Effects of Culture Conditions on Heterogeneity and the Apical Junctional Complex of the ARPE-19 Cell Line

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PURPOSE. ARPE-19 is a spontaneously transformed cell line of human RPE that is widely studied. This report examines its suitability for studying the tight junctions of the RPE.

METHODS. ARPE-19 was maintained in standard medium or one of three reduced-serum medium formulations. The expression and distribution of cytoskeletal and junctional proteins were examined by immunocytochemistry, immunoblot analysis, and the reverse transcription–polymerase chain reaction. Barrier function was measured as the transepithelial electrical resistance (TER) and the transmonolayer diffusion of horseradish peroxidase (HRP).

RESULTS. Unlike the original reports using passage-15 to -20 cells, commonly available strains of ARPE-19 exhibited a heterogeneous mixture of elongate and polygonal cells. Actin was distributed in stress fibers rather than circumferential bands. The TER was low, and the permeability of HRP was high. The expression of claudins and cytokeratins was heterogeneous. Partial differentiation could be induced in subsets of cells by manipulating the growth medium. A common effect was an increase in the expression of JAM-A, AF-6, and PAR-3 that correlated with a redistribution of actin filaments. This effect was accompanied by a 10X decrease in the permeability of HRP, but a minimal effect on TER.

CONCLUSIONS. The properties of ARPE-19 appear to be changing in ways that may depend on how the cells are maintained and passaged. Caution should be exercised in comparing data between laboratories and in interpreting studies in which only a subset of cells may respond to experimental stimuli. Specialized media promoted the maturation of the adherens junction, but only a partial maturation of the tight junctions.

No cultured cell perfectly models the tissue from which it was derived, simply because cells need to interact with their environment to maintain a native phenotype. Adaptation to a new environment drives phenotypic alterations. One of the most difficult properties to retain in epithelial cell culture is the barrier function performed by tight junctions. Tight junctions encircle each cell of the monolayer to form an occluding seal between neighbors that retards diffusion across the paracellular spaces. The properties of tight junctions vary among epithelia, as evidenced by differences in the transepithelial electrical resistance (TER). Relatively few culture models exhibit an in vivo–like TER, but the transformed cell lines that retain tight junctions have been invaluable in understanding this extraordinarily complex structure. For the retinal pigment epithelium (RPE), several primary and secondary culture models have been devised that preserve the function of tight junctions, but the best of these are based on human fetal RPE, or animal models in which the tissue is scarce. To study RPE tight junctions, it would be valuable to have a transformed cell line of RPE that retains barrier function.

The ARPE-19 cell line is a spontaneously transformed line of human RPE that has a normal appearing karyotype. The original reports demonstrated that with time in culture, ARPE-19 adopted the cobblestone appearance of a simple epithelial monolayer. Under special growth conditions, ARPE-19 exhibited a low TER and a polarized secretion of FGF. To establish a high-TER cell line, these investigators attempted to subclone cells from ARPE-19. Unfortunately, they were unsuccessful, because the clones rapidly senesced. The low TER and inability to subclone the cells suggests a mixture of similar, but distinct, cellular phenotypes that work synergistically to maintain the culture. Such microheterogeneity may change with continued passage in culture in a way that depends on culture conditions. If so, this would add to the difficulty of comparing data among laboratories.

To improve the functionality of ARPE-19 tight junctions, we examined how culture conditions affect the protein composition of the apical junctional complex. This complex forms a circumferential band that joins the neighboring cells of a monolayer together. Besides a role in signal transduction, the adherens junction portion of the complex provides mechanical strength, whereas the tight junction portion retards diffusion across the paracellular spaces of the monolayer. Commonly, ZO-1 and occludin are used to establish the presence of tight junctions. These are important, but insufficient, criteria. ZO-1 is found in all cells where it participates in different types of cell junctions. Cogent examples are the ependyma and RPE in the early phase of development. In each case, ZO-1 is found in an apical junctional complex that lacks tight junctions. Similarly, occludin is expressed in some cells that lack tight junctions, such as primordial RPE cells and the precursors of astrocytes and neurons in culture. In these circumstances, occludin may function as a regulator of the TGF-β receptor. In cultures of chick RPE, zonulae rings of ZO-1 and occludin are evident, even when the tight junctions are discontinuous and therefore, nonfunctional. Therefore, we examined additional proteins of the complex. Claudins are a family of transmembrane proteins that help determine the selectivity of the paracellular pathway. JAMA (formerly JANM-1) and its ligands, AF-6 and PAR-3, are involved in the assembly of the apical junctional complex.
We observed increased heterogeneity in the morphology of currently available passages of ARPE-19 (passages 21–25), from three independent sources. Accordingly, we also examined the expression of several cytoskeletal proteins including actin and cytokeratin under various culture conditions. We found that we were able to advance the maturation of the apical junctional complex, but the expression of claudins, cytokeratins, and melanin was heterogeneous, and the increase in TER was modest.

