Improvement of Storage Medium for Cultured Human Retinal Pigment Epithelial Cells Using Factorial Design

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Storage of human retinal pigment epithelium (hRPE) can contribute to the advancement of cell-based RPE replacement therapies. The present study aimed to improve the quality of stored hRPE cultures by identifying storage medium additives that, alone or in combination, contribute to enhancing cell viability while preserving morphology and phenotype. hRPE cells were cultured in the presence of the silk protein sericin until pigmentation. Cells were then stored for 10 days in storage medium plus sericin and either one of 46 different additives. Individual effects of each additive on cell viability were assessed using epifluorescence microscopy. Factorial design identified promising additive combinations by extrapolating their individual effects. Supplementing the storage medium with sericin combined with adenosine, L-ascorbic acid and allopurinol resulted in the highest cell viability (98.6 ± 0.5%) after storage for three days, as measured by epifluorescence microscopy. Flow cytometry validated the findings. Proteomics identified 61 upregulated and 65 downregulated proteins in this storage group compared to the unstored control. Transmission electron microscopy demonstrated the presence of melanosomes after storage in the optimized medium. We conclude that the combination of adenosine, L-ascorbic acid, allopurinol and sericin in minimal essential medium preserves RPE pigmentation while maintaining cell viability during storage.

Age-related macular degeneration (AMD) is a leading cause of blindness in the developed world and is characterized by impairment and loss of the retinal pigment epithelium (RPE). Due to the lack of treatment options for the dry type of AMD, which affects 85% of patients, replacement of the RPE has been proposed as a future therapy for this disease. Expectations for the application of RPE transplants to treat retinal diseases are high, and several studies have shown that this approach can restore subretinal anatomy and improve visual function. A recent review by Nommiste et al. covers several proof-of-principle studies investigating the efficacy of different cell sources and transplantation techniques. The RPE cells used in these studies are derived from primary human stem cells, induced pluripotent stem cells and several other sources, and have been transplanted to the subretinal space by means of suspensions, strips or patches on coated polymers. While the strategies for cell replacement are improving, the production of cell sheets that fulfill the requirements for transplantation is complex and will likely lead to centralization of specialized culture laboratories. The ability to store RPE successfully is necessary in order to transport the tissue from the culture laboratories to the transplantation clinics and make widespread use of RPE replacement therapies possible. An established storage method would not only allow for transportation, but also make quality control and microbiological testing of the tissue possible. With continued improvement of RPE tissue engineering approaches, and more than 20 million patients suffering from AMD...
Results
Effect of Individual and Combined Storage Medium Additives on Viability of hRPE. hRPE were seeded in complete EpiCM on Nunclon Δ surface plates and cultured for two days before replacing EpiCM with modified DMEM (hereafter named <differentiation medium>) containing 1% sericin for 14 days. The cells had then developed pigmentation as demonstrated earlier\(^1\), and were stored for 10 days in storage medium plus sericin and either one of 46 different additives. The control group, containing sericin, was stored without additional additives. Cell survival following 10 days of storage in all 47 experimental groups (N = 3) was assessed by calcein-acetoxymethyl ester (CAM) fluorescence using ImageJ software to measure the culture well area covered by CAM-stained live hRPE cells. The results are presented in Fig. 1. Cells stored in MEM containing 1% sericin served as the control. Control cells covered 73.5 ± 22.3% of the culture well area. In comparison, cells that had not been stored covered 99.2 ± 0.1% of the culture well area. No single storage medium additive contributed to increasing the CAM-stained culture well area significantly compared to the control. One-way ANOVA revealed that two additives significantly reduced cell viability (carnosine and glutathione), while the Student’s t-test revealed that four additives significantly reduced cell viability (carnosine, glutathione, defereroxamine mesylate, and protease inhibitor cocktail) compared to the control.

To investigate whether combinations of additives could increase cell viability further, the five additives which provided the largest CAM fluorescence area (adenosine, allopurinol, β-glycerophosphate, L-ascorbic acid, and taurine) were selected for factorial design experiments. Normality of the data was confirmed by Design-Expert, as was absence of significant outliers on residuals plots. The data on percentage of cell viability was then power transformed as recommended by the Design-Expert software before subsequent analysis. A significant model including all possible additive combinations was computed by Design-Expert software (Stat-Ease) using ANOVA (P = 0.047). No single additive supplemented individually to the storage medium had a significant impact on cell viability in the factorial design experiments. The combined effects of sericin, adenosine, allopurinol and L-ascorbic acid, however, provided the highest desirability regarding both CAM-stained culture well area and cell viability in the factorial design experiments. Most of the additives have, to our knowledge, never been tested in the current setting. The effects of a total of 32 different combinations of the five most promising additives were simulated using a factorial design experiment. The single best combination of additives was selected for further study by additional experiments to assess its effects on phenotype and morphology.

