Published in final edited form as:

Exp Eye Res. 2009 January ; 88(1): 99–105. doi:10.1016/j.exer.2008.10.013.

Mouse Retinal Pigmented Epithelial Cell Lines retain their phenotypic characteristics after transfection with Human Papilloma Virus: A new tool to further the study of RPE biology

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Abstract

Development of immortalized mouse retinal pigmented epithelial cell (RPE) lines that retain many of their in vivo phenotypic characteristics, would aid in studies of ocular diseases including age related macular degeneration (AMD). RPE cells were isolated from 16 month old (estrogen receptor knockout) ERKO α and ERKO β mice and their C57Bl/6 wild type littermates. RPE65 and cellular retinaldehyde binding protein (CRALBP) expression, in vivo markers of RPE cells, were detected by real-time RT-PCR and western analysis. We confirmed the presence of epithelial cell markers, ZO1, cytokeratin 8 and 18 by immunofluorescence staining. In addition, we confirmed the distribution of actin filaments and the expression of ezrin. To develop cell lines, RPE cells were isolated, propagated and immortalized using human papilloma virus (HPV) 16 (E6/E7). RPE-specific markers and morphology were assessed before and after immortalization. In wildtype littermate controls, there was no evidence of any alterations in the parameters that we examined including MMP-2, TIMP-2, collagen type IV, and estrogen receptor (ER) α and ER β protein expression and ER copy number ratio. Therefore, immortalized mouse RPE cell lines that retain their in vivo phenotype can be isolated from either pharmacologically or genetically manipulated mice, and may be used to study RPE cell biology.

Keywords

mouse retinal pigmented epithelial cells; estrogen receptors; extracellular matrix; immortalization

Introduction

The study of in vivo pathology has been greatly advanced through the utilization of isolated cells from the tissue of interest. In particular, the complexity of the intact eye has made ocular cell culture an attractive tool to study eye disease. To date many studies on human retinal diseases have utilized the retinal pigmented epithelial (RPE) cell line ARPE-19 (Dunn et.al, 1996) or human cell lines that proliferate for a finite number of passages. These cells retain

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their differentiated characteristics in vitro, but obtaining human eyes suitable for cell culture and the physiologic differences between donors makes studies with human material difficult at best.

There is increasing interest in mouse models of ocular disease including age related macular degeneration (AMD) (Ambati et.al, 2003;Cousins et.al, 2002;Cousins et.al, 2003;Elizabeth Rakoczy et.al, 2006;Malek and Cousins, 2006) Although a method has been established for propagating and growing primary mouse RPE cells (Gibbs et.al, 2003), the ability to sustain cultures of mouse RPE cells derived from these models would be highly advantageous. To this end, multiple cell types including epithelial cells have been immortalized with the E6/E7 open reading frames of the human papilloma virus (HPV) 16 (Fontijn et.al, 1995;Loeuillet et.al, 2001). Ideally RPE immortalization would produce cell lines that retain their cobblestone morphology as well as other epithelial cell characteristics including polarization and protein markers.

Our group has focused on the role of extracellular matrix (ECM) turnover dysregulation and how it contributes to the development of early dry AMD (Cousins et.al, 2003;Elliot et.al, 2006;Marin-Castano et.al, 2006). We have previously shown that feeding C57/Bl6 mice a high fat diet coupled with blue light exposure to the eye leads to sub-retinal deposit formation in middle age ovariectomized or 16 month old female mice (Cousins et.al, 2003). These data suggested that estrogens may play a role in preventing ECM alterations and encouraged us to elucidate the precise role of the two estrogen receptor subtypes in this process. Therefore we isolated and propagated mouse RPE cell lines from C57Bl/6 wildtype littermates and genetically altered estrogen receptor knockout (ERKO) mice. The resulting cell lines have been characterized with regard to morphology, cell surface proteins, and other epithelial specific markers.

Materials and Methods: Isolation RPE sheets from mice

The following method was modified from an existing method developed for rat RPE cell isolation (Yue et.al, 1991). Intact eyes were removed quickly from 8 week and 16 month old C57/Bl6 mice (Jackson Laboratories, Bar Harbor, ME) and 16 month old male and female ERKO mice (kindly provided by Ken Korach, NIEHS, NC) following anesthesia. The guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research were followed, and the Division of Veterinary Resources approved all experiments. The eyes were washed twice in Dulbecco's modified eagle's medium (DMEM) containing high glucose, followed by an enzymatic digestion in 2% Dispase in DMEM for 45 minutes at 37°C. Eyes were washed two times in growth medium (GM) consisting of DMEM high glucose, 10% FBS, 1% penicillin/streptomycin, 2.5mM-L-Glutamine and 1% non-essential aminoacids. After washing, the eyes were transferred into fresh GM medium for dissection.

