In Vitro Differentiation Capacity of Telomerase Immortalized Human RPE Cells

Lakshmi Rambhatla,1 Choy-Pik Chiu,1 Randolph D. Glickman,2 and Cheryl Rowe-Rendleman1,3

PURPOSE. To investigate conditions promoting the differentiation of cultured human retinal pigment epithelial (RPE) cells and assess the differentiation potential of telomerase-immortalized RPE cells.

METHODS. Sera!ry passed RPE 340 (parental) cells have limited replicative ability and senesce after 50 to 60 population doublings (PDs). RPE 340 cells transfected with the catalytic component of human telomerase (hTERT) have an extended lifespan. RPE 340 and hTERT-transfected RPE (hTERT-RPE) cells were maintained at confluence without serum for up to 12 weeks. Morphologic, immunocytochemical, flow cytometric, and spectrophotometric analyses were performed to examine the extent of RPE differentiation.

RESULTS. Parental RPE 340 and hTERT-RPE cells underwent growth arrest and differentiated in the absence of serum. In early-passage parental (PD 11) and hTERT-RPE (PD 115 and PD 300) cells, serum deprivation for 4 weeks or more induced terminal differentiation as characterized by mature, growth-arrested, confluent sheets of polygonal and melanized cells that demonstrated diminished 5'-bromo-2'-deoxyuridine (Brdu) uptake and positive reactivity with antibodies to cellular retinaldehyde-binding protein (CRALBP), cytokeratin, and vimentin. Reintroduction of serum at 4 or 8 weeks allowed the cells to reenter the cell cycle and demelanize. Midpassage (PD 25) parental cells, however, were irreversibly arrested at G1 after 8 weeks of serum deprivation.

CONCLUSIONS. Cultured parental and hTERT-RPE cells express RPE-associated proteins and become stably melanized when density is arrested in the absence of serum. Moreover, hTERT-immortalized RPE cells retain the capacity to undergo terminal differentiation in vitro, even after long-term culture. (Invest Ophthalmol Vis Sci. 2002;43:1622–1630)

The retinal pigment epithelium (RPE) is a major component of the blood-retinal barrier and supports the physiological and biochemical renewal of photoreceptor outer segments and visual cycle intermediates. Defects in these functions have been implicated in the development of a number of retinal degenerative diseases, such as macular degeneration. The ability to study RPE physiology and function would benefit from having relevant cell culture models. Insofar as their usefulness in the physical and biochemical analysis of RPE cells, primary cultures of human RPE cells are limited by their finite proliferative potential and variable compositions. Commonly used practices for harvesting perinatal and adult RPE cells often introduce nonepithelial cells (e.g., Müller glia, melanocytes, and choroidal endothelial cells) that readily proliferate along with RPE cells in culture.1–3 The presence of these cells in primary and serially passaged cultures may not be obvious, because, similar to RPE cells, they may have pigment and express similar antigens or may be obscured because they grow beneath or through the clusters of RPE cells in the culture dish. The differing metabolic capacities and protein expression profiles of nonepithelial cells in culture can confound the analysis of RPE behavior in functional studies.4–7 Furthermore, a growing body of evidence indicates that except for the occasional spontaneous mutant strain,8,9 cells that grow out of primary cultures do not maintain a mature RPE phenotype (e.g., cuboidal shape, melanization, or RPE-specific antigen expression) during serial passages in culture.10,11 Either at log phase growth or after confluence, these early cultures (2–4 weeks) elaborate a variety of cellular morphologies (fusiform,stellate, and cuboidal) and express only some of the proteins associated with terminally differentiated RPE cells.12 Thus, the alteration in gene expression13 and absence of polarization or cell-cell attachment in these cultures makes the in vitro evaluation of RPE physiology difficult.14

Telomerase, a ribonucleoprotein complex, has been shown to regulate the replicative lifespan of most human somatic cells by maintaining the telomeres at the ends of chromosomes.15,16 We have previously described the isolation of immortalized RPE cell clones using the catalytic component of human telomerase, hTERT.17 In this article we describe a culture condition that induced RPE cell maturation and also demonstrate that the clonal hTERT-immortalized RPE cells maintained differentiation characteristics on long-term culture. The combination of infinite proliferation capacity and stable differentiation potential makes these cells promising candidates for the study of RPE physiology and for therapeutic applications.