Validation of Viability Data Using Flow Cytometry. The viability of hRPE stored in the optimal mix (MEM supplemented with sericin, adenosine, allopurinol and L-ascorbic acid) for three days was validated using flow cytometry with propidium iodide (PI) staining. PI passes through permeable cell membranes of dead cells and stains double-stranded DNA. PI bound to 3.1 ± 0.5% of control cells and 7.8 ± 2.5% of stored cells (P < 0.05), yielding a viability of 96.8 ± 0.5% and 92.1 ± 2.5%, respectively (Fig. 4C). While the difference was statistically significant (P = 0.03), these results support the CAM fluorescence area viability data showing only a small change in cell loss in cultures stored using the optimal combination of additives.

pH Measurement. pH of the storage medium was assessed using pH indicator paper and demonstrated pH in the physiological area (pH = 7.4).

Morphology of Optimal Combination hRPE. Both light microscopy, scanning electron microscopy and transmission electron microscopy were performed to investigate the effect of the optimal combination of storage medium additives on the morphology of hRPE. Control cells were cultured to confluence and obtained the characteristic morphology comprising a hexagonal cell shape and cytoplasmic pigmentation (Fig. 5). The same features were observed in hRPE cells that had been stored for three days using the optimal combination of storage medium additives, indicating that hRPE can be stored in this additive combination while retaining a classic RPE morphology.

Worldwide\(^1\), an upcoming need for improved storage and transportation methods for cultured RPE is anticipated. An above-freezing temperature storage system as suggested by our research group circumvents the need for cryoprotectants, which are known to inflict freezing injury to tissues at both high and low cooling rates\(^18\)–\(^20\).

After testing nine different storage temperatures between 4 °C and 37 °C, we found that hRPE cultures stored at 4 °C in a storage medium containing 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES)- and sodium bicarbonate-buffered Minimum Essential Medium (MEM) preserved the greatest number of viable cells (unpublished data). An earlier study showed that the addition of 1% sericin to the cell culture medium enhanced hRPE cell maturation, most notably by increasing cell pigmentation\(^11\). The MEM storage medium is a defined basal medium that mainly consists of inorganic salts, vitamins and glucose. We therefore investigated the effects of supplementing this medium with many different sericin additives, including sericin to preserve the differentiated state of the cells. The effects of the 46 individual supplements on viability of hRPE cell cultures were analyzed after ten days of storage at 4 °C. Some additives were selected based on their known or proposed effects on viability or antioxidant function in cultures of RPE or other cell types\(^31\)–\(^32\), while others were chosen based on effects demonstrated in pilot experiments. Most of the additives have, to our knowledge, never been tested in the current setting. The effects of a total of 32 different combinations of the five most promising additives were simulated using a factorial design experiment. The single best combination of additives was selected for further study by additional experiments to assess its effects on phenotype and morphology.
Transmission electron microscopy demonstrated that the degree of melanization in cells stored in the optimal additive combination maintained or even exceeded that of control cells (Fig. 6A–E), thereby supporting the findings made by light microscopy. Intercellular tight junctions were present both between control cells and between cells stored in the optimal additive combination (Fig. 6B,D). Microvilli were demonstrated in both groups, both by transmission and scanning electron microscopy (Fig. 6A,B,F,G).

Proteomic Analysis of hRPE Using the Optimal Additive Combination. Proteomic analysis was performed to investigate the effect of the optimal combination of storage medium additives on the hRPE proteome. hRPE cells stored in the optimal storage medium combination were compared to control cells that had not been stored. Of 3902 identified proteins, 126 were differentially expressed applying t-test with \( P < 0.05 \) (Tables 1 and 2). A total of 65 proteins (1.7%) were downregulated during storage for three days in the optimal additive mix, while 61 proteins (1.6%) were upregulated during storage in the optimal additive mix (Fig. 7). The distribution of differentially expressed proteins was similar between the groups (Fig. 8).

The cytoskeleton-related proteins ezrin and desmoplakin, and peroxiredoxins 2 and 3, important antioxidant enzymes of the cytosol and mitochondria, respectively, were upregulated during storage. Expression of vinculin and microtubule-associated protein 4 was reduced during storage. Vinculin is a membrane-associated protein that functions as a multiprotein linker to the actin cytoskeleton, while microtubule-associated protein 4 is involved in crosslinking of microtubules to actin filaments. The expression levels of several proteins associated...
with important RPE functions were specifically analyzed. The list of proteins was selected based on their known roles in visual pigment generation, phagocytosis and adhesion of RPE. Only one of the selected proteins important for specific RPE functions had significantly changed regulation in the stored cells compared to the control (Table 3). Tyrosinase was slightly, but significantly downregulated in stored cells compared to control cells (fold change 0.8; \( P < 0.01 \)).

