HANNA HIIDENMAA

Differentiation of Human Pluripotent Stem Cells Towards Functional Retinal Pigment Epithelium for Future Therapeutic Applications

ACADEMIC DISSERTATION
To be presented, with the permission of the Board of the BioMediTech of the University of Tampere, for public discussion in the Auditorium of Finn-Medi 5, Biokatu 12, Tampere, on December 12th, 2014, at 12 o’clock.

UNIVERSITY OF TAMPERE
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Differentiation of Human Pluripotent Stem Cells
Towards Functional Retinal Pigment Epithelium
for Future Therapeutic Applications

Acta Universitatis Tamperensis 2001
Tampere University Press
Tampere 2014
Abstract

The light-detecting retina is located at the back of the eye and consists of two layers: a complex network of sensory cells (neural retina) and a monolayer of retinal pigment epithelial (RPE) cells. RPE cells form a tight epithelium that maintains retinal functions in several ways. Thus, RPE dysfunction affects the health of the entire retina, causing the impairment or even loss of vision. Retinal diseases caused by dysfunctional RPE affect every third person aged 75 and older, and current treatments for these diseases are unsatisfactory. However, a cell therapy in which dysfunctional cells are replaced with healthy ones is believed to be a promising future treatment for retinal degenerations. In addition, in vitro models resembling authentic RPE would increase our understanding of RPE physiology, diseases, and pharmacokinetics.

Human pluripotent stem cells (hPSCs) are able to divide without limit and can be differentiated in vitro into any cell type of human body. Studies have shown that hPSC-derived RPE (hPSC-RPE) has several characteristics of authentic RPE, thus hPSCs have been promoted as one of the most potent sources of RPE cells. Consequently, the first clinical trials related to the safety and suitability of hPSC-RPE for the treatment of retinal diseases have been launched. However, the invocation of hPSC-RPE cells in the previously mentioned applications still presumes the wide characterization of the cells, the development of more efficient and safer methods for the production of functional hPSC-RPE, and standardized protocols for hPSC-RPE transplantations into the subretinal space of the eye.

This dissertation aimed to evaluate and improve protocols for hPSC-RPE differentiation, characterization, and transplantation for future approaches. Firstly, it was shown that putative RPE cells arise spontaneously from numerous hPSC lines that are maintained using human fibroblast feeder cells. The tendency to spontaneous differentiation varied between hPSC lines. Although the resulting differentiation efficacy remained moderate, RPE cells arose relatively quickly in vitro when compared with the natural development of human RPE. Increased understanding of RPE differentiation will help to improve methods for hPSC-RPE production. Secondly, it was demonstrated that a xeno-free culture medium is proper for hPSC-RPE differentiation. The avoidance of animal-derived
components in the production protocol is important because they may transfer pathogens to the host.

For the development of hPSC-RPE applications, it is remarkably important to identify the properties and quality of hPSC-RPE cells. A broad characterization panel showed that the cells differentiated in this dissertation work resembled native human RPE cells based on morphology, pigmentation, gene and protein expression, and cell polarization. Functionality analyses demonstrated that hPSC-RPE cells had the ability to phagocytize photoreceptor outer segments and to secrete RPE-related growth factor. In addition, they had similar electrophysiological and permeability properties as their native counterparts. Furthermore, it was shown that hPSC-RPE cells expressed efflux proteins, which are significant for the active transportation of molecules, e.g. drugs, through the blood–retina barrier. An increased understanding of the efflux proteins in the RPE is significant for the development of drugs for eye diseases.

Epithelial integrity is essential for a functional RPE, thus transplantation of the RPE as a tight epithelium is the most natural way to transplant hPSC-RPE cells into a diseased eye. Lastly, polyimide (PI) biomembrane was studied as a scaffold for hPSC-RPE. The mechanical properties of PI satisfy the requirements for an RPE scaffold. Additionally, the biocompatibility of PI has been proven in other solutions, which increases the promise of its suitability for retinal transplantations. PI as such was not suitable for supporting hPSC-RPE propagation; thus, different biopolymers for cell adhesion enhancement were evaluated. Many of the studied biopolymers improved hPSC-RPE cell adhesion and maturation on PI. Consequently, PI can be considered as a potential scaffold for hPSC-RPE in future applications, including cell therapy.

To conclude, this dissertation has increased understanding of hPSC-RPE differentiation and characteristics; this knowledge can be utilized in future applications with these cells. In addition, these results have contributed to the generation of a functional and transplantable hPSC-RPE free of animal-derived materials, which improves the safety and quality of future cell therapy.
Tiivistelmä


Tämän väitöskirjatutkimuksen tavoitteena oli kehittää menetelmiä, joiden avulla voidaan edesauttaa kantasoluista erilaistettujen RPE-solujen hyödyntämistä tulevaisuuden sovelluksissa. Ensiksi osoitettiin, että RPE-soluja saadaan erilaistetut spontaanisti useista ihmisen fibroblastien avulla ylläpidetyistä erittäin monikykyisistä kantasolulinjoista. Spontaani alttius erilaistusta RPE-soluiksi vaihteli solulinjojen välillä. Lopullinen erilaistamistehokkuus jäi alhaiseksi, vaikka silmän


Tämä tutkimus on yhtäältä lisännyt ymmärrystä ihmisen erittäin monikykyisten kantasolujen erilaistamisesta RPE-soluiksi sekä näiden solujen ominaisuuksista, ja toisaalta edesauttanut RPE-mallin syntymistä sekä turvallisen ja toimivan hoitokeinon kehitystä verkkokalvon rappuemasairauksiin.
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List of original communications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals (I–III):


* Hiidenmaa, née Vaajasaari

**Authors contributed equally

# Publication has been previously included in the doctoral dissertation “Delivery of biologics to the retinal pigment epithelium” by Astrid Subrizi, University of Helsinki, 2014.

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This dissertation contains some unpublished data, which is highlighted separately in the text.
Abbreviations

AASDH, amino adipate-semialdehyde dehydrogenase, (NRPS988)
ABCB1 ATP-binding cassette, sub-family B (MDR/TAP), member 1
ABCC ATP-binding cassette, sub-family C (CFTR/MRP)
ACTC1 actin, alpha, cardiac muscle 1
AFP alpha-fetoprotein
AMD age-related macular degeneration
ATP adenosine triphosphate
BCRP breast cancer resistance protein, (ABCG2)
bFGF basic fibroblast growth factor
BEST1 bestrophin-1
BioMediTech Institute of Biosciences and Medical Technology
BRB blood retina barrier
BrdU bromodeoxyuridine
BSA bovine serum albumin
Calcein-AM calcein-acetoxymethyl
CD36 platelet glycoprotein-4
CD133 Prominin-1
c-Myc myc proto-oncogene protein
CRALBP retinaldehyde binding protein 1, (RLBP1)
6-CF 6-carboxyfluorescein
Dkk1 dickkopf-1
DMEM Dulbecco's Modified Eagle Medium
EB embyoid body
ECM extracellular matrix
ELISA enzyme-linked immunosorbent assay
EMT epithelial mesenchymal transition
ESRRB estrogen-related receptor beta
FACS fluorescence-activated cell sorting
FBS fetal bovine serum
feRPE fetal RPE
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GMP</td>
<td>good manufacturing practice</td>
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<td>GUSB</td>
<td>glucuronidase, beta</td>
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<tr>
<td>hESC</td>
<td>human embryonic stem cell</td>
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<td>HEK293</td>
<td>Human Embryonic Kidney 293 cells</td>
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<td>hFF</td>
<td>human foreskin fibroblasts</td>
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<td>hiPSC</td>
<td>human induced pluripotent stem cell</td>
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<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>hPSC</td>
<td>human pluripotent stem cell</td>
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<tr>
<td>hPSC-RPE</td>
<td>human pluripotent stem cell-derived retinal pigment epithelium</td>
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<tr>
<td>HSA</td>
<td>human serum albumin</td>
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<tr>
<td>ICM</td>
<td>inner cell mass</td>
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<tr>
<td>IF</td>
<td>immunofluorescence</td>
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<td>1,4,5-trisphosphate</td>
</tr>
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<td>in vitro fertilization</td>
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<tr>
<td>Ki67</td>
<td>antigen KI-67</td>
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<tr>
<td>KO-DMEM</td>
<td>KnockOut Dulbecco's Modified Eagle Medium</td>
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<tr>
<td>Ko-SR</td>
<td>KnockOut™ Serum Replacement</td>
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<tr>
<td>KLF</td>
<td>krüppel-like factor</td>
</tr>
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<td>LIN28</td>
<td>lin-28 homolog A</td>
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<tr>
<td>LRAT</td>
<td>lecithin retinol acyltransferase</td>
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<tr>
<td>MEF</td>
<td>mouse embryonic fibroblasts</td>
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<tr>
<td>MEM-NEAA</td>
<td>Minimum Essential Medium non-essential amino acids</td>
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<tr>
<td>MERTK</td>
<td>tyrosine-protein kinase Mer</td>
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<tr>
<td>miRNA</td>
<td>micro RNA</td>
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<tr>
<td>MITF</td>
<td>microphthalmia-associated transcription factor</td>
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<tr>
<td>MRP</td>
<td>multidrug resistance associated proteins</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>Na/K-ATPase</td>
<td>sodium/potassium-transporting ATPase</td>
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<tr>
<td>NANOG</td>
<td>nanog homeobox</td>
</tr>
<tr>
<td>NIC</td>
<td>nicotinamide, vitamin B3</td>
</tr>
<tr>
<td>NUBP1</td>
<td>nucleotide binding protein 1</td>
</tr>
<tr>
<td>OTX</td>
<td>orthodenticle homeobox gene</td>
</tr>
<tr>
<td>PAX6</td>
<td>paired box 6</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>PEDF</td>
<td>pigment epithelium-derived factor</td>
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<td>P-GP</td>
<td>p-glycoprotein</td>
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<tr>
<td>PI</td>
<td>polyimide</td>
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<tr>
<td>PLGA</td>
<td>poly (lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PMEL</td>
<td>premelanosome protein</td>
</tr>
<tr>
<td>PSA-NCAM</td>
<td>polysialylated neuronal cell adhesion molecule</td>
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<td>POS</td>
<td>photoreceptor outer segments</td>
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<tr>
<td>POU5F1</td>
<td>POU domain, class 5, transcription factor 1, (OCT3/4)</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RAX</td>
<td>retina and anterior neural fold homeobox</td>
</tr>
<tr>
<td>RCS</td>
<td>Royal College of Surgeons</td>
</tr>
<tr>
<td>ROCK</td>
<td>rho-associated coiled-coil forming protein serine/threonine kinase</td>
</tr>
<tr>
<td>RPE</td>
<td>retinal pigment epithelium</td>
</tr>
<tr>
<td>RPE65</td>
<td>retinal pigment epithelium-specific 65 kDa protein</td>
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<tr>
<td>RPLP0</td>
<td>ribosomal protein, large, P0</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>RT</td>
<td>room temperature</td>
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<tr>
<td>SCED</td>
<td>single cell enzymatic differentiation</td>
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<tr>
<td>SIX3</td>
<td>six homeobox 3</td>
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<tr>
<td>SOX</td>
<td>sex determining region Y –box</td>
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<tr>
<td>SRY</td>
<td>sex determining region Y</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TEP</td>
<td>transepithelial electrical potential</td>
</tr>
<tr>
<td>TER</td>
<td>transepithelial electrical resistance</td>
</tr>
<tr>
<td>TF</td>
<td>transcription factor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor β</td>
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<tr>
<td>TJs</td>
<td>tight junctions</td>
</tr>
<tr>
<td>TRA-1-81</td>
<td>tumor related antigen-1-81</td>
</tr>
<tr>
<td>TYR</td>
<td>tyrosinase</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VSX</td>
<td>visual homeobox 2, (CHX10)</td>
</tr>
<tr>
<td>WB</td>
<td>Western blotting</td>
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<tr>
<td>ZO-1</td>
<td>zona occludens protein 1</td>
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1 Introduction

The retinal pigment epithelium (RPE) is located in the inner part of the eye. It regulates the homeostasis of the neural retina, and has an essential role in maintaining retinal health. Degenerative diseases of the retina, such as age-related macular degeneration (AMD), are the leading cause of central vision defects in the Western world (Kinnunen et al., 2012). Currently, there are no curative treatments for these diseases. Vision is considered the most important of the human senses. Consequently, it is important to find solutions for diseases that threaten vision. (Tucker et al., 2014).

It is hoped that human pluripotent stem-derived RPE cells (hPSC-RPE) cells will revolutionize the treatment of degenerative diseases of the retina. Human PSCs provide an unlimited supply of RPE cells (Hirami et al., 2009; Klimanskaya et al., 2004; Thomson et al., 1998). RPE-like cells are derived from various hPSC lines using different methods (Rowland et al., 2012). Furthermore, hPSC-RPE have many important characteristics of native human RPE, and their functionality has been proven in several studies (Bharti et al., 2011; Rowland et al., 2012).

Human PSC-RPE cells can be used to replace diseased cells at the back of the eye, and also as physiologically relevant in vitro cell model for studies of retinal diseases and for drug screening (Carr et al., 2013; Melville et al., 2013; Tucker et al., 2014). The first clinical trials using hPSC-RPE have been initiated (Bharti et al., 2014; Schwartz et al., 2012). However, many challenges remain to be overcome in order to ensure the safety and efficacy of hPSC-RPE cell therapy. Firstly, RPE differentiation protocols should be standardized and scaled up, and in terms of the safety of cell therapy, it is important that animal-derived and undefined components are replaced with safer alternatives, such as human-derived factors and recombinant proteins (Rowland et al., 2012; Unger et al., 2008). Secondly, RPE identity must be defined, and it must be shown that hPSC-RPE cells have the characteristics as well as functional properties of RPE (Bharti et al., 2011). Thirdly, the successful transplantation of hPSC-RPE cells into the retina still requires the development of a standardized surgical procedure, including a cell carrier system (Stanzel et al., 2012). Epithelial integrity is vital for RPE function, thus it is suggested that transplantation of hPSC-RPE as an intact cell sheet would improve
the integration of the transplant into the host tissue (Diniz et al., 2013; Kamao et al., 2014).

The objectives of this study were to investigate the differentiation of hPSCs towards RPE, to develop methods to enhance the safety of future hPSC-RPE transplants, to study the characteristics and functionality of hPSC-RPE, and to find a potential cell carrier for future applications of hPSC-RPE, such as cell therapy and \textit{in vitro} RPE modelling.
2 Literature review

2.1 The eye and the retina

The human eye is a small but complex organ that detects light and converts it into electro-chemical impulses. The eye consists of several specialized tissues (Figure 1). The transparent cornea covers the front of the eyeball. Together with the lens and iris, it forms the optic apparatus. Most of the refraction of light occurs in the cornea, the lens focuses the light onto the retina, and the iris regulates the amount of light entering the eye. In the middle and at the back of the eye, the eye wall has three layers. The outermost layer is the protective sclera. The middle layer is formed by the highly vascularized choroid, which supplies nutrients to the innermost layer: the photosensitive retina. Near the centre of the retina is the macula, which has the highest spatial resolution. (Bharti et al., 2011; Marmor and Wolfensberger, 1998).