METHODS

Cell Culture

Parental human RPE 340 cells were isolated from a 1-year-old donor.18 From these cells, hTERT-expressing cell clones were produced with a plasmid vector containing the human telomerase catalytic subunit (hTERT) and electroporation, as described previously.17 Cells were passaged in RPE medium composed of Dulbecco’s minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS) and were maintained in low-humidity conditions in an incubator at 37°C and 10% CO2. Early and midpassage (population doublings [PD] 11 and PD 25) parental RPE 340 cells and clonally selected T58 hTERT-RPE cells (PD 115 and PD 300) were used in these experiments. Cultures were plated at a density of 1 × 104 cells in 75-cm2 tissue culture flasks with RPE medium containing serum. After 48 hours, the cells were fed...
suspensions, as shown in Figure 2A.

This OD was extracted from the wavelength scans of the cellular visually observed degree of pigmentation in the cells (see Fig. 2A). A small relative peak at 550 nm that appeared to correlate with the melanin absorption spectrum, which is itself rather featureless. From not corresponding to any particular diagnostic absorption peak in the cellular melanin content,19 this wavelength in fact does correspond to an indicator of cellular melanin content,19 this wavelength in fact does correspond to any particular diagnostic absorption peak in the melanin absorption spectrum, which is itself rather featureless. From measurements of several pigmented primary RPE cell lines, we found a small relative peak at 550 nm that appeared to correlate with the visually observed degree of pigmentation in the cells (see Fig. 2A). Based on this data, we used OD550 as an empirical indicator of melanization. This OD was extracted from the wavelength scans of the cellular suspensions, as shown in Figure 2A.

Spectrophotometric Assay for Cellular Melanization
Aliquots containing $1 \times 10^6$ cells were prepared as a suspension in 0.5-mL Dulbecco’s phosphate-buffered saline (DPBS). The sample was placed in a 1-cm glass cuvette, and a wavelength scan of optical absorption from 350 to 700 nm was made in a spectrophotometer (DU 640; Beckman, Palo Alto, CA), referenced to DPBS alone. Although some previous studies have used optical density of 475 nm (OD475) as an indicator of cellular melanin content,19 this wavelength in fact does not correspond to any particular diagnostic absorption peak in the melanin absorption spectrum, which is itself rather featureless. From measurements of several pigmented primary RPE cell lines, we found a small relative peak at 550 nm that appeared to correlate with the visually observed degree of pigmentation in the cells (see Fig. 2A). Based on this data, we used OD550 as an empirical indicator of melanization. This OD was extracted from the wavelength scans of the cellular suspensions, as shown in Figure 2A.

Immunocytochemistry
Cells were plated on sterile coverslips and fixed for 0.5 hour in 1:1 (vol/vol) mixture of acetone and methanol at $-20^\circ$C and blocked in 10% goat serum in 0.1 M phosphate buffer (pH 7.2) for 2 hours. Antibodies tested included anti-cellular retinaldehyde-binding protein (CRALBP; a generous gift from John C. Saari, University of Washington, Seattle, WA) diluted 1:200 in blocking buffer, and anti-vimentin (Dako, Glostrup, Denmark) diluted 1:500 in blocking buffer, and anti-pan cytokeratin (Biomedica, Foster City, CA) used as supplied. Cells were incubated with primary antibody overnight at 4°C and rinsed. The secondary and tertiary antibodies, biotinylated goat anti-mouse IgG and streptavidin Texas red (Vector Laboratories, Burlingame, CA), respectively, were each diluted 1:500 in phosphate buffer containing 5% goat serum. Cells were then incubated with secondary and tertiary antibody for 1 and 0.5 hours, respectively. Photograph-microscopy was performed at $\times$400 final magnification on a microscope (Labophot; Nikon, Tokyo, Japan) equipped with epifluorescence excitation at $\lambda = 540$ to $580 \text{ nm}$.

