Cultured Human Retinal Pigment Epithelial (hRPE) Sheets: A Search for Suitable Storage Conditions

Ayyad Zartasht Khan[1,2], Tor Paaske Utheim[1,3,4,5,6,7,8], Sjur Reppe[1], Leiv Sandvik[9], Torstein Lyberg[1], Borghild Barth-H Roald[2,10], Ibrahim Basim Ibrahim[2], and Jon Roger Eidet[1,3]

[1] Department of Medical Biochemistry, Oslo University Hospital, Kirkeveien 166, P.O. Box 4956, Nydalen, 0424 Oslo, Norway;
[2] Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, P.O Box 1171, Blindern, 0318 Oslo, Norway;
[3] Department of Ophthalmology, Oslo University Hospital, Kirkeveien 166, P.O. Box 4956, Nydalen, 0424 Oslo, Norway;
[4] Department of Ophthalmology, Drammen Hospital, Vestre Viken Hospital Trust, Dronninggata 28, P.O. Box 800, 3004 Drammen, Norway;
[5] Department of Ophthalmology, Stavanger University Hospital, Stavanger, Norway;
[6] Department of Plastic and Reconstructive Surgery, Oslo University Hospital, Kirkeveien 166, P.O. Box 4956, Nydalen, 0424 Oslo, Norway;
[7] Department of Clinical Medicine, Faculty of Medicine, University of Bergen, Bergen, Norway;
[8] Institute of Oral Biology, Faculty of Dentistry, University of Oslo, Sognsvannsveien 10, P.O. Box 1052, 0316 Oslo, Norway;
[9] Department of Periodontology, Institute of Clinical Odontology, Faculty of Dentistry, University of Oslo, Sognsvannsveien 10, P.O. Box 1052, 0316 Oslo, Norway;
[10] Department of Pathology, Oslo University Hospital, Kirkeveien 166, P.O. Box 4956, Nydalen, 0424 Oslo, Norway.

ABSTRACT

The advancement of human retinal pigment epithelial cell (hRPE) replacement therapy is partly dependent on optimization of cell culture, cell preservation, and storage medium. This study was undertaken to search for a suitable storage temperature and storage medium for hRPE. hRPE monolayer sheets were cultured under standard conditions at 37°C and then randomized for storage at six temperatures (4°C, 16°C, 20°C, 24°C, 28°C, and 37°C) for seven days. After revealing a suitable storage temperature, hRPE sheets were subsequently stored with and without the silk protein sericin added to the storage medium. Live dead assay, light microscopy, transmission electron microscopy, pH, and phenotypic expression of various proteins were used to assess cell cultures stored at different temperatures. After seven days of storage, hRPE morphology was best preserved at 4°C. Addition of sericin to the storage medium maintained the characteristic morphology of the preserved cells, and improved pigmentation and levels of pigmentation-related proteins in the cultured hRPE sheets following a seven-day storage period at 4°C.

1. INTRODUCTION

Loss of healthy retinal pigment epithelial (RPE) cells can lead to visual impairment and blindness. Diseased RPE cells may lose pigment, proliferate, migrate, and transdifferentiate into other cell types (Kuznetsova, et al., 2014), thereby playing a key role in the development of several eye diseases, including age-related macular degeneration (AMD), Stargardt macular dystrophy (SMD) and some forms of retinitis pigmentosa (RP) (Jager, et al., 2008; Kuznetsova, et al., 2014; Schwartz, et al., 2014). These conditions are among the leading causes of vision loss and blindness in our part of the world (Friedman, et al., 2004; Schwartz, et al., 2014). The number of people with AMD is estimated to be 20-25 million people worldwide (Cavallotti & Cerulli, 2008). In line with the expected demographic changes, the prevalence of these sight-threatening eye diseases is predicted to increase in the coming years (Cavallotti & Cerulli, 2008). Patients with RPE dysfunction-related diseases experience significant reductions in quality of life (Casten & Rovner, 2008), reduced ability to participate in valued activities (Rovner & Casten, 2002), and heavily rely on rehabilitative services. Diseases involving RPE cells therefore represent a major medical and socioeconomic challenge.

The retinal pigment epithelium is a hexanocuboidal monolayer of pigmented cells that is essential for the maintenance and survival of the photoreceptor cells, and in regulating the integrity of the choroidal capillaries (Strauss, 2005). RPE transplantation aims to reverse vision loss and has emerged as a promising treatment modality for several eye disorders, including AMD, SMD, and RP (da Cruz, et al., 2007). Recently, clinical studies using transplantation of human *ex vivo* cultured RPE cells have been reported (Schwartz, et al., 2014). Interestingly, several studies have revealed that transplantation of RPE cells into different areas of the brain successfully reverse parkinsonian deficits in rats, monkeys, and patients with Parkinson disease (Stover, et al., 2005; Watts, et al., 2003; Yin, et al., 2012). In addition, studies on the plasticity of RPE stem cells have revealed their ability to transform into osteogenic, chondrogenic, adipogenic, and myogenic cell

types (Salero, et al., 2012). Thus, RPE cell replacement may have curative potential in a wide array of disorders, and is not limited to diseases solely related to vision.

While the potential of RPE cell transplantation as a therapeutic strategy in improvement of several diseases has been clearly established by the extensive research in this field over the past two decades, a considerable amount of work is still required to refine the tissue engineering process. This includes not only optimization of cell culture and storage protocols to maximize the quality of target tissue prior to transplantation, but also exploration of additives that can promote neuroprotection of cells adjacent to the transplanted tissue. One such additive, sericin ($C_{30}H_{40}N_{10}O_{16}$), a protein produced by *Bombyx mori* (silkworm), has been used as a serum substitute and an additive for cell cultures (Cao & Zhang, 2017). We have previously reported that sericin promotes pigmentation of cultured RPE by inducing the NF-κB pathway and upregulates genes related to pigmentation (Eidet, et al., 2016). In the present study, we aimed to explore if sericin could benefit in RPE preservation.