**MATERIALS AND METHODS**

**Cell Culture**

ARPE-19 cells were obtained from the American Type Culture Collection (passage 22; Manassas, VA) or were gifts of Colin Barnstable (passage 24; Yale University) or Nancy Philp (passage 21; Thomas Jefferson University, Philadelphia, PA). The cells were used at passages 24 to 26 and were maintained in tissue culture flasks in DMEM-F12 (Invitrogen, Carlsbad, CA) containing 2% fetal bovine serum (FBS; Invitrogen) until they were postconfluence for several weeks. Then, the cells were harvested and plated in DMEM-F12, 2% FBS on glass coverslips or laminin-coated clear filters (Transwell; Fisher Scientific, Hampton, NH) at a density of 1.8 × 10⁵ cells/cm². The filters subdivide the culture dish into two medium chambers to enable studies of transmonolayer permeability. Electrodes may be placed in the apical and basal chambers to measure the TER, or a tracer may be placed in the apical chamber to measure its diffusion into the basal chamber. The cultures rapidly became confluent. At 4 days after confluence, the culture medium was changed to the desired medium: (1) DMEM-F12 was supplemented with 1% FBS. This medium yielded cultures with native morphology in earlier studies.5 (2) Triple medium (a 1:1:1 mix of Ham F12, MCBD 131, and Medium 199 [Invitrogen]) was supplemented with ITS⁻, an insulin-transferrin-selenium-BSA–linoleic acid mixture (BD Biosciences, Bedford, MA); 1-glutamate; and 0.5% to 5% FBS. Triple medium was formulated with the purpose of establishing RPE-endothelial cell cocultures. (3) SF3 medium was a serum-free medium supplemented with ITS⁻ for cultures of chick RPE.4 (4) JTT medium is a low-serum medium, supplemented with ITS⁻ and 0.2% FBS that promoted the expression of RPE-specific proteins and was generously supplied by Joyce Tombran-Tink (University of Missouri, Kansas City, MO). Culture media were changed weekly.

**Assays of Junctional Permeability**

The permeability of cultures maintained on filters was measured in triplicate as the TER and the permeation of horseradish peroxidase (HRP). The TER was measured at 33°C (Endohm electrodes; World Precision Instruments, Sarasota, FL). This temperature was easier to maintain than 37°C and avoided the phase transition that occurs in tight junctions at ambient temperatures.23 Measurements were made in a modified SF3 in which the bicarbonate was replaced with 20 mM HEPES (pH 7.2).4 The resistance of filter was subtracted and the measurement reported as ohms × square centimeter.

For assays of HRP permeability, transcytosis was blocked by the inclusion of 10 mM ammonium chloride. Cultures were precubicated in culture medium containing 50 µg/ml HRP (type VI HRP; Sigma-Aldrich, St. Louis, MO) to both media chambers for 1 hour. The basal side of the filter was quickly rinsed six times in culture medium that lacked HRP. The appearance of the apical HRP in the basal chamber was monitored by taking two 5-µL aliquots at 30, 60, and 90 minutes. The 5-µL aliquots were assayed by adding freshly prepared 195-µL substrate, which contained 400 µg/ml o-phenylenediamine (Sigma-Aldrich), 0.012% H₂O₂, 0.05 M citric acid, and 0.1 M phosphate (pH 5.0). After a 15-minute incubation at room temperature, the reaction was terminated by 50 µL of 0.3 M H₂SO₄. The absorbance at 492 nm was determined with a microplate reader (Bio-Rad Laboratories, Hercules, CA). Concentrations were calculated from a standard curve. Permeation was reported as microliters per square centimeter.23

