

A Novel Serum-Free Method for Culturing Human Prenatal Retinal Pigment Epithelial Cells

David M. Gamm,^{1,2} J. Nicholas Melvan,¹ Rebecca L. Shearer,¹ Isabel Pinilla,³ Grzegorz Sabat,⁴ Clive N. Svendsen,^{1,5} and Lynda S. Wright¹

PURPOSE. Established techniques for culturing primary human retinal pigment epithelial (RPE) cells have facilitated the laboratory investigation of this multipurpose retinal cell layer. However, most culture methods involve the use of animal serum to establish and maintain RPE monolayers, which can complicate efforts to define and study factors involved in the maturation and function of these cells. Therefore, this study was conducted to develop a simple, serum-free system to propagate and sustain human RPE *in vitro*.

METHODS. RPE was dissected from human prenatal donor eyes and cultured in serum-free defined medium containing the commercially formulated supplement B27 or N2. Cultures were grown initially as adherent tissue sections or suspended spherical aggregates and later expanded and maintained as monolayers. PCR, Western blot analysis, and immunocytochemistry were used to monitor gene and protein expression in established cultures, followed by examination of secretory products in RPE conditioned medium by ELISA and mass spectrometric analysis.

RESULTS. In medium supplemented with B27, but not N2, RPE could be expanded up to 40,000-fold over six passages and maintained in culture for more than 1 year. In long-term cultures, typical cellular morphology and pigmentation were observed, along with expression of characteristic RPE markers. RPE monolayers also retained proper apical-basal orientation and secreted multiple factors implicated in the maintenance of photoreceptor health and the pathogenesis of age-related macular degeneration.

CONCLUSIONS. Monolayer cultures of human prenatal RPE can be grown and maintained long term in the total absence of serum and still retain the phenotype, gene and protein expression profile, and secretory capacity exhibited by mature RPE cells.

From the ¹Waisman Center, and the Departments of ²Ophthalmology and Visual Sciences, ³Anatomy and Neurology, and ⁴Genetics/Biotechnology Center, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin; and the ⁵Department of Ophthalmology, Miguel Servet University Hospital and Aragones de Ciencias de la Salud, Zaragoza, Spain.

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Corresponding author: David M. Gamm, Department of Ophthalmology and Visual Sciences, University of Wisconsin School of Medicine and Public Health, T607 Waisman Center, 1500 Highland Avenue, Madison, WI 53705; dgamm@wisc.edu.

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The homogenous, relatively nondescript appearance of RPE on the light microscopic level belies a complex cellular organization and function that is critical for the preservation of outer retinal health and activity.¹ In addition, mounting evidence points to an important role for the RPE in the development of the neurosensory retina.^{2,3} The study of the RPE has been aided by advancements in culture techniques that encourage the adoption of characteristic RPE properties found *in vivo*. Such properties include, among others, formation of a pigmented, compact monolayer of polygonal cells connected by tight junctions, expression of specialized proteins, preservation of cellular orientation, and an ability to secrete multiple factors.⁴⁻¹¹ However, the demonstration of some or all of these features *in vitro* has necessitated the use of animal serum and/or intricate combinations of chemical and protein constituents not available in prefabricated media supplements.

The presence of serum in cell culture medium can be problematic, because it contains numerous partially characterized or undefined factors that vary in concentration between commercial preparations.^{7,12-15} The existence of a defined, serum-free growth and maintenance medium would be of particular value for the straightforward identification of factors released by the RPE *in vitro*. It would also provide a simplified, reproducible environment for studying the maturation and physiology of the RPE layer and its response to pharmacologic treatments. Last, it would facilitate the use of cultured human RPE in clinical studies by reducing exposure of cells to animal products before transplant. Some protocols allow for the reduction or removal of serum from RPE medium preparations after initial attachment or once the cells reach confluence.¹⁶⁻¹⁹ However, this practice may alter cell survival and function and does not eliminate the exposure of proliferating RPE cell populations to serum.^{14,20} To address this problem, Tezel and Del Priore¹² developed a chemically defined, serum-free medium that supported the initial attachment and growth to confluence of adult human RPE cells. However, production of the customized serum-free medium required the separate addition of numerous components and did not use commercially formulated supplements. Furthermore, the cultured RPE cells underwent limited characterization *in vitro*. Thus, the impact of the serum-free environment on the expression of critical RPE proteins remained unknown.

Sources of human RPE for experimentation and clinical use have grown to include primary prenatal^{6,8,17,21} and postnatal^{7,12,22,23} tissue, transformed cell lines,²⁴⁻²⁷ and embryonic stem (ES) cells.²⁸ These sources differ widely in their expansion potential, degree of differentiation, and propensity to display RPE-like characteristics. For example, the commonly used ARPE-19 cell line can be passaged indefinitely but does not remain pigmented or reliably express certain RPE-selective proteins, such as bestrophin.^{9,27,28} Postnatal human RPE, on the other hand, has limited expansion potential, but retains a relatively mature phenotype in culture.^{7,12} Human ES cells are theoretically in limitless supply and can produce cells with a

gene and protein expression pattern and appearance similar to primary RPE cultures with little contamination from other cell types.²⁸ However, the functional competence of ES cell-derived RPE is yet to be established²⁹ and, similar to other sources of RPE, they are grown in the presence of serum. Cultures of primary human prenatal RPE have the benefit of possessing a greater growth capacity than adult RPE while still exhibiting many of the known features of mature RPE in vitro.^{6,17} There are also disadvantages to the culture of human prenatal RPE, including supply limitations and ethical concerns regarding its use.

With these issues in mind, we sought to develop a simple, serum-free method for culturing human prenatal RPE by using commercially formulated medium supplements. B27, a supplement that contains numerous factors of potential importance for RPE growth and maturation,^{6,8,12,18,30} supported the adherence, propagation, and passaging of RPE on laminin-coated tissue culture plastic. In contrast, RPE cultures exhibited minimal expansion in the presence of N2 supplement, which includes only a fraction of the components present in B27.^{30,31} Primary attachment and outgrowth of RPE cells was improved by implementing a method in which explants were cultured initially as suspended spheroids. Resulting pigmented spheroids consistently adhered to laminin-coated tissue culture plastic and produced high yields of RPE cells. The morphology, gene and protein expression profile, polarity, and secretory capacity of cells cultured in this manner were similar to that reported for RPE grown in the presence of serum.^{6,11}

METHODS

Serum-Free RPE Monolayer Culture

RPE was isolated from human eyes between 10 and 16 weeks of gestation according to protocols approved by the NIH, the Institutional Review Board at the University of Wisconsin-Madison, and the University of Washington. Eyes were shipped from the University of Washington Birth Defects Laboratory overnight at 4°C in sterile-filtered transport medium (30 mM KCl, 5 mM glucose, 0.24 mM MgCl₂, 1.95 mM NaH₂PO₄ · 6H₂O, 20 mM Na₂HPO₄ · 2H₂O, and 20 mM lactic acid, followed by adjustment to pH 7.2 with KOH pellets and 300 mOsm with approximately 140 millimoles sorbitol; all chemicals obtained from Sigma-Aldrich, St. Louis, MO) and dissected on arrival. Whole eyes were maintained in ice-cold dissection medium (DM; 70% DMEM containing 4.5 g/L D-glucose (catalog no. 11965; Invitrogen, Carlsbad, CA), 30% F12 nutrient mixture containing L-glutamine (catalog no. 11765; Invitrogen), and 1% antibiotic-antimycotic solution (catalog no. 14240; Invitrogen), whereas the attached periorcular tissues were carefully removed and discarded. The remaining intact globes were rinsed twice in DM, and the anterior segment of the eye and the vitreous were removed. The eye cup was then washed two times with DM, and the detached neural retina was removed in its entirety with forceps. If the neural retina had not spontaneously detached after removal of the vitreous, a 1-mL syringe with a 25-gauge, 5/8-in. needle was used to irrigate with DM gently under the peripheral retina, to separate it fully from the underlying RPE. After two more DM washes, the remaining RPE and attached choroid were peeled from the underlying sclera with forceps, being careful to avoid the ciliary margin region. At this point, the RPE-choroid explants were treated in one of three ways.