The retina consists of two distinct parts: the neural retina and the retinal pigment epithelium (RPE) (Figure 1). The retinal layers are in close contact with each other, and the interphotoreceptor matrix fills the space between them (Strauss, 2005). The neural retina is a complex network of several light-detecting cell types. Photoreceptor cells – the rods and cones – detect light and convert light energy into electrical impulses that are transferred to the brain via the optic nerve. Visual signals are processed and amplified in the retina, but actual visual perception occurs in the visual cortex of the brain (Baylor, 1996). In contrast to the neural retina, the structure of the underlying RPE is simple; it is a monolayer of tightly packed cuboidal cells with a dense pigmentation (Marmor and Wolfensberger, 1998). Bruch’s membrane separates the RPE from the choroid (Booij et al., 2010). RPE cells express a specific apico-basal polarization. Long apical microvilli form a close connection with the photoreceptors, while the intensely folded basal side is responsible for nutrient uptake. Melanin granules are located in the apical compartments of the RPE cells, while the nuclei and mitochondria are found in the basal part. (Burke and Hjelmeland, 2005; Marmor and Wolfensberger, 1998; Marmorstein et al., 1998).
2.1.1 Development of the retina

Human eye development begins with the formation of the optic vesicles during the fourth developmental week (Figure 2). The optic vesicles arise bilaterally from the neuroepithelium of the primitive forebrain (O'Rahilly, 1975). Orthodenticle homeobox genes (OTX) -1 and -2, paired box 6 (PAX6), retina anterior neural fold homeobox (RAX), and six homeobox 3 (SIX3) are key factors for these early stages of eye development (Fuhrmann et al., 2014; Martinez-Morales et al., 2001; Muranishi et al., 2012). Next, the optic vesicles migrate and connect with the surface ectoderm. This interaction triggers the thickening of the surface ectoderm, resulting in the formation of the lens placode (Fuhrmann, 2010). The lens placode and optic vesicle invaginate, forming the lens vesicle and the bilayered optic cup. At this stage, the developmental pathway of the presumptive retinal layers separates and can be distinguished by the expression of microphthalmia-associated transcription factor (MITF) in the developing RPE and visual homeobox 2 (VSX, CHX10) in the presumptive retina (Fuhrmann, 2010; Hirashima et al., 2008; Hodgkinson et al., 1993). Although the developmental pathways of the retinal layers separate in the optic cup, they stay closely connected throughout their development. (Fuhrmann et al., 2014).

Interactions between surrounding tissues guide the patterning of the optic cup (Fuhrmann et al., 2014). The surface ectoderm induces the generation of the neural
retina in the distal part of the optic vesicle via fibroblast growth factor (FGF) signalling (Pittack et al., 1997). FGF signalling induces VSX2 expression in prospective neural retina cells and inhibits RPE generation (Bharti et al., 2008). In chicks, activin A secreted by extra ocular mesenchyme activates transforming growth factor β (TGFβ) signalling, which induces RPE fate and inhibits the development of the neural retina (Fuhrmann et al., 2000). MITF expression in the presumptive RPE is initiated in the dorsal optic vesicle (Bharti et al., 2008). MITF induces the genes that are important for melanin production, and hence it has a fundamental function in melanin-producing cells. OTX2 is another important gene for RPE melanogenesis. (Fuhrmann et al., 2014; Martinez-Morales et al., 2004).

The retina develops relatively early in human development. The RPE is fully maturated in the eighth developmental week, whereas maturation of the neural retina continues for several years after birth (Hu, Simon and Sarna 2008). Although retinal development is widely studied, studies are mainly done on animals, so the exact activation mechanisms of the signalling pathways that direct retinal development in humans are still unknown in part; for instance, we still do not know how MITF is regulated during retinal development (Fuhrmann et al., 2014).

Figure 2. The development of the RPE. Eye development starts in the primitive forebrain with the formation of optic the vesicle following the formation of the bilayered optic cup.

2.1.2 Functions of the RPE

Despite its simple structure, the RPE is a highly specialized epithelium that is vital for the functioning of the neural retina (Marmor and Wolfensberger, 1998). The RPE enhances visual acuity by absorbing scattered light into its apical melanosomes (Strauss, 2005). Another way the RPE maintains visual functions is the regeneration and recycling of a visual pigment in the process called the visual cycle. In the photoreceptors, 11-cis-retinal is bound to a G protein-coupled receptor (opsin) forming rhodopsin (Buczyilko et al., 1996; Hargrave et al., 1993).
Light absorption by opsin causes the conformation change of 11-cis-retinal into all-trans-retinol, which elicits opsin activation, and finally the photon’s energy is transferred into electrical signals (Baylor, 1996). In the RPE, all-trans-retinol is first reisomerized back into 11-cis-retinal conformation, which is transported back to the photoreceptors. Lecithin retinol acyltransferase (LRAT), Retinaldehyde-binding protein 1 (CRALBP, RLBP1) and Retinal pigment epithelium-specific 65 kDa protein (RPE65) are mainly responsible for the visual cycle (Bunt-Milam and Saari, 1983; Moiseyev et al., 2005; Strauss, 2005).

Furthermore, the RPE supports the renewal of the photoreceptors by phagocytizing damaged photoreceptor outer segments (POS) and recycling essential substances back to the photoreceptors (Young and Bok, 1969). The phagocytosis is regulated by the circadian rhythm (LaVail, 1976), light exposure, and the following receptors: the αVβ5-Integrin receptor, Tyrosine-protein kinase Mer (MERTK), and Platelet glycoprotein 4 (CD36) (D’Cruz et al., 2000; Finnemann et al., 1997; Gal et al., 2000; Ryeom et al., 1996). POS phagocytosis, high oxygen consumption, and light exposure cause remarkably high oxidative stress in the retina (Strauss, 2005; Tate et al., 1995). The RPE protects the retina against oxidative stress with its melanin, antioxidants and lysosomes (Boulton and Dayhaw-Barker, 2001). The RPE transports ions and molecules, such as water, nutrients, and metabolites between the neural retina, the subretinal space, and the choroid, making it metabolically significant (Strauss, 2005).

Lastly, the RPE secretes numerous growth factors, such as FGF, TGFβ, Vascular endothelial growth factor (VEGF) and Pigment epithelium-derived factor (PEDF) (Adamis et al., 1993; Jablonski et al., 2000), as well as immunosuppressive factors such as interleukins. The dialogue with the immune system via interleukins together with the isolation of the inner retina by the blood retina barrier (BRB) also makes the RPE an immunologically significant tissue (Strauss, 2005).

2.1.2.1 Barrier properties of the RPE

Ocular functions require a specific environment, which is maintained by the BRB. The BRB consists of two major components, an inner BRB formed by the endothelium of the retinal blood vessels, and an outer BRB formed by the RPE (Cunha-Vaz, 2004). The outer BRB maintains the homeostasis of the retinal environment by preventing and regulating the transportation of solutes between the choroid and the retina (Runkle and Antonetti, 2011). The tight junctions (TJs) of the RPE are fundamental components of the outer BRB (Rizzolo et al., 2011).
TJs are formed gradually during RPE development and their main components in the RPE are zona occludens protein 1 (ZO-1), N-cadherin, and Claudin-3 and -19 (Harhaj and Antonetti, 2004; Peng et al., 2011; Peng et al., 2013; Rizzolo, 2007). Prominent TJs form a structural barrier between the apical and basal compartments of the RPE, resulting in the specific polarization of the RPE, which is defined by protein and organelle distribution, growth factor secretion, and cell membrane structure differences (Bertolotti et al., 2014). The tightness of the TJs can be evaluated by measuring transepithelial electrical resistance (TER). TER values are highly dependent on the machinery used and are approximates, since membrane folding and microvilli are impossible to take into account when defining the surface area (Rizzolo, 2014).

Active transport is another key function of the BRB (Runkle and Antonetti, 2011). The membrane transporter proteins can be divided to influx and efflux proteins based on the direction of their transportation. Efflux proteins belong to the ATP-binding cassette transporter family that actively transports substances out from cells (Sharom, 2008). The efflux proteins, such as Multidrug resistance-associated proteins (MRP, ABCC), P-glycoprotein (P-GP, MDR1), and the breast cancer resistance protein (BCRP) have a significant role in the pharmacokinetics of the eye, but their exact mechanism of action is still poorly understood (Dahlin et al., 2013). In addition, a functional and reliable in vitro model for BRB does not yet exist (Vellonen et al., 2014). Increased knowledge about efflux protein functions would be valuable in understanding the pharmacokinetics of the eye and in the generation of more potent treatments for ocular diseases (Mannermaa et al., 2009; Vellonen et al., 2014).

2.1.3 Age-related macular degeneration

As demonstrated in the preceding chapters, the RPE has an essential role in maintaining retinal health. Therefore, it is obvious that a dysfunctional RPE causes the degeneration of photoreceptor cells and leads to the impairment of vision. An RPE defect is involved in the early stages of many retinal diseases, such as age-related macular degeneration (AMD), Stargardt’s disease, and retinitis pigmentosa (Kinnunen et al., 2012; Ramsden et al., 2013).

AMD is one of the leading causes of blindness in industrialized countries (Klein and Klein, 2013). The prevalence of AMD is likely to increase in the future due to rising life expectancy; the predicted number of AMD patients worldwide in 2020 is
almost 200 million (Wong et al., 2014). AMD occurs in two forms: dry AMD (atrophic AMD) and the more progressive but rarer wet AMD (exudative AMD). AMD is a multifactorial disease; it emerges through a combination of genetic and environmental factors in a way that is not fully understood. Accumulation of extracellular aggregates – the drusens – between the RPE and Bruch’s membrane is often the first sign of dry AMD (Kinnunen et al., 2012). Due to the drusens, the RPE detaches from Bruch’s membrane, which eventually leads to RPE cell death. In about 10% of AMD cases, the pathogenesis progresses as a choroidal neovascularization, causing wet AMD. Abnormal angiogenesis leads to rapid loss of vision. (Holz et al., 2014; Kinnunen et al., 2012).

Progression of wet AMD can be prevented with VEGF inhibitors (Holz et al., 2014). However, the treatment is not curative; it requires repeated dosing and monitoring, which can cause massive expenses and may burden the inner eye. In addition, in 20% of cases vision loss still occurs after VEGF inhibitor treatments, and some patients lose the improvements gained in visual acuity (Gibson and Gibson, 2014). The more common dry AMD has currently no proven treatment. One of the most promising ways of preventing AMD progression is to replace diseased RPE cells with their healthy counterparts in the early stage of pathogenesis. Human primary RPE, fetal RPE (feRPE) and a transformed RPE cell line as well as some non-RPE cells, such as retinal progenitors and neural stem cells, are considered a potential source of RPE cells (Blenkinsop et al., 2012; da Cruz et al., 2007; Ramsden et al., 2013). Despite promising results, issues of quality, availability, and ethics, not to mention technical problems, restrict the use of these cells (da Cruz et al., 2007; Lund et al., 2001).

2.2 Human pluripotent stem cells

Human PSCs are able to self-renew indefinitely and differentiate into cells of all germ layers (Tanabe et al., 2014; Yamanaka et al., 2008). The limitless differentiation capacity of hPSCs has generated enormous interest from biomedical science (Melville et al., 2013). Human PSC lines are derived from early embryos or by reprogramming the pluripotent machinery of somatic cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Thomson et al., 1998). Human PCS lines can be used as a foundation for fully differentiated cells and tissues (Yamanaka et al., 2008). These cells can be exploited in regenerative medicine, for drug screening, and as an in vitro model for studying human physiology, pathogenesis, and
toxicology (Kujala et al., 2012; Melville et al., 2013; Meyer et al., 2009; Schwartz et al., 2014).

2.2.1 Establishment of human embryonic stem cell lines

When Thomson et al. (1998) described the derivation of the first stable human embryonic stem cell (hESC) lines; they also defined three criteria for hESCs: derivation from the preimplantation embryo, prolonged undifferentiated proliferation, and the stable differentiation potential to form derivatives of all three germ layers even after prolonged culture. Sixteen years later, over 1500 hESC lines have been registered with the International Stem Cell Registry (http://www.iscr-admin.com).

In general, hESC lines are derived from the inner cell mass (ICM) of human blastocyst stage embryos, but morula or even single blastomeres can also be used (Klimanskaya et al., 2006; Unger et al., 2008). An isolated ICM is placed on supportive feeder cell layers. Mitotically inactivated mouse embryonic fibroblast (MEF) cells and human foreskin fibroblasts (hFF) are the most used feeder cells (Crocco et al., 2013). Recently, hESC lines have also been successfully derived without feeder cells (Rodin et al., 2014). The ICM is propagated in a special culture medium for a few passages to gain a developmentally stable hESC line. Basic fibroblast growth factor (bFGF) is a critical component for the maintenance of pluripotency (Vallier et al., 2005). When an hESC line is finally established, the cells are tightly packed into colonies (Hoffman and Carpenter, 2005; Thomson et al., 1998). The morphology of the colony changes rapidly when the cells differentiate.

Legislation on the use of human embryos for the derivation of hPSC lines varies between countries (Hyun et al., 2008). In Finland, couples undergoing in vitro fertilization (IVF) treatment can donate excess embryos for research. These surplus embryos are often poor quality, and thus the establishment of new hESC lines is challenging (Skottman, 2010).

2.2.1.1 The culture of hESC lines

The pluripotent stage is very fragile, and the maintenance of hESCs is laborious and costly. Human PSCs require bFGF and substrata to support pluripotency (Vallier et al., 2005). Traditionally, hPSCs were cultured on MEFs with fetal bovine serum (FBS) (Thomson et al., 1998). However, undefined or non-human
components in hESC cultures should be avoided because they cause safety risk if the cells are transplanted into humans (Hoffman and Carpenter, 2005; Martin et al., 2005). FBS is therefore often replaced with the more constant and better-defined KnockOut serum replacement (Ko-SR) supplemented with human recombinant bFGF. However, Ko-SR is still not optimal for clinical applications, since it contains bovine serum albumin (BSA) (Hoffman and Carpenter, 2005). Additionally, MEFs can be substituted with feeder cells of human origin, such as hFF (Hovatta et al., 2003) or feeder-independent culture conditions (Rodin et al., 2014; Vallier et al., 2005). Extensive variation in the derivation and culture methods of hESCs also results in high variation in the quality and specific properties of the hESC lines (Allegrucci and Young, 2007).