**Qualitative and Semiquantitative Characterization of Tight Junction Gene Expression**

Total RNA was isolated using the RNasy Protect kit (Qiagen, Valencia, CA), according to the manufacturer’s protocols. RNA was treated with DNase I (Qiagen) to remove any contaminating genomic DNA and reverse transcribed to cDNA (Script cDNA Synthesis Kit; Bio-Rad Laboratories). For nonquantitative studies, the polymerase chain reaction (30 cycles) was used to amplify the cDNA of interest, which was visualized on ethidium bromide gels. For quantitative measurements, real-time RT-PCR was performed (SYBR Green PCR Master Mix; Bio-Rad) in a 96-well optical reaction plate (Bio-Rad Laboratories). For each primer pair, a standard curve was generated using cloned cDNA. Data from multiple samples were normalized using ribosomal RNA (18S) primers and competitors (Ambion, Austin, TX), according to the manufacturer’s instructions. Table 1 indicates the primers that were used.

**Immunofluorescence**

The subcellular distribution of tight junctional proteins was determined by indirect immunofluorescence. The cultures were fixed with 2% paraformaldehyde in PBS for 20 minutes at room temperature or in 100% ethanol at 4°C for 30 minutes and labeled as described previously.4,18 Rabbit polyclonal antibodies against claudin-1, AF-6, and PAR-3 and mouse monoclonal antibodies against ZO-1, cytokeratin 7, cytokeratin 18, and β-tubulin were purchased from Zymed (South San Francisco, CA). Mouse monoclonal antibody against JAM-A (BV16, IgG1) was a gift of Elisabetta Dejana (Milano, Italy). ML-grade secondary antibodies (specifically prepared for multiple-label protocols) conjugated with HRP, Cy2, and Cy3 dyes were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Nuclei were labeled with DAPI, and actin filaments were revealed with phalloidin-Bodipy 581/591 (Invitrogen). Fluorescence images were acquired with a fluorescence microscope (Axioskop; Carl Zeiss, Inc., Thornwood, NY), or a confocal microscope (FluoView 500; Olympus, Melville, NY).

**Immunoblot Analysis**

The cultures were washed with cold PBS, lysed with ice cold lysis buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 300 mM dithiothreitol [DTT], 0.001% bromphenol blue) containing a protease inhibitor cocktail (Halt; Pierce, Rockford, IL), and immediately frozen in liquid nitrogen. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford MA). Immunoblots were developed using HRP-conjugated secondary antibodies and chemiluminescence reagent (ECL; GE Healthcare, Arlington Heights, IL). The monoclonal antibody to cytokeratin 7, OV-TL 12/30 (Zymed), yielded three bands on immunoblot, as described previously.23

**RESULTS**

**Heterogeneity of the Cultures**

Each strain of ARPE-19 tested (passages 24–26) lacked the cobblestone morphology originally reported for ARPE-19 (passages 15–20).5 Each strain displayed cords of elongate cells intermixed with spread, polygonal cells when cultured under standard conditions of DMEM-F12 medium supplemented with 1% to 10% fetal calf serum (Fig. 1). Polygonal cells were favored when cells were plated at high density, but after a week in culture many elongate cells were observed. A range of microscopic fields can be appreciated by examining the DMEM-F12 images in Figures 1, 3–6, 8, and 9. A more polygonal morphological could be recovered if confluent cultures were switched from DMEM-F12 to one of the other media tested, fed only once per week, and cultured for several months. Low or no
A subset of cells by 6 months (Fig. 3). Many cells in the bright-field image were not seen, because they lack pigment, as demonstrated by staining the cells for actin or ZO-1. The ZO-1 and circumferential bundles of actin revealed a more orderly, polygonal array than the cultures at 2 weeks. By contrast, cultures maintained in DMEM-F12 with 1% FBS, exhibited little melanin, actin was assembled in stress fibers, and cell morphology was irregular.