Method 1: Isolated RPE Sheets. Some explants were incubated in DM containing 2% Dispase (catalog no. 17105-041; Invitrogen) for 30 minutes at 37°C and washed twice in DM before small sheets of RPE were teased away from the choroid with forceps and irrigation. The isolated RPE sheets were collected and further chopped into 200- μ m sections with a McIlwain automated tissue chopper (Mickle Laboratory Engineering Co., Ltd., Guildford, UK), as described previously for primary cortical and retinal tissue.^{32,33} After the tissue sections were chopped, they were placed on laminin-coated (0.001%,

catalog no. L2020; Sigma-Aldrich) tissue culture plastic (flasks or wells) in a minimal volume of serum-free RPE medium (SFRM), consisting of DM supplemented with either 2% B27 (50 \times solution, catalog no. 17504; Invitrogen) or 1% or 2% N2 (100 \times solution, catalog no. 17502; Invitrogen), designated SFRM-B27 or SFRM-N2, respectively.

Method 2: RPE-Choroid Explants. Alternatively, RPE-choroid explants were immediately chopped into 200- μ m sections without exposing them to proteolytic enzymes or manipulating them further with forceps. After examining the chopped explant sections under a dissection microscope, only those containing prominent sheets of attached RPE were subsequently placed onto laminin-coated tissue culture plastic. As described earlier, chopped explants were cultured exclusively in SFRM-B27 or SFRM-N2.

Method 3: Pigmented Spheroids. Based on observations of cell cultures established from the previous two methods, a modification of method 2 was devised in which chopped RPE-choroid explants were initially placed in suspension culture in SFRM-B27. Nonpigmented and pigmented spherical tissue aggregates formed within hours, with many partially pigmented spheroids becoming uniformly pigmented over 2 to 4 weeks in culture. At various times, darkly pigmented spheroids were removed from suspension culture by using a glass Pasteur pipette and were plated onto laminin-coated tissue culture plates in SFRM-B27 or SFRM-N2.

Regardless of the method used, all cultures were maintained at 37°C and 5% CO₂, and 50% to 75% of the cell culture medium was exchanged every 1 to 2 days. Within 24 to 48 hours of initial plating, expanding monolayers of RPE cells were observed to emanate from attached tissue sections or pigmented spheroids. With method 2, occasional colonies also arose that contained dense collections of discrete, multilayered choroidal fibroblasts. The contaminating colonies were marked on the outside of the flask and manually removed with a Pasteur pipette under a dissecting microscope. After approximately 4 to 7 days, the remaining tissue sections or spheroids were easily detached with simple irrigation and collected, leaving the surrounding monolayer colonies of RPE cells attached to the flasks. Detached pigmented spheroids could be replated on laminin-coated plastic at least three times with no observable decrease in initial RPE production, or stored in liquid nitrogen in serum-free, cell-freezing medium (catalog no. C6295; Sigma-Aldrich).

After removal of the tissue sections or spheroids, the remaining adherent RPE monolayer colonies were treated with cell-detachment medium (Accutase, catalog no. SCR005; Chemicon, Temecula, CA) for 10 to 15 minutes at 37°C until all cells had rounded up and begun to lift off the plastic. Dissociated RPE cells were harvested by gently irrigating the remaining loosely attached cells to lift them off the plastic with a glass Pasteur pipette and transferring the cell suspension to a 15-mL conical tube. After a 3-minute centrifugation at 1000 rpm, the cell detachment solution overlying the cell pellet was removed, and the cells were resuspended in 10 mL of SFRM-B27 or SFRM-N2. The cells were pelleted and resuspended a second time, counted on a hemocytometer, and plated on laminin-coated tissue culture plastic at a density of $\geq 50,000$ cells/cm². When cultures reached confluence (3–7 days after plating), they could be passaged until a growth plateau was reached, and evidence of active cell division was no longer evident by light microscopy (up to six passages). At each passage, a portion of the cells were designated for long-term observation and study or for serial passaging to establish growth curves. Excess RPE cells were stored as aliquots in liquid nitrogen after dissociation and later rethawed to establish additional cultures as needed.

RPE Growth Determination

Growth curves were obtained by serially passaging RPE cultures within 24 hours of reaching confluence and determining the total number of cells present per tissue culture flask or well using a hemocytometer and trypan blue dye exclusion. Initial cell counts at day 0 (i.e., passage 1) were obtained after dislodging and removing adherent primary tissue sections or pigmented spheroids and harvesting the surrounding

TABLE 1. Primers Used for RT-PCR

Gene Amplified	Forward	Reverse	Size (bp)
<i>RPE65</i>	GCC CTC CTG CAC AAG TTT GAC TTT	AGT TGG TCT CTG TGC AAG CGT AGT	259
Bestrophin	ATT TAT AGG CTG GCC CTC ACG GAA	TGT TCT GCC GGA GTC ATA AAG CCT	359
<i>CRALBP</i>	TTC GGC ATG GTA CCT GAA GAG GAA	ACT GCA GCC GGA AAT TCA CAT AGC	349
<i>MerTK</i>	AGC CTG AGA GCA TGA ATG TCA CCA	TGT TGA TCT GCA CTC CCT TGG ACA	234
<i>VWF</i>	AAA GAG TCT CCA TGC CCT ATG CCT	TTC ACA CCA CTG TTC TCC ACT GCT	284
<i>PEDF</i>	AAT CCA TCA TTC ACC GGG CTC TCT	TGC ACC CAG TTG TTG ATC TCT TGC	253
<i>NGF</i>	AGC GTC CGG ACC CAA TAA CAG TTT	GCA GTG TCA AGG GAA TGC TGA AGT	213
<i>IGF1</i>	TGA GCT GGT GGA TGC TCT TCA GTT	TAC TTC CTT CTG GGT CTT GGG CAT	241
<i>VEGF</i>	AGG GCA GAA TCA TCA CGA AGT GGT	TCT GCA TGG TGA TGT TGG ACT CCT	218
<i>EGF</i>	ATG AGC AAT TGG TGG TGG ATG CTG	TTC CAG CCA CCT CTG AAG ACC AAA	345
<i>FGF2</i>	AAG AGC GAC CCT CAC ATC AAG CTA	ATA CTG CCC AGT TCG TTT CAG TGC	237
<i>TGFB1</i>	TGC TAA TGG TGG AAA CCC ACA ACG	ATT TCT GGT ACA GCT CCA CGT GCT	189
<i>BDNF</i>	TCC ACT GAG CAA AGC CGA ACT TCT	TTG CCT TGT CCG TGG ACG TTT ACT	234

monolayer skirt of RPE cells for subsequent passage. All counts were performed in triplicate, and the mean was used to establish the increase (*n*-fold change) in cell quantity for each passage. Doubling times were calculated from exponential growth curves plotted for each culture (Prism ver. 3.02; GraphPad Software, San Diego, CA). Cumulative cell counts at later passages were extrapolated to account for the fraction of cells stored in liquid nitrogen.

BrdU Incorporation

Acutely dissociated RPE cells from three separate cultures were plated onto poly-L-lysine (0.01%; Sigma-Aldrich) and laminin-coated glass coverslips (50,000 cells/coverslip) and incubated in SFRM-B27, with or without 20 ng/mL FGF2 (catalog no. GF003; Chemicon). After 2 days in culture, bromodeoxyuridine (BrdU; Sigma-Aldrich) was added to the media to a final concentration of 0.2 μ M. Twenty-four hours later, the RPE cells were fixed for 10 minutes in ice-cold methanol, washed in PBS, and prepared for immunocytochemistry.

RNA Isolation and cDNA Synthesis

Total RNA was isolated (RNeasy MiniKit; Qiagen, Valencia, CA) using the company's protocol, which included an optional DNase I treatment step. Individual RNA samples were treated a second time with amplification grade DNase I (Invitrogen) before cDNA synthesis, which was performed with a kit (Superscript III First-Strand Synthesis System for RT-PCR; Invitrogen). All reactions included control samples in which reverse transcriptase enzyme was not added to the reaction.

RT-PCR

PCR reactions were performed by combining master mix (Promega, Madison, WI), 10 μ m each of the appropriate forward and reverse primers (Table 1), and 1:40 diluted cDNA template. Samples were initially denatured at 95°C for 5 minutes followed by 35 cycles of PCR amplification (95°C for 15 seconds, 60°C for 30 seconds, 72°C for 1 minute) and a final extension at 72°C for 10 minutes. PCR products were visualized on a 1.5% agarose gel containing 0.1% ethidium bromide.