2.2.1.2 Characterization of hESC lines

The stable diploid karyotype of hESC lines and their pluripotency – meaning the limitless ability to generate all three germ layers – must be confirmed after derivation and regularly during the lifespan of the hESC lines. Pluripotency is often studied by teratoma formation in immunodeficient mice (Hoffman and Carpenter, 2005). A more straightforward but more imperfect way to study differentiation potential without the sacrifice of an animal is to perform spontaneous in vitro differentiation in three-dimensional embryoid bodies (EBs) (Itskovitz-Eldor et al., 2000; Watanabe et al., 2005). EBs are multicellular complexes where cells can cooperate in the same way as in the early embryo (Keller, 1995). Furthermore, pluripotency can be examined by the expression of genes and proteins defining pluripotency at the molecular level, such as POU domain, class 5, transcription factor 1 (POU5F1, OCT3/4) (Takeda et al., 1992), Nanog homeobox (NANOG) (Chambers et al., 2003), Sex-determining region Y (SRY)-box 2 (SOX2) (Stevanovic et al., 1994), Krüppel-like factors 2 and 4 (KLF2, KLF4) (Wani et al., 1999; Yet et al., 1998), and Estrogen-related receptor beta (ESRRB) (Giguere et al., 1988; Hoffman and Carpenter, 2005). Telomerase and alkaline phosphate activities are also often studied when pluripotency is evaluated. The characteristics of hESCs must remain after long-term in vitro culture. (Hoffman and Carpenter, 2005).
2.2.2 Human induced pluripotent stem cells

Yamanaka and Takahashi made a ground-breaking discovery in showing that differentiated adult cells can be genetically manipulated back to the pluripotent stage to have similar characteristics as ESCs (Takahashi and Yamanaka, 2006). These cells are called induced pluripotent stem cells (iPSCs). At first, Yamanaka and Takahashi reprogrammed the pluripotent machinery of mouse fibroblasts using four transcription factors – Myc proto-oncogene protein (c-Myc), Pou5f1, Sox2, and Klf4 – which are nowadays known as Yamanaka factors (Takahashi and Yamanaka, 2006). Soon thereafter, the same was done with human cells (hiPSC) (Takahashi et al., 2007). Since then, iPSC lines have been generated from numerous tissues with different sets of transcription factors (Li et al., 2009). Initially, the reprogramming was initiated using integrative viral vectors, but safer non-integrative vectors have now been developed (Fusaki et al., 2009; Kim et al., 2009; Warren et al., 2010). The reprogramming success varies according to the tissue type and the reprogramming method (Borooah et al., 2013). Generally, hiPSC lines are maintained and characterized in the same way as hESC lines.

2.2.3 Comparison of hESCs and hiPSCs

At first, it seemed that hiPSCs were identical to hESCs. However, several groups have recently reported differences between them (Bai et al., 2013; Buchholz et al., 2009; Chin et al., 2009). The comparison is challenging, since even different hESC lines are known to have extensive differences in their characteristics, such as their differentiation capacity (Osafune et al., 2008; Toivonen et al., 2013). Nevertheless, obvious differences between hESCs and hiPSCs are due to origin and establishment method (Figure 3).

Human iPSC lines can be derived from adult tissues, e.g. skin fibroblasts, whereas hESC lines are derived from human embryos, where ethical concerns are obviously significantly greater. Attempts have been made to overcome the ethical problems related to hESC line derivation by establishing hESC lines from blastomeres (Klimanskaya et al., 2006; Rodin et al., 2014). Blastomeres can be isolated without destroying the embryo. However, the later use of the manipulated embryo is questionable. It is a matter of debate as to which is the greater ethical problem: the later use of attacked embryos or the destruction of the excess embryos.
Although the generation of hiPSC lines does not create the same ethical problems as the derivation of hESC lines, the clinical safety of hiPSC lines raises other kinds of issues. First, the epigenetic memory may remain if the reprogramming of pluripotency is insufficient (Hu et al., 2010; Watanabe et al., 2013). This may result in an insufficient differentiation capacity or cause abnormal growth in the host (Hussein et al., 2011; Toivonen et al., 2013). Another safety aspect is related to genetic manipulation methodology. Possible reactivation of transgenes after transplantation increases the risk of tumorigenesis (Tanabe et al., 2014). Methods to enhance the efficiency and safety of reprogramming methods are under investigation (Borooah et al., 2013). Non-integrative transfection methods, such as the Sendai virus (Fusaki et al., 2009), DNA plasmids (Takahashi et al., 2007) protein transduction (Kim et al., 2009), mRNA (Warren et al., 2010), and small molecules (Lin et al., 2013) would increase the safety of hiPSC lines. Despite these advancements, current methods are still not sufficient to warrant the clinical safety of hiPSC lines (Borooah et al., 2013).

One absolute advantage of iPSC technology is the possibility to create autologous hPSC lines. The autologous hPSC lines provide a unique tool for personalized medicine where particular features of individuals can be studied (Jin et al., 2011; Kujala et al., 2012; Tucker et al., 2014). This may revolutionize the treatment of certain rare diseases and help us to understand their pathogenesis (Tucker et al., 2014). Initially, it was hoped that immune rejection, which has been observed with hESCs (Utermohlen et al., 2009), could also be avoided using autologous hiPSC lines. However, it has later been shown that even more immunogenic problems are seen with autologous hiPSCs than with hESCs (Carr et al., 2009b; Zhao et al., 2011). In addition, treatment of genetic diseases with autologous cells is problematic since transplanted cells have the same genetic background, including the mutation causing the treated illness (Hussein et al., 2011). Respectively, hESCs may also carry a genetic disease. However, in the case of age-related diseases, such as AMD, it is unlikely that the transplanted cells would generate the disease during the patient’s lifetime. Methods to correct the disease-causing mutation have been developed (Borooah et al., 2013). These problems can be reduced by the creation of cell banks consisting of a wide range of screened hPSC lines with different human leukocyte antigen (HLA) haplotypes (Zimmermann et al., 2012). Cell banking would enable the careful selection and validation of cell lines (Osakada et al., 2009). Since there are still many open questions in the use of both hPSC types, more studies are needed to confirm which is the best hPSC type for each purpose.
Figure 3. The establishment of the hPSC lines and the generation of hPSC-RPE. Human PSC lines can be derived from human embryos or by genetic manipulation of somatic cells. RPE differentiation can be initiated either spontaneously by removing external factors from the culture medium or by directing the differentiation using a supplemental factor or specific substrata. After the differentiation, pigmented cells are selected; further maturation of the RPE is performed adherently. Finally, a polarized hPSC-RPE monolayer can be used to treat retinopathies or to model the human retina.

2.3 Human pluripotent stem cell-derived RPE cells

The RPE is an interesting tissue from the viewpoint of regenerative medicine for several reasons. Firstly, there is an emerging need for RPE cells, since vision is considered the most important of the human senses and treatments for impaired
vision are insufficient. Secondly, the RPE is a relatively simple tissue to differentiate and reconstruct. Thirdly, the eye is an immune-privileged organ, thus hypothesizing the better survival of a cell transplant, although the immune privilege of the eye is questionable at the diseased stage. Finally, the retina is surgically accessible and the eye can be monitored visually, which enables post-transplantation monitoring (Tucker et al., 2014). In recent studies, hPSC-RPE cells have been shown to enhance visual acuity in animals and humans (Lu et al., 2009; Lund et al., 2001; Schwartz et al., 2012; Vugler et al., 2008).

Besides the clinical need for RPE cells, in vitro cultured RPE cells are desirable for modelling the retina (Borooah et al., 2013). An increased understanding of the pathogenesis of retinal diseases, eye pharmacokinetics, drug screening (Vellonen et al., 2014), and human eye development would help us to find answers to many unsolved questions, and decrease the number of ethically questionable and expensive animal studies (Hornof et al., 2005). Current immortalized RPE cell lines lack many features of authentic RPE, and animal models are not sufficient for the human retina (Melville et al., 2013; Tucker et al., 2011). A functional and authentic RPE model of human origin would be a valuable tool for retinal studies.

2.3.1 Differentiation of RPE cells from hPSCs

RPE cells have been successfully differentiated in several laboratories, both from hESCs and hiPSCs (Hirami et al., 2009; Klimanskaya et al., 2004; Rowland et al., 2012). The steps of hPSC-RPE generation are illustrated in Figure 3. Spontaneous differentiation based on the removal of bFGF from the basic hPSC culture medium in EBs or adherently on feeder cells is the simplest method to generate RPE cells from hPSCs (Klimanskaya et al., 2004) (Table 1). The exact mechanism of spontaneous differentiation is not understood (Munoz-Sanjuan and Brivanlou, 2002). On average, pigmented foci appear within one month of the initiation of spontaneous differentiation (Table 1). Spontaneous differentiation produces a heterogeneous cell population and the amount of RPE cells remain low even after long-term differentiation (Buchholz et al., 2013).

Several groups have developed protocols to improve RPE differentiation efficacy and to standardize the process. The most fundamental directed differentiation protocols are introduced in Figure 4. These protocols are often divided into two stages; the generation of neuroectodermal cells, and the following RPE induction (Meyer et al., 2011; Zhu et al., 2011). Guiding the cells to certain
pathways can be done either by inducing known developmental pathways or by the inhibition of competitive signalling cascades. Despite the fact that the extracellular matrix (ECM) has an important role in many cellular processes, such as cell differentiation (Gong et al., 2008), few comparative studies exist on cell–ECM interactions during differentiation (Rowland et al., 2013; Sorkio et al., 2014). As seen from Figure 4, existing RPE differentiation protocols vary significantly from each other, and only a few protocols are adopted by different laboratories (Kokkinaki et al., 2011; Krohne et al., 2012; Kuroda et al., 2012; Zhu et al., 2011). Although Ko-SR is often used in RPE differentiation, the basal medium, inductive molecules, time window, and substrata vary between protocols (Buchholz et al., 2013; Gong et al., 2008; Idelson et al., 2009; Maruotti et al., 2013; Meyer et al., 2009; Meyer et al., 2011; Osakada et al., 2009; Rowland et al., 2013).

Besides the development of more efficient RPE differentiation protocols, the maintenance of retinal precursors would shorten the RPE generation process. Cho et al. cryopreserved cystic, optic vesicle-like structures that were successfully directed to form RPE after thawing (Cho et al., 2012). Banking of the cells at the precursor stage would facilitate the mass production of hPSC-RPE, which is important if the cells are intended for use in treating retinal diseases.

The possibility of reprogramming totally differentiated cells straight into another mature cell type (direct conversion) (Qiang et al., 2011; Vierbuchen and Wernig, 2011) would simplify the generation of the aimed-for cells. If the cells could be produced by the direct conversion method, the slow and demanding differentiation stage could be avoided. Recently, RPE-like cells have also been generated from human fibroblasts using the direct conversion method (Zhang et al., 2014). These cells expressed RPE-related proteins, but their functionality remained unsolved (Zhang et al., 2014). However, more studies are needed to prove the clinical relevance of the cells produced by direct conversion (Borooah et al., 2013).
Table 1. Summary of the published spontaneous RPE differentiations.

<table>
<thead>
<tr>
<th>HPSC CULTURE</th>
<th>RPE DIFFERENTIATION AND CULTURE</th>
<th>Number of hESC lines</th>
<th>Number of hPSC lines</th>
<th>Feeder cells / substrata</th>
<th>Spontaneous differentiation method (duration of differentiation)</th>
<th>Culture medium during:</th>
<th>After spontaneous differentiation</th>
<th>Onset of pigment</th>
<th>Used of xenomaterials</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>0 MEF</td>
<td>EBs and adherent (2-3 months)</td>
<td>Seeded on gelatin / laminin</td>
<td>1) RPEbasic 2) RPEbasic + FBS + bFGF</td>
<td>in 3-8 wks</td>
<td>Plasmanate, MEF, FBS</td>
<td>Ko-SR, dFGF, FBS</td>
<td>Hirokawa et al. 2004, Klimanskaya et al. 2006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3 MEF</td>
<td>EB (8 days)</td>
<td>Seeded on gelatin</td>
<td>1) RPEbasic 2) Miller medium</td>
<td>in 2-3 wks</td>
<td>Ko-SR, FBS</td>
<td>Phillips et al. 2012</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4 MEF, HA27 or Matrigel</td>
<td>Adherent (60-90 days)</td>
<td>Seeded on gelatin</td>
<td>1) RPEbasic 2) UMEM high glu, Ko-SR, FBS (bFGF until reached confluence)</td>
<td>in 3-5 wks</td>
<td>MEF, Ko-SR</td>
<td>Phillips et al. 2012</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1 MEF</td>
<td>Adherent (126 days)</td>
<td>Seeded on Matrigel (GF-) / gelatin</td>
<td>1) RPEbasic 2) RPEbasic / Miller medium</td>
<td>in 1-4 wks</td>
<td>MEF, Ko-SR, FBS</td>
<td>Phillips et al. 2012</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0 NSF</td>
<td>Adherent</td>
<td>Seeded on Matrigel / gelatin</td>
<td>1) RPEbasic 2) KO-DMEM high glu, Ko-SR, FBS</td>
<td>in 4-6 wks</td>
<td>Ko-SR, FBS</td>
<td>Harness et al. 2011</td>
<td></td>
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</table>

**Notes:**

2.3.1.1 Differentiation of three-dimensional structures

The next level in cell differentiation is the generation of more complex tissues, including several cell types or even whole organs. A ground-breaking study demonstrated the formation of the entire optic cup from mouse ESCs (Eiraku et al., 2011). Later, this was repeated with human cells (Nakano et al., 2012; Phillips et al., 2012). In addition, three-dimensional structures that resemble the early stages of eye morphogenesis have been reported in several studies (Cho et al., 2012; Meyer et al., 2009; Phillips et al., 2012). These results are very encouraging for the whole field of regenerative medicine, giving hope of the possibility of creating entire organs from hPSCs.
The most fundamentally directed RPE differentiation protocols. The EB culture and adherent cultures are highlighted with different colours. Constant medium components are described in the upper row of each protocol and below the variables and inducing factors.