Long-term cultures maintained in SF3 comprised squamous and low cuboidal cells. The cuboidal cells were densely packed with melanin granules and exhibited microvilli. Confocal images were focused on the apical junctional complex. These were found in two different focal planes. The more apical focal plane captured the apical junctional complex of the taller cells. The cell borders, ZO-1 colocalized with circumferential bands of actin, as evidenced by the yellow signal. Punctate actin suggested the presence of microvilli (Fig. 4A). When focused more basally, the junctional complexes of the neighboring squamous cells were evident. Besides the junctions, actin was evident in the cytoplasm of the squamous cells, but the cytoplasm of the taller cells was darkened by the densely packed melanin granules. A reconstruction of the x-z plane (Fig. 4B) demonstrated that in the taller cells, rich with melanin, ZO-1 was confined to the apical portion of the lateral membrane. Actin was evident in this apical junctional complex (rendering the junctions yellow) and in the position of microvilli along the apical membrane. In the squamous portions of the monolayer, ZO-1 was observed along the entire lateral border and along the basal membrane.

By contrast, long-term cultures maintained in DMEM-F12 were composed entirely of squamous cells. Actin and ZO-1 failed to colocalize, as evidenced by the green signal at the cell borders. The ZO-1 was aligned along tortuous cell borders, as opposed to the straight borders observed in SF3. Actin was mostly arranged in stress fibers that were most in focus in the basal focal plane. The XZ-plane revealed a squamous monolayer, with no evidence of microvilli.

### Table 1. Primers for PCR

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<th>Gene</th>
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<th>Downstream</th>
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The Response of Actin Cytoskeleton and Barrier Function to Culture Conditions

The barrier properties of the cultures were measured as the TER and the transmonolayer diffusion of HRP. Two weeks after confluence, the TER was only 20 to 25 Ω-cm² (Table 2), which was little more than the TER of a layer of fibroblasts (~10 Ω-cm²). Under the most favorable growth conditions (SF3 or JTT medium), the TER increased to as much as 30 to 45 Ω-cm² (at 33°C) after 6 weeks to 6 months in culture. In contrast to the TER, a roughly 10× decrease in the permeability to HRP was observed after 6 weeks in culture (Table 2). Permeability to HRP is a much less stringent measure of barrier function that can be achieved with partially formed junctions.19 Again, this change in permeability failed to occur in DMEM-F12. A common feature of the low-serum specialized medium was the inclusion of a commercial mixture of insulin, transferrin, and selenium (ITS⁺). When this mixture was added to DMEM-F12 with 1% FBS, the permeability to HRP decreased in line with the other media (P<sub>HRP</sub> = 0.0002 μL/cm²·s). This treatment had no effect on TER, but did cause a rearrangement of actin filaments, as discussed later.

To understand the low functionality of the junctions, we first looked at three proteins involved in the assembly of the apical junctional complex, JAM-A, AF-6, and PAR-3, and at actin filaments whose subcellular distribution is affected by a properly assembled junction. The actin filaments of ARPE-19 maintained in DMEM-F12 were arranged in stress fibers that are more typical of fibroblasts than epithelia. These filaments gradually rearranged into the circumferential bands common to epithelia when the culture medium was replaced. ARPE-19 was examined after 2 and 6 weeks in the culture medium described in the legend to Figure 5. Low-magnification fields were selected to show cords of elongate cells running through areas of polygonal cells. After 2 weeks in culture, actin filaments were still arranged in stress fibers that interconnected many cells. The high-magnification views shown for triple medium are representative of cells maintained in the other media. When viewed at high magnification, the borders between the cells, as revealed by ZO-1, were jagged and tortuous.

After 6 weeks in culture, the actin cytoskeleton appeared unchanged in cells maintained in DMEM-F12 medium. By contrast, actin was rearranged in the other media. Stress fibers were still present, but circumferential bands of actin became evident at the cell borders. In addition, ZO-1 at the cell borders appeared more linear. Despite these improvements, there were still many elongate cells, and the TER remained low. The trend toward more actin distributed to circumferential bands continued over 6 months in culture in non-DMEM-F12 media (see Figs. 4, 5, 9). In DMEM-F12, much of the actin remained in stress fibers, and high magnification views showed that the lateral membranes, revealed by ZO-1 staining, was more tortuous than in the other media (see also occludin and claudin, Fig. 9).