Tissue engineering laboratories require specialized facilities and are subject to high safety and quality standards. A limited number of laboratories are able to meet these requirements. This will likely be a barrier to the potentially widespread future use of tissue engineered RPE cells in the clinic. Optimization of storage temperature for cultured cells is of importance because it can enable transportation of the cultured cells from centralized laboratories to clinics worldwide (Van Buskirk, et al., 2004). Additionally, it can facilitate sufficient time for quality control and microbiological testing (Van Buskirk, et al., 2004). However, the optimal temperature for short-term storage of normal and differentiated RPE cell cultures has not yet been established.

In the present study, we: (a) aimed at identifying a suitable storage temperature for human retinal pigment epithelial (hRPE) cells, and (b) investigated if an alternative storage medium additive could improve the pre-transplantation hRPE cell quality. Based on

literature on storage of cultured epithelial cells (Utheim, et al., 2007) and an RPE cell line (Pasovic, et al., 2013b), we hypothesized that storage temperatures between 4°C and 37°C differently affect the viability, phenotype and morphology of cultured hRPE cells.

2. MATERIALS AND METHODS

2.1. Materials

We obtained normal hRPE and complete epithelial cell medium (EpiCM) from ScienCell Research Laboratories (San Diego, CA). Dulbecco's Modified Eagle's Medium (high glucose, with pyruvate; hereafter named DMEM) and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma Aldrich (St Louis, MO). Nunclon Δ surface 96-well plates, pipettes and other routine plastics were obtained from VWR International (West Chester, PA). Rabbit polyclonal anti-tyrosinase (clone H-109) and premelanosomal protein-17 (Pmel17; clone B1510) antibodies were acquired from Santa Cruz Biotechnology (Dallas, TX). Mouse monoclonal anti-cellular retinaldehyde-binding protein (CRALBP; clone B2) was from Abcam (Cambridge, UK). Mouse monoclonal anti-proliferating cell nuclear antigen (PCNA; clone PC10) was purchased from DAKO (Glostrup, Denmark). Secondary fluorescein isothiocyanate (FITC) and cyanin 3 (Cy3) conjugated anti-mouse or anti-rabbit antibodies were from Abcam. Mouse anti-zonula occludens 1 (ZO-1; clone L1212) and Live/Dead viability assay were from Invitrogen (Carlsbad, CA).

2.2. Cell Culture

Third passage hRPE cells were seeded (7.000 cells/cm^2) in complete EpiCM on Nunclon Δ surface 96-well plates and cultured under routine conditions with 95% air and 5% CO_2 at 37°C as described elsewhere (Eidet, et al., 2016), with the following modification: after two days, EpiCM was replaced with DMEM containing 4.5 g/l glucose and pyruvate, 1 % sericin, and 10.000 U penicillin/10 mg streptomycin at a final concentration of 1%

(Ahmado, et al., 2011). The culture medium was changed every two days, and the hRPE cells were maintained in culture for a total of nine days.

2.3. Cell Storage

Following the nine-day culture period, we replaced the culture medium with a storage medium consisting of 0.3 ml Minimum Essential Medium (MEM), 25 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 22.3 mM sodium bicarbonate, and 50 μg/mL gentamycin. Twenty-one multiplates (three for each storage temperature, and three unstored multiplates) were randomized for storage at six temperatures (4°C, 16°C, 20°C, 24°C, 28°C, and 37°C) or processed immediately for analyses without storage (control). The cultures were sealed with parafilm and stored for seven days in custom-built storage containers without CO_2 supply. The storage containers and temperature stability during storage have been previously described (Pasovic, et al., 2013b). Additionally, the temperature inside each storage container was checked daily throughout the experiments.

2.4. Light Microscopy

Three representative photomicrographs from each culture group were captured using a Leica DM microscope and Canon EOS 5D mark II camera at 200x magnification. To assess the fraction of pigment-containing cells in light microscopy photomicrographs, two investigators counted the number of pigment-containing cells and related it to the total number of cells to obtain the percentage of pigment-containing cells. The investigators were blinded to the origin of the different cultures.

2.5. Live/Dead Viability Assay

After storage, the cultures were incubated at 37°C for 30 minutes with phosphate-buffered saline (PBS) containing 1.0 μM calcein-acetoxymethyl ester (CAM) and ethidium homodimer-1 (EH-1). Photomicrographs were captured at 200x magnification at pre-

138 determined locations in the culture wells using a Nikon Eclipse Ti fluorescence microscope
139 with a DS-Qi1 black-and-white camera and a motorized stage.

140 2.6. Immunofluorescence

141 Following seven-day storage, the cells were fixed in 100 % ice-cold methanol for 15
142 minutes and subsequently washed three times with fresh PBS. Fixed cells were incubated
143 for 45 minutes at room temperature in a blocking buffer consisting of 10 % goat serum, 1
144 % bovine serum albumin (BSA), 0.1 % Triton X-100, 0.05 % Tween-20, and 0.05 %
145 sodium azide in PBS. Cells were then incubated overnight at 4°C with primary antibodies
146 diluted in blocking buffer (Table 1). FITC-conjugated and Cy3-conjugated secondary
147 antibodies were diluted (1:3000 and 1:250 respectively) in PBS with 1% BSA and
148 incubated for one hour at room temperature. The cultures were thereafter rinsed three
149 times in PBS and incubated with 1 μg/mL DAPI in PBS to stain cell nuclei before a final
150 wash with PBS. Photomicrographs were captured at 200x magnification at pre-determined
151 locations in the culture wells using a Nikon Eclipse Ti fluorescence microscope with a DS-
152 Qi1 black-and-white camera and a motorized stage. The exposure length and gain was
153 maintained at a constant level for all samples and the fluorescence intensities of the FITC
154 or Cy3 fluorochromes, which were conjugated to the secondary antibodies, were within the
155 dynamic range of the camera.

