Appropriately differentiated ARPE-19 cells regain phenotype and gene expression profiles similar to those of native RPE cells

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Purpose: The RPE cell line ARPE-19 provides a dependable and widely used alternative to native RPE. However, replication of the native RPE phenotype becomes more difficult because these cells lose their specialized phenotype after multiple passages. Compounding this problem is the widespread use of ARPE-19 cells in an undifferentiated state to attempt to model RPE functions. We wished to determine whether suitable culture conditions and differentiation could restore the RPE-appropriate expression of genes and proteins to ARPE-19, along with a functional and morphological phenotype resembling native RPE. We compared the transcriptome of ARPE-19 cells kept in long-term culture with those of primary and other human RPE cells to assess the former’s inherent plasticity relative to the latter.

Methods: ARPE-19 cells at passages 9 to 12 grown in DMEM containing high glucose and pyruvate with 1% fetal bovine serum were differentiated for up to 4 months. Immunocytochemistry was performed on ARPE-19 cells grown on filters. Total RNA extracted from ARPE-19 cells cultured for either 4 days or 4 months was used for RNA sequencing (RNA-Seq) analysis using a 2 × 50 bp paired end protocol. The RNA-Seq data were analyzed to identify the affected pathways and recognize shared ontological classification among differentially expressed genes. RPE-specific mRNAs and miRNAs were assessed with quantitative real-time (RT)–PCR, and proteins with western blotting.

Results: ARPE-19 cells grown for 4 months developed the classic native RPE phenotype with heavy pigmentation. RPE-expressed genes, including RPE65, RDH5, and RDH10, as well as miR-204/211, were greatly increased in the ARPE-19 cells maintained at confluence for 4 months. The RNA-Seq analysis provided a comprehensive view of the relative abundance and differential expression of the genes in the differentiated ARPE-19 cells. Of the 16,757 genes with detectable signals, nearly 1,681 genes were upregulated, and 1,629 genes were downregulated with a fold change of 2.5 or more differences between 4 months and 4 days of culture. Gene Ontology analysis showed that the upregulated genes were associated with visual cycle, phagocytosis, pigment synthesis, cell differentiation, and RPE-related transcription factors. The majority of the downregulated genes play a role in cell cycle and proliferation.

Conclusions: The ARPE-19 cells cultured for 4 months developed a phenotype characteristic of native RPE and expressed proteins, mRNAs, and miRNAs characteristic of the RPE. Comparison of the ARPE-19 RNA-Seq data set with that of primary human fetal RPE, embryonic stem cell–derived RPE, and native RPE revealed an important overall similar expression ratio among all the models and native tissue. However, none of the cultured models reached the absolute values in the native tissue. The results of this study demonstrate that low-passage ARPE-19 cells can express genes specific to native human RPE cells when appropriately cultured and differentiated.

The RPE, a polarized monolayer of highly differentiated epithelial cells, positioned strategically between the neural retina and the vascular choriocapillaris, performs crucial functions that are necessary to maintain the function and survival of the photoreceptors and other retinal cells. These functions include the formation of the blood–retinal barrier by tight junctions, transporting nutrients to the neural retina, regulating ion, pH, and fluid homeostasis in the subretinal space, absorbing excess light, scavenging free radicals and reactive oxygen species (ROS), regenerating the visual chromophore 11-cis retinal, and phagocytosing and degrading rod outer segments that are undergoing circadian shedding [1]. Because of these functions, the integrity of the RPE is critical for retinal function, and damage or malfunction of the RPE may play a central role in the pathogenesis of various retinal diseases, such as retinitis pigmentosa and age-related macular degeneration (AMD) [2,3].

In vitro cultures of human RPE cells present an attractive model for studying the physiology and pathophysiology of native tissue. Although much work has been done using immortalized cell lines created from several species, including the rat, pig, and human [4-6], these RPE culture models generally fail to preserve many of their specialized characteristics and the ability to recapitulate functional
characteristics and gene expression patterns exhibited by the RPE in vivo. Primary cultures of human fetal RPE (fhRPE) have been used widely as they are known to retain many characteristics of the native human RPE [7-9], while primary cultures of the adult human RPE are considered the most physiologically mature form to study RPE in vitro [10]. However, primary RPE cells cultured from different donors may exhibit physiological differences and lose their capacity to redifferentiate after a limited number of expansions, also losing important RPE characteristics [11]. An important disadvantage of culturing human primary RPE cells is that human eyes are often difficult to obtain. Another disadvantage is the genetic variability inherent in the use of cells from different donors.

ARPE-19, a human RPE cell line established by Dunn et al. [5] from a single individual, has been used as an alternative to native RPE as these cells exhibit epithelial cell morphology and express several genes specific for the RPE, such as RPE65, a protein preferentially and abundantly expressed in the RPE [12], and cellular retinaldehyde-binding protein (CRALBP), a retinoid-binding protein involved in the regeneration of visual pigment [13]. These cells perform many of the known functions of the human RPE, including assimilation of photoreceptor outer segments (POS) by phagocytosis [5,14]. In addition, these cells have been widely used to study various aspects of cell growth and differentiation [15-18]. However, it has become difficult to replicate some differentiated characteristics of the native RPE because ARPE-19 cells lose their specialized properties after multiple passages. Epithelial-mesenchymal transition (EMT) is thought to play an important role in this process in which epithelial cells shed their epithelial characteristics and acquire migratory mesenchymal cell-like properties [19]. MicroRNAs, single-stranded noncoding small (about 21 nucleotides) RNA molecules, have been shown to play a role in cellular differentiation by regulating EMT and vice versa [20,21]. Several studies have shown that culture conditions can be a potential confounding influence on the phenotype of these cells [7,18]. ARPE-19 cells cultured in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose and pyruvate have recently been shown to be an excellent model to study human RPE cell function [22]. Although these cells cultured in this medium have been shown to restore pigmentation and the expression of mature RPE cell markers with western blotting, little is known about the overall gene expression profile of these cells. Therefore, we used RNA sequencing (RNA-Seq) to carry out a whole transcriptome analysis on ARPE-19 cells cultured in DMEM with high glucose and pyruvate to elucidate how the changes in gene expression might account for RPE epithelial and cellular characteristics. In this study, we observed that long-term culture induces epithelial phenotype differentiation with the dark pigmentation characteristic of RPE in ARPE-19 cells in addition to the expression of genes and proteins preferentially expressed in the RPE. In addition, miR-204 and miR-211, the two most RPE-enriched miRNAs and inducers of epithelial differentiation [8], are highly increased along with microphthalmia-associated transcription factor (MITF), the master regulator of RPE differentiation. Strikingly, RPE65 mRNA expression was greatly increased. The RNA-Seq analysis uncovered more than 3,000 differentially expressed genes. Cross-referencing with the reported transcriptome analyses of RPE cells from human embryonic stem cells, fetal tissues, and native RPE cells showed that most genes specific to RPE function are upregulated in the differentiated ARPE-19 cells. These observations suggest that the transcriptome of the differentiated ARPE-19 cells closely resembles that of the native human RPE. This study shows that ARPE-19 cells can be used to model the cellular functions of RPE, provided that low-passage cultures are appropriately cultivated and differentiated.

METHODS

Cells and culture conditions: The human retinal pigment epithelial ARPE-19 cell batch used in these experiments was validated by the American Type Culture Collection (ATCC) Cell Line Authentication Service (Promega, Madison, WI) using tandem repeat analysis plus the Amelogenin gender determining locus and was a perfect match for the ATCC human cell line CRL-2302 (ARPE-19) [23]. The cells (two to three passages after authentication) were grown on Primaria® tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose, L-glutamine, and 1 mM sodium pyruvate supplemented with 1% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml; DMEM + high glucose/pyruvate/1% FBS; Thermo Fisher Scientific, Grand Island, NY), as described previously [22]. Sodium pyruvate and other media components were freshly added during the media preparation, and the prepared media were used within a week or two. The cells were maintained at 37 °C in a humidified environment of 5% CO₂ and were allowed to grow for 4 days or 4 months with media exchange performed twice a week.

Immunocytochemistry and confocal microscopy: ARPE-19 cells grown for 4 months on laminin-coated Transwell® filter inserts were blocked for 30 min with 1% bovine serum albumin (BSA) and stained for 1 h at room temperature with the following antibodies: rabbit anti-zenula occludens-1 (ZO-1, 1:200; Thermo Fisher Scientific, Waltham, MA, Cat # 61–7300), rabbit anti-claudin-2 (1:100; Thermo Fisher
After the sequencing was completed, the reads were aligned to External RNA Controls Consortium (ERCC) sequences to assess the success of the library construction and sequencing. A subset of the reads (about 1 million reads) were aligned to the base call files were converted into FASTQ files using Illumina Software (CASAVA) and Expression Analysis developed open source programs. To prepare the reads for alignment, the sequencing adapters and other low-quality bases were clipped. The reads were aligned to External RNA Controls Consortium (ERCC) sequences to assess the success of the library construction and sequencing. A subset of the reads (about 1 million reads) were aligned to other spiked-in control sequences (PhiX and other Illumina controls used during library preparation), residual sequences (globin and rRNA), and poly-A/T sequences that persisted after clipping. The reads were also aligned to a sampling of intergenic regions to assess the level of DNA contamination. On average, about 98% of the sort-pair-end reads was mapped using the CLC genomics workbench on the GRCh38.

RNA-Seq library preparation and sequencing: The ARPE-19 cells cultured as described for either 4 days or 4 months were used for total RNA extraction using the miRNeasy Mini Kit (Qiagen, Valencia, CA). The RNA samples were submitted to Expression Analysis, Inc., for RNA-Seq analysis (Q^3 Solutions, Morrisville, NC). Briefly, stranded and rRNA depleted cDNA libraries were prepared from the total RNA samples using the TruSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, CA). This method preserves mRNA and other noncoding RNA species, including lncRNA, snRNA, and snoRNA. The cDNA libraries were analyzed for size distribution using an Agilent Bioanalyzer (Agilent, Santa Clara, CA) and then normalized to 2 nM in preparation using the KAPA Library Quant Kit (# KK4824, KAPA Biosystems, Wilmington, MA). The libraries were sequenced using a 2 × 50 bp paired end protocol with the median number of 87.3 million reads across all samples on the Illumina HiSeq 2500 (Illumina).

Data processing: After the sequencing was completed, the base call files were converted into FASTQ files using Illumina Software (CASAVA) and Expression Analysis developed open source programs. To prepare the reads for alignment, the sequencing adapters and other low-quality bases were clipped. The reads were aligned to External RNA Controls Consortium (ERCC) sequences to assess the success of the library construction and sequencing. A subset of the reads (about 1 million reads) were aligned to other spiked-in control sequences (PhiX and other Illumina controls used during library preparation), residual sequences (globin and rRNA), and poly-A/T sequences that persisted after clipping. The reads were also aligned to a sampling of intergenic regions to assess the level of DNA contamination. On average, about 98% of the sort-pair-end reads was mapped using the CLC genomics workbench on the GRCh38.

p5 human genome release. The differential gene expression analysis (DESeq2) software package was used to identify differentially expressed transcripts [24].

Gene read counts, derived from three biologic replicates, were averaged and subjected to paired Student t test analysis. Expression levels were given as fragments per thousand bases of gene per million bases mapped (FPKM) for normalization. The basic statistical calculations were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA.). To identify the affected pathways and recognize shared ontological classification among differentially expressed genes, the set was examined with Gene Set Enrichment Analysis (GSEA) [25] as implemented with Pathway Studio Desktop Platform Version 9.0 (Elsevier Inc., Philadelphia, PA). The FPKM values of the RNA-Seq data from the human native RPE isolated from the nasal, temporal, and macular regions of the RPE/choroid in human eye tissue were downloaded for comparison analysis [26]. Row reads from human fetal RPE (hFRPE) and RPE derived from the H1 and H9 human embryonic stem cell (hESC) lines were downloaded from the Sequence Read Archive (SRA). Ingenuity pathway analysis (IPA) and DAVID were used for pathway analysis [27]. The data obtained were used to compare the expression levels of the genes in the RNA-Seq data set with the RNA-Seq data sets from the hESC-derived RPE and hFRPE (GSE36695), as well as the native RPE from the nasal, temporal, and macular regions of the RPE/choroid [26,28]. Scatter plots with regression lines were generated using GraphPad for each comparison. The Spearman and Pearson correlation coefficients were calculated for more than 20,000 genes.

