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Isolation and Culture of Primary Mouse Retinal Pigment Epithelial (RPE) Cells with Rho-Kinase and TGF β R-1/ALK5 Inhibitor

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background:

Primary RPE cells could be a reliable model for representing *in vivo* status of RPE compared with cell lines. We present a protocol for *in vitro* isolation and culture of primary RPE cells from C57BL mice.

Material/Methods:

We used C57BL mice ages 7 days to 4 months. The RPE layer was separated from the neural retina layer by digestion with 2% Dispase for 45 min and scraped off from the choroid after 25-min incubation in 37°C. Collected RPE sheets were gently pipetted up into smaller sheets. RPE sheets were transferred into well plates and cultured *in vitro* for 2 weeks. To inhibit epithelial-mesenchymal transition (EMT) of RPE cells, we used Y27632 and Repsox to treat cultured primary RPE cells.

Results:

RPE cells isolated from C57BL mice maintained pigmented and hexagonal morphology in culture. However, long-term *in vitro* culture lead to the periphery cells of a RPE sheet becoming mesenchymal-like cells. In contrast to the control group, Y27632 and Repsox, which are inhibitors of Rho-kinase or TGF β R-1/ALK5, promoted primary RPE cells to maintain epithelial-like morphology and eventually become confluent.

Conclusions:

RPE cells isolated from C57BL mice could be a powerful cell model to study the biological function of RPE. Especially, C57BL mice with different defective genetic background resulting in ocular diseases, would expand the genome type of RPE cells. The method presented here could be an efficient and applicable technique to obtain large numbers of primary RPE cells that maintain some characteristics of *in vivo* RPE.

MeSH Keywords:

Mice, Inbred C57BL • Primary Cell Culture • Retinal Pigment Epithelium

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Background

The retinal pigment epithelium (RPE), located between photoreceptor and Bruch's membrane, is a monolayer structure composed of hexagonal cells [1]. The function of RPE cells includes light absorption, nourishment of photoreceptors, phagocytosis of photoreceptor outer segment (POS), maintenance of the blood-retinal barrier, and secretion of various growth factors [2].

RPE dysfunction can cause many diseases leading to blindness, such as age-related macular degeneration (AMD), retinitis pigmentosa (RP), and diabetic retinopathy (DR) [3–5]. The study of the mechanism of these diseases needs *in vitro* culture of primary RPE, as the immortalized cell line loses some critical characteristics of RPE cells. It is also difficult to get human RPE due to the limited donor resources. On the other hand, with the extensive application of genetic-modified mice in biological fields, using primary mouse RPE cells has the advantage of maintaining the genetic *in vivo* features.

Early in 1978, scientists were searching for methods to culture mouse primary RPE cells. It was Edwards who first successfully isolated rat RPE cells by storing the enucleated eye for 6–24 h in a balanced salt solution prior to treatment with 0.1% trypsin [6]. However, because mouse eyes are too small and have fewer cells, and the cells from older mice cannot proliferate well, we have not yet established a method which is quick and easy to operate [7–10]. RPE cells isolated by most of current methods gradually lose pigmentation, polygonal shape, and transepithelial electrical resistance (TER) [11].

Hence, we describe an efficient protocol for primary culture of mouse RPE cells. We compared 2 different methods: single-cell culture and cell sheets culture. Furthermore, we used a small molecular cocktail, Y27632 and Repsox. The results demonstrated that cultured mouse primary RPE cells can maintain the RPE-specific epithelial morphology.

Material and Methods

Procedure of mouse primary RPE cell isolation and culture

Our method was simplified and optimized from a previous protocol established by Gibbs [12]. C57BL/6 mice ages 7 days to 4 months were sacrificed by cervical dislocation. The age of the mice was not critical to the success of our experiment. Intact eyes with optic nerve were cut out gently using curved scissors and immediately dipped in a 3.5-cm petri dish containing fresh DMEM-F12 (GIBCO, NY, USA) + 5% penicillin-streptomycin (P/S) (GIBCO, NY, USA) on ice. Eyes were then washed twice (5 min each) under a sterile hood. With the help of a dissecting stereomicroscope, we used Dumont #5 tweezers

and angled scissors to cut down all the muscles and tissues around the eye ball, and then we washed again to clean away the remaining blood and tissues before placing the eye balls in 2% Dispase (GIBCO, NY, USA) in DMEM-F12 medium for 45 min at 37°C in a 5% CO₂-aerated incubator.

