Characterization of a Spontaneous Mouse Retinal Pigment Epithelial Cell Line B6-RPE07

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PURPOSE. A spontaneously arising retinal pigment epithelial (RPE) cell line (B6-RPE07) was cloned from a primary culture of mouse RPE cells and maintained in culture for more than 18 months. Morphologic and functional properties of this cell line have been characterized.

METHODS. The morphology of the B6-RPE07 cells was examined by phase-contrast light microscopy, electron microscopy, and confocal microscopy. Barrier properties were measured by the flux of fluorescence from the apical to the basolateral compartment of culture chambers. The abilities of the cells to bind/phagocytose photoreceptor outer segments (POS) were determined by confocal microscopy, electron microscopy, and flow cytometry. Cytokine/chemokine secretion was measured by cytometric bead array. The expression of visual cycle proteins was determined by RT-PCR and Western blotting.

RESULTS. In standard culture conditions, B6-RPE07 cells display cobblestone morphology. When cultured on three-dimensional (3D) collagen gel–coated membranes, B6-RPE07 cells exhibit a monolayer epithelial polarization with apical surface microvilli. Immunohistochemistry of B6-RPE07 cultures revealed a high expression of pan-cytokeratin. B6-RPE07 cells also expressed the retinal pigment epithelium-specific marker CRALBP, but not RPE65. Cell junction proteins ZO-1 and β-catenin, but not Claudin-1/3 or occludin-1, were observed in B6-RPE07 cells. B6-RPE07 cells are able to bind, phagocytose, and digest POS. Finally, B6-RPE07 cells produce high levels of IL-6 and CCL2.

CONCLUSIONS. This is the first report of a mouse RPE cell line with morphology, phenotype, and function similar to those of in vivo mouse RPE cells. This cell line will be a valuable resource for future RPE studies, in particular for in vivo gene modification and transplantation studies. (Invest Ophthalmol Vis Sci. 2008;49:3699–3706) DOI:10.1167/iovs.07-1522

The neural-derived retinal pigment epithelial (RPE) cells, positioned between the neural retina and the vascular choriocapillaris, are central to retinal homeostasis and to many common retinal diseases. The retinal pigment epithelium constitutes the outer aspect of the blood-retina barrier (BRB) and controls the passage of metabolites to and from the circulatory system. RPE cells phagocytose and digest membranes shed from photoreceptor cell outer segments and play important roles in the maintenance of visual function and the survival of photoreceptor cells. In addition, RPE cells have an important immunoregulatory role, on the one hand contributing to ocular immune privilege by the elaboration of immunosuppressive cytokines (e.g., TGF-β and its activating molecule, thrombospondin), the expression of regulatory proteins such as CD59 and complement factor H, and the production of immunomodulatory mediators such as nitric oxide and on the other hand playing a proinflammatory role, with the secretion of a range of cytokines and chemokines, when appropriately activated. The role of RPE cells as a local source of complement factor H, an important complement regulatory factor, has particular significance for the pathogenesis of age-related macular degeneration (AMD), the commonest cause of blindness in the industrialized world. Dysfunction of RPE cells can lead to devastating effects on retinal function and in some diseases, such as AMD, can lead to photoreceptor cell death. RPE cell transplantation has been considered as one of the possible approaches for AMD treatment and, in fact, autograft of RPE cells is in clinical trials. In pathologic conditions, RPE cells produce various cytokines and chemokines which may underlie the roles of the retinal pigment epithelium in the pathogenesis of various ocular diseases, including uveoretinitis.

Our understanding of RPE cell function, in particular the role of the retinal pigment epithelium in ocular abnormalities such as retinal inflammation, degeneration, and neovascularization, remains limited. Tissue culture of retinal pigment epithelium provides a system for study of the specialized functions and characteristics of the retinal pigment epithelium under controlled conditions. Primary cultures of RPE cells from various species have been used for RPE research. In addition, primary cultures of retinal pigment epithelium have been shown to retain many normal physiological functions, including blood-retina barrier function, the capacity to phagocytose photoreceptor outer segments and the ability to transport viral proteins in a polarized fashion. However, experiments performed on primary cultures of retinal pigment epithelium may require customized cell purification, and such primary cultures exhibit considerable physiological variability from experiment to experiment, based simply on donor differences. An alternative is the use of cell lines, RPE cell lines from human and other species are available commercially. Some cell lines have been spontaneously created during the passage of primary cell culture, such as the human RPE cell lines D407 and ARPE19 and the rat RPE cell line BPEI-1, whereas other cell lines have been intentionally created by transformation with oncogenes or viral proteins such as the rat RPE cell line RPE-J, which was transfected with the SV40 virus. No mouse RPE cell line, however, is commercially available for experimental studies. The use of the mouse as an experimental model has many advantages, such as enhanced genetic definition, increased range of available reagents, and the possibility of modifying a specific gene (knockout or overexpression). In this study, we have generated and characterized a continuous mouse RPE cell line, B6-RPE07, which arose...
spontaneously in our laboratory from a primary culture of C57BL/6 mouse RPE cells.

