Storage of Cultured Retinal Pigment Epithelium for Transplantation

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Når det kjem til stykket

År ut og år inn har du site bøygd yver bøkene,
du har samla deg meir kunnskap
enn du treng til ni liv.
Når det kjem til stykket, er det
so lite som skal til, og det vesle
har hjarta alltid visst.
I Egypt hadde guden for lærdom
hovud som ei ape.

Olav H. Hauge (1908-1994)
Norwegian poet
2. ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AMD</td>
<td>Age-related macular degeneration</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATF6</td>
<td>Activating transcription factor 6</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAM</td>
<td>Calcein-acetoxyymethyl ester (CAM)</td>
</tr>
<tr>
<td>CEC</td>
<td>Cultured epidermal cell</td>
</tr>
<tr>
<td>CHOP</td>
<td>DNA-damage-inducible transcript 3</td>
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<tr>
<td>CSRNP1</td>
<td>Cysteine-serine-rich nuclear protein 1</td>
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<td>Cy3</td>
<td>Cyanine dye 3</td>
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<tr>
<td>DADLE</td>
<td>[D- Ala2, D-Leu5]-encephalin</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DSC1</td>
<td>Desmocollin-1</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FC</td>
<td>Fold change</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FOS</td>
<td>FBJ murine osteosarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>GAS6</td>
<td>Growth arrest-specific 6</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HGjE</td>
<td>Human conjunctival epithelial cells</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>hESC</td>
<td>Human embryonic stem cell</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>HIT</td>
<td>Hibernation induction trigger</td>
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<tr>
<td>HLEC</td>
<td>Human limbal epithelial cell</td>
</tr>
<tr>
<td>HOK</td>
<td>Human oral keratinocytes</td>
</tr>
<tr>
<td>hRPE</td>
<td>Human retinal pigment epithelium</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INHBA</td>
<td>Inhibin beta A</td>
</tr>
<tr>
<td>IPE</td>
<td>Iris pigment epithelium</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
</tr>
<tr>
<td>IRBP</td>
<td>Interphotoreceptor retinal-binding protein</td>
</tr>
<tr>
<td>IRE1α</td>
<td>Endoplasmic reticulum to nucleus signaling 1</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JSH-23</td>
<td>4-methyl-1-N-(3-phenylpropyl)benzene-1,2-diamine</td>
</tr>
<tr>
<td>LRAT</td>
<td>Lecithin retinol acyltransferase</td>
</tr>
<tr>
<td>MAP2K3</td>
<td>Mitogen-activated protein kinase kinase 3</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>MERTK</td>
<td>MER proto-oncogene, tyrosine kinase</td>
</tr>
<tr>
<td>MFG-E8</td>
<td>Milk fat globule-EGF factor 8 protein</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NHCM</td>
<td>Normal human choroidal melanocytes</td>
</tr>
<tr>
<td>NPD1</td>
<td>Neuroprotectin D1</td>
</tr>
<tr>
<td>OCA2</td>
<td>Oculocutaneous albinism II</td>
</tr>
<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEDF</td>
<td>Pigment epithelium-derived factor</td>
</tr>
<tr>
<td>PERK</td>
<td>Protein kinase R-like endoplasmic reticulum kinase</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PR</td>
<td>Photoreceptor</td>
</tr>
<tr>
<td>PRKCB</td>
<td>Protein kinase C, β</td>
</tr>
<tr>
<td>RBP</td>
<td>Retinol binding protein</td>
</tr>
<tr>
<td>RCS</td>
<td>Royal College of Surgeons</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RF111</td>
<td>Ring finger protein 111</td>
</tr>
<tr>
<td>RLBP1</td>
<td>Retinaldehyde binding protein 1</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigment epithelium</td>
</tr>
<tr>
<td>RPE65</td>
<td>Retinal pigment epithelium 65</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SFRP4</td>
<td>Secreted frizzled-related protein</td>
</tr>
<tr>
<td>SMAD7</td>
<td>Smad family member 7</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
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<tr>
<td>TLE1</td>
<td>Transducin-like enhancer of split 1</td>
</tr>
<tr>
<td>TRIB1</td>
<td>Tribbles homolog 1</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient receptor potential vanilloid</td>
</tr>
<tr>
<td>TYR</td>
<td>Tyrosinase</td>
</tr>
<tr>
<td>TYRP1</td>
<td>Tyrosinase-related protein 1</td>
</tr>
<tr>
<td>TYRP2</td>
<td>Tyrosinase-related protein 2</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula occludens 1</td>
</tr>
</tbody>
</table>
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5. INTRODUCTION

5.1 THE RETINAL PIGMENT EPITHELIUM IN HEALTH AND DISEASE

5.1.1 Structure and Function of the Healthy Retinal Pigment Epithelium

The retinal pigment epithelium (RPE) is a monolayer of pigmented cells located behind the neurosensory retina (1), separated from the sclera by its basal lamina (Bruch’s membrane), the choriocapillaris and the choroid (2) (Figure 1). Apical pseudopodial processes of the RPE stretch upward and surround the outer segments of rods and cones embedded in the interphotoreceptor matrix (2). Photoreceptors (PRs) are highly metabolically active cells, which continuously shed disciform segments of their outer membranes. The RPE’s position in the midst of this bustling environment forces it to serve a multitude of functions essential for maintaining vision – most importantly, ensuring PR function by phagocytosis of PR outer segments. In addition, the RPE and Bruch’s membrane are crucial for transporting nutrients from the choroid to the retina, for maintaining volume and chemical composition of the subretinal space, and for absorbing scattered light (3-8). In addition, they form the blood-retinal barrier (9).

Another crucial function of the RPE is its role in the visual cycle through the regeneration of the visual pigment rhodopsin. As incoming light meets the eye, it passes through the cornea, the anterior chamber of the eye, the lens, the vitreous and then traverses the many cell layers of the retina before it reaches the PRs. As the PRs absorb the light, the rhodopsin chromophore 11-cis-retinal is photoisomerized from cis to trans formation, eventually leading to the generation of all-trans-retinal (10). Each activated pigment catalyzes the formation of several hundred GTP from GDP, thereby initiating the process of amplification in visual excitation (11) that is followed by transduction to the optic nerve and further processing by the visual cortex. PR membrane discs containing all-trans-retinal are shed into the interphotoreceptor matrix and phagocytized by the RPE. Each RPE cell then contributes in isomerizing all-trans-retinal back to 11-cis-retinal, leading to the regeneration of the rhodopsin
molecule (8) so that the process can be repeated. The 11-cis-retinal is transported back to the PRs through binding to interphotoreceptor retinal-binding protein (IRBP) in the subretinal space. The exact mechanisms as to how 11-cis-retinal leaves the RPE and enters the PRs is not clearly understood (12).

Figure 1. Anatomy of the retina. The retinal pigment epithelium is situated between the neurosensory retina and the choriocapillaris. Courtesy of Dr. Magnus Fritzvold, Akershus University Hospital, Norway. Published in a PhD thesis by Raeder and Utheim in 2009 (13).

The continuous clearance of shed PR outer segments by the RPE is important not only because of regeneration of visual pigment, but also because it prevents the accumulation of debris, which can trigger a chronic inflammatory response. RPE
cells are impressive phagocytes; it is estimated that each RPE cell ingests 1 million outer segment discs in the course of a lifetime (8). This action is vital to PR repair because PRs do not produce the detoxifying enzyme glutathione reductase and are under constant stress from free oxygen radicals (3, 8). The outer segments that are internalized by the RPE are rich in docosahexaenoic acid (DHA), a substrate to the synthesis of neuroprotectin D1 (NPD1). NPD1 is a docosanoid that contributes in protecting both the RPE and the PRs from oxidative damage (8, 14, 15).

Another feature of the RPE that serves a photoprotective function in the retina is its production of melanin pigment. Melanin contributes in maintaining equilibrium of the retinal environment by protecting from oxidative stresses (16-18) and detoxification of peroxide, as well as binding zinc and drugs (18). In addition, the presence of melanin in the RPE prevents internal reflection of incoming light from the sclera back to the retina (19).

The RPE monolayer is held together by adherens and tight junctions. Adherens junctions connect actin filaments between the cells, while tight junctions block diffusion through paracellular spaces and thereby establish a concentration gradient across the RPE cell layer. This gradient is necessary for the control of fluid, ion and glucose transport, and for maintaining the competence of the blood-retinal barrier (20). The presence of tight junctions is critical for the establishment of the blood-retinal barrier (9, 12), which, together with the retinal microvasculature, protects the subretinal space and thereby renders it an immune-privileged site (21).

The concentration gradient established by tight junctions is utilized in generation of a Na⁺ ion gradient in combination with Na⁺-K⁺-ATPase proteins. Na⁺ concentration in the subretinal space increases, which is crucial for the function of the photoreceptor dark current and for transport of solutes through symporters and antiporters of the RPE (20), such as the GLUT channels.

In addition to the physical effects of the blood-retinal barrier, inherent properties of the RPE make it an immune-privileged tissue (21-23). An organic site is considered immunologically privileged if foreign tissue implanted to this site is relatively resistant to immunological rejection and enjoys prolonged survival (24). RPE cells have been shown to express CD95L and can induce
apoptosis in activated human T cells and thereby suppress the humoral immune response (23). In addition, the RPE cell layer secretes TGF-β (25) and other immunomodulatory factors which contribute in creating an immunosuppressive environment (22).

5.1.2 Age-Related Macular Degeneration

5.1.2.1 Epidemiology
Age-related macular degeneration (AMD) constitutes a leading cause of blindness in the developed world (26), with 20-25 million people affected worldwide (27). The number of individuals affected by the disease in the USA is expected to double in the coming decades (28), and a similar trend can be expected worldwide as a result of the current global demographic and epidemiological transitions.

The diagnostic criteria of AMD are detailed below, but in general terms, two main categories of AMD are recognized and popularly coined “dry” and “wet” AMD: The nonvascular (nonexudative, atrophic or “dry”) form affects the majority of patients (85–90 %), while the neovascular (exudative, or “wet”) form affects the minority (10–15 %) (29). However, the neovascular form is responsible for more cases of severe visual loss or legal blindness (29). Both categories can coexist in the same patient, and either one can progress into the other (2).

5.1.2.2 Aetiology and Pathophysiology
AMD is a degenerative eye disease affecting the macula and resulting in loss of central vision. A common factor in the pathogenesis of both nonvascular and neovascular AMD is a diseased RPE and Bruch’s membrane (3), but the pathophysiology is complex and multifactorial. In essence, the hallmark findings of nonvascular AMD are drusen and geographic atrophy, while choroidal neovascular membranes, hemorrhage, accumulation of subretinal fluid, RPE detachment and fibrosis are distinctive features of neovascular AMD (26, 30).
Drusen are glycoprotein-covered deposits in the macula and peripheral retina (2, 26). The origin of drusen is unknown, but dying PRs and RPE cells are substrates to their formation. They appear between the basement membrane of the RPE and the inner collagenous layer of Bruch’s membrane (2). In advanced AMD, drusen may fade or become resorbed in areas of geographic atrophy.

In nonvascular AMD, it is postulated that abnormalities in the enzymatic activity of aged RPE cells lead to an accumulation of metabolic by-products that interfere with normal metabolism, which over time leads to progressive degeneration (31). The RPE is a post-mitotic cell layer originating from a layer of neuroectodermal cells (6) and is mitotically silent throughout life. Hence, any loss of RPE cells will inflict a larger phagocytic load on the remainder. This increases the metabolic demands on the RPE cells, further aggravating the process (31). The continuous phagocytosis of photoreceptor outer segments by aging RPE cells leads to the accumulation of the undegradable metabolite lipofuscin in lysosomes. Lipofuscin accumulation exacerbates the problem by blocking the function of lysosomal enzymes, thereby inhibiting autophagy (32, 33).

In vascular AMD, defects of Bruch’s membrane allow choroidal neovascular complexes to enter the subretinal space by choroidal neovascularization (27). These anomalous blood vessels leak and can cause submacular hemorrhage, leading to detachment of the RPE or neuroretina from Bruch’s membrane, causing visual distortion known as metamorphopsia.

Throughout the lifetime, Bruch’s membrane calcifies and doubles in thickness, reducing the transport rate of fluids and nutrients across the membrane (2). The accumulation of extracellular deposits around Bruch’s membrane can trigger a chronic local inflammation, where the injured RPE serves as a source of cytokines, vascular endothelial growth factor (VEGF) and other triggering factors (24, 34). Hageman et al. have hypothesized that chronic inflammation at the RPE-Bruch’s membrane interface initiates dendritic cell recruitment and activation, and leads to the accumulation of RPE debris, lipofuscin and drusen formation (34). Inflammatory processes may also precipitate choroidal neovascularization when the VEGF and chemokine-
triggered influx of inflammatory cells lead to a disruption of Bruch’s membrane, thereby allowing new vessels to enter the subretinal space (24).

The presence of a functional RPE is crucial for the preservation of the outer nuclear layer in the presence of disciform degeneration due to AMD (35). The fibrosis seen in neovascular AMD is often termed a disciform scar, and is the end-stage of a process with alteration of the RPE where fibrous tissue with neovascularization proliferates to the extent of partially or totally replacing the neuroretina (36).

