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The use of cultured human fetal retinal pigment epithelium in studies of the classical retinoid visual cycle and retinoid-based disease processes

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ABSTRACT

Human fetal retinal pigment epithelium (hfRPE), when harvested by mechanical dissection and cultured initially under low calcium conditions, will proliferate and tolerate cryopreservation for future use. Cryopreserved cells can be subsequently thawed and cultured in standard calcium and in the presence of appropriate nutrients to a high state of differentiation, allowing recapitulation of multiple *in vivo* functions. In this review we briefly discuss some of our previous studies of the classical retinoid visual cycle and introduce current studies in our laboratory that involve two new areas of investigation; the dynamic response of the receptor for retinol binding protein, STRA6 to the addition of holo-retinol binding protein to the culture medium and the protective complement-based response of hfRPE to the ingestion of toxic byproducts of the visual cycle. This response is studied in the context of genotyped hfRPE expressing either predisposing or protective variants of complement factor H.

1. Introduction

The retinal pigment epithelium, although post-mitotic in the adult eye, is capable of replication *in vivo* under appropriate conditions. It is also endowed with the ability to re-enter the cell cycle when removed from the eye and placed in cell culture. Thus, since the early 1970's, vision scientists have been studying the cell biology of this important cellular monolayer *in vitro* (Albert et al., 1972; Mannagh et al., 1973). These efforts produced mixed results for several decades. Common among the difficulties encountered was dedifferentiation of the RPE from its normal cobblestone appearance to a fusiform phenotype that was neither morphologically nor functionally normal. Immortalization of RPE cultures through the use of large T antigen (Nabi et al., 1993) also produced cells that were less than optimal in appearance and function. Even RPE cells that transformed spontaneously (Dunn et al., 1996), although useful for certain studies, do not carry out the full repertoire of healthy RPE *in vivo*. However since 2001, the vision science community has had access to detailed RPE culture protocols that provide an opportunity to study the many facets of RPE *in vivo* function. An initial methods paper (Hu and Bok, 2001), followed by two modifications of human fetal RPE culture conditions (Maminishkis et al., 2006; Sonoda et al., 2009) have pushed the field forward. As far as we have ascertained, all three of these culture methods produce highly differentiated RPE that is useful for a variety of studies. Here we describe our method for producing large quantities of human RPE cells that can be readily frozen and used for multiple experiments. This also allows comparison of cell function among genotypic variants. We emphasize the utilization of these cultures for the study of the classical retinoid visual cycle and its toxic byproducts in a dish but also give examples of other processes that can be readily studied *in vitro* using these cultures.

2. Methods and materials

2.1. RPE culture

Our methods for the non-enzymatic harvesting of RPE from pairs of human fetal eyes, initial culture establishment and expansion of the cell population in low calcium medium (0.5 mM), freezing, thawing, differentiation and use in selected experiments

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are fully documented in two publications (Hu and Bok, 2001, 2010). The latter reference more specifically addresses the use of these cultures for the study of the basolateral uptake of Vitamin A (retinol) from retinol-binding protein (RBP), intracellular transport and processing by retinoid binding proteins and enzymes and enhanced secretion of 11-*cis* retinal into the apical culture medium in the presence of interphotoreceptor retinoid-binding protein (IRBP). The protocols outlined in these two publications are suitable for all experiments described in this review. Therefore, the fine details will not be repeated here. In brief, human fetal RPE (hfRPE) is typically cultured in 12 mm Millicell™ HA chambers whose nitrocellulose support membrane has been coated with recombinant mouse laminin (Hu and Bok, 2010). The differentiation medium is Eagles Minimum Essential Medium containing 1.8 mM calcium and the special additives that we have shown to be important for the generation of highly differentiated cultures. These additives include bovine retinal extract, which enhances the transepithelial resistance essential for rigorous transport studies.

2.2. Retinol-binding protein incubation

Cultured RPE cells are grown for two months in Millicell™ HA culture wells. Apo-retinol-binding protein (apo-RBP) is generated by bleaching recombinant holo-retinol-binding protein (holo-RBP) at 328 nm in a spectrofluorometer (Hu and Bok, 2010). The apo-RBP or holo-RBP are added at a concentration of 2.3 μM to the basal side of each culture well and incubation is carried out for various periods of time. In the example shown here, incubation proceeded for 3 h.