**Discussion**

The present study indicates that the storage viability of hRPE cells can be increased by supplementing the serum-free MEM-based storage medium containing sericin with a combination of three additional additives, while maintaining a differentiated morphology and with only slight phenotypic changes. A total of 47 individual proteins were specifically analyzed and only one, tyrosinase, was found to have significantly changed regulation in stored cells compared to control cells.
additives were studied, including 32 combinations of the five most promising additives using a full-factorial design experiment. Herein, the five most promising storage media additives (adenosine, allopurinol, β-glycerophosphate, L-ascorbic acid and taurine) were investigated simultaneously. Compared to one-factor-at-a-time (OFAT) studies, factorial experiments have several advantages. First, they require less time, material, and number of experiments, making them more cost-effective. Second, they yield better estimates of the effects of each factor because all observations are used to calculate the effect of each individual variable. Third, they reveal interactions between factors and thus permit the exploration of optimal combinations over the entire repertoire of substances. Hence,

Figure 4. Viability of hRPE stored in the optimal combination of storage medium additives for three days. hRPE were analyzed by (A, B) quantitative fluorescence and (C) flow cytometry. (A) Cell viability as measured by area of calcein-acetoxymethyl ester (CAM) fluorescence, demonstrating similar results between groups (N = 6). Error bars represent the standard deviation of mean values. (B) Representative photomicrographs demonstrating similar CAM labeling between groups. (C) Representative flow cytometry plots of dead cells by propidium iodide exclusion in control cells and cells stored for three days (N = 3). The plots demonstrate a relatively low cell death rate in the stored group.
compared to OFAT studies, which vary only one factor at a time, factorial experiments simultaneously inspecting several factors are far more efficient when analyzing the effect of two or more variables.

The full-factorial experiment revealed that adenosine, allopurinol, and L-ascorbic acid together provided the most desirable additive combination with regard to cell viability. This finding was controlled using CAM fluorescence measurements and validated by flow cytometry. The combined effects of these additives on hRPE storage have not been described earlier, but their individual effects on many cellular processes have been widely studied. Adenosine is a purine nucleoside which has been shown to participate in the regulation of inflammatory responses by limiting inflammatory tissue destruction. Adenosine binds G protein-coupled adenosine receptors, and A<sub>3</sub> receptor activation has been demonstrated to protect retinal cultures against neurodegeneration. Activation of the ATP receptor P2X7 is known to induce death of retinal ganglion cells, but simultaneous intravitreal injection of an A<sub>3</sub> receptor agonist can prevent the P2X7-associated cell death. P2X7 overactivation results in dysregulated calcium signaling and is involved in the age-related dysfunction and degeneration of RPE cells. This suggests that overactive purinergic signaling may contribute to the geographic atrophy seen in dry AMD. Whether the beneficial effect of adenosine on preventing P2X7-associated cell death is responsible for providing increased hRPE cell viability, or other mechanisms are at play, warrants further study.

Allopurinol is a xanthine oxidase inhibitor that reduces the production of uric acid and is being investigated for management of reperfusion injury. It has been shown to prevent postasphyxial changes in newborn pig retinas and has been successfully used in the treatment of autoimmune uveitis in an experimental setting. Allopurinol administered to RPE cell cultures in high doses has been demonstrated to prevent free-radical-induced cell damage. Its proposed effect on quenching free radicals might have contributed to enhancing cell viability of cultured hRPE cells during storage in the present study.

It has been established that high levels of antioxidant vitamins can significantly reduce the risk of advanced AMD and its associated vision loss in patients with intermediate or advanced AMD. The addition of ascorbic acid to primary RPE cell cultures in vitro has been demonstrated to provide a dose-related downregulation of early-response proteins that are triggered by oxidative stress. In a study using the RPE cell line ARPE-19, however, ascorbic acid was not shown to protect the cells from hydroxyl radical induced cell death. Yet other studies have shown that ascorbic acid supplementation can protect RPE cells from hypoxic damage and reduce vision cell loss from damaging light. However, the latter effect might be attributable to ascorbic acid preventing excessive shedding of rod outer segments upon light exposure. The effect of ascorbic acid in the present study might be similar to that of allopurinol in that it reduces the oxidative stress burden.

Our research group recently demonstrated that sericin induces melanogenesis of hRPE cells through activation of the NF-κB pathway. Sericin has been shown to inhibit tyrosinase, and proteomic analysis in the present study confirmed that tyrosinase expression is slightly reduced in cells stored in the optimal additive combination in the presence of sericin. The expression of other pigment-related proteins (premelanosome protein 17,
tyrosinase related protein 1 and tyrosinase related protein 2) was maintained during storage using the optimal additive combination. Tyrosinase is the main rate-limiting melanogenesis enzyme, catalyzing the formation of dihydroxyphenylalanine (L-DOPA) from L-tyrosine. However, light microscopy and TEM demonstrated the presence of melanized cells and melanosomes in stored cell cultures. While phase contrast and transmission electron microscopy can determine the presence of melanosomes, these are not satisfactory methods by which to objectively determine the level of pigmentation. Future studies warrant the use of other methods, i.e. spectrophotometry or modified scanning devices as demonstrated by Lane et al.