Using microdissection scissors and an upright dissection microscope, a circular incision was made around the ora serrata of each eye. The posterior eyecup containing the neural retina and the lens were placed in fresh GM medium and incubated for 20 minutes at 37°C in 10% CO₂ incubator to facilitate separation of the RPE sheets from the neural retina.

After removal of the RPE sheets from the neural retina, intact sheets of RPE cells were peeled and collected in an eppendorf tube. RPE cells were centrifuged at 1500 rpm for 5 minutes and resuspended in GM medium. The cell suspension (0.5ml) was added to the upper chamber of a 12-well plate, containing polycarbonate transwell inserts with a 3.0µm pore diameter. Growth on the transwells aids in the preservation of cellular polarity.

GM medium was added to the lower well (1ml). Cells were cultured at 37°C in 10% CO₂ for 10 days, with a change of medium (GM) every other day. After 10 days the cells were washed

with versene for 3 minutes and then trypsinized for 4 minutes to detach the cells from the insert. The cells were collected in a tube, centrifuged at 1000 rpm for 5 minutes and resuspended in DMEM/F:12 10% FBS PEN/strep, l-glutamine, sodium bicarbonate. The cells were plated in 24 well plates until they reached confluence, at which time they were trypsinized and grown in a larger flask.

Viability Assay

Isolated sheets of RPE cells were tested for viability using a viability kit from Molecular Probes. The live/dead viability kit (Molecular Probes) is a two color assay which identifies live versus dead cells on the basis of membrane integrity and esterase activity. The combine solution was added to the tissue for 45 minutes at room temperature immediately after isolation. Using fluorescent microscopy, viability was assessed based on color, live cells in green and dead cells in red.

Immortalization

Cells were plated in 24 well plates and allowed to come to semi confluence. Media was removed and a replication-deficient retrovirus containing human papiloma virus 16 E6/E7 DNA with a gentamycin resistant gene, was added to each well in the presence of polybrene (4 µg/ml). 24 hours later the medium was changed and gentamycin (800 µg/ml) added for 3 days to select those cells which have incorporated the virus (Fontijn et.al, 1995). Following treatment the surviving cells were passaged and characterized. A portion of the same cell culture was grown in a sister well to confluence, but was not subjected to immortalization and served as controls for the characterization experiments. To assure the presence of HPV, we utilized an antibody against HPV16 E6 (Santa Cruz Biotechnology, CA).

Growth Curves

Cells were plated at equal density in medium containing 10% serum in 24-well plates. Twenty-four hours later, cells were counted to determine plating efficiency. Cell number was assessed on days 1–5 and continued until day 15 with a Coulter Z1 cell counter (Beckman Coulter, Hialeah, FL).

EM

RPE cells were grown on German Glass cover slips (Carolina Biologicals) to confluence. The monolayers were fixed in 2% glutaraldehyde/100mM Sucrose/0.05M PO₄³⁻ for 24–48 hours. This was followed by rinsing and post fixation with 2% Osmium/0.1M PO₄³⁻ for 1 hour. The samples were further dehydrated through graded ethanol, embedded in Epon -812 and polymerized overnight at 64°C. Thick and ultrathin sections (0.7–1.0 µm) were cut on a microtome (MT-2; Porter Blum, Hatfield, PA). Thick sections were stained with toluidine blue and examined by light microscopy. Ultrathin sections were stained with 4% uranyl acetate and lead citrate and examined with a transmission electron microscope (model CX-100; JEOL, Tokyo, Japan).

Immunohistochemistry

RPE cells were plated in either 24 well plates or 8 well chamber slides for 7–10 days prior to fixation. Cells were washed in PBS and fixed with either 4% paraformaldehyde, methanol or 10% TCA for 10 minutes depending on the antibody staining to be performed. This was followed by three washes with PBS for 10 minutes to remove excess fixative solution and blocking for 1 hour at room temperature with a solution containing 5% BSA and 1% Triton in PBS. The blocking solution was removed, the slide was washed with PBS for 5 minutes and