156 2.7. ImageJ Analysis

157 A combined CAM/EH-1 viability assay permitted the simultaneous quantification of CAM-
158 stained viable and EH-1-stained dead cells using custom-made macros with the freely
159 available software ImageJ (National Institutes of Health, Bethesda, MD). In brief, unevenly
160 transmitted light (rolling=50) was subtracted from all 16-bit photomicrographs using the
161 “Subtract Background”-command in ImageJ before they were converted to binary photos,
162 as reported elsewhere (Khan, et al., 2016). The “Analyze particles”-command was then

used to automatically count the number of EH-1 stained nuclei. The culture well area covered by CAM-stained cells was automatically measured using the “Area Fraction”-command.

Phenotype was assessed by measuring mean fluorescence intensity within regions of interest (ROI) by custom-made macros for ImageJ (Figure 1). First, unevenly transmitted light was removed using the “Subtract Background”-command. Then, ROI were automatically created around each nucleus in the binary DAPI-stained images. These selections were then restored in the 16-bit PCNA-stained photomicrographs and expression of the nuclear marker PCNA was subsequently measured within the image area covered by the DAPI-stained cell nuclei (Khan, et al., 2016). For the cytosolic markers CRALBP, Pmel17 and tyrosinase, the selections created around the DAPI-stained cell nuclei were enlarged by ImageJ to enclose the cytosolic area. This was facilitated using the “Voronoi”-command. Expression of CRALBP, Pmel17 and tyrosinase was then measured similarly in the cytosol. By using this method, we were able to normalize for difference in cell density in each photomicrograph.

2.8. Transmission Electron Microscopy

The unstored control and stored cultures of hRPE cells were processed for transmission electron microscopy (TEM) analysis as previously described (Raeder, et al., 2007). In brief, ultrathin sections (60–70 nm thick) were cut on a Leica Ultracut Ultramicrotome (Leica, Wetzlar, Germany) and examined using a CM120 transmission electron microscope (Philips, Amsterdam, the Netherlands).

2.9. pH Measurement

pH of cell cultures was assessed manually at room temperature using pH indicator paper.

2.10. Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics for Macintosh version 22.0 (IBM Corp, Armonk, NY). The Kruskal-Wallis test for nonparametric analysis of variance

was employed for comparing three or more groups, while the Mann-Whitney U-test was used to compare two groups. A two-tailed *P*-value less than 0.05 was considered significant. Data are expressed as mean ± standard deviation. For all experiments, the number of N (or the number of independent samples) relates to the number of wells.

3. RESULTS

3.1. Effect of Storage Temperature on Cultured Human Retinal Pigment Epithelial Cell Viability

To study the impact of different storage temperatures on hRPE cell survival, we measured the culture well area covered by CAM-stained live hRPE cells. Except for storage at 24°C, which resulted in a reduced area of live cells (92.69% ± 6.19%; *P*=0.03), no significant reduction in live cells was found at the various storage temperatures compared to the unstored control (Figure 2). Storage at the three lowest temperatures (4°C, 16°C and 20°C) typically, but not significantly, showed lower cell death values compared to control (60% ± 18%; *P*=0.11; 82% ± 12%; *P*=0.34; 68% ± 5%; *P*=0.11, respectively), whereas storage at the three highest temperatures (24°C, 28°C and 37°C) typically, but not significantly, showed the highest cell death values compared to control (159% ± 15%, *P*=0.49; 127% ± 15%, *P*=0.99; 100% ± 8%, *P*=0.99, respectively). The lowest cell death count was observed at 4°C. The live dead assay indicates that storage temperature affects viability of cultured hRPE cells.

3.2. Effect of Storage Temperature on Cultured Human Retinal Pigment Epithelial Cell Phenotype

To assess the expression of various phenotypic markers in the cell cultures, immunocytochemistry was employed, and protein levels were objectively measured using ImageJ (Figure 3). The level of Pmel17 in percentage of the control, a transmembrane glycoprotein in pigmented cells (Berson, et al., 2001), was reduced across all storage

temperatures compared to the control (P<0.05). The highest level of this protein was observed at 16°C (62% ± 0.08%), while the lowest level was seen at 4°C (30% ± 0.13%). The level of CRALBP in percentage of the control, another marker for differentiated hRPE (Strauss, 2005), was highest at 16°C (111% ± 0.01%; P<0.05). The level of tyrosinase in percentage of the control, a rate-regulating melanogenesis enzyme (Winder, et al., 1993), was reduced at all storage temperatures compared to the control (P<0.05). The highest level of this enzyme after storage was seen at 37°C (61% ± 0.001%), while the lowest levels were at 20°C (19% ± 0.01%). The level of PCNA, a proliferation marker, was preserved at all storage temperatures, compared to the control. Collectively, no single storage temperature consistently showed the most favorable RPE phenotype.

3.3. Cell Morphology

Light microscopy was performed to investigate the effect of storage temperature on the morphology of stored hRPE cells. Prior to storage, the differentiated hRPE cells were grown to confluence and they displayed characteristic hRPE morphology, *i.e.* hexagonal cell shape, adequate cell-cell contact, and cytoplasmic pigmentation distinctive to RPE (Figure 4A). After storage, morphology most similar to control was observed at 4°C (Figure 4B). At higher temperatures (16°C, 20°C, 24°C, 28°C, and 37°C) (Figure 4C-G), increased intercellular space was observed and cells appeared to lose their characteristic hexagonal shape. Cell shrinkage was observed at 37°C (Figure 4G). In addition, extracellular pigmented granules were visible in cultures stored at temperatures above 4°C. The fraction of pigment-containing cells was assessed (Table 2).