<table>
<thead>
<tr>
<th>Week</th>
<th>Protocol Details</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>EB in GMEM + 20-15 % Ko-SR, NIC, ActA</td>
</tr>
<tr>
<td>2</td>
<td>EB in DMEM/F12 Noggin, Dkk1</td>
</tr>
<tr>
<td>3</td>
<td>DMEM/F12 + 15 % Ko-SR On d5 --&gt; 5% CO2 / 20% O2</td>
</tr>
<tr>
<td>4</td>
<td>Reference</td>
</tr>
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</table>

Adherent on PA6 cells in GMEM + 10% Ko-SR --> neural progenitors

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<tr>
<th>Week</th>
<th>Protocol Details</th>
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<tbody>
<tr>
<td>3</td>
<td>Adherent on Matrigel or Bruch’s membrane in GMEM + 10% Ko-SR Puryvate Onset of pigmentation</td>
</tr>
<tr>
<td>4</td>
<td>Maruotti et al. 2013</td>
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<th>Week</th>
<th>Protocol Details</th>
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<tbody>
<tr>
<td>1</td>
<td>EB in DMEM/F12 + 20% Ko-SR d21 Adherent on poly-D-lysine/laminin/fibronectin GMEM +10 % Ko-SR in 9 wks</td>
</tr>
<tr>
<td>2</td>
<td>Adherent on Matrigel/VN-PAS in TeSR1</td>
</tr>
<tr>
<td>3</td>
<td>Osakada et al. 2008 and 2009</td>
</tr>
<tr>
<td>4</td>
<td>Idelson et al. 2009</td>
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EB in DMEM/F12 +20 % Ko-SR

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<tr>
<th>Week</th>
<th>Protocol Details</th>
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<tr>
<td>6</td>
<td>Adherent on laminin in DMEM/F12 Selection NIC d0-15: ROCKi                      d1-21 Dkk1, Lefty or alternatively  CKI-7 and SB-431542</td>
</tr>
<tr>
<td>7</td>
<td>EB in Ko-DMEM, 14% Ko-SR d20-25 selection --&gt; EB  in DMEM/F12</td>
</tr>
<tr>
<td>8</td>
<td>Meyer et al. 2009 and 2011 in 4 to 7 wks</td>
</tr>
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</table>

Compared ECM proteins: mouse lam-111, rat lam-332, human lam-332, human fibronectin, human vitronectin, rat collagen I, human C-I, mouse and human C-IV, bovine elastin and Gelatin and Matrigel

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<th>Week</th>
<th>Protocol Details</th>
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<tbody>
<tr>
<td>2</td>
<td>Adherent selection in RPEbasic on several ECM proteins for 73-87 days</td>
</tr>
<tr>
<td>3</td>
<td>in 5 wks</td>
</tr>
<tr>
<td>4</td>
<td>in 2 wks</td>
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Adherent differentiation in RPEbasic on several ECM proteins for 73-87 days

<table>
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<tr>
<th>Week</th>
<th>Protocol Details</th>
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<tbody>
<tr>
<td>1</td>
<td>EB in DMEM/F12 Adherently on Matrigel in DMEM/F12+B27, N2, NEAA for 14 days</td>
</tr>
<tr>
<td>2</td>
<td>RPE selected based on morphology --&gt; Plated on Matrigel coated transwell membranes as single cells</td>
</tr>
<tr>
<td>3</td>
<td>DMEM high Glucose, 1% FBS, Glutamax, sodium puryvate for 30 days</td>
</tr>
<tr>
<td>4</td>
<td>OSAKADAY, 2009</td>
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2 wks

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<th>Week</th>
<th>Protocol Details</th>
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<tbody>
<tr>
<td>1</td>
<td>EB in DMEM/F12 + 20% Ko-SR</td>
</tr>
<tr>
<td>2</td>
<td>Adherent on laminin in DMEM/F12</td>
</tr>
</tbody>
</table>

Selection NIC d0-15: ROCKi                      d1-21 Dkk1, Lefty or alternatively  CKI-7 and SB-431542
2.3.2 Expansion and maturation of hPSC-RPE cells

Maturation of hPSC-RPE is conducted by mimicking the authentic retinal environment. Studies with RPE cells from other sources, such as ARPE-19 (a commercially available RPE cell line) or feRPE, are a good basis for defining optimal culture conditions for hPSC-RPE. To obtain a pure RPE population, putative RPE cells are selected from differentiation cultures. Most often, the selection is done manually by selecting the pigmented foci (Harness et al., 2011; Kamao et al., 2014; Klimanskaya, 2006; Maruotti et al., 2013) or by certain culture conditions that favour hPSC-RPE growth (Buchholz et al., 2013). After the selection, pigmented cells are seeded onto a substrata that resembles Bruch’s membrane or contains its components, such as gelatin (Buchholz et al., 2009; Carr et al., 2009a; Zhu et al., 2011), poly-D-lysine (Hirami et al., 2009; Osakada et al., 2009), laminin (Idelson et al., 2009), fibronectin (Zhu et al., 2011), collagen I (Kamao et al., 2014) or Matrigel (Krohne et al., 2012; Torrez et al., 2012). After the cell seeding, hPSC-RPE cells lose their cobblestone morphology as well as their pigmentation. Within a few weeks, these characteristics are regained. Human PSC-RPE cells have a tendency to lose their ability to re-establish pigmentation after a few passagings (Buchholz et al., 2009; Feng et al., 2010; Singh et al., 2013a). This phenomenon depends on the cell line and passaging rate, and it is more often detected with hiPSC-RPE cells than with hESC-RPE. However, it was recently shown that the hPSC-RPE passage could be extended by Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK) inhibition (Croze et al., 2014).

Often hPSC-RPE cells are maintained in a culture medium that is designed for the culture of pluripotent stem cells. An hPSC culture medium without bFGF (RPEbasic) works surprisingly well for hPSC-RPE cells (Buchholz et al., 2009; Carr et al., 2009b; Klimanskaya et al., 2006). Another commonly used hPSC-RPE expansion medium, Miller medium, has been developed for feRPE (Maminishkis et al., 2006). The main components of Miller medium are MEM α-modified basal medium, FBS, taurine, triiodothyronine, hydrocortisone and N1 supplement, including insulin, transferrin, sodium selenite, putrescine, and progesterone (Maminishkis et al., 2006). In addition, among others, pyruvate (Ahmado et al., 2011a), linoleic acid, vitamin A and E (Gamm et al., 2008), biotin (Hu and Bok,
2001), and ROCK inhibitor (Croze et al., 2014; Kokkinaki et al., 2011) have been used to improve the maturation of hPSC-RPE.

2.3.3 Human PSC-RPE characterization

Several studies have proven that putative hPSC-RPE cells share many characteristics with authentic human RPE (Table 2). The characteristics of hPSC-RPE vary depending on the differentiation and culture method, and even morphologically similar hPSC-RPE cells establish dissimilar maturation stages (Hu et al., 2010; Liu et al., 2014; Rizzolo, 2014; Zhu et al., 2011). Controversial dissimilarities have also been found between hPSC-RPE and native RPE cells (Liao et al., 2010). In comparative studies, hPSC-RPE cells have been shown to more closely resemble fRPE than adult human RPE (Klimanskaya et al., 2004; Liao et al., 2010; Lu et al., 2009), except in one study, where hiPSC-RPE cells more closely resembled human adult RPE than fRPE (Kamao et al., 2014). Comparisons of hPSC-RPE with their native counterparts produce valuable knowledge about the true nature of hPSC-RPE. Due to the variety of existing putative RPE cells, it is important to define RPE identity (Bharti et al., 2011; Rizzolo, 2014), and, moreover, to improve quantitative methods to identify the different maturation stages of the hPSC-RPE cells. Buchholz et al. suggested the creation of a systematic characterization panel for hPSC-RPE to ensure the stable quality of the generated hPSC-RPE cells. The panel should include at least the following: gene and protein analyses, a quantitative phagocytosis assay, TER measurements, a growth factor secretion study, a retinoid metabolism assay, and functionality in an animal model (Buchholz et al., 2009).
Table 2. Summary of the published hPSC-RPE characterization methods. The characterization methods are classified based on the number of studies they have been used in (frequency of used method). The first of otherwise significant studies are used as a reference.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Analyse method and characteristics of the hPSC-RPE</th>
<th>Frequency of used method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>miRNA expression: Pattern during RPE differentiation</td>
<td>&lt;2</td>
<td>Hu et al. 2010</td>
</tr>
<tr>
<td></td>
<td>DNA methylation (RAX, OTX2, MITF, Pax6)</td>
<td>&lt;5</td>
<td>Harness et al. 2011</td>
</tr>
<tr>
<td><strong>Electrophysiology</strong></td>
<td>Voltage clamp: functional voltage-gated transporters, membrane potential 25.50-27.38 mV</td>
<td>&lt;5</td>
<td>Kokkinaki et al. 2011</td>
</tr>
<tr>
<td><strong>ATP response</strong></td>
<td>IP3 and Ca influx increased to 58 nM after 100 μM ATP flux</td>
<td>&lt;5</td>
<td>Meyer et al. 2011, Singh et al. 2013</td>
</tr>
<tr>
<td><strong>Autofluoresence</strong></td>
<td>hESC-RPE did not autofluoresce at any stage examined</td>
<td>&lt;5</td>
<td>Vugler et al. 2008</td>
</tr>
<tr>
<td><strong>Functionality in animal models</strong></td>
<td>Different mouse and rat models (RCS rat most often used model) and one monkey study</td>
<td>&gt;10</td>
<td>Lund et al. 2006, Lu et al. 2009, Carr et al. 2009, Hu et al. 2012, Kamao et al. 2014</td>
</tr>
</tbody>
</table>

IF: immunofluorescence; WB: Western blotting; FACS: fluorescence-activated cell sorting; POS: photoreceptor outer segments

2.3.4 Human PSC-RPE transplantation studies

2.3.4.1 Cell injection method

The effectiveness of hPSC-RPE cell therapy has tentatively been evaluated in animal studies (Carr et al., 2009a; Idelson et al., 2009; Krohne et al., 2012; Lu et al., 2009; Lund et al., 2006; Maruotti et al., 2013; Vugler et al., 2008; Zhu et al., 2013). The most widely used animal model is the Royal College of Surgeons (RCS) rat with rapid retinal degeneration due to a MerTK mutation (Mullen and LaVail, 1976). Human PSC-RPE cells injected as a single cell suspension into the subretinal space of RCS rats decelerated the degeneration of the neural retina and improved visual acuity (Carr et al., 2009b; Lu et al., 2009; Lund et al., 2006; Zhu et al., 2013). Injection is an easy and atraumatic method of administering hPSC-RPE.
cells (Carr et al., 2013). Human PSC-RPE cells were detected in the host retina even several months after the transplantations, but their supportive effect deteriorated with time (Carr et al., 2009b; Idelson et al., 2009; Krohne et al., 2012; Lu et al., 2009; Lund et al., 2006; Vugler et al., 2008). It has been speculated that the supportive effect in these transplantation studies is partly due to the trophic factors secreted by the transplanted cells or the macrophages of the host, but at the same time, the injected cells internalized POS, which is crucial for the health of the retina (Carr et al., 2009b; Idelson et al., 2009; Vugler et al., 2008). It is unknown how well xeno-transplants in animal models resemble treatments aimed at humans (Bharti et al., 2014).

2.3.4.2 Cell sheet transplantation

Cell surveillance and integration into the host retina was improved when hPSC-RPE cells were transplanted as polarized cell sheets (Diniz et al., 2013; Kamao et al., 2014; Kanemura et al., 2014). Bruch’s membrane is often damaged in retinal degenerations; therefore, a scaffold substituting the function of Bruch’s membrane would complete the treatment. Consequently, a scaffold material for hPSC-RPE transplantation should mimic the properties of Bruch’s membrane. Bruch’s membrane is a dynamic, 2-5 µm-thick pentalaminar structure that mainly consists of collagens, elastins, laminin, and fibronectin (Booij et al., 2010; Ramrattan et al., 1994). The thickness and permeability of Bruch’s membrane varies with age, pathological stage, and retinal localization (Booij et al., 2010).

A clinically exploitable scaffold material would support the formation of tight hPSC-RPE in vitro with the proper apico-basal polarization and the characteristics of the authentic RPE. The absolute requirements for an RPE scaffold are at least the following: biocompatibility, porosity, and mechanical properties that not only enable the handling of the cell sheet and transplantation but also the integration of the transplant into the retina (Lu et al., 2012). In addition, the optimal thickness of the RPE scaffold should be less than 11 µm (Coffey et al., 2009). One of the debated properties for the hPSC-RPE scaffold material is biostability. A biostable membrane would provide constant support for the cells, while a biodegradable membrane might release toxic by-products but at the same time improve integration of the transplant by providing better permeability, which is likely to be critical for retinal health (Stanzel et al., 2014).

Several biomaterials, including collagen, poly (lactic-co-glycolic acid) (PLGA), and the ECM of RPE, have been studied in vitro with human adult and feRPE cells
Hynes and Lavik, 2010). Human RPE sheet transplantations have been studied in vivo using polyesters (Stanzel et al., 2012; Stanzel et al., 2014), collagen (Thumann et al., 2009), and fibrinogen (Oganesian et al., 1999). Sheet transplantation studies with hPSC-RPE are limited to parylene-C in rats (Diniz et al., 2013; Hu et al., 2012) and studies without a scaffold using ECM produced by differentiated hiPSC-RPE in mice, rats and monkeys (Kamao et al., 2014; Kanemura et al., 2014).

Dissociated cells may redifferentiate after subretinal injection, which makes sheet transplantation a more controlled and safer method for transplanting hPSC-RPE than cell injections. In addition, the required cell quantity is much lower with sheet transplantation than in subretinal injections (Diniz et al., 2013). The disadvantages of transvitreal transplantation of the cell sheet are the size of the trauma and the demands of the surgical procedure. Improved surgical techniques have been developed to ease RPE sheet transplantations into the back of the eye (Stanzel et al., 2012; Thumann et al., 2009).

2.3.4.3 Clinical trials with hPSC-RPE

Several hPSC-RPE-based cell therapies to treat retinal degenerations are proposed and three clinical trials have already commenced (Bharti et al., 2014; Cyranoski, 2014; Schwartz et al., 2014). In the proposals, the cell source is distributed evenly, half aiming to use hESC-RPE and half autologous hiPSC-RPE; whereas the sheet transplantation method is more frequently aimed to use than the cell suspension method (Bharti et al., 2014). The first clinical trials were announced by Advanced Cell Technology (ACT, Marlborough, USA) to treat nine patients with Stargardt’s macular dystrophy and nine with dry AMD using submacular injections of hESC-RPE cell suspension (NCT01344993, 2011; NCT01345006, 2011). In these studies, the hESC-RPE cells were well tolerated and there were no serious adverse safety signals attributed to the transplanted hESC-RPE cells and possible therapeutic improvement was observed (Schwartz et al., 2014; Schwartz et al., 2012). In a clinical trial by the Japanese group led by M. Takahashi, an autologous hiPSC-RPE cell monolayer was implanted into the subretinal space of an AMD patient very recently (Cyranoski, 2014; http://www.cdb.riken.jp/). In this study, the transplanted monolayer was supported by an ECM produced by hiPSC-RPE as described by (Kamao et al., 2014).
2.3.4.4 Future challenges

There are still several issues to resolve before hPSC-RPE cells can be routinely used in the treatment of retinal diseases (Bharti et al., 2014; Stanzel et al., 2012). Firstly, there are questions related to the safety and efficacy of hPSC-RPE transplants. Animal-derived components such as FBS and MEFs, which are generally used in the establishment, culture and differentiation of hPSCs, may transfer non-human pathogens to the patient and cause immune reactions (Martin et al., 2005; Sakamoto et al., 2007). Transplants containing living material are difficult to sterilize, hence it is critical to avoid materials that may transfer pathogens to the patient. Another concern related to hPSC-RPE safety is tumorigenicity; pluripotent cells form specific tumours called teratoma when they are transplanted into immunodeficient mice (Hoffman and Carpenter, 2005). Although, tumour formation has not been observed in animal studies or in the human trials with hPSC-RPE cells, residual pluripotent cells in the transplant may cause malignant teratocarcinoma (Carr et al., 2009b; Kanemura et al., 2014; Krohne et al., 2012; Lund et al., 2006; Schwartz et al., 2014). Currently, most purification methods are based on visual observation following the enrichment of pigmented cells by repeated passaging (Maruotti et al., 2013; Singh et al., 2013a). Therefore, it is critical to develop purification methods to ensure the homogeneity of the transplanted cells. Kuroda et al., (2012) compared three methods of detecting the amount of hPSCs cells in the hPSC-RPE culture and found a qPCR-based follow-up of the lin-28 homolog A (LIN28) marker to be suitable. However, this methodology alone is still insufficient for clinical use. Correspondingly, genetic manipulation increases the risk of tumour formation. For example, the reactivation of transgenes, such as oncogenic c-MYC, can lead to the generation of cancer (Okita and Yamanaka, 2011). Consequently, permanently integrated vectors should be avoided when hiPSC lines are generated (Bharti et al., 2014). Many important characteristics of hPSC-RPE are still questionable; therefore, clinically used cells should be precisely studied and characterized. Likewise, culture conditions should be carefully selected and even validated for each cell line separately. (Harness et al., 2011; Liao et al., 2010; Singh et al., 2013a; Tanabe et al., 2014).
3 Aims of the study

The main aim of this dissertation was to study the RPE differentiation of hPSC lines cultured on hFF feeder cells and to evaluate the characteristics of the differentiated hPSC-RPE cells. The specific focus was on the generation of a clinically exploitable hPSC-RPE transplant, thus one aim was to avoid the use of undefined and non-human materials. The specific aims of the sub-studies were:

1. To evaluate the tendency of several hPSC lines maintained on hFF feeder cells to differentiate spontaneously towards functional RPE (Study I).