5.1.2.3 Classification and Diagnostic Criteria
Several different classifications have been used both in clinical studies and in clinical practice, without an international consensus to diagnostic criteria. A clinical classification system of AMD was developed in 2013 by the Beckman Initiative for Macular Research Classification Committee (37), and is the one currently adapted by international guidelines like BMJ Best Practice (38). It classifies the disease into normal aging changes and four disease categories (early, intermediate and late AMD): individuals > 55 years with small drusen (< 63 µm) are considered to have normal aging changes; individuals with medium drusen (≥ 63–< 125 µm) are considered to have early AMD; individuals with large drusen (≥ 125 µm) or with pigmentary abnormalities associated with medium drusen are considered to have intermediate AMD; individuals with lesions associated with neovascular AMD or geographic atrophy are considered to have late AMD.

The classification is helpful in assessing an individual’s risk for developing late AMD. According to the Committee, the five-year risk of developing late AMD is estimated to 0.5 % for normal aging changes and to 50 % for intermediate changes (37).

The diagnosis of AMD is clinical and based on the presence of characteristic findings on slit lamp eye examination. One takes into consideration the amount and size of drusen, as well as areas of geographic atrophy seen as areas of depigmentation or increased pigmentation. Fluorescein dye retinal angiography is used to expose choroidal neovascular vessels leaking
fluorescein, in contrast to normal retinal vessels. Indocyanine green angiography is typically used to visualize the choroidal vasculature, and can identify occult vascular membranes (39). The presence of sub- and intraretinal fluid is evaluated using optical coherence tomography (OCT) (Figure 2). Vascular abnormalities such as neovascularization (abnormal flow), non-perfusion (absence of flow), dilated vessels and aneurysms can be visualized by OCT angiography (40). Fundus autofluorescence allows the identification of lipofuscin particles (41).

Figure 2. OCT image of a patient with vascular AMD. The image demonstrates cystoid macular edema with drusenoid pigment epithelial detachment. Courtesy of Dr. Bektesevic, The University Hospital of North Norway, Tromsø.

5.1.2.4 Risk Factors
Age is the strongest risk factor for development of AMD (36). A causal relationship between smoking and AMD has also been confirmed (42). The direct causation between smoking and retinal damage is not fully understood, although oxidative insults to the retina have been suggested (36). Tobacco smoking has been showed to reduce the macular pigment density in a dose-response relationship (43), which might offer some explanation: Macular pigment has, as discussed above, the ability to protect against oxidative damage both by
absorbing incoming light and by acting as an antioxidant. Among other modifiable risk factors to the development of AMD are reduction in body mass index and treatment of hypertension (36).

There is also a strong genetic component to the development of AMD (44), and first-degree relatives of AMD patients are at increased risk (45). Ethnic differences in prevalence have been described, with the largest prevalence in persons of European descent and the lowest in persons of African descent (46). AMD is also strongly associated with an allelic variant of complement factor H, a finding that suggests a causality between alterations in complement factor H expression and the pathogenesis of AMD (47).

5.1.2.5 Clinical Presentation
AMD is a disease of insidious nature. In its early stages, it gives very few symptoms (36) and is rarely diagnosed before irreversible damage to the retina has occurred. Although AMD develops over months and years, the neovascular form can give sudden and profound visual loss because of subretinal hemorrhage (26, 29). As mentioned above, AMD primarily affects high-acuity central vision, which is required for fine-detailed tasks such as driving, reading, recognizing faces and other activities of daily living (48) (Figure 3). Blurring and distortion of near vision are the most frequent earliest changes reported by patients with neovascular AMD. Metamorphopsia (straight line distortion) due to accumulation of submacular fluid can be evaluated by use of the Amsler grid (30).

The psychosocial consequences of AMD are also significant, and patients with AMD report lower quality of life and greater emotional distress than visually healthy controls and visually healthy controls with other chronic illnesses (49). Approximately 30% of patients with AMD suffer from clinical depression, which is a major cause of disability and adds to the disease burden of these patients, further reducing quality of life (50). Intriguingly, the risk of depression is greater in patients with only one diseased eye than in patients with both diseased eyes (51). This might be a consequence of perceived uncertainty and worry surrounding future vision loss in patients with one diseased eye (51).
In this context, the many advantages of improving treatment and prognosis for AMD patients become apparent, as such interventions would not only improve the lives of individuals but also save additional costs of care borne by the health system.

Figure 3. The visual field in AMD. A. Schematic representation of a normal visual field. B. Schematic representation demonstrating a central scotoma.

5.1.2.6 Established Treatment Approaches
While more than 85 % of AMD patients suffer from the nonvascular form of the disease, the current treatment modalities can only address the issue of neovascularization. For advanced cases, and for nonvascular AMD, there is as yet no satisfactory cure (27, 52). Risk factor modification and high-dose supplementation of antioxidants and minerals remain the only evidence-based approaches for secondary prevention of nonvascular AMD (53), and there are currently no verified treatment options (52). For early and intermediate neovascular AMD, several treatment options exist. The mainstay of treatment has been antagonism of VEGF by intraocular injection of anti-angiogenic
medication such as bevacizumab or ranibizumab, which have proven to be equally effective in preventing vision loss and improving visual acuity in patients with early neovascular AMD (54-57). The newer anti-VEGF drug aflibercept has been demonstrated equally effective as ranibizumab in a recent Cochrane review (58). Treatment with ranibizumab has been shown to be superior to photodynamic therapy with verteporfin (59). However, the need for monthly monitoring and repeat treatment creates a huge burden on resources. An additional drawback is that 20% of patients treated with anti-VEGF have been shown to lose vision over time (60). These findings have dampened the initial enthusiasm for biological treatment. Furthermore, anti-VEGF treatment in neovascular AMD has been shown to exacerbate the development of RPE and macular atrophy and choroidal atrophy in these patients (61, 62). It is unclear whether the disease progression can be attributed to pharmacological side effects or the natural progression of the disease process (62).

5.1.3 Stargardt Disease and Fundus Flavimaculatus

5.1.3.1 Aetiology
Stargardt disease and fundus flavimaculatus are considered two different manifestations of the same disease (63), caused by autosomal recessive mutations affecting the ABC4R gene on the short arm of chromosome 1 (63-65). The ABC4R gene encodes the RmP protein, a retina-specific ATP-binding cassette transporter (64). The RmP protein plays an important role in retinoid cycling between the PRs and the RPE (65). Malfunction of the protein over time leads to slowing of the retinoid cycle kinetics and an abnormal deposition of lipofuscin in the RPE, followed by degeneration of RPE and PRs (66).

5.1.3.2 Clinical Presentation
Both Stargardt disease and fundus flavimaculatus present with macular degeneration and loss of visual acuity, but in different periods of life. In Stargardt disease, symptoms start in the first or second decade, while fundus flavimaculatus starts later (63). Hence, Stargardt disease is the most common
form of juvenile macular degeneration (67, 68) with an estimated prevalence of 1:8000–1:10000 (68, 69).

5.1.3.3 Treatment Approaches
At present, no cure and no effective treatment modalities exist for these hereditary macular dystrophies. The only secondary preventive measure suggested thus far is the use of darkness as a possible treatment, owing to the discovery that A2E, the major chromophore of lipofuscin, accumulates faster in light than in darkness (70).

5.2 TRANSPLANTATION OF THE RETINAL PIGMENT EPITHELIUM

5.2.1 History of RPE Transplantation

5.2.1.1 Background
Given the limitations of the existing treatments for macular diseases described above, current research points to tissue engineering and cell transplantation as the most promising future treatment options for both AMD and the hereditary macular dystrophies (52, 71-75). Numerous studies have been conducted, using several animal models, many different cell sources and a variety of surgical techniques. Some of the most notable approaches are presented below, however, a full historical background of RPE transplantation is beyond the scope of this thesis.

5.2.1.2 Animal Models
Transplantation of RPE cells has been performed in several animal models, of which the Royal College of Surgeons (RCS) rat is the most widely applied (76-83). The RCS rat offers an indirect cause of PR degeneration due to a disrupted phagocytic ability of RPE cells caused by a mutation in the MERTK gene (84). Other animal models include mice (85), rabbits (72, 86, 87), pigs (88) and cats (89). With the demonstration of long-term survival of RPE cell transplants in various animal models, transplantation was envisioned to offer the prospect of a
single intervention cure. Preclinical studies such as these paved the way for clinical trials in patients.

5.2.1.3 Transplantation of RPE in Humans

Several clinical trials for RPE transplantation are currently underway. Schwartz et al. performed the first study to examine the safety and tolerability of hESC-RPE transplantation in humans without finding evidence of tumorigenicity (67, 90). In contrast to using RPE suspensions for transplantation, The London Project to Cure Blindness (LPCB) have commenced a clinical trial investigating the efficacy of transplanting hESC-derived RPE cultured on a polyester membrane (91). So far, one patient has been treated. The same group is currently planning another trial investigating the treatment of AMD patients using iPSC-derived RPE monolayers. A California-based company, Regenerative Patch Technologies, has launched two clinical trials: one focusing on the treatment of patients with AMD, the other on patients with retinitis pigmentosa (92). The AMD trial was only recently approved and will be investigating the transplantation of hESC-derived retinal sheets into the macula of patients with dry AMD. The retinitis pigmentosa trial has recently advanced to phase 2. In this phase, the company will investigate the effect of transplanting a suspension of retinal progenitor cells by injection into the subretinal space of 70 patients. No results have been published thus far.

5.2.2 Delivery of RPE to the Subretinal Space

5.2.2.1 Transplantation of RPE Suspensions

Several techniques for RPE cell transplantation have been attempted, including injection of RPE cell suspensions to the subretinal space (76, 79, 82, 83, 93, 94), transplantation of full- or partial-thickness RPE-choroid sheet grafts, or RPE-Bruch’s membrane complexes (72, 73, 87, 93, 95) (Figure 4.). There are advantages and disadvantages to every technique. In general, the injection of RPE cell suspensions is considered an easier procedure with less adverse events
than the transplantation of grafts, given the smaller surgical access route. The main disadvantage of cell suspension transplantation is that a limited number of disorganized cells are spread on an impaired basal lamina (72, 93), with the risk of cell multilayering and development of subretinal fibrosis (96). The irregular distribution of transplanted RPE cells from suspensions has been shown to result in increased risk of graft failure and damage to adjacent PRs (86). Failure of the cells to grow on a diseased or aged Bruch’s membrane is considered a major disadvantage of this method (75, 97).

**Figure 4. Transplantation of RPE suspensions and monolayers.** A. First, a detachment is made using a jet stream of fluid to separate the neurosensory retina from the RPE monolayer. B. A suspension of RPE cells is injected into the subretinal space. C. An RPE monolayer attached to a natural or constructed carrier substrate is introduced to the subretinal space.
The transplantation of RPE cell suspensions to the subretinal space of RCS rats has induced chronic immune rejections (98) despite the immunological privilege of the subretinal space (21). In the case of choroidal neovascularization, this can be explained by breached competence of the blood-retinal barrier. It has been speculated that transplantation of autologous RPE sheets will result in a lesser triggering of the immune response than the transplantation of cell suspensions – the latter thought to be less immune privileged and prone to induce a more powerful immune reaction (21).

5.2.2.2 Scaffolds for RPE Transplantation

Both natural materials (grafts) and bioimplants can serve as scaffolds for the transplantation of RPE cell sheets/monolayers. Successful transplantation of an RPE cell layer is likely to exhibit several advantages over cell suspension transplantation, as it implies the insertion of an organized cell layer on a functional Bruch’s membrane (72, 93). This enables appropriate implantation and orientation of an organized RPE cell layer into the retina (52, 99, 100) and circumvents several of the complications associated with the use of RPE cell suspensions (72, 93, 100, 101). Autologous partial-thickness grafts have shown the ability both to sustain photoreceptor survival and commence early repair of debrided RPE (87).

Van Zeelburg et al. (102) did a seven-year follow-up study of 131 patients (133 eyes) who underwent autologous choroid-RPE-grafting after vitrectomy and excision of choroidal neovascular membranes. Five percent of patients had a best-corrected visual acuity of more than 20/40. However, the surgical procedure has been described as extremely demanding with significant risk of complications (75).

For RPE cells grown as a monolayer, the use of a substrate is considered necessary for appropriate implantation and orientation of the transplant (52, 99), as well as prevention of apoptosis (103). Several studies point to the damaged host Bruch’s membrane as one of the limiting factors for adherence of transplanted cells (97, 104-106). A preclinical study comparing the RPE cell suspension and monolayer transplantation techniques in rats concluded that
transplantation of monolayers offered increased survival and better host integration (107).

Platform devices and specialized scaffolds have been constructed in order to ease the delivery of ultra-thin RPE grafts into the subretinal space (80, 108, 109). Scaffolds are implants that provide a suitable structure for delivery of cells, and can be constructed from biomaterials that may originate from natural or synthetic polymers (110). In a study by Koss et al. (111), hESC-derived RPE were pre-grown on a biocompatible membrane before subretinal implantation. Another group has constructed a synthetic polyethylene glycol (PEG)–grafted nanofiber surface, which provides a physiologically accurate environment for the culture of RPE cells. This surface is also stable for longer storage periods (112).

5.2.3. Sources of RPE Cells for Transplantation

5.2.3.1 Autologous Cell Sources

Autologous transplantation of RPE-choroid graft sheets from the midperipheral retina has been demonstrated in rabbits (87) and humans (93, 95, 104, 113, 114). Falkner-Radler et al. compared autologous RPE-choroid transplantation to autologous RPE suspension transplantation and found that the two methods yielded comparable anatomical and functional outcomes (93).