**Materials and Methods**

**RPE Cell Isolation, Culture, Passage, and Cloning**

B6-RPE07 was originated from a 12-week-old healthy female C57BL/6 mouse, which was obtained from the Medical Research Facility at the Medical School of the University of Aberdeen. All procedures adopted conformed to the regulations of the Animal License Act (UK) and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The mouse was killed by CO2 inhalation; eyes were removed under sterile condition then placed in ice-cold Dulbecco modified Eagle medium (DMEM; Gibco BRL, Paisley, UK) and transported to the tissue culture laboratory, where they were dissected. The primary RPE culture was carried out using a previously described method with a slight modification.40–41 Briefly, the cornea, lens, and retina were removed, and the posterior eyecups were immersed in 0.5% (wt/vol) trypsin-EDTA (ICN Flow, Irvine, CA) at 37°C for 1 hour, RPE cells were then released from the basement (Bruch) membrane by gentle aspiration with 0.25% trypsin-EDTA digestion (5 minutes at 37°C) at a split ratio of 1:6. During cell passages, the RPE cells gradually lost their melanin granules, as has been observed previously with other RPE cell lines.36–39 By the sixth passage, almost no melanin granule could be seen by macroscopic inspection of the dishes. A remarkable morphologic change accompanied by an accelerated growth kinetic was noticed by the eighth passage. All cells showed a uniform, epithelioid morphology at this stage and reached confluence after subculture at a 1:6 dilution in complete DMEM using 0.25% trypsin-EDTA to detach the cells. During cell passages, the RPE cells were cultured in complete DMEM for another 6 days, by which time they had reached confluence. The cells were then subcultured at a 1:3 dilution in complete DMEM using 0.25% trypsin-EDTA to detach the cells. During cell passages, the RPE cells gradually lost their melanin granules, as has been observed previously with other RPE cell lines.36–39 By the sixth passage, almost no melanin granule could be seen by macroscopic inspection of the dishes. A remarkable morphologic change accompanied by an accelerated growth kinetic was noticed by the eighth passage. All cells showed a uniform, epithelioid morphology at this stage and reached confluence after subculture at a 1:6 dilution every 4 days. The spontaneously transformed RPE cells were further cloned by limited dilution in 96-well plates. The wells that contained a single cell were marked, and clones were selected for the appearance of an epithelioid morphology. Nine clones were obtained. Among them clone 7, named B6-RPE07, was chosen for further study.

**Routine Conditions for B6-RPE07 Cell Culture and Passage**

B6-RPE07 cells were routinely cultured in T75 flasks (Nunc, Roskilde, Denmark) in complete DMEM at 5% CO2 at 37°C and passaged with 0.25% trypsin-EDTA digestion (5 minutes at 37°C) at a split ratio of 1:6 every 4 days. Culture media were changed every other day. In some experiments, the cells were cultured in serum-free epithelial medium (Quantin 286; PAA Laboratories Ltd., Somerset, UK). Cells were cultured in our laboratory for more than 18 months (750 doublings) after cloning. Subculturing of the B6-RPE07 cells continues.

**Cell Growth Characters**

Cell growth rates were determined by placing a single-cell suspension (1 × 104 cells/mL/well) into two 24-well plates at normal culture conditions. Culture medium was changed every 2 days by replacing 500 μL old medium with the same amount of fresh medium. Cell numbers were counted using a hemocytometer each day from three wells after attaching the cells from the well by 0.25% trypsin-EDTA digestion.