2. To develop a xeno-free and defined differentiation protocol for hPSC-RPE cells (Study I).

3. To characterize the molecular properties and functionality of the differentiated hPSC-RPE cells (Studies I, II and III).

4. To study the expression and functionality of efflux proteins in hPSC-RPE (Study II).

5. To find a proper biomaterial for the hPSC-RPE culture and for future transplantation studies (Study III).
4 Materials and methods

4.1 Culture of hPSC lines

Altogether five hESC lines and one hiPSC line were used in the original articles (Figure 5) (Rajala et al., 2010; Skottman, 2010). The FiPS5-7 line was generated by Professor Timo Otonkoski’s group from human fibroblasts using the following factors: *POU5F1*, *SOX2*, *NANOG*, and *LIN28* (Rajala et al, 2010). Prior to the experiments, all hPSC lines were cultured on mitotically inactivated hFF feeder cells (CRL-2429, ATCC) in a basic hESC culture medium consisting of KnockOut Dulbecco’s Modified Eagle Medium (KO-DMEM), 20% KnockOut serum replacement (Ko-SR), 2 mM Glutamax, 0.1 mM 2-mercaptoethanol (all from Life Technologies, Paisley, UK), 1% Minimum Essential Medium non-essential amino acids (MEM-NEAA), 50 U/ml Penicillin/Streptomycin (both from Cambrex Bio Science, Walkersville, MD, USA), and 8 ng/ml human bFGF (R&D Systems Inc., Minneapolis, MN, USA or PeproTech, NJ, USA). All hPSC lines were regularly characterized as described in (Skottman, 2010).

4.2 RPE differentiation methods

RPE differentiation was performed using three different methods: spontaneous differentiation on hFF, spontaneous differentiation in EBs, or in xeno-free culture conditions (Figure 5). The spontaneous differentiations were initiated by removing bFGF from the basic hESC culture medium one week after the passaging of the pluripotent colonies (Studies I, II, III). The spontaneous differentiation medium is referred to as RPEbasic in this study (Table 3). Adherent spontaneous differentiation was initiated on hFF with cell lines Regea06/040 and Regea08/017 (not included in the original communications). In the adherent differentiation, hPSC colonies were allowed to grow to superconfluence on hFF until a sufficient amount of pigmented foci appeared. Alternatively, the differentiation was done in EBs. For the EB formation, undifferentiated hPSC colonies were manually cut and placed onto low attachment well plates. In Study I, the RPEbasic medium was
compared with the xeno-free medium (RPEregES), which was modified from the previously described RegES (Rajala et al., 2010). The composition of RPEregES is introduced in Table 3 and the consistency of the RegES concentrate is introduced in Supplemental Table 1.

When a sufficient quantity of pigmented cells were amassed, the pigmented areas were manually dissected from the EBs using a surgical scalpel, and seeded onto collagen IV-coated well plates (Sigma-Aldrich, St. Louis, MO, USA) or permeable mouse collagen IV BD Biocoat cell culture inserts (BD Biosciences, San Jose, CA, USA) (Study I and II). In Study III, track etched polyimide (PI) biomembrane (ipCELLCULTURE™, it4ip s.a.; Seneffe, Belgium) (thickness 24 µm, pore diameter 1 µm, 2.2 x 10⁷ pores/cm²) was studied as an hPSC-RPE cell carrier. The following biopolymers were studied for the enhancement of cell growth on the PI biomembrane: laminins from mouse (BD Biosciences) and human placenta (Sigma-Aldrich), synthetic laminin peptide (Millipore), heparin sulphate from porcine (Sigma-Aldrich), bacterial hyaluronic acid (Lifecore Biomedical), collagen I and IV both from human placenta (Sigma-Aldrich), humanized CELLstart™ (Gibco-Invitrogen), MaxGel™ from human ECM (Gibco-Invitrogen), and HyStem™ (Bacillus subtilis, bovine, porcine) (Glycosan Biosystem) (Study III).

Table 3. The compositions of the RPEbasic and RPEregES culture media used in RPE differentiation in this dissertation.

<table>
<thead>
<tr>
<th>RPEbasic</th>
<th>RPEregES</th>
</tr>
</thead>
<tbody>
<tr>
<td>KO-DMEM</td>
<td>KO-DMEM</td>
</tr>
<tr>
<td>Kc-SR (15 %)</td>
<td>RegES concentrate (20 %)</td>
</tr>
<tr>
<td>Glutamax (2 mM)</td>
<td>Glutamax (2 mM)</td>
</tr>
<tr>
<td>2-mercaptopethanol (0.1 mM)</td>
<td>2-mercaptopethanol (0.1 mM)</td>
</tr>
<tr>
<td>MEM-NEAA (1%)</td>
<td>MEM-NEAA (1%)</td>
</tr>
<tr>
<td>Penicillin/Streptomycin (50 U/ml)</td>
<td>Penicillin/Streptomycin (50 U/ml)</td>
</tr>
<tr>
<td></td>
<td>Human recombinant insulin (10 mg/ml)</td>
</tr>
<tr>
<td></td>
<td>Activin A (0.005 mg/ml)</td>
</tr>
<tr>
<td></td>
<td>Trace elements B (0.736 mg/ml)</td>
</tr>
<tr>
<td></td>
<td>Trace elements C (0.736 mg/ml)</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Cambrex Bio Science</td>
<td>Cambrex Bio Science</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>House-made, see supplemental table</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>CAMBrex Bio Science</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>Cellgro</td>
</tr>
<tr>
<td>Life Technologies</td>
<td>Cellgro</td>
</tr>
</tbody>
</table>

Abbreviations are presented on page 11.

4.3 Differentiation efficacy analysis methods

In Study I, RPE differentiation efficacy was studied by recording the onset of pigmented cells and the percentage of pigment-containing EBs from the total number of EBs 21–30 days after the removal of bFGF. RPE morphology and pigmentation were regularly monitored using a Nikon SMZ 1000 stereomicroscope.
and a Nikon Eclipse TE2000-S phase contrast microscope (Nikon Instruments, The Netherlands).

Figure 5. The generation of hPSC-RPE in this study. The establishment and characterizations of the six studied hPSC lines are described by Skottman (2010) and Rajala et al. (2010). RPE differentiation was initiated either adherently on hFF feeder cells or in suspension as EBs. RPEbasic, the spontaneous differentiation medium, was studied with both methods whereas xeno-free RPEregES was studied only in EB cultures. Pigmented areas were manually selected and maturated adherently until they formed an intensely pigmented tight epithelium. The fluid-filled dome-shaped structures indicate initiated fluid transportation on non-permeable culture plates.
4.4 Cell characterization methods

4.4.1 Reverse transcriptase PCR

Gene expressions of differentiated cells were studied using the reverse transcriptase polymerase chain reaction (RT-PCR) method (Studies I, II, III). The PCR protocol is described in details in the original publications. The studied genes are shown in the Table 4.

Table 4. The expressions of the genes studied.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene name</th>
<th>Function of the encoded protein</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Participates in nuclear events, here used as endogenic control</td>
<td>I, II, III</td>
</tr>
<tr>
<td>NANOG</td>
<td>nanog homeobox</td>
<td>TF / hPSC proliferation and self-renewal, prevents differentiation</td>
<td>I, III</td>
</tr>
<tr>
<td>POU5F1</td>
<td>POU class 5 homeobox 1 pseudogene 5</td>
<td>TF / controls embryonic development. Critical for hPSC pluripotency</td>
<td>I, II, III</td>
</tr>
<tr>
<td>SOX10</td>
<td>SRY (sex determining region Y)-box 10</td>
<td>TF / directs embryonic development, and specification of neural crest cells to melanocytes</td>
<td>I</td>
</tr>
<tr>
<td>AFP</td>
<td>alpha-fetoprotein</td>
<td>Binding protein in early fetal development, here used as marker for endoderm</td>
<td>I</td>
</tr>
<tr>
<td>ACTC1</td>
<td>actin, alpha, cardiac muscle 1</td>
<td>Cell motility in cardiac muscle cells, here used marker for mesoderm</td>
<td>I</td>
</tr>
<tr>
<td>OTX2v1</td>
<td>orthodenticle homeobox 2 (variant 1)</td>
<td>TF / regulates eye development and pigmentation of RPE</td>
<td>I</td>
</tr>
<tr>
<td>PAX6</td>
<td>paired box 6</td>
<td>TF / important for eye development</td>
<td>I, II, III</td>
</tr>
<tr>
<td>MITF</td>
<td>microphthalmia-associated transcription factor</td>
<td>TF / regulates cell differentiation and melanogenesis</td>
<td>I, II, III</td>
</tr>
<tr>
<td>RAX</td>
<td>retina and anterior neural fold homeobox</td>
<td>TF / regulates retinal fate determination also expressed in adult retina</td>
<td>I, II, III</td>
</tr>
<tr>
<td>BEST1</td>
<td>bestrophin-1</td>
<td>Forms calcium-sensitive chloride channels in the epithelia / RPE</td>
<td>I, II</td>
</tr>
<tr>
<td>PEDF</td>
<td>pigment epithelium derived factor</td>
<td>Inhibits angiogenesis, secreted by RPE</td>
<td>I, II</td>
</tr>
<tr>
<td>PMEL</td>
<td>premelanosome protein</td>
<td>Biogenesis of melanosomes</td>
<td>I, II</td>
</tr>
<tr>
<td>RPE65</td>
<td>retinal pigment epithelium-specific protein 65kDa</td>
<td>Regenerates visual pigment in visual cycle</td>
<td>I, II, III</td>
</tr>
<tr>
<td>TYR</td>
<td>tyrosinase</td>
<td>Catalyzes the conversion of tyrosine to melanin</td>
<td>I, II, III</td>
</tr>
</tbody>
</table>

TF: transcription factor; hPSC: human pluripotent stem cell; RPE: retinal pigment epithelium

4.4.2 Quantitative PCR

Quantitative PCR (qPCR) was used to analyse relative gene expression levels between the chosen reference sample and the studied samples (Studies I and II). In Study I, qPCR was used to study the expression of FiPS5-7-derived RPE cells
more precisely. The \textit{POU5F1} (Hs00999632_g1) expression levels of hiPSC-RPE cells were compared with their undifferentiated counterparts to assess the level of \textit{POU5F1} expression in the end-point sample. In addition, the fluctuation of \textit{RAX} (Hs00429459_m1) expression was compared at four time points (d0, d7, d44, d72).

In \textbf{Study II}, qPCR was used to compare the expression levels of genes coding efflux proteins. The compared cell samples were: undifferentiated hPSCs, three morphological distinguished hPSC-RPE cells (fusiform, epithelioid, cobblestone), immortalized RPE cell lines (ARPE-19 and D407), the human embryonic kidney 293 cell line (HEK293), and hFF (CCD-1112Sk, CRL-2429™, ATCC®, Teddington, UK). A later human adult RPE sample was included in these analyses (not included in original communication). D407 was used as a reference sample in analyses of \textit{MRP1}, -2, -3, -4, -5, \textit{BCRP} and \textit{P-GP}, whereas HEK293 was used as reference sample for the \textit{MRP6} gene. The studied genes were \textit{MRP1} (\textit{ABCC1}) (Hs00219905_m1), \textit{MRP2} (\textit{ABCC2}) (Hs00155123_m1), \textit{MRP3} (\textit{ABCC3}) (Hs00358656_m1), \textit{MRP4} (\textit{ABCC4}) (Hs00195260_m1), \textit{MRP5} (\textit{ABCC5}) (Hs00981071_m1), \textit{MRP6} (\textit{ABCC6}) (Hs00184566_m1), \textit{BCRP} (\textit{ABCG2}) (Hs01053790_m1), and \textit{P-GP} (\textit{ABCB1}) (Hs00184500_m1).

In both studies, qPCR was performed using the standard protocol for the ABI Prism 7300 instrument with TaqMan chemistry (Applied Biosystems Foster City CA, USA) \hspace{1em} \textbf{(Studies I and II)}. Relative expression analysis was performed with 2$^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). Glyceraldehyde 3-phosphate dehydrogenase (\textit{GAPDH}) (Hs99999905_m1) was used as an endogenic control gene in qPCR analyses. To assess the reliability of \textit{GAPDH} for these samples, the results were analysed with different housekeeping genes: nucleotide-binding protein 1 (\textit{NUBP1}), ribosomal protein, large, P0 (\textit{RPLP0}), glucuronidase beta (\textit{GUSB}), and aminoadipate-semialdehyde dehydrogenase (\textit{AASDH}, \textit{NRPS988}). This analysis showed that \textit{GAPDH}, \textit{NUBP1}, and \textit{RPLP0} are the most stable in this cell material. \textit{AASDH} and \textit{GUSB} expression was different from all other studied housekeeping genes. Since comparative data analyses using the geometric mean of \textit{GAPDH}, \textit{NUBP1}, and \textit{RPLP0} in normalization gave the analogous result for \textit{GAPDH} alone, the analyses were performed only with \textit{GAPDH}.

4.4.3 \hspace{1em} Immunofluorescence

RPE-related protein expression and their subcellular localization were evaluated with immunofluorescence analyses. The studied proteins are summarized in Table
5. The detailed protocol is described in the original publications (Studies I, II and III).