An alternative autologous cell source is iris pigment epithelium (IPE), which is more easily available and possesses some of the RPE-specific functions. However, studies indicate that IPE cells are both less efficient than RPE at phagocytosis (115) and have a lower degradative capacity of PR outer segments (116). Attempts at autologous IPE transplantation have hitherto not resulted in improved visual acuity (117, 118).

5.2.3.2 Transplantation of RPE Allografts

As mentioned above, AMD is often complicated by choroidal neovascularization. Algvere et al. (119) investigated the transplantation of homologous fetal RPE as a monolayer patch following resection of choroidal neovascular membranes. The study demonstrated the successful survival of the transplant three months
postoperatively. However, with the inclusion of only three patients, it serves as a proof-of-principle study and its clinical effects cannot be extrapolated. A later study by the same group showed a high rejection rate and demonstrated the risks of host-graft rejection in transplantation of allogeneic cells in the absence of immunosuppressive therapy (120). In a study by Tezel et al., systemic immunosuppression prevented the rejection of transplanted allografts, but did not improve visual function (121).

5.2.3.3 RPE Cell Lines

ARPE-19 is a spontaneously immortalized RPE cell line derived in 1986 from the eyes of a 19-year-old male who died in a motor vehicle accident (122). The cell line has been thoroughly characterized and found to be structurally and functionally similar to RPE in vivo (7), and is therefore frequently used as a model of RPE function. The cell line has also been transplanted by suspension to the subretinal space of RCS rats (76, 77, 79, 81, 82), yielding visual function rescue in the same order as that achieved using hESCs (83) without evidence of inflammation or uncontrolled growth (79). The advantage of using cell lines lies in the possibility of expanding them as required and testing them prior to transplantation.

5.2.3.4 Human Embryonic Stem Cells

Both human embryonic stem cell (hESC)-derived RPE and primal stem cell-derived RPE have been tested in preclinical studies of animal models (78, 83, 111, 123-126) with promising results regarding visual acuity. These results paved the way for clinical studies in patients (67, 90). However, several concerns have been raised concerning the safety and possible tumorigenicity of hESC-derived cell sources. Schwartz et al. transplanted hESCs into the eyes of patients with AMD and Stargardt disease and found no evidence of adverse proliferation, rejection, or serious ocular or systemic safety issues (67, 90).
5.2.3.5 Induced Pluripotent Stem Cells

The feat of developing functional RPE derived from induced pluripotent stem cells (iPSC) has been convincingly demonstrated (71, 127-129) Use of iPSC RPE cells for transplantation purposes has been investigated by some research groups (130-132). These cells provide the possibility of using autologous cell sources, but harbour the same safety issues as hESCs, namely that of tumorigenicity. However, in a novel approach described by Stadtfeldt et al. (133), nonintegrating adenoviruses can be used for the generation of so-called adeno-iPSCs, an approach in which tumor formation so far has not been observed. Whether adeno-iPSCs can be utilized in RPE transplantation remains to be elucidated.

The London Project to Cure Blindness is planning to commence a clinical trial in which the effect of transplantation of iPSC-derived RPE monolayers will be studied. Results are eagerly awaited, but the successful generation of iPSCs for use in AMD is a complex and very costly procedure, estimated to require 7 months to develop and approximately $1,000,000 per procedure (134, 135).

5.2.4 Risks Associated with RPE Transplantation

The transplantation of RPE cells to the subretinal space, particularly RPE cell sheets, is a demanding surgical procedure that harbors several risks. Surgical complications include hemorrhage, retinal detachment, cataract formation, and infections including endophthalmitis. In cases where allografts are transplanted, immunosuppressive therapy should be administered to avoid graft rejection, which can increase the risk of infections. Several research groups are using hESC-derived RPE or iPSC-derived RPE, where there is a known risk of tumorigenicity.

5.3 PRESERVATION OF RETINAL PIGMENT EPITHELIUM

5.3.1 Benefits of Banking

In corneal transplantation, the development of storage techniques has been a huge success, simplifying surgery logistics, enabling quality control and tissue
transportation, and providing worldwide tissue availability. With the advancement of RPE cell replacement therapy, and with 20-25 million known sufferers from AMD worldwide (27), a great need for improved storage methods for cultured RPE is likely to emerge.

### 5.3.2 Cryopreservation of Ocular Tissue

To our knowledge, the idea of tissue banking of RPE for transplantation was first coined by Valtink et al. (136), who established a storage facility for cryopreserved RPE cell suspensions in Hamburg, Germany. In their facility, donated RPE cells are propagated in culture, before one aliquot is cryopreserved and the other half is genotyped and morphologically characterized (136) for future reference. While serially cultured cells and cell lines are prone to genetic variation, banking of cryopreserved tissue offers the possibility of capturing a single desired phenotype (137). The method is also well established and relatively cost-effective. Although it offers a long-term solution to storage, there are known disadvantages to the method.

Cryopreservation inflicts freezing injury to tissues at both high and low cooling rates; high cooling rates cause direct damage from the formation of ice crystals, while lower cooling rates cause secondary damage by an increase in solute concentrations due to the gradual formation of ice (138). Optimally, tissues should be cooled at a particular rate balancing the two mechanisms of damage (139). To minimize damage caused by cooling, cryoprotectants are routinely added to the cryopreservation media. Glycerol or dimethyl sulfoxide (DMSO) are the most frequently used (139) and act by reducing the rate of ice formation. Vitrification circumvents the problems of ice crystallization, but requires high concentrations of cryoprotectants, which in itself causes more osmotic stress and toxic damage than can be tolerated by most cells (138, 140). In addition, xenobiotic compounds like fetal bovine serum (FBS) are routinely added to increase viability.

Although cryopreservation of cells is known to yield acceptable post-thawing viability rates (139, 141), cryopreservation has been reported to cause oxidative stress (142), irreversible changes to the cellular enzymatic function,
and induce telomere shortening and early cellular senescence (143). Membrane injury occurs by cooling below 5°C–15°C as a result of phase changes in the lipid bilayer (144, 145). It has also been demonstrated that cryopreservation increases the generation time of normal human choroidal melanocytes (NHCMs) 10.8-fold after thawing (146). Adding to this, the knowledge that cryopreservation necessitates the use of biofreezers or liquid nitrogen tanks for storage and transportation, as well as the need for thawing and propagation of thawed cells by the recipient clinic, warrants the development of alternative methods of storage.

5.3.3 Above-Zero Short-Term Storage of RPE
While cryopreservation is commonly used for the long-term storage (months to years) of RPE cell suspensions, a short-term storage method (e.g. a few days) would be sufficient for the purpose of transporting RPE grafts or propagated RPE cell layers to transplantation clinics. To date, however, no such protocol is available, and the optimal temperature for the short-term storage of RPE cells has not been established.

Due to strict regulatory demands (147, 148), the development of a suitable storage method will be essential to enable the transportation of viable cell constructs from centralized laboratories to operating theatres (147). Some have raised concerns over the current lack of international guidelines and common standards to risk management in the case of bioengineered ocular tissue (149). As pointed out by Utheim in a recent review covering transplantation and storage of cultured oral mucosa, the establishment of storage procedures may help standardize the culture protocol across international borders, particularly in cases where one culture unit provides cultured tissue to multiple clinics (150) (Figure 5).

In order to make RPE transplantation a realistic future treatment option, it is essential to establish a viable method for tissue banking of cultured RPE cells, with the imperative goal of avoiding xenobiotic components (5, 151, 152). The short-term serum-free and xenobiotic-free storage protocol proposed by our research group for limbal epithelial cells, addresses this matter (153). In essence,
given the correct media and temperature conditions, limbal epithelial cells can be stored without the addition of xenobiotic components. The method also offers the possibility of microbiological testing of stored tissue (154), enhancing the safety of the procedure. Implementation of this approach to the storage of RPE would also allow for pharmacologic or genetic modifications as well as expansion of the RPE cell layer before transplantation. Knowing that the transition from its natural environment and into primary cell culture often leads to morphological changes to the RPE, such as shortening and loss of apical microvilli, loss of basal infolds and alterations in surface protein distribution (6), the storage process offers an opportunity to augment the therapeutic effect of the cultured RPE transplant (72, 73, 155), aiming for cells that are able to fulfil the tasks of a differentiated RPE in vivo (5, 52, 152). Given the high level of expertise needed, this further adds to the notion that the process should be centralized in order to ensure acceptable quality. Consequently, centralization is likely to create a greater need for storage solutions for transportation.
Figure 5. Culture, storage and transportation of cultured RPE cells. Harvested cells (A) are expanded in vitro (B) and cultured under defined, optimal conditions avoiding xenobiotic compounds (C) before being placed in a custom-made storage system (D). Cells can then be transported (E) to surgical clinics offering treatment to patients worldwide (F). Courtesy of Dr. Sehic, Department of Oral Biology, University of Oslo, Norway. Published by Utheim et al. in 2016 (156).

5.3.4 Storage Media and Additives

5.3.4.1 Choice of Storage Medium

The maintenance of viability of stored cells is of critical importance if the graft is to survive and function optimally when transplanted into the subretinal space. Traditionally, ocular tissue preservation has focused predominantly on the storage of whole corneas, which are either cold-stored at 4°C or organ cultured at 31–37°C (157). However, whether these temperatures are optimal for maintaining RPE cell quality remains to be elucidated. Results from our research group on the storage of various cell types indicate that this might not be the case. In 2007, Raeder et al. discovered that organ culture storage of cultured human limbal epithelial cells (HLECs) was superior at 23°C compared to 37°C and Optisol-GS storage at 5°C (154). Later, Eidet et al. showed that the serum-free short-term storage viability of human conjunctival epithelial cells (HCjE) was similar for Optisol-GS and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered Minimum Essential Medium (MEM) storage (158). These findings were later supported by Jackson et al. who demonstrated excellent survival of cultured epidermal cell (CEC) sheets stored in xenobiotic-free HEPES-buffered MEM (159). Islam et al. (160) assessed the effect of nine different storage temperatures on the viability, morphology and phenotype of cultured human oral keratinocytes (HOK) stored in HEPES- and bicarbonate-buffered MEM. They found that cell viability was best preserved in the culture groups stored at 12°C and 16°C, as opposed to the typical 4°C and 37°C options.

MEM is a relatively cheap, xenobiotic-free and simple medium consisting of inorganic salts, amino acids, vitamins and glucose. As mentioned above, it has proven to be equally effective as more expensive alternatives for short-term
storage purposes of different cell types. Moreover, its simple composition is beneficial for research purposes, as it eases the interpretation of the individual effects of any added substances. Based on these considerations, MEM was chosen as the basis medium for the storage experiments performed in the current study.

5.3.4.2 Supplementation with Storage Medium Additives

In one of the papers presented in this thesis, the individual effects of a total of 46 different substances on storage of RPE cells is discussed. Most of the additives have, to our knowledge, never been tested in the current setting. An earlier study by our group showed that the addition of 1% sericin to the cell culture medium enhanced maturation of hRPE cells, most notably by increasing cell pigmentation (161). Therefore, sericin was supplemented to the storage medium of all groups in the study presented in Paper IV. Some additives were selected based on their known or proposed cell supportive effects in cultures of RPE or other cell types (14, 15, 161-170), while others were chosen based on effects demonstrated in pilot experiments.
6. AIMS OF THE PRESENT STUDY

The present study aspires to aid in the development of a novel storage technique as a part of a regenerative approach to treat patients suffering from age-related macular degeneration worldwide. First, we aim to identify the hitherto unknown optimum storage temperature for the conservation of RPE viability, morphology and phenotype. Second, we seek to investigate the changes in gene expression profiles of stored RPE cells in an effort to understand the mechanisms underlying temperature differences in cell viability, and describe how the genetic machinery of RPE phagocytosis, pigment synthesis, visual cycle and adhesion are affected by the basic environmental influence of temperature. Third, we seek to improve the preservation of cultured RPE cells by exploring various storage medium additives.
7. MATERIALS AND METHODS