The mislocalized actin filaments and elongate cells suggested that the apical junctional complex was not properly assembled. Therefore, we examined proteins, besides ZO-1, that help assemble junctions and could help recruit actin to the complex. JAM-A is necessary for assembly of junctions and links to actin via the adaptor proteins ZO-1 and AF-6. JAM-A also recruits the adaptor protein PAR-3 to the maturing tight junction.22,23 A signal for AF-6 and JAM-A was detected at cell borders in each culture condition in postconfluence cells (Fig. 6). By contrast, PAR-3 was not recruited to the junctions before 6 weeks in culture and only in cultures that rearranged actin filaments.

To determine whether the steady state levels of the mRNAs for these proteins increased, total RNA was isolated from postconfluent cells. In DMEM-F12, the amount of mRNA was stable between 3 and 6 weeks for AF-6, JAM-A, and PAR-3 (Fig. 7). By contrast, these mRNAs gradually increased in JTT medium. A check of SF3 medium at 6 weeks revealed a similar increase. As noted earlier and in Table 2, the supplementation of DMEM-F12 with 1% FBS with ITS⁺ decreased the permeation of HRP with no effect on TER. Under these growth conditions, there was an increase in the mRNAs for AF-6, JAM-A, and PAR-3 and a concurrent rearrangement of actin fibers (Figs. 7, 8).

Effect of Culture Conditions on the Expression of Occludin and the Claudins

The expression of occludin and claudin-1 and -2 was examined in long-term cultures. ARPE-19 was maintained in DMEM-F12, JTT, or SF3 medium for 3 months. At high magnification, the lateral borders revealed by ZO-1 were most tortuous and the
cell shape most irregular in DMEM-F12 (Fig. 9). The same cells were colabeled to reveal actin filaments. Circumferential bands of actin that colocalize with ZO-1 were most evident in JTT or SF3 media.

Occludin is an important regulator of tight junctions.\textsuperscript{10,21} Like ZO-1, occludin was expressed at cell borders uniformly across the culture. The intensity of occludin staining was unexpectedly diminished in culture medium that favored a polygonal morphology, melanization and circumferential bands of actin (Fig. 9). The TER was highest in SF3 where occludin expression was weakest. These cells were colabeled to reveal claudin-1. Unlike occludin, claudin-1 expression was variable across the culture. Notably, the expression of claudin-1 at cell borders was lowest in SF3 and highest in DMEM-F12 with 1% FBS. There was no apparent correlation between cell shape and the expression of cytokeratin-7. Bar, 50 μm. (B) Protein extracts of 2- and 6-week cultures were analyzed by immunoblot. The three bands for cytokeratin-7 were expected for this monoclonal antibody.\textsuperscript{26} β-Tubulin was used as a loading control.

### Figure 2.

The expression of cytokeratins was heterogenous in all culture media. (A) Cells were cultured as indicated and stained with DAPI (nuclei, blue) and either antibodies to cytokeratin-7 or cytokeratin-18 (green). More than 95% of the cells were positive for cytokeratin-18, but only 40% to 50% of the cells were positive for cytokeratin-7. These results were unaffected by time in culture or culture medium, but the distribution of the cytokeratins was more diffuse in SF3 than in DMEM-F12 with 1% FBS. There was no apparent correlation between cell shape and the expression of cytokeratin-7. Bar, 50 μm. (B) Protein extracts of 2- and 6-week cultures were analyzed by immunoblot. The three bands for cytokeratin-7 were expected for this monoclonal antibody.\textsuperscript{26} β-Tubulin was used as a loading control.