2.3. Challenge of RPE cultures with wild type or Abca4$^{-/-}$ photoreceptor outer segments

Outer segments from BALB/c (wild type) and Abca4$^{-/-}$ mice (containing A2E precursors) are isolated from animals 3–6 months of age. Following dissection of the retinas, they are placed in Hanks' balanced salt solution (HBSS, Invitrogen) containing 45% sucrose and gently vortexed. The sheared outer segments are sedimented at 10,000×g for 10 min at 4 °C, washed twice with HBSS and resuspended in DMEM (Sigma). The outer segments (OS) from 2 BALB/c or 2 Abca4$^{-/-}$ mice are then added to genotyped, differentiated RPE cultures and incubation is continued for 15 h. After removing the OS and washing with 37 °C DMEM, full strength human serum is added to the basal compartment of the chambers for 2 h. The cells are then washed once with HBSS, fixed, embedded in agarose, sectioned and stained with antibodies for confocal microscopy.

2.4. Vibratome sectioning

For confocal microscopy of monolayer cross-sections, the method of Hale and Matsumoto (2002) provides sections that are superior to cryosections. Following excision from its plastic chamber, the nitrocellulose support film and its attached cells are fixed at room temperature in 4% formaldehyde buffered with 0.1 M phosphate. They are embedded in agarose (Type XI, low gelling temperature, Sigma–Aldrich, St. Louis, MO). 50 μm sections are cut in a vibratome (VT1000s, Leica Microsystems, Germany).

2.5. Antibodies

The following primary antibodies were used for images displayed in this review. Rabbit polyclonal to human STRA6 (1:100, Abcam, Cambridge, MA), mouse anti-RPE65 (1:250, Millipore), rabbit anti-LRAT polyclonal antibodies prepared by us (Ruiz et al., 1999) and mouse anti-C5b-9 antibodies (1:50 Dako).

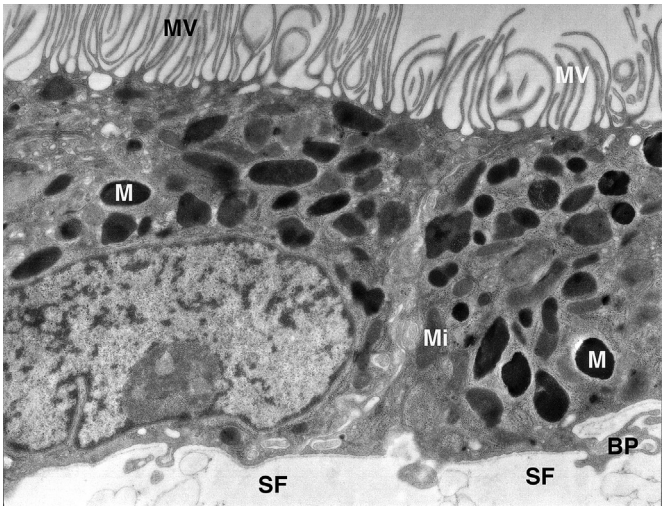

Fig. 1. Electron micrograph of cultured RPE. The apical surface of the cell monolayer is at the top, where abundant microvilli (MV) project into the culture medium. Melanin granules (M) are abundant after 2 months of culture and mitochondria (Mi) are largely displaced toward the basal part of the cell, the natural position for these organelles *in vivo*. Basal processes (BP) project into the nitrocellulose support film (SF).

2.6. Immunofluorescence

The fixed cells are permeabilized with TritonX-100 (0.1%) in phosphate buffered saline (PBS) for 10 min. After blocking with 5% goat serum and 1% bovine serum albumin, the cells are exposed to rabbit polyclonal anti human STRA6 antibodies (1:100, Abcam, Cambridge, MA) in blocking solution at 37 °C for 2 h. After washing three times with PBS, the cells are exposed to goat anti-rabbit IgG conjugated to Alexa-488 for 1 h at room temperature. The cells and their supports are then mounted on glass slides and covered with 5% N-propyl gallate in glycerol to protect from fluorescence fading. Images are captured with an Olympus Fluoview FV1000 confocal scanning laser microscope.

3. Results and discussion

3.1. Phenotype of differentiated, cultured human RPE

By two months in culture the monolayers are highly polarized with abundant apical microvilli facing the culture medium in the upper chamber of the Millicell (Fig. 1). The basal surface of the cell layer is tightly bound to the nitrocellulose support film, sometimes penetrating into the meshwork of the film by a micron or more. The cells are highly melanized and abundant mitochondria are mostly displaced toward the basal region of the cell. Intercellular junctional complexes are visible in appropriately aligned sections.

We know from studies of the RPE *in vivo* that a normal-appearing RPE phenotype does not necessarily guarantee normal function. For example, even in the presence of selected gene disruption, the RPE can be morphologically normal but lack the essential biosynthetic function of LRAT (Ruiz et al., 2007). Nonetheless, normal morphology is an essential starting point. Experimentally immortalized and spontaneously immortalized RPE cells uniformly lack essential morphological features such as abundant microvilli (Nabi et al., 1993; Davis et al., 1995; Dunn et al., 1996). RPE cells cultured under the conditions described here have all of the standard features observed *in vivo*. For example, our previous