7.1 CULTURE AND STORAGE OF RETINAL PIGMENT EPITHELIUM

7.1.1 Cell Culture Media and Reagents

Cells from the adult RPE cell line ARPE-19 were obtained from the American Type Culture Collection (ATCC) (Manassas, VA), while primary hRPE and complete epithelial cell medium (EpiCM) were obtained from ScienCell Research Laboratories (San Diego, CA). Dulbecco’s Modified Eagle’s Medium (DMEM): Nutrient Mixture F12, fetal bovine serum (FBS), bovine serum albumin (BSA), trypsin-EDTA, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium bicarbonate, gentamycin, phosphate-buffered saline (PBS), Triton X-100, penicillin, streptomycin, propidium iodide (PI), N1 growth supplement, taurine, triiodothyronine, non-essential amino acids and 4’,6-diamidino-2-phenylindole (DAPI) was purchased from Sigma-Aldrich (St. Louis, MO). The Minimum essential medium (MEM) and calcein-acetoxymethyl ester (CAM) was purchased from Invitrogen (Carlsbad, CA). Nunclon Δ-surface multidishes, glass coverslips, pipettes and other routine plastics were supplied by VWR International (West Chester, PA). The primary antibodies used included mouse anti-RPE65 antibody obtained from Abcam (Cambridge, UK), mouse anti-PCNA antibody from DAKO (Glostrup, Denmark) and rabbit anti-cleaved caspase-3 (Asp 175) antibody from Cell Signaling Technology (Danvers, MA). The secondary antibodies used were FITC conjugated to goat anti-mouse IgG and Cy3 conjugated to goat anti-rabbit IgG, both purchased from Abcam (Cambridge, UK). Nunclon T25 and T75 flasks, pipettes and other routine plastics were obtained from VWR International (West Chester, PA). The miRNeasy Mini Kit containing the QIAzol Lysis Reagent was from Qiagen (Venlo, the Netherlands). Acrodisc syringe filters for sterile-filtration were purchased from Pall Corporation (Port Washington, New York). The 47 additives used in the study are listed in Table 1.
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<th>Additive</th>
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<tr>
<td>1</td>
<td>Adenosine</td>
<td>5 mM</td>
<td>Sigma</td>
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<tr>
<td>2</td>
<td>Allopurinol</td>
<td>1 mM</td>
<td>Sigma</td>
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<tr>
<td>3</td>
<td>Arachidonic acid</td>
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<td>Sigma</td>
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<td>4</td>
<td>β-glycerophosphate</td>
<td>10 mM</td>
<td>Sigma</td>
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<tr>
<td>5</td>
<td>BSA</td>
<td>1 %</td>
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<tr>
<td>6</td>
<td>Capsazepine</td>
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<tr>
<td>7</td>
<td>Carnosine</td>
<td>20-50 mM</td>
<td>Sigma</td>
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<td>8</td>
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<td>Sigma</td>
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</tr>
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<td>14</td>
<td>Genistein</td>
<td>30 μM</td>
<td>Sigma</td>
</tr>
<tr>
<td>15</td>
<td>Glutathione</td>
<td>3 mM</td>
<td>Sigma</td>
</tr>
<tr>
<td>16</td>
<td>Glycerol</td>
<td>10 mg/mL</td>
<td>Sigma</td>
</tr>
<tr>
<td>17</td>
<td>HGF</td>
<td>100 ng/mL</td>
<td>Sigma</td>
</tr>
<tr>
<td>18</td>
<td>Hydrocortisone</td>
<td>3 ng/mL</td>
<td>Sigma</td>
</tr>
<tr>
<td>19</td>
<td>IGF1</td>
<td>100 ng/mL</td>
<td>Sigma</td>
</tr>
<tr>
<td>20</td>
<td>Imidazole</td>
<td>200 mM</td>
<td>Sigma</td>
</tr>
<tr>
<td>21</td>
<td>Insulin</td>
<td>5 μg/mL</td>
<td>Sigma</td>
</tr>
<tr>
<td>22</td>
<td>JSH-23</td>
<td>5 μM</td>
<td>Sigma</td>
</tr>
<tr>
<td>23</td>
<td>Kolliphor 188</td>
<td>0.05 %</td>
<td>Sigma</td>
</tr>
<tr>
<td>24</td>
<td>L-ascorbic acid</td>
<td>50 μg/mL</td>
<td>Sigma</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>28</td>
<td>Laevulose</td>
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<td>Koch Light Laboratories</td>
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<td>29</td>
<td>Leukemia Inhibitory Factor</td>
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<tr>
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<tr>
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<td>Sigma</td>
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<tr>
<td>36</td>
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<tr>
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<td>Sigma</td>
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<tr>
<td>42</td>
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<td>Sigma</td>
</tr>
<tr>
<td>43</td>
<td>Sucrose</td>
<td>10 mg/mL</td>
<td>BDH</td>
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<tr>
<td>44</td>
<td>Taurine</td>
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<tr>
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<td>Sigma</td>
</tr>
<tr>
<td>46</td>
<td>Triiodothyronine</td>
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<td>Sigma</td>
</tr>
<tr>
<td>47</td>
<td>Urea</td>
<td>10 mg/mL</td>
<td>Merck</td>
</tr>
</tbody>
</table>
Table 1. Additives used in Paper IV. Abbreviations: BSA: bovine serum albumin; DADLE: [D-Ala2, D-Leu5]-Enkephalin; DHA: docosahexaenoic acid; HGF: hepatocyte growth factor; IGF1: insulin-like growth factor 1; JSH-23: 4-methyl-1-N-(3-phenylpropyl)benzene-1,2-diamine; PEDF: pigment epithelium-derived factor.

7.1.2 Culture of RPE
As described in Papers I-III, adult human retinal pigment epithelial (ARPE-19) cells were routinely cultured in 95 % air and 5 % CO2 at 37°C in DMEM/F12 medium containing 10 % FBS, 50 units/mL penicillin and 50 µg/mL streptomycin. The cells were seeded (5000 cells/cm²) on Nunclon Δ-surface multi-dishes and glass coverslips. The culture medium was changed on the second day and confluent cultures were obtained on the third day. Control cultures, which were not subjected to subsequent storage, were then immediately processed for the various analyses.

A modified culture method was employed in Paper IV. Herein, third passage hRPE were seeded (20,000 cells/cm²) in complete EpiCM on 96-well Nunclon Δ 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 (161). The culture medium was changed every two or three days.

7.1.3 Storage of RPE

7.1.3.1 Storage Medium
In Paper I, ARPE-19 cells were cultured on multi-dishes for three days, upon which the culture medium was replaced by storage medium consisting of 1mL MEM, 25 mM HEPES, 22.3 mM sodium bicarbonate and 50 µg/mL gentamycin. Thereafter, the cultures were randomized for storage at nine temperatures (4°C, 8°C, 12°C, 16°C, 20°C, 24°C, 28°C, 32°C and 37°C) for seven days in custom-built storage containers without CO2 supply.
In Papers II and III, ARPE-19 cultures were handled similarly, but stored at either 4°C, 16°C or 37°C before being processed for further analyses.

In Paper IV, hRPE 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 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.

### 7.1.3.2 Storage Medium Additives

In Paper IV, a total of 46 different additives (Table 1) 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 hRPE cultures were stored at 4°C for ten days, without change or addition of storage medium. The storage containers were custom-built as explained below. pH measurements of the storage medium were performed using pH indicator paper.

### 7.1.3.3 Storage System

The storage containers were made from polystyrene and were kept in a cold-room which maintained an ambient temperature below 4°C. All containers were equipped with: 1) an electronic temperature display that enabled control of the storage temperature inside each box; 2) a heater that increased the temperature inside the box from the ambient room temperature (<4°C) to the desired storage temperature; 3) a highly sensitive thermometer that continuously regulated the heater; 4) and a small fan that ensured a homogeneous temperature inside the box by circulating the air. The stability of the temperature inside the storage containers was confirmed in a pilot study (Figure 6). The temperature inside each storage container was checked regularly throughout all experiments.
Figure 6. Pilot study assessing temperature variations in storage units. The temperature inside seven of the storage containers was noted at 86 consecutive time points throughout 20 hours to assess the magnitude of the variation of the set temperature. The maximum deviation was -0.8 to +1.0°C. Published by Pasovic et al. in 2013 (171).

7.2 VIABILITY OF STORED RETINAL PIGMENT EPITHELIUM

7.2.1 Cell Viability Assay using CAM/EH-1

Cell viability was studied in Paper I using a CAM viability assay and in Paper IV using a CAM/EH-1 viability assay. CAM is enzymatically cleaved into the green fluorescent calcein inside living cells (Figure 7A) (172). EH-1 is a membrane-impermeable dye that binds to the DNA of dead cells.

7.2.2. Viability Analysis using a Microplate Fluorometer

In Paper I, cell viability after one week of storage was determined using a microplate fluorometer. The cells were incubated for one hour in PBS containing 1 μM CAM and the CAM fluorescence was measured by a microplate fluorometer (Fluoroskan Ascent, Thermo Scientific; Waltham, MA) with the excitation/emission filter pair 485 nm/538 nm. The viability was determined for different temperatures (N = 6 for 4°C, 8°C and 24°C–37°C; N = 12 for 12°C–20°C). Three-day cultured cells that were not subjected to storage, but instead immediately analysed with CAM, served as controls.

To determine the reliability of the CAM measurements obtained by the microplate reader, a standard curve was made. Using a cell counter (Scepter 2.0
Cell Counter, Merck Millipore; Billerica, MA), cell suspensions with increasing cell concentrations were seeded in multi-dishes and left for two hours to ensure cell attachment. The cells were then incubated with the CAM reagent as described above to stain the attached cells. The CAM fluorescence was thereafter measured by the microplate reader. The number of seeded cells correlated highly with the measured CAM fluorescence, thereby showing great accuracy of the microplate reader measurements (Pearson’s $r=0.984$; $P < 0.001$) (Figure 7E).

Figure 7. Cell viability analysis. A calcein-acetoxyethyl ester (CAM) reagent, which exclusively stains living cells, was used to analyze cell survival. To validate the method, control cells and methanol-fixed cells were incubated with PBS containing 1µM CAM (green). (A) Control cells were CAM+ (green). (B) Corresponding phase contrast micrograph to A. (C) Fixed cells were CAM-. (D) Corresponding phase contrast micrograph to C. (E) Cells were seeded in multi-dishes in increasing concentrations and incubated for two hours to ensure attachment to the substrate. The CAM reagent was added to the cells for one hour, and the CAM fluorescence was measured with a microplate fluorometer. The number of seeded cells correlated significantly with the measured CAM fluorescence, thereby proving great accuracy of the microplate reader measurements. Published by Pasovic et al. in 2013 (171).
7.2.3. Cell Viability Assay using Quantitative Immunofluorescence

In Paper IV, cell viability was determined using quantitative immunofluorescence. 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. 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.

7.2.4. Factorial Design

A factorial design experiment was employed in Paper IV. It 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 used 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).

7.2.5. Validation of Cell Viability using Flow Cytometry

Flow cytometry was used to validate the viability results in Paper IV. Cells were cultured in T25 cell culture flasks following the aforementioned protocol. Control cells and cells stored in the optimal additive combination (1 % sericin, 5 mmol/L adenosine, 50 µg/mL L-ascorbic acid and 1 mM allopurinol) 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).

7.3 MORPHOLOGY OF STORED RETINAL PIGMENT EPITHELIUM

7.3.1 Scanning Electron Microscopy

In the study presented in Paper I, ARPE-19 cells were cultured on glass coverslips, and the samples were stored at nine temperatures for seven days before being processed for scanning electron microscopy (SEM) as previously described (N = 4) (173). In brief, stored cultures were fixed in 2.5 % glutaraldehyde solution, dehydrated in ethanol, and dried in compliance with the critical point method (Polaron E3100 Critical Point Drier; Polaron Equipment Ltd, Watford, UK). The control cultures were processed for SEM without delay after the three-day culture period. Coating of the samples with a 30 nm thick layer of platinum in a Polaron E5100 sputter coater was done prior to photographing with an XL30 ESEM electron microscope (Philips, Amsterdam, Netherlands).
7.3.2 Transmission Electron Microscopy

In the study presented in Paper IV, both unstored hRPE 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 (173). In essence, a Leica Ultracut Ultramicrotome (Leixa, Wetzlar, Germany) was used to cut ultrathin sections, which were examined using a CM120 transmission electron microscope (Philips, Amsterdam, the Netherlands).

7.4 PHENOTYPE OF STORED RETINAL PIGMENT EPITHELIUM

7.4.1 Immunocytochemistry

7.4.1.1 Preparations

Cells were cultured in 24-well multi-dishes and stored at 12°C, 16°C and 20°C (Paper I) for one week as described above. Samples were subsequently prepared for immunocytochemical characterization by 15 minutes of methanol fixation at room temperature. Permeabilizing/blocking solution containing PBS with 1% BSA and 0.2% Triton X-100 was added for a 30-minute incubation. Control cells were processed for immunocytochemistry immediately after the three-day culture period.

Anti-ZO-1 (1:50), anti-RPE65 (1:200), anti-PCNA (1:1000) and anti-cleaved caspase-3 (1:400) antibodies were diluted in blocking solution (PBS with 1% BSA). Primary antibodies were omitted from the negative controls. Samples were incubated overnight at 4°C. Goat anti-mouse FITC-conjugated secondary antibodies (diluted 1:250 in blocking solution) and goat anti-rabbit Cy3-conjugated secondary antibodies (diluted 1:10000 in blocking solution) were added for one hour at room temperature. Specimens were washed three times in PBS, with the addition of 1 µg/mL DAPI during the last wash to stain the cell nuclei. Positive control cultures for caspase-3 included incubating cells with 1
µM staurosporine for 24 hours. Treatment with staurosporine is expected to trigger expression of caspase-3 and induce cell apoptosis (174).

To visualize the actin cytoskeleton, samples were fixed in 4 % formaldehyde for 10 minutes, permeabilized with PBS containing 0.1 % Triton-X, and stained with PBS containing 25 µL/mL Alexa Fluor® 568 phalloidin methanolic stock solution. After incubating for 20 minutes in room temperature, specimens were washed in PBS and stained with DAPI.

7.4.1.2 Fluorescence Quantification by Interobserver Agreement

The specimens were studied using a Nikon Eclipse Ti fluorescence microscope and photographed at x200 magnification with a DS-Qi1 black-and-white camera. Photomicrographs were captured at five predetermined positions in each culture using a motorized microscope stage. The exposure length and gain was maintained at a constant level for all samples, and the image brightness was within the dynamic range of the camera. Two blinded and independent investigators assessed expression of the various markers in five photomicrographs in each culture (N = 8 (repeated twice, 4 each)). For the RPE65, PCNA and caspase-3 markers, the number of positive cells/total number of cells x 100 % was calculated. Assessment of interobserver agreement between the two investigators demonstrated high reliability of the phenotypic data (Table 2).