### Figure 3.

In SF3 medium, prolonged time in culture promoted melanization and a polygonal morphology. ARPE-19 were maintained in SF3 or DMEM-F12 with 1% FBS for 6 months and viewed using bright-field or fluorescence optics, as indicated. Bright-field images indicated the presence of melanin in a subset of cells, but only in the SF3 cultures. Staining of actin and ZO-1 revealed an organized, cobblestone morphology in SF3, but a more disordered monolayer in DMEM-F12. Bar, 50 μm.
FIGURE 4. In SF3 medium, prolonged time in culture promoted a cuboidal morphology in melanin-rich cells. (A) ARPE-19 was maintained, as described in Figure 3 and double labeled for ZO-1 (green) and actin (red). Where filamentous actin and ZO-1 colocalized, the combined signal was yellow. Confocal images were taken in the apical and basal focal planes. In the apical plane of the SF3 cultures, only the taller cells were visible. In a more basal plane, ZO-1 and actin were colocalized in circumferential bands of shorter cells, but in taller cells, this signal was absent because the focal plane was below the apical junctional complex. Further, cytoplasmic actin was not visible, because of quenching by the melanin granules. In DMEM-F12 with 1% FBS, the apical plane showed ZO-1 alone in the apical junctional complex (green), whereas stress fibers came into focus in the basal plane. (B) The x-z planes were reconstructed along the blue lines in (A). The cells in the monolayer in SF3 were taller than those in DMEM-F12, especially where melanin was concentrated and actin and ZO-1 colocalized at the apical end of the lateral membranes (yellow dots). In the taller cells, a thick, discontinuous band of actin was also observed along the free border (apical membrane) of the cells, which suggested the presence of microvilli. By contrast, a squamous monolayer was evident in DMEM-F12. Actin was found throughout the cells and often failed to colocalize with ZO-1 (green dots). Arrowheads: basal membrane. Bar: (A) 20 μm; (B) 10 μm.

TABLE 2. Barrier Properties of ARPE-19 Cultured on Laminin-Coated Filters

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<th>JTT</th>
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APRE-19 cells were plated at confluent densities in DMEM/F12, 2% FBS for 1 week and switched to DMEM/F12, 1% FBS, JTT, 0.2% FBS, or SF3 for the time indicated. TER (Ω·cm<sup>2</sup>) and P<sub>H<sub>RP</sub></sub> (μL/cm<sup>2</sup>) were measured on triplicate filters. The standard error for three to nine experiments was approximately 5% (TER) or 7% (P<sub>H<sub>RP</sub></sub>).

* P < 0.01 for DMEM/F12 vs. SF3.
F12. The decrease in claudin expression was confirmed by immunoblot analysis. Relative to cultures maintained in DMEM-F12, steady levels of claudin 1 decreased in the media that we tested (Fig. 10). The decrease occurred within a week or two and was not affected by the serum concentration. Similar results were obtained by real-time RT-PCR (data not shown).

The low expression of claudin-1 in cultures with higher TER suggested the presence of other claudins. Claudin-2 was not evident at cell borders in cultures of DMEM-F12, but was evident in subpopulations of cells maintained in SF3 (Fig. 9). The distribution along the lateral membranes was punctate in contrast to the uniform appearance of ZO-1.

The variable expression of claudin-1 and -2 in different culture medium prompted us to examine the expression of a larger panel of claudins by RT-PCR (Fig. 11; Table 3). Claudin-1 and -2 were expressed by all culture media. As demonstrated above, expression of the mRNA did not necessarily indicate localization to the tight junction or uniform distribution among the cells of the culture. Using real-time RT-PCR, significant changes in expression were not observed as a function of time after 2 weeks in culture (data not shown).

Claudin-6, -9, -11, and -12 were also expressed in each culture condition but the expression of claudin-3, -4, -14, -15, and -16 depended on serum concentration and the base medium. For comparison, we included data from investigators in another laboratory who used standard culture conditions. They reported additional variability.

**DISCUSSION**

Culture models of the RPE are inherently heterogeneous, simply because the RPE monolayer exhibits regional variation. For example, heterogeneity in the expression of adhesion molecules and the polarity of Na⁺,K⁺-ATPase has been described in bovine RPE. Though microheterogeneity observed in vivo, the apical junctional complex has to conform to a basic structural plan and functionality that allows the entire epithelium to function as a blood-retinal barrier. The core of the barrier is tight junctions, which encircle each cell to retard transmonolayer diffusion through the paracellular spaces. Although there may well be small variations in semiselectivity
and permeability, each cell has to participate in the formation of this complex.