A phenotypic marker for tight junction proteins, zonula occludens (ZO-1) was used to assess the integrity of cell-cell junctions between different storage groups (Figure 5). Control cultures not subjected to storage displayed profound ZO-1 staining (Figure 5A). Following storage, cell-cell contact and ZO-1 staining were best preserved at 4°C and

16°C (Figure 5B and C). Normal ZO-1 mediated cell-cell adhesion was generally not seen at 28°C and 37°C (Figure 5F and G). Intercellular space appeared to increase proportionally with storage temperature (Figure 5B-G). Collectively, morphology after storage was best maintained at 4°C.

3.4. pH Measurements

Enzymes necessary for cellular function are most active at physiological pH (Boron & Boulpaep, 2012). Hence, maintaining physiological pH is important in cell preservation. pH in the storage medium was therefore manually assessed in all groups at the end of the storage period by pH indicator paper. All pH measurements showed physiological pH (pH=7.4).

3.5. Optimization of Storage by a Serum-Free Additive

Using the optimal storage temperature based on the data reported above, *i.e.* 4°C, hRPE cells were subsequently stored at 4°C with and without the silk protein sericin added to the storage medium. The cells were cultured and stored with the same protocol described above. After seven days of storage, live/dead assay showed no significant difference between the two groups (P=0.13). However, higher levels of tyrosinase was detected in cultures stored with sericin (141% ± 1%) in comparison to the cultures stored without sericin (100% ± 1%; P=0.03) (Figure 6A-C). In support of the results showing increased levels of the pigmentation-related protein tyrosinase in sericin-stored cultures, a higher level of premelanosome protein Pmel17 was detected in the cultures stored with 1 % sericin (252% ± 8%) compared to cells stored without sericin (100% ± 6%; P=0.01) (Figure 6D-F). Both storage groups (+/- sericin) maintained morphology typical to hRPE as described above, demonstrating hexagonal cell shape and adequate cell-cell contact (Figure 6G-H). However, supplementing the storage medium with sericin appeared more effective in maintaining cell pigmentation (Figure 6H). Melanosomes at different stages of development were observed using TEM (Figure 7). Hence, these results show that storage

medium supplied with the silk protein sericin does not alter hRPE viability or morphology compared to control, but improves hRPE phenotype and pigmentation.

4. DISCUSSION

In this study, hRPE cell viability, phenotype, morphology, and pH status following seven days of storage at six different temperatures ranging from 4°C through 16°C, 20°C, 24°C, 28°C, and 37°C, were assessed. We demonstrate that storage temperature affects cell viability, phenotype, and morphology of cultured hRPE cells. Our results suggest that pigmented hRPE cells are best preserved at a storage temperature of 4°C. We also show that the silk protein sericin does not alter viability nor morphology compared to control, but improves phenotype and pigmentation of cultured hRPE cells following a seven-day storage period at 4°C.

In the present study, hRPE was stored at six different storage temperatures ranging from 4°C to 37°C (4°C, 16°C, 20°C, 24°C, 28°C, and 37°C). A broader temperature range, *e.g.* from -196°C, or -20°C, to 50°C could have been investigated, however, our selection of temperatures was based on 1) relevant previous scientific literature (Pasovic, et al., 2013a; Utheim, et al., 2007) and 2) practical considerations related to ease of hospital-hospital transportation, intra-hospital transportation, and long-distance transportation between tissue engineering laboratories and clinics.

Maintaining high viability of cultured hRPE cells following storage is critical in hRPE cell replacement. High viability of the cells in the graft increases the success of transplantation after transfer of the graft into the subretinal space (Wright, 2013). In a previous study by our research group on the storage of the spontaneously immortalized RPE cell line ARPE-19, cell viability varied considerably depending on storage temperature (Pasovic, et al., 2013b). In the current study, however, storage temperature did not affect cell viability to the same extent, with the exception of storage at 24°C. In contrast to the differentiated and pigmented normal hRPE cells used in the current study,

the ARPE-19 cells used in the previous study were from a non-pigmented cell line which is relatively undifferentiated. Additionally, the ARPE-19 cells were cultured in a DMEM:F12-based medium with 10% fetal bovine serum. Thus, the differences in cell type (normal cells versus cell line), degree of differentiation/pigmentation, and culture media may explain the dissimilarity in the results of the previous and present studies.

We demonstrate that storage temperature impacts cell viability in cultured hRPE cells. The lowest cell viability following storage was seen at 24°C. Though not significant, our data following storage further showed a tendency for relatively few dead cells in cultures stored at temperatures between 4°C to 20°C and a relatively higher number of dead cells in cultures stored at 24°C. This is in agreement with previous studies reporting room temperature-induced cell death in several cell lines (Shimura, et al., 2000) and in stored cultures of conjunctival epithelial cells (Khadka, et al., 2015). Some have hypothesized that the change of cellular oxygen consumption at room temperatures compared to other temperatures (4°C - 37°C) generates reactive oxygen species (ROS), and that ROS subsequently induces cell death (Shimura, et al., 1997; Shimura, et al., 2000).

Compared to the control, we show that 4°C was the best storage temperature for maintaining cellular morphology. hRPE preserved at this temperature displayed classic hRPE morphology, as demonstrated by the hexagonal cells of homogeneous size and shape. Cell-cell contact, visualized by ZO-1, was also conserved, which contributes to the maintenance of cell polarity (Van Itallie & Anderson, 2006). Moreover, ample hRPE cell-cell contact plays a key role in upholding the *in vivo* differences of the chemical composition between the subretinal extracellular space and the choroid (Maminishkis, et al., 2006).