Quantitative real-time (RT)–PCR: ARPE-19 cells cultured for either 4 days or 4 months were used for total RNA and miRNA extraction using the RNeasy Protect Mini Kit (Qiagen) and the mirVana miRNA isolation kit (Thermo Fisher Scientific), respectively. Total RNA (2 μg) was reverse transcribed using the high-capacity cDNA archive kit (Thermo Fisher Scientific). The universal cDNA synthesis kit (Exiqon, Woburn, MA) was used for cDNA synthesis from miRNA. Five microliters of cDNA was used as a template for PCR, and each PCR reaction (20 μl) was performed on the ViiA™ 7 Real-Time PCR System using various TaqMan® (Thermo Fisher Scientific) or LNA probes (Exiqon; Table 1). The manufacturer’s default thermal cycling conditions were followed (40 cycles of 1 s at 95 °C and 20 s at 60 °C). GAPDH or RNUIAI were used as the reference gene for mRNA or miRNA, respectively. The results were expressed as n-fold induction or inhibition in gene expression relative to endogenous control calculated using the ΔΔCt method.
Western blot analysis: The human RPE cells cultured for either 4 days or 4 months were washed twice with PBS (1X; 155 mM NaCl, 1.06 mM KH$_2$PO$_4$, 2.97 mM Na$_2$HPO$_4$·7H$_2$O, pH 7.4, #10010-023, Gibco, Thermo Fisher Scientific), and cell extracts were prepared using cell lysis buffer according to the manufacturer's recommendation (Cell Signaling, Danvers, MA). Cell lysates containing equal amounts of protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) using 4–12% NUPAGE Bis-Tris gels and then transferred to a nitrocellulose membrane using the iBlot® Dry Blotting System (Thermo Fisher Scientific). After blocking in 5% non-fat milk in Tris-buffered saline (TBS) containing 0.01% Tween-20 for 1 h, the membranes were incubated overnight at 4 °C with the following primary antibodies: anti-rabbit RPE65 (1:10,000, PETLET), anti-rabbit RDH5 (1:5,000, Abcam: ab101457), anti-rabbit RLBP1 (1:40,000, gift of J. Saari, University of Washington), anti-rabbit LRAT (1:5000, Abcam: ab137304), anti-mouse bestrophin (1:5,000, Abcam: ab2182), anti-mouse MERTK (1:1,000, Santa Cruz: SC-365499), anti-mouse Tyrosinase (1:1,000, Santa Cruz: SC-20035), anti-rabbit E-cadherin mAb (1:1,000, Cell Signaling: #3195), anti-mouse vimentin (1:1,000, Santa Cruz: SC-6260), and anti-rabbit ZEB1 (1:1,000, Cell Signaling: #3396). Peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G (IgG) antibody was used as the secondary antibody, and the immunocomplexes were visualized with a chemiluminescence method using the enhanced chemiluminescence (ECL) Prime Western Blotting Detection Kit (GE Healthcare Life Sciences, Marlborough, MA). The blots were then stripped and reprobed with mouse monoclonal anti-α-tubulin antibody (Cell Signaling: #3873, 1:20,000).

Table 1. Real-time RT–PCR Primers/Probes for mRNA and miRNA Expression Analysis.

| Gene Symbol | Source              | Assay Type | Assay ID/Catalog No. |
|-------------|---------------------|------------|---------------------|
| RPE65       | ThermoFisher Scientific | TaqMan     | Hs01071462_m1       |
| LRAT        | ThermoFisher Scientific | TaqMan     | Hs00428109_m1       |
| RLBP1       | ThermoFisher Scientific | TaqMan     | Hs00165632_m1       |
| RDH5        | ThermoFisher Scientific | TaqMan     | Hs01123934_g1       |
| RDH10       | ThermoFisher Scientific | TaqMan     | Hs00416907_m1       |
| BEST1       | ThermoFisher Scientific | TaqMan     | Hs00188249_m1       |
| TYR         | ThermoFisher Scientific | TaqMan     | Hs00165976_m1       |
| MERTK       | ThermoFisher Scientific | TaqMan     | Hs01031973_m1       |
| KL          | ThermoFisher Scientific | TaqMan     | Hs00183100_m1       |
| TYRO3       | ThermoFisher Scientific | TaqMan     | Hs00170723_m1       |
| AXL         | ThermoFisher Scientific | TaqMan     | Hs01064444_m1       |
| CDH1        | ThermoFisher Scientific | TaqMan     | Hs01023894_m1       |
| CDH2        | ThermoFisher Scientific | TaqMan     | Hs00983056_m1       |
| CDH3        | ThermoFisher Scientific | TaqMan     | Hs00999915_m1       |
| TJP1        | ThermoFisher Scientific | TaqMan     | Hs01551861_m1       |
| VIM         | ThermoFisher Scientific | TaqMan     | Hs00958111_m1       |
| CCDN1       | ThermoFisher Scientific | TaqMan     | Hs00765553_m1       |
| MITF        | ThermoFisher Scientific | TaqMan     | Hs0117294_m1        |
| TRPM1       | ThermoFisher Scientific | TaqMan     | Hs00931865_m1       |
| TRPM3       | ThermoFisher Scientific | TaqMan     | Hs00257553_m1       |
| GAPDH       | ThermoFisher Scientific | TaqMan     | Hs99999905_m1       |
| miR-204-5p  | Exiqon              | SYBR Green  | # 206072            |
| miR-211-5p  | Exiqon              | SYBR-Green  | # 204009            |
| RNU1A1      | Exiqon              | SYBR-Green  | # 308015            |
room temperature and washed once with PBS. The ARPE-19 cells were plated on 12-well, 12 mm diameter Transwell® inserts at a density of 300,000 cells/cm² and were allowed to grow for 4 months with media exchange performed twice a week. Transepithelial electrical resistance (TER) was measured using STX3 chopstick electrodes, and the net TER values (Ω × cm²) were calculated by subtracting the value of blank from the value recorded for each individual filters plated with cells and multiplying the background subtracted TER values by the area of the Transwell® filter membranes. A minimum of six independent Transwell® filters were used in each experimental condition and repeated in two separate experiments.

Retinoid analysis: All procedures involving retinoids were conducted under dim red light. ARPE-19 cells were grown on Transwell® polyester semipermeable filters (Corning, NY), 0.4 μm pore size, for 4 months in DMEM + high glucose/pyruvate/1% FBS medium. The cells were then washed twice with serum-free media, treated with or without 4 μM all-trans retinol given basally (the outer chamber) in serum-free media and then incubated overnight in a humidified environment of 5% CO₂ at 37 °C. Serum-free media from the apical (inner) chamber were collected, and the retinoids were extracted in the presence of hydroxylamine. Briefly, serum-free media were mixed with 1 ml of freshly made hydroxyamine buffer (50 mM MOPS, 10 mM NH₄OH, pH 6.5) and with 1 ml ethanol and then incubated in the dark for 30 min at room temperature. Following this, the retinoids were extracted twice with the addition of 4 ml hexane, vortexing, and centrifugation (2,000 × g, 8 min). The upper hexane phases were collected and pooled, and solvent was evaporated under a gentle stream of argon. The dried samples were dissolved in 50 μl hexane and then analyzed with high-performance liquid chromatography (HPLC). In addition, the cells were washed, harvested, and then homogenized in PBS (pH 7.4) using a disposable microtissue homogenizer (BioMasher II, Warrington, PA). The cell homogenates were extracted into hexane (as above). Retinoloxime standards were prepared following the protocol of Garwin and Saari [29]. Briefly, 0.5 μmol authentic all-trans-retinal (Sigma-Aldrich Corp., St. Louis, MO), or 11-cis-retinal (Toronto Research Chemicals, Toronto, Canada), dissolved in 1 ml ethanol was incubated in the presence of 10 mM hydroxyamine, 10 mM MOPS, pH 6.5, for 30 min in the dark at room temperature. The newly formed retinaloximes were extracted twice with the addition of 4 ml hexane. The upper hexane phases were combined and solvent was evaporated under a gentle stream of argon. Standards and samples were separated on a LiChrospher Si-60 (5 μm; ES Industries, West Berlin, NJ) normal-phase column using a 4.5% ethyl acetate, 0.5% dioxane (v/v) in the hexane mobile phase at a flow rate of 1.2 ml/min. Absorbance was monitored at 365 and 325 nm for the presence of retinaloxime and retinylester, respectively. Data were analyzed using Empower 3 software (Waters Corp., Milford, MA).

**STRING interaction analyses:** Potential interactions among selected genes were analyzed using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), a database and web resource dedicated to protein–protein interactions, including physical and functional interactions [30]. The gene IDs of the up- and downregulated genes were used as an input in the online database to find the associations derived from several computational predicted and experimentally determined functional associations and protein–protein interactions. The result was graphically displayed with different colored edges indicating the type of evidence.

**Statistical analysis:** All values are expressed as mean ± standard deviation (SD), n = 4. For statistical significance, the paired Student t test in Excel was used. A p-value of less than 0.05 denotes statistically significant differences. The results shown are representative of three or more independent experiments.

**RESULTS**

**ARPE-19 cells exhibit RPE characteristics after prolonged culture:** Maintenance of native RPE characteristics by ARPE-19 cells depends on how they are cultured and passaged. In addition, these cells undergo dedifferentiation during culture, and this particular phenomenon appears to be a product of cell substrate and growth media [18,22,31]. Figure 1 illustrates the typical growth of the ARPE-19 cells cultured on 100 mm Primaria® Petri dishes. Initially, the ARPE-19 cells cultured in DMEM with 4.5 g glucose and 1 mM sodium pyruvate and supplemented with 1% FBS (DMEM + high glucose/pyruvate/1% FBS) show non-epithelial morphology while they are subconfluent and mitotically active (Figure 1A). They attain morphology reminiscent of fibroblasts after 4 days of reaching confluence (Figure 1B) and remain in elongated fibroblast morphology for more than 2 weeks under these culture conditions. However, the cells grown in culture for 1 month exhibit islands of hexagonally packed epithelial-like cells characteristic of the RPE (Figure 1C). As the cell cultures mature, they increasingly exhibit characteristics of the RPE, such as well-defined cell borders and an increase in pigmentation (indicative of de novo synthesis of melanin pigment). This differentiation required 3–4 months after cultures reach a confluent density. The long-term cultures developed features characteristic of the native RPE, including the typical cobblestone epithelial morphology and heavy pigmentation often visible to the naked eye (Figure 1D).
1D), an important structural feature characteristic of native RPE cells. These cells remain well differentiated and form a monolayer of pigmented cells resembling differentiated RPE cells in vivo even after a year in culture (data not shown).

To characterize further the phenotypic changes associated with differentiation, we examined the existence of tight junctions (Figure 2B) between adjacent cells, an important structural feature of native RPE monolayers [32], in the ARPE-19 cells cultured for 4 months. We examined the expression and localization of the tight junction-associated protein ZO-1, claudin-2, and actin with immunocytochemistry and confocal microscopy. These cells express the tight junction complex protein ZO-1, predominantly localized to the cell edges within the cultured RPE cells, suggesting that these differentiated RPE culture form continuous cell–cell contact with tight junctions. Claudin-2 expression was also localized to the tight junction complex uniformly across the monolayer in the differentiated RPE cells (Figure 2D,E) and was detected along with ZO-1 in the junctional complex (Figure 2F, merged). In addition, the actin cytoskeleton in these cells is distributed at the cell periphery along the tight junctions, closely resembling that of the native RPE (Figure 2C). We also observed the expression of PMEL (also known as PMEL17), an integral membrane protein exclusively expressed in pigmented cells, in the differentiated ARPE-19 cells (Figure 2A). In addition, we used the Millicell® electrical resistance system to measure the TER, a common methodology for measuring the barrier function that is often taken as an indicator of the permeability and resistance of tight junctions [33]. ARPE-19 cells plated on laminin-coated Transwell® filters were grown in DMEM + high glucose/pyruvate/1% FBS for the duration of the experiment. The
barrier properties of the culture were measured via TER, and a mean level of $126 \pm 26 \text{ \Omega cm}^2$ TER ($n = 12$) was observed in the cells cultured for 4 months.