In a study by Vugler et al., investigating RPE cells differentiated from human embryonal stem cells (HESC-RPE), a larger number of stage 4 melanosomes were displayed; however, these cells were of a different origin and were cultured under very different conditions than used in the present study. For instance, the HESC-RPE were cultured on Matrigel for five weeks. Polarization was evident with basally oriented nuclei like in our cells, but apical microvilli were more developed in this study than is shown in our cultures. Both the cell source and culture length might be of essence in order to further enhance differentiation. Ultrastructure is presented in great detail in a study by Carr et al., who demonstrated that co-culture of HESC-RPE with human retina leads to maturation-associated morphological alterations. Herein, the presence of melanosomes, tight junctions and microvilli is demonstrated. Similar findings are made in control cells and cells stored in the optimal additive combination in this study (Fig. 6).
| Gene Symbol | Gene Description | Biological function |
|-------------|------------------|---------------------|
| ACOX1       | Peroxisomal acyl-CoA oxidase 1 | Desaturation of acyl-CoA to 2-trans-enoyl-CoA |
| ALDH1A8A    | Delta-1-pyruvate-5-carboxylate synthase | Synthesis of proline, ornithine and arginine |
| AP2A1       | AP-2 complex subunit alpha-1 | Component of the adaptor protein complex 2; clathrin-dependent endocytosis |
| ARHGAP1     | Rho GTPase-activating protein 1 | GTPase activator for Rho, Rac and Cdc42 |
| ARMT1       | Protein-glutamate O-methyltransferase | Formation of gamma-glutamyl methyl ester residues |
| BCLAF1      | Bcl-2-associated transcription factor 1 | Death-promoting transcriptional repressor |
| DAZAP1      | DAZ-associated protein 1 | RNA-binding protein; possibly required in spermatogenesis |
| DBI         | Acetyl-CoA-binding protein | Possible intracellular carrier of acetyl-CoA esters |
| DBT         | Liposomial acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial | Conversion of alpha-keto acids to acetyl-CoA and CO₂ |
| DDX23       | Probable ATP-dependent RNA helicase DDX23 | Pre-mRNA splicing |
| DNAJC3      | DnaJ homolog subfamily C member 3 | Unfolded protein response during endoplasmic reticulum stress |
| DSSP        | Desmosplakin | Anchoring of intermediate filaments to desmosomes |
| EIF6        | Eukaryotic translation initiation factor 6 | Prevents the association of the 60S ribosomal subunit with the 40S subunit |
| EPM2AIP1    | EPM2A-interacting protein 1 | Unknown |
| EZR         | Ezrin | Connection of cytoskeletal structures to the plasma membrane; formation of microvilli |
| FARS8       | Phenylalanine-tRNA ligase beta subunit | Regulatory tRNA ligase beta subunit |
| FBN2        | Fibrillin-2 | Component of extracellular calcium-binding microfibrils; regulation of elastic fibers |
| FLOT1       | Flotillin-1 | Possible scaffolding protein within caveolar membranes; formation of caveolae |
| FUCA2       | Plasma alpha-L-fucosidase | Hydrolyzation of glycoproteins |
| GATM        | Glycine amidinotransferase, mitochondrial | Synthesis of creatine precursor guanidinoacetate |
| GDAP2       | Ganglioside-induced differentiation-associated protein 2 | Unknown |
| HIST1H4A    | Histone H4 | Core nucleosome component |
| HNRNPD      | Heterogeneous nuclear ribonucleoprotein D0 | RNA-binding protein |
| HNRNPH1     | Heterogeneous nuclear ribonucleoprotein H | Pre-mRNA processing |
| HNRNPM      | Heterogeneous nuclear ribonucleoprotein M | Pre-mRNA binding protein |
| HSDB17B2    | Estradiol 17-beta-dehydrogenase 2 | Interconversion of testosterone and androstenedione; estradiol and estriol |
| KIAA1468    | LinH domain and HEAT-repeat-containing protein KIAA1468 | Unknown |
| KTNR        | Kinetin | Kinesin-driven vesicle motility |
| LRPPRC      | Leucine-rich PPR motif-containing protein, mitochondrial | Nuclear and mitochondrial RNA metabolism |
| LRRCA8A     | Volume-regulated anion channel subunit LRRCA8A | Essential component of the volume-regulated anion channel |
| LRSAM1      | E3 ubiquitin-protein ligase LRSAM1 | Regulation of signaling pathways, cell adhesion, self-ubiquitylation, and cargo sorting during receptor endocytosis |
| MRPL28      | 39S ribosomal protein L28, mitochondrial | Component of the 39S mitochondrial ribosome subunit |
| MRRF        | Ribosome-recycling factor, mitochondrial | Release of ribosomes from mRNA |
| MYH9        | Myosin-9 | Cytokinesis, cell shape, secretion |
| MYO7A       | Unconventional myosin-VIIa | Intracellular movements |
| NUDT19      | Nucleoside diphosphate-linked moiety X motif 19 | Hydrolysis of various CoA esters |
| NUP155      | Nuclear pore complex protein Nup155 | Component of the nuclear pore complex |
| PDHB        | Pyruvate dehydrogenase E1 component subunit beta, mitochondrial | Conversion of pyruvate to acetyl-CoA and CO₂ |
| POLR2E      | DNA-directed RNA polymerases I, II, and III subunit RPABC1 | Subunit of RNA polymerase II |
| PRCP        | Lysosomal Pro-X carboxypeptidase | Cleavage of C-terminal amino acids |
| PRDX2       | Peroxisiredoxin-2 | Involved in redox regulation of the cell |
| PRDX3       | Thioredoxin-dependent peroxide reductase, mitochondrial | Involved in redox regulation of the cell |
| PRKCSH      | Glucosidase 2 subunit beta | Beta-subunit of glucosidase II, an ER glycan-processing enzyme |
| PRKDC       | DNA-dependent protein kinase catalytic subunit | DNA double-strand break repair |
| PRPF8       | Pre-mRNA-processing-splicing factor 8 | Assembly of spliceosomal proteins |
| PRPS2       | Ribose-phosphate pyrophosphokinase 2 | Synthesis of phosphoribosylpyrophosphate (PRPP), essential for nucleotide synthesis |
proteins have been associated with clinical syndromes involving the skin, heart, hair and immune system. Ezrin is a cortical cytoskeleton protein which localizes to epithelial microvilli. Loss of ezrin function as demonstrated in ezrin knockout mice leads to substantial reduction in RPE apical microvilli and retarded photoreceptor development. Desmoplakin is necessary for the anchoring of keratin at cell-cell contacts, and thus important for the regulation of desmosomal adhesion strength. It functions as a tumor suppressor, and a decrease in desmosomal protein expression is associated with poor prognosis in several cancers. Loss-of-function mutations in desmosomal proteins have been associated with clinical syndromes involving the skin, heart, hair and immune system.