We then transferred the eye balls to a dish with growth medium (GM) consisting of DMEM-F12 +10%FBS+5% P/S before carefully washing the eye balls with a p200 micropipette to neutralize the enzyme activity of the Dispase.

Intact eyes were put in a new petri dish with GM for dissection under a laminar airflow hood. An upright dissection microscope was used; the eyes, fixed with Dumont #5 tweezers, were cut by 8-cm Vannas scissors along the corneal sclera edge. We gently removed and discarded the cornea, iris epithelium, and lens. When the lens and iris are removed, it was possible to simultaneously bring out the retinal layer, although some of the RPE cells would stick to the retina. The retina and the posterior sclera were put in GM and incubated for 25 min in a 37°C and 5% CO₂ incubator to help the separation of the neural retina from RPE. At this point, the RPE layer should be completely separated from the neural retina. If some RPE cells are noted to tightly adhere to the neural retina, it is still possible to use the tweezers to peel them off. Care should be taken to avoid damage to the retinal structure; otherwise, it would be easy to cause contamination of the neural retina cells.

After removal of the retina, the RPE layer was attached to the choroid. We used tweezers to make the optic nerve as the center and cut the remaining cup-like structure into a 4-leaf shape. Therefore, a single layer of RPE cells, located above the choroid, can be clearly observed under the microscope at this time. From the inside to outside, the optic nerve to the corneal limbus can be carefully scraped off the RPE sheet with an ophthalmic scraper, and then the scraped RPE debris can be transferred into a new petri dish with a p200 micropipette. The RPE fragments on each flap were scraped and collected with the same method. After collection, we transferred the RPE sheets into a new petri dish by p200 micropipette; this dilution can be used to reduce the contamination of other type cells, especially neural retina and choroid.

The collected RPE sheets were placed in a 1.5-ml EP tube prior to centrifugation at 1000 rpm for 5 min at room temperature. The supernatant was discarded and then the RPE cells were gently resuspended in a 1-ml complete medium (DMEM-F12+15%FBS (Hyclone) +2% P/S). As some of the RPE fragments would be large, we needed to use a p200 micropipette to pipette the sheets gently about 10 times. We put cells from 2 mouse eyes into o1 well of the 4-well plate with 500 µL complete medium. Cells were confirmed to be evenly distributed under a microscope before transferring into a 37°C 5% CO₂ incubator.