*Differentiation-induced changes in gene expression:* To determine whether changes in gene expression might account for acquisition of the RPE phenotype, we performed a whole transcriptome RNA-Seq analysis of the RPE cells kept in culture for 4 days and for 4 months. The ARPE-19 cells were seeded at low density, and cells were collected at 4 days while subconfluent or at 4 months when they had acquired the typical cobblestone epithelial morphology with heavy pigmentation. RNA-Seq yielded approximately 84.1 million on-target reads from the median number of 87.3 million actual reads for each of the six cDNA libraries (three from the cells cultured for 4 days and three from the cells cultured for 4 months). The ARPE-19 cells were plated on laminin-coated Transwell® filter membrane at a density of $3 \times 10^5$ cells/cm$^2$ and grown for 4 months as outlined in the Methods section. Rabbit anti-zonula occludens-1 (ZO-1), mouse anti-premelanosome protein (PMEL), mouse anti-claudin-2 primary antibodies, and Alexa Fluor secondary antibodies were used to immunostain the ARPE-19 cells at 4 days post-confluency; rhodamine-phalloidin was used to stain actin. A: PMEL immunostaining correlates with the presence of melanosome pigmentation. B: ZO-1 immunostaining shows good junctional development and localization at the cell borders containing a mixture of elongated and polygonal cells. C: Actin immunostaining reveals circumferential actin distribution throughout the height of the cells. D: Claudin-2 immunostaining shows tight junction localization uniformly across the monolayer. E: ZO-1 immunostaining shows good junctional development and localization at the cell borders regardless of the secondary antibody used. F: Claudin-2 immunofluorescent labeling shows colocalization with ZO-1 in the tight junction complexes (merged image of D and E). Images were taken using an Andor Revolution XD spinning disk confocal microscope using the 40X Plan Fluor oil immersion objective. Scale bar = 15 μm.
for 4 months) obtained from the pooled cultured cells. The reads were aligned to the human genome issue hg38 using the CLC genomics workbench. The differential gene expression analysis (DESeq2) software package was used to identify differentially expressed transcripts [24]. Detailed RNA-Seq data associated with this publication are available through the Gene Expression Omnibus (GEO; GSE88848). Overall, an average of 98% of the reads aligned to the transcriptome. The gene compilation for this particular analysis consists of 27,939 genes and 77,612 isoforms. Volcano plots were used to visualize the differential gene expression between the RPE cells cultured for 4 days and for 4 months and the distribution of the fold changes and the p-values of the 27,939 genes (Figure 3). To identify genes that are differentially expressed, a fold-change criterion was employed with the mean fold change between 4 months versus 4 days to be ≥2.5 or ≤-2.5. Of the 16,736 genes with detectable signals, about 1,681 (9.96%) genes were upregulated, and approximately 1,629 (9.72%) genes were downregulated with a fold change of 2.5 or more differences between 4 months and 4 days. The genes were then ranked by their greatest fold change to better characterize differentially expressed genes with critical functions within the RPE.

Table 2 comprises the genes most highly upregulated in the differentiated RPE cells cultured for 4 months, genes previously identified as RPE marker genes, or genes with functions characteristic of differentiated RPE cells [34-38]. The database is based upon Gene Ontology and scientific literature curated by Pathway Studio/Elsevier. Gene Ontology analysis showed that these upregulated genes are associated with, for example, visual cycle, phagocytosis, pigment synthesis, cell differentiation, and RPE-related transcription factors. Among the most highly upregulated genes, ranked by fold change, are the pigmentation-related genes, DCT (OMIM 191275), TRPM1 (OMIM 603576), OCA2 (OMIM 611409), TYRPI (OMIM 115501), and TYR (OMIM 606933). The gene with the largest increase (2,840 fold) was DCT, an important regulatory enzyme that plays a pivotal role in the biosynthesis of melanin [39]. All the genes associated with the visual cycle, RPE65 (OMIM 180069), LRAT (OMIM 604863), RLBP1 (OMIM 180090), RDH5 (OMIM 601617), RBP1 (OMIM 180260), and RDH10 (OMIM 607599), were also highly increased. In particular, RPE65 had a more than 1,460-fold increase in gene expression in the differentiated RPE cells cultured for 4 months. Genes that have been reported to be associated with phagocytosis were also highly increased in the RPE cells cultured for 4 months. In particular, the expression of MERTK (OMIM 604705), an indispensable receptor for POS internalization [40], and ITGAV (OMIM 193210; integrin αv), which promotes POS recognition and binding [41], were increased by 17- and 12-fold, respectively.

Genes whose expression correlates with the state of confluence, and are preferentially expressed in differentiated cells, were highly increased in the RPE cells cultured for 4 months. In particular, the expression of death-associated protein-like 1 (DAPLI; Gene ID: 92196), abundantly expressed in the retina/RPE transcriptome and involved in early stages of epithelial differentiation [42], was increased by more than 1,900-fold in the differentiated RPE cells. Genes known to be abundantly expressed in the RPE, and that play a protective role in RPE function, such as PEDF (OMIM 172860; 190-fold) and cystatin C (CST3, OMIM 604312; 44-fold), were also highly increased. The expression of claudin 2 (CLDN2; OMIM 300520), which plays an important role in determining tight junction selectivity and permeability, was significantly upregulated (210-fold) in the differentiated ARPE-19 cells. In addition, with the RT–PCR analysis, we confirmed that the expression of claudin 3 (OMIM 602910; CLDN3, ∆CT = 29) and claudin 19 (OMIM 610036; CLDN19, ∆CT = 35), the major claudin of human RPE, were slightly increased in the differentiated ARPE-19 cells, supporting the RNA-Seq observations (data not shown). However, the level of expression of claudin 3 and claudin 19 was lower than that of claudin 2 (∆CT = 26). E-cadherin (CDH1; OMIM 602910) and P-cadherin (CDH3; OMIM 114021), epithelial markers expressed in the native RPE monolayer, were highly increased compared to the RPE cells in culture for 4 days. Several RPE-associated transcription factors, such as PAX6 (which synergizes with MITF to activate the expression of genes involved in pigment biogenesis [43]), SOX9 (a key regulator of visual cycle genes [44]), OTX2 (which regulates RPE specification during retina development [45]), and MITF [46,47], were all observed to be significantly upregulated in the differentiated ARPE-19 cells cultured for 4 months that resembled the native RPE phenotype.

Table 3 shows selected genes that were downregulated with a mean fold change of ≤ 2.0 in the differentiated ARPE-19 cells that were cultured for 4 months. The majority of the most down-regulated genes play a role in the cell cycle and its regulation, based on the Gene Ontology analysis. For example, in the RPE cells cultured for 4 months, the expression of transforming growth factor beta 1 (TGFB1; OMIM 190180), a major inducer of EMT [48], decreased by more than 21-fold compared to the cells that were cultured for 4 days. In addition, the expression of cyclin-dependent kinase 1 (CDK1; OMIM 116940), an important factor in determining the cell cycle timing of mitosis by interacting with cyclin
B, decreased by more than 31-fold in the differentiated RPE cells.

**RPE differentiation promotes expression of RPE-specific visual cycle genes and proteins:** The expression of key enzymes of the visual cycle is widely used as a marker for RPE-specific differentiation. We examined the expression of visual cycle genes in the ARPE-19 cells cultured for 4 days or 4 months with RT–PCR (Figure 4A). All genes associated with the visual cycle (RPE65, CRALBP, LRAT, RDH5, and RDH10) showed increased expression in the differentiated ARPE-19 cells cultured for 4 months, supporting the RNA-Seq observations. In particular, the mRNA expression of RPE65 was increased more than 10,000-fold in the ARPE-19 cells cultured for 4 months compared with the cells cultured for 4 days.
| Gene | Gene Name | Biological Function | p-value | 4 Days | 4 Months | FC 4M/4D |
|------|-----------|---------------------|---------|--------|----------|----------|
| **Vitamin A Metabolism** | | | | | | |
| RPE65 | Retinal Pigment Epithelium-Specific Protein 65kDa | Visual pigment regeneration | 1.32E-05 | 0.01 | 9.75 | 1462.50 |
| TTR | Transthyretin | Retinol transport | 1.04E-05 | 0.00 | 3.98 | 1326.67 |
| RGR | Retinal G Protein Coupled Receptor | Receptor for retinal | 5.43E-04 | 0.02 | 15.98 | 959.00 |
| RLBPI | Retinaldehyde Binding Protein 1 | Retinol regeneration | 2.50E-04 | 0.39 | 198.37 | 504.32 |
| BCMO1 | Beta-Carotene 15,15-Monoxygenase 1 | Beta-carotene metabolism | 8.39E-06 | 0.13 | 35.68 | 281.66 |
| RDH5 | Retinol Dehydrogenase 5 | Retinal biosynthesis | 2.76E-04 | 4.02 | 100.39 | 24.95 |
| LRA | Lecithin Retinol Acyltransferase | Esterification of all-trans-retinol | 6.55E-04 | 0.41 | 9.13 | 22.44 |
| RBP1 | Retinol Binding Protein 1, | Cellular Retinol transport | 1.37E-04 | 12.32 | 37.44 | 3.04 |
| STRA6 | Stimulated by Retinoic Acid 6 | Retinol transport | 1.38E-02 | 0.12 | 0.25 | 2.14 |
| RDH10 | Retinol Dehydrogenase 10 | Retinol biosynthesis | 1.65E-05 | 90.34 | 181.83 | 2.01 |
| **Phagocytosis** | | | | | | |
| COLEC12 | Collectin Sub-Family Member 12 | Phagocytosis | 2.32E-05 | 0.64 | 94.98 | 148.40 |
| PLA2G5 | Phospholipase A2, Group V | Phagocytosis | 1.42E-07 | 0.12 | 10.88 | 93.29 |
| TUB | Tubby Bipartite Transcription Factor | Phagocytosis | 5.67E-06 | 0.01 | 0.45 | 67.00 |
| MERTK | MER Proto-Oncogene, Tyrosine Kinase | Phagocytosis | 1.73E-05 | 0.42 | 7.00 | 16.81 |
| ITGAV | Integrin, Alpha V | Signal transduction | 5.63E-04 | 3.08 | 35.91 | 11.65 |
| **Pigmentation** | | | | | | |
| DCT | Dopachrome Tautomerase | Melanin synthesis | 1.50E-04 | 0.02 | 47.34 | 2840.20 |
| TRPM1 | Transient Receptor Potential Cation Channel, M1 | Melanin synthesis | 4.01E-06 | 0.01 | 36.23 | 2717.26 |
| OCA2 | Melanocyte-Specific Transporter Protein | Melanosome maturation | 1.01E-03 | 0.00 | 4.06 | 1352.22 |
| TYRP1 | Tyrosinase-Related Protein 1 | Melanin biosynthesis | 1.28E-06 | 3.77 | 3749.82 | 995.53 |
| TRPM3 | Transient Receptor Potential Cation Channel, M3 | Calcium signaling | 6.89E-05 | 1.50 | 647.81 | 432.84 |
| TYR | Tyrosinase | Melanin biosynthesis | 6.43E-08 | 0.77 | 273.39 | 356.60 |
| PMEL | Premelanosome Protein | Biogenesis of melanosomes | 6.45E-05 | 1.27 | 136.65 | 107.60 |
| SLC45A2 | Solute Carrier Family 45, Member 2 | Melanin biosynthesis | 1.04E-04 | 1.42 | 69.92 | 49.12 |
| **Cell Differentiation** | | | | | | |
| DAPL1 | Death Associated Protein-Like 1 | Epithelial cell differentiation | 1.67E-05 | 0.34 | 663.95 | 1933.85 |
| CP | Ceruloplasmin (Ferroxidase) | Iron metabolism | 2.33E-04 | 0.51 | 136.22 | 265.36 |
| DSC1 | Desmocollin 1 | Cell adhesion | 4.30E-06 | 0.12 | 30.33 | 245.92 |
| CLDN2 | Claudin 2 | Tight junction | 2.06E-03 | 0.02 | 3.51 | 210.8 |
| SERPINF1 | PEDF | Anti-angiogenesis | 1.61E-05 | 7.76 | 1478.63 | 190.46 |
| FGFR2 | fibroblast growth factor R2 | Cell cycle | 2.28E-05 | 0.12 | 17.51 | 142.00 |
| Gene      | Gene Name                          | Biological Function     | p-value  | 4 Days | 4 Months | FC 4M/4D |
|-----------|------------------------------------|-------------------------|----------|--------|---------|----------|
| BEST1     | Bestrophin 1                       | Electron transport      | 3.41E-04 | 0.30   | 13.06   | 44.01    |
| CST3      | Cystatin c                         | Protease inhibitor      | 1.35E-04 | 66.06  | 2013.58 | 30.48    |
| CDH3      | Cadherin 3, P-Cadherin              | Cell adhesion           | 1.44E-03 | 0.02   | 0.50    | 24.83    |
| CDH1      | Cadherin E                         | Cell adhesion           | 8.79E-05 | 0.04   | 0.57    | 13.23    |
| **RPE-related Transcription Factor** |                                   |                         |          |        |         |          |
| TFEC      | Transcription Factor                | Transcriptional regulation | 1.91E-07 | 0.08   | 4.63    | 57.88    |
| SOX9      | SRY -Box 9                         | Transcriptional regulation | 1.21E-03 | 4.90   | 21.77   | 4.44     |
| OTX2      | Orthodenticle Homeobox 2           | Sensory organ development. | 8.13E-07 | 38.35  | 150.33  | 3.92     |
| MITF      | Microphthalmia-Associated          | Melanocyte development  | 1.21E-07 | 50.91  | 119.18  | 2.34     |
| CRX       | Cone-Rod Homeobox                  | Transcriptional regulation | 8.82E-03 | 1.07   | 1.33    | 1.25     |
| PAX6      | Paired Box 6                       | Eye development         | 1.79E-02 | 28.42  | 33.87   | 1.19     |