** Colony-Formation Assay**

A colony formation assay was carried out in a soft agar culture system. In a 55-mm culture dish, 2 mL of 0.6% agar (Sigma, Poole, UK) in DMEM culture was placed in the bottom and left to gel. A total of 5 × 103 cells in 2 mL of 0.5% agar in complete DMEM were seeded on the top of the 0.6% agar layer and set at room temperature for 10 minutes to gel. One milliliter fresh culture medium was then added on top of the gel and cultured at 37°C for 3 weeks with regular medium replacement (twice a week). Culture dishes were examined by phase-contrast microscopy every 3 days.

**Photomicroscopy**

Cell morphology of cultured RPE cells was observed by phase-contrast microscopy throughout the experiment. Images were captured using an microscope (IMT-2; Olympus, Tokyo, Japan) fitted with a digital camera (Jenoptik Laser, Optik System GmbH, Germany). Images were processed using the ProgRes C14 Imaging software (Jenoptik Laser).

**Electron Microscopy**

B6-RPE07 cells were cultured on Transwell-COL filters (0.4-μm pore size, 6.5-mm diameter; Corning, Fisher Scientific, Loughborough, UK). Two weeks later samples were collected and processed for electron microscopy. In one experiment, cells were also incubated with photoreceptor outer segment tips for 2 hours at 37°C. Samples were briefly washed with PBS and then fixed with 2.5% buffered glutaraldehyde (Fisher Scientific) at 4°C for 48 hours. After thorough washing, the samples were passed through a graded ethanol series, transferred to propylene oxide, and embedded in resin (Epon 812; Ted Pella, Redding, CA). Ultrathin sections were stained with uranyl acetate and lead citrate and were examined using a transmission electron microscope (CM10; Philips, Eindhoven, The Netherlands).

**Immunofluorescence Staining**

Single B6-RPE07 cell suspension (1 × 105) were seeded in 8-chamber slides (Nalge Nunc International, Rochester, NY) and were cultured for 4 days. Confluent B6-RPE07 cells were washed briefly with cold PBS and then fixed with 100% ice-cold methanol (Fisher Scientific) for 10 minutes. After thorough washing, samples were permeabilized with 0.5% (vol/vol) Triton X-100 in Tris-buffered saline (TBS) for 5 minutes at room temperature. Samples were incubated with rabbit anti-ZO1 (1:100 in TBS; Zymed, South San Francisco, CA), goat anti–β-catenin (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti–claudin-1 (1:100; Stratachem Scientific Ltd., Newmarket, Suffolk, UK), rabbit anti–claudin-3 (1:100; Zymed), mouse anti–occludin-1 (1:100; Zymed), rabbit anti–pan cytokeratin (1:50; ICN Pharmaceuticals, Costa Mesa, CA), mouse anti–cytokeratin 19 (1:50; DAKO, Glostrup, Denmark), rabbit polyclonal anti–cytokeratin 12 (1:50; Santa Cruz), rabbit anti–GFAP (1:100, Abcam, Cambridge, UK), rabbit anti–integrin β5 (1:100; Abcam), biotinylated anti-mouse CD34 (1:100; Biolegend, San Diego, CA), or biotinylated anti-mouse CD51 (integrin αv, RMV-7, 1:100; BD Biosciences, Oxford, UK) for 1 hour. After washing, samples were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti–rabbit immunoglobulin (1:200; Zymed), goat anti–mouse immunoglobulin (1:200; Zymed), or donkey anti–sheep/goat immunoglobulin (1:100; Serotec, Oxford, UK), or FITC-conjugated streptavidin (1:200; BD Biosciences) and propidium iodide (PI; 1:200; Molecular Probes, Eugene, OR) for another hour. After thorough washing, the immunofluorescence-stained samples were mounted with mounting medium (Vectorshield; Vector Laboratories, Burlingame, CA) and examined under a fluorescence microscope (LSM510 META; Carl Zeiss, Göttingen, Germany).

To determine the apical Na+/K+ -ATPase and basal bestrophin expression in polarized B6/RPE cells, a single-cell suspension of B6-RPE07 was seeded in 6-mm cell culture inserts (0.4-μm pore size; Corning, Fisher Scientific) in serum-free epithelial medium (Quantin 286; PAA Laboratories Ltd.) for 4 to 5 days. After the cells had reached confluence, insert membranes were fixed in ice-cold 100% methanol for 10 minutes. Membranes were permeabilized with 1% Triton X-100 for 5 minutes and were blocked with 5% BSA for 30 minutes. Rabbit anti-Na+/K+ -ATPase (1:100; Santa Cruz Biotechnology) or rabbit anti-
mouse bestrophin (1:100; Abcam) was added to the membrane and incubated at room temperature for 1 hour, followed by FITC-conjugated goat anti-rabbit immunoglobulin (1:200; Zymed) and PI for another hour. After thorough washing, the membranes were mounted with mounting medium (Vectorshield; Vector Laboratories) and examined by confocal microscopy.

