁴⁰ as well as in native tissue.^{57,58} Efforts to understand why cultured RPE express Na^+K^+ -ATPase on both membranes in culture are worthwhile. One hypothesis is that photoreceptors may have a role in sorting Na^+K^+ -ATPase to the apical membrane, perhaps by creating a signal, such as cytokine release, or a cell-cell interaction that triggers apical polarization/segregation for which there is some evidence.¹⁰¹ However, fhRPE in culture without photoreceptors still exhibit preferential apical localization of Na^+K^+ -ATPase, suggesting that either a media component may be required for adult cultures to achieve preferential apical polarization²⁵ or that a maturation or aging component prevents preferential Na^+K^+ -ATPase localization.

Another difference observed between fetal and adult cultured RPE is the difference in direction of the epinephrine R_T response. Typically, epinephrine depolarizes the resistance of apical and basolateral membranes due to the presence of α -1 adrenergic receptors. Apical epinephrine first depolarizes the basolateral membrane by increasing Cl^- conductance, which is followed by a decrease in the apical membrane K^+ conductance.⁶⁴ Here, we find in ahRPE, TEP increases as expected from native RPE data; however, R_T also increases, whereas in fhRPE, R_T decreases, consistent with the epinephrine response (opening of basolateral membrane Cl^- channels) in fhRPE. However, the increase in TEP observed in ahRPE, in response to apical epinephrine, is consistent with the activation of apical membrane α -1 adrenergic receptors in ahRPE cultures.

Retinal pigment epithelium marker gene expression of ahRPE compared to genetically matched nhRPE, and non-genetically matched fhRPE was compared. Overall the expression of the majority of genes between ahRPE and nhRPE was not significant. Of the significant differences, 90% showed higher expression in ahRPE than nhRPE suggesting that the cultures exhibit native-like RPE identity. Of the two genes that were significantly decreased in ahRPE compared to nhRPE, one is *Claudin-16*, a tight junction protein. Interestingly, *Claudin-19* is significantly expressed at higher levels in ahRPE than nhRPE suggesting a change in the ratio of *Claudin-19/Claudin-16* in the tight junction complex.¹⁰² We investigated further this question regarding relative expression of tight junctions. We focused on the question: what is the relative ratio among the claudins within ahRPE and fhRPE? We analyzed tight junction associated gene expression relative to each other within each given cell type (Supplementary Fig. S1B). This analysis demonstrated no significant difference in the ratios of various tight junction-associated genes between ahRPE and fhRPE. Therefore, ahRPE has all the capability to make tight junctions similar to fhRPE at least at the transcriptome level. Further work will be required to examine whether proper subcellular localization is occurring, especially those that have signaling functions.

All of the transcription factors involved in RPE development that we measured (*PAX6*, *LHX2*, *SOX9*, *CRX*) had a greater than 5-fold higher expression in fhRPE over ahRPE. This difference may be explained by fetal cells retaining more immature features than adult. The transcription factors *MITF* and *OTX2* regulate many genes central to RPE function and surprisingly are expressed most highly in ahRPE. Why they would be higher than nhRPE is not understood. One possibility is that it is due to the cell division of ahRPE, because this involves robust levels of transcription.¹⁰³ Alternatively, it could be due to the improved health of ahRPE compared to native tissue that inevitably undergoes declines after death. Future studies investigating the epigenetic regulation of these genes will likely provide further insight into the gene expression differences between ahRPE, fhRPE, and nhRPE.

Adult human RPE are known to dedifferentiate when cultured. The methods used for the ahRPE cultures studied here create conditions that reduce the likelihood RPE will permanently change while fostering the preservation of native RPE physiology.⁴³ We have demonstrated many hallmarks of differentiated RPE including expression of Bestrophin-1, TER, tight junction protein expression, apical MCT1 expression, polarized cytokine secretion, expression of visual cycle genes, demonstration of RPE specific ion channels, and fluid transport. Electrophysiology data suggest that all of the conductances tested seem present, some of which at a lower functional capacity than fetal and/or young adult RPE. To further define the differentiation state, future work will examine the presence of channels that are typically lost upon culture or gained because of it. L-type Ca^{2+} channels, for example, are only expressed for a maximum of 9 days in culture in adult rat RPE.¹⁰⁴ Some evidence suggests that RPE in culture transdifferentiate toward a neural phenotype, expressing low-voltage gated Ca^{2+} channels similar to channels found near synaptic terminals, and TTX sensitive Na^{+} channels.^{105,106} Determination of the presence of these RPE conductances and others, including $\text{Na}^{+}/\text{H}^{+}$ exchanger, and M-type K^{+} will provide a better understanding of the differentiation state of ahRPE cultures.

Using the methodology presented here, we routinely obtained between 1.0×10^6 and 5×10^6 RPE cells/donor globe pair. During passages 0 and 1, these cells undergo approximately six to eight population doublings among passage 0 and passage 1, resulting in at least 6.2×10^7 RPE

cells/donor, which display the presented characteristics. A second passage can produce 5×10^8 RPE cells/donor (not shown). We successfully obtained RPESC-derived RPE monolayer cultures from patients with AMD, diabetic retinopathy, and glaucoma, and anticipate that this method will be effective for patients with other RPE-related diseases. The amount of ahRPE cells obtained is not only sufficient to carry out the various kinds of experiments shown here, but also can be useful for future studies requiring tens of millions of cells, such as chromatin immunoprecipitation sequencing.

A major improvement in the expansion and culture of fetal human RPE with preserved native physiology,^{15,25–27} has led to a surge in understanding RPE physiology, gene expression, and function. When pairing this method with gene editing technologies, such as homologous recombination, RPE-related diseases caused by single gene mutations can be studied in the flexible context of an in vitro system. However, multifactorial diseases, such as AMD, with genetic and epigenetic components, are more difficult to study and model using fetal human RPE. Determining the optimal model to study AMD pathophysiology is premature, as each model contributes to understanding the relationship between RPE function and disease in its own way. The results shown here, using a recently described culture method,⁴³ provide a new model useful for the in vitro study of adult and aged RPE, RPE disease, and RPE transplantation.

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