Transcript fold change and adjusted p-value of transcripts between 4 days and 4 months cultured RPE cells, and were considered significantly changed with adjusted p<0.05 and fold change of at least 2.0.
Table 3. Selected downregulated genes in the differentiated RPE cells.

| Gene       | Gene Name                          | Biological Function | p-value  | 4 Days | 4 Months | FC 4M/4D |
|------------|------------------------------------|---------------------|----------|--------|----------|----------|
| **Cell Cycle** |                                    |                     |          |        |          |          |
| TGFBI      | Transforming Growth Factor          | Cell cycle          | 2.01E-06 | 417.62 | 19.64    | 0.047    |
| SERPINE1   | Serpin Peptidase                   | Inhibitor of fibrinolysis | 1.48E-05 | 255.38 | 13.21    | 0.052    |
| TAGLN      | Transgelin                         | Cell cycle          | 3.11E-06 | 181.82 | 8.52     | 0.047    |
| NPPB       | Natriuretic Peptide B              | Cell proliferation  | 2.07E-06 | 162.61 | 0.24     | 0.001    |
| CDK1       | Cyclin-Dependent Kinase 1          | Cell cycle          | 6.96E-05 | 37.73  | 1.22     | 0.033    |
| CPA4       | Carboxypeptidase A4                | Proliferation       | 6.31E-07 | 37.03  | 1.39     | 0.038    |
| UBE2C      | Ubiquitin-Conjugating Enzyme       | Cell cycle          | 1.03E-06 | 20.57  | 0.17     | 0.008    |
| CENPF      | Centromere Protein F               | Cell cycle          | 4.56E-04 | 20.27  | 1.98     | 0.098    |
| CEP55      | Centrosomal Protein 55 kDa         | Cell cycle          | 1.21E-05 | 18.57  | 0.31     | 0.017    |
| CDC6       | Cell Division Cycle 6              | Cell cycle          | 2.06E-05 | 15.16  | 0.72     | 0.047    |
| CCNB2      | Cyclin B2                          | Cell cycle          | 1.41E-05 | 12.76  | 0.45     | 0.036    |
| BUB1       | BUB1 Serine/Threonine Kinase       | Cell cycle          | 4.44E-06 | 10.99  | 0.58     | 0.053    |
| BIRC5      | Baculoviral IAP Repeat             | Cell cycle          | 2.84E-05 | 10.17  | 0.13     | 0.013    |
| CCNE2      | Cyclin E2                          | Cell cycle          | 3.27E-05 | 9.71   | 0.46     | 0.047    |
| CDC20      | Cell Division Cycle 20             | Cell cycle          | 9.49E-06 | 8.60   | 0.24     | 0.028    |
| MKI67      | Marker of Proliferation K-67       | Cell cycle          | 2.19E-04 | 5.45   | 0.16     | 0.029    |
| AURKB      | Aurora Kinase B                    | Cell Cycle          | 5.15E-08 | 5.12   | 0.13     | 0.025    |
| E2F1       | E2F Transcription Factor 1         | Cell cycle          | 1.29E-07 | 4.38   | 0.28     | 0.064    |
| PLK1       | Polo-Like Kinase 1                 | Cell cycle          | 5.67E-05 | 2.91   | 0.12     | 0.041    |
| CDC25C     | Cell Division Cycle 25C            | Cell cycle          | 1.71E-04 | 2.31   | 0.08     | 0.033    |
| **Transcription Factors** |                                    |                     |          |        |          |          |
| ANKRD1     | Ankyrin Repeat Domain 1            | Transcription       | 1.49E-07 | 326.21 | 7.39     | 0.023    |
| HIST2H3C   | Histone Cluster 2 H3c              | Transcription       | 6.75E-07 | 66.71  | 1.12     | 0.017    |
| TOP2A      | Topoisomerase (DNA) II Alpha       | Transcription       | 6.18E-05 | 37.41  | 0.74     | 0.020    |
| PTTG1      | Pituitary Tumor-Transforming 1     | Transcription       | 1.99E-05 | 31.15  | 1.78     | 0.057    |
| JUN        | Jun Proto-Oncogene                 | Transcription       | 4.00E-05 | 29.84  | 2.79     | 0.093    |
| RRM2       | Ribonucleotide Reductase M2        | Transcription       | 1.06E-05 | 14.39  | 0.71     | 0.049    |

Transcript fold change and adjusted p-value of transcripts between 4 days and 4 months cultured RPE cells, and were considered significantly changed with adjusted p<0.05 and fold change of at least 2.0.
Figure 4. Expression of visual cycle genes increases in the differentiated RPE cells. ARPE-19 cells grown for either 4 days or 4 months were used for total RNA and protein extractions and were used for real-time quantitative PCR and western blotting, respectively, as described in the Methods section. A: Real-time PCR analysis of RPE-specific mRNA expression. The values are mean ± standard deviation (SD), n = 4. *p<0.001 compared with control. B: Western blot analysis of the expression of the RPE-specific proteins. α-Tubulin expression shows that the amount of protein used in the different samples is similar.
To assess the effect of these increases in the mRNA expression of the visual cycle genes on their respective protein expression in the differentiated cells, we performed western blot analysis. Immunoreactive bands specific for CRALBP, RDH5, and LRAT were detected only in the cells cultured for 4 months and not in the RPE cells cultured for 4 days (Figure 4B). The observed increases in the expression of the visual cycle protein correlated well with the increase in mRNA expression. However, expression of the RPE65 protein was undetectable on immunoblots in the differentiated and un-differentiated RPE cells (Figure 4B).

Retinoid analysis: As we observed the expression of proteins, except RPE65, that are essential for the retinoid visual cycle in the differentiated ARPE-19 cells cultured for 4 months, we examined whether the RPE cells grown for 4 months in Transwell® inserts were able to take up all-trans retinol and convert it to all-trans retinyl esters. To observe the ester formation, ARPE-19 cells grown for 4 months in the Transwell® inserts were incubated with or without 4 μM all-trans retinol added into the basal chamber, and the plates were incubated at 37 °C for 16 h in the dark. Following extraction, the retinoids in the ARPE-19 cells were analyzed with HPLC and monitored at 325 nm. As shown in Figure 5A, the formation of all-trans retinyl esters was evident with detectable amounts of all-trans retinyl esters in treated cells. The observed formation of the all-trans retinyl esters was corroborated by the observed chromatogram of authentic all-trans retinyl palmitate standard and its corresponding absorbance spectrum (Figure 5B). No traceable all-trans retinyl ester peak was observed in the untreated control cells.

RPE differentiation increases the expression of melanogenesis and phagocytosis genes and proteins: We examined several RPE-specific genes expressed in native RPE cells. As seen in Figure 6A, bestrophin-1 (BEST1; OMIM 607854), a calcium-sensitive chloride channel predominantly expressed in the basolateral membrane of the RPE [49], was highly increased (about 100-fold) in the differentiated ARPE-19 cells. Tyrosinase (TYR), a rate-limiting enzyme responsible for melanin biosynthesis in the RPE [50], was increased close to 10,000-fold, and the expression of the klotho (KL; OMIM 604824) gene, a regulator of melanin biosynthesis [51], was also increased greatly, indicating that these cells are able to produce the melanin pigment under these culture conditions, consistent with the observed dark pigmentation. In addition, expression of MER tyrosine kinase (MERTK), a receptor found at the apical surface of the RPE that plays a critical role in phagocytosis of shed ROS [52], was increased by more than 15-fold in the differentiated RPE cells. However, the expression of TYRO3 (OMIM 600341) and AXL (OMIM 109135), fellow TAM family members with MERTK, was downregulated in the cells cultured for 4 months.

Western blot analysis was performed to corroborate the RNA results (Figure 6B). Corresponding to the mRNA expression, the bestrophin-1 protein was increased in the differentiated RPE cells. Similarly, the tyrosinase and MERTK proteins were detected in the cells cultured for 4 months and not in the cells cultured for 4 days.

Differentiated RPE cells exhibit epithelial specific gene expression: To further characterize the phenotypic changes associated with differentiation, we examined the expression of established markers typical for nonepithelial and epithelial cells. We found that mRNA expression of E-cadherin (CDH1), an epithelial morphoregulatory protein [53], was highly increased in the ARPE-19 cells cultured for 4 months (Figure 7A) as was E-cadherin protein expression (Figure 7B). Similarly, the mRNA expression of P-cadherin (CDH3), an epithelial cadherin coexpressed with E-cadherin [53], was also highly increased (Figure 7A). However, the expression of N-cadherin (CDH2; OMIM 114020), which is typically found in nonepithelial cells [53], was decreased in the cells cultured for 4 months. In addition, the expression of another widely used nonepithelial marker vimentin [54] was also decreased. The vimentin (VIM; OMIM 193060) mRNA and protein were highly expressed in the cells cultured for 4 days, and the expression decreased in the differentiated cells cultured for 4 months (Figure 7A,B). Interestingly, in addition to the predicted 57 kDa protein band for vimentin, we noticed two additional bands of lower intensity and lower molecular weight in the cells cultured for 4 months.

An important structural feature that cultured RPE cells should retain is the barrier function performed by tight junctions. ZO-1 is an important regulator of tight junctions [19]. Here, we observed that the expression of TJPI (OMIM 601009; Figure 7A), the gene that codes the tight junction protein ZO-1, was significantly increased in the cells cultured for 4 months. The higher level of TJPI would correlate with the acquired epithelial morphology characteristic of the native quiescent RPE cells. Expression of cyclin D1 (CCND1; OMIM 168461), an activator of cell cycle progression, which regulates progression of cells from the G1 to S phase [55], was decreased in the RPE cells cultured for 4 months compared with the cells cultured for 4 days (Figure 7A).

Differentiation increases the expression of miR-204 and miR-211: MiR-204 and -211, the two most highly expressed miRNAs in primary cultures of human fetal RPE, play a critical role in RPE epithelial barrier function and differentiation [8,56]. Therefore, we examined miR-204 and miR-211 expression in the differentiated RPE cells with RT–PCR. As
Figure 5. Retinyl ester synthesis in the differentiated ARPE-19 cells. Cells cultured for 4 months on Transwell® permeable membranes were incubated for 16 h with or without 4 μM all-trans retinol provided via the outside (basal) chamber. The cells were harvested, and the extracted retinoids were then analyzed with normal-phase high performance liquid chromatography (HPLC). A: Chromatogram overlay of retinoid extracts from ARPE-19 cells treated with (black) or without (red) all-trans retinol. The inset shows the absorbance spectrum from the peak observed in the treated ARPE-19 cells (black). B: Chromatogram of the authentic all-trans retinyl palmitate standard and its corresponding absorbance spectrum (inset).
Figure 6. Differentiation of RPE cells increases non-visual cycle gene expression. ARPE-19 cells grown for either 4 days or 4 months were used for total RNA and protein extractions and were used for real-time quantitative PCR and western blotting, respectively, as described in the Methods section. A: Real-time PCR analysis of RPE-specific non-visual cycle mRNA expression. The values are mean ± standard deviation (SD), n = 4. *p<0.001 compared with control. B: Western blot analysis of the expression of the RPE-specific non-visual cycle proteins. α-Tubulin expression shows that the amount of protein used in different samples is similar.
Figure 7. Epithelial-specific gene expression is increased in the differentiated ARPE-19 cells. ARPE-19 cells grown for either 4 days or 4 months were used for total RNA and protein extractions. The samples were then used for real-time quantitative PCR and western blotting, respectively, as described in the Methods section. 