Quantitative PCR

Quantitative PCR was performed on a thermocycler (Opticon 2; Bio-Rad, Hercules, CA, with SYBR Green 2 \times master mix; Applied Biosystems, Foster City, CA) and 300 picomoles of the following forward and reverse primers in 20- μ L reactions: β -actin: (forward) 5'-GCGAGAA-GATGACCCAGATC-3', (reverse) 5'-CCAGTGGTACGGCCAGAGG-3'; bestrophin: (forward) 5'-CAA GCT GCT ATA TGG CGA GTT-3', (reverse) 5'-GCC AGC CTA TAA ATA AAG CGG AT-3'. Reverse transcription reactions were diluted 1:40, and 4 μ L were used per reaction. Samples from five individual cultures were initially denatured at 95°C for 15 minutes followed by 40 cycles of PCR amplification (95°C for 20 seconds, 55°C for 30 seconds, 72°C for 30 seconds) with a plate reading after every extension. Melting curves were determined to confirm amplification of the expected fragment. In addition, standard curves were measured for all primer sets by serial dilution of reverse transcriptase reactions, and efficiencies were calculated to ensure that all primer sets had similar efficiencies of amplification. Each reaction was performed in triplicate and C_T values were obtained by averaging the results. The relative differences (*n*-fold) in gene expression of a characteristic marker of mature RPE, bestrophin, was determined across cultures by using β -actin as an internal control for each reaction set (to determine ΔC_T values) and arbitrarily designating one culture as a reference (to determine $\Delta\Delta C_T$ values). Data are presented as the mean relative change \pm SEM of three separate qPCR amplification reactions for each culture.

Cell Lysate Preparation

RPE cells were solubilized in modified RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 0.7% Triton X-100, 0.3% Nonidet P-40, 1% deoxycholate, 1 mM PMSF, 2 mM NaVO₃) and 1:100 protease inhibitor cocktail for use with mammalian cell and tissue extracts (catalog no. P8340; Sigma-Aldrich) containing 104 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 80 μ M aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64. Lysates were sonicated and total protein was quantified (DC Protein Assay; Bio-Rad).

RPE-Conditioned Medium Preparation

Tissue culture flasks containing confluent RPE monolayers were washed three times with DM followed by a 24-hour incubation in DM. Thereafter, RPE-conditioned medium (RPE CM) was collected, and 1:200 protease inhibitor cocktail for use in tissue culture medium (catalog no. P1860; Sigma-Aldrich) was added. Conditioned medium was then concentrated (up to 80-fold) using centrifugal filter devices (Amicon; Millipore, Billerica, MA) with a 3-kDa cutoff according to the manufacturer's instructions. Total protein was then quantified (DC Protein Assay; Bio-Rad).

Western Analysis

Twenty micrograms of protein from RPE cell lysates or concentrated RPE-conditioned medium was collected from RPE monolayers. Protein samples were separated on 4% to 20% gradient Tris-Cl gels (Bio-Rad), electroblotted onto PVDF membranes and stained with Ponceau red to confirm transfer. Membranes were blocked with 5% nonfat dry milk and 2.5% BSA in TBST for 1 hour at room temperature followed by consecutive 1 hour incubations at room temperature with primary antibody in TBST+1.5% BSA and HRP-conjugated secondary antibody in TBST+1% nonfat dry milk. Primary antibodies used for Western blot analysis were directed against RPE65 (mouse monoclonal, 1:2000; Chemicon), bestrophin (mouse monoclonal, 1:500; Chemicon), CRALBP (mouse monoclonal, 1:50,000, gift of John Saari, University of Washington, Seattle), ezrin (rabbit polyclonal, 1:1000; Cell Signaling Technologies, Danvers, MA), occludin (rabbit polyclonal, 1:250; Zymed, Carlsbad, CA), claudin-10 (rabbit polyclonal, 1:250; Zymed), PEDF (mouse monoclonal, 1:500; Chemicon), VEGF (rabbit polyclonal, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), and FGF2 (mouse monoclonal, 1:500; Upstate Biotechnology, Charlottesville, VA). Protein bands were visualized by chemiluminescence (ECL or ECL Plus Western Blot Analysis Detection Kit; GE Healthcare, Chalfont St. Giles, UK).

Enzyme-Linked Immunosorbent Assay

During routine passaging of RPE cultures, 10^6 cells were plated into each laminin-coated well of a six-well plate in SFRM-B27. Cultures were maintained with daily medium exchange for 4 to 6 weeks until cultures displayed mature RPE cell morphology and pigmentation. Cultures were then washed three times with DM followed by a 24-hour incubation in SFRM-B27. Thereafter, RPE CM was collected and 1:200 protease inhibitor cocktail (catalog no. P1860; Sigma-Aldrich) was added. Conditioned medium was then concentrated threefold with the centrifugal filter devices (Amicon Centriplus; Millipore) with a 3-kDa cutoff according to the manufacturer's instructions. Levels of VEGF and FGF2 protein were quantified in triplicate by ELISA (R&D Systems, Minneapolis, MN) using the manufacturer's protocols. PEDF levels were similarly quantified by ELISA (Chemicon), with the exception that the concentrated RPE CM was first treated with urea, per the manufacturer's protocol. The plated RPE cells from each well were then dissociated and counted by using a hemocytometer to express results as nanograms of growth factor produced per day per million cells (mean \pm SEM). A minimum of three assays were performed for each growth factor.

Immunocytochemistry

During routine passaging of proliferating RPE cultures, 30,000 to 50,000 cells were plated in SFRM-B27 onto poly-L-lysine and laminin-coated glass coverslips in 24-well plates. Cultures were maintained in SFRM-B27 with daily medium exchange for 4 to 8 weeks until cultures displayed mature RPE cell morphology and pigmentation. Cells were fixed with 4% paraformaldehyde for 20 minutes and washed with phosphate-buffered saline (PBS). Fixed cell cultures were permeabilized for 10 minutes in 0.2% Triton X-100 in PBS, blocked with 5% normal goat serum and 0.2% Triton X-100 in PBS for 30 minutes, and processed for immunocytochemistry with primary antibodies to be-

strophin (1:100), CRALBP (1:2000), ezrin (1:100), occludin (1:100), RPE65 (1:100), and ZO-1 (rabbit polyclonal, 1:100, Zymed). After they were rinsed with PBS, the cells were incubated for 30 minutes with secondary antibodies conjugated to Alexa 488 or Alexa 546 (1:1000; Invitrogen-Molecular Probes, Eugene OR). Thereafter, Hoechst 33258 (1:10,000 in PBS) was added for 5 minutes to visualize nuclei, followed by mounting in antifade reagent (Prolong Gold; Invitrogen-Molecular Probes). Serial confocal image sections were collected with a laser scanning fluorescence confocal microscope (model C1; Nikon Corp., Tokyo, Japan) and the resulting imaging data were used to reconstruct cross-sectional z scans and rendered volume images (EZ-C1 software; Nikon Corp.). For BrdU immunostaining, coverslips were exposed to primary antibody directed against BrdU (rat monoclonal, 1:300; Accurate Chemical, Westbury, NY), washed, and incubated with 1:2000 anti-rat secondary antibody conjugated to Cy3 (Jackson Laboratories, West Grove, PA), followed by Hoechst 33258. To count BrdU-positive cells, digital images of immunostained cells were taken using a high-resolution camera (SPOT RT Slider; Diagnostic Instruments, Sterling Heights, MI). Five to 10 random fields ($\times 40$ magnification) were counted from at least three separate coverslips per experiment. The percentage of BrdU incorporation was expressed as the mean \pm SEM for three separate experiments, and the results were analyzed using Student's two-tailed, unpaired *t*-test.

Two-Dimensional Gel Electrophoresis

Protein samples from RPE CM were concentrated to 919 $\mu\text{g}/\text{mL}$ and mixed with five volumes of precooled (-20°C) precipitation solution (10% trichloroacetic acid in acetone). Proteins were precipitated overnight at -20°C , pelleted by centrifugation for 15 minutes at 12,000g (4°C), washed twice with ice-cold acetone, pelleted again, and washed once more with 50% ice-cold acetone in water to remove any residual salt contamination. The pellet was dried to remove excess acetone but was prevented from completely drying, to facilitate subsequent resolubilization. The sample was reconstituted in isoelectric focusing (IEF) buffer (8 M urea, 4% CHAPS, 40 mM Tris-HCl [pH 9.0], 100 mM dithiothreitol [DTT], and 0.2% ampholyte [Bio-Lyte 3/10; Bio-Rad], with trace bromophenol blue).

