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Research article

Characterization of a spontaneously generated murine retinal pigmented epithelium cell line; a model for in vitro experiments

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ABSTRACT

Retinal pigmented epithelium (RPE), the outermost layer of the retina, has a key role in maintaining retinal cells' functions. Severity of the culture of RPE cells has exerted many limitations to both *in vitro* and *in vivo* studies and its therapeutic applications. Therefore, establishment of RPE cell lines with high proliferative potential can considerably improve study of RPE cell biology. Here we report generation of a spontaneously immortalized murine RPE cell line in primary mouse RPE cell culture. Founded colonized cells were picked up and expression of RPE and retinal progenitor cells' (RPC) markers were studied using immunocytochemistry (ICC). Emerged cells cultured over 35 passages and population doubling times in different serum concentrations were calculated. We also investigated the ability of cells for becoming transfected by calcium-phosphate method and for becoming infected by adeno-associated virus serotype 2 (AAV2) using flow cytometry. Data showed that the cobblestone constituent cells expressed RPE65, cytokeratin and ZO1 and moreover several progenitor markers such as Pax6, Sox2, Nestin and Chx10. It revealed that, despite primary RPE cells, the newly emerged cells were easily transfectable and were highly infectable when compared with HEK293T cells. Our data indicated that the emerged mouse RPE cell line pretended RPC-like phenotype and also simultaneously expressed RPE markers. It would be a promising model for leading studies on RPE and RPC cells and substantially confirmed the great RPE plasticity and its invaluable potential in research studies.

1. Introduction

The retinal pigmented epithelial (RPE) layer is a monolayer of highly polarized pigmented cells located outermost on the retina and plays a key role in maintaining retina function. This layer as a metabolic gatekeeper mediates a bidirectional transport between the neural retina and the vascular choriocapillaris that regulates the chemical composition and volume of the subretinal space [9,11,15]. These cuboidal hexagonal cells are in charge of a critical role in metabolic and cellular supporting of the retinal photoreceptors [12,24,30,36]. In mammalian, RPE cells, such as other retinal cells, enter post-mitotic phase in early development and get non-proliferative throughout the life [25], but several studies have demonstrated the plasticity of these cells *in vitro* [3,11,41]. RPE cells in non-human organisms, such as amphibians and chick and mouse embryo show the high capacity of self- regeneration and differentiation potential [18,40]. In humans, in some pathological conditions such as proliferative vitreoretinopathy (PVR), plasticity of RPE cells has clearly proved [2]. Furthermore, some studies showed some subpopulations of RPE primary cells can enter into mitotic phase and gain multipotential capacity [28].

Defects in RPE cells that occur in diseases such as retinitis pigmentosa (RP) and age-related macular degeneration (AMD) can lead to various ocular impairments. Hopes for treatment of degenerative diseases mostly are based on gene and cell therapies [14,45]. Along with clinical objectives, the unique retina structure and superior development of retinal cells have triggered creditable researches in this field.

In many studies, RPE cells are isolated and cultured *in vitro*. RPE cells generally loss their morphological and functional characteristics and get fibroblast-like or fusiform variants in figures. Although these primary cells express RPE specific markers such as RPE65 and Cytokeratin, they do not exhibit epithelial morphology *in vitro* and specify limited proliferation rate and involve senescence features very soon in cultures. Generation of RPE cells that

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are keeping their appreciable features, as like as RPE cells *in vivo*, and are maintaining characterizations to reproduce experimental results would be crucial for advancement of retinal cell biology and *in vitro* production of retinal cells. For these purposes some researches groups have generated induced RPE cell lines [8,23] or modified culture methods to gain functional and epithelial-like cells [22,34]. Generation of human RPE cells from pluripotent cells is one of the promising ideas in this field [4,5,16,26,29,33]. Isolation of spontaneously generated cell lines is possible from some primary cells in different organisms such as human [35] , bovine [46] and mice [13,20,21,44]. This method helps to maintain cell context without oncogene delivery or using different methods to produce the desired cell type. Due to differences in Hayflick limit in organisms [6,42], generation of spontaneously cell lines is more likely in mice than in human cells. However, spontaneously generated RPE cell lines from different organisms including mice [9] , human [10] and bovine [19] have been reported previously.

We could detect hexagonal colonized cells in mouse RPE primary cultures and assessed them for their behavior as an alternative substitution for RPE cells. To confirm this assumption we characterized the isolated cells for RPE and retinal progenitor cells' (RPC) markers. We analyzed the proliferation potential and the ability of isolated cells for transgene tolerance by calcium-phosphate transfection and adeno associated virus serotype 2 (AAV2) infection. We reported that the generated mouse RPE cell line pretended RPC-like phenotype and can be used for prospective studies.

2. Materials and methods

2.1. Animals and care

RPE cells were isolated from about 2 months NMRI female mice. The animals were obtained from National Institute of Genetic Engineering and Biotechnology (NIGEB) animal house and all experiments were performed under laboratory animal welfare and ethical rules. The mice were housed under normal conditions of 12 h dark/12 h light, 20–25 °C temperature and free access to water and food.