A: Real-time PCR analysis of epithelial- and mesenchymal-specific mRNA expression. The values are mean ± standard deviation (SD), n = 4. *p<0.001 compared with control.

B: Western blot analysis of the expression of the epithelial- and mesenchymal-specific proteins. α-Tubulin expression shows that the amount of protein used in different samples is similar.
Figure 8. Expression of genes and miRNAs involved in regulating RPE differentiation is increased. ARPE-19 cells grown for either 4 days or 4 months were used for total RNA extractions. The samples were then used for real-time quantitative PCR, as described in the Methods section. A: Real-time PCR analysis of RPE-specific miRNAs, miR-204 and miR-211, expression. B: Real-time PCR analysis of MITF and its target genes TRPM1 and TRPM3 mRNA expression. The values are mean ± standard deviation (SD), n = 4. *p<0.001 compared with control.
shown in Figure 8A, the ARPE-19 cells cultured for 4 months showed high expression of miR-204-5p and miR-211-5p. A more than 3,500-fold increase in miR-211-5p expression was observed in the cells cultured for 4 months compared with the cells cultured for 4 days, while miR-204-5p was increased by more than 2,000-fold. These data support our observation that the RPE cells grown for 4 months differentiated into an epithelial phenotype.

As miR-211 is contained within an intron of the transient receptor potential cation channel, subfamily m, member 1 (TRPM1) gene, and miR-204 is contained within an intron of the transient receptor potential cation channel, subfamily m, member 3 (TRPM3; OMIM 608961) gene, we measured the expression of TRPM1 and TRPM3 and found that both were highly increased in the ARPE-19 cells cultured for 4 months compared with the ARPE-19 cells cultured for 4 days (Figure 8B). TRPM1 and TRPM3 are regulated by MITF, therefore, it was not surprising that MITF expression was also greatly increased in the ARPE-19 cells cultured for 4 months compared with the ARPE-19 cells cultured for 4 days (Figure 8B).
Protein network visualization shows functional associations of differentially expressed genes: Potential interactions between the selected up- and downregulated differentially expressed genes from the RPE cells cultured for 4 months and 4 days were examined through STRING to determine associations between the differentially expressed genes. As shown in Figure 9, the network visualized in STRING illustrates that nearly all of the core gene set was organized into tight relationships among molecules; 67 of 72 genes were closely connected in a single network. The complex network could be segregated into four modules, involved in visual cycle, phagocytosis, melanin biosynthesis, and cell cycle regulation. The cell cycle regulation module contains polo-like kinase 1 (PLK-1; OMIM 602098), an early trigger for the G2/M transition [57], cyclin B2 (CCNB2; OMIM 602755), which plays a key role in TGFβ-mediated cell cycle control [58], and mitotic checkpoint serine/threonine-protein kinase BUB1 (BUB1; OMIM 602452), a central component of the mitotic checkpoint for spindle assembly (SAC) [59].

Differentiation increases RPE-specific gene expression: Previous studies have identified several specific genes that are preferentially expressed in human RPE compared to other tissues or cell types and suggested that they can be used to validate the progress of RPE differentiation and function [34,35]. Therefore, we performed a whole transcriptome comparison (> 20,000 genes) between the differentiated ARPE-19 cells data set and the reported transcriptomes from the hESCs and hfRPE, and native RPE from the macular regions of the RPE/choroid. The number of GO biological processes pathway terms was significantly enriched in these transcripts, and the top 18 are summarized in Table 4. Interestingly, of these 18 GO biological processes, four GO terms were highly increased (Yellow/Red, p-value ≥ 1.00E-06 to E-64) in all the data sets analyzed. Significant terms included translation and oxidative phosphorylation indicating that the most overrepresented pathways reflect the main property of metabolically active RPE cells. However, the levels of the mitochondrion-related process (electron transport, respiration, etc.) were low in the RPE cells cultured for 4 months and the HF (Blue, p-value ≤ 1.00E+00) compared to the native RPE. In addition, some GO biological processes are statistically significant only in the RPE models but not in the native tissue.

To explore genes that are preferentially expressed in human RPE, we assessed trends in gene expression across the data set of the ARPE-19 cells cultured for 4 months and the RNA-Seq data sets from the H1, H9, HF, and RPE from the macular, nasal, and temporal regions of the RPE/choroid punches. As shown in Figure 11A, the expression level of key visual cycle genes, such as RPE65 and LRAT, was low in the ARPE-19 cells cultured for 4 months among all six samples analyzed, while the expression of RLBP1 in the ARPE-19 cells cultured for 4 months was statistically significantly higher compared to that in the H1, H9, and HF. Significantly, the expression of RPE65, RLBP1, RBP1, BEST1, and LRAT was much higher in the native RPE cells compared to the H1, H9, and HF RPE cells. This indicates the high degree of functional enrichment in this set of genes in native tissue. When we cross-referenced the RNA-Seq data for several genes highly expressed in the native RPE (such as SERPINF1, PMEL, TYR, MERTK, and
MITF), we found these genes were highly expressed and even at levels similar to those of the native RPE cells (Figure 11B), corroborating the validity of the ARPE-19 cell differentiation phenotype.

**DISCUSSION**

In this paper, we show that, in addition to developing many of the structural and physiological properties characteristic of the native human RPE, long-term cultured ARPE-19 cells show an increase in genes preferentially expressed in the native RPE and epithelium. Gene Ontology analysis showed that in the cells cultured for 4 months the genes associated with visual cycle, phagocytosis, pigment synthesis, cell differentiation, and RPE-related transcription factors increased while the majority of the downregulated genes are associated with cell cycle and proliferation. Furthermore, this increase in the expression of the mRNA of the visual cycle and epithelial genes is translated into a corresponding increase in
protein expression. In addition, the two most RPE-enriched miRNAs and inducers of epithelial differentiation, miR-204 and miR-211, were increased in the RPE cells cultured for 4 months. Taken together, our data indicate that low-passage ARPE-19 cells grown for 4 months in DMEM + high glucose/pyruvate/1% FBS can be an important cell culture model for studying the physiology and pathophysiology of the native RPE in vitro.

ARPE-19 cells grown to sub-confluence or confluence for a short duration are the most commonly used system to study the RPE function in vitro. However, this presents a challenge to reproducibility as ARPE-19 cells adopt a variable phenotype depending upon the culture conditions [18,37,38,60]. Here, we showed that low-passage ARPE-19 cells grown in DMEM + high glucose/pyruvate/1% FBS for 4 months differentiated into a stable cobblestoned epithelial phenotype characteristic of native RPE in addition to reprising gene expression patterns commonly found in native RPE, primary RPE cultures, and hESC-derived RPE. In addition, heterogeneity and non-epithelial characteristics were not seen when ARPE-19 cells at passage numbers between 9 and 14 were used. Importantly, the study specifies that several months are required for the cultured cells to attain their epithelial phenotype after confluency, in common with various previous studies [5,18,22]. In this regard, lengthening the period of the in vitro cultures of induced pluripotent stem (iPS)-derived RPE cells to 18 weeks allowed for enhanced morphological differentiation [61]. Interestingly, even with extended culture, the composition of the medium is important to this differentiation process. Thus, ARPE-19 cells cultured similarly for 4 months in 1:1 DMEM and Ham's F12 (DMEM/F12) medium with high glucose and pyruvate supplemented with 1% FBS did not differentiate into an epithelial morphology (data not shown). This correlates well with the original report that ARPE-19 cells, even at higher passage numbers (22 to 28) differentiated into an epithelial phenotype when cultured in DMEM + high glucose/pyruvate/1% FBS for 3 months but did not in the typical DMEM/F12 medium with high glucose and pyruvate and 1% FBS [22].

In the present study, the expression of proteins that play a central role in inducing the epithelial phenotype was highly increased. In particular, cadherins (cell adhesion molecules that form adherens junctions) were highly increased in the ARPE-19 cells grown beyond confluency [62,63]. In

| GO Biological Process                                                                 | ARPE-19 | HF   | RPE/CHOR Macular |
|--------------------------------------------------------------------------------------|---------|------|------------------|
| Translational elongation                                                             | 6.80E-59<sup>a</sup> | 2.00E-61<sup>a</sup> | 3.90E-64<sup>a</sup> |
| Translation                                                                           | 3.30E-41<sup>a</sup> | 1.30E-33<sup>a</sup> | 2.80E-36<sup>a</sup> |
| Generation of precursor metabolites and energy                                       | 2.10E-14<sup>a</sup> | 2.50E-08<sup>a</sup> | 2.00E-21<sup>a</sup> |
| Oxidative phosphorylation                                                            | 6.40E-09<sup>a</sup> | 3.20E-06<sup>a</sup> | 2.20E-15<sup>a</sup> |
| Electron transport chain                                                              | 1.00E+00<sup>a</sup> | 1.00E+00<sup>a</sup> | 7.90E-10<sup>a</sup> |
| Cellular respiration                                                                  | 1.00E+00<sup>a</sup> | 1.00E+00<sup>a</sup> | 2.30E-07<sup>a</sup> |
| Mitochondrial electron transport, NADH to ubiquinone                                  | 1.00E+00<sup>a</sup> | 1.00E+00<sup>a</sup> | 4.80E-07<sup>a</sup> |
| Mitochondrial ATP synthesis coupled electron transport                               | 1.00E+00<sup>a</sup> | 1.00E+00<sup>a</sup> | 1.00E-06<sup>a</sup> |
| Response to inorganic substance                                                      | 1.00E+00<sup>a</sup> | 1.80E-06<sup>a</sup> | 9.40E-07<sup>a</sup> |
| Anti-apoptosis                                                                        | 1.00E+00<sup>a</sup> | 1.20E-07<sup>a</sup> | 7.00E-06<sup>a</sup> |
| Response to organic substance                                                        | 1.00E+00<sup>a</sup> | 7.80E-06<sup>a</sup> | 4.00E-09<sup>a</sup> |
| Negative regulation of protein metabolic process                                     | 1.00E+00<sup>a</sup> | 1.00E+00<sup>a</sup> | 6.70E-09<sup>a</sup> |
| Oxidation reduction                                                                  | 3.30E-07<sup>a</sup> | 1.00E+00<sup>a</sup> | 8.00E-06<sup>a</sup> |
| Intracellular transport                                                               | 2.30E-09<sup>a</sup> | 9.90E-09<sup>a</sup> | 1.00E+00<sup>a</sup> |
| Intracellular protein transport                                                       | 2.60E-08<sup>a</sup> | 1.60E-07<sup>a</sup> | 1.00E+00<sup>a</sup> |
| Cellular protein localization                                                        | 4.00E-08<sup>a</sup> | 1.90E-07<sup>a</sup> | 1.00E+00<sup>a</sup> |
| Cellular macromolecule localization                                                  | 4.80E-08<sup>a</sup> | 1.60E-07<sup>a</sup> | 1.00E+00<sup>a</sup> |
| Protein localization                                                                 | 21.50E-06<sup>b</sup> | 4.70E-06<sup>b</sup> | 1.00E+00<sup>b</sup> |