For isoelectric focusing, 17 cm, pH 3 to 10 IPG strips (ReadyStrip; Bio-Rad) were rehydrated with 500 and 1200 μg of protein sample overnight at room temperature. The isoelectric focusing was performed in an IEF cell (Protean; Bio-Rad) at 20°C with a current limit of 30 $\mu\text{A}/\text{strip}$ for a total of 55,000 V/h (1-hour equilibration at 250 V, 3-hour slow ramp at 3000 V, 4-hour slow ramp at 10,000 V, and final fast ramp at 10,000 V for 45 V/h). Focused IPG strips were stored at -80°C until second-dimension analysis.

Before second-dimension analysis, the strips were equilibrated for 30 minutes in SDS equilibration solution (6 M Urea, 2% SDS, 30% glycerol, 50 mM Tris-HCl [pH 8.8], with trace bromophenol blue) containing 1% wt/vol DTT, then transferred to SDS equilibration solution containing 2.5% IEF and incubated for an additional 30 minutes. The second dimension was performed overnight (Protean II xi system; Bio-Rad) with 1-mm-thick, precast, 8% to 16% gradient SDS polyacrylamide gels (Bio-Rad) as follows: 1-hour stacking at 25 V (start: 8 mA, end: 7 mA), 1-hour stacking at 50 V (start: 16 mA, end: 15 mA), 2-hour resolving at 100 V (start: 31 mA, end: 25 mA), 16-hour resolving at 80 V (start: 19 mA, end: 10 mA), 0.5-hour resolving at 300 V (start: 40 mA, end: 38 mA).

After electrophoresis, the gels were rinsed in water, fixed for 3 hours in $\text{MeOH}/\text{H}_2\text{O}/\text{CH}_3\text{COOH}$ (50%:45%:5%) and stained overnight with colloidal Coomassie blue (SimplyBlue SafeStain; Invitrogen). After they were stained, the gels were washed in water three times for 30 minutes to remove any background staining, and the image was captured on a GS-800 calibrated densitometer (Bio-Rad). Spots of interest were manually excised with a scalpel and transferred to siliconized tubes for subsequent proteolytic digestion and mass spectrometric analysis.

TABLE 2. Composition of B27, N2, and Customized Serum-Free Medium Supplements

Component	B27	N2	Tezel and Del Priore ¹²
Bovine serum albumin	Y	Y	Y
Transferrin	Y	Y	Y
Insulin	Y	Y	Y
Progesterone	Y	Y	N
Putrescine	Y	Y	Y
Sodium selenite	Y	Y	N
Biotin	Y	N	N
L-Carnitine	Y	N	N
Corticosteroid	Y*	N	Y†
Ethanolamine	Y	N	N
D(+)-galactose	Y	N	N
Glutathione (reduced)	Y	N	N
Linolenic acid	Y	N	N
Linoleic acid	Y	N	Y
Vitamin A derivative	Y‡	N	Y§
Selenium	Y	N	Y
T3 (triiodo-L-thyronine)	Y	N	Y
DL- α -Tocopherol (vitamin E)	Y	N	N
DL- α -tocopherol acetate	Y	N	N
Catalase	Y	N	N
Superoxide dismutase	Y	N	N
Follicle stimulating hormone	N	N	Y
Epidermal growth factor	N	N	Y
Basic fibroblast growth factor	N	N	Y

* Corticosterone.

† Hydrocortisone.

‡ Retinyl acetate.

§ all-*trans* Retinoic acid.

Enzymatic Digestion and Mass Spectrometric Analysis

In gel digestion and mass spectrometric analysis were performed at the Mass Spectrometry Facility (Biotechnology Center, University of Wisconsin-Madison). Coomassie G-250 (colloidal)-stained gel pieces were destained completely in 1:1 methanol:H₂O containing 100 mM NH₄HCO₃, dehydrated once for 10 minutes in 1:1 acetonitrile (ACN):H₂O with 25 mM NH₄HCO₃ and again for 1 minute in 100% ACN, and dried for 5 minutes (Speed-Vac). The dried gel pieces were rehydrated and reduced in 25 mM DTT (in 25 mM NH₄HCO₃ buffer) for 30 minutes at 56°C, alkylated with 55 mM iodoacetamide (in 25 mM NH₄HCO₃ buffer) in darkness at room temperature for 30 minutes, washed twice in H₂O for 1 minute each, equilibrated in 25 mM NH₄HCO₃ for 1 minute, dehydrated once for 10 minutes in 1:1 ACN:H₂O containing 25 mM NH₄HCO₃ and again for 1 minute in 100% ACN, dried, and rehydrated with 20 μ L of 20 ng/ μ L trypsin solution (Sequence Grade Modified; Promega) containing 25 mM NH₄HCO₃. The digestion was conducted overnight (18 hours) at 37°C and subsequently terminated by acidification with an equal volume of 2.5% trifluoroacetic acid (TFA). Peptides generated from the digestion were extracted with an equal volume of 0.1% TFA and vigorous vortexing for 15 minutes, followed by the addition of an identical volume of 70:25:5 ACN:H₂O:TFA and repeated vortexing. The peptide solution was collected and dried completely in a Speed-Vac, resuspended in 50 μ L of 0.1% TFA, and solid phase extracted (ZipTip C18 pipette tips; Millipore). Peptides were eluted off the C18 column with 60:40:0.2 ACN:H₂O:TFA directly onto 384-well plates (Opti-TOF Applied Biosystems) and recrystallized with 0.75 μ L of matrix (10 mg/mL α -cyano-4-hydroxycinnamic acid in 50:35:0.1 acetone/CAN/TFA). Peptide map fingerprint result-dependent MS/MS analysis was then performed (4800 Matrix-Assisted Laser Desorption/Ionization-Time of Flight-Time of Flight [MALDI/TOF-TOF] mass spectrometer; Applied Biosystems). Peptide fingerprints were generated by scanning a 700- to 4000-Da mass range using 1000 shots acquired from 20 randomized regions of the sample

spot at 3600 intensity of an on-axis laser in positive reflectron mode (OptiBeam; Applied Biosystems). The 10 most abundant precursors (excluding trypsin autolysis peptides and sodium/potassium adducts) were selected for subsequent tandem MS analysis, during which 2000 total shots were taken with 4200 laser intensity. Postsource decay (PSD) fragments from the precursors of interest were isolated by timed-ion selection and reaccelerated into the reflectron to generate the MS/MS spectrum. Raw data was deconvoluted (GPS Explorer software; Applied Biosystems) and submitted for peptide mapping and MS/MS ion search analysis against a nonredundant National Center for Biotechnology Information (NCBI; Bethesda, MD) database with an in-house licensed Mascot search engine (Matrix Science, London, UK).

RESULTS

Role of Serum-Free Medium Containing B27 Supplement in Primary Human RPE Culture Expansion and Maintenance

Human prenatal RPE was dissected as isolated sheets (method 1) or RPE-choroid explants (method 2), chopped into 200- μ m sections, and placed in laminin-coated tissue culture plastic in serum-free RPE medium (SFRM) containing DMEM with high glucose, Ham's F12, and either B27 (SFRM-B27) or N2 (SFRM-N2) supplement. B27 supplement was chosen for this study because it is commercially formulated and contains many constituents present in customized RPE medium preparations and/or deemed important for optimal RPE growth and maintenance (Table 2).^{6,8,12,18,30} N2 supplement, which contains a limited subset of factors present in B27,^{30,31} was used for comparison.

With either method, initial outgrowth of cells was observed in the presence of SFRM-B27 or SFRM-N2, but on subsequent passages, substantial culture expansion occurred with SFRM-B27 only (Fig. 1). No cell expansion was seen with cultures maintained in SFRM without B27 or N2 (data not shown). The doubling time of these RPE cell cultures in SFRM-B27 over the first three passages was 1.84 ± 0.30 days ($n = 4$), and growth could be maintained for a maximum of six passages over 3 to 4 weeks, producing an approximately 40,000-fold increase in the number of cells. After passaging and plating at a density of 50,000 cells/cm², the cells became confluent within 5 days, adopted a characteristic RPE morphology, and became increas-

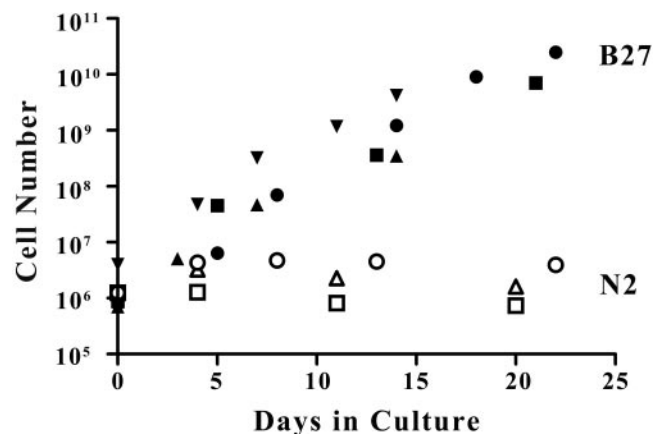


FIGURE 1. Growth potential of representative RPE cultures in serum-free medium containing either B27 (filled symbols) or N2 (open symbols) supplement. Cultures were derived from isolated RPE sheets (method 1; circles), RPE-choroid explants (method 2; squares) or first (up triangles) or second (down triangles) platings of pigmented spheroids (method 3). Data points reflect cumulative cell counts obtained after each culture passage.