BrdU Assay
hTERT-RPE cells were seeded on sterile glass coverslips and grown in 24-well plastic tissue culture plates. Cell proliferation was assessed by incorporation of 5′-bromo-2′-deoxyuridine (BrdU) into cellular DNA, using a cell proliferation kit (In Situ; Roche Molecular Biochemicals, Indianapolis, IN). Adherent cells were incubated in 10 µM BrdU for 0.5 hours at 37°C. The cells were fixed in ethanol on the coverslip, and the manufacturer’s protocol for BrdU staining with a fluorescein-conjugated secondary antibody was followed. Cells were also counterstained with 10 µM Hoechst 33342 for 2 minutes (Molecular Probes, Eugene, OR) to reveal all cell nuclei in the population. The cells were imaged with a charge-coupled device (CCD) integrating camera (Cohu, San Diego, CA) fitted to a microscope (BX-60; Olympus, Lake Success, NY) with epifluorescence. Fluorescence parameters (center wavelengths) for imaging the Hoechst 33342-stained cells were $\lambda_{\text{ex}} = 340 \text{ nm}$, $\lambda_{\text{em}} = 450 \text{ nm}$, where Ex is excitation and Em is emission, and for the BrdU-stained cells were $\lambda_{\text{ex}} = 490 \text{ nm}$, and $\lambda_{\text{em}} = 530 \text{ nm}$. After image acquisition, counts of the number of BrdU-stained and total (Hoechst 33342-stained) nuclei were obtained by performing a bright-objects count on the images by computer (ImagePro software; Media Cybernetics, Silver Spring, MD).

Flow Cytometry Analysis of Cell Cycle
To evaluate the proliferative potential of parental and telomerase-expressing cells, RPE cultures were grown to confluence and held stationary in serum-free medium for 8 weeks. After recovery for 10 days in serum-supplemented medium, $1 \times 10^6$ cells were harvested and fixed in 75% methanol at 4°C overnight. The cell pellet was washed two times in phosphate-buffered saline (PBS; pH 7.2) and resuspended in 1 mL PBS containing RNase A (100 µg/mL) and pro-
Senescence-Associated β-Galactosidase Assay
Cultures grown for 8 weeks in serum-free medium were replated in the presence of serum at low density on 0.8 cm² chamber slides (Nunc, Naperville, IL). Adherent cells were fixed for 2 minutes in 0.2% glutaraldehyde in PBS. To detect senescence-associated lysosomal activity, fixed cells were incubated overnight at 37°C in a solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl₂ buffered at pH 6 with 40 mM citric acid-sodium phosphate. The positive control reaction to detect nonspecific lysosomal β-galactosidase activity was performed in citric acid-sodium phosphate buffer adjusted to pH 4.0. Cells were rinsed in PBS and mounted in aqueous medium (Aquamount; Lerner Laboratories, Pittsburgh, PA). Bright-field photomicroscopy was then performed (Labophot; Nikon, equipped with a SPOT CCD cooled camera; Diagnostic Instruments, Inc., Sterling Heights, MI).

RESULTS

Morphologic Differentiation and Melanization
In culture, parental RPE cells are capable of a finite number of cell divisions, reaching replicative senescence at PD52 to PD65. In this work, RPE cultures were maintained at low humidity to maintain a more sterile environment to retard fungal contamination during long-term cultures. Senescence in cells grown under these conditions occurred at PD45 to PD50 and was accompanied by the characteristic morphologic changes, decreased replicative potential, and expression of senescence-associated β-galactosidase activity.²⁰ (data not shown). The telomerase-overexpressing subclone T58, in contrast, has apparently infinite proliferative potential and has been maintained in vitro for more than 500 PDs. Parental RPE 340 and hTERT-RPE cultures grown to confluence and maintained in medium containing serum for 2 to 4 weeks were multilaminar, as determined by focusing through cultures at the light microscopic level. The cultures were populated with morphologically different cell types with elongated fibroblastic, flattened stellate, and cuboidal shapes (Fig. 1, left).