<table>
<thead>
<tr>
<th>Markers</th>
<th>Specificity</th>
<th>Investigator criteria for positive staining</th>
<th>Investigator A and B agreement</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Significance level of correlation (r) between investigators</td>
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<td>RPE65</td>
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<td>Stained cytosol</td>
<td><em>P</em> &lt; 0.001</td>
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<tr>
<td>PCNA</td>
<td>Proliferating cells (nucleus)</td>
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<td><em>P</em> &lt; 0.001</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Apoptotic cells (mainly cytosol)</td>
<td>Stained cytosol</td>
<td><em>P</em> = 0.044</td>
</tr>
</tbody>
</table>

CI = confidence interval; *r* = Pearson’s correlation coefficient

Table 2. Interobserver agreement of ARPE-19 cell characterization.
7.4.2 Whole Genome Transcript Profiling

7.4.2.1 RNA Extraction and Microarray Hybridization
Cultured ARPE-19 cells that had been stored for seven days at 4°C, 16°C, and 37°C, as well as control cultures that had not been stored, were rinsed with PBS and directly lysed with QIAzol Lysis Reagent (Papers II and III). A final amount of 150 ng of total RNA was subjected to GeneChip HT One-Cycle cDNA Synthesis Kit and GeneChip HT IVT Labeling Kit, following the manufacturer’s protocol for whole genome gene expression analysis (Affymetrix, Santa Clara, CA, USA). Microarray analyses were performed using the Affymetrix GeneChip Human Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA), which contains approximately 28,000 gene transcripts. Biotinylated and fragmented single stranded cDNAs were hybridized to the GeneChips. The arrays were washed and stained using FS-450 fluidics station (Affymetrix). Signal intensities were detected by Hewlett Packard Gene Array Scanner 3000 7G (Hewlett Packard, Palo Alto, CA, USA).

The scanned images were processed using the Affymetrix GeneChip Command Console software and the CEL files were imported into Partek Genomics Suite software (Partek, Inc. MO, USA). The Robust Multichip Analysis algorithm was applied for generation of signal values and normalization. Gene transcripts with maximal signal values of less than 32 across all arrays were removed to filter for low and non-expressed genes, reducing the number of gene transcripts to 17,684.

7.4.2.2 Microarray Data Analysis
Gene networks and canonical pathways representing key genes were identified using Ingenuity Pathways Analysis (www.ingenuity.com). Briefly, the data set containing gene identifiers and corresponding fold changes and P values was uploaded onto the web-delivered application and each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Functional analysis identified the biological functions and/or diseases that were most significant to the data sets. Fisher’s exact test was performed to calculate a P value determining the probability that each biological function and/or disease assigned to the data set was due to chance alone. The data sets
were mined for significant pathways with the Ingenuity Pathways Analysis library of canonical pathways, and networks were generated using graphical representations of the molecular relationships between genes and gene products.

### 7.4.3 Polymerase Chain Reaction (PCR)

The differential gene expression data were validated for selected transcripts (CSRNP1, PRKCB, CHOP, TYRP1, DSC1 and GLUT12) using TaqMan® Gene Expression Assays and the Applied Biosystems® ViIA™ 7 Real-Time PCR system (Applied Biosystems, Life Technologies) (Papers II and III). Briefly, 200 ng of total RNA was reverse transcribed using qScript™ cDNA Super Mix (Quanta Biosciences) following the manufacturer’s instructions. After completion of cDNA synthesis, 1/10th of the first strand reaction was used for PCR amplification. A total of 9 µl of diluted cDNA (diluted in H₂O), 1 µl of selected primer/probes TaqMan® Gene Expression Assays (Life Technologies) and 10 µl TaqMan® Universal Master Mix (Life Technologies) were used following the manufacturer’s instructions. Transducin-like enhancer of split 1 (TLE1) was used as endogenous control due to low coefficient of variation (CV) (0.444) in the Affymetrix study. Each gene was run in duplicates. TaqMan® Gene Expression Assays (Life Technology) used assays detecting CSRNP1 (Hs01042624_m1), PRKCB (Hs00176998_m1), CHOP (Hs00358796_g1), TYRP1 (Hs00167051_m1), DSC1 (Hs00245189_m1), GLUT12 (Hs01547015_m1) and TLE1 (Hs00270768_m1).

### 7.4.4. Proteomics

The proteome analyses were performed as described elsewhere (175). 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 (176) 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 (177). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis 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.

7.5 STATISTICAL ANALYSIS

A one-way analysis of variance (ANOVA) with Tukey’s post hoc comparisons (IBM SPSS Statistics for Macintosh version 19.0 and 22.0) (IBM Corp, Armonk, NY) was used for statistical evaluation of the results from the viability and immunocytochemistry analyses (Papers I and IV). Pearson’s correlation and a paired sample T-test were utilized to calculate observer agreement of the immunocytochemistry data (Paper I) and to compare two groups in the hRPE viability analysis (Paper IV). In the microarray analysis (Papers II and III), expression comparisons of different group profiles were performed using a 1-way ANOVA model. The results were expressed as fold changes (FC) and P values. In the PCR analysis, P values were calculated using Student’s T-test in Microsoft Excel using delta Ct values. Normalized target gene expression levels (FC) were calculated using the formula: $2^{-\Delta\Delta Ct}$ (Papers II and III). P values below 0.05 were considered significant for all analyses.
8. SUMMARY OF RESULTS (PAPERS I–IV)

**Paper I** describes the significance of temperature on the viability, morphology and phenotype of stored ARPE-19 cultures. The aim of this study was to identify the optimal storage temperature for the conservation of ARPE-19 cells. ARPE-19 cells were cultured on plastic multi-dishes following a standard protocol, sealed and randomized for storage in HEPES-buffered MEM at 4°C, 8°C, 12°C, 16°C, 20°C, 24°C, 28°C, 32°C and 37°C for seven days. The number of live cells after seven days of storage, as indicated by the CAM fluorescence measurements, was reduced at all storage temperatures compared to the control. Storage at 16°C conserved the highest number of live cells (48.7 ± 9.8%; \( P < 0.01 \) compared to 4°C, 8°C, and 24°C–37°C; \( P < 0.05 \) compared to 12°C). The cellular ultrastructure, as determined by SEM, was best maintained at 12°C, 16°C and 20°C storage, with mostly preserved intercellular adhesion, intact apical microvilli and only occasional apoptotic cells. At storage temperatures below 12°C and above 20°C, the majority of cells showed signs of cell damage and apoptosis. Immunocytochemistry revealed that the expression of actin, ZO-1, PCNA, caspase-3 and RPE65 was maintained after storage at 16°C. We conclude that storage at 12°C, 16°C and 20°C is optimal for maintenance of RPE cell viability, morphology and phenotype.

**Paper II** describes a microarray study where the expression of cell survival genes in ARPE-19 cells at three storage temperatures (4°C, 16°C and 37°C) is analyzed. The aim of this study was to identify gene expression profiles of stored RPE cells that could shed light on the mechanisms underlying temperature-dependent differences in cell survival during storage. The analysis revealed that the gene expression profiles of cell cultures stored at different temperatures cluster into separate groups, with 4°C cultures clustering closest to the unstored control. The 4°C group displayed the least change in total gene expression after storage (157 differentially expressed genes), while the 16°C and 37°C groups showed a much larger change in regulation (1787 and 1357 differentially expressed genes, respectively). At 16°C, the expression of several genes with proposed tumor suppressor functions was markedly increased.
Changes in regulation of several known signaling pathways and of oxidative stress markers were discovered at both 16°C and 37°C. The findings were validated by PCR. We conclude that ARPE-19 cultures stored at 16°C show the greatest propensity to modulate their gene expression profile in a manner that supports cell survival during storage.

**Paper III** builds on paper II, and aims to depict the details of storage temperature-induced effects on the expression patterns of genes critical to important RPE functions. DNA microarray technology was used to determine the expression levels of several key genes involved in phagocytosis, pigment synthesis, the visual cycle, adherens and tight junctions, and glucose and ion transport. In ARPE-19 cells stored at 4°C and 16°C, key genes from all categories were maintained close to control levels. Cells stored at 37°C displayed changes in the regulation of a larger subset of genes related to phagocytosis, adherens and tight junctions. The findings were validated by PCR. We conclude that RPE cultures stored at 4°C and 16°C for one week are capable of maintaining the expression levels of genes important for key RPE functions close to control levels.

**Paper IV** aims to improve the storage protocol for RPE cells by exploring various storage medium additives. The individual effects of 47 different additives on hRPE cell viability were determined using a CAM viability assay. The most promising additive combination was identified using a full-factorial design model. Epifluorescence microscopy demonstrated that supplementation of 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. The finding was validated by flow cytometry. Proteomics analysis identified few phenotypic changes between the hRPE group stored in the optimized medium compared to the unstored control, indicating equilibrium of the hRPE proteome during storage. Phase contrast microscopy and transmission electron microscopy demonstrated the presence of pigmentation and 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 hRPE pigmentation while maintaining cell viability during storage.
9. DISCUSSION

9.1 METHODOLOGICAL CONSIDERATIONS

9.1.1 Choice of Cell Source

9.1.1.1 Use of the ARPE-19 Cell Line

The ARPE-19 cell line was used in the studies presented in papers I-III. The cell line is widely used, given its recognition for displaying significant functional differentiation, forming polarized epithelial monolayers and tight junctions with barrier properties, as well as expressing several RPE-specific markers. These traits have made the cell line indispensable in RPE research over the last decades (7, 178). However, the ARPE-19 cell line does not mirror all the functions and characteristics of native RPE (179-181). Some studies have demonstrated a relatively lower expression of some RPE-specific transcripts in ARPE-19 cells compared to native RPE cells (182), while others have not (183). Native RPE in its natural state exhibits considerable regional variation, and thus any culture models of the RPE will be inherently heterogeneous (179, 184, 185). This diversity is further augmented in culture models and cell lines, which can exceed the normal variation described in RPE in vivo (179, 186-188). For strains of the ARPE-19 cell line, these changes in characteristics are attributable to differences in culture protocols in different laboratories over several years and are aggravated with increasing passages (179, 181). A recent study by Samuel et al. (189) presented a culture protocol in which ARPE-19 cells were cultured for a prolonged period (four months), thereby regaining phenotype and gene expression profiles similar to those of native human RPE cells. Since morphology and function of ARPE-19 cells can change during subcultivation, caution should be exercised when interpreting data acquired from experiments utilizing cultures from a high passage number (179, 181, 182). Therefore the ARPE-19 cells used in the current study have all been cultivated from passages lower than 4 after acquisition from the vendor.

ARPE-19 cells are capable of phagocytizing PR outer segments (4, 190, 191). However, there are some differences between ARPE-19 cells and primary
hRPE cultures. While both require the integrin receptor αvβ5 for the binding and internalization of outer segments (7, 190), there is a difference at the level of promoter strength, which yields a higher transcriptional activity in ARPE-19 (192). In a comprehensive review on RPE phagocytosis in culture comparing several RPE cell sources and cell lines including ARPE-19, Mazzoni et al. (193) conclude that no RPE model is clearly superior to others for phagocytosis studies. Human RPE are known to not produce melanin under regular culture conditions in vitro (19, 194, 195), and the ARPE-19 cell line has been shown to lack pigmentation when maintained under conditions similar to those described herein (179, 196).

9.1.1.2 Use of Primary Human RPE Cells

A study conducted by our research group shows that pigmentation can be induced in human RPE cells in vitro by the addition of the silk protein sericin to the culture medium (161). In that study, hRPE were cultured in the presence of sericin for up to 12 days. This contrasts to the study by Samuel et al. mentioned above, where a culture period for as long as four months was necessary to obtain pigmentation of ARPE-19 cells in lieu of sericin (189). Whether the addition of sericin to the culture medium might shorten the time of maturation of ARPE-19 cells, remains to be elucidated.

Primary human RPE cells were employed in Paper IV. These cells were obtained from a vendor that distributes hRPE cells to scientific laboratories worldwide. Primary hRPE was included in order to assess storage of pigmented RPE cells. Based on results obtained in a previous and unpublished study, the pigmented hRPE was only stored at 4°C, in contrast to the ARPE-19 cells.

9.1.2 Choice of Culture Medium and Substrate

In the studies presented in this thesis, RPE cells were cultured on glass or plastic culture dishes. This was done in order to reduce the culture variables to a minimum and allow the isolation of the individual impacts of storage temperatures and storage medium additives on cultured cells. Following that line of thought, the MEM storage medium was chosen in part for its simplicity. In
support of this study design, it has been shown that differences in the ARPE-19 transcriptome can be attributed to culture conditions, and that gene expression by cultured RPE cells is substrate dependent (197). It has been shown that culture of ARPE-19 cells on plastic substrates results in superior maintenance of a phenotype close to native RPE cells (198), yielding a functional profile of differentially expressed genes (198). Furthermore, the global expression profile of ARPE-19 cells can be directed towards that of primary RPE cells by withdrawing serum (180). In our studies, cells were cultured and stored on plastic, and the storage medium MEM contained no xenobiotic components (Papers I-III). In Paper IV, cells were cultured in a differentiation medium containing sericin and then stored in MEM with a total of 46 different, individually supplemented additives. The final optimal storage medium contained a combination of five storage medium additives including sericin.