the primary antibody added (RPE-65 a kind gift from Dr. Michael Redmond was used at 1:1000), (cytokeratin 8 and 18 a kind gift from Dr. Boris Stanzel were used respectively at 1:2000 and 1:1000) and ZO-1 (Invitrogen cat# 33-9100 at 1 µg/ml). Cells were also stained with ezrin monoclonal antibody (Covance; 5 µg/ml). Ezrin is an actin-binding protein crucial for morphogenesis of apical microvilli and basolateral infoldings in RPE cells. Cells were incubated overnight with primary antibodies or exposed to the antibody against ZO-1 or rhodamine phalloidin for 1 hour, washed and mounted. Following an overnight incubation with the primary antibody, the cells were rinsed three times with PBS for 10 minutes, and the secondary antibody was added for 2 hours. (Alexa Fluor 546 goat anti rabbit or Alexa Fluor 546 goat anti mouse; 4 mg/ml). The cells were then washed five to six times in PBS, mounted and examined with a Leica microscope.

Western Blot analysis

Western analysis was performed as previously described (Elliot et.al, 2006). Briefly cells were plated in T75 flasks and allowed to grow to confluence. The cells were maintained at confluence for a period of one week at which time they were collected into lysis buffer containing a protease inhibitor cocktail. Extraction of protein and a protein assay was performed to determine loading amounts. 50 µg of protein was electrophoresed on a 10% polyacrylamide gel for RPE65 (Chemicon) and cellular retinaldehyde-binding protein (CRALBP, Abcam). For ERα 40 µg of protein was loaded and for ERβ 100 µg of protein was immunoprecipitated as previously described (Elliot et.al, 2006). All western blots were exposed to actin to normalize for loading variability. In addition, cells were also tested for the presence of SV2, to rule out contamination with neural cells, rhodopsin, and PKCα, markers of photoreceptors and bipolar rods respectively.

Real-time RT-PCR

Real-time RT-PCR was performed as previously described on isolated RPE cells before and after immortalization (Elliot et.al, 2006). Briefly, real-time RT-PCR reactions were performed using the TaqMan One Step RT-PCR Master Mix reagent kit and the ABI Prism 7700 sequence detection system (PerkinElmer Applied Biosystems) in a total volume of 50 µl of reaction mixture. A TaqMan ribosomal probe RNA control reagent kit was used to detect the 18S ribosomal RNA gene, which represented an endogenous control. Baseline mRNA expression of RPE65, MMP-14, MMP-2, TIMP-2, collagen type I and type IV and ERα and ERβ was determined. Each sample was normalized to the 18S transcript content.

Flow Cytometry

Cells were analyzed by flow cytometry using a FACScan (Beckton Dickinson). Briefly, cells were trypsinized, and fixed in 70% ethanol overnight. Ethanol was removed by multiple PBS washes and 50 µg/ml of propidium iodide was added. Briefly, cells were measured by width and area and plotted by area. Following analysis, the amount of DNA, which reflects the number of cells or the stage of the cell cycle, was quantified by incorporation of nucleic acid stains (propidium iodide).

Results and summary: Viability

The majority of the cells on the isolated sheets were viable as assessed by the live/dead assay (Figure 1A). The cell lines were propagated to obtain a bank of frozen cell lines. In addition, cells have been frozen and thawed multiple times without noticeable changes in morphology. Immortalization of mouse RPE cell lines had no effect on morphology of the cell lines. They retained their cobblestone appearance and were contact inhibited (Fig 1B and C). Spontaneous transformation of the cell lines was ruled out by the presence of HPV protein (Figure 1D).

Growth Curves

The initial doubling time for most cell lines was 16–24 hours as has been described for ARPE-19 cells (Dunn et.al, 1996). As the cells approached confluency, cell number increased (Figure 2A) at a slower rate, although there was variability between cell lines. The cells were maintained in a monolayer for 7–14 days before peeling off in sheets.

Specific Markers of RPE cells

To confirm the presence of *in vivo* RPE markers, we performed real time RT-PCR and western analysis of cultured cells (Fig 3). The mRNA expression of RPE65 was consistent between individual cell lines (Table 1). The protein expression of RPE65 and CRALBP, another marker of RPE cells expressed *in vivo*, was evident in non-transformed and immortalized RPE cells (Fig 3). Expression of these molecules have been shown on human ARPE-19 cell lines (Dunn et.al, 1996). An interesting finding was the differential regulation of RPE65 in the absence of ER β (lanes 3 and 7) even in the absence of injury *in vivo* (lane 3). In addition, SV2 expression, a neural cell marker, PKC α and rhodopsin were negative.