2.2. RPE cells isolation and culture

Animals were killed by cervical dislocation. Eyes enucleated and placed in 1x phosphate buffer saline (PBS) supplemented with 2 × penicillin/streptomycin. Under stereo microscope in culture room, cornea and lens were removed. The retinas, after a brief mechanical digestion with scalpel, were transferred to 1 U/ml of dispase I solution (Invitrogen, Brussels, Belgium) and incubated for 50 min in 37 °C. Following centrifugation, the pletted cells were cultured in DMEM/F12 medium (Biowest, Nuaillé, France) supplemented with 20% FBS. The medium was changed every 2–3 days. The cells were subcultured in a ratio of 1:3, by using 0.25% Trypsin (w/v; Sigma, Munich, Germany)–1 mM EDTA (Merck, Darmstadt, Germany). In the third passage, spontaneously generated RPE colonies were picked up with scratching method by a pipette tip. To achieve single cell culture, colonies were trypsinized and then subcultured in DMEM/F12 medium supplemented with penicillin/streptomycin and 10% FBS.

2.3. Immunocytochemistry

Using standard techniques, primary cells were grown on coverslips until semi-confluence. Cultures were washed with PBS (pH 7.4) and fixed for 10 min with chilled methanol at 4 °C. Non-specific binding was blocked by incubating with 5% (w/v) bovine serum albumin (BSA) in PBS for 1 h at room temperature. Then, the cells were incubated with primary antibodies (all antibodies from Santa Cruz Inc., TX, USA): mouse anti-cytokeratin 8/18 1:100 (sc-52325), rabbit anti-RPE65 1:100 (sc-32893), rabbit anti-ZO1 1:100 (sc-10804), goat anti-pax6 1:100 (sc-7750), goat anti-sox2 1:100 (sc-17320), goat anti CHX10 1:100 (sc-21690), rabbit *anti*-nestin 1:100 (sc-20978). Incubation was performed in 0.5% BSA, overnight at 4 °C. Secondary antibodies rhodamine-conjugated anti-rabbit 1:400 (sc-2091) and FITC-conjugated anti-mouse 1:200 (sc-2010), anti-goat 1:200 (sc-2024), anti-rabbit 1:200 (sc-2012) were applied for 2 h at room temperature. Finally, the cell nuclei were stained with 1 mg/ml of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Santa Cruz Inc., TX, USA) for 3 min in the dark at room temperature (RT). The coverslips were mounted onto slides using fluorescence mounting media (glycerol 80%, PBS 10%, and phenyl diamine 10% [w/v]). The negative controls were processed as described above but without any primary antibody. An Axiophot Zeiss fluorescence microscope equipped with a 460 nm filter for DAPI and a 520 nm filter for the FITC was used to observe and taking images of the cells.

2.4. Growth curve tracing and population doubling time calculation

1000 cells /cm2 were plated in 24-well plate in different serum concentrations (2.5%, 5% and 10%). The cells were counted, as triplex, every day (zero day was the first day after seeding) with trypan blue dye exclusion method until cultures reached to confluency. After drawing the growth curves, comparisons were done by Student's T-test and $P < 0.05$ was considered significant. To calculate population doubling time (PDT) the following formula was used. $PDT = 1/[3.32(logN2-logN1)/T2-T1]$.

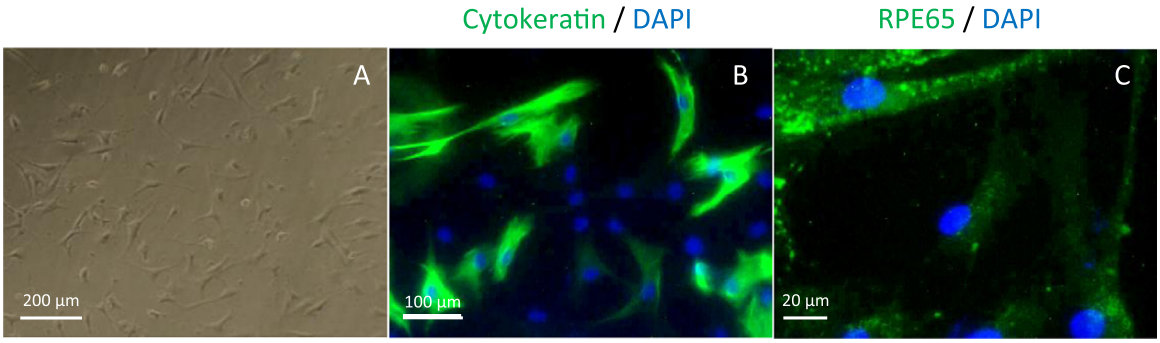

Fig. 1. Isolation and characterization of mouse RPE cells. (A) Image of the primary RPE cells at the first passage under standard *in vitro* culture conditions. Morphologically, these cells were fibroblast-like. (B-C) Merged images were immunofluorescently labeled for RPE markers'specific antibodies including Cytokeratin (B) and RPE65 (C) and DAPI nuclear staining (blue).