Although several laboratories have investigated the cultivation of RPE cells on artificial substrates, aiming to identify carrier materials that could be directly transplanted into the subretinal space (80, 100, 199), there is currently no consensus with regard to the future use of culture substrates for the transplantation of RPE.

9.2 DISCUSSION OF THE RESULTS

9.2.1 Effect of Storage Temperature on Viability of ARPE-19 Cells

Paper I demonstrated that the viability of stored RPE cells is heavily dependent on temperature, and that the temperature interval 12°C–20°C was optimal for cell preservation (Figure 8). Tezel and coworkers assessed the viability of adult primary RPE cell sheet grafts after four days of storage at 4°C (96). They used a calcein and ethidium homodimer viability kit similar to ours, but employed cell counting rather than a microplate fluorometer. They found that the ratio of live cells to the total cell number decreased to 32.4 % after four days. We used the cell line ARPE-19 and stored the cells for seven days instead of four (Paper I). In line with the findings by Tezel et al., the 4°C storage group showed a great drop in the number of live CAM-retaining cells compared to the control. Notably, we compared the number of live cells after storage with the number of live cells in
the control, whereas Tezel and coworkers compared the ratio of live cells to the total cell number in the stored cultures. A later study presented in Paper IV and discussed below, showed excellent maintenance of hRPE viability during storage at 4°C by supplementation with a combination of storage medium additives.

Figure 8. Viability of stored ARPE-19 cells. Cultured ARPE-19 cells were stored for seven days at 4°C, 8°C, 12°C, 16°C, 20°C, 24°C, 28°C, 32°C and 37°C, and viability was assessed with a calcein-acetoxymethyl ester reagent. The bar chart shows the percentage of viable cells after storage compared to control cells (100 %).* P < 0.01 compared to 4°C, 8°C, 28°C and 37°C; P < 0.05 compared to 24°C and 32°C. † P < 0.01 compared to 4°C, 8°C and 24°C–37°C; P < 0.05 compared to 12°C. ‡ P < 0.01 compared to 4°C, 8°C and 24°C–37°C. Error bars represent the standard deviation of mean values. Published by Pasovic et al. in 2013 (171).

The finding that the best storage temperature for cultured ARPE-19 cells is approximately midway between the traditional temperatures for cell culture (37°C) and cold storage (4°C) is supported by studies on other epithelial cell
types performed by our research group. Raeder et al. reported that the storage of cultured human limbal epithelial cells at 23°C is superior to storage at both 5°C and 31°C (154), while another study reported that cultured human conjunctival epithelium maintained viability after four days of storage at 23°C in HEPES-MEM (158). A later study on the effects of different storage temperatures on viability, morphology and phenotype of human conjunctival epithelium concluded that storage at 12°C was optimal for the maintenance of this cell type (200). Another study on storage of cultured epidermal cell sheets also concluded that storage at 24°C was superior to 4°C and 37°C for preservation of viable cells at two-weeks storage (159), whereas temperatures 12°C, 16°C and 24°C all preserved high cell viability during one-week storage. Hypothermia has been shown to reduce both ARPE-19 cell metabolism and vascular endothelial growth factor secretion in a temperature-dependent fashion (201). We speculate that ARPE-19 cell metabolism is reduced at 16°C and that this contributes to improved cell survival.

### 9.2.2 Effect of Storage Temperature on Morphology of ARPE-19 Cells

Morphology of ARPE-19 cells stored at different temperatures was assessed by SEM (Paper I). An epithelial morphology similar to that demonstrated previously with subcultures of this cell line (7) was revealed. Cellular ultrastructure was best preserved at storage temperatures between 12°C and 20°C, although increased intercellular spacing was seen after storage at all temperatures (Figure 9). Some of the intercellular gaps, however, represented microcracks due to critical point drying as part of sample preparation for scanning electron microscopy (202). Apical microvilli seemed to be preserved during storage at 12°C–20°C. Loss of apical microvilli can occur with aging (203), causing unfavorable effects on the RPE cell functions and accelerating degenerative processes in the retina (203, 204). Hence, the temperature-specific preservation of microvilli demonstrated in our study further emphasizes the need for careful temperature control during ARPE-19 cell storage.
Figure 9. Morphology of stored ARPE-19 cells. Photomicrographs of control cells and cells stored for seven days at 4°C, 8°C, 12°C, 16°C, 20°C, 24°C, 28°C, 32°C and 37°C, were captured by a scanning electron microscope. An epithelial cobblestone morphology can be seen in the control (A, B) and this was best maintained after storage at 12°C, 16°C and 20°C (G-L). ARPE-19 cells demonstrate apoptotic morphological alterations like shrinkage and membrane blebbing after storage at temperatures below 12°C (C-F) and above 20°C (M-T). Images are representative of three independent samples. Scale bars: 100µm (black); 20µm (white). Black arrowheads: shrinkage and membrane blebbing. White arrowheads: microcracks representing artifacts due to sample preparation. Published by Pasovic et al. in 2013 (171).

9.2.3 Effect of Storage Temperature on Phenotype of ARPE-19 Cells

Paper I demonstrated the maintenance of actin, ZO-1, PCNA, caspase-3 and RPE65 expression in cells that had been stored at 16°C (Figure 10). Only cells stored at 12°C, 16°C and 20°C were phenotypically characterized.
9.2.3.1 Effect on the Actin Cytoskeleton of ARPE-19 Cells

Phalloidin-Alexa 568 was used to visualize the actin cytoskeleton to ensure normal epithelial characteristics of the employed ARPE-19 cells. The actin cytoskeleton is involved in many cellular functions, affecting both cell adhesion, morphogenesis and phagocytosis (205). Stress fibers are contractile bundles of actomyosin that are assembled when cells encounter mechanical stress (205). Their presence in vivo is usually confined to muscle cells and myofibroblasts in dermal wound tissue (206), but they are common in epithelial cells cultured in vitro (206). Formation of actin stress fibers in the ARPE-19 cell line has been reported earlier, and it has been demonstrated that the cells’ propensity for developing these fibers depends both on culture length and the composition of the culture medium (179).

The staining revealed that prior to storage, the actin filaments were mostly arranged in circumferential bands, but that a subset of cells were elongated and displayed stress fiber formation. After storage at 12°C, 16°C and 20°C, actin staining revealed no stress fiber formation, indicating both preservation and even promotion of normal epithelial characteristics of ARPE-19 cells stored in serum-free HEPES-MEM (Figure 10A). In support of this finding, Luo et al. (179) have reported a reduced tendency of stress fiber formation in ARPE-19 cells cultured in serum-free medium when compared to cells cultured in serum-supplemented medium.

9.2.3.2 Effect on Intercellular Tight Junctions of ARPE-19 Cells

Staining with anti-ZO-1 antibody was performed to assess the presence of intercellular tight junctions. The marker localized to cell borders between all apposed cells, indicating a tight junction organization typical of native RPE and revealing a cobblestone morphology with predominantly polygonal cells in all groups (Figure 10B).
9.2.3.3 Effect on RPE65 Expression of ARPE-19 Cells

The RPE65 antibody, localizing to the retinal pigment epithelium-specific 65 kDa protein, was used to detect expression of this essential RPE differentiation marker (196). RPE65 encodes a retinoid isomerohydrolase which functions in the visual cycle by production of 11-cis retinal (12). Expression levels were similar between control cultures and groups stored at 12°C, 16°C and 20°C, indicating preservation of the differentiated RPE phenotype during storage at these temperatures (Figure 10C-D).

9.2.3.4 Effect on PCNA Expression of ARPE-19 Cells

Proliferating Cell Nuclear Antigen (PCNA) is involved in cell cycle progression and DNA replication, among other vital functions. It is highly expressed during the S phase of the cell cycle (207, 208). Staining with anti-PCNA antibody revealed that the PCNA expression of ARPE-19 cells was maintained after storage at 12°C and 16°C, but increased after storage at 20°C (Figure 10E-F). Maintenance of PCNA expression during storage has also been reported for cultured human conjunctival epithelium kept for seven days at 23°C in HEPES-MEM (158).

According to Rieder and Cole, transition through the G₂ and M phases of mitosis comes to a halt when the temperature is lowered to approximately 16-20°C, thereby prolonging the cell cycle (209). The tendency of decreasing PCNA expression with storage temperature could therefore be related to inhibition of the G₂/M-transition occurring below these temperatures. Upon heating, cells that have been stored at 19°C proliferate at an even higher rate than that of control cells maintained at 37°C (209). The RPE cell layer is mitotically inactive in vivo (210), but has the ability to grow by cell enlargement if damage occurs (72, 210). Both transplanted freshly harvested RPE and transplanted cultured RPE are capable of proliferating in vivo, but proliferation is halted upon close apposition to the neural retina, indicating an effect of the neural retina in stalling RPE cell proliferation (89). Thus, it can be expected that the stored RPE cells will eventually stop dividing following transplantation. Some initial proliferative
activity may be advantageous, as it could enable transplanted cells to cover exposed areas of Bruch’s membrane (96, 103).
Figure 10. Phenotype of stored ARPE-19 cells. Cultured ARPE-19 cells were stored at 12°C, 16°C and 20°C for seven days, and expression of actin, ZO-1, RPE65, PCNA and caspase-3 was assessed. The percentage of cells expressing RPE65, PCNA and caspase-3 was quantified by two independent and blinded investigators. (A) Photomicrographs showing immunostaining with phalloidin-Alexa 568 used to visualize actin filaments (red). Nuclei were stained with DAPI (blue). (B) Photomicrographs showing immunostaining of ZO-1 (green). Nuclei were stained with DAPI (blue). (C) Photomicrographs showing immunostaining of RPE65 (red). Nuclei were stained with DAPI (blue). (D) Bar chart demonstrating RPE65 expression in stored and control cells. Expression of RPE65 was maintained after storage at all three temperatures. (E) Photomicrographs showing immunostaining of PCNA (red) in control and stored cells. Nuclei were stained with DAPI (blue). (F) Bar chart displaying the percentage of PCNA+ cells in the control cultures and in the storage groups. PCNA expression was maintained at 12°C and 16°C, and increased after storage at 20°C compared to the control. (G) Photomicrographs of cells stained with anti-caspase-3 antibody (red). Nuclei were stained with DAPI (blue). (H) Bar chart showing the percentage of caspase-3+ cells. There was no increase in caspase-3+ cells after storage compared to control. Original magnification of all photomicrographs: x200. All error bars: standard deviation of mean values. Published by Pasovic et al. in 2013 (171).

9.2.3.5 Effect on Caspase-3 Expression of ARPE-19 Cells

Sequential activation of caspases is a central process in cell apoptosis, and caspase-3 is a frequently used marker to detect apoptotic cells (211). In paper I, we did not detect an increase in caspase-3+ apoptotic cells after storage. However, the number of CAM+ live cells after storage dropped to less than 50% compared to the control (Figure 10G-H). The low percentage of caspase-3+ cells can be explained by the dead cells’ tendency to detach and wash away during rinsing prior to immunostaining. In support of this assumption, we found very few cells demonstrating an apoptotic morphology after storage at 12°C, 16°C and 20°C.
9.2.4 Effect of Storage Temperature on the Gene Expression Profile of ARPE-19 Cells

9.2.4.1 Effect of Storage Temperature on Genes Related to Cell Survival
Storage of cultured ARPE-19 cells at different temperatures can induce a wide range of effects on the cellular gene expression profiles (Paper II and Paper III). The overall gene expression profile of cultures stored at 4°C clustered closest to the controls and differed markedly from cultures stored at 16°C and 37°C (Figure 11). The compact internal clustering of each culture group strengthened the reliability of the analysis, and PCR validated it further.

Figure 11. Principal component analysis (PCA) mapping. The PCA shows that the different groups of cell cultures form four separate and independent clusters, indicating a clear distinction in gene expression patterns across cell culture groups. The three culture replicates in the 37°C storage group clustered less tightly than the respective culture replicates of the other groups. This might indicate a greater spread of data between replicates stored at 37°C. The false discovery rate was set to 1%. Published by Pasovic et al. in 2016 (212).
Single genes that exhibited particular increases in expression were evaluated. At 16°C storage, the FOS, CSRNP1, and TRIB1 genes were the three most upregulated genes (13.8-, 12.7- and 7.5-fold, respectively). FOS expression has been associated with metaplasia of skin (213) and bone (214), but also with favorable prognosis in ovarian cancer (215), mammary carcinoma (216), liver carcinoma (217), thyroid tumors (218) and gastric cancer (219). CSRNP1 functions as a tumor suppressor, and its expression is decreased in lung cancer, hepatocellular carcinoma, and colorectal carcinoma (220). Its overexpression, as shown in the 16°C storage group, has been reported to halt cell cycle progression at mitosis (221). TRIB-1 overexpression has also been shown to reduce the number of cell divisions (222). Whether TRIB-1 functions as a tumor suppressor (223) or an oncogene (224) is a matter of ongoing debate. The increased transcription of both CSRNP1 and TRIB1 at 16°C storage might, through their individual effects on halting mitosis, contribute to the preservation of cells seen after storage at this temperature.