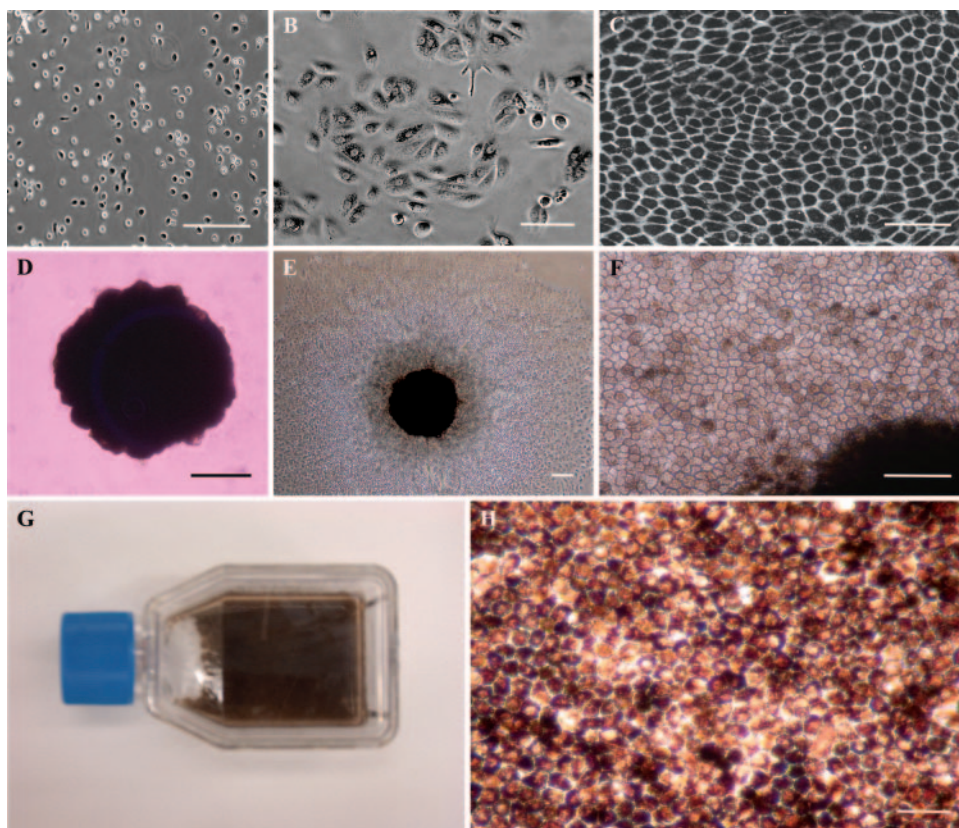


FIGURE 2. Phase-contrast light microscopic images of cultured RPE established from isolated sheets of primary tissue (method 1) on days 0 (A), 1 (B), and 28 (C) after third passage in SFRM-B27. (D) A typical pigmented spheroid (method 3) derived from sectioned RPE-choroid sheets after 25 days in suspension culture in SFRM-B27. (E) Low- and (F) high-power images of the same spheroid shown in (D) 6 days after attachment to laminin-coated tissue culture plastic. (G) Low- and (H) high-power images of a T-12.5 flask containing third-passage RPE (method 3) 6 weeks after seeding. Scale bars: (B, C, H) 20 μm ; (A, F) 50 μm ; (D, E) 200 μm .

ingly compacted and pigmented over the ensuing 6 weeks (Figs. 2A–C). RPE monolayers were difficult to dissociate at this late stage and could not be expanded further.

Although similar growth characteristics were observed in RPE cultures established using method 1 or 2, disadvantages were also encountered with both approaches. In method 1, chopped sections of isolated, primary RPE would frequently fail to adhere to the laminin-coated plastic, while in method 2, contaminating choroidal cell colonies (distinguished by their fibroblast morphology and tendency to form multilayers¹⁰) or mixed choroidal/RPE cell colonies were regularly seen after initial plating (Supplementary Fig. S1A; supplementary figures are online at <http://www.iovs.org/cgi/content/full/49/2/788/DC1>). When present, these contaminating colonies required manual removal from culture flasks before subsequent passaging. Therefore, in an effort to promote primary tissue adherence and minimize early choroidal cell infiltration, a modification of method 2 was used. In this third method, dissected RPE-choroid explants were first grown as suspended spheroids in SFRM-B27 until most of them adopted a uniform, darkly pigmented appearance (Fig. 2D). Often, light microscopy could clearly identify a continuous surface layer of RPE on these spheroids (Supplementary Figure S1B). The pigmented spheroids readily adhered to laminin-coated tissue culture plastic and gave rise to rapidly enlarging monolayers of RPE cells with rare choroidal cell contamination (Figs. 2E, 2F). RPE cells generated in this manner could be dissociated, expanded and used to establish long-term monolayer cultures in T-12.5, -25, and -75 flasks (Figs. 2G, 2H; Supplementary Figs. S1C, S1D). Furthermore, the doubling time (1.59 ± 0.14 days; $n = 3$) and morphology of cell cultures established from pigmented spheroids (method 3) were essentially indistinguishable from those originally obtained from isolated RPE sheets (method 1) or RPE-choroid explants (method 2). Pigmented spheroids recovered after initial plating could be

stored frozen or replated at least three times, to establish additional RPE cultures as needed. RPE cultures generated from reused spheroids retained similar growth characteristics (Fig. 1) and morphologic features (Supplementary Figs. S1E, S1F) exhibited by spheroids after initial plating. Based on these collective findings, we elected to use RPE cultures established by method 3 for subsequent experiments, except where otherwise indicated.

Enhancement of RPE Proliferation by the Addition of FGF2

Previous reports suggested that the inclusion of FGF2, a mitogen not present in B27 supplement, could significantly improve cell proliferation in serum-containing RPE cultures.^{17,34} This finding prompted Tezel and Del Priore¹² to include FGF2 in their serum-free RPE culture medium preparation (Table 2). To determine whether the addition of FGF2 to SFRM-B27 could enhance RPE cell division compared with SFRM-B27 alone, 24-hour BrdU incorporation was measured in the presence or absence of FGF2 beginning 2 days after culture passage (a period corresponding to peak cell proliferation). Cultures ($n = 3$) maintained in SFRM-B27 with 20 ng/mL FGF2 demonstrated $37.8\% \pm 2.8\%$ BrdU incorporation, compared with $19.6\% \pm 2.6\%$ in parallel cultures grown without FGF2 ($P = 0.003$; Fig. 3). Thus, although SFRM-B27 is capable of supporting substantial human RPE growth in vitro, its proliferative effects can be augmented with the addition of one or more defined factors.