The table shows GO biological process common to the 1000 most highly expressed genes analyzed from RNA-Seq data of differentiated RPE cells and RPE cells from primary human fetal (HF) eye and macular region of native RPE/Choroid. p-value of 1 was assigned to pathways not overrepresented, and “a”, “b” and “c” denotes the changes in p-values; a < b < c.
Figure 11. Comparison of visual cycle and RPE signature gene expression. Fragments per thousand bases of gene per million bases mapped (FPKM) values obtained from RNA sequencing (RNA-Seq) of the ARPE-19 cells cultured for 4 months (ARPE-19 4M), RPE derived from human embryonic stem cells (H1 and H9) and fetal eyes (HF), and native RPE from the nasal (RPE/CHOR_NAS), temporal (RPE/CHOR_TMP), and macular (RPE/CHOR_MAC) regions of the RPE/choroid were used for the analysis. **A**: Comparison of the expression of the visual cycle genes. **B**: Comparison of the expression of RPE-specific genes. The x-axis indicates the expression levels of the genes analyzed. The y-axis indicates the transcript expression levels quantified as FPKM on a $\log_{10}$ scale.
particular, the expression of E-cadherin (CDH1), a critical inducer of epithelial morphogenesis, was highly increased in the RPE cells cultured for 4 months compared with the cells cultured for 4 days. Western blot analysis showed that the E-cadherin protein was present only in the ARPE-19 cells cultured for 4 months, similar to an earlier observation that E-cadherin was detectable only in the cultures of primary human RPE cells maintained at confluence for several weeks [53]. Furthermore, an increase in P-cadherin (CDH3) expression, an epithelial cadherin coexpressed with E-cadherin and required for human RPE viability [53,64], in the cells cultured for 4 months further correlates with the observed epithelial phenotype. N-cadherin (CDH2), typically found in nonepithelial cells, was decreased in the cells cultured for 4 months, consistent with the reduced coexpression of N-cadherin with E-cadherin [65]. However, epithelial cells have an inherent plasticity to transition into mesenchymal cells (e.g., fibroblasts) via EMT, and back to epithelia via mesenchymal to epithelial transition (MET), either fully or partially [19]. The expression of vimentin, a canonical mesenchymal indicator, was markedly decreased in the ARPE-19 cells cultured for 4 months suggesting that MET may mediate the epithelial differentiation. The decrease in vimentin protein in the cells cultured for 4 months underscores the loss by the cells of their migratory property and their acquisition of polarity and expression of tight junction protein ZO-1, a structural feature of native RPE monolayers. However, downregulation of ZO-1 and E-cadherin expression by TGF-β in ARPE-19 cells has been associated with EMT [66,67]. Correspondingly, we found that TGF-β expression was downregulated in the ARPE-19 cells cultured for 4 months, further supporting a role for MET in the epithelial phenotype differentiation. In addition, we observed with immunofluorescence microscopy that claudin-2, a minor tight junction protein, colocalizes with ZO-1 uniformly across the monolayer in the differentiated ARPE-19 cells. It is known that the formation of tight junctions is an essential component of the epithelial monolayer barrier formation [68]. However, modulation of the RPE tight junction by minor claudins needs further evaluation to understand the mechanism through which they might regulate the barrier properties [69,70]. Recently, it has been shown that overexpression of either claudin-3 or claudin-19, the major claudins in native human RPE, improved the barrier function, increased the expression of genes related to RPE, and partially restored native RPE phenotype to high-passage ARPE-19 cells cultured for 2 weeks [71]. Whether these phenomena could be observed in the differentiated ARPE-19 cells needs further investigation.

In addition to developing the typical cobblestoned morphology of native RPE, the ARPE-19 cells cultured for 4 months were heavily pigmented to the naked eye. Pigmentation is a hallmark of native RPE, and an important indicator of phenotypic maturity of these cells in culture [72,73], requiring functional melanosomes containing melanogenesis enzymes, including tyrosinase (TYR), tyrosinase-related protein (TYRP1), and L-dopachrome tautomerase (DCT). Although melanin pigments occur in human fetal RPE cultures [79], the most common phenotype deficiency of immortalized adult human RPE cells has been the lack of melanin biosynthesis under common culture conditions [50]. In the present study, mRNA and protein expression of tyrosinase, PMEL, a key component of mammalian melanosome biogenesis, and DCT, which helps to regulate eumelanin and pheomelanin levels [39], were all highly increased in the ARPE-19 cells cultured for 4 months. The present results are in contrast to earlier observations that melanogenesis occurs more reliably in cultured fetal RPE cells [9,74]. Specialized media or substrates have been shown to play a role in inducing melanogenesis and differentiation. In particular, pyruvate has been reported to increase pigmentation in non-RPE cells [75,76].

We grew ARPE-19 cells on plastic as ARPE-19 cells grown on a plastic surface have a closer expression profile to native RPE than cells grown on protein matrices [36]. We used RNA-Seq as it allows for greater sensitivity and a broader range of fold changes than microarrays. The most highly upregulated genes included RPE65, DCT, and DAPL1. However, many downregulated genes are involved in cell cycle, proliferation, and transcription factors that play a central role in cell cycle and apoptosis. The presence of RPE65 [77-79] is of particular importance as this gene plays a critical role in the retinoid cycle and is a marker for terminally differentiated RPE cells. Even then, while the mRNA expression of RPE65 was highly increased (about 10,000 fold), we were unable to detect the RPE65 protein in these cells. This result is consistent with numerous studies that have demonstrated RPE65 mRNA in ARPE-19 cells with no detectable level of the RPE65 protein [5,38,60]. A decrease in the RPE65 protein was also observed in the primary cultures of monkey and bovine [12] RPE cells. In contrast, Ahamado et al. [22] observed the RPE65 protein in addition to RPE65 mRNA in ARPE-19 cells cultured for 3 months in DMEM + high glucose/pyruvate/1% FBS. Other than RPE65, the increased expression of other visual cycle genes [1], LRAT, RDH5, and RBP1 also known as CRALBP (retinaldehyde-binding protein 1), correlated with increases in protein expression, confirming the degree of differentiation of the ARPE-19 cells cultured for 4 months. In addition, the expression of bestrophin-1, another important marker of terminal RPE maturation [80], was highly increased in the ARPE-19 cells.
cultured for 4 months, and the increase was translated to a corresponding increase in the protein. Previously, the bestrophin-1 protein had been shown to accumulate only in cells of fetal origin [9,74]. A recent study also showed a more than fourfold increase in bestrophin-1 mRNA expression when the ARPE-19 cells grown for 2 weeks were transfected exogenously with either claudin-3 or claudin-19 [71]. However, the observed increase in BEST1 mRNA expression was not translated into a corresponding increase in the bestrophin-1 protein [71].

It is evident from these observations that ARPE-19 cells grown for 4 months in DMEM + high glucose/pyruvate/1% FBS express proteins, except RPE65, that play a central role in the classical retinoid visual cycle performed by the RPE, a signature feature of the RPE monolayer [81]. This is in line with observations that RPE culture conditions can be crucial for the expression of proteins essential for the retinoid visual cycle [82]. However, it has been shown earlier that RPE can be morphologically normal but lack the essential activity of LRAT, the sole enzyme responsible for RPE retinyl ester formations needed for vitamin A storage and as the substrate for the isomerase of the visual cycle [82,83]. Here we observed that the ARPE-19 cells grown on Transwell<sup>®</sup> inserts for 4 months developed morphology approximating that of the native RPE and are able to synthesize all-trans retinyl esters from all-trans retinol provided in the basal chamber, indicative of an active LRAT enzyme. Accumulation of all-trans retinyl ester with no detectable 11-cis retinal is attributed to the lack of the RPE65 protein. This may be due to post-transcriptional regulation of RPE65 expression during cell culture as previously described [12].

Another important function of RPE is the daily phagocytosis and clearance of shed POS disks [84]. Phagocytosis by RPE cells in culture is highly dependent on the expression of the RPE phagocytosis molecular machinery and on the degree of RPE differentiation or polarization. We found that several genes implicated in phagocytosis were highly increased in the differentiated ARPE-19 cells, including integrin alpha-V, cathepsin D, and MERTK, and confirmed the expression of the MERTK protein with western blotting. Integrins (integral membrane proteins that function as an αβ heterodimer), present in the apical surface of the RPE, are responsible for POS binding to the RPE [14]. Mice or rats lacking αvβ5 integrin or Merkt have profound abnormalities in cytosolic phagocytic signaling with severely reduced POS binding and engulfment [40,85-88]. The expression level of MERTK also plays an important role in mediating phagocytosis in human primary RPE cells [51]. Furthermore, cathepsin D, a major lysosomal enzyme that degrades internalized POS, was also increased in the differentiated ARPE-19 cells cultured for 4 months. Thus, taken together these data indicate that the ARPE-19 cells cultured for 4 months are competent to phagocytose and degrade POS in vitro.

Cell differentiation requires highly regulated gene expression mediated by specific transcription factors. We saw increases in several transcription factors known to promote differentiation, survival, proliferation, and melanogenesis in RPE. Transcription factor EC (TFEC), belonging to the family of basic helix-loop-helix-zipper transcription factors, such as MITF, TFE3, and TFEB, was highly increased in the ARPE-19 cells cultured for 4 months. TFEC regulates osteoclast differentiation in mice, forming heterodimers with MITF to activate target genes [89]. Apart from binding to TFEC, MITF stimulates the expression of its target genes as a homodimer or through cross-regulation with transcription factors, such as PAX6, OTX2, and SOX9 [90], considered the key regulators for RPE specification together with MITF. We found increased expression of the KL gene that is expressed in primary cultured human RPE and that regulates MITF and TYR, further supporting the similarities of differentiated ARPE-19 cells to primary human RPE cells. Further, the mRNA expression of TRPM1 and TRPM3, tightly controlled by MITF, was highly increased in the differentiated RPE cells. MITF also regulates the transcription of melanogenesis genes (including TYR, TRP1, and DCT) and synergistically interacts with OTX2 and SOX9, both increased in differentiated ARPE-19 cells, to activate the transcription of several visual cycle genes [44]. Thus, MITF together with OTX2, PAX6, and SOX6 may be responsible for the changes (synthesis of melanin, epithelial phenotype differentiation, and upregulation of the RPE-specific genes) in these cells. The potential interactions between the up- and downregulated genes visualized in the STRING action view (Figure 9) also illustrates that nearly all of the core gene set was organized into tight relationships among the molecules (visual cycle, phagocytosis, melanogenesis, and cell cycle regulation) underlying the differentiation.

In addition to transcriptional control, regulation of mRNA translation by miRNA presents yet another level of regulation that affects cell fate and various biologic processes. RPE-specific miRNAs could be involved in RPE maturation related to the expression of genes involved in the visual cycle pathway and pigmentation [91,92]. Using microarray analysis, our laboratory showed that many miRNAs with the inherent ability to regulate cell growth, differentiation, and development are normally expressed in ARPE-19 cells [93]. Wang et al. [8] showed that miR-204 and -211 are the two most highly expressed miRNAs in primary cultures of human fetal RPE.
and play a critical role in maintaining epithelial barrier function and cell physiology. Adijanto et al. showed directly that transfection of pre-miR-204/211 into hfRPE cells promoted differentiation, while the addition of miR-204/211 inhibitors caused dedifferentiation [56]. We found that the expression of miR-204 and miR-211 were greatly increased in the ARPE-19 cultured for 4 months, suggesting the involvement of miR-204/211 in mediating its differentiation.

Finally, we compared the RNA-Seq data set to two previously reported transcriptome analyses of RPE cells, one of RPE derived from hESCs and hfRPE and the other of native RPE isolated from the macular, nasal, and temporal regions of the RPE/choroid [26,28]. This comparison is of interest because these RNA-Seq data sets can provide potential molecular markers for assessing the integrity and function of RPE and to validate the progress of RPE differentiation [35]. There is about 37% (565 genes; union of 1,511 genes) similarity in gene expression between the ARPE-19 RNA-Seq data and the transcriptome analyses of hESCs and hfRPE. When compared with the RNA-Seq data for the macular regions of the RPE/choroid and hfRPE, there was a 23% (409 genes; union of 1,735 genes) similarity in gene expression. Gene Ontology analysis indicated that included among these genes were visual cycle proteins, epithelial markers, and transporters, such as RPE65, RBP1, BEST1, CDH3, and SLC6A20. This indicates that, in addition to morphology, the transcriptome of the differentiated ARPE-19 cells resembles that of other reported RNA-Seq derived expression data sets. Although significant similarity in gene expression was observed between all cell culture models, the absolute expression values of the genes specific to RPE functions were quite low compared to that of native RPE. In particular, the expression values of the genes involved in the RPE part of the visual cycle were comparatively greatly decreased even in hfRPE, considered a well-regarded model of RPE function, compared with native RPE. It is possible that the observed difference in gene expression could be due to mitochondrial dysfunction associated with cultured RPE cells. The GO biological process comparison showed low expression of the genes related to mitochondrial function in the cultured RPE cells compared with the native RPE. Mitochondrial dysfunction resulted in a shift in cell metabolism and was accompanied by the loss of RPE-specific genes [94]. In addition, we cannot rule out that the relative lack of maturity of the RPE cells derived from either stem cell or fetal RPE compared to native adult RPE is another possible reason for the observed differences in gene expression. This possibility is supported by an earlier observation that the RPE transcriptome dynamically changes during the outer blood–retinal barrier development [95]. However, the moderate difference observed between the ARPE-19 cells cultured for 4 months with the hESC-derived RPE and primary hfRPE cells is not unexpected considering the experimental design and the differences in the culture conditions used in the respective analyses. In addition, it is possible that the observed difference could be due to the ARPE-19 cells being grown on a plastic surface as it has been shown that human fetal RPE cells grown on Transwell® filters produce confluent RPE with morphological and physiological characteristics of the intact RPE monolayer [9]. However, irregular morphology in hESC-derived RPE cells has been observed in human extracellular matrix-coated Transwell® filters that promoted differentiation of hfRPE [28]. RPE cells grown on a plastic surface were shown previously to have a closer expression profile to native RPE than cells grown on protein matrices [36].