At 37°C storage, desmocollin-1 and the protein kinase C gene PRKCB were upregulated 8.3- and 4.7-fold, respectively. Desmocollins constitute the adhesive proteins of the desmosome cell-cell junction (225) and are required for cell adhesion. Loss of function of desmocollins is associated with skin barrier defects (226) and metastasis of cancer cells (227). The role of DSC1 in tumorigenesis has not been thoroughly elucidated, but its isoform DSC3 has been described as a tumor suppressor in lung (228), breast (229), oral (230) and colorectal (231) cancer. PRKCB is involved in diverse cellular signaling pathways and is a critical regulator of cell proliferation and survival (232). The PRKCB protein has been reported to be involved in apoptosis, but whether alterations in its activity enhance or suppress apoptosis appears to depend on the initiating signal as well as the specific cell type (233-235).

9.2.4.2 Effect of Storage Temperature on Genes Associated with Cellular Stress
Oxidative stress can lead to the accumulation of unfolded or otherwise damaged proteins in the cellular endoplasmic reticulum (236). In turn, this can lead to triggering of the unfolded protein response (UPR) in the endoplasmic reticulum,
which aims to restore normal function by inhibiting general transcription, degrading misfolded proteins, and increasing the production of molecular chaperones involved in protein folding (236, 237). However, a failed attempt at the UPR can lead to cellular apoptosis. After storage at 37°C, three of the important UPR-associated genes were upregulated, and CHOP displayed a 4-fold increase (Paper II). CHOP signaling has been shown to induce cellular apoptosis (237, 238). Thus, these findings indicate the activation of the UPR in cells stored at 37°C, implying the presence of oxidative stress in cultures stored at this temperature. This is in line with earlier studies from our group, showing increased cell death in both ARPE-19 cultures and human conjunctival epithelial cultures stored at 37°C (171, 200).

9.2.4.3 Effect of Storage Temperature on the Regulation of Signaling Pathways
Five of the most well-known signal transduction pathways were analyzed to determine whether they are influenced by cell storage (Paper II). The Wnt, Janus kinase/signal transducer and activator of transcription (JAK/STAT), transforming growth factor beta (TGF-β), bone morphogenetic protein (BMP), and Hedgehog signaling pathways were selected for analysis. Cells stored at 37°C showed the most comprehensive changes in regulation of key signaling pathways. At this temperature, expression levels of seven genes related to the Wnt pathway were changed more than 1.5-fold. Notably, secreted frizzled-related protein (SFRP4), the soluble modulator of Wnt signaling, displayed a 12.6-fold downregulation. Lesser changes were noted for genes related to the JAK/STAT, TGF-β, Hedgehog, and BMP pathways. For cells stored at 16°C, there was increased activation of the JAK/STAT pathway, likely associated with the 13.8-fold upregulation of FOS. Lesser changes occurred in the TGF-β and BMP pathways. After storage at 4°C, only the c-FOS gene related to the JAK/STAT signaling pathway showed more than 1.5-fold change in expression compared to control cultures.
9.2.4.4 Effect of Storage Temperature on Genes Associated with Key RPE Functions

We investigated the effect of storage temperature on important cellular functions of ARPE-19 cells by comparing the expression levels of genes associated with phagocytosis, pigment synthesis, visual cycle, adherens and tight junctions, and glucose and ion transport (Paper III). The results are presented in Figures 12–13. We found no changes in the transcription of phagocytosis-associated genes after storage at 4°C and 16°C compared to control cells. Expression changes in genes involved in the recognition, engulfment, and degradation phases of RPE phagocytosis (239) could be identified following storage at 37°C. Two receptor ligand pairs are recognized for exhibiting key roles in the molecular machinery of RPE phagocytosis. These include the receptor tyrosine kinase MerTK and its secreted ligands Gas6 and Protein S, as well as the integrin receptor αVβ5 and its secreted ligand MFG-E8 (193). Loss of function of integrin αVβ5 or MFG-E8 renders the phagocytic ability of RPE cells irresponsive to light stimulation (240, 241), while loss of function of MerTK or both Gas6 and Protein S destroys the phagocytic ability altogether (242, 243). With the exception of Protein S, expression of all of these important genes was maintained during storage at all temperatures. Protein S was the most differentially expressed phagocytosis gene in our dataset, with a 1.7-fold increase in expression at 37°C storage. Protein S is a vitamin K-dependent serum protein that, in addition to its effects in the coagulation cascade, has a notable stimulatory effect on phagocytosis of PR outer segments (244). Although the differences in expression of the remaining phagocytosis associated genes were modest, these results may indicate a slightly disrupted phagocytic ability in cells stored at 37°C.

The expression of genes associated with pigment synthesis in the RPE was also evaluated. Human RPE cells do not produce melanin in vitro under regular culture conditions (19, 194, 195), and the ARPE-19 cell line is known to lack pigmentation when maintained under conditions similar to those described in the current study (179, 196). Four genes have been described as key contributors in the melanin biosynthesis pathway: TYR, TYRP1, TYRP2, and P gene (OCA2) (195). Smith-Thomas et al. (19) found that primary human RPE cells failed to express TYRP2, and that a very low percentage of the cells
expressed TYRP1, but only if cultured for more than 3 weeks. Lu et al. (195) found that human RPE cultured under standard conditions failed to express any of the four key genes of melanin synthesis mentioned above. However, we were able to detect both OCA2 and TYRP1 in all culture groups, as well as several other genes related to pigment synthesis. The largest individual change noted for pigment synthesis-related genes in our dataset was the 3.6-fold upregulation of the TYRP1 gene in cultures stored at 37°C. Expression of OCA2 was maintained at control levels in all temperatures. Loss of function of OCA2 and TYRP1 is associated with oculocutaneous albinism type 2 and 3, respectively, while no mutations of the TYRP2 gene have been reported (195).

A string of proteins contribute in the visual cycle, and the expression levels of critically important genes such as cellular retinol binding protein 1 (RBP1, also known as CRBP1), lecithin retinol acyltransferase (LRAT), cellular retinaldehyde binding protein 1 (RLBP1, also referred to as CRALBP) and cellular retinol binding protein 5 (RBP5) were maintained at control levels during storage at all three temperatures. This indicates that important components of the visual cycle can be preserved under the storage conditions used in this study.

Cell-cell adhesion is important for maintaining the correct RPE phenotype (245, 246), and loss of cell-cell contact can initiate epithelial-mesenchymal transition and the onset of cell proliferation (246). Adult RPE is mitotically quiescent, and contact inhibition is assumed to explain this phenomenon (3, 247, 248). Cultures stored at 4°C and 16°C did not differ from controls in regard to expression of adherens junction genes. Cultures stored at 37°C, however, showed a differential regulation of five adherens junction genes, among them an 8.3-fold upregulation of DSC1 and a change in expression of several cadherins. These changes might indicate a slight perturbation of adherens junction properties after 37°C storage. This group also showed the largest expression changes of tight junction genes, mostly down-regulation. This might indicate a loss of integrity of the intercellular junction in cells stored at 37°C compared to control cells. The classic tight junction proteins ZO-1 and occludin did not display any changes in expression levels after storage at any of the three temperatures.
Several GLUT proteins have been described, and GLUT1 has been identified as the most abundantly expressed isoform in RPE cells (249). Minor expression of GLUT3 (249, 250) and GLUT5 (249) in RPE cells has also been reported. All three were identified in our cells, in addition to eight other isoforms. The dominant expression of GLUT1 was demonstrated by an expression level 4 to 8-fold above that of GLUT3 and an expression level 100-fold above that of GLUT5 in all storage groups. This is in line with existing gene expression studies on native RPE (249-251). Expression of GLUT1 was maintained at control levels during storage at all temperatures. Given the dominant role of this transporter in RPE cells, the maintenance of its expression in all storage groups indicates a preservation of glucose transport function after storage. In an earlier study by Takagi et al. (249), the addition of FBS to the culture medium was shown to increase the expression of GLUT1 in human RPE cells. Based on this observation, one might anticipate a downregulation of this isoform when replacing the FBS-containing growth medium with a xenobiotic-free storage medium. However, that was not the case in our cultures. Expression of GLUT3 was increased 2-fold after storage at 16°C. GLUT3 is a highly effective glucose transporter, displaying both a higher affinity for glucose than GLUT1, -2, and -4, and a fivefold greater transport capacity than GLUT1 and -4 (252). Its expression has been identified in several cell types characterized by very specific and high metabolic demand, such as neurons and placental trophoblasts (252-254). Its expression in neurons increases in an activity-related manner, providing more glucose when confronted with an increased demand (252). One can speculate whether this strategy is utilized by ARPE-19 cells stored at 16°C and if it contributes to preserving a larger number of viable cells compared to other temperatures where GLUT3 expression remains unchanged.

Active transport of Na+ across the apical membrane of RPE cells creates a high Na+ concentration in the subretinal space, which is crucial for the photoreceptor dark current and for transport of solutes through symporters and antiporters of the RPE (20). Three isoforms of each of the Na+-K+-ATPase α and β subunits were identified, and most were expressed close to control levels in all storage groups. The same isoforms were identified in a recent study on native RPE (251).
Figure 12. Heat map diagrams 1. Heat maps demonstrating a selection of the most important genes related to RPE phagocytosis, pigment synthesis, adherens junctions and visual cycle, respectively. The color scale illustrates the relative expression level of ARPE-19 mRNAs: green color represents a high expression level; orange color represents a low expression level. Published by Pasovic et al. in 2015 (255).
Figure 13. Heat map diagrams 2. Heat maps demonstrating a selection of the most important genes related to RPE tight junctions, glucose transportation and Na-K-ATPase, respectively. The color scale illustrates the relative expression level of ARPE-19 mRNAs: green color represents a high expression level; orange color represents a low expression level. Published by Pasovic et al. in 2015 (255).

9.2.4.5 Considerations Regarding the Gene Expression of Cultures Stored at 4°C
We were intrigued to find that the microarray analyses revealed an expression profile of ARPE-19 cultures stored at 4°C which closely resembled that of unstored control cultures (Paper II and Paper III). This was unexpected given the low viability found in ARPE-19 cells stored at 4°C (Paper I). When considering these findings, it is essential to note that the generated gene expression values represent that of the remaining viable cells of the culture from each storage
temperature – meaning that the expression changes in cells which had died and detached from the substrate were not included in the analysis. The number of viable ARPE-19 cells at 4°C dropped to less than 4% of the control group (Paper I) (171). This finding can have at least two explanations: First, the cultures stored at 4°C contain a large number of dead and dying cells, which have a tendency to detach and be washed away, for example when rinsing the cell layer with PBS prior to addition of the QIAzol Lysis Reagent. Second, adhesion of ARPE-19 cells seems to be severely affected during 4°C storage, resulting in the loss of otherwise viable and well-functioning cells from the monolayer.

It is known that temperature has a crucial effect on the adhesive abilities of several cell types (256-259). An early study by Juliano and colleagues (259) demonstrated that the rapid adhesion of mammalian cells to glass surfaces at 25°C is completely inhibited when the temperature drops to 4°C. This supports the latter explanation model. Recent unpublished data from our research group demonstrates improved viability of ARPE-19 cells following storage at 4°C by implementing a radical change in the culture protocol in order to improve cell adhesion prior to storage. These findings further support our hypothesis that cellular adhesion is severely affected at low storage temperatures. In Paper IV, we demonstrate excellent viability of hRPE cultures stored at 4°C in the presence of several additives. Whether the drastically improved storage quality of hRPE cells at 4°C compared to that of ARPE-19 cells is related to inherent adhesive properties of hRPE cells, or to effects of storage medium additives, is yet to be determined.

9.2.5 Effect of Storage Medium Additives on Viability of Primary hRPE

9.2.5.1 Viability Using a Full-Factorial Design Model

Paper IV demonstrated that the number of viable cultured hRPE cells following storage can be increased by adding the combination of adenosine, L-ascorbic acid and allopurinol to our serum- and xenobiotic-free MEM-based storage medium containing sericin. A total of 47 individual additives were studied using CAM area fluorescence measurements (Figure 14).
Figure 14. Viability of primary hRPE cell cultures after storage at 4°C as measured by area of CAM fluorescence. The control line drawn from the black diamond represents the area of CAM fluorescence obtained in the control group, where 1% sericin was added to the MEM-based storage medium. Other bar points are representations of CAM area fluorescence for each additive supplemented to MEM in the presence of 1% sericin. Resulting effects are displayed as either increasing or decreasing CAM area fluorescence compared to the control line. The addition of carnosine, deferroxamine mesylate, glutathione or the protease inhibitor cocktail to the storage medium significantly reduces cell viability as measured by CAM area fluorescence (P < 0.05). Submitted by Pasovic et al. in 2017.
After defining the additives yielding highest CAM area fluorescence, 32 combinations of the five most promising additives were analyzed 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. The full-factorial experiment revealed that adenosine, allopurinol and L-ascorbic acid together provided the most desirable additive combination regarding cell viability (Figure 15 and 16).

![Factorial design analysis](image)

**Figure 15.** Factorial design analysis illustrated by ramp charts. Factorial design analysis of the five most promising additives providing a ramp display showing...
individual graphs for each additive in the most desirable storage medium combination. Presence of additive was set as “1”, while absence of additive was set as “-1”. The dot on each ramp represents the factor setting or response prediction for the resulting combination. Submitted by Pasovic et al. in 2017.