**Phagocytosis of Photoreceptor Outer Segment Tips by B6-RPE07**

Bovine photoreceptor outer segments (POS) were isolated by sucrose gradient centrifugation, as described previously.\(^7\)\(^{-22}\) Isolated POS aliquots were stored at −80°C at a concentration of 10^9/mL. For RPE phagocytosis assay, POS were washed free of sucrose using PBS. POS were then unlabeled or labeled with 5-(4,6-dichlorotriazin-2-yl) amino fluorescein (500 μM/mL; Sigma). B6-RPE07 cells were grown on Transwell-COL filters (Corning; Fisher Scientific) or 24-well plates and were incubated with 1 × 10^4/mL POS for different times. Cells grown on Transwell-COL filters were fixed with 2.5% glutaraldehyde and prepared for transmission electron microscopy. Cells grown in 24-well plates were quenched with 2% trypan blue to exclude extracellularly bound POS and then were collected by 0.25% trypsin-EDTA for flow cytometry.

Cells incubated with labeled POS were analyzed directly by flow cytometry. Positive cells were selected using an anti-rhodopsin antibody (gift from confocal microscopy.

**Permeability Assay**

B6-RPE07 cell barrier function was determined by measuring the apical-to-basolateral movement of sodium fluorescein.\(^43\) A single-cell suspension (1 × 10^4 in 100 μL) was grown on Transwell inserts (0.4-μm pore size, 12-mm diameter; Corning, Fisher Scientific) and placed in 12-well plates, which thus formed an upper and a lower chamber system containing 1 mL complete DMEM. Half the culture medium from the inserts and bottom wells was replaced with the same amount of fresh medium every 2 days. After 4 weeks’ culture, 100 μL of 50 μg/mL sodium fluorescein (Sigma) was added to the upper chamber (inserts), and 100 μL medium was collected from the basolateral chamber at 5, 10, 15, 30, 60, and 180 minutes after the tracer dye was added. At each time point, three wells were used for fluorescein measurement. The fluorescein was quantified by fluorescent light (Fluorolight 100; Dynex Technologies, Worthing, UK) with computer software (Biolinx; Dynex Technologies). Human retinal pigment epithelial cell line ARPE-19 was used as a positive control. Inserts without cells served as negative controls.

**Western Blotting**

Total cell protein was isolated from confluent B6-RPE07 cells or primary cultured RPE cells of a C57BL/6 mouse using cell lysis buffer (50 mM Tris-HCl, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA). Twenty micrograms of protein was loaded on the minigel (NuPAGE; Invitrogen, Paisley, UK) according to the manufacturer’s instructions and was transferred electrophoretically to nitrocellulose membranes (Invitrogen). After overnight blocking with 5% fat-free milk in TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4), the membranes were incubated with the following primary antibodies: monoclonal mouse anti-opsin (1:2000, Sigma), rabbit anti-mouse cellular retinoid-binding protein (CRBP; 1:200; Floi135; Santa Cruz Biotechnology), rabbit anti-mouse cellular retinaldehyde-binding protein (CRALBP; 1:2000; Abcam), mouse anti-RPE65 (1:5000; Novus Biologicals, Littleton, CO), rabbit anti-mouse hBest (1:500; Abcam), rabbit anti–occludin (1:500; Zymed), and rabbit anti–claudin-1 (1:500; Stratex Scientific Ltd.). Secondary antibodies used were horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin or HRP-conjugated goat anti-rabbit immunoglobulin, secondary antibody (1:2000; DAKO). Membranes were visualized with enhanced chemiluminescence (ECL Plus Detection Kit; Invitrogen).