In summary, our study provides a detailed description of RPE-specific gene expression in differentiated ARPE-19 cells. Most importantly, our data reveal a striking similarity in overall gene expression between these ARPE-19 cells and primary and native human RPE cells, indicating that well-differentiated ARPE-19 cells can provide a useful tool for studying different RPE cellular functions in vitro. This study also showed the increased expression of important proteins normally found in the RPE, including visual cycle proteins, in the differentiated ARPE-19 cells. Surprisingly, however, the RPE65 protein was undetectable even in the fully differentiated cells, despite the abundant RPE65 mRNA expression. Given this level of functionality, it is apparent that culture conditions are crucial to gene expression profiles. Although care must be used when interpreting and extrapolating gene expression changes between cultured and native RPE cells, we conclude that appropriately cultured and differentiated low-passage ARPE-19 cells can be functional models for RPE studies. Finally, the results observed for the low-passaged ARPE-19 cells can be used as a standard for comparison for cultures of late passage ARPE-19 cells.

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REFERENCES

1. Strauss O. The retinal pigment epithelium in visual function. Physiol Rev 2005; 85:845–81. [PMID: 15987797].

2. Bhutto I, Lutty G. Understanding age-related macular degeneration (AMD): relationships between the photoreceptor/retinal pigment epithelium/Bruch’s membrane/choriocapillaris complex. Mol Aspects Med 2012; 33:295-317. [PMID: 22542780].
3. Phelan JK, Bok D. A brief review of retinitis pigmentosa and the identified retinitis pigmentosa genes. Mol Vis 2000; 6:116-24. [PMID: 10889272].

4. Greenwood J, Pryce G, Devine L, Male DK, dos Santos WL, Calder VJ, Adamson P. SV40 large T immortalised cell lines of the rat blood-brain and blood-retinal barriers retain their phenotypic and immunological characteristics. J Neuroimmunol 1996; 71:51-63. [PMID: 8982103].

5. Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. Exp Eye Res 1996; 62:155-69. [PMID: 8698076].

6. Toops KA, Tan LX, Lakkaraju A. A detailed three-step protocol for live imaging of intracellular traffic in polarized primary porcine RPE monolayers. Exp Eye Res 2014; 124:74-85. [PMID: 24861273].

7. Hu J, Bok D. A cell culture medium that supports the differentiation of human retinal pigment epithelium into functionally polarized monolayers. Mol Vis 2001; 7:14-9. [PMID: 11182021].

8. Wang FE, Zhang C, Maminishkis A, Dong L, Zhi C, Li R, Zhao J, Majerciak V, Gaur AB, Chen S, Miller SS. MicroRNA-204/211 alters epithelial physiology. FASEB J 2010; 24:1552-71. [PMID: 20056717].

9. Maminishkis A, Chen S, Jalickee S, Banzon T, Shi G, Wang FE, Ehalt T, Hammer JA, Miller SS. Confluent monolayers of cultured human retinal pigment epithelium exhibit morphology and physiology of native tissue. Invest Ophthalmol Vis Sci 2006; 47:3612-24. [PMID: 16877436].

10. Stern JH, Temple S. Stem cells for retinal replacement therapy. Neurotherapeutics 2011; 8:736-43. [PMID: 21948217].

11. Adjiento J, Philip NJ. Cultured primary human fetal retinal pigment epithelium (hfRPE) as a model for evaluating RPE metabolism. Exp Eye Res 2014; 126:77-84. [PMID: 24485945].

12. Hamel CP, Tsilou E, Peiffer BA, Hooks JJ, Detrick B, Redmond TM. Molecular cloning and expression of RPE65, a novel retinal pigment epithelium-specific microsomal protein that is post-transcriptionally regulated in vitro. J Biol Chem 1993; 268:1571-7. [PMID: 8340400].

13. Eisenfeld AJ, Bunt-Milam AH, Saari JC. Localization of retinoid-binding proteins in developing rat retina. Exp Eye Res 1985; 41:299-304. [PMID: 3905423].

14. Finnemann SC, Bonilha VL, Marmorstein AD, Rodriguez-Boulan E. Phagocytosis of rod outer segments by retinal pigment epithelial cells requires alpha(v)beta5 integrin for binding but not for internalization. Proc Natl Acad Sci USA 1997; 94:12932-7. [PMID: 937778].

15. Alizadeh M, Wada M, Gelfman CM, Handa JT, Hjelmeland LM. Downregulation of differentiation specific gene expression by oxidative stress in ARPE-19 cells. Invest Ophthalmol Vis Sci 2001; 42:2706-13. [PMID: 11581219].

16. Chen S, Samuel W, Fariss RN, Duncan T, Kutty RK, Wiggert B. Differentiation of human retinal pigment epithelial cells into neuronal phenotype by N-(4-hydroxyphenyl)retinamide. J Neurochem 2003; 84:972-81. [PMID: 12603822].

17. Zhang C, Baffi J, Cousins SW, Csaky KG. Oxidant-induced cell death in retinal pigment epithelium cells mediated through the release of apoptosis-inducing factor. J Cell Sci 2003; 116:1915-23. [PMID: 12668724].

18. Luo Y, Zhuo Y, Fukushima M, Rizzolo LJ. Effects of culture conditions on heterogeneity and the apical junctional complex of the ARPE-19 cell line. Invest Ophthalmol Vis Sci 2006; 47:3644-55. [PMID: 16877439].

19. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest 2009; 119:1420-8. [PMID: 19487818].

20. Diaz-Lopez A, Moreno-Bueno G, Cano A. Role of microRNA in epithelial to mesenchymal transition and metastasis and clinical perspectives. Cancer Manag Res 2014; 6:205-16. [PMID: 24812525].

21. Gregory PA, Berg AT, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol 2008; 10:593-601. [PMID: 18376396].

22. Ahmad A, Carr AJ, Vugler AA, Semo M, Gias C, Lawrence JM, Chen LL, Chen FK, Turowski P, da Cruz L, Coffey PJ. Induction of differentiation by pyruvate and DMEM in the human retinal pigment epithelium cell line ARPE-19. Invest Ophthalmol Vis Sci 2011; 52:7148-59. [PMID: 21743041].

23. Poliakov E, Strunnikova NV, Jiang JK, Martinez B, Parikh T, Lakkaraju A, Thomas C, Brooks BP, Redmond TM. Multiple A2E treatments lead to melanization of rod outer segment-challenged ARPE-19 cells. Mol Vis 2014; 20:285-300. [PMID: 24644403].

24. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014; 15:550. [PMID: 25516281].

25. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci USA 2005; 102:15545-50. [PMID: 16199517].

26. Whitmore SS, Wagner AH, DeLuca AP, Drack AV, Stone EM, Tucker BA, Zeng S, Braun TA, Mullins RF, Scheetz TE. Transcriptomic analysis across nasal, temporal, and macular regions of human neural retina and RPE/choroid by RNA-Seq. Exp Eye Res 2014; 129:93-106. [PMID: 25446321].

27. Huang W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009; 4:44-57. [PMID: 19139596].

28. Peng S, Gan G, Qiu C, Zhong M, An H, Adelman RA, Rizzolo LJ. Engineering a blood-retinal barrier with human embryonic stem cell-derived retinal pigment epithelium:
transcriptome and functional analysis. Stem Cells Transl Med 2013; 2:534-44. [PMID: 23734062].
29. Garwin GG, Saari JC. High-performance liquid chromatography analysis of visual cycle retinoids. Methods Enzymol 2000; 316:313-24. [PMID: 10800683].
30. Franceschini A, Szklarczyk D, Frankild S, Kuhn M, Simonovic M, Roth A, Lin J, Mingez P, Bork P, von Mering C, Jensen LJ. STRING v9.1: protein-protein interaction networks, with increased coverage and integration. Nucleic Acids Res 2013; 41:D808-15. [PMID: 23203871].
31. Grisanti S, Guidry C. Transdifferentiation of retinal pigment epithelial cells from epithelial to mesenchymal phenotype. Invest Ophthalmol Vis Sci 1995; 36:391-405. [PMID: 7531185].
32. Madara JL. Regulation of the movement of solutes across tight junctions. Annu Rev Physiol 1998; 60:143-59. [PMID: 9558656].
33. Rizzolo LJ, Peng S, Luo Y, Xiao W. Integration of tight junctions and Claudins with the barrier functions of the retinal pigment epithelium. Prog Retin Eye Res 2011; 30:296-323. [PMID: 21704180].
34. Booij JC, ten Brink JB, Swagemakers SM, Verkerk AJ, Essing AH, van der Spek PJ, Bergen AA. A new strategy to identify and annotate human RPE-specific gene expression. PLoS One 2010; 5:e9341-10. [PMID: 20479888].
35. Strunnikova NV, Maminishkis A, Barb JJ, Wang F, Zhi C, Sergeev Y, Chen W, Edwards AO, Stambolian D, Abeасis G, Swaroop A, Munson PJ, Miller SS. Transcriptome analysis and molecular signature of human retinal pigment epithelium. Hum Mol Genet 2010; 19:2468-86. [PMID: 20360305].
36. Tian J, Ishibashi K, Handa JT. The expression of native and cultured RPE grown on different matrices. Physiol Genomics 2004; 17:170-82. [PMID: 14982971].
37. Cai H, Del Priore LV. Gene expression profile of cultured adult compared to immortalized human RPE. Mol Vis 2006; 12:1-14. [PMID: 16464697].
38. Tian J, Ishibashi K, Honda S, Boylan SA, Hjelmeland LM, Handa JT. The expression of native and cultured human retinal pigment epithelial cells grown in different culture conditions. Br J Ophthalmol 2005; 89:1510-7. [PMID: 16234463].
39. Körner AM, Pawelek J. Dopachrome conversion: a possible control point in melanin biosynthesis. J Invest Dermatol 1980; 75:192-5. [PMID: 6774031].
40. Feng W, Yasumura D, Mattes MT, LaVail MM, Vollrath D. Mertk triggers uptake of photoreceptor outer segments during phagocytosis by cultured retinal pigment epithelial cells. J Biol Chem 2002; 277:17016-22. [PMID: 11861639].
41. Finnemann SC, Nandrot EF. MerTK activation during RPE phagocytosis in vivo requires alphaVbeta5 integrin. Adv Exp Med Biol 2006; 572:499-503. [PMID: 17249615].
42. Sun L, Ryan DG, Zhou M, Sun TT, Lavker RM. EEDA: a protein associated with an early stage of stratified epithelial differentiation. J Cell Physiol 2006; 206:103-11. [PMID: 15920738].
43. Raviv S, Bharti K, Rencus-Lazar S, Cohen-Tayar Y, Schyr R, Evantal N, Mesheror E, Zilberberg A, Idelson M, Reubinoff B, Grebe R, Rosin-Abresfeld R, Lauderdale J, Luty G, Arneheiter H, Ashery-Padan R. PAX6 regulates melanogenesis in the retinal pigmented epithelium through feed-forward regulatory interactions with MITF. PLoS Genet 2014; 10:e1004360-[PMID: 24875170].
44. Masuda T, Wahlin K, Wan J, Hu J, Maruotti J, Yang X, Iacovelli J, Wolkow N, Kist R, Dunafey JL, Qian J, Zack DJ, Esumi N. Transcription factor SOX9 plays a key role in the regulation of visual cycle gene expression in the retinal pigment epithelium. J Biol Chem 2014; 289:12908-21. [PMID: 24634209].
45. Martinez-Morales JR, Signore M, Acampora D, Simeone A, Bovolenta P. Ott genes are required for tissue specification in the developing eye. Development 2001; 128:2019-30. [PMID: 11493524].
46. Tsukiji N, Nishihara D, Yajima I, Takeda K, Shibahara S, Yamamoto H. Mitf functions as an in vivo regulator for cell differentiation and proliferation during development of the chick RPE. Dev Biol 2009; 326:335-46. [PMID: 19100253].
47. Mochii M, Mazaki Y, Mizuno N, Hayashi H, Eguchi G. Role of Mitf in differentiation and transdifferentiation of chicken pigmented epithelial cell. Dev Biol 1998; 193:47-62. [PMID: 9466887].
48. Nawshad A, Lagamba D, Polad A, Hay ED. Transforming growth factor-beta signaling during epithelial-mesenchymal transformation: implications for embryogenesis and tumor metastasis. Cells Tissues Organs 2005; 179:11-23. [PMID: 15942189].
49. Bakall B, Marmorstein LY, Hoppe G, Peachey NS, Wadels M, Marmorstein AD. Expression and localization of bestrophin during normal mouse development. Invest Ophthalmol Vis Sci 2003; 44:3622-8. [PMID: 12882816].
50. Reinisalo M, Putula J, Mannermaa E, Urtti A, Honkakoski P. Regulation of the human tyrosinase gene in retinal pigment epithelial cells: the significance of transcription factor orthodenticle homeobox 2 and its polymorphic binding site. Mol Vis 2012; 18:38-54. [PMID: 22259223].
51. Kokkinaki M, Abu-Asab M, Gunawardena N, Ahern G, Javidnia M, Young J, Golestaneh N. Klotho regulates retinal pigment epithelial cell. J Neurosci 2013; 33:16346-59. [PMID: 24107965].
52. Tsou WI, Nguyen KQ, Calarena DA, Garforth SJ, Antes AL, Smirnov SV, Almo SC, Birge RB, Kotenko SV. Receptor tyrosine kinases, TYRO3, AXL, and MER, demonstrate distinct patterns and complex regulation of ligand-induced activation. J Biol Chem 2014; 289:25750-63. [PMID: 25074926].
53. Burke JM, Cao F, Irving PE, Skumatz CM. Expression of E-cadherin by human retinal pigment epithelium: delayed expression in vitro. Invest Ophthalmol Vis Sci 1999; 40:2963-70. [PMID: 10549658].
54. Mendez MG, Kojima S, Goldman RD. Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition. FASEB J 2010; 24:1838-51. [PMID: 20097873].