To determine whether removal of serum or growth factors may induce terminal differentiation, we next examined the survival and phenotype of parental and hTERT-RPE cells maintained in medium without serum. Replicative age of the parental culture was found to be the major factor affecting long-term survival under these conditions. Cultures that had accumulated longer than 25 PDs survived for only 6 to 8 weeks in serum-free medium, whereas early-passage parental (PD11) and hTERT-RPE cells survived for at least 12 weeks. After 2 to 4 weeks in serum-free medium, the density of spreading fibroblastic processes in cultures of hTERT and parental RPE cells diminished. Many cells interposed between the RPE layer and culture dish had long, thin processes that retracted and became less prominent with time under these conditions. This may be consistent with the death of other more serum-sensitive cell types in the culture (e.g., Müller glia).²¹,²² The surviving monolayer of confluent cells appeared to be more homogeneous in shape, size, and overall density. These cells were more reminiscent of a cuboidal epithelium (Fig 1, right) and the morphology remained stable in the young parental and hTERT-RPE cultures for up to 12 weeks. By contrast, overt signs of deterioration, such as the formation of small foci and the appearance of empty patches, occurred throughout the midpassage parental cultures after 8 weeks in serum-free medium.

Long-term survival in serum-free medium was also accompanied by an increase in cellular melanin. After 4 weeks, confluent early-passage parental RPE and hTERT-RPE cultures exhibited clusters of lightly melanized cells that became more prevalent by 8 weeks. From 8 to 12 weeks, melanization appeared denser and, eventually, by 12 weeks, the entire plate of cells was uniformly melanized. Clusters of melanized cells, however, did not appear in midpassage parental RPE cultures (PD25) until 8 weeks, and these cultures did not form uniformly melanized, confluent sheets of cells. Similarly, RPE cultures maintained at confluence in serum-containing medium showed some melanization if maintained for more than 8 to 12 weeks, but they never formed uniformly melanized sheets with a cuboidal epithelial morphology (data not shown).

Accumulation of intracellular melanin results in a general increase in OD across the optical spectrum from ultraviolet to infrared. In addition, there is often a small but consistent peak

FIGURE 2. Absorption spectra (350–800 nm) of RPE cells. (A) Spectrophotometric scans of bovine RPE cells, parental RPE cells, and hTERT-RPE cells are shown. A prominent peak at 550 nm appeared in heavily melanized bovine primary RPE after approximately 4 weeks in culture (not passaged). Smaller, but discernible, peaks near 550 nm appeared in the lightly melanized parental and hTERT-RPE cells grown for 2 weeks in the absence of serum. The 550-nm peak was not present in hTERT-RPE cells grown for 2 weeks in serum-supplemented medium. (B) Photomicrograph of hTERT-RPE cells maintained for 10 weeks in culture at confluence in serum-deprived medium. Clusters of cells with obvious pigmentation were observed scattered throughout the culture plate. Magnification, ×200.

A

B

Downloaded From: http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932914/ on 06/04/2018
Expression of RPE-Associated Proteins

Young parental and hTERT-RPE cells that had been grown for 8 weeks in medium without serum were evaluated for the expression of RPE-associated proteins with antibodies against pan-cytokeratin, vimentin, and CRALBP. Positive staining was observed in all RPE cultures tested. Final magnification, ×400.

<table>
<thead>
<tr>
<th>Expression of RPE-Associated Proteins</th>
</tr>
</thead>
</table>
| Young parental and hTERT-RPE cells that had been grown for 8 weeks in medium without serum were evaluated for the expression of cyto-keratin, vimentin, and CRALBP, by immunohistochemistry (Figs. 3A, 3B). As expected, the epithelial cell marker cytokeratin was detected in all RPE cultures. The marker appeared in 100% of cells and did not distinguish among the various morphologic types. The pattern of pan-cytokeratin staining was identical in both hTERT and parental RPE cells. Vimentin-positive intermediate fibers were also detected in both parental RPE and hTERT-RPE cultures. Fluorescent staining elucidated a delicate pattern of mostly longitudinal fibers in early-passage parental and hTERT-RPE cells. Vimentin-like fibers in midpassage parental RPE cells (PD25) were characterized by thicker and shorter branch-like spicules that decorated the cells in a longitudinal pattern. CRALBP cross-reacts with cytoplasmic elements in RPE cells and Müller glia. We observed that CRALBP stained the cytoplasm of parental and hTERT-RPE in a speckled pattern that was concentrated in the perinuclear region of the cells. The density of speckling appeared consistent among early-passage parental and hTERT-RPE cells but decreased in the midpassage parental RPE cells. For all three markers, the pattern of immunoreactive staining was similar in cells maintained in log phase growth, at confluence without serum, or on re-entry into the cell cycle after reintroduction of serum (data not shown). These results therefore suggest that the expression pattern of these three RPE-associated proteins in parental and hTERT-RPE cells is not dependent on the cell cycle status.