Additionally, cytosolic pigmentation was best maintained at 4°C. The pigmentation of hRPE cells is critical to normal visual function (Strauss, 2005) for two main reasons:

First, absorption of out of focus light by the RPE improves the quality of vision (Strauss, 2005). Second, the retina is almost continually exposed to ultraviolet light - a major factor in the generation of ROS (Oduntan & Masige, 2011). ROS in turn cause oxidative damage to cells and tissue and contribute to the pathogenesis of AMD (Oduntan & Masige, 2011). The pigmentation of RPE facilitates UV light absorption (Strauss, 2005), and is therefore necessary for the protection of vision. Levels of the pigmentation-associated proteins Pmel17 and tyrosinase were not the highest in cells stored at 4°C compared to other storage groups, but light microscopy showed more pigmented cells at this storage temperature in comparison to higher storage temperatures. The latter finding is somewhat unexpected since enzyme activity, e.g. the activity of tyrosinase, is related to temperature and becomes reduced below 37°C (Boron & Boulpaep, 2012; Duarte, et al., 2012). However, a possible cause of the lower percentage of pigment-containing cells found at higher storage temperatures could be poorer cell integrity at these temperatures with subsequent loss of pigment granules to the extra-cellular space.

The increased intercellular distance observed at higher temperatures (16°C, 20°C, 24°C, 28°C, and 37°C) might partly be attributed to the temperature-dependent membrane permeability which allows transfer of molecules that subsequently cause osmolality-related edema at higher temperatures, visualized in the cultures as increased intercellular distance (Quinn, 1988). High membrane permeability is associated with high metabolic activity because when there is an increase in membrane permeability and influx/efflux of molecules, transmembrane ATPases increase in activity to ensure normal membrane potential. These ATPases, including the sodium-potassium exchanger (Na+/K+ATPase) consume substantial energy, and thus force the cells to up-regulate their metabolic activity. Although normal, and even necessary *in vivo* (Boron & Boulpaep, 2012), this hypothesized increase in membrane permeability may not be beneficial in cell preservation, as the aim of cell storage is to keep the cellular activity (and thus the metabolic activity) at the very

minimum, while still maintaining resemblance to the native tissue to assure proper cell function upon transplantation. Hence, based on our results, the storage temperature that best resembles native tissue in morphology is 4°C.

Supplementing the storage medium with the silk protein sericin at 4°C did not affect hRPE viability, but increased the levels of the pigment-related proteins Pmel17 and tyrosinase. Pmel17-expression is reported to correlate with melanin content (Kwon, et al., 1991). The level of this protein was increased in cultures stored with 1% sericin in comparison to the control cultures stored in storage medium without sericin. Consistent with the phenotypic analysis, cultured hRPE cells stored with sericin appeared to be more pigmented than the hRPE stored without sericin. Tyrosinase catalyzes the formation of dihydroxyphenylalanine (L-DOPA) from L-tyrosine (Reinisalo, et al., 2012). L-DOPA is thereafter converted into melanin (Wang & Hebert, 2006), which has several important functions, including protection from ultraviolet radiation (Sanyal & Zeilmaker, 1988), inhibition of lipid peroxidation (Memoli, et al., 1997), and neutralization of molecules toxic to the cell (Double, 2006). The potential ability of sericin to enhance levels of tyrosinase in hRPE is therefore beneficial in RPE tissue engineering. Our group has recently demonstrated that sericin induces melanogenesis through activation of the NF-kß pathway (Eidet, et al., 2016). This is in contrast to the previously described anti-tyrosinase properties of this protein (Aramwit, et al., 2010; Chlapanidas, et al., 2013). Since sericin is a mix of proteins encoded by three different genes with different splice variants, the difference in extraction techniques between the cited studies and the sericin employed in our experiment may serve as a possible explanation. Further research is therefore needed to investigate the specific peptides or pathways through which sericin has the observed melanin-inducing effect.

The photomicrographs of cell cultures in the present study were automatically analyzed using ImageJ software in combination with custom-built macros (*i.e.* automated

series of ImageJ commands). In addition to better reproducibility, the application of ImageJ macros has some other benefits over manual assessment; it facilitates objective assessment that is free from human error and it uses a simple algorithm that can be applied to large sample series, thereby reducing analysis time.

In conclusion, this study demonstrates that cultured normal and pigmented hRPE cells, if stored between 4°C-37°C, are best preserved when stored at 4°C with sericin added to the storage medium. Optimization of hRPE cell preservation is essential for the future advancement of hRPE cell replacement therapy.

5. FIGURE LEGENDS

Figure 1: To assess the expression of various phenotypic markers in the cell cultures, immunofluorescence was employed (A), and measurement of mean fluorescence intensity using custom-made macros for ImageJ was performed as an indication of protein levels. First, uneven image illumination was removed using the “Subtract Background”-command. Then, each DAPI-stained nucleus was separated from the background noise by utilizing the “Make binary”-function. Thereafter, bordering nuclei were separated by the “Watershed”-command in order to avoid computation of adjoining nuclei as a single nucleus. The resulting selections of cell nuclei (B) were then restored in the 16-bit PCNA-stained photomicrographs and expression of the nuclear marker PCNA was subsequently measured within the image area covered by the cell nuclei. For the cytosolic markers Pmel17, CRALBP and tyrosinase, the selections created around the DAPI-stained nuclei were enlarged by ImageJ to enclose the cytosolic area (C). This was facilitated using the “Voronoi”-command. Expression of Pmel17, CRALBP and tyrosinase was then measured in the cytosol (D). Original magnification: 200x. Scale bars: 20 μm.