**RT-PCR for RPE65 and Cellular Retinaldehyde-Binding Protein Expression**

Cells were harvested from confluent B6-RPE07 cultures, and total RNA was isolated (RNeasy Mini Kit; Qiagen Ltd., West Sussex, UK). Complementary DNA (cDNA) was synthesized in a reaction consisting of 5 μg total RNA, 1 μg oligo-dT primer (Thermo Electron GmbH, Ulm, Germany), 5 mM nucleotide mixture (Roche Diagnostics, Mannheim, Germany), 20 mM dithiothreitol, and 200 U reverse transcriptase (Invitrogen).

Each PCR was carried out in a 25-μL volume with 1 μL synthesized cDNA, 200 μM each nucleotide, 1 μM each primer, 0.125 U Taq DNA polymerase (Promega, Southampton, UK), and 2 mM MgCl2 in 10× thermostable DNA polymerase buffer (Promega). DNA was denatured at 94°C, annealed at 55°C, and extended at 72°C, with a total of 35 reaction cycles. Amplified PCR products were electrophoresed in a 1.8% agarose gel and photographed under ultraviolet light. Amplification of housekeeping gene β-actin was used as a positive control, and water-replaced cDNA was used as a negative control. Primer sequences were as follows: RPE65 forward primer 5'-ATGATCGAGAAGAGGATTGTC-3' and reverse primer 5'-CTGCTTTTCACTGGAGGATCATC-3'; CRALBP forward primer 5'-CAAGAGGGCAGTATGTCACAGC-3' and reverse primer 5'-GAAGAGTTCAAGTACTGGAAGCT-3'; β-actin forward primer 5'-CTCTTGTGGCGGTCACAC-3' and reverse primer 5'-ACACCTTGTTGCCATAGG-3'.

**Cytokine/Chemokine Measurement**

B6-RPE07 cells were cultured in 24-well plates. After they reached confluence, cells were washed with PBS and were replaced with fresh culture medium (with or without 10% fetal calf serum). After 24-hour incubation, supernatants were collected, and total protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA). Cytokines TNF-α, IFN-γ, IL-4, IL-6, IL-10, IL-12, and chemokine MCP-1 were measured (FACS Cytometric Bead Array kit; BD Biosciences). Cytokine/chemokine levels in the supernatant were expressed as picogram cytokine per microgram total protein.

**RESULTS**

**Morphology of B6-RPE07**

Primary cultures of the mouse RPE cells used in this study grew as an irregularly shaped monolayer with areas of dense pigmentation (Fig. 1A). Some fibroblastic cells were also seen in the cultures. After a few successive subcultures using differential transmittance-EDTA purifications, the cultures were highly epithelial in shape and contained no visible melanin (Fig. 1B). When they reached confluence, the cultures displayed a typical “cobblestone” morphology (Fig. 1B). Subcloned B6-RPE07 cells maintained the cobblestone morphology (Fig. 1C).

Electron microscopy of B6-RPE07 cells cultured on Transwell-COL filters revealed many features of RPE morphology, including apical microvilli (Figs. 2A, 2B), numerous dense bodies in the cytoplasm (Fig. 2C), and apical electron-dense structures (indicative of apical tight junctions; Fig. 2D). However, cells cultured in normal culture medium (10% FCS in DMEM) tended to overlap each other and to have epithelial characteristics that were less polarized (Fig. 2A) than cells cultured in serum-free epithelial medium (Quantin 286; Fig. 2E).

**Cell Growth Characteristics**

In normal culture conditions (10% FCS in DMEM, in 5% CO₂ at 37°C), after passage, B6-RPE07 cells had a 1.4-day delay in...
growth (Fig. 3), representing the recovery time of the cells because of the “passage insult.” After that delay, cells went through a rapid growth phase with a maximum growth rate of $1 \times 10^5$ cells/mL/day (Fig. 3). Cells reached a plateau phase by day 5 (Fig. 3), indicating a contact (density)-inhibition growth characteristic of B6-RPE07 cells.

To investigate whether B6-RPE07 cells were tumorigenic, we cultured the cells in soft agar to test their anchorage-independent growth properties. No cell clone was observed after 3 weeks' culture, suggesting that B6-RPE07 cells do not have anchorage-independent growth ability.