Markers of epithelial cell junctions and polarity

ZO-1 (Figure 4A), cytokeratin 8 (Figure 4B) and cytokeratin 18 (data not shown) were also expressed on RPE sheets (data not shown), and isolated cells in culture similar to rat RPE-J cells (Nabi et.al, 1993). Importantly all of these markers were present up to 32 passages after immortalization. Actin filaments were distributed predominately about the cell periphery as described for other epithelial cells (Figure 4C). Ezrin immunostaining (Figure 5A and B) revealed partial localization to the apical surface on RPE cell lines by Z-scans performed through the monolayer (Figure 5C). We found that mouse RPE cell lines expressed ezrin in a time dependent manner (Figure 5D).

Electron Microscopy

EM revealed the presence of junctions (Figure 6) in all RPE cell lines studied. This is in agreement with ARPE-19 cells grown on matrigel coated transwell filters which possess junctional complexes (Dunn et.al, 1996).

PCR

We found that mRNA expression for MMP-2, TIMP-2, MMP-14, collagen type IV and ER α /ER β ratio did not differ between immortalized and non-immortalized **wildtype** cultures of RPE cells (Table 2). Comparisons were only made on wildtype cell lines for this part of the characterization since we found a difference in expression of extracellular matrix molecules between ERKO cell lines and wildtype littermates as a consequence of the presence or absence of ER α or ER β (Elliot et.al, 2008).

Flow Cytometry

We found no difference between immortalized and non immortalized cell lines in the number of cells entering the stages of the cell cycle. $55.3 \pm 11\%$ of immortalized cells were in G1, $13 \pm 4.5\%$ in S, and $27 \pm 13\%$ in G2. Non-immortalized cells had $53 \pm 15.4\%$ in G1, $16.7 \pm 5.7\%$ in S, and $23.5 \pm 6.9\%$ in G2 (N=3).

Western blot analysis ER subtypes

Western analysis revealed the presence of both ER subtypes in wildtype cells (Figure 7A and B) as we have previously shown in human ARPE-19 cells and primary human RPE cells (Marin-Castano et.al, 2003).

Summary

We have successfully established mouse RPE cell lines from C57Bl/6 male and female mice. In addition we have cell lines derived from male and female ERKO α and ERKO β mice. The cell lines retain their cobblestone morphology and are contact inhibited at confluence. RPE cells *in vitro* expressed *in vivo* epithelial cell proteins including ZO1, expressed at tight junctions, actin filaments, in a pattern consistent with epithelial cells, and cytokeratin 8 and 18. Importantly, RPE65 and CRALBP, molecules synthesized by differentiated RPE *in vivo*, (Bunt-Milam and Saari, 1983; Hamel et.al, 1993) were expressed by all of the cell lines tested. Expression of these molecules has been shown in human ARPE-19 cells and rat RPE-J cells (Dunn et.al, 1996; Nabi et.al, 1993). SV2, a marker of neural cells, as well as Rhodopsin, a photoreceptor marker, were not expressed by our cell lines. Furthermore immortalization of RPE cells using the E6/E7 open reading frames of the HPV16 did not alter any *in vivo* markers or characteristics tested.

To study some of the aspects of *in vivo* pathology, RPE cell lines should ideally maintain their *in vivo* epithelial properties *in vitro*. We found that ezrin, a membrane organizing protein (Hayashi et.al, 1999) was expressed in the cell lines in a time dependent manner. It should be noted that these cell lines do not remain in a monolayer for longer than 20 days which may prevent total differentiation. Confocal microscopy revealed that ezrin was not fully localized to the apical membrane, although EM studies showed that junctions were present in all cell lines tested. Importantly most of the cell lines we tested in this study were isolated from aging mice, and recent studies have suggested that RPE acquire subtle but important age-related changes in phenotype (Binder et.al, 2007; Burke, 2008).

We and others have described the retention of the *in vivo* phenotype in other cell types. For example diabetic glomerular mesangial cells and fibroblasts retain their diabetic phenotype *in vitro* (Elliot et.al, 1993; Trevisan et.al, 1992). Therefore, RPE cells that are altered *in vivo* by oxidant injury or other genetic manipulations exhibit stable changes over multiple passages. We have propagated many of our cell lines for up to 32 passages, however it can not be ruled out, as in any cell culture experiments that increased passage number will lead to alterations of phenotype.