Cell Cycle Analysis by BrdU Uptake and Flow Cytometry

To determine the proliferative response of RPE cells to serum, we quantitated BrdU incorporation and analyzed the cell cycle profiles of parental and hTERT-RPE cells that had been maintained in serum-free medium. The number of dividing cells decreased steadily over time in postconfluence cultures of hTERT-RPE cells, as demonstrated by BrdU uptake in confluent cultures maintained in serum-free conditions for 1 and 8 weeks (Fig. 4A). Quantitative analysis of BrdU-positive nuclei shows that approximately 30% of cells were still dividing 1 week after reaching confluence. This proportion steadily declined to almost 0% by 6 weeks (Fig. 4B).

Using flow cytometry, we found that the cell cycle profiles were almost identical between parental and hTERT-RPE cells after 8 weeks in serum-free medium (Fig. 5). The large peak at G1, representing 94% and 97% of parental RPE and hTERT-RPE cells, respectively, and the near absence of cells with S- or G2-phase nuclei are consistent with growth arrest and synchronization at G1 in both parental and hTERT-RPE cells (Figs. 5A, 5B). The capacity of hTERT-RPE to undergo growth arrest in the absence of serum is consistent with our previous finding that the infinite capacity for proliferation in these cells does not abolish this important checkpoint arrest.

The proliferative potential of cultures that had been arrested at G1 was assessed by replating parental and hTERT-RPE cells in medium containing 10%, FBS. The cell cycle profiles of parental RPE cultures at PD11 and PD25 were compared with those of hTERT-RPE cells at PD114 and PD300 (Figs. 5C–F; Table 1). The growth profiles of the early-passage parental and hTERT-RPE cells were similar. The predominant population of G1-arrested cells in PD11 RPE was diminished by 25%. This was accompanied by an increase of 12% in both S- and G2-phase nuclei. The hTERT-RPE cells behaved similarly, except that the increase in proliferating cells was 22%. It is important to note that the cells became less melanized after reentry into the cell cycle. Midpassage (PD 25) parental RPE cells, however, remained arrested in G1 and did not enter the cell cycle on introduction of serum. These cells remained melanized, indi-

![Figure 3. Immunohistochemical staining of parental (A) and hTERT-RPE (B) cell cultures. RPE cells were assayed for expression of RPE-associated proteins with antibodies against pan-cytokeratin, vimentin, and CRALBP. Positive staining was observed in all RPE cultures tested.](image-url)
cating that the midpassage parental culture was terminally arrested after 8 weeks in serum-free medium. These results thus suggest that early-passage parental and hTERT-RPE cells maintain similar cell cycle responses to serum.19

**Senescence-Associated β-Galactosidase Assay**

In most growing cells, there is positive β-galactosidase activity in lysosomal compartments that is optimal at acidified pH.20 Thus, at pH 4, both parental (PD11 and PD25) and hTERT-RPE (PD114 and PD300) cells in log phase growth produced a blue reaction product (Figs. 5B, 6A, ). It was observed that serum deprivation for 8 weeks followed by reintroduction of serum, induced the senescence-associated β-galactosidase with optimal pH activity at pH 6 in midpassage (PD25), but not in the early-passage, parental (PD11) RPE cells. This is in agreement with the flow cytometry result (Fig. 5). Furthermore, the hTERT-RPE cultures at PD114 and PD300 had similarly low senescence-associated β-galactosidase activity as the young parental cells under the same conditions (Figs. 6C–F). This suggests that midpassage RPE cells become prematurely senescent in response to the stress imposed by serum removal and do not re-enter the cell cycle.