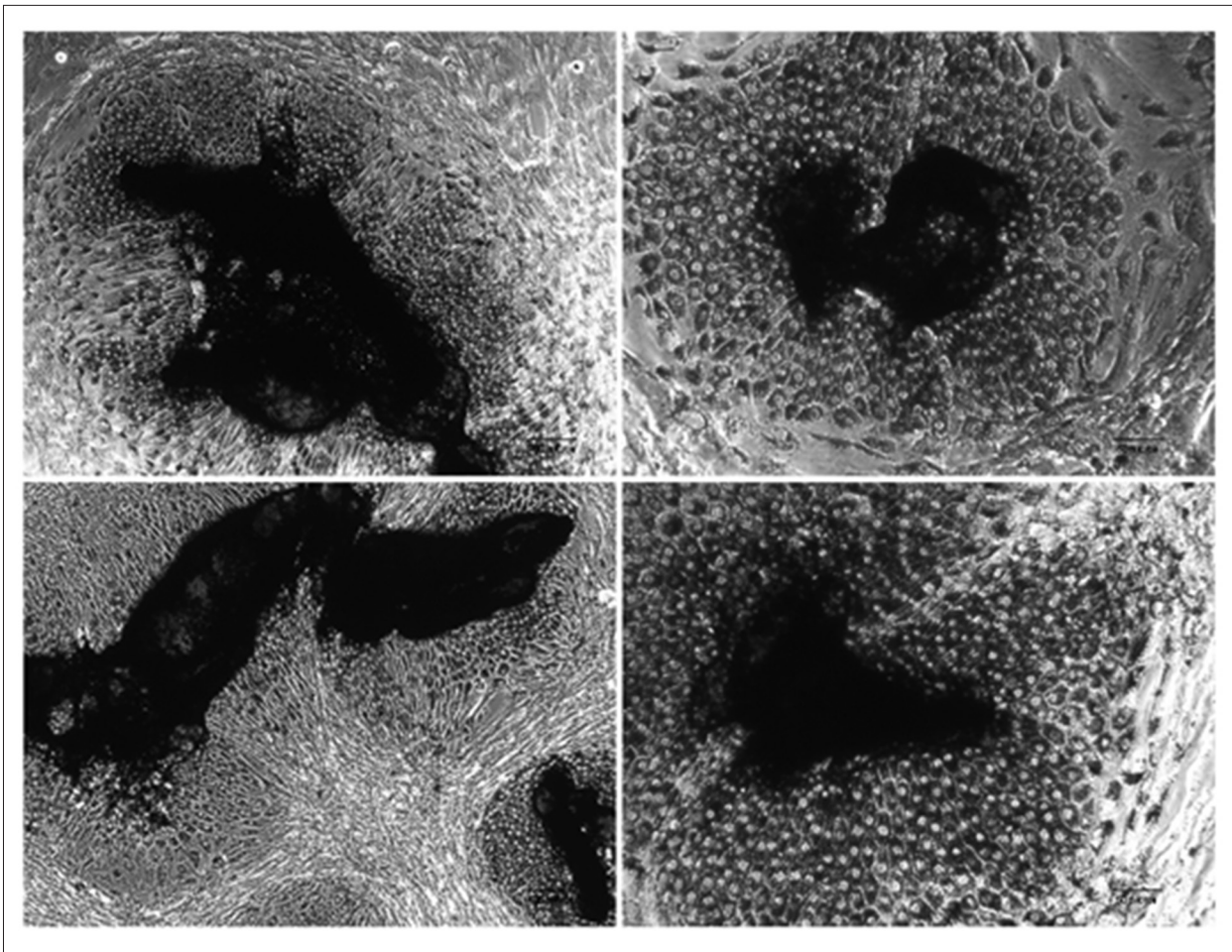


Figure 1. Morphology of primary C57BL/6 mouse RPE cells grown as cell sheets after seeding at 2 weeks, central cells maintained pigmented particles and polygonal morphology but peripheral cells transformed into fibroblast cells. Scale, left: 100 μm , right: 50 μm .

The culture dish was kept in undisturbed condition for at least 48 h to allow cell attachment to the dish. Culture medium was half-changed every 2 days through removing half of the culture medium in the dish and then adding half of the fresh culture medium.

Results

Morphology of the mouse primary RPE cell

Since the loss of cell-cell tight junctions will lead to epithelial-mesenchymal transition (EMT) of RPE, RPE sheets or RPE clusters method was used in our primary culture. RPE cells migrated out from cultured RPE sheets at 2 weeks. Central cells maintained polygonal morphology and pigment particles (Figure 1), but peripheral cells turned into mesenchymal-like cells.

Those cells that grew too far from others in the culture dish also contained pigment particles but failed to establish cell-cell junctions and showed no polygonal morphology (Figure 2).

To facilitate the cultured mouse primary RPE cells, to maintain the RPE specific morphology, and to proliferate steadily, we treated the cells with small molecules combination, Y27632 (50 μM , Sigma) and Repsox (10 μM , Selleck) (RY). Cell morphology was analyzed under the bright-field microscope. The results showed that the RPE cells in the RY-treated group proliferated well and maintained epithelial-like morphology, while those in the control group transformed into fibroblast-like cells. We also found that those newly-split RPE cells did not contain pigmented particles, but they presented a polygonal morphology in the RY-treated group, which indicates that RY effectively inhibits EMT and promotes proliferation of primary RPE cells (Figure 3).