Heterogeneity that crosses the boundary of normal variation has been described in cultures of RPE. Fibroblastic and epithelial cells were isolated from secondary cultures of human RPE. Despite the expression of junctional proteins, the fibroblast-like cells failed to form an apical junctional complex. Rak et al. found that by breaking and reforming calcium-dependent cell–cell junctions, all cells in the cultures would form adherens junctions and synthesize melanin granules, but fully functional tight junctions failed to appear. These data demonstrate that all the cells in the culture were derived from RPE, but that RPE can manifest a heterogeneity in culture that exceeds the heterogeneity observed in vivo.

Our first indication of heterogeneity in ARPE-19 was the mixture of elongate and polygonal cells. Unlike the original reports based on earlier passages of the cell line, this heterogeneity did not resolve after months in culture, even in low serum, unless we used specialized media formulations.

![Figure 6](image-url) **Figure 6.** JAM-A and PAR-3 redistributed to cell borders in JTT and SF3 media. Postconfluent cells were maintained in DMEM-F12 with 1% FBS; JTT; 0.2% FBS; or SF3 for 2 months. Subcellular localization was determined by indirect immunofluorescence. AF-6 was concentrated at cell borders in each medium, but cytoplasmic staining for JAM-A appeared higher in DMEM-F12 medium. PAR-3 was not detectable at cell borders until 6 weeks and only in JTT and SF3 media. Bar, 10 μm.

![Figure 7](image-url) **Figure 7.** Proteins associated with junction assembly and cell polarity slowly increased in culture media that induced a rearrangement of actin filaments. Total mRNA was isolated from cultures maintained in the indicated medium for 3 or 6 weeks. Quantitative, real-time RT-PCR was used to amplify the mRNA for AF-6, JAM-A, and PAR-3. The reaction product was normalized to the level of expression determined in the 3-week cultures maintained in DMEM-F12 with 1% FBS. *Statistically significant difference from DMEM-F12 at 3 weeks (P < 0.05).*

![Figure 8](image-url) **Figure 8.** Supplementation with ITS rearranged the distribution of actin filaments. Cultures were maintained for 6 weeks in DMEM-F12 with 1% FBS with or without ITS, as indicated. Bar, 10 μm.
like the cultures of adult RPE noted earlier, subclones of the elongate and polygonal cells survived only a few weeks in culture. This finding was consistent with earlier reports that cells could not be subcloned from ARPE-19. The second indication was the heterogeneity of the expression of the cytokeratins. RPE normally expresses cytokeratin-18, but can express cytokeratin-7 in pathologic conditions. We found that most ARPE-19 expressed cytokeratin-18, but a large minority population also expressed cytokeratin-7. There was no apparent correlation between the expression of cytokeratin-7 in ARPE-19 and the adoption of an elongate morphology. Nor was there any correlation between the expression of cytokeratin-7 and the heterogenous distribution of claudins (discussed later). A third indication of heterogeneity was that only a subpopulation of cells could be induced to express melanin granules and shift from a flattened, squamous morphology to a taller, low cuboidal morphology. In contrast to the rearrangement of actin filaments discussed later, melanin formation took 3 to 6 months. This morphology sharply contrasts with the high cuboidal or columnar morphology demonstrated in the first report of ARPE-19 in DMEM-F12, 1% serum. We do not question the results of that earlier study or the studies of others that have replicated those findings. The strains of ARPE-19 that we acquired have passed through several hands and have different histories of how they were maintained and passaged. We want only to note the care that investigators should follow in characterizing their strain of ARPE-19 and comparing their results with those of others.

The heterogeneity of cell shape may be related to the maturity of the apical junctional complex, a mixture of tight, gap, and adherens junctions. Mature junctions are associated
with an underlying, circumferential band of actin filaments. Under standard culture conditions, actin filaments were organized in stress fibers instead of circumferential bands. Further, when viewed in the plane of the monolayer, the apical junctional complex followed a tortuous path. Because the circumferential bands are contractile and exert a purse stringlike tension of the apical junctional complex, this cortical structure would favor a polygonal, rather than elongate, morphology. In standard culture conditions of DMEM-F12 (5% or 1% FBS), the atypical arrangement that we observed persisted 6 months after confluence. The other culture media used in this study restored the circumferential distribution of actin filaments. This rearrangement would be a prerequisite to the observed conversion of a tortuous to a more linear apical junctional complex and the gradual shift to a polygonal cell shape. The functional correlate of this more organized junctional complex was the observed decrease in permeability to HRP. However, the low TER and the heterogeneous claudin distribution indicates that the tight junctions never fully developed.