**DISCUSSION**

Primary cultures of human RPE cells are excellent tools for pharmacodynamic and physiological evaluation of drug and

![Figure 4. BrdU uptake in hTERT-RPE cells maintained in serum-free medium. (A) Immunohistochemical staining for BrdU incorporation. Top left: BrdU stained hTERT-RPE cells at 1 week in culture. Top right: same cells, counterstained with Hoechst 33342. Bottom left: BrdU-stained hTERT-RPE cells at 8 weeks in culture. Bottom right: same cells, counterstained with Hoechst 33342 at 8 weeks in culture. There was virtually no proliferation in these cells after 8 weeks in confluent culture. (B) Quantitative analysis. The number of BrdU- and Hoechst 33342–positive nuclei was obtained by counting five microscopic fields per sample. The mean ± SD of the samples is shown. The percentage of BrdU-positive nuclei was also indicated for each time point.]
cell interactions at the choroid-RPE-photoreceptor layer. These cultures are usually well differentiated and melanized, but the mature characteristics of RPE cells are lost when the cells divide in vitro, limiting their use in functional analyses. We have previously shown that hTERT-RPE have extended lifespans and exhibit a nontumorigenic phenotype. Specifically, expression of exogenous telomerase in RPE cells maintains telomeres and prevents replicative senescence in culture. Consequently, lifespan in culture is extended without any alterations in the population-doubling time, and the cells retain normal growth control in response to serum deprivation, high cell density, G1 and G2 phase blockers, and spindle inhibi-

![Figure 5](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932914/)

**Figure 5.** Cell cycle analysis of parental and hTERT-RPE cells. The percentage of cells in G1, S, and G2/M was determined by flow cytometry. Parental (PD11) and hTERT-RPE cells (PD114) were arrested in the G1 phase after they were held at confluence in the absence of serum for 8 weeks (A, B). When early-passage parental (PD 11) and hTERT-RPE cells were reacclimated to serum for 10 days, the bimodal distribution of cells in G1 and G2 obtained the indicated reentry into the cell cycle (C, D). Midpassage RPE (PD25) remained arrested after reintroduction of serum (E). In contrast, later-passage hTERT-RPE (PD300) cells were still capable of responding to serum after 8 weeks of growth arrest (F).

| Table 1. Growth and Differentiation in Parental and hTERT-RPE Cultures in Serum-Free Medium |
|---------------------------------|---------------------------------|
| **Parental RPE** | **hTERT-RPE** |
| | | PD11 | PD25 | PD114 | PD300 |
| | | 4 wk | 8 wk | 4 wk | 8 wk | 4 wk | 8 wk |
| Serum-free medium | + + | ++ | + | + |
| Replated in 10% FBS medium | + | + | + | + |
| Melanin | + + | ++ | + | + |
| Senescence-Associated β-Galactosidase assay | + | + | + | + |
| Resumption of Cell Cycle | + | + | + | + |

Parental RPE and hTERT-RPE cells at different PDs were arrested in serum-free conditions for 8 weeks and the extent of melanization was assessed visually. The cells were then reintroduced to serum-containing medium for 10 days and melanization, resumption of cell cycle, and senescence-associated β-galactosidase activity were determined. −, +, and ++ refer to the degree of melanization in the cells. Differences in behavior were observed between midpassage PD25 parental RPE (highlighted in bold) and the early-passage parental RPE (PD11) or hTERT-RPE (PD114 and PD300).
cultures became densely melanized and morphologically less diverse. RPE associated proteins were also stably expressed in these serum-free cultures. It is not clear whether this is a result of selective growth of RPE or of a change in phenotype of other cells. In either case, our results indicate that this method effectively enriches the RPE component from a mixed monoclonal population of primary cells, making them more desirable for physiological and pharmacodynamic measurements.