Table 5. The expressions of the proteins studied in the immunofluorescence analyses.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Protein name</th>
<th>Function of the protein in RPE</th>
<th>Localization in RPE</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>MITF</td>
<td>Microphthalmia associated transcription factor</td>
<td>TF / regulates melanogenesis and differentiation</td>
<td>Nucleus</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Bestrophin-1</td>
<td>Bestrophin-1</td>
<td>Forms calcium-sensitive chloride channels</td>
<td>Basolateral membrane</td>
<td>I, II, III</td>
</tr>
<tr>
<td>CRALBP</td>
<td>Cellular retinaldehyde-binding protein</td>
<td>Retinoid carrier in visual cycle</td>
<td>Cytoplasm</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Na/K-ATPase</td>
<td>Sodium/potassium-transporting ATPase</td>
<td>Active transporter of sodium and potassium</td>
<td>Apical membrane</td>
<td>I, II, III</td>
</tr>
<tr>
<td>RPE65</td>
<td>Retinal pigment epithelium-specific 65 kDa protein</td>
<td>Regenerates visual pigment in visual cycle</td>
<td>Cytoplasm linked to cell membrane</td>
<td>I, III</td>
</tr>
<tr>
<td>KI-67</td>
<td>Antigen KI-67</td>
<td>Associates cell proliferation</td>
<td>Nucleus of dividing cells</td>
<td>I, III</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zona occludens protein 1</td>
<td>Formation of tight junctions</td>
<td>Cell membrane / junctions</td>
<td>I, III</td>
</tr>
<tr>
<td>MERTK</td>
<td>Tyrosine-protein kinase Mer</td>
<td>Regulates POS phagocytosis</td>
<td>Apical membrane</td>
<td>III</td>
</tr>
<tr>
<td>Laminin</td>
<td>Laminin</td>
<td>ECM protein</td>
<td>ECM</td>
<td>III</td>
</tr>
<tr>
<td>Collagen I</td>
<td>Collagen I</td>
<td>ECM protein</td>
<td>ECM</td>
<td>III</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>Collagen IV</td>
<td>ECM protein</td>
<td>ECM</td>
<td>III</td>
</tr>
<tr>
<td>MRP-1</td>
<td>Multidrug resistance-associated protein-1</td>
<td>Efflux protein</td>
<td>Cell membrane</td>
<td>I</td>
</tr>
<tr>
<td>MRP-4</td>
<td>Multidrug resistance-associated protein-4</td>
<td>Efflux protein</td>
<td>Cell membrane</td>
<td>I</td>
</tr>
<tr>
<td>MRP-5</td>
<td>Multidrug resistance-associated protein-5</td>
<td>Efflux protein</td>
<td>Cell membrane</td>
<td>I</td>
</tr>
<tr>
<td>Opsin</td>
<td>Opsin (rat)</td>
<td>Visual pigment</td>
<td>In cone photoreceptor</td>
<td>III</td>
</tr>
</tbody>
</table>

TF: transcription factor; ECM: extra-cellular matrix; POS: photoreceptor outer segment

4.4.4 PEDF secretion analyse method

PEDF secretion was studied with enzyme-linked immunosorbent assay (ELISA) (Study I). Culture medium was collected after two to three days cell culture from ten pigmented and non-pigmented EBs. The total cell amount was not defined. The collections were performed at four time points (d49, d70, d97, d230) for RPEbasic cells and at two time points (d49, d98) for RPEregES cells. PEDF concentrations of media samples were analysed with Chemikine PEDF Sandwich ELISA kit (Millipore) following manufacturer’s instructions.

4.4.5 Phagocytosis assays

Ability of the hPSC-RPE cells to phagocytose POS was studied either using porcine POS (Study I) or rat retina explant culture (Study III). Porcine POS were isolated using sucrose gradient and then FITC labelled (0.04 µg/µl). POS were incubated with differentiated pigmented and non-pigmented EBs for 16 hours. (Study I).

The retinas of non-dystrophic RCS-rats were isolated for retinal explant coculture. Pigmented cells matured on PI were cultured with the retinas in B27/N2
medium for two days (Johnson and Martin, 2008). After the co-culture, the rat retinas were discarded and the cells were stained with rat anti-Opsin (O48869, Sigma-Aldrich) /donkey anti-rabbit Alexa 488 fluorescent dye (Study III). Filamentous actins were stained with TRITC-Phalloidin (P1951, Sigma-Aldrich) and nuclei stained with DAPI after the cells were exposed to POS or rat retina. The internalization of POS was studied using sectional scanning with Zeiss LSM 700 confocal microscope (Studies I and III).

4.4.6 Western blotting

The expression of ATP-dependent efflux transporter proteins MRP1, -4, and -5, was studied with Western blot (Study II) from ARPE-19 and hESC -derived cells. Alpha-tubulin was used as internal control. Details of the protocol are found from original article (Study II).

4.4.7 Calcein-acetoxymethyl and the cell viability test

Efflux protein activity was assessed with calcein-acetoxymethyl (calcein-AM) assay (Study II). The effect of four efflux protein inhibitors: cyclosporine A (15 mM) (Calbiochem, La Jolla, CA, USA), progesterone (200 mM) (Sigma-Aldrich), verapamil (500 mM) (ICN Biomedicals, Irvine, CA, USA), and MK571 (100 mM) (Cayman Chemicals, Ann Arbor, MI, USA) on calcein-AM metabolism was measured after 20 min incubation with a Victor 1420 Multilabel Counter (Wallac, Turku, Finland). In addition, cell viability was measured from the same cells using Live/Dead Viability/Cytotoxity kit for Mammalian cells (Invitrogen, Life Technologies Carlsbad, CA, USA).

4.4.8 Bioelectrical measurements

The integrity of the cell layer and the tightness of the epithelium were studied by measuring TER (Studies I and III). In Study I, the development of TER was measured using MilliCell electrical resistance system volt-ohm meter with stick electrodes (Millipore). In Study III, the TER was calculated from transepithelial electrical potential (TEP) values. TEP was measured using Ussing/diffusion chamber (Harvard Apparatus, MA, USA) equipped with glass barrel Ag/AgCl
electrodes (World Precision Instruments, FL, USA) and a voltage-current clamp (VCC MC 6, Physiologic Instruments, CA, USA). The measurements were conducted under open-circuit conditions. Current pulses of ± 5 μA were applied for two seconds across the cell layer, and the changes in TEP values were monitored. The TER values of the responding membrane without cells were subtracted to gain final TER values in both studies.

4.4.9 Permeability assay

In Study III, epithelial permeability was studied in an Ussing/diffusion chamber (Harvard Apparatus, MA, USA) using 6-carboxyfluorescein (6-CF) (0.0377 mg/ml, 100 μM, 376 Da) (Sigma-Aldrich, MO, USA). The samples were collected from a donor and receptor chamber at 15 min intervals up to 180 min. The 6-CF concentrations were measured after fluorescence analyses using a 96-well fluorescence plate reader (Varioskan Flash, Thermo Scientific, MA, USA) at 485 nm excitation and 530 nm emission wavelength settings. The diffusion of 6-CF across the hESC-RPE monolayers was characterized by calculating the apparent permeability coefficient (Papp, cm/s) as Papp = dC/dt/(60 C₀A), where dC/dt is the slope of the linear portion of the permeability curve (nmol/min), C₀ is the initial concentration in the donor chamber (nmol/cm³) and A is the exposed surface area of the RPE monolayer (0.28 cm²).

4.4.10 Statistical Analyses

In Study II, the statistical significance for differences in relative gene expressions of the efflux-protein related genes was determined with an analysis of variance (ANOVA) with Bonferroni’s correction using PASW Statistics version 18. P-values < 0.05 were considered statistically significant and p-values < 0.01 highly significant. A statistical analysis for the calcein-AM study was performed with a one-sample t-test using PASW Statistics version 18. P-values < 0.05 - 0.01 were considered significant and p-values < 0.01 highly significant.
4.5 Ethical considerations

The Institute of Biosciences and Medical Technology (BioMediTech, formerly Regea) has approval from the National Authority for Medicolegal Affairs, Finland to conduct research with human embryos (Dnro 1426/32/300/05), and a supportive statement from the local ethics committee of the Pirkanmaa Hospital District Finland (R05149) to derive and expand hESC lines from surplus embryos not used in the treatment of infertility by donating couples, and to use these lines for research purposes (R05116). A human iPSC line (FiPS5-7) was generated at the University of Helsinki with the permission of the Ethics Committee of the University of Helsinki. No new hPSC lines were established for the studies conducted in this dissertation.
5 Summary of the results

5.1 The evaluation of RPE differentiation methods

5.1.1 Efficacy of the studied differentiation methods

In this dissertation, three methods were used to differentiate hPSCs towards RPE: spontaneous differentiation adherently on hFF, spontaneous differentiation in EBs using the RPEbasic medium, and xeno-free differentiation using the RPEregES medium (Figure 5). Pigmented cells with a cobblestone morphology were successfully differentiated in all three methods. Firstly, adherent spontaneous differentiation was initiated on hFF in RPEbasic with two cell lines: Regea06/040 (n=3) and Regea08/017 (n=2) (not reported in the original communications). The onset of pigmentation occurred on day nine and 22, respectively, and the amount of pigmentation was relatively good, as can be seen on Figure 5. However, differentiating cells often detached from hFFs during the long-term differentiation that was required to produce a sufficient quantity of pigmented cells. Consequently, this differentiation method was discarded from the future differentiation experiments and analyses.

Secondly, we studied spontaneous EB differentiation with six hPSC lines. All studied hPSC lines generated pigmented cells in RPEbasic, but the differentiation efficacy varied between cell lines. The differentiation efficacy was followed firstly by the onset of pigmentation and secondly by defining the percentage of pigment-containing EBs from the total amount of EBs on days 21–30 (Study I/Table3). The onset of pigmentation was detected between days 10–21 depending on the cell line. However, the standard deviation was five days on average. The percentage of pigment-containing EBs varied from 6.5% to almost 25% between the cell lines.

Thirdly, we studied RPE differentiation in EBs using the xeno-free RPEregES medium with the Regea08/023 and FiPS5-7 cell lines (Study I). In RPEregES, the first pigmented cells were observed on day seven with Regea08/023, and on day six with the FiPS5-7 cell line (Study I/Table3). The degrees of pigmentation
between 23–28 days of differentiation were respectively 45% for the Regea08/023-RPE cells and 37% for the FiPS5-7-RPE cells (Study I/Table3).

5.1.2 Maturation of hPSC-RPE

To enrich hPSC-RPE and for further formation of the RPE monolayer, the pigmented parts of EBs were manually dissected and seeded either onto collagen IV-coated well plates or porous cell culture inserts. On average, it took 50 days at a minimum to gain a sufficient quantity of pigmented cells for the enrichment. The quantity of pigmented cells did not increase remarkably after this time point (not reported in the original communication). After the cell seeding, the cells underwent an epithelial mesenchymal transition (EMT)-like process; cells first lost their pigment and took on a fusiform morphology, then started to regenerate pigment and became rounded until they reached the typical cobblestone morphology with a high degree of pigmentation. Clear, fluid-filled dome-like structures often appeared during the long-term culture on the culture wells, but not on porous culture inserts (Figure 5) (not reported in the original communication).

5.1.3 Gene expression during differentiation

Spontaneous differentiation in EBs was also studied by comparing the gene expression profiles of five hPSC lines at different time points (d0, d7, d28, d44, d52, d72) (Study I/Table 4). Undifferentiated cells (d0) expressed pluripotency markers NANOG and POU5F1, but also many genes related to the differentiated cells as follows: SOX10, ACT, PMEL, and PEDF for Regea08/023 and FiPS5-7; and RAX, MITF, and RPE65 for Regea08/017 (Study II). After seven days of differentiation, the cells expressed several genes regulating RPE development and functions (PAX6, RAX, MITF, RPE65, BEST1, OTX2v1, PMEL, and PEDF) (Study I/Table 3). TYR expression was initiated on average by day 44 by the latest of the studied genes. The differences between cell lines detected by RT-PCR were minor and observed only in the expression of RAX, RPE65, BEST1, and TYR. RAX expression by the hiPSC line (FiPS5-7) was more precisely studied with qPCR, since the expression was incoherent and diverse across the hESC lines (Study I/Figure 4). Another difference in gene expression profiles was in RPE65 expression, which was at the earliest expressed by day seven (Regea08/056-RPE) and at the latest by day 44 (Regea08/013-RPE). The same expression profile was
studied at three time points (d0, d7, d44) in Reges08/023-RPE and FiPS5-7-RPE differentiated in xeno-free RPEregES. Gene expressions were coherent between the RPEbasic and RPEregES differentiation methods (Study I/Figure 7).

Gene expression end-point analysis (d196) for RPEbasic was made on Regea08/023-RPE and FiPS5-7-RPE after over 100 days of adherent maturation on collagen IV-coated well plates. These cells had intense pigmentation and a cobblestone morphology. Besides the studied markers for mature RPE (PAX6, MITF, RPE65, TYR, BEST1, PMEL, PEDF, and OTX2v1), both cell lines’ derivatives expressed melanocyte-related SOX10 (Study I/Figure 5). In addition, FiPS5-7-RPE cells expressed the pluripotency markers NANOG and POU5F1, as well as actin, alpha cardiac muscle 1 (ACTC1), a marker for mesodermal differentiation (Study I). The POU5F1 expression level of FiPS5-7-RPE at different time points was further studied with qPCR (Study I/Figure 5). Pluripotency markers were not detected in hESC-RPE by RT-PCR (Studies I, II and III).

5.1.4 Protein expression of mature hPSC-RPE

For immunofluorescence analyses, the pigmented parts of EBs, differentiated in RPEbasic or RPEregES, were dissociated and seeded onto porous collagen IV-coated cell culture inserts. Human PSC-RPE cells were matured for 32 days on the porous inserts. Like gene expression, RPE-related protein expression (MITF, CRALBP, Na/K-ATPase, Bestrophin-1, ZO-1, and RPE65) was coherent between RPEbasic and RPEregES-differentiated hPSC-RPE (Study I/Figures 3, 6 and 7). Nevertheless, in RPEregES there were still some Antigen KI-67 (Ki67) -expressing cells, which were not detected in the confluent cultures generated with RPEbasic. The subcellular localization of the studied proteins was correct. The distribution of Bestrophin-1 on the basolateral side of the cells and the Sodium/potassium-transporting ATPase (Na/K-ATPase) on the apical side of the cells reflected the polarization of the epithelium. (Study I)

5.1.5 Functional analyses

PEDF secretions by pigmented and non-pigmented EBs differentiated in Regea08/023 and FiPS5-7 using RPEbasic and RPEregES were compared (Study I). Pigmented cells secreted PEDF at all studied time points (d49, d70–80, d97,
d230–250), as opposed to the non-pigmented cells, which did not secrete measurable levels of PEDF. PEDF secretion by pigmented EBs in RPEbasic varied from 1.7 ng/ml to 548.0 ng/ml and from 13.8 ng/ml to 251.8 ng/ml between time points in Regea08/023-RPE and FiPS5-7-RPE cells, respectively. PEDF secretion by pigmented cells differentiated with RPEregES increased from 16.2 ng/ml to 182.4 ng/ml between days 49 and 97. However, the cell quantity was not equal between the replicative measurements, thus these results are only qualitative. (Study I).

The ability of hPSC-RPE to phagocytize POS was verified from the Regea08/023-RPE- and FiPS5-7-RPE-differentiated spontaneous EB method (RPEbasic) (Study I). FITC-labelled porcine POS were internalized by pigmented hiPSC-RPE and hESC-RPE after 77 days of differentiation (Study I/Figure 6). A major quantity of POS was enriched in cell junctions.

The formation of epithelial integrity after seeding pigmented hPSC-RPE onto porous inserts was evaluated with TER measurements using a MilliCell electrical resistance system volt-ohm meter. The TER values of Regea08/023-RPE and FiPS5-7-RPE differentiated in RPEbasic increased during the 60-day follow-up period in the weekly recordings as follows: Regea08/023-RPE from 6 Ωcm² to 311 Ωcm², and FiPS5-7-RPE from 6 Ωcm² to 74 Ωcm² (Study I).