ECM components of RPE cells isolated from wildtype littermate control mice have not been altered by immortalization. This is important for our future studies utilizing these cell lines to determine therapeutic options to target ECM dysregulation that may occur in early dry AMD. We found that immortalization did not change any of the trimolecular complex components necessary for MMP-2 activation nor collagen type I or type IV mRNA expression. Importantly, the ratio of ER α /ER β and the ER subtype protein expression also was not altered by immortalization as has been shown with other immortalization vectors such as SV40.

Studies are ongoing involving the role of estrogens in controlling ECM turnover in RPE and as expected deletion of either ER subtype has profound effects on the expression of ECM molecules (Elliot et.al, 2008). Future studies of mice treated with compounds that target estrogen receptor activation as well as ECM components will benefit from this powerful technique. In addition, studies on other RPE related physiology and pathophysiology will be greatly advanced by the ability to isolate mouse RPE cell lines.

Acknowledgements

This work was supported in part by National Institutes of Health National Eye Institute Grant RO1 EY14477-04 (SJE and SWC). We would like to thank Hong Yu for the gift of HPV and Jason Fritz for technical assistance with the confocal microscopy.

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Figure 1. Mouse RPE sheets are viable and isolated cells retain their cobblestone appearance
A. RPE sheets are viable after isolation. Live cells fluoresce bright green, whereas dead cells fluoresce red. Comparison of immortalized mouse RPE cells (B) and human RPE cells (C). Mouse RPE cells retain their cobblestone appearance in vitro and have a similar appearance to human RPE cells, D. HPV protein was expressed by all cell lines tested as determined by Western analysis.

Figure 2. Growth Curve of representative RPE cell lines

Cells were plated in 10% containing medium and counted following a 24 hour attachment. Wells were counted on days noted on the x axis of the graph. Shown are two representative RPE cell lines (□) (●)

Figure 3. RPE65 and CRALPB, markers of RPE cells, are expressed in mouse immortalized cells
Western analysis for RPE65 and CRALPB was performed on immortalized, passage 20 RPE cell lysates as described in methods. Lanes 1 and 2: wt RPE cells no injury; lane 3: ERKO β no injury, lane 4; ERKO α no injury, lane 5: wt cells isolated from eyes injured in vivo, lane 6: ERKO α isolated from eyes injured in vivo, lane 7: ERKO β isolated from eyes injured in vivo, and lane 8 wt cells non transfected no injury. The same blot was stripped and reprobed with an antibody to actin to ensure equal loading of each lane.

Figure 4. Immunofluorescent localization of ZO1 (A) and Cytokeratin 8 (B) and Actin (C) are expressed in the pattern characteristic for epithelial cells, on mouse RPE cells in vitro

A: ZO1 positive bands are localized between RPE cells. B and C: Cytokeratin and actin filaments localize to the periphery of the cell. Magnification x 40.

Figure 5. Ezrin expression

A and B are XY projections of the entire 3D stack. C is a XZ reconstruction of a 10-voxel thick section (Slidebook 4.2) Light Blue DAPI (DNA), red, ezrin. Bars: AB 20 μ m C., μ m 10. D. Western blot analysis of three representative cell lines (#1 and #3; wildtype cell lines, #2; ERKO β cell line) revealed the presence of ezrin protein which increased over time.

Figure 6. Junctions are present in immortalized mouse RPE cells

Mouse RPE cell lines were grown on coverslips for 10 days and fixed for EM as described in methods. Representative photograph of RPE cell. Upper insets depict a single "kiss" tight junction. Scale bar 2 μ M

Figure 7. ER α (A) and ER β (B) protein expression in mouse RPE cells is not altered by immortalization

Western analysis of RPE cell lysates as described in methods. A. Lanes 1–3 represent three individual cell lines. B. Lanes 1–4 represent four individual cell lines. Rec; recombinant protein. Arrow indicates band of interest, 65kDa for ER α and 57kDa for ER β .

Table 1

mRNA expression of RPE65 in mouse cell lines

Cell Line	RPE65/18s
2214 ERKO β	1.75
2229 wtERKO β	1.69
2211 wtERKO α	1.78
2231 ERKO α	1.73
2221 ERKO α	1.9

Table 2
RPE mRNA expression before and after immortalization (representative wildtype cell lines)

Ratio molecule/18s	RPE65	ER α /ER β ratio(copy number)	MMP-2	TIMP-2	MMP-14	Collagen IV
Primary	1.87	2.6	84	123	6.3 $\pm$ 1.5	159
Immortalized cells	1.9	2.6	91	127	5.9 $\pm$ 1.3	152