Figure 2: Cultured human retinal pigment epithelial cells (hRPE) were stored for seven days at 4°C, 16°C, 20°C, 24°C, 28°C, and 37°C. Viability was objectively quantified using

ImageJ to measure calcein-acetoxymethyl ester (CAM)/ethidium homodimer-1 (EH-1) fluorescence. (A) The bar chart shows culture well area covered by live cells normalized to the unstored control cultures (100%). N=3 (three wells per storage temperature). Data are expressed as mean ± standard deviation. * *P*=0.028 compared to the unstored control. (B) The bar chart displays number of dead cells normalized to the unstored control cultures (100%). N=3 (three wells per storage temperature). Data are expressed as mean ± standard deviation. (C) The bar chart shows percentage of dead cells in each culture condition. N=3 (three wells per storage temperature). Data are expressed as mean ± standard deviation.

Figure 3: Cultured human retinal pigment epithelial cells (hRPE) were stored for seven days at 4°C, 16°C, 20°C, 24°C, 28°C, and 37°C. Cellular protein levels after storage were compared to the unstored control (100%) by measuring mean fluorescence intensity within the image area covered by cells. The bar charts show the mean fluorescence intensity of immunofluorescence stainings with anti-premelanosomal protein-17 (Pmel17) (A), cellular retinaldehyde binding protein (CRALBP) (E), tyrosinase (I), and proliferating cell nuclear antigen (PCNA) (M) antibodies. Photomicrographs show anti-Pmel17 (B-D; red), anti-CRALBP (F-H; red), anti-tyrosinase (J-L; red), and anti-PCNA (N-P; green). Photomicrographs B, F, J, and N are representative of control cultures, whereas D, G, L, and O are representative of cultures with the highest protein levels relative to control. Photomicrographs C, H, K, and P are representative of cultures with the lowest protein levels relative to control. Nuclei were stained with DAPI (blue). N=3 (three wells per storage temperature). Data are expressed as mean ± standard deviation. Original magnification: 200x. * *P*<0.05 compared to all other groups. ** *P*<0.05 compared to all other groups. *** *P*<0.05 compared to all other groups. Scale bars: 20 μm.

Figure 4: Photomicrographs of cultured human retinal pigment epithelial cells (hRPE) stored for seven days at 4°C (B), 16°C (C), 20°C (D), 24°C (E), 28°C (F), and 37°C (G). Photomicrograph A is representative of unstored control cultures. Cells stored at 4°C best resembled native tissue morphology as demonstrated by hexagonal cell shape, ample cell-cell contact, and characteristic cytosolic pigmentation (arrows). Images are representative of three wells per storage condition. Original magnification: 200x. Scale bars: 20 μm.

Figure 5: Immunofluorescence staining of tight junction protein zonula occludens (ZO-1) (green) in cultured human retinal pigment epithelial cells (hRPE) showing presence of tight-junctions between cells following storage at 4°C (B), 16°C (C), 20°C (D), and 24°C (E). Small areas with absence of intercellular contact were observed at storage temperatures 16°C (C), 20°C (D), and 24°C (E). Normal ZO-1 mediated cell-cell adhesion was generally not seen at 28°C (F) and 37°C (G). Intercellular space is indicated by asterixs. Nuclei were stained with DAPI (blue). All micrographs are representative of three wells per storage condition. Original magnification: 200x. Scale bars: 20 μm.

Figure 6: To study the effect of the silk protein sericin on preservation of cultured human retinal pigment epithelial cells (hRPE), cellular expression levels of tyrosinase, a key enzyme responsible for melanin biosynthesis in hRPE, and premelanosomal protein-17 (Pmel17), a transmembrane glycoprotein in pigmented cells, were objectively measured using immunofluorescence in addition to obtaining light microscopy photomicrographs. (A) The bar chart shows the level of tyrosinase in hRPE cultures stored with or without 1 % sericin for seven days at 4°C. Data are expressed as mean ± standard deviation. N=4 (four wells per storage condition). *P=0.026. Immunostaining of anti-tyrosinase (red) in hRPE cultures stored for seven days without (B) and with (C) sericin. Images are representative of four wells per storage condition. Nuclei were stained with DAPI (blue).

Original magnification: 200x. Scale bars: 20 μm. (D) The bar chart shows the level of Pmel17 in hRPE cultures stored with or without 1 % sericin for seven days at 4°C. Data are expressed as mean ± standard deviation. N=4 (four wells per storage condition). **P=0.014. Immunostaining of anti-Pmel17 (red) in hRPE cultures stored for seven days without (E) and with (F) sericin. Images are representative of four wells per storage condition. Original magnification: 200x. Scale bars: 20 μm. Intracellular pigment in the cultures stored with sericin (H) appeared to be more abundant than in the group without sericin (G). Photomicrographs are representative of three wells per storage condition. Original magnification: 200x. Scale bars: 20 μm.

Figure 7: (A) Transmission electron microscopy (TEM) image of cultured human retinal pigment epithelial cells (hRPE) stored for seven days at 4°C with 1% sericin added to the storage medium showing a polarized hRPE cell with tight junctions (TJ) and melanosomes at different stages (I-IV). (B) TEM image of hRPE stored for seven days at 4°C without sericin added to storage medium. The images are representative of three culture wells per storage condition. TJ: tight junction, I: melanosome stage I, II: melanosome stage II, III: melanosome stage III, IV: melanosome stage IV. Scale bars: 2 μm.

6. Competing Interests

A patent application has been filed by the authors on the use of sericin in culture media based on previous data.

7. REFERENCES

AHMADO, A., CARR, A.-J., VUGLER, A.A., SEMO, M.A., GIAS, C., LAWRENCE, J.M., CHEN, L.L., CHEN, F.K., TUROWSKI, P., DA CRUZ, L. & COFFEY, P.J. (2011). Induction of Differentiation by Pyruvate and DMEM in the Human Retinal Pigment Epithelium Cell Line ARPE-19. Investigative ophthalmology & visual science **52**(10), 7148-7159.