Expression of RPE-Selective Genes and Proteins in Serum-Free Culture

RT-PCR was used to examine the gene-expression profile of RPE monolayers ($n = 3$) grown and maintained exclusively in SFRM-B27. Complementary DNA was generated from long-term RPE monolayer cultures, all of which expressed the RPE-

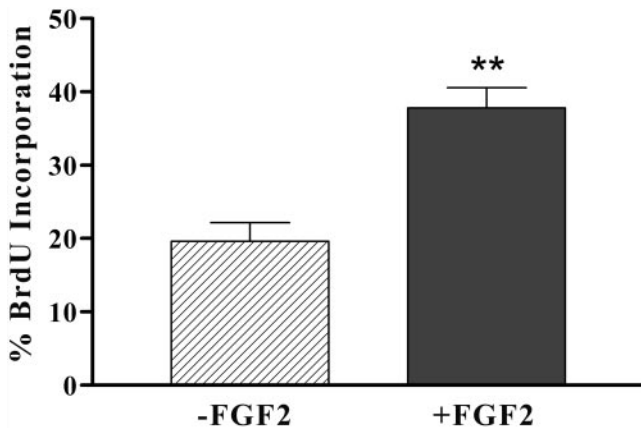


FIGURE 3. Percentage of BrdU incorporation into RPE cultured in SFRM-B27 without (*hatched bar*) or with (*solid bar*) 20 ng/mL FGF2. Results are expressed as the mean \pm SEM of results of four experiments on three separate cultures (** $P < 0.005$).

specific gene *RPE65*, as well as bestrophin, cellular retinaldehyde-binding protein (*CRALBP*), and receptor tyrosine kinase Mer (*MerTK*), whereas von Willebrand factor, an endothelial cell marker, was absent (Fig. 4A). PCR results obtained from cultures established by using method 1 or 2 showed the same pattern of gene expression (data not shown). Quantitative PCR analysis further demonstrated <2 -fold variations in bestrophin gene expression levels across multiple cultures ($n = 5$) of different methods of origin, age in culture, passage number, and spheroid plating number (Supplementary Fig. S2).

Western blot analysis performed on cell lysates obtained from RPE monolayer cultures ($n = 3$) revealed protein expression of RPE65, bestrophin, and CRALBP in all samples (Fig. 4B). Other RPE proteins prominently expressed *in vivo* were also present in cell lysates, including ezrin and the tight junction proteins claudin-10 and occludin (Fig. 4B). Of note, occludin was often found in a low-molecular-weight form, although higher-molecular-weight forms indicative of protein phosphorylation³⁵⁻³⁷ were also present on these blots.

Apical-Basal Polarity and Tight Junction Protein Expression in RPE Cells in Serum-Free Culture

RPE cells are polarized *in vivo*, with their apical surface directed toward the outer segments of photoreceptors and their basal surface facing the choriocapillaris.^{1,6,8,38} This orientation is necessary for the RPE layer to perform many of its functions properly *in vivo*, including photopigment recycling, outer segment phagocytosis, ion and fluid transport, and directed factor secretion.¹ To assess the polarity of cultured RPE cells grown and maintained in SFRM-B27, we performed immunocytochemistry and confocal microscopy using antibodies directed against RPE proteins expressed in the apical cell membrane (ezrin), in the basolateral membrane (bestrophin), or within the cytoplasm (CRALBP and RPE65). As shown in Figures 5A-D, these proteins were predominantly localized to their expected regions within the cultured RPE cells.

Another important structural feature of native RPE monolayers is the existence of tight junctions between adjacent cells, which are key components of the outer blood-retina barrier.³⁸ We investigated the expression and localization of the tight junction proteins zonula occludens (ZO)-1 and occludin, both of which demonstrated localized intercellular staining (Figs. 5E, 5F). A third junctional complex protein, claudin-10, was not consistently detected in the intercellular space by immunocytochemistry (data not shown). Together, these re-

sults reveal that SFRM-B27 can support the development and maintenance of proper RPE cell orientation, as well as the expression and localization of some, but probably not all, of the tight junction proteins found in native RPE.

Multiple Protein Factors Secreted by RPE Cells in Serum-Free Culture

RPE cells secrete numerous growth factors and other proteins that impact photoreceptor health and function.^{1,4,6,11} The capacity of serum-free RPE monolayer cultures to produce and secrete specific growth factors was investigated by RT-PCR, Western blot analysis, and ELISA. First, we examined gene expression of a host of growth factor genes known to be expressed in the RPE, including pigment epithelium-derived growth factor (*PEDF*), nerve growth factor (*NGF*), insulin-like growth factor 1 (*IGF1*), vascular endothelial growth factor (*VEGF*), epidermal growth factor (*EGF*), FGF2, transforming growth factor β 1 (*TGF β 1*), and brain-derived growth factor (*BDNF*; Fig. 6A). These genes were expressed in our standard serum-free conditions in all cultures tested ($n = 3$). To determine whether selected growth factor gene transcripts are subsequently translated and released, CM was collected from these

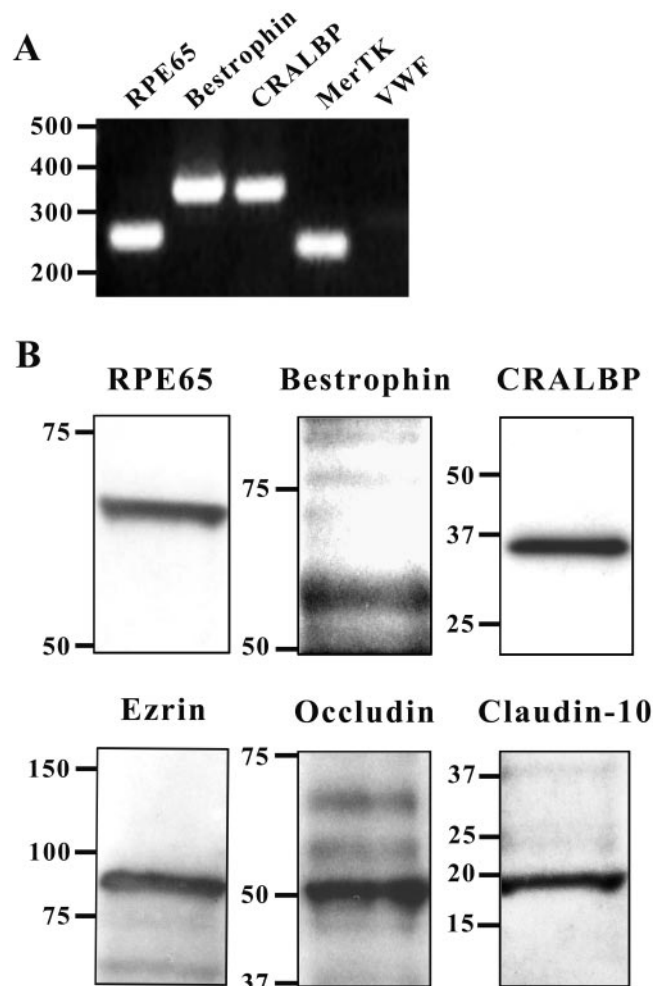


FIGURE 4. Expression of characteristic RPE genes (A) and proteins (B) in serum-free cultures as determined by RT-PCR and Western blot analysis ($n \geq 3$), respectively. VWF was used as a marker for endothelial cell contamination. Results were consistent across all cultures tested, regardless of the method of origin (methods 1-3), time spent in culture (2-12 months), or number of passages (2-5).

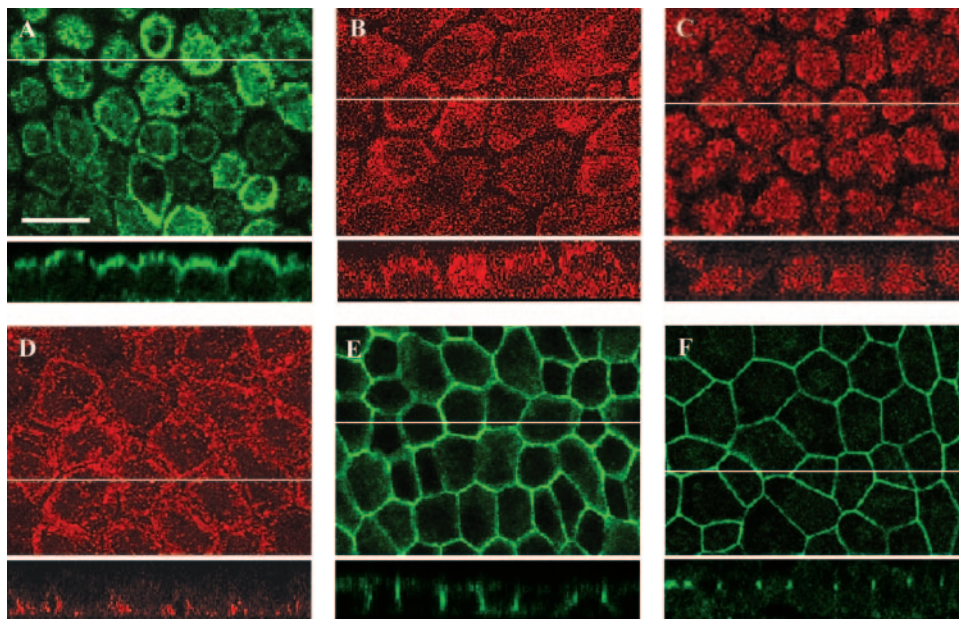


FIGURE 5. Immunocytochemistry staining and confocal imaging of RPE cultures (passage 2 or 3) grown exclusively in SFRM-B27. *Top*: an en face view of an RPE monolayer presented as a maximum-intensity projection through the z -axis. *Bottom*: cross-section through the z -plane of multiple optical slices at the location indicated by the white reference line in the corresponding *top* portion. Ezrin (A), CRALBP (B), RPE65 (C), bestrophin (D), occludin (E), and ZO-1 (F). Scale bar, 10 μ m.