**Melanization In Vitro**

A general observation has been that primary RPE cells progressively lose their melanosome content with time in culture. This may result from dilution of the melanosomes as the cells divide and are successively passed. A related reason may be that there is little or no new melanin synthesis in daughter cells, probably because cultured RPE cells (in contrast to uveal melanocytes or choroidal melanocytes) have very low tyrosinase activity. Melanin in RPE melanosomes may also be degraded normally as part of a turnover process, although the extent of such turnover in RPE cells is unknown. A pigmented RPE cell line is highly desirable for studying such processes as photosensitized reactions in pigmented cells, as well as for examining the hypothesized photoprotective versus photodamage-promoting role of melanin. Several methods have been described for increasing the pigment content of RPE cells in culture—for example, by feeding RPE cultures melanin granules or lipofuscin granules or by exposing the cultures to medium containing insulin and/or elevated bicarbonate. In the method reported in this study, no specific external factors are used to promote melanization. The melanization process may be accelerated by maintaining the cultures in a serum-free medium. If this is undesirable, the RPE cultures may simply be maintained at confluence for a prolonged time (at least 2 months, data not shown). This technique has been applied to RPE cells stably immortalized by exogenous telomerase expression and may be more generally applicable. Porcine RPE cells have been reported to become partly melanized if kept in culture for at least seven weeks after becoming confluent.

The activity of tyrosinase, an enzyme involved in melanin synthesis, would be expected to accompany the increasing melanization of the hTERT-RPE cells. An attempt was made to demonstrate the presence of tyrosinase in these RPE cells by immunohistochemical staining with an antityrosinase antibody. We were surprised that only a very small number (<1%) of cultured hTERT-RPE cells showed positive tyrosinase staining (data not shown). We obtained a similar finding in heavily pigmented primary cultures of baboon RPE cells. Our results suggest that tyrosinase activity may be downregulated soon after melanin is produced, or that most tyrosinase is present as a proenzyme and is processed into functional enzyme only in small amounts. In fact, tyrosinase has rarely if ever been detected in cultured RPE cells. Abul-Hassan et al. have demonstrated that, whereas the tyrosinase promoter is upregulated in cultured RPE cells, the enzyme activity itself is not detected, possibly because of a failure of a post-translational regulatory mechanism in RPE cells in culture. Despite the inability to detect tyrosinase directly, it was obvious from visual inspection that some of the cells in our cultures were becoming pigmented (Fig. 2B), a change that was also manifested in their increasing OD (Fig. 2A).

**Role of Telomerase**

Telomerase has been demonstrated to regulate the replicative lifespan of human somatic cells through telomere maintenance. Ectopic expression of hTERT has been shown to im-
mortalize fibroblasts, RPE cells, and endothelial cells.\textsuperscript{17,46} The increase in replicative capacity is not associated with a malignant phenotype or any alterations in normal differentiated functions in vitro or in vivo. For example, hTERT-immortalized human endothelial cells form angiogenic webs on synthetic basement membrane, hTERT-immortalized human fibroblasts form normal skin equivalents with keratinocytes in SCID mice, and hTERT-immortalized bovine adrenocortical cells rescue adrenalecetomized animals.\textsuperscript{25,46,47}

We have previously demonstrated that hTERT-transfected RPE cells that have been grown in serum have similar morphology and protein expression pattern identical with that of early-passage parental RPE cells in culture.\textsuperscript{48} Under those conditions, RPE cells remain small, unpigmented, and replicatively active and demonstrate appropriate cell–cell contact.\textsuperscript{49} In the current study, cultured hTERT-immortalized RPE cells maintained a normal RPE phenotype and the capacity to accumulate melanin when grown under serum-free conditions. The epithelial organization, protein expression, cell cycle distribution, and capacity for melanization of hTERT-RPE more strongly resemble that of early-passage rather than late-passage RPE. These results indicate that telomerase-expressing RPE cells retain a phenotype characteristic of early-passage RPE cells, thus supporting the utility of telomerase in cell and gene therapy approaches for RPE-related diseases.

**Acknowledgments**

The authors thank Donghee Choi and Neeru Kumar for expert technical assistance and Melissa Fisher for help with graphic presentation.

**References**