ARAMWIT, P., DAMRONGSAKKUL, S., KANOKPANONT, S. & SRICHANA, T. (2010). Properties and antityrosinase activity of sericin from various extraction methods. Biotechnology and applied biochemistry **55**(2), 91-98.

BERSON, J.F., HARPER, D.C., TENZA, D., RAPOSO, G. & MARKS, M.S. (2001). Pmel17 initiates premelanosome morphogenesis within multivesicular bodies. Molecular biology of the cell **12**(11), 3451-3464.

BORON, W.F. & BOULPAEP, E.L. (2012). *Medical Physiology, 2e Updated Edition: with STUDENT CONSULT Online Access*. Elsevier Health Sciences.

CAO, T.T. & ZHANG, Y.Q. (2017). The potential of silk sericin protein as a serum substitute or an additive in cell culture and cryopreservation. Amino acids **49**(6), 1029-1039.

CASTEN, R. & ROVNER, B. (2008). Depression in Age-Related Macular Degeneration. Journal of visual impairment & blindness **102**(10), 591-599.

CAVALLOTTI, C. & CERULLI, L. (2008). *Age-related changes of the human eye*. Totowa, N.J.: Humana Press.

CHLAPANIDAS, T., FARAGO, S., LUCCONI, G., PERTEGHELLA, S., GALUZZI, M., MANTELLI, M., AVANZINI, M.A., TOSCA, M.C., MARAZZI, M., VIGO, D., TORRE, M.L. & FAUSTINI, M. (2013). Sericins exhibit ROS-scavenging, anti-tyrosinase, anti-elastase, and in vitro immunomodulatory activities. International journal of biological macromolecules **58**, 47-56.

DA CRUZ, L., CHEN, F.K., AHMADO, A., GREENWOOD, J. & COFFEY, P. (2007). RPE transplantation and its role in retinal disease. Progress in retinal and eye research **26**(6), 598-635.

DOUBLE, K.L. (2006). Functional effects of neuromelanin and synthetic melanin in model systems. Journal of neural transmission **113**(6), 751-756.

DUARTE, L.T., TIBA, J.B., SANTIAGO, M.F., GARCIA, T.A. & FREITAS BARA, M.T. (2012). Production and characterization of tyrosinase activity in Pycnoporus sanguineus CCT-4518 Crude extract. Brazilian Journal of Microbiology **43**(1), 21-29.

EIDET, J.R., REPPE, S., PASOVIC, L., OLSTAD, O.K., LYBERG, T., KHAN, A.Z., FOSTAD, I.G., CHEN, D.F. & UTHEIM, T.P. (2016). The Silk-protein Sericin Induces Rapid Melanization of Cultured Primary Human Retinal Pigment Epithelial Cells by Activating the NF-kappaB Pathway. Sci Rep **6**, 22671.

FRIEDMAN, D.S., O'COLMAIN, B.J., MUNOZ, B., TOMANY, S.C., MCCARTY, C., DE JONG, P.T., NEMESURE, B., MITCHELL, P., KEMPEN, J. & EYE DISEASES PREVALENCE RESEARCH, G. (2004). Prevalence of age-related macular degeneration in the United States. Archives of ophthalmology **122**(4), 564-572.

JAGER, R.D., MIELER, W.F. & MILLER, J.W. (2008). Age-related macular degeneration. The New England journal of medicine **358**(24), 2606-2617.

KHADKA, A., IACOVELLI, J. & SAINT-GENIEZ, M. (2015). PGC1a rescue retinal pigment epithelial cells from oxidative damage: implication for age-related macular degeneration. The FASEB Journal **29**(1 Supplement).

KHAN, A.Z., UTHEIM, T.P., JACKSON, C.J., REPPE, S., LYBERG, T. & EIDET, J.R. (2016). Nucleus Morphometry in Cultured Epithelial Cells Correlates with Phenotype. Microscopy and microanalysis: the official journal of Microscopy Society of America, Microbeam Analysis Society, Microscopical Society of Canada **22**(3), 612.

KUZNETSOVA, A.V., KURINOV, A.M. & ALEKSANDROVA, M.A. (2014). Cell models to study regulation of cell transformation in pathologies of retinal pigment epithelium. Journal of ophthalmology **2014**, 801787.

KWON, B.S., CHINTAMANENI, C., KOZAK, C.A., COPELAND, N.G., GILBERT, D.J., JENKINS, N., BARTON, D., FRANCKE, U., KOBAYASHI, Y. & KIM, K.K. (1991). A melanocyte-specific gene, Pmel 17, maps near the silver coat color locus on mouse chromosome 10 and is in a syntenic region on human chromosome 12. Proceedings of the National Academy of Sciences of the United States of America **88**(20), 9228-9232.

MAMINISHKIS, A., CHEN, S., JALICKEE, S., BANZON, T., SHI, G., WANG, F.E., EHALT, T., HAMMER, J.A. & MILLER, S.S. (2006). Confluent Monolayers of Cultured Human Fetal Retinal Pigment Epithelium Exhibit Morphology and Physiology of Native Tissue. Investigative ophthalmology & visual science **47**(8), 3612-3624.

MEMOLI, S., NAPOLITANO, A., D'ISCHIA, M., MISURACA, G., PALUMBO, A. & PROTA, G. (1997). Diffusible melanin-related metabolites are potent inhibitors of lipid peroxidation. Biochimica et biophysica acta **1346**(1), 61-68.

ODUNTAN, O. & MASIGE, K. (2011). A review of the role of oxidative stress in the pathogenesis of eye diseases. African Vision and Eye Health **70**(4), 191-199.

PASOVIC, L., UTHEIM, T.P., MARIA, R., LYBERG, T., MESSELT, E.B., AABEL, P., CHEN, D.F., CHEN, X. & EIDET, J.R. (2013a). Optimization of Storage Temperature for Cultured ARPE-19 Cells. Journal of ophthalmology **2013**, 216359.