Table 1. Significantly upregulated proteins during storage (low in control cells).

| Gene Symbol | Gene Description | Biological function |
|-------------|------------------|---------------------|
| PTCD3       | Pentatricopeptide repeat domain-containing protein 3, mitochondrial | Mitochondrial RNA-binding protein |
| PTPRA       | Receptor-type tyrosine-protein phosphatase alpha | Regulation of integrin signaling, cell adhesion and proliferation |
| RAB7A       | Ras-related protein Rab-7a | Key regulator in endo-lysosomal trafficking |
| RPL18       | 60S ribosomal protein L18 | Component of the ribosomal 60S subunit |
| RPL37A      | 60S ribosomal protein L37a | Component of the ribosomal 60S subunit |
| SLC25A3     | Phosphate carrier protein, mitochondrial | Transport of phosphate groups from the cytosol to the mitochondrial matrix |
| SOSTDC1     | Sclerostin domain-containing protein 1 | Bone morphogenetic protein antagonist |
| SRP54       | Signal recognition particle 54 kDa protein | Transfer of presecretory protein from ribosomes to TRAM (translocating chain-associated membrane protein) |
| STAG2       | Cohesin subunit SA-2 | Component of the cohesin complex |
| SUCLG2      | Succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial | Citric acid cycle |
| SULT1A1     | Sulfotransferase 1A1 | Sulfate conjugation of catecholamines, phenolic drugs and neurotransmitters |
| TOR1A       | Torsin-1A | Protein folding, processing, stability and localization |
| TRA2B       | Transformer-2 protein homolog beta | Pre-mRNA splicing |
| UBA1        | Ubiquitin-like modifier-activating enzyme 1 | Ubiquitin conjugation |
| VPS18       | Vacuolar protein sorting-associated protein 18 homolog | Vesicle-mediated protein trafficking to lysosomal compartments |

Pyruvate has been shown to induce pigmentation of ARPE-19 cells cultured in DMEM with high glucose. In our study, the basic storage medium was supplemented with pyruvate, which might have contributed further to the increased pigmentation demonstrated in both the current and earlier studies by our research group. Although several culture protocols using hESCs or iPSCs have successfully produced differentiated and pigmented RPE cells, they are usually more time-consuming. The use of sericin might contribute in shortening the culture period. The focus on the differentiation process is critical, as its efficiency is considered crucial to the economic feasibility of regenerative therapy using RPE cells.

The expression of the tight junction protein ZO-1 was maintained during storage, as demonstrated by proteomics analysis and transmission electron microscopy. Cultured cells established the classic hexagonal distribution of mature hRPE monolayers. The RPE, being a polarized monolayer, is dependent on functional intercellular tight junctions to maintain high transepithelial resistance, secure cellular barrier function and regulate paracellular permeability. Hence, the present study confirms earlier findings, but still indicates that hRPE cells can retain features of a mature phenotype when stored in the optimal additive combination.

The cytoskeleton-related proteins ezrin and desmoplakin were upregulated during storage. Ezrin is a cortical cytoskeleton protein which localizes to epithelial microvilli. Loss of ezrin function as demonstrated in ezrin knockout mice leads to substantial reduction in RPE apical microvilli and retarded photoreceptor development. Desmoplakin is necessary for the anchoring of keratin at cell-cell contacts, and thus important for the regulation of desmosomal adhesion strength. It functions as a tumor suppressor, and a decrease in desmosomal protein expression is associated with poor prognosis in several cancers. Loss-of-function mutations in desmosomal proteins have been associated with clinical syndromes involving the skin, heart, hair and immune system.

Upregulation of these proteins during storage might indicate that stored cells maintain robust cytoskeletal functions.