RPE monolayers, concentrated, and analyzed by Western blot ($n = 3$). All growth factors examined (PEDF, VEGF, and FGF2) were present in RPE CM (Fig. 6B). Quantification of PEDF, VEGF, and FGF2 in RPE CM by ELISA ($n \geq 3$) revealed secretion rates of 2453 ± 1099 ng/ 10^6 cells/d, 0.94 ± 0.05 ng/ 10^6 cells/d, and 0.054 ± 0.005 ng/ 10^6 cells/d, respectively (Fig. 6C).

In addition to growth factors, RPE cells release a host of regulatory proteins with diverse functions into their surrounding microenvironment.¹¹ The defined, serum-free medium used for this study allowed straightforward identification of major proteins present in the human RPE secretome. Concentrated RPE CM was separated by 2-D gel electrophoresis, and isolated protein spots were subjected to tandem mass spectrometry. We identified the 12 most abundant proteins in RPE CM by this method, including proteins involved in signaling, metal binding, and immune regulation, in addition to one carry-over from B27 supplement (bovine serum albumin; Table 3). Thus, cultured RPE retains a strong capacity to secrete a variety of factors without the need for serum supplementation.

DISCUSSION

Numerous culture methods and media preparations have been described that support the initial attachment, limited growth, and long-term maintenance of primary human RPE cells in vitro. Early media formulations were relatively simple, primarily using a base medium supplemented with a high percentage of serum.^{22,23,39-45} A drawback of using serum supplementation in culture media is the ambiguous introduction of signaling molecules, including hormones and cytokines, and other known and unknown factors with the potential to influence cell behavior.^{12,13,44} With the development of more complex and precise RPE media preparations, less reliance was placed on undefined additives.^{6,8,18,45} Some of these tailored medium recipes called for the presence of a low percentage (1%–5%) of animal serum and/or tissue extract throughout the culture period,^{6,8} whereas in others, high levels of serum (15%–20%) were present at initial seeding.^{6,18} By contrast, Tezel and Del Priore¹² developed a chemically defined culture medium that supported attachment, proliferation, and three serial passages of adult human RPE in the total absence of serum. However, characterization of the cultured RPE cells was limited to mor-

phology and cytokeratin expression, and preparation of the customized serum-free medium required assembly of 12 separate components (including 7 growth factors and hormones) in addition to the base medium.

Comparison of the formulas used in successful low or no serum medium preparations for human RPE cultures reveals certain commonalities. All include a base medium (typically MEM or DMEM supplemented with amino acids and inorganic salts), transferrin and insulin, and most contain corticosteroid, triiodothyronine, selenium, and putrescine.^{6,8,12,18} Many of these factors are necessary for the growth and maintenance of mammalian cells in general. Others, such as triiodothyronine and corticosteroid, are potentially important for RPE cell metabolism, fluid flux, and tight junction formation.⁴⁶⁻⁴⁸ Additional factors used in various RPE-specific media include EGF, FGF2, follicle stimulating hormone, retinoic acid, linoleic acid, ascorbic acid, progesterone, and taurine. These factors have been shown to affect RPE cell adhesion, proliferation, survival, and morphology, along with other critical cellular functions.^{12,17,18,49-51} Most of these substances are present in B27 supplement, a highly augmented form of N2 initially designed to support the maintenance of hippocampal neurons and neuronal cell lines.⁵² Subsequently, B27 was found to promote expansion of embryonic and adult neural stem cells in serum-free neurosphere cultures.^{30,53} Given its composition and proven efficacy in serum-free cultures of other neuroectodermal cell types, we predicted that B27 would also support RPE growth and long-term survival. However, the fact that B27 alone could eliminate the need for serum to establish and expand RPE cultures was unanticipated, since certain mitogens absent from B27 are often used to promote cell growth in other serum-free media preparations.^{12,18,32,33} Even so, mitogens can be used to enhance the growth potential of human RPE in SFRM-B27, as demonstrated by the increased cell proliferation observed after addition of FGF2. The effects of FGF2 supplementation on other aspects of RPE cell culture (e.g., differentiation) are currently being investigated by using our serum-free system.

In the present study, a single medium was used for all phases of RPE culture. Other culture systems use different formulations for the adherence, expansion, differentiation and/or maintenance of RPE cells in vitro.^{6,8} Phenotypic maturation of RPE monolayers in SFRM-B27, on the other hand,

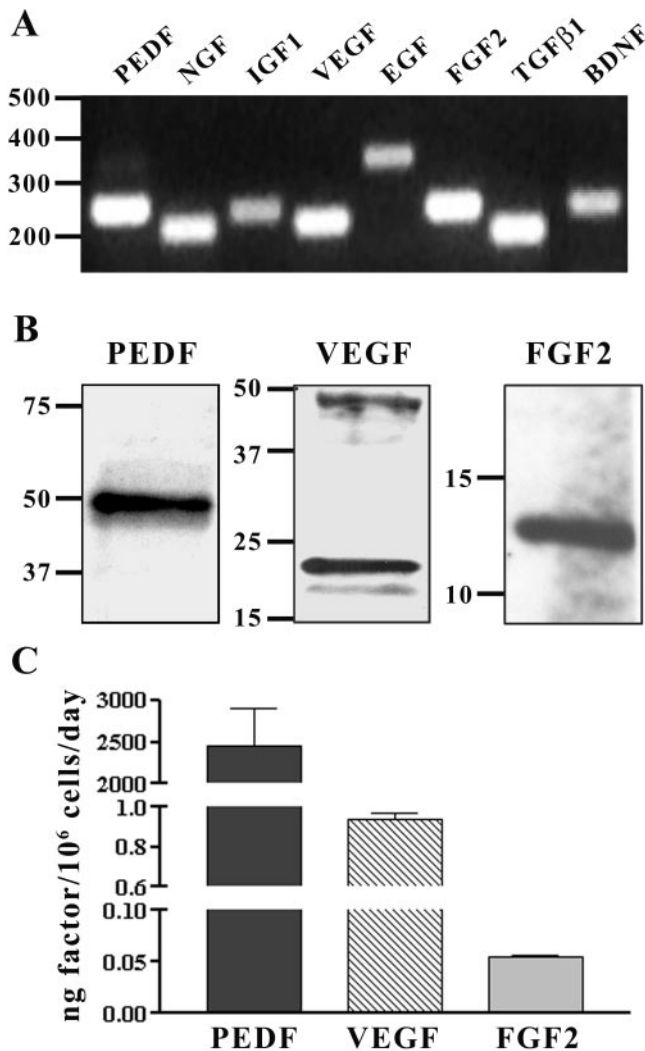


FIGURE 6. (A) Expression of selected growth factor genes in serum-free RPE cultures as determined by RT-PCR. (B) Western blot analysis of conditioned medium from serum-free RPE cultures reveal the presence of secreted growth factors. (C) Quantification of PEDF, VEGF, and FGF2 levels in serum-free RPE conditioned medium. Results were consistent across all cultures tested ($n \geq 3$), regardless of method of origin (methods 1-3), time spent in culture (2-12 months), or the number of passages (2-5).

occurred spontaneously over time after the cells reached confluence, as evidenced by replicative senescence, adoption of a compact, polygonal morphology, and repigmentation.

The profound difference in growth potential between serum-free RPE cultures treated with B27 versus N2 supplement may prove useful in the identification of candidate factors important for RPE proliferation. There are 15 B27 components lacking in N2 supplement, five of which (triiodothyronine, vitamin A derivative, corticosteroid, selenium, and linoleic acid) were also incorporated in the serum-free RPE medium developed by Tezel and del Priore.¹² The availability of defined, serum-free RPE culture protocols should facilitate the future investigation of the roles of these and other factors in human RPE growth.