Figure 16. Factorial design analysis illustrated by a cube plot. The cube plot demonstrates the predicted response as a function of the three additives that created the most desirable effect. The plot shows how three factors (B, D, E) combine to affect the response. All values are predicted. Maximum desirability is reached at settings B+, D+ and E+ (allopurinol, L-ascorbic acid and adenosine). Submitted by Pasovic et al. in 2017.

9.2.5.2 Validation of Viability of Primary hRPE by Flow Cytometry

The full-factorial experiment revealed that adenosine, allopurinol and L-ascorbic acid together provided the most desirable additive combination regarding cell viability. This finding was controlled using CAM fluorescence measurements, which showed that viability after three days of storage was similar between control cells that had not been stored (N = 3) and cells stored in the optimal additive combination (N = 5), with a mean CAM fluorescence area of $99.2 \pm 0.1 \%$
and $98.6 \pm 0.5\%$, respectively (Figure 17A). The results were validated by flow cytometry, which showed that the percentage of viable cells was $96.8 \pm 0.5\%$ and $92.1 \pm 2.5\%$, respectively (Figure 17B). This is a much higher storage viability compared to that demonstrated in a similar study on storage of hRPE at 4°C by Tezel and colleagues. In that study, hRPE cell viability was $51\%$ after storage for three days (96). One should note, however, that cells were stored for three days in our final additive combination experiment, and not for ten days, as in the original viability experiment where individual additives were analyzed. A study investigating the storage of hRPE cells in the best additive combination for a total of 10 days is necessary in order to obtain a completely robust assessment.

Figure 17. Viability of primary hRPE stored at 4°C in the optimal combination of additives. hRPE were analyzed after three days of storage by A) quantitative fluorescence and B) flow cytometry. A) Cell viability as measured by area of CAM
fluorescence, demonstrating similar results between groups. B) Representative flow cytometry plots of dead cells by propidium iodide exclusion in control cells and cells stored for three days. The plots demonstrate a relatively low cell death rate in the stored group. Submitted by Pasovic et al. in 2017.

9.2.6 Effect of Storage Medium Additives on Morphology of Primary hRPE

9.2.6.1 Light Microscopy

Light microscopy demonstrated the presence of melanized hRPE cells in cultures that had been stored in the optimal additive combination. Cultured cells established the classic hexagonal distribution of mature hRPE monolayers (Figure 18).
Figure 18. Melanization of hRPE cells demonstrated by light microscopy. The microphotographs are representative samples from light microscopy observations before and after three days of hRPE storage at 4°C using the best additive combination. Melanized hRPE cells are visible in both groups and demonstrate the classic hexagonal distribution of mature hRPE monolayers. Submitted by Pasovic et al. in 2017.

9.2.6.2 Transmission Electron Microscopy

TEM confirmed the presence of melanosomes in hRPE cell cultures that had been stored in the optimal additive combination (Figure 19). Hence, the morphology findings in the present study indicate that hRPE cells can retain features of a mature phenotype when stored in the optimal additive combination.

<table>
<thead>
<tr>
<th>Melanosome stages</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>Pre-melanosomes, nonpigmented vacuoles (not shown)</td>
</tr>
<tr>
<td>Stage II</td>
<td>Internal striations (not shown)</td>
</tr>
<tr>
<td>Stage III</td>
<td>Some melanin pigment deposited onto the striations</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Fully melanized</td>
</tr>
</tbody>
</table>

Figure 19. Melanosomes in stored hRPE cells. Representative TEM image of hRPE cells stored in the best additive storage combination at 4°C for three days, demonstrating the presence of melanosomes at stage III (some melanin pigment deposited onto internal striations) and stage IV (fully melanized). Submitted by Pasovic et al. in 2017.
9.2.7 Effect of Storage Medium Additives on Phenotype of Primary hRPE

Proteomic analysis was performed to investigate the effect of the optimal combination of storage medium additives (adenosine, allopurinol and L-ascorbic acid) on phenotype of hRPE stored at 4°C. A total of 126 proteins were differentially expressed during storage for three days, of which 65 proteins (1.7%) were downregulated and 61 proteins (1.6%) were upregulated. A thorough discussion of all differentially regulated proteins is beyond the scope of this chapter. Of note, however, is the differential expression of two upregulated cytoskeleton-related proteins: Ezrin is a cortical cytoskeleton protein which localizes to epithelial microvilli (260). Loss of ezrin function as demonstrated in ezrin knockout mice leads to substantial reduction in RPE apical microvilli and retarded photoreceptor development (260). Desmoplakin is necessary for the anchoring of keratin at cell-cell contacts (261), and thus important for the regulation of desmosomal adhesion strength (262). It functions as a tumor suppressor (263), and a decrease in desmosomal protein expression is associated with poor prognosis in several cancers (264-266). Loss-of-function mutations in desmosomal proteins have been associated with clinical syndromes involving the skin, heart, hair and immune system (267-270). Upregulation of these proteins during storage might indicate that stored cells are able to maintain robust cytoskeletal functions. A proteomic analysis is in itself not sufficient to thoroughly characterize the cytoskeletal properties of stored hRPE.

Integrity of intercellular tight junctions is critical for fundamental RPE functions, like maintaining a high transepithelial resistance, securing cellular barrier function and regulating paracellular permeability (271-274). The expression of the tight junction protein ZO-1 was maintained during storage of hRPE in the optimal medium. This finding was supported by the light microscopy analysis, which demonstrated that cultured cells established the typical hexagonal distribution of mature hRPE monolayers.
9.2.8 Possible Effects of the Optimal Additive Combination on Primary hRPE

9.2.8.1 Effect of Sericin

The development of a serum-free cell storage medium is important due to the many risks associated with the use of FBS, including the potential transmission of animal pathogens (5), batch-to-batch variability and introduction of animal proteins during transplantation (275). While FBS has long been considered a necessity for maintaining viability in cryopreservation (276) and hypothermic storage at 4°C (277), it can be omitted if antioxidants are included in the storage medium (277). The combined effects of the additives comprising the optimal storage medium combination presented herein have not been described earlier, but their individual effects on many cellular processes have been widely studied. Based on effects discovered in earlier studies by our group sericin was included in the storage medium of all tested additives. Sericin is one of the main constituents of silk, which is produced by the silkworm, *Bombyx mori* (278). Sericin has shown promising qualities in cryopreservation of human and animal cell cultures, by successfully replacing FBS (279, 280) and reducing the need for DMSO (279, 281). Sericin has also been reported to display antioxidant properties by suppressing lipid peroxidation and inhibiting tyrosinase function (278). Its effect on maturation and pigmentation of hRPE cells has been discussed earlier in this thesis.

9.2.8.2 Effect of Adenosine

Adenosine is a purine nucleoside which has been shown to participate in the regulation of inflammatory responses by limiting inflammatory tissue destruction (282). Adenosine binds G protein-coupled adenosine receptors (283), of which there are several subtypes. Activation of the A3 receptor subtype has been demonstrated to protect retinal cultures against neurodegeneration (284). Activation of the ATP receptor P2X7 is known to induce death of retinal ganglion cells, but simultaneous intravitreal injection of an A3 receptor agonist can prevent the P2X7-associated cell death (285). P2X7 overactivation results in
dysregulated calcium signaling and is involved in the age-related dysfunction and degeneration of RPE cells (286). This suggests that overactive purinergic signaling may contribute to the geographic atrophy seen in dry AMD (287). The activation of adenosine receptors and inhibition of P2X7 is considered clinically relevant for the prevention of cell death in several eye diseases, including AMD (287). 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.

9.2.8.3 Effect of Allopurinol

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 (288) and has been successfully used in the treatment of autoimmune uveitis in an experimental setting (289). Allopurinol administered to RPE cell cultures in high doses has been demonstrated to prevent free-radical-induced cell damage (290). Its proposed effect on quenching free radicals might have contributed to enhancing cell viability of cultured hRPE cells during storage in the present study.

9.2.8.4 Effect of L-Ascorbic Acid

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 (53). 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 (291). In a study using ARPE-19 cells, however, ascorbic acid was not shown to protect the cells from hydroxyl radical induced cell death (292). Yet other studies have shown that ascorbic acid supplementation can protect RPE cells from hypoxic damage (293) and reduce vision cell loss from damaging light (294). However, the latter effect might be attributable to ascorbic acid
preventing excessive shedding of rod outer segments upon light exposure (295). The effect of ascorbic acid in the present study might be similar to that of allopurinol in that it reduces the oxidative stress burden. More research is needed in order to characterize the effects of these storage medium additives in further detail.

9.3 Strengths and Limitations of the Studies

9.3.1 Study Designs

All studies were designed as randomized controlled trials in order to reduce bias. Apart from the factorial design analysis presented in Paper IV, all studies analyzed one-factor effects while other variables were kept constant. Compared to one-factor-at-a-time studies, factorial experiments have several advantages (296). 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, compared to one-factor-at-a-time 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 analyses presented herein are generally based on a relatively low number of replicates. Therefore, generalizability to larger groups is not necessarily given. A consequence of using small samples is that some significant results might be missed due to underpowered studies.

9.3.2 Analyses

In Paper I, the comparisons of expressed proteins were subjectively assessed. In an effort to lessen the chance of observer bias, two independent and blinded investigators performed the analyses. Other methods, like microarray and proteomics analyses, provide a strengthened analysis of the quantification of

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expressed DNAs and proteins, and were therefore used in later studies (Papers II-IV).

9.3.3 Generalizability of the Results

The studies have been performed using two different sources of RPE cells, namely the ARPE-19 cell line (Papers I-III) and primary hRPE cells (Paper IV). *In vitro* studies are in any case not directly transferable to humans, and the potential drawbacks to using cell lines compared to primary cells has been discussed earlier in this section. While Paper I demonstrated highest viability of ARPE-19 cells stored at 16°C and very low viability for cells stored at 4°C, later studies by our research groups have demonstrated improved results for 4°C storage for primary hRPE cells (unpublished results). The reason for this finding has yet to be explained, but according to our unpublished results it seems like primary and differentiated (pigmented) hRPE might withstand storage at 4°C better than the unpigmented cell line ARPE-19. One could speculate whether the increased melanin content in the primary hRPE following culture in differentiation medium might be of significance. It has been shown that melanin can act as an antioxidant (16-18), and thus might protect hRPE cells stored at 4°C. This theory warrants further analyses, e.g. by storing differentiated ARPE-19 (using the differentiation medium mentioned earlier in this thesis) at different temperatures.

9.4 Ethics

All human material used in the studies presented herein were acquired from international vendors. The ethics requirements which otherwise apply to the use of animal or human tissues are therefore not applicable to these studies.
10. FUTURE PERSPECTIVES

The studies presented herein provide a novel approach to the development of a storage protocol for RPE cells. The methods used are, however, not directly clinically applicable, and further studies on the validation of our storage technique using several clinically applicable RPE cell sources and carrier substrates are warranted. Future studies aimed at identifying storage conditions and substrates for RPE cell maintenance could further refine the technology to improve cell survival and key RPE functions following storage.
11. CONCLUSIONS

11.1 General Conclusion

The overarching aim of this thesis was to aid in the development of a novel storage technique for RPE as part of a regenerative approach to treat patients with AMD. In accordance with this objective, we first aimed to identify the optimum storage temperature for RPE cells. We demonstrated that cultured ARPE-19 cells are best preserved during storage at 16°C, with regard to both viability, morphology and phenotype. Secondly, we aimed to discover mechanisms underlying temperature-dependent differences in cell survival by analysing the changes in gene expression profiles of stored RPE cells. We found that ARPE-19 cultures stored at 16°C modulated their gene expression profile in a manner that supports cell survival during storage. Changes in regulation of several signaling pathways and of oxidative stress markers were discovered. ARPE-19 cells stored at 16°C maintained the expression levels of RPE-specific transcripts important for phagocytosis, pigment synthesis, the visual cycle and adhesion. Thirdly, we tried to improve the preservation of RPE cultures by exploring various storage medium additives. We discovered that the storage viability of primary hRPE cells can be increased by the addition of a combination of sericin, adenosine, L-ascorbic acid and allopurinol to the storage medium. Knowledge of the influence of storage temperature and a large number of storage medium additives on RPE viability, morphology and phenotype might aid in the development of a future storage technique for RPE cells.

11.2 Conclusions of the Individual Papers

Paper I  
Storage at 12°C, 16°C and 20°C is optimal for maintenance of ARPE-19 cell viability, morphology, and phenotype. The study shows that the preservation of ARPE-19 cells is critically dependent on storage temperature.
**Paper II**  ARPE-19 cultures stored at 16°C show the greatest propensity to modulate their gene expression profile in a manner that supports cell survival during storage. Cultures stored at 4°C cluster closest to control cultures and display the least change in gene expression after storage.

**Paper III**  ARPE-19 cultures stored at 4°C and 16°C for one week can maintain the expression levels of key genes involved in phagocytosis, pigment synthesis, the visual cycle, adherens and tight junctions, and glucose and ion transport close to control levels.

**Paper IV**  The combination of adenosine, L-ascorbic acid, allopurinol and sericin in MEM preserves hRPE pigmentation while maintaining cell viability close to control levels during storage at 4°C. Antioxidant effects might be central to increasing cell viability during storage.
12. CONFLICT OF INTEREST

Lara Pasovic, Jon Roger Eidet and Tor Paaske Utheim have filed a patent application for a novel RPE storage medium based on the results described in this thesis.
13. REFERENCES


14. PAPERS