5.2 Expression of efflux proteins in the studied cells

In Study II, efflux protein expression, localization, and functionality were studied from undifferentiated hESCs and from three morphologically distinguished hESC-RPE cells – fusiform, epithelioid, and cobblestone – and compared with ARPE-19, D407, hFF, and HEK-293 cell lines (Study II). The RT-PCR profile showed that Regea08/017-RPE fusiform, epithelioid, and cobblestone cells, and likewise ARPE-19 had an equal expression of PAX6, RAX, MITF, and RPE65, whereas TYR expression was observed only in hESC-derived cells. Of the studied eye-related genes, D407 expressed only MITF, and hFF had a faint expression of PAX6. The undifferentiated cells were the only cells to show expression of the pluripotency markers POU5F1 and NANOG (Study II/Figure 1).

In the original communication, we compared the expression of genes coding the efflux proteins of Regea08/017-derived cells, D407, HEK-293, ARPE-19, and hFF cells. In addition, the comparison was later widened to adult human RPE. In this later analysis, the expression of the same genes by adult human RPE were
compared with cobblestone Regea08/017-RPE, ARPE-19, D407, and HEK293 (not reported in the original communication). The gene and protein-level expressions of efflux proteins are summarized in Table 6. The key points of these results are introduced below. A relative gene expression comparison showed that the expression of genes coding efflux proteins fluctuated during hPSC-RPE maturation (Study II/Figure 2). Expression of MRP1, MRP4, MRP5, and MRP6 was higher in hPSC-RPE cells than in ARPE-19 and D407. Statistically significant differences were observed in MRP1 expression between fusiform (4-fold) and epithelioid (5-fold) cells in comparison with D407. MRP2 expressions were significantly lower in all studied samples compared with the D407 cell line, and BCRP expression levels were at an almost undetectable level in all other cells except for D407. MRP3 gene expression was at similar level as the fusiform and epithelioid hESC-RPE, ARPE-19, and D407 cell lines, whereas cobblestone hESC-RPE had a lower MRP3 expression (Study II/Figure 2). Extensional qPCR analysis showed that human adult RPE had a similar trend in the expression of MRP2, MRP3, MRP5, MRP6, BCRP, and P-GP as cobblestone hPSC-RPE; whereas the expressions of MRP1 and MRP4 by human adult RPE were more similar to ARPE-19 than to hPSC-RPE (Table 6).

Western blot analysis showed that MRP1, MRP4, and MRP5 were expressed at protein level by all studied samples, namely ARPE-19, fusiform, epithelioid, and cobblestone hPSC-RPE. The other efflux proteins were not studied due to the lack of a functional antibody. Immunofluorescence was used to verify the localization of MRP1, MRP4, and MRP5 proteins in ARPE-19, fusiform, epithelioid, and cobblestone hPSC-RPE. The overall intensity of immunofluorescence staining remained low. Despite a positive expression in Western blot analyses, the studied proteins were not detected in ARPE-19 cells by immunological staining. Immunofluorescence analyses indicated that MRP1, MRP4, and MRP5 protein expression increased and became correctly localized with hPSC-RPE maturation (Study II/Figure 4). MITF, CRALBP, and Na/K-ATPase describe the maturation stage of the studied cells and help to define the localization of the MRPs.

In addition, efflux pump activity in ARPE-19, fusiform and cobblestone hESC-RPE was studied with a calcine-AM assay. ARPE-19 showed efflux pump activity seven days after plating but the activity was lost with cell maturation (Study II/Figure 5). The fusiform cell had greater efflux pump activity than the cobblestone hPSC-RPE. Undifferentiated Regea08/017 and hFF did not show efflux pump activity.
Table 6. Summary of the results of efflux protein expressions at gene and protein level by undifferentiated Regea08/017; fusiform, epithelioid and cobblestone Regea08/017-RPE; human adult RPE, ARPE-19, D407, HEK-293, and hFF. The expression values of qPCR are presented as fold regulations (the standard deviation from three biological replicates of hPSC-RPE is represented in brackets), the reference sample for each gene is bolded.

<table>
<thead>
<tr>
<th>Relative gene expression</th>
<th>Regea08/017</th>
<th>Fusiform hPSC-RPE</th>
<th>Epithelioid hPSC-RPE</th>
<th>Cobblestone hPSC-RPE</th>
<th>ARPE-19</th>
<th>Human adult RPE</th>
<th>D407</th>
<th>HEK293</th>
<th>hFF</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRP1</td>
<td>1.2 (0.2)</td>
<td>4.1 (1.4)</td>
<td>5.0 (1.3)</td>
<td>3.3 (1.1)</td>
<td>1.0</td>
<td>0.7</td>
<td>1</td>
<td>1.6</td>
<td>1.9</td>
</tr>
<tr>
<td>MRP2</td>
<td>0.0 (0.0)</td>
<td>0.0 (0.0)</td>
<td>0.0 (0.0)</td>
<td>0.01 (0.0)</td>
<td>0.0</td>
<td>0.1</td>
<td>1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>MRP3</td>
<td>0.02 (0.0)</td>
<td>1.1 (0.6)</td>
<td>1.5 (1.5)</td>
<td>0.2 (0.3)</td>
<td>0.5</td>
<td>0.2</td>
<td>1</td>
<td>UD</td>
<td>0.2</td>
</tr>
<tr>
<td>MRP4</td>
<td>0.4 (0.1)</td>
<td>1.8 (0.4)</td>
<td>2.5 (1.1)</td>
<td>1.5 (0.6)</td>
<td>0.3</td>
<td>0.1</td>
<td>1</td>
<td>378.0</td>
<td>0.5</td>
</tr>
<tr>
<td>MRP5</td>
<td>1.0 (0.3)</td>
<td>3.4 (1.4)</td>
<td>4.1 (1.0)</td>
<td>22.1 (14.9)</td>
<td>0.3</td>
<td>49.5</td>
<td>1</td>
<td>1.8</td>
<td>0.1</td>
</tr>
<tr>
<td>MRP6</td>
<td>0.4 (0.0)</td>
<td>1.3 (0.5)</td>
<td>4.3 (0.6)</td>
<td>4.7 (2.5)</td>
<td>0.01</td>
<td>1.9</td>
<td>0.3</td>
<td>1</td>
<td>0.0</td>
</tr>
<tr>
<td>BCRP</td>
<td>0.4 (0.2)</td>
<td>0.0 (0.0)</td>
<td>0.1 (0.3)</td>
<td>0.0 (0.1)</td>
<td>0.0</td>
<td>0.0</td>
<td>UD</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>P-GP</td>
<td>0.01 (0.0)</td>
<td>1.6 (0.8)</td>
<td>2.2 (0.9)</td>
<td>0.08 (0.1)</td>
<td>UD</td>
<td>0.3</td>
<td>1</td>
<td>1.3</td>
<td>0.0</td>
</tr>
</tbody>
</table>

WB: Western blotting; IF: Immunofluorescence; hPSC-RPE: human pluripotent stem cell-derived retinal pigment epithelium; hFF: human foreskin fibroblasts; UD: undetected; NA: not analysed.

5.3 Cell growth on polyimide biomembrane

In Study III, we evaluated the suitability of polyimide biomembrane (PI) as an hPSC-RPE (Regea06/040-RPE and Regea08/017-RPE) cell carrier. Cell attachment on uncoated PI was poor. Several biopolymers were studied to enhance cell growth (listed in the methods section). Synthetic laminin peptide, HS, and HA did not improve cell attachment to PI, whereas all the other coatings enhanced the hPSC-RPE cell adherence to PI and supported the formation of pigmented hPSC-RPE (Study III/Figure 1). The qualities of the protein coatings were studied with immunofluorescence, showing that the proteins form an even coating on the PI. Human PSC-RPE formed an even, strongly pigmented epithelium on HyStem™-coated PI. However, hPSC-RPE detached from HyStem™-coated PI during processing of the cell sheet, probably due to the gel-like structure of HyStem™.

Maturation of hPSC-RPE on coated PI was studied by profiling RPE characteristics using morphology observations, RT-PCR, and immunofluorescence. Human PSC-RPE cultured on coated PI expressed several RPE-related genes (PAX6, RAX, MITF, RPE65, BEST1, PMEL, PEDF, and TYR) and similarly POU5F1 expression was not detected by RT-PCR (Study III/Figure 3). Moreover,
these cells had a positive expression and the correct localization of following RPE-related proteins: MITF, CRALBP, Na/K-ATPase, and MERTK (Study III/Figure 4). Localization of Na/K-ATPase on the apical side of the cells indicated the polarization of hPSC-RPE. The confluent cultures did not contain dividing cells according to Ki67 expression.

Epithelial integrity was studied with TER and a permeability assay. TER values of the highly pigmented cells cultured on the PI membrane were 40–80 ±12 Ω cm². In this study, TER values were measured using an Ussing/diffusion chamber. The paracellular permeability of 6-CF ranged from 1.3 to 1.5 ±0.6x10⁻⁵ cm/s. (Study III). Moreover, hPSC-RPE functionality on coated PI was studied with a phagocytosis assay by co-culturing the cells with rat retina explant. Rat Opsin staining inside the hESC-RPE cultured on the coated PI membrane confirmed functionality of the cells (Study III/Figure 4).
6 Discussion

6.1 Spontaneous differentiation of hPSC lines towards RPE

6.1.1 Spontaneous differentiation on hFF feeder cells and in EBs

The first aim of this study was to evaluate the tendency of hPSC lines maintained on hFF feeder cells to differentiate spontaneously towards RPE. In this study, spontaneous differentiation was initiated either adherently on hFF or in EBs by the removal of bFGF from the basic hPSC culture medium. Generally, spontaneous RPE differentiation is performed on MEF feeder cells (Table 1). It has been stated that spontaneous differentiation on feeders is a more efficient way to generate RPE cells than the EB method (Vugler et al., 2008). The exact role of feeder cells in RPE differentiation is undetermined. Feeder cells secrete a variety of factors into the growth medium, such as activin A, which regulates RPE differentiation (Eiselleova et al., 2008; Idelson et al., 2009; Kokkinaki et al., 2011). Feeder cells may also provide mesenchymal support, which is known to be important during authentic RPE development (Fuhrmann et al., 2000).

To minimize the use of animal-derived components, our group used hFF feeder cells as the culture matrix for undifferentiated hPSC lines (Skottman, 2010). For the first time, in this present study, hFF feeder cells were also used instead of MEFs in the RPE differentiation. In this study, the amount of pigmented cells was similar in both spontaneous differentiation methods, the adherent hFF differentiation, and in the EB method. However, the cells often detached from the hFF feeder cells during the differentiation, resulting in the loss of the whole cell patch. A similar finding was noted with other human fibroblast feeder cells (hs27x) (Rowland et al., 2012). Based on these findings, it can be suggested that human fibroblast feeder cells are less suitable for long-term differentiation of RPE than MEFs. In addition, long-term maintenance of the confluent multi-layered cultures leads to substantial culture medium consumption, requiring daily feedings with the maximum amount of culture medium; this increases the expenses and the risk of contaminations. As a conclusion, in this study we showed that hPSCs cultured on
hFF feeder cells were able to differentiate spontaneously towards RPE. Furthermore, spontaneous differentiation in EBs was more efficient than spontaneous differentiation adherently on hFF feeder cells.

6.1.2 RPE differentiation rate using spontaneous EB differentiation

The tendency of five hPSC lines to generate RPE spontaneously in EBs were compared. The RPE differentiation tendencies were evaluated by recording the onset of pigmentation and the amount of pigmented EBs, and by studying the expression of RPE-developmental genes. All five hPSC lines generated RPE-like cells spontaneously, but the RPE differentiation tendency varied between cell lines and also between replicative experiments within individual hPSC lines (Study I/Table3), as also seen in other studies (Liao et al., 2010; Meyer et al., 2009). The gene expression profiles of different hPSC lines did not vary significantly. However, qPCR would have been a more optimal method for comparing these expression profiles.

In our studies, the first pigmented cells were observed on average two weeks after the removal of bFGF. In other spontaneous RPE differentiation studies, pigmentation was observed one to eight weeks after bFGF removal (Table 1). During human development, RPE melanogenesis starts around day 40 (O'Rahilly, 1975). The co-expression of PAX6, RAX, and MITF was detected already on day seven by RT-PCR, which confirmed that eye differentiation starts relatively quickly in vitro; this was also the case in other studies with hPSCs (Table 1) (Buchholz et al., 2013; Meyer et al., 2009). Besides early eye-field markers, genes regulating the functions of mature RPE cells (RPE65, BEST-1, OTX2, PMEL, and PEDF) were also expressed early in the differentiation. Since RPE cells have been differentiated spontaneously from several hPSC lines cultured in the diverse conditions used by different laboratories (Table 1), it is speculated that RPE is a primary fate (Bharti et al., 2011). Our results support previous studies that have shown that hPSC lines may favour certain developmental pathways (Mikkola et al., 2006; Toivonen et al., 2013). It has been shown that even a single ICM as a split can produce hPSC lines with a variable differentiation potential (Lauss et al., 2005). Presumably, the spontaneous tendency to follow the RPE fate of the individual hPSC line is already determined in the derivation by the source cells.

Nevertheless, the quick progression of the differentiation indicated the heterogeneity of the undifferentiated hPSC colonies. Later, we noticed that the
single cell enzymatic differentiation (SCED) method (Ellerstrom et al., 2010) produced a more homogenous hPSC population (unpublished results). Comparative fluorescence-activated cell sorting (FACS) analyses of SCED and mechanically passaged hPSC colonies showed that the expression of the pluripotency marker tumour-related antigen (TRA-1-81) was 95% and 51%, respectively. Furthermore, the percentage of polysialylated neuronal cell adhesion molecule (PSA-NCAM)-positive cells was 14% in mechanically passaged hPSC colonies, indicating the initiation of neural differentiation (unpublished results). Signs of the initiated neural differentiation were also detected by the faster onset of pigmentation by the mechanically passaged cells (unpublished results), as also observed by (Klimanskaya et al., 2006). In the present study, all hPSC colonies were passaged mechanically. The quality of the starting material had an impact on the differentiation rate, which may explain the variation between the replicative experiments. Since some of the cells had already started to differentiate on d0 when the EBs were formed, it seems that hFF feeder cells may also have had an effect on the early stages of EB differentiation. In a later study, we noticed that the hFF-conditioned culture medium induced a better RPE differentiation when compared with the RPEbasic medium (Hongisto et al., 2012). The supportive effect of feeder cells was similarly obtained using supplemental activin A, but at a significantly lower concentration than in other RPE differentiation studies (Hongisto et al., 2012; Idelson et al., 2009; Meyer et al., 2011). The rate of RPE differentiation in this study would most probably have been different if the hPSC lines had been passaged with the SCED method. It has been previously reported that an altered karyotype affects differentiation capacity (Mikkola et al., 2006). Due to regular karyotyping, we can ensure that variations between replicative experiments were not due to karyotype abnormalities.