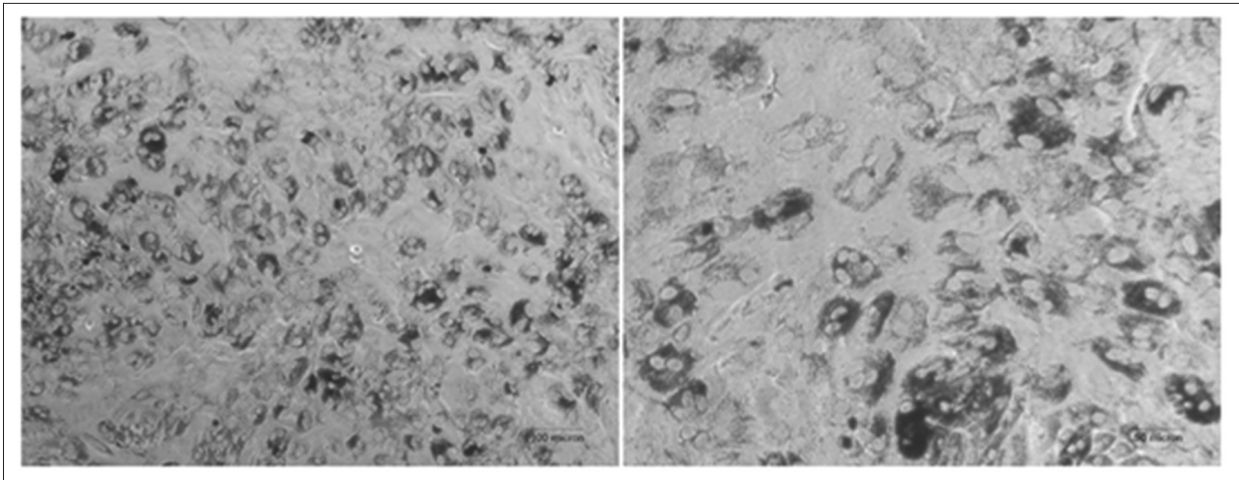


Figure 2. Morphology of primary C57BL/6 mouse RPE cells grown as single cells after seeding at 2 weeks; no cell-cell junctions were observed. Scale, left: 100 µm, right: 50 µm.

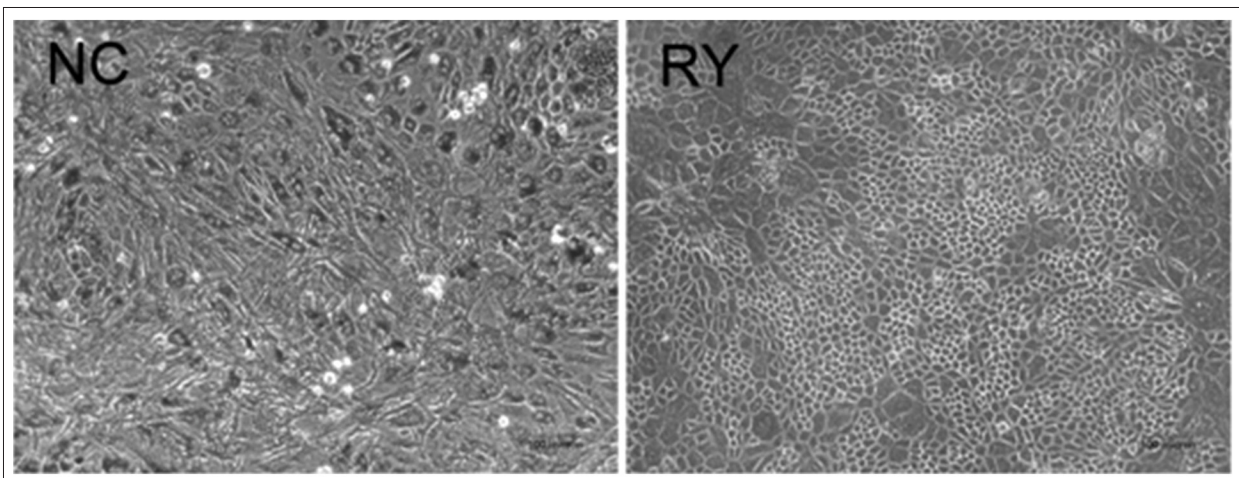


Figure 3. Morphologies of primary C57BL/6 mouse RPE cells at 10 days in control and RY group. All cells in the RY group showed a uniform, epithelial-like morphology and reached a high confluence. Scale: 100 µm.

Discussion

Mouse models have been extensively used to mimic various retinal diseases, including oxidative stress model, AMD model, and degenerative retinal degeneration model [13–15]. Primary mouse RPE cells have also been extensively used to study the function of RPE *in vitro*, but most of the isolated primary RPE cells are mainly cultured into single cells by trypsin digestion [16]. Although these RPE cells presented epithelial morphology, they lacked cell-cell tight junctions and a polygonal shape. With the increasing time of *in vitro* culture, they would become mesenchymal-like cells and lose pigmentation.

Here, we optimized a method for culturing mouse retinal pigment epithelial cells with the use of Dispase to detach the neural retina from the RPE layer and mechanically peeling off the RPE from the choroid. This method is easier, faster, and is reproducible,

requiring little special surgical skill. This may provide an advantage over existing methods, for example, compared with previous protocols that were designed for mice ages 10 to 15 days [3,9–11]. This method could be used to successfully collect RPE cells from mice at any age from 7 days to 4 months. This method does not require use of special reagents, such as some protocols that used complicated growth medium, including DMEM-high glucose plus 10% fetal calf serum, 1% P/S, 2.5 mM L-Glutamine, and 1×MEM non-essential amino acids. Our method only uses DMEM-F12 plus 15% FBS and 2% P/S. For the washing buffer, we only require DMEM-F12 plus 5% P/S, as opposed to the previous one using Ca^{2+} - and Mg^{2+} - free Hank's balanced salt solution (Gibbs et al., 2003). Furthermore, our protocol is effective and efficient because it takes less than 3 h and cells can maintain their polygonal morphology and pigmentation for 2 weeks. The presented protocol does not require transwell and pre-coated dishes with matrigel or laminin, as previously proposed [17,18].

In this optimized method, RPE cells maintain a better epithelial-like morphology when cultured in RPE sheets. However, with long-term *in vitro* culture, cells located on the periphery of RPE clusters were also susceptible to EMT. Y-27632 is a Rho-kinase inhibitor and has previously been shown to prevent the fibrosis of RPE cells [19]. It has been reported that TGF β -induced EMT in lens epithelial cells can be reversed in the presence of Y-27632 [20]. Repsox, a TGF- β antagonist, is demonstrated to have a reprogramming function and lead to EMT-MET [21]. We further used these 2 small molecules together to treat cultured mouse RPE cells. Our results show that the combination treatment of Repsox and Y27632 inhibits the EMT of RPE cells and maintains RPE epithelial-like morphology.

To successfully isolate and culture RPE cells, some critical steps need to be followed. Firstly, the digestion time of Dispase is very crucial. We recommend 45 min because if the time is shorter, we cannot separate the neural retina from the RPE layer, and if longer, the RPE layer with the neural retina can be detached together from the choroid. Secondly, confluence of

cells at seeding is fundamental for the formation of the RPE monolayer. Furthermore, RPE sheet is the key for the cells to achieve tight junctions; thus, the RPE cells need to be gently pipetted about 10 times. Finally, we have to make sure that the cells are evenly distributed without any bubbles.

Conclusions

We provide an efficient and reliable protocol for isolation and culture of mouse primary RPE cells. The cultured cells maintain RPE characteristics such as polygonal morphology and pigmentation. This method should be useful in studying functions of RPE cells from mouse models with various genetic defects that can result in retinal degeneration diseases.

Conflict of interests

None.

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