Third passage hRPE cells were employed in this study. The increased tendency of epithelial to mesenchymal transition with increasing passages of RPE cells has been demonstrated by Grisanti et al. They showed a large disparity between passage 2 RPE and passage 10 RPE, where cells of the higher passages transdifferentiate and lose differentiated RPE properties. While there is a wide consensus regarding the advantages of using early-passage RPE cells to avoid this phenomenon in culture, an exact passage number has not been defined. In conclusion, the current study demonstrates that the storage medium additive combination of sericin, adenosine, allopurinol and L-ascorbic acid successfully maintains hRPE cell viability during storage while preserving the characteristic hRPE morphology and proteome. The effects of the individual additives are not thoroughly understood, but previous research points to free radical scavenging mechanisms as possible explanations for these findings.

Future studies should investigate the effect of increased storage duration on hRPE cells in the optimal combination medium, and ideally expand the scope to RPE derived from different sources, including primary human stem cells and induced pluripotent stem cells. This could provide valuable knowledge when establishing a storage protocol for clinical use.
| Gene Symbol | Gene Description | Biological function |
|-------------|------------------|---------------------|
| ABCA1       | ATP-binding cassette sub-family A member 1 | Transmembrane transport |
| ATP1A1      | Sodium/potassium-transporting ATPase subunit alpha-1 | Hydrolysis of ATP coupled with the exchange of sodium and potassium ions across the plasma membrane |
| ATP1A3      | Sodium/potassium-transporting ATPase subunit alpha-3 | Hydrolysis of ATP coupled with the exchange of sodium and potassium ions across the plasma membrane |
| ATP6V1C2    | V-type proton ATPase subunit C2 | Subunit of the vacuolar ATPase |
| CD2AP       | CD2-associated protein | Adapter protein between membrane proteins and the actin cytoskeleton |
| COASY       | Bifunctional coenzyme A synthase | CoA biosynthetic pathway |
| COPZ1       | Coatamer subunit zeta-1 | Binds dilysine motifs, reversibly associates with Golgi non-clathrin-coated vesicles |
| CTTN        | Src substrate cortactin | Organization of the actin cytoskeleton |
| EIF2S2      | Eukaryotic translation initiation factor 2 subunit 2 | Early protein synthesis |
| EIF4A3      | Eukaryotic initiation factor 4A | ATP-dependent RNA helicase |
| FAH         | Fumarase-lactate dehydrogenase | Tyrosine catabolism |
| FAM234A     | Protein ITFG3/Protein FAM234A | Unknown |
| FERMT2      | Fermitin family homolog 2 | Scaffolding protein, activates integrin |
| FND3A       | Fibronectin type-III domain-containing protein 3A | Spermatid-Sertoli adhesion in spermatogenesis |
| G3BP2       | Ras GTase-activating protein-binding protein 2 | Probable scaffold protein, may be involved in mRNA transport |
| GDI2        | Rab GDP dissociation inhibitor alpha | Regulates the GDP/GTP exchange reaction of Rab proteins |
| GLYR1       | Putative oxosorosidase GLYR1 | Promotes KDM1B demethylase activity |
| GNPD1A      | Glucosamine-6-phosphate isomerase 1 | Conversion of D-glucosamine-6-phosphate into D-fructose-6-phosphate and ammonium |
| GOLM1       | Golgi membrane protein 1 | Unknown |
| GPPX        | Probable glutathione peroxidase 8 | Unknown |
| HDCC2       | HD domain-containing protein 2 | Unknown |
| HS3B1B      | 3-hydroxyacyl-CoA dehydrogenase type-2 | Mitochondrial RNA maturation |
| KIF5B       | Kinesin-1 heavy chain | Distribution of mitochondria and lysosomes |
| KPNA2       | Importin subunit alpha-1 | Nuclear protein import |
| KPNB1       | Importin subunit beta-1 | Nuclear protein import |
| LDHB        | L-lactate dehydrogenase B chain | Synthesizes lactate from pyruvate |
| LIMCH1      | LIM and calponin homology domains-containing protein 1 | Unknown |
| LPL         | Lipoprotein lipase | Hydrolysis of triglycerides of chylomicrons and very low density lipoproteins |
| LRRN1       | Leucine-rich repeat neuronal protein 1 | Unknown |
| MAP4        | Microtubule-associated protein 4 | Promotes microtubule assembly |
| MARS        | Methionine-tRNA ligase, cytoplasmic | Ligation of methionine to tRNA molecules |
| MAT2B       | Methionine adenosyltransferase 2 subunit beta | Regulatory subunit of S-adenosylmethionine synthetase 2 |
| MPI         | Mannos-6-phosphate isomerase | Mannosyl transfer reactions |
| MRPL2       | 39S ribosomal protein L2, mitochondrial | Component of the 39S mitochondrial ribosome subunit |
| MYO1D       | Unconventional myosin 1d | Intracellular movement |
| MYRIP       | Rab effector MyRIP | Melanosome transport |
| NDUF83      | NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 3 | Mitochondrial respiratory chain NADH dehydrogenase |
| NHP2        | H/ACA ribonucleoprotein complex subunit 2 | Ribosome biogenesis |
| NOV         | Protein NOV homolog | Cell proliferation, adhesion, differentiation |
| PARK7       | Protein DJ-1 | Oxidative stress and cell death protection |
| PDAPI       | 28kDa heat- and acid-stable phosphoprotein | PDGFA-stimulated fibroblast growth |
| PLS3        | Plastin-3 | Actin-bundling protein of intestinal microvilli, stereocilia, fibroblast filopodia |
| RABGAP1     | Rab GTase-activating protein 1 | Unknown |
| RHOBT2      | Mitochondrial Rho GTase 2 | Mitochondrial trafficking |
| RPS13       | 40S ribosomal protein S13 | Component of the ribosomal 40S subunit |
| RTCB        | tRNA-splicing ligase RtcB homolog | Subunit of tRNA-splicing ligase |
| SCEP1P1     | Retinoid-inducible serine carboxypeptidase | Unknown |
| SEC23B      | Protein transport protein Sec. 23B | ER-derived vesicle transport |
| SLC1A5      | Neutral amino acid transporter B0 | Sodium-dependent amino acid transport |