Besides recording the onset of pigmented cells, the pigmentation rate was estimated by calculating the percentage of pigment-containing EBs. After one month of spontaneous differentiation, 10–40% of EBs contained pigmented areas of varying sizes. After approximately two months of differentiation, the hPSC-RPE reached the plateau stage in pigment generation. At this point, the cells could be seeded for adherent maturation. These results are consistent with other studies (Table 1). However, one should bear in mind that the comparison of pigmentation rate with other studies is problematic since the evaluation is based on visual perception and thus highly dependent on the observer. In addition, the evaluation of the pigmentation degree is suggestive, since the sizes of pigmented areas vary significantly. Quantification of the amount of pigmented cells as done by Rowland
and co-authors (Rowland et al., 2012) or the expression of certain RPE-specific proteins, such as MITF – using cytospin analysis method, for instance – would be a more optimal way to evaluate the rate of RPE differentiation. However, hPSC-RPE cells are very tightly bound to each other and a large number of pigmented cells would be lost during cell dissociation. Due to variation in this methodology between the published studies, a global comparison of RPE differentiation efficacies is challenging. In the present study, one person made pigmentation rate evaluations for all cell lines, and thus, comparison within this study is reliable. Altogether, it can be concluded that the generation of a mature hPSC-RPE monolayer is a slow and inefficient process.

A directed and more efficient RPE differentiation protocol would be optimal to enable standardized mass-production of hPSC-RPE. Many groups have developed these protocols (Figure 4). However, these protocols are rarely adopted by other laboratories, and hPSC-RPE cells for proposed clinical trials are mainly differentiated using spontaneous differentiation (Bharti et al., 2014), which indicates that the current directed differentiation protocols are not superior to spontaneous differentiation, or that they have some other issues complicating their clinical use. Due to the diversity of the hPSC lines, it seems that the protocols should be individually optimized for each of the hPSC lines (Zhu et al., 2013). Spontaneous differentiation is a simple and cost-effective method for producing RPE cells when compared to the current directed differentiation protocols that require a variety of expensive inducing factors and are still relatively slow (Figure 4). Moreover, RPE enrichment after directed differentiation can be done with the serial expansion of hPSC-RPE (Sugino et al., 2011). Although spontaneous differentiation is a long-term process, after repetitive passaging, the amount of generated cells is competitive with the current directed differentiation protocols (Table 1, Figure 4).

6.2 Xeno-free differentiation of RPE

In this study, for the first time, we introduced RPE differentiation method without animal derived materials using xeno-free culture medium RPEregES (Study I). Serum-free B27® supplement is often used as serum replacement in RPE differentiation protocols (Meyer et al., 2011; Rowland et al., 2012). However these protocols contain other xeno-materials. Most cell lines used in RPE differentiation studies are contaminated with animal derived components, such as MEF and FBS.
The avoidance of undefined and xeno-materials is important if the cells are aimed to be used for clinical purposes (Brafman et al., 2009; Gamm et al., 2008; Rodin et al., 2014). In the present study, hPSC-RPE generated using RPEregES, had a typical RPE morphology, they expressed several genes and proteins related to RPE fate and secreted PEDF growth factor. Generation of pigmented cells was even more efficient in RPEregES than in RPEbasic (Study I/Table 3). However this remains still to be confirmed statistically. RPEregES is modified from RegES culture medium which was originally developed for undifferentiated hPSC culture (Rajala et al., 2010) (Supplemental Table 1). Possible inductive effect in RPEregES may be due to supplemental activin A, which has been previously demonstrated as RPE inducer (Fuhrmann et al., 2000; Hongisto et al., 2012; Idelson et al., 2009). In addition, insulin and transferrin, other supplements of RPEregES, are included in mostly used RPE culture media (Maminishkis et al., 2006). To find optimal RPEregES consistency for RPE differentiation and maturation, the necessity of each component could be studied by separate exclusion. Definition of factors needed for RPE differentiation would increase understanding of RPE development. Furthermore, KO-DMEM basal medium, which was used in this study, is optimized to support pluripotency, and thus another basal medium could be more optimal for RPE maturation. Finally, other supplemental components which may favour RPE growth, such as taurine, triiodo-thyronin and pyruvate (Ahmado et al., 2011b; Duncan et al., 1999; Gabrielian et al., 1992) would be interesting to study. RPEregES medium has batch-to-batch variation since it contains undefined human serum albumin (HSA) and is manually prepared. Defined human recombinant albumin should be studied to replace undefined HSA for improving the stability of RPEregES.

The RPEregES protocol enables the RPE differentiation from hPSCs without xeno-materials. Nevertheless, the initial derivation and undifferentiated culture of the hPSC lines has been done using culture medium containing Ko-SR that includes BSA, and FBS is still used in the culture medium of hFF. Feeder cell independent and xeno-free hPSC culture methods are nowadays widely used and even derivation of new hESC lines in xeno-free culture conditions without feeder cells has been done (Rodin et al., 2014). The RPE generation method developed here would be truly defined and xeno-free, if hPSC lines used would be also initially established using xeno-free and defined medium (RegES) and maintained without feeder cells or on feeder cells cultured in xeno-free conditions.
6.3 Characteristics of hPSC-RPE

6.3.1 Phenotype and molecular properties

The phenotype of the hPSC-RPE cells resembled authentic RPE. After replating, the cells lost their pigment and underwent morphological alterations – from fusiform to hexagonal cells; a similar phenomenon has also been observed in adult RPE cells when cultured in vitro (Sonoda et al., 2009). The hexagonal cells had an intense pigmentation. Melanin production is rarely observed in immortalized RPE cell lines, such as ARPE-19 (Dunn et al., 1996; Kanuga et al., 2002). The heterogeneity in the amount of melanin among the hPSC-RPE cells on the same culture plates was comparable to the mosaicism seen in human adult RPE cultures (Burke and Hjelmeland, 2005; Sonoda et al., 2009). The degree of pigmentation has been shown to effect hPSC-RPE characteristics (Rowland et al., 2013; Schwartz et al., 2012; Sorkio et al., 2014). In addition, hyperpigmentation is known to involve RPE pathogenesis (Bonilha, 2008). Hyperpigmented cells do not typically polarize or develop high TER (Sonoda et al., 2009; Sorkio et al., 2014). Thus, it would be interesting combine a quantitative study on melanin production with other analysis methods, such as TER measurements.

After phenotypic observations, we evaluated the expression of typical RPE-related genes. Putative hPSC-RPE cells expressed all of the studied RPE genes (Studies I, II and III). All hPSC lines expressed TYR last. Furthermore, comparative analyses showed that TYR was not expressed by the studied immortalized RPE cell lines ARPE-19 and D407 (Study II). It is known that the RPE maturation begins with the activation of TYR promoter (Strauss, 2005). Correspondingly, RPE65 seems to be a watershed in RPE maturity (Buchholz et al., 2009; Zhu et al., 2011). Consequently, TYR and RPE65 could be appropriate markers for RPE maturity. In addition, it is important to show the absence of non-RPE genes. Of the evaluated non-RPE genes, both hESC-RPE and hiPSC-RPE cells expressed SOX10, which directs embryonic development and cell determination, including, for example, the melanocytes’ specification from the neural crest cells (Bondurand et al., 1999; Huber et al., 2003). This indicated the existence of other cell lineages even after long-term differentiation (196 days). In addition, hiPSC-RPE cells showed a positive expression of the pluripotency markers NANOG and POU5F1 even 196 days post-differentiation. Even though more precise analyses with qPCR showed that POU5F1 expression was remarkably
lower in hiPSC-RPE than in their pluripotent counterparts, this finding highlights the need for cell purification before cell transplantations to minimize the risk of tumorigenesis. Recently, it has been shown with the studied hiPSC line FiPS5-7 that retroviral transduction might cause reactivation of transgenes specifically in RPE differentiation (Toivonen et al., 2013); this most probably also happened in this study. Besides gene expression, the cells had a positive expression and the proper localization of all the studied RPE-related proteins. Distribution Na/K-ATPase and Bestrophin-1 on different parts of cell membrane demonstrated the polarization of the epithelium (Studies I, II and III).

Since the permeability of Bruch’s membrane is critical for RPE well-being, porous culture inserts were used to enhance the maturation and polarization of the RPE. This study is one the first to use porous cell culture inserts in hPSC-RPE maturation. It is likely possible that RPE cells need their natural environment for definitive maturation (Bharti et al., 2011; Carr et al., 2009b; Vugler et al., 2008). Fluid filled dome-like structures in RPE cultures are indicators of launched fluid transportation and a functional BRB (Aronson, 1983; Buchholz et al., 2009; Burke et al., 1996; Feng et al., 2010; Hu et al., 2010). We observed these domes in hPSC-RPE cultures on well plates but not on porous inserts. The lack of domes when using porous cell culture inserts indicated that the size of the pores were adequate for fluid transportation from the basal side of the hPSC-RPE.

6.3.2 Functionality of hPSC-RPE

Besides the phenotype and molecular properties, we studied the functional properties of the generated hPSC-RPE cells. The main functions of RPE are light absorption, POS phagocytosis, the visual cycle, growth factor secretion, BRB formation, and the active transportation of solutes through the BRB. PEDF secretion by hPSC-RPE cells was verified for the first time in this dissertation (Study I).

The ability to POS phagocytize was shown after exposing the hPSC-RPE to isolated porcine POS (Study I) and by co-culturing with rat retina explant (Study III). In our studies, POS were mostly bound to cell junctions, and significantly fewer quantities of POS were detected inside the cells, although in the same study engulfment receptor MERTK was clearly detected on the apical membrane of hPSC-RPE. In another study, it was noted that POS is more tightly bound by hPSC-RPE than by feRPE, but the amount of internalized POS was equal (Liao et
This may suggest that hPSC-RPE cells have an imbalance between POS-binding and ingestion-related proteins (Liao et al., 2010; Westenskow et al., 2012). In our later studies, the amount of internalized POS increased when the culture medium was supplemented with FBS (unpublished results). FBS supplies ligands for phagocytosis-related receptors αVβ5-integrin and MERTK. In the native retina, these ligands are located in the interphotoreceptor matrix and trigger the function of the phagocytosis receptors (Mazzoni et al., 2014).

The positive ZO-1 staining, the generation of fluid-filled domes, epithelial permeability and the TER values indicated the formation of the BRB. TJs are formed by a complex of over one hundred different proteins, thus ZO-1 alone is not an optimal marker for functional TJs (Rizzolo, 2007). Additional Claudin-19 staining, for example, would give a more reliable description of the stage of the TJs. However, in this study, ZO-1 staining was combined with TER measurements and epithelial permeability (study III). TER values increased logically with the epithelial maturation. TER values measured using a volt-ohm meter with stick electrodes varied from 6 Ωcm² to 311 Ωcm² in hESC-RPE and from 6 Ωcm² to 74 Ωcm² in hiPSC-RPE respectively (Study I). TER values on the PI biomembrane measured using an Ussing chamber with implanted electrodes were remarkable lower (40–80 ± 12 Ωcm²) (Study III). Rizzolo summarized that TER values measured from cultured human fetal and adult RPE using stick electrodes vary between 200–1500 Ωcm² and 300–800 Ωcm² respectively when using an Ussing chamber. TER values measured in this study are still consistent with measurements for human adult RPE, at 36–148 Ωcm² (Quinn and Miller, 1992). The TER values are lower when the cells have been cultured in a serum-free medium (Rizzolo, 2014). Generally, the limitation for the epithelial integrity of hPSC-RPE is set at 300 Ωcm² (Kamao et al., 2014; Singh et al., 2013a; Zhu et al., 2011; Zhu et al., 2013). However, the measurement machinery has a significant impact on TER values, thus the comparison of TER values gained in different circumstances is problematic (Rizzolo, 2014). TER values measured using an Ussing chamber with implanted electrodes, as done in Study III, is considered the most reliable method for TER measurements (Rizzolo, 2014). The barrier properties of the differentiated cells could have been improved by using a longer maturation period.

Altogether, hPSC-RPE cells are broadly characterized in several laboratories. Based on current knowledge, we can conclude that the hPSC-RPE cells differentiated in this study correspond to the mainstream of hPSC-RPE cells in having several important characteristics of authentic human RPE. However, a
worldwide comparison of hPSC-RPE cells is difficult since RPE authenticity is still undefined (Bharti et al., 2011; Buchholz et al., 2009). Therefore, it is important to continue the general discussion initiated by Bharti et al. about the definition of RPE cell identity (Bharti et al., 2011).

6.3.3 Efflux protein expression of hPSC-RPE

Efflux proteins have a high impact on drug transportation through the RPE. An increased understanding of the pharmacokinetic properties of RPE is invaluable, especially for drug discovery for retinal diseases; unfortunately, this knowledge is currently elusive (Dahlin et al., 2013). Study II was the first study on efflux protein expression by hPSC-RPE.

We compared the expression of efflux proteins of four morphologically distinguished hPSC types with other cell lines (D407, HEK-293, ARPE-19) with the known expression of these proteins, and later also with human adult RPE. Relative qPCR analyses showed that efflux protein expressions fluctuated during RPE maturation. In addition, based on qPCR analyses, the efflux protein expression of hPSC-RPE was more similar to adult human RPE than to the spontaneously transformed human RPE cell lines, namely ARPE-19 and D407. In general, however, the expression of these proteins corresponded to other studies on human RPE cells (Dahlin et al., 2013; Mannermaa et al., 2009). Western blot analysis showed that the hPSC-RPE and ARPE-19 cell lines expressed the studied efflux proteins also at protein level. The lack of signals by ARPE-19 in the immunofluorescence analysis indicated that the proteins produced were not functional. Localization of these proteins varied between hPSC-RPE samples. A calcein-AM assay demonstrated that the expressed efflux proteins MRP-1 or P-GP were also functional in fusiform and cobblestone hPSC-RPE. RPE polarization is vitally important for drug transportation in the RPE (Zhu et al., 2011), hence the tightness of the epithelium, e.g. TER value, could have been a better way to select hPSC-RPE cells for this study than morphology. Of the studied genes, MRPI, MRP5, BCRP, and P-GP were among the ten most expressed in the human retina, but BCRP, for instance, was localized in the inner BRB and the neural retina and not in the RPE (Dahlin et al., 2013).

This study showed that hPSC-RPE resembled human native RPE well. The study has produced novel data about efflux proteins related to the pharmacokinetic
properties of hPSC-RPE. These results can be used as a starting point for the further development of an *in vitro* BRB model for hPSCs. A more precise study about functionality of these proteins, as well as a broader comparison with different human adult RPE samples, would be invaluable.

### 6.4 Polyimide biomembrane as scaffold for hPSC-RPE

The functionality of the PI biomembrane was studied to find a proper biomaterial for hPSC-RPE. Such a biomaterial should support the generation of the polarized epithelium, be able to be used as a cell carrier in future applications, such as *in vitro* cell culture model systems, or be suitable for cell transplantation into the subretinal space. PI is permeable, its mechanical properties allow the handling of the material, and it was well tolerated in the subretinal space in animals (Julien et al., 2011; Richardson et al., 1993). In addition, PI is already used in human applications (Kane et al., 2008).

This study shows that the PI biomembrane is a promising scaffold for hPSC-RPE. Other published biomaterial studies using hPSC-RPE cells studies are still restricted to parylene-c (Diniz et al., 2013) and ECM produced by hPSC-RPE (Kamao et al., 2014). Besides these studies, at least one patent application is concerning a scaffold material for hPSC-RPE applications (Coffey et al., 2009). The principal requirement for an optimal cell carrier material is that it must support cell growth. At first, cell attachment was poor on uncoated PI. Therefore, we studied different biopolymers to enhance cell adherence and growth on PI. Similarly to two other recent studies on hPSC-RPE ECM interactions (Rