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Novel method for the rapid isolation of RPE cells specifically for RNA extraction and analysis

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ABSTRACT

RPE cells are involved in the pathogenesis of many retinal diseases. Accurate analysis of RPE gene expression profiles in different scenarios will increase our understanding of disease mechanisms. Our objective in this study was to develop an improved method for the isolation of RPE cells, specifically for RNA analysis. Mouse RPE cells were isolated using different techniques, including mechanical dissociation techniques and a new technique we refer to here as "Simultaneous RPE cell Isolation and RNA Stabilization" (SRIRS method). RNA was extracted from the RPE cells. An RNA bioanalyzer was used to determine the quantity and quality of RNA. qPCR was used to determine contamination with non-RPE-derived RNA. Several parameters with a potential impact on the isolation protocol were studied and optimized. A marked improvement in the quantity and quality of RPE-derived RNA was obtained with the SRIRS technique. We could get the RPE in direct contact with the RNA protecting agent within 1 min of enucleation, and the RPE isolated within 11 min of enucleation. There was no significant contamination with vascular, choroidal or scleral-derived RNA. We have developed a fast, easy and reliable method for the isolation of RPE cells that leads to a high yield of RPE-derived RNA while preserving its quality. We believe this technique will be useful for future studies looking at gene expression profiles of RPE cells and their role in the pathophysiology of retinal diseases.

1. Introduction

The retinal pigment epithelium (RPE) is a versatile monolayer of cells located between the retina and the choroidal vasculature. A wide range of metabolic pathways are constantly active in RPE cells, and are essential to maintaining the delicate homeostasis of the retina. Among its many functions, the RPE is involved in the phagocytosis of shed photoreceptor outer segments, the metabolism of retinol, the formation of the extracellular matrix, the formation of the outer blood-retinal barrier, and the transport of nutrients and debris to and from the retina (Thumann et al., 2006). As a consequence, changes affecting RPE cell function or their metabolic activity can have very serious effects on vision. Age-related macular degeneration is just one of many diseases in which changes in RPE function play a major role. Exploring the gene expression profile of these very complex cells under different conditions may hold important clues as to the pathogenesis of retinal diseases. However, there have been significant challenges to analyzing mRNA expression of RPE cells reliably. The first step to achieve such a goal is the isolation of RPE cells from posterior eye cups. This has been done for at least 4 decades using different techniques. These techniques include mechanical disruption techniques (with brushes (Saari et al., 1977) or forceps (Wang et al., 1993; Liu et al., 2010), enzymatic dissociation (Mannagh et al., 1973; Flood et al., 1980; Edwards, 1982, 1977; Sakagami et al., 1995; Jaffe et al., 1990) and freeze-thawing followed by fluid flushing (Donita Garland and Eric Pierce – personal communication/ARVO 2010 poster #2590, A379). Clearly, these techniques are very useful for specific purposes including RPE culturing, or studying sub-RPE structures (Garland and Pierce, personal communication). However, there may be some concerns using these techniques when the goal is to isolate RNA for gene expression studies.

Isolating RPE cells from posterior eye cups in a manner that preserves RNA quality, quantity and gene expression profile is challenging. Enzymatic treatments with hyaluronidase, collagenase, trypsin and/or proteinase K involve prolonged incubations. This could in theory affect both the quality of the RNA and also the mRNA expression profile. Mechanical techniques may increase the risk of significant contamination from choroid, and may also affect the mRNA quality and expression pattern. Wang et al. reported that

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RNA quality from ocular tissues preserved in RNAlater was superior to incubation on ice or snap freezing techniques (Wang et al., 2001). However, as we demonstrate here, RNAlater poses significant problems when isolating RNA from RPE cells. This study reports the development of a new technique for the isolation of RPE cells specifically for the purpose of RNA isolation and analysis. This technique is simple, fast, and results in the isolation of RPE cells with high yield and purity. Importantly, it also preserves the quality of the mRNA.

2. Materials, supplies and detailed methods

2.1. Animals

Adult C57BL/6 wild type mice were used for these experiments. The mice were bred and kept in a barrier animal facility at UT Southwestern Medical Center under normal lighting conditions with 12 h on, 12 h off cycles. All animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision research and approved by the institutional animal care and use committee (IACUC) of the UT Southwestern Medical Center.

2.2. RPE cell isolation by mechanical dissociation method

Mice were anesthetized with a ketamine-xylazine cocktail (100 mg/kg ketamine, 5 mg/kg xylazine). The first eye was enucleated and was immediately placed on a gauze soaked with PBS. Vannas scissors were used to open the eye just behind the limbus, and the anterior segment was discarded (cornea, iris and lens). The retina was carefully removed, exposing the RPE. The posterior eye cup (sclera-choroid-RPE) was then transferred into a small petri dish and was flash frozen in liquid nitrogen. PBS (200 μl) was then flushed onto the inside of the posterior eye cup using a pipette with a 200 μl tip. This forceful flushing was repeated 20 times, reusing the same PBS. The PBS (approximately 200 μl), containing brown clumps of released material (presumably RPE cells), was transferred into a 2 ml centrifuge tube, and 1.8 ml of RNAlater (Qiagen catalog # 76104) was added. At this time the second eye was enucleated and the same procedure repeated in order to isolate the RPE cells into a different 2 ml tube. After 10 min in the RNAlater:PBS (9:1), the entire contents of the 2 ml tube were filtered through a Microcon centrifugal filter (Millipore Cat# 42416) according to the manufacturer's protocol. The recovered pigmented material was then used for RNA extraction as described below.

2.3. RPE cell isolation and RNA extraction using SRIRS (simultaneous RPE isolation and RNA stabilization) method

Mice were anesthetized with a ketamine-xylazine cocktail (100 mg/kg ketamine, 5 mg/kg xylazine). After enucleation, the anterior segment and retina were removed as described above. The remaining posterior eye cup was quickly dipped in PBS in order to quickly washout any adherent debris. It was then immediately transferred into a 1.5 ml microcentrifuge tube containing 200 μl of RNAprotect cell reagent (Qiagen cat. 76526). It took roughly 1 min from the time of enucleation to the transfer into the RNAprotect-containing microcentrifuge tube. The eye cup was incubated for 10 min at room temperature. The tube was briefly agitated to ensure most of the RPE cells were released and then the eye cup was removed. Centrifugation was then performed for 5 min at 2500 rpm (685× g) to pellet the RPE cells, which were then subjected to total RNA extraction using the RNeasy Micro Kit (Qiagen cat. 74004) per the manufacturer's instructions. In brief, 75 μl of lysis buffer (RLT) were added and the sample was homogenized with a disposable pestle grinder (Fisher 03-392-106), and then centrifuged for 2 min at 16,000× g. The supernatant was transferred to a new tube, and 70% ethanol (1:1 volume) was added. The entire sample was transferred to an RNeasy MinElute spin column. In the final SRIRS protocol a DNA digestion step using DNase I was performed, per manufacturer's protocol, directly on the RNeasy MinElute spin column. We used 30 units of DNase I per sample and incubated for 15 min at room temperature. The RNA was eluted with RNase-free water. We used an RNA carrier during our isolation protocol due to the low concentration of RPE RNA isolated from the cells. For the initial experiments (Figs. 1 and 2) we used a poly-A carrier RNA (included in RNeasy Micro Kit). However, for the last experiments we switched to an *Escherichia coli* ribosomal carrier RNA (Roche cat 206938) in order to avoid interference with the cRNA generation needed for the Illumina gene expression microarray assay (microarray data is not shown here).

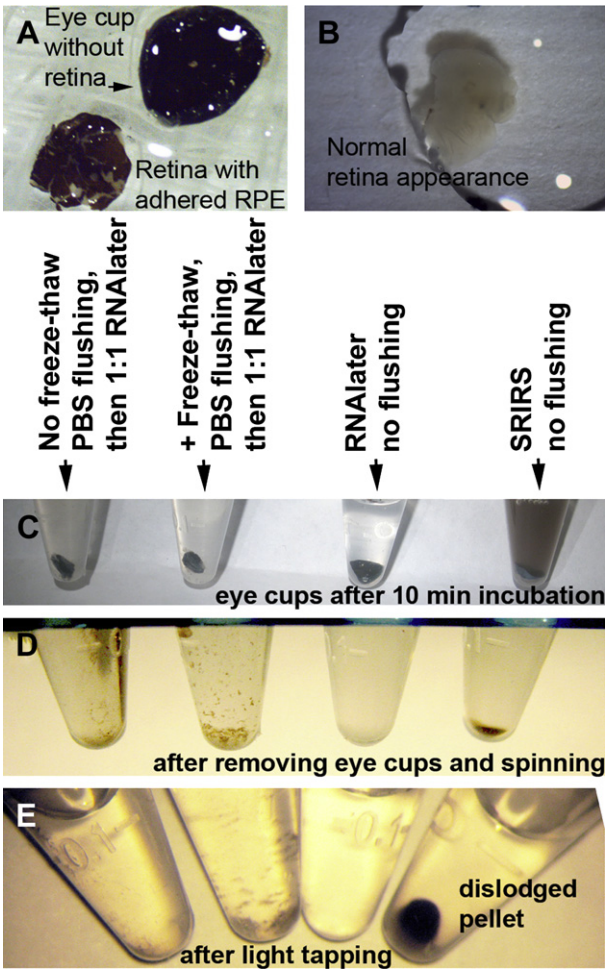

Fig. 1. Photographic documentation of steps in the different RPE isolation protocols. (A) Exposure of the eye cup to RNAlater before removing the retina leads to the tight adherence of RPE cells to the retina. (B) There is no detectable adherence of RPE cells to the retina when the eye is exposed to RNAprotect (as part of the SRIRS protocol) instead of RNAlater prior to the removal of the retina. (C) Posterior eye cups (after removing the retina) were used for RPE isolation. There is a marked increase in the release of RPE cells using the SRIRS protocol (last tube on the right) vs. mechanical isolation using PBS flushing (first two tubes) or simple exposure to RNAlater (third tube). RNAlater was added to the first two tubes (1:1 by volume) after the PBS flushing. (D) Centrifugation leads to a well-formed pellet only in the SRIRS tube. (E) Another view of the tubes (tubes are lying down) after a light tapping has released the pellet in the last tube, demonstrating the size/density of the pellet in the SRIRS sample.