**Phenotype of B6-RPE07 Cells**

Cytokeratin intermediate filaments are epithelial-specific markers. Immunostaining of B6-RPE07 cells (passage [P] 12 and P60) showed that pan-cytokeratin was highly expressed in the cytoplasm of all B6-RPE07 cells, with denser expression in the perinuclear region (Fig. 4A). B6-RPE07 cells also expressed a certain degree of cytokeratin 19 (Fig. 4B) but no cytokeratin 12 (Fig. 4C). Isotype control staining revealed no positive signals (Fig. 4D). The barrier function of RPE cells is considered highly dependent on the integrity of specialized tight junctions. Immunostaining of B6-RPE07 cells with various tight junction proteins, including claudin-1 (Fig. 4G), claudin-3 (Fig. 4H), and occludin-1 (Fig. 4I), were not detected in B6-RPE07 cells by confocal microscopy, even when cells were cultured in serum-free epithelial medium (Quantin 286). B6-RPE07 cells were negative for GFAP (Fig. 4J). When B6-RPE07 cells were cultured on insert membranes in serum-free epithelial medium (Quantin 286), an epithelial polarization marker, Na$^+/K^+$-ATPase, was detected at the apical and lateral junction areas of B6-RPE07 cells (Fig. 4K). The tight junction protein ZO-1, however, was detected mainly at the apical junctions under these conditions (Fig. 4L). This observation is in agreement with previous studies in rat46 and chick29 RPE cells.

**Function of B6-RPE Cells**

**Phagocytosis of Bovine POS by B6-RPE07 Cells.** Daily phagocytosis of the shed tips of renewing POS tips is one of the main functions of RPE cells in vivo. Exposure of the B6-RPE07 cultures to isolated bovine POS indicated that cells from early passages (P12) and late passages (P60–P80) were equally able to ingest POS (Figs. 6A, 6B). Electron microscopic examination revealed many phagosomes in B6-RPE07 cells fed with POS (Fig. 6C). A time-course study indicated that ingested POS were digested by B6-RPE07 cells over 3 to 5 days (Fig. 6D).

Because the cell surface scavenger receptor CD36 and integrin $\alpha_v\beta_3$ are involved in RPE phagocytosis,47,48 we examined the expression of these molecules in B6-RPE07 cells. Confocal microscopy revealed a certain level of CD36, integrin $\alpha_v$ (CD51), and $\beta_3$ on B6-RPE07 cells cultured in DMEM/F12...
medium or serum-free epithelial medium (Quantium 286) but not on cells cultured in normal DMEM (Figs. 6E–G). Isotype control staining showed no positive signals (Fig. 6H).

**Blood-Retina Barrier Function.** The functional barrier properties of the RPE cells grown to confluence on Transwell inserts were evaluated by measuring the apical to basolateral permeability of sodium fluorescein. During the 180-minute sodium fluorescein incubation, little sodium fluorescein moved through the RPE monolayer into the bottom well compared with negative controls (Fig. 7), indicating that the B6-RPE07 cell monolayer maintained a certain barrier function. The barrier function of the B6-RPE07 cells, however, was not as strong as the ARPE19 cells under the same culture conditions (Fig. 7). The lower barrier function of B6-RPE07 cells correlated with the incomplete expression of tight junction proteins (Figs. 4E, 4F). These results suggested that B6-RPE07 cells maintain rudimentary tight junctions under normal culture conditions.

**Cytokine/Chemokine Secretion by B6-RPE07 Cells**

RPE cells are able to secrete various cytokines and chemokines involved in retinal/choroidal homeostasis or disease. In normal culture conditions, B6-RPE07 cells secreted a large amount of MCP-1, and this production was further enhanced when cells were cultured in serum-free medium (Fig. 8). In addition, IL-6 was also secreted at a detectable level in B6-RPE07 cultures (Fig. 8).

**FIGURE 4.** Immunocytochemistry of B6-RPE07 cells. (A–J) B6-RPE cells were cultured in eight-well slides and stained for pan cytokeratin (A), cytokeratin 19 (B), cytokeratin 12 (C), rabbit immunoglobulin (D), ZO-1 (E), β-catenin (F), claudin-1 (G), claudin-3 (H), occludin-1 (I), GFAP (J), and PI and were examined by confocal microscopy. (K, L) B6-RPE07 cells were cultured in serum-free epithelial medium on Transwell membrane and stained for Na+/K+/ATPase (K) or ZO-1 (L) and PI and were observed by confocal microscopy. Na+/K+/ATPase was stained positive in the perijunctional region of the lateral and apical membranes (K, arrows), whereas ZO-1 was detected mainly in the apical junction areas (L, arrows). Images in A–J are reconstructions of a series of Z-stack images. In all images, red indicates nuclei stained with PI, and green indicates specific antigens. Scale bars: 20 μm (A–J); 10 μm (K, L).