55. Dey A, Li W. Cell cycle-independent induction of D1 and D2 cyclin expression, but not cyclin-Cdk complex formation or Rb phosphorylation, by IFNgamma in macrophages. Biochem Biophys Acta 2000; 1497:135-47. [PMID: 10838167].

56. Adijanto J, Castorino JJ, Wang ZX, Maminishkis A, Grunwald GB, Philp NJ. Microphthalmia-associated transcription factor (MITF) promotes differentiation of human retinal pigment epithelium (RPE) by regulating microRNAs-204/211 expression. J Biol Chem 2012; 287:20491-503. [PMID: 22523078].

57. Holtrich U, Wolf G, Brauning A, Karn T, Bohme B, Rubsam-Waigmann H, Strehhardt K. Induction and down-regulation of PLK, a human serine/threonine kinase expressed in proliferating cells and tumors. Proc Natl Acad Sci USA 1994; 91:1736-40. [PMID: 8127874].

58. He J, Choe S, Walker R, Di Marzio P, Morgan DO, Landau NR. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. J Virol 1995; 69:6705-11. [PMID: 7474080].

59. Bolanos-Garcia VM, Blundell TL. BUB1 and BUBR1: multifaceted kinases of the cell cycle. Trends Biochem Sci 2011; 36:141-50. [PMID: 20888775].

60. Sharma RK, Orr WE, Schmitt AD, Johnson DA. A functional profile of gene expression in ARPE-19 cells. BMC Ophthalmol 2005; 5:25. [PMID: 16262907].

61. Westenskow PD, Moreno SK, Krohne TU, Kurihara T, Zhu S, Zhang ZN, Zhao T, Xu Y, Ding S, Friedlander M. Using flow cytometry to compare the dynamics of photoreceptor cell death in the mouse retina. Invest Ophthalmol Vis Sci 2012; 53:6282-90. [PMID: 22871841].

62. Stockinger A, Eger A, Wolf J, Beug H, Foisner R. E-cadherin regulates cell growth by modulating proliferation-dependent beta-catenin transcriptional activity. J Cell Biol 2001; 154:1185-96. [PMID: 11564756].

63. Halbleib JM, Nelson WJ. Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. Genes Dev 2006; 20:3199-214. [PMID: 17158740].

64. Sprecher E, Bergman R, Richard G, Lurie R, Shalev S, Petronius D, Shalata A, Anbinder Y, Leibu R, Perlman I, Cohen N, Szargel R. Hypotrichosis with juvenile macular dystrophy is caused by a mutation in CDH3, encoding P-cadherin. Nat Genet 2001; 29:134-6. [PMID: 11544476].

65. Burke JM. Epithelial phenotype and the RPE: is the answer blowing in the wind? Prog Retin Eye Res 2008; 27:579-95. [PMID: 18775790].

66. Lee J, Ko M, Joo CK. Rho plays a key role in TGF-beta-induced cytoskeletal rearrangement in human retinal pigment epithelium. J Cell Physiol 2008; 216:520-6. [PMID: 18314880].

67. Li H, Wang H, Wang F, Gu Q, Xu X. Snail involves in the transforming growth factor beta1-mediated epithelial-mesenchymal transition of retinal pigment epithelial cells. PLoS One 2011; 6:e23232.[PMID: 21853110].

68. Rizzolo LJ. Development and role of tight junctions in the retinal pigment epithelium. Int Rev Cytol 2007; 258:195-234. [PMID: 17338922].

69. Furuse M, Furuse K, Sasaki H, Tsukita S. Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. J Cell Biol 2001; 153:263-72. [PMID: 11309408].

70. Van Itallie CM, Fanning AS, Anderson JM. Reversal of charge selectivity in cation or anion-selective epithelial lines by expression of different claudins. Am J Physiol Renal Physiol 2003; 285:F1078-84. [PMID: 13129853].

71. Peng S, Wang SB, Singh D, Zhao PY, Davis K, Chen B, Adelman RA, Rizzolo LJ. Claudin-3 and claudin-19 partially restore native phenotype to ARPE-19 cells via effects on tight junctions and gene expression. Exp Eye Res 2016; 151:179-89. [PMID: 27593915].

72. Boulton M, Rozanowska M, Rozanowski B. Retinal photodamage. J Photochem Photobiol B 2001; 64:144-61. [PMID: 11744401].

73. Seagle BL, Rezai KA, Kobori Y, Gasyna EM, Rezaei KA, Norris JR Jr. Melanin photoprotection in the human retinal pigment epithelium and its correlation with light-induced cell apoptosis. Proc Natl Acad Sci USA 2005; 102:8978-83. [PMID: 15951427].

74. Gamm DM, Melvan JN, Shearer RL, Pinilla I, Sabat G, Svendsen CN, Wright LS. A novel serum-free method for culturing human prenatal retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 2008; 49:788-99. [PMID: 18235029].

75. Turowski P, Adamson P, Sathia J, Zhang JJ, Moss SE, Aylward GW, Hayes MJ, Kanuga N, Greenwood J. Basement membrane-dependent modification of phenotype and gene expression in human retinal pigment epithelial ARPE-19 cells. Invest Ophthalmol Vis Sci 2004; 45:2786-94. [PMID: 15277505].

76. Stanzel BV, Espana EM, Gruterich M, Kawakita T, Parel JM, Tseng SC, Binder S. Amniotic membrane maintains the phenotype of rabbit retinal pigment epithelial cells in culture. Exp Eye Res 2005; 80:103-12. [PMID: 15652531].

77. Jin M, Li S, Moghrabi WN, Sun H, Travis GH. Rpe65 is the retinoid isomerase in bovine retinal pigment epithelium. Cell 2005; 122:449-59. [PMID: 16096063].

78. Moiseyev G, Chen Y, Takahashi Y, Wu BX, Ma JX. RPE65 is the isomerase involved in the visual cycle. Proc Natl Acad Sci USA 2005; 102:12143-8. [PMID: 16116091].

79. Redmond TM, Poliakov E, Yu S, Tai JY, Lu Z, Gentleman S. Mutation of key residues of RPE65 abolishes its enzymatic role as isomerohydrolase in the visual cycle. Proc Natl Acad Sci USA 2005; 102:13658-63. [PMID: 16150724].
80. Marmorstein AD, Kinnick TR. Focus on molecules: bestrophin (best-1). Exp Eye Res 2007; 85:423-4. [PMID: 16720022].

81. Hu J, Bok D. The use of cultured human fetal retinal pigment epithelium in studies of the classical retinoid visual cycle and retinoid-based disease processes. Exp Eye Res 2014; 126:46-50. [PMID: 24060345].

82. Ruiz A, Ghyselinck NB, Mata N, Nusinowitz S, Lloyd M, Dennefeld C, Champon P, Bok D. Somatic ablation of the Lrat gene in the mouse retinal pigment epithelium drastically reduces its retinoid storage. Invest Ophthalmol Vis Sci 2007; 48:5377-87. [PMID: 18055784].

83. Batten ML, Imanishi Y, Maeda T, Tu DC, Moise AR, Bronson D, Possin D, Van Gelder RN, Baehr W, Paleczewski K. Lechithin-retinol acyltransferase is essential for accumulation of all-trans-retinyl esters in the eye and in the liver. J Biol Chem 2004; 279:10422-32. [PMID: 14684738].

84. Finnemann SC. Focal adhesion kinase signaling promotes phagocytosis of integrin-bound photoreceptors. EMBO J 2003; 22:4143-54. [PMID: 12912913].

85. Finemann SC. Focal adhesion kinase signaling promotes phagocytosis of integrin-bound photoreceptors. EMBO J 2003; 22:4143-54. [PMID: 12912913].

86. Hu G, Huang K, Yu J, Gopalakrishna-Pillai S, Kong J, Xu H, Liu Z, Zhang K, Xu J, Luo Y, Li S, Sun YE, Iverson LE, Xue Z, Fan G. Identification of miRNA signatures during the differentiation of hESCs into retinal pigment epithelial cells. PLoS One 2012; 7:e37224. [PMID: 22848339].

87. Ohana R, Weiman-Kelman B, Raviv S, Tamm ER, Pasmanik-Chor M, Rinon A, Netaney D, Shamir R, Solomon AS, Ashery-Padan R. MicroRNAs are essential for differentiation of the retinal pigmented epithelium and maturation of adjacent photoreceptors. Development 2015; 142:2487-98. [PMID: 26062936].

88. Kutty RK, Samuel W, Jaworski C, Duncan T, Nagiini CN, Raghavachari N, Wiggert B, Redmond TM. MicroRNA expression in human retinal pigment epithelial (ARPE-19) cells: increased expression of microRNA-9 by N-(4-hydroxyphenyl)retinamide. Mol Vis 2010; 16:1475-86. [PMID: 20806079].

89. Zhao C, Yasumura D, Li X, Matthes M, Lloyd M, Nielsen G, Ahern K, Snyder M, Bok D, Duniae J, LaVail MM, Vollrath D. mTOR-mediated dedifferentiation of the retinal pigment epithelium initiates photoreceptor degeneration in mice. J Clin Invest 2011; 121:369-83. [PMID: 21135502].

90. Rizzolo LJ, Chen X, Weitzman M, Sun R, Zhang H. Analysis of the RPE transcriptome reveals dynamic changes during the development of the outer blood-retinal barrier. Mol Vis 2007; 13:1259-73. [PMID: 17679949].