Continued
## Methods

**Supplies.** Primary hRPE and complete epithelial cell medium (EpiCM) were purchased from ScienCell Research Laboratories (San Diego, CA). Dulbecco’s Modified Eagle’s Medium (high glucose, with pyruvate; hereafter named DMEM), Minimal Essential Medium, heat-inactivated fetal bovine serum (FBS), N1 growth supplement, tauroine, triiodothyronine, non-essential amino acids, glutamine-penicillin-streptomycin, hydrocortisone, propidium iodide (PI), phosphate-buffered saline (PBS) and 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma Aldrich (St Louis, MO). Nunclon Delta surface plates, pipettes and other routine plastics were purchased from VWR International (West Chester, PA). The calcein-acetoxymethyl ester (CAM)/ethidium homodimer 1 (EH-1) viability kit was purchased from Invitrogen. The 47 additives used in the study are listed in Supplementary Information, Table S1.

**Culture and Preparation of Cells.** Third passage hRPE were seeded (20,000 cells/cm²) in complete EpiCM on 96-well Nunclon Delta surface plates and cultured under routine conditions of 95% air and 5% CO₂ at 37°C. After two days, EpiCM was replaced with modified DMEM (hereafter named «differentiation medium») containing 4.5 g/L glucose, pyruvate, 1% sericin, and 1% penicillin-streptomycin. Cells were then cultured for 14 days in differentiation medium until pigmentation, as demonstrated in an earlier study. The culture medium was changed every two or three days.

**Storage of hRPE cells.** Cells were cultured in the differentiation medium for 14 days, until cells were confluent and ≥20% of cells were pigmented as visually determined by phase contrast microscopy. The differentiation

| Gene Symbol | Gene Description | Biological function |
|-------------|------------------|---------------------|
| SLC7A5      | Large neutral amino acids transporter small subunit 1 | L-leucine transport across the blood-retinal barrier |
| SMARCD1     | SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 1 | Chromatin remodeling |
| SPR         | Septaperin reductase | Reduction of pteridine derivatives |
| TKFC        | Triokinase/FMN cyclase | Dihydroxyacetone phosphorylation |
| TNKS1BP1    | 182 kDa tankyrase-1-binding protein | Colocalizes with chromosomes in mitosis |
| TPRN        | Taperin | Sensory epithelial protein associated with autosomal recessive deafness. |
| TSPAN4      | Tetraspanin-4 | Cell surface glycoprotein binding to integrin |
| TWF2        | Twinfilin-2 | Actin-binding protein involved in motile and morphological processes |
| TXNL1       | Thioredoxin-like protein 1 | Active thioredoxin |
| TYR         | Tyrosinase | Formation of pigments, melanin production from tyrosine |
| UBE2O       | E2 ubiquitin-conjugating enzyme | Mono-ubiquitination of target proteins |
| UNCI1D      | Protein unc-13 homolog D | Vesicle maturation during exocytosis |
| USP5        | Ubiquitin carboxyl-terminal hydrolase 5 | Cleaves multiubiquitin polymers |
| VCL         | Vinculin | Actin binding protein involved in cell-matrix adhesion and cell-cell adhesion |
| VPS25       | Vacular protein-sorting-associated protein 25 | Sorting of ubiquitinated membrane proteins during endocytosis |
| YAP1        | Transcriptional coactivator YAP1 | Critical regulatory target of the Hippo signaling pathway |

Table 2. Significantly downregulated proteins during storage (high in control cells).
Figure 8. Distribution of protein functions. Gene ontology pie chart showing the distribution of protein functions in hRPE before (A) and after (B) storage according to their molecular functions as determined using Scaffold software with NCBI annotations.

Table 3. Effect of storage on the expression of some proteins associated with RPE-specific functions. Fold change represents changes in cells stored for three days compared to control cells.
medium was then removed and the cultures were rinsed with PBS before addition of storage medium. The storage medium consisted of 0.3 mL MEM, 25 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 22.3 mM sodium bicarbonate, 50 μg/mL gentamycin, and 1% sericin. A total of 46 different additives were individually supplemented to the storage medium and sterile-filtered (pore size 0.2 μm) before being added to the culture wells (N = 3) using a Biomek® 4000 Laboratory Automation Workstation (Beckman Coulter, Inc., Brea, CA). All cultures were stored at 4 °C for ten days, without change or addition of storage medium. The storage containers were custom-built as reported elsewhere. pH measurements of the storage medium were performed using pH indicator paper.