**FIGURE 5.** Expression of visual cycle proteins in B6-RPE07 cells. (A) mRNA was extracted from B6-RPE07 cells. RPE65 and CRALBP gene expression was examined by RT-PCR. A single band of (397 bp) CRALBP was detected in B6-RPE07 cells. No clear RPE65 band was detected. Results represent three independent experiments. M, DNA ladder marker. (B–D) Western blot of CRALBP (B), RPE65 (C), and CRBP-1 (D) in B6-RPE07 cells (lane 1) and primary cultures of mouse RPE cell (lane 2). (E, F) Expression of bestrophin in B6-RPE07 cells. (E) B6-RPE07 cells were cultured in serum-free epithelial medium on Transwell membrane and stained for bestrophin. Z-stack confocal image shows the basal expression of bestrophin (arrow) in B6-RPE07 cells. (F) Western blot shows the expression bestrophin in B6-RPE07 cells (lane 1) and primary cultures of mouse RPE cell (lane 2). Scale bar, 20 μm (E).
**DISCUSSION**

Increasing evidence suggests that RPE cells are actively involved in retinal inflammation and degeneration. They do so not only by maintaining retinal homeostasis but more so by active secretion of soluble factors, including cytokines/chemokines and complement components. It is believed that many more novel biologic functions of RPE cells critical for the development of retinal diseases are yet to be revealed, and the use of RPE cultures is an essential tool in developing our understanding of these processes. Primary culture of RPE cells from various species is not technically difficult and is widely used in many studies. Although primary cultures of RPE cells have many similarities in their in vivo conditions, both in morphology and in phenotype, they also pose the problems of limited availability and heterogeneity. The heterogeneities may be attributed to the ages of the donors, the techniques used for RPE culture, and the durations of subculture; therefore, stabilized continuous cultures of RPE cells would greatly assist in reducing these variables. Several RPE cell lines have been reported in the literature, either of human or rat origin, such as human cell lines ARPE-19 and D407, rat cell lines RPE-J and BPE-1. These cell lines have been used broadly in the investigation of outer blood-retina barrier function, tight junction assembly, and RPE cell transplantation. In this study we

**FIGURE 6.** Phagocytosis of POS by B6-RPE07 cells. (A, B) B6-RPE07 cells were cultured in 24-well plates and fed with 1 × 10⁷/mL FITC-labeled POS. Sixteen hours later, cells were quenched with trypan blue, and single-cell suspensions were prepared. Samples were then (A) cytopun for confocal microscopy or (B) prepared for flow cytometry. (A) Reconstructed z-stack images show intracellular locations of POS. (B) Mean fluorescence intensity (MF) of POS in early-passage cells (P12) and late-passage cells (P60). (C) B6-RPE07 cells were cultured on Transwell-COL filters and fed with 1 × 10⁷/mL POS. Two hours later samples were prepared for TEM. Arrows: phagosomes with POS. (D) B6-RPE07 cells were cultured in 24-well plates and fed with 1 × 10⁷/mL of unconjugated POS for 2 hours. At different time points, cells were collected, and single-cell suspensions were prepared. Samples were then stained for rhodopsin and analyzed by flow cytometry. N = 3, *P < 0.05, **P < 0.01 compared with control non-POS-fed cells. Data represent two independent experiments. (E–H) B6-RPE07 cells were cultured in eight-well slides and stained for CD36 (E), integrin αv (F), integrin β3 (G), and rabbit immunoglobulin (H) and were observed by confocal microscopy. Images shown are reconstructions of a series of z-stack images.

**FIGURE 7.** Apical-to-basolateral paracellular permeability of B6-RPE07 cells. 1 × 10⁴ B6-RPE07 cells were cultured on Transwell-COL filters. Four weeks later, 100 µL of 50 µg/mL sodium fluorescein was added to the inserts, and 140 µL medium was collected from the basolateral chamber at different time points. The fluorescein was quantified. N = 3, *P < 0.001 compared with control non–cell-cultured wells. †P < 0.05 compared with ARPE19 cells. Data represent one of three repeated experiments.