PASOVIC, L., UTHEIM, T.P., MARIA, R., LYBERG, T., MESSELT, E.B., AABEL, P., CHEN, D.F., CHEN, X. & EIDET, J.R. (2013b). Optimization of storage temperature for cultured ARPE-19 cells. Journal of ophthalmology **2013**.

QUINN, P.J. (1988). Effects of temperature on cell membranes. Symposia of the Society for Experimental Biology **42**, 237-258.

RAEDER, S., UTHEIM, T.P., UTHEIM, O.A., CAI, Y., ROALD, B., LYBERG, T., KJELDSEN-KRAGH, J., RAMSTAD, H., MESSELT, E. & NICOLAISSEN, B., JR. (2007). Effect of limbal explant orientation on the histology, phenotype, ultrastructure and barrier function of cultured limbal epithelial cells. Acta ophthalmologica Scandinavica **85**(4), 377-386.

REINISALO, M., PUTULA, J., MANNERMAA, E., URTTI, A. & HONKAKOSKI, P. (2012). Regulation of the human tyrosinase gene in retinal pigment epithelium cells: the significance of transcription factor orthodenticle homeobox 2 and its polymorphic binding site. Molecular vision **18**, 38-54.

ROVNER, B.W. & CASTEN, R.J. (2002). Activity loss and depression in age-related macular degeneration. The American journal of geriatric psychiatry : official journal of the American Association for Geriatric Psychiatry **10**(3), 305-310.

SALERO, E., BLENKINSOP, T.A., CORNEO, B., HARRIS, A., RABIN, D., STERN, J.H. & TEMPLE, S. (2012). Adult human RPE can be activated into a multipotent stem cell that produces mesenchymal derivatives. Cell stem cell **10**(1), 88-95.

SANYAL, S. & ZEILMAKER, G.H. (1988). Retinal damage by constant light in chimaeric mice: implications for the protective role of melanin. Experimental eye research **46**(5), 731-743.

SCHWARTZ, S.D., REGILLO, C.D., LAM, B.L., ELIOTT, D., ROSENFELD, P.J., GREGORI, N.Z., HUBSCHMAN, J.P., DAVIS, J.L., HEILWELL, G., SPIRN, M., MAGUIRE, J., GAY, R., BATEMAN, J., OSTRICK, R.M., MORRIS, D., VINCENT, M., ANGLADE, E., DEL PRIORE, L.V. & LANZA, R. (2014). Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. Lancet.

SHIMURA, M., ISHIZAKA, Y., YUO, A., HATAKE, K., OSHIMA, M., SASAKI, T. & TAKAKU, F. (1997). Characterization of room temperature induced apoptosis in HL-60. FEBS Letters **417**(3), 379-384.

SHIMURA, M., OSAWA, Y., YUO, A., HATAKE, K., TAKAKU, F. & ISHIZAKA, Y. (2000). Oxidative stress as a necessary factor in room temperature-induced apoptosis of HL-60 cells. Journal of leukocyte biology **68**(1), 87-96.

STOVER, N.P., BAKAY, R.A., SUBRAMANIAN, T., RAISER, C.D., CORNFELDT, M.L., SCHWEIKERT, A.W., ALLEN, R.C. & WATTS, R.L. (2005). Intrastriatal implantation of human retinal pigment epithelial cells attached to microcarriers in advanced Parkinson disease. Archives of neurology **62**(12), 1833-1837.

STRAUSS, O. (2005). The retinal pigment epithelium in visual function. Physiological reviews **85**(3), 845-881.

UTHEIM, T.P., RAEDER, S., UTHEIM, O.A., CAI, Y., ROALD, B., DROLSUM, L., LYBERG, T. & NICOLAISSEN, B. (2007). A novel method for preserving cultured limbal epithelial cells. The British journal of ophthalmology **91**(6), 797-800.

VAN BUSKIRK, R.G., BAUST, J.M., SNYDER, K.K., MATHEW, A.J. & BAUST, J.G. (2004). Hypothermic storage and cryopreservation. BioProcess Int **2**(10).

VAN ITALLIE, C.M. & ANDERSON, J.M. (2006). Claudins and epithelial paracellular transport. Annual review of physiology **68**, 403-429.

WANG, N. & HEBERT, D.N. (2006). Tyrosinase maturation through the mammalian secretory pathway: bringing color to life. Pigment cell research / sponsored by the European Society for Pigment Cell Research and the International Pigment Cell Society **19**(1), 3-18.

WATTS, R.L., RAISER, C.D., STOVER, N.P., CORNFELDT, M.L., SCHWEIKERT, A.W., ALLEN, R.C., SUBRAMANIAN, T., DOUDET, D., HONEY, C.R. & BAKAY, R.A. (2003). Stereotaxic intrastriatal implantation of human retinal pigment epithelial (hRPE) cells attached to gelatin microcarriers: a potential new cell therapy for Parkinson's disease. Journal of neural transmission. Supplementum (65), 215-227.

595 WINDER, A., KOBAYASHI, T., TSUKAMOTO, K., URABE, K., AROCA, P., KAMEYAMA, K. & HEARING, V.
596 (1993). The tyrosinase gene family--interactions of melanogenic proteins to regulate
597 melanogenesis. Cellular & molecular biology research **40**(7-8), 613-626.
598 WRIGHT, B. (2013). *Corneal regenerative medicine : methods and protocols*. New York: Springer.
599 YIN, F., TIAN, Z.M., LIU, S., ZHAO, Q.J., WANG, R.M., SHEN, L., WIEMAN, J. & YAN, Y. (2012).
600 Transplantation of human retinal pigment epithelium cells in the treatment for Parkinson
601 disease. CNS neuroscience & therapeutics **18**(12), 1012-1020.

602