Although SFRM-B27 supported RPE growth from isolated sheets of human RPE (method 1) or RPE-choroid explants (method 2), there were technical limitations to each of these approaches. Primary attachment was inconsistent with isolated RPE sheets, and early choroidal contamination was seen in

explant cultures by light microscopy. However, we noted that chopped RPE-choroid explants that did not immediately plate down would persist as suspended spheroids in SFRM-B27 and become uniformly pigmented over time. Conversely, nonadherent RPE sheets degraded in culture, consistent with previous reports.^{54,55} Over time, the explant-derived, pigmented spheroids spontaneously attached to the culture plastic surface and cells of a nearly exclusive RPE morphology emanated from them. We postulate that the relative lack of choroidal contamination in these cultures is due to the development of a layer of RPE cells on the spheroid surface, which may sequester choroidal fibroblasts from substrate after plating. Electron microscopic studies are currently under way to investigate this possibility in more depth. RPE monolayers established from the pigmented spheroid method (method 3) could be passaged and expanded in a manner identical with those established from isolated RPE sheets or RPE-choroid explants. These findings were similar to those of Aronson,²¹ who first noted that rounded RPE-choroid fragments could generate RPE cultures after plating. Later, Rezaei et al.⁵⁴ and Gabrielian et al.⁵⁶ showed that human fetal RPE could be grown on free-floating spheroidal polymer scaffolds in medium containing 15% FBS. After attachment of the RPE-coated polymer spheres to culture plastic, RPE cells proliferated from the site of adhesion and formed monolayers. Therefore, culturing primary RPE initially as suspended spheroids on a natural (choroid) or synthetic substrate promotes subsequent cell attachment and proliferation in the absence or presence of serum, respectively.

Once grown to confluence in SFRM-B27, monolayer RPE cultures expressed markers indicative of mature RPE cells. Furthermore, immunocytochemical analysis revealed that the normal apical-basal polarity of RPE cells was preserved, along with the expression of characteristic junctional complex proteins. An exception was claudin-10, a tight junction protein shown to be expressed discontinuously by human RPE cultured in the presence of serum.⁶ In the present study, claudin-10 was detected in RPE lysates by Western blot but could not be unequivocally localized to the junctional complex by immunocytochemistry. Occludin expression also did not appear to be restricted to apical junctions, although it was clearly present along the lateral RPE membranes. A possible explanation for these observations is that SFRM-B27 lacks the elements necessary to assemble the complete complex. However, tight junction formation in cultured RPE cells can also be inhibited by serum,⁵⁷ suggesting that multiple unknown factors, both positive and negative, can influence this process.⁵⁸ The existence of serum-free RPE culture protocols should facilitate the search for these and other factors involved in the development and regulation of RPE structure and function in vitro.

A defined, serum-free environment is also conducive, although not essential, to the study of cellular secretion in vitro. Recently, An et al.¹¹ examined the secretome profile of adult human RPE cultured in the presence of serum by labeling newly synthesized proteins with isotope-tagged amino acids. The authors detected a wide range of proteins in spent RPE medium, most of which are known to be secreted. In the present study, numerous discrete protein spots were visible after 2-D gel electrophoresis of concentrated RPE CM from serum-free prenatal cultures. Mass spectrometric analysis of the major protein spots subsequently led to the identification of 12 individual proteins. Six of these were also found in the study by An et al.¹¹: PEDF, galectin-3 binding protein, prostaglandin D2 synthase, complement subcomponents 1s and 1r, and β -actin. Of the remaining six prominent proteins, four are known to be produced by RPE (cystatin C, transthyretin, cathepsin D, and ceruloplasmin), whereas the ocular expression of another (al-cadein α -1) has not been documented. The final identified constituent in our study, bovine serum albumin, represents a

TABLE 3. Proteins Identified by Mass Spectrometry in the Conditioned Medium of Serum-Free RPE Cultures

Protein	Accession Number	Mass (kDa)	pI	Mascot Score*	Protein Coverage (%)
Cystatin C	gi 73535286	13.3	8.75	485	66
Transthyretin	gi 1181953	13.8	5.55	549	80
Prostaglandin D2 synthase	gi 55962673	21.0	7.66	196	47
β -Actin	gi 14250401	41.0	5.56	376	44
Cathepsin D	gi 16740920	44.5	6.10	141	31
Pigment epithelium-derived factor	gi 30585311	46.4	5.97	502	40
Galectin-3 binding protein	gi 16041761	65.3	5.13	303	19
Albumin [<i>Bos taurus</i>]	gi 162648	69.2	5.82	364	37
Complement component 1, s subcomponent	gi 34785163	76.6	4.86	174	21
Complement component 1, r subcomponent	gi 62896521	80.2	5.89	112	18
Alcadin α -1	gi 54125663	108.6	4.83	383	22
Ceruloplasmin	gi 1942284	120.0	5.41	287	24

* Mascot scores greater than 78 are significant ($P < 0.05$).

carry-over contaminant from B27 supplement. This finding is consistent with an earlier report in which serum contaminants were present in RPE CM despite multiple washings and incubation with serum-free medium before sample collection.¹¹ Thus, short-term removal of serum from RPE culture medium is not sufficient to eliminate serum components in subsequent experiments.

Most of the proteins identified by our mass spectrometric analysis are secreted and have the potential to influence retinal development, homeostasis, and/or disease. PEDF is an inhibitor of retinal angiogenesis^{59,60} with neuroprotective and neurotrophic properties⁶⁰ that is secreted by RPE before and after birth.^{61,62} Galectin-3 binding protein is apically secreted and promotes integrin-mediated cell adhesion and binds numerous extracellular proteins, including fibronectin.^{63,64} Prostaglandin D2 synthase, an enzyme postulated to function as a retinoid transporter,⁶⁵ is released into the interphotoreceptor matrix where it is taken up by photoreceptors. The roles of complement proteins C1s and C1r are less clear, although other complement proteins have been implicated in the pathogenesis of age-related macular degeneration (AMD).^{66,67} Cystatin C is a secreted cysteine protease inhibitor thought to be involved in neurodegeneration, neuroprotection, and central nervous system (CNS) repair.^{68,69} It is highly expressed in fetal and adult RPE and is postulated to regulate photoreceptor degradation during development and after trauma or ischemia.^{68,70-72} Cystatin C may indirectly regulate the activity of cathepsin D, a major lysosomal protease responsible for the degradation of phagocytosed photoreceptor outer segments.^{68,73} Although the proenzyme form of cathepsin D (52 kDa) is secreted by RPE,⁷⁴ its precise role after release into the extracellular environment is not known. Transthyretin is a homotetrameric serum transport protein for thyroxine and triiodothyronine that also interacts with retinol binding protein.⁷⁵ In the eye, it is known to be secreted in abundance by RPE,⁷⁵ predominantly from the apical surface,^{76,77} but it is also found in AMD drusen deposits.⁷⁸ Ceruloplasmin serves as an antioxidant, converting ferrous iron to the less dangerous ferric form.^{79,80} It has a secreted form that is present within the retina and vitreous,⁷⁹⁻⁸¹ and the absence of ceruloplasmin has been linked to free radical injury and degenerative diseases within the brain and retina.^{79,82} Alcadeins indirectly associate with amyloid β -protein precursor (APP)^{83,84} and are thought to function physiologically in concert with APP, a molecule implicated in the pathogenesis of Alzheimer disease and AMD.⁸⁵ Like APP, alcadin α -1 is a transmembrane protein whose extracellular domain is subject to cleavage and release into the extracellular space. However, the present analysis discovered only the unprocessed form of alcadin α -1 in RPE CM.

Although PEDF was the only growth factor identified as a major secreted protein by 2-D gel electrophoresis, RPE is known to secrete a host of other growth factors as well.^{1,4,60} It is likely that these other molecules are more labile than PEDF or are secreted at levels too low to detect on Coomassie- or silver-stained 2-D gels, even after concentration of RPE CM. However, the potential for serum-free RPE cultures to produce and secrete multiple growth factors, including VEGF and FGF2, was confirmed in this study by PCR, Western blot, and ELISA. Thus, human RPE cultured exclusively in SFRM-B27 retains its ability to secrete a host of growth factors implicated in the maintenance of retinal health and function.

Altogether, the results confirm that human RPE cultures can be established, expanded, and maintained in the complete absence of serum. By taking advantage of the commercially formulated B27 supplement, we simplified the preparation of serum-free medium without adversely affecting RPE cell morphology, protein expression, or secretory capacity. Additions to this minimal serum-free medium formulation or alterations in the culture technique may optimize the method further. However, the current protocol should facilitate examination of RPE development, structure, function, and disease in a defined, reproducible culture environment.

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