**FIGURE 8.** B6-RPE07 cytokine/chemokine production. B6-RPE07 cells were cultured in 24-well plates. After confluence, cells were washed with PBS and replaced with fresh medium with or without 10% FCS. After another 24-hour culture, supernatants were collected and analyzed by CBA. N = 3, *P < 0.01 compared with CCL2 level produced by cells cultured in the presence of 10% FCS. Data represent two independent experiments.
have developed and characterized a mouse RPE cell line, B6-RPE07, which arose spontaneously from an 8-week-old C57BL/6 mouse RPE culture, and we have subsequently cloned this line by limiting dilution.

Characterization of the B6-RPE07 cell line shows that the cell line expresses specific cytokeratins, has a typical cobblestone morphology, forms a certain degree of intercellular tight junctions (rudimentary tight junctions) under normal culture conditions, adopts a polarized morphology in 3D culture in serum-free epithelial cell culture medium, and expresses Na+/K+-ATPase on the lateral and apical junction areas and bestrophlin in the basal membrane region once polarized, all which indicate an epithelial phenotype. B6-RPE cells also express CRALBP, which is only expressed in RPE cells and Müller cells in the eye. However, B6-RPE07 cells do not express GFAP, indicating that they are not of glial (Müller) cell origin. These results suggest that B6-RPE cells are of RPE origin. In vivo, RPE cells are interdigitated with photoreceptor outer segments in the interphotoreceptor matrix. RPE cells characteristically contain electron-dense particles such as melanin granules, phagosomes, and lipofuscin granules. Melanin was observed in primary cultures of B6-RPE cells but not in subcloned B6-RPE07 cells. However, electron microscopy study revealed dense bodies similar to pre-melanosomes in B6-RPE07 cells (Fig. 2C). Lack of melanin has been reported in many other established RPE cell lines, including human RPE cell lines ARPE1937 and ARPE1937v. Whether melanin could be induced in B6-RPE cells by altering the culture conditions, as has been reported in a rat RPE cell line, BPE1, requires further investigation.

Phagocytosis of POS is an essential function of RPE cells in physiological conditions. Phagocytosis is mediated by specific receptors, including αvβ3, αvβ5, CD36, and the mannose receptor. Phagocytosed POS tips form phagosomes and ultimately are digested by RPE cells. B6-RPE07 cells express CD36 and integrin αvβ6 and are able to bind and phagocytose POS. This function of B6-RPE07 cells does not change after prolonged (over 12 months) in vitro culture. Phagocytosed POS formed phagosomes in B6-RPE07 cells and were digested in 3 longed (over 12 months) in vitro culture. Phagocytosed POS in B6-RPE07 cells and were digested in 3 months.

Under normal culture conditions, B6-RPE07 cells constitutively produce high levels of CCL2, similar to primary cultured RPE cells. CCL2 production was further enhanced when B6-RPE07 cells were cultured in the absence of serum. CCL2 is a chemokine important for monocyte/macrophage migration. During inflammation, increased levels of CCL2 at the site of inflammation direct specific monocyte subsets to migrate. Constitutive expression of CCL2 by RPE cells indicates that this chemokine may play an important role in retinal homeostasis. Mice deficient in CCL2 or its cognate receptor, CCR2, develop retinal changes similar to those of human age-related macular degeneration. Although the exact link between the CCL2/CCR2 pathway and AMD pathogenesis remains to be elucidated, the observation suggests that in the absence of CCL2/CCR2, retinal homeostasis is disturbed during aging. This stabilized mouse RPE cell line (B6-RPE07) could thus be of great value for the studies of retinal inflammatory diseases, including uveoretinitis and macular degeneration, as well as for studies of RPE physiology such as retinoid metabolism.

To the best of our knowledge, B6-RPE07 is the first characterized mouse RPE cell line. It has many features of in vivo mouse RPE cells, in particular its ability to produce various cytokines and chemokines. Using the same mouse RPE cell line, we showed previously that complement factor H is produced at high levels in normal culture conditions. Because of the wide availability of retinal disease mouse models, particularly models of retinal degeneration, and the advanced in vitro gene modification technique, we believe that B6-RPE07 will serve as an invaluable tool in dissecting the immunobiological functions of RPE cells and their possible contributions to various ocular diseases. B6-RPE07 cells will have great value in experimental studies, particularly in vivo experimental treatment studies including RPE cell transplantation.

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References