The TER reported in this study in DMEM-F12 with 1% FBS was similar to the original report of TER in this medium. The specialized medium that we used did not yield as high a TER (90 Ω·cm²), as the specialized medium in that study. There are two factors that contribute to this difference. First, that study was performed at ambient temperature, which could increase the TER significantly. Second, the heterogenous morphology raises the possibility that the apical junctional complex, which regulates cell size and proliferation, was also heterogeneous. Heterogeneity of the tight junctions was manifest by a nonuniform distribution of claudin-1 and -2. Only a subset of cells expressed and distributed enough claudin-1 or -2 to cell borders for the claudin to be detected. Some cells distributed claudin to only a subset of its cell–cell contacts. Because the claudins bind claudins of a neighboring cell, it is likely that claudin accumulated only at the lateral membranes that were shared with a neighbor that expressed that same, or a compatible, claudin. The expression of the claudins was very dependent on culture conditions, which would explain why our results differ from those of other laboratories. Because suitable antibodies do not exist for many of the claudins that are expressed in RPE, we cannot say whether all cells in the monolayer express one subset of claudins or another. Nonetheless, our data indicated that there is heterogeneity of claudin expression. Although claudin-1 and occludin expression decreased in certain culture conditions, the TER rose. This is nonparadoxical, given that occludin plays an ill-defined role in regulating cell size and proliferation.

**Figure 10.** Claudin expression was affected by base media, but not serum concentration. (A) Protein was extracted from cultures maintained in DMEM/12, 1% FBS; JTT; 0.2% FBS; or SF3 for 6 weeks and equal amounts of protein were immunoblotted. Claudin 1 expression was much lower in the SF3 and JTT cultures than in the DMEM-F12 cultures. (B) Cultures were maintained in triple medium supplemented as indicated, for various periods before protein was extracted. The level of expression was not affected by the concentration of serum. Actin or β-tubulin was immunoblotted as a loading control.

**Figure 11.** Expression of claudin mRNA was dependent on the culture medium. Total RNA was isolated 2 weeks after confluence and the indicated claudin or occludin was amplified with the primers described in Table 1.
latory role and there are many claudins to substitute for claudin 1. The difficulty in using ARPE-19 to understand this problem is the large diversity of claudins expressed and the heterogeneity of claudin expression from cell to cell within the monolayer. The sensitivity of claudin expression to culture conditions makes the comparison of data among laboratories that much more of a challenge.

ARPE-19 is a dynamic cell line composed of a heterogeneous mix of RPE-related phenotypes. This heterogeneity presents a challenge. Genomic analysis demonstrates the sensitivity of ARPE-19 to culture conditions.40–42 Even in the most favorable culture conditions, with the best-behaved strains of ARPE-19, there are significant differences between the genes expressed by ARPE-19 and native RPE.40 Standardizing experimental conditions becomes difficult, because (1) differentiation of ARPE-19 in culture is a long, drawn-out affair with different features differentiating at different rates, (2) differentiation depends on plating density and the state of differentiation when the cells were harvested for plating, and (3) differentiation depends on the medium composition and feeding schedule. Another complication is exemplified by the heterogeneous expression of claudins, cytokeratins, and melanin-pathway enzymes. What percentage of the reported differences in gene expression are exhibited in all cells of the population and how many differences occur only in subsets of cells within the population? Heterogeneity raises the possibility that different subtypes may be enriched over time in a manner dependent on how the cells are maintained and passaged. Careful attention to these details revealed the value and limitations of this cell line for studying the apical junctional complex. The present study suggests we are studying the transition between the maturation of the adherens junction and the assembly of the tight junctions.43–45 The regulators of JAM-A, AF-6, and actin distribution of the adherens junction and the assembly of the tight junctions suggests we are studying the transition between the maturation of the adherens junction and the assembly of the tight junctions.43–45 The regulators of JAM-A, AF-6, and actin distribution should be the targets of future investigations. These proteins assembled before the PAR-3 polarity complex was recruited to the tight junctions in what might be regarded as a stalled maturation of the tight junctions.

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References