Viability Analysis using Quantitative Immunofluorescence. Cell viability was analyzed after 10 days of storage by incubating the stored cells with PBS containing 1.0 μM CAM and 1.0 μM EH-1 for 30 min. CAM is enzymatically cleaved into the green fluorescent calcein inside living cells. EH-1 is a membrane-impermeable dye that binds to DNA of dead cells. Area of fluorescence was calculated for all additive groups using epifluorescence microscopy and custom-made macros with ImageJ software (National Institutes of Health, Bethesda, MD). In detail, photomicrographs were captured at 200x magnification at five predetermined locations in each culture well using a Nikon Eclipse Ti fluorescence microscope (Nikon Instruments, Tokyo, Japan) with a DS-Qi1 black-and-white camera (Nikon Instruments) and a motorized microscope stage. Identical exposure length and gain were used for all compared groups, while keeping the image brightness within the camera’s dynamic range. ImageJ software was used to subtract unevenly transmitted light from all 16-bit photomicrographs using the “Subtract Background” -command. All photomicrographs were converted to binary photos before the “Area Fraction” -command was used to measure the culture well area covered by CAM-stained cells. The number of EH-1 stained nuclei was automatically counted using the “Analyze Particles” -command (Fig. 9).

Factorial Design. A factorial design experiment is a complex statistical design offering the possibility to study more than one factor at a time by creating a simulation of combined factor effects. Factorial design using Design-Expert (Stat-Ease, Inc., Minneapolis, MN) was employed to identify the most promising combination of storage medium additives. The five best additives from the viability analysis were included as independent variables (adenosine, allopurinol, β-glycerophosphate, L-ascorbic acid and taurine), with area of CAM fluorescence and the number of dead cells as the two dependent variables. The combined results of two end points were studied. However, the «Importance» tool was employed to set relative priorities for the two variables. The importance of viability (CAM fluorescence area) was emphasized over cell death (number of dead cells). The two-level full-factorial design included replicates of all 32 possible combinations of the five additives. Data were fitted to a full quadratic model. ANOVA was used to calculate the adjusted significance of both models (viability and death) in Design-Expert (P = 0.0047 and P = 0.036, respectively).

Flow Cytometry. Flow cytometry was used to validate the viability results. Cells were cultured in T25 cell culture flasks following the aforementioned protocol. Control cells (N = 3) and cells stored in the optimal additive combination (1% sericin, 5 mmol/L adenosine, 50 μg/mL L-ascorbic acid and 1 mM allopurinol) (N = 3) for three days were compared. Propidium iodide (PI), which binds to double-stranded DNA of dead cells, was added to the culture medium of both culture groups at a concentration of 2.5 μg/300 μL sample and cells were returned to the incubator for 15 minutes. Cells were then rinsed with PBS, trypsinized for 2–3 minutes, then washed and re-suspended in ice-cold HBSS + 4% FBS. Samples were kept on ice and analyzed using the BD Accuri C6 bench top flow cytometer. PI is excited by the 588 nm laser and is detected in filter 616//23 (FL3).

Transmission Electron Microscopy. Both unstored cultures and samples of hRPE stored for three days in MEM storage medium with the optimal additive combination (1% sericin, 5 mmol/L adenosine, 50 μg/mL L-ascorbic acid and 1 mM allopurinol) were processed for transmission electron microscopy (TEM) analysis as described earlier. In essence, a Leica Ultracut Ultramicrotome (Leixa, Wetzlar, Germany) was used to cut ultrathin sections, which were examined using a CM120 transmission electron microscope (Phillips, Amsterdam, the Netherlands).

Statistical Analysis. Statistical analysis was performed using IBM SPSS Statistics for Macintosh version 22.0 (IBM Corp, Armonk, NY). A one-way analysis of variance with Tukey’s post-hoc comparisons was used for statistical evaluation of the viability results. The Student’s t-test was used to compare two groups. P values below 0.05 were considered significant.

Proteomics. The proteome of hRPE cells stored in the optimal storage medium combination was analyzed and compared to control cells that had not been stored. The proteome analyses were performed as previously described. Briefly, the proteins of cell lysates were digested in-solution with trypsin. The generated peptides were analyzed by LC-MS using a nano-UHPLC connected to a Q Exactive mass spectrometer. Proteins were identified using the Mascot search engine and Scaffold software (version Scaffold, 4.7.3, Proteome Software Inc., Portland, OR) was used for further data analysis and label-free quantification. Scaffold was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS alone were grouped to satisfy the
principles of parsimony. Distribution of protein functions in hRPE before and after storage was determined using Scaffold software with annotations downloaded from the NCBI web database.

Data availability. The datasets generated and analyzed during the current study are available from the corresponding author on request.

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**Author Contributions**

L.P., T.P.U., C.J. and J.R.E. supervised the project. L.P., S.R., A.Z.K., B.T. and J.R.E. performed the experiments. L.P., S.R., A.Z.K., B.T., E.M. and J.R.E. analyzed the data. L.P., T.P.U., J.P.B. and J.R.E. wrote the manuscript. All authors reviewed the manuscript.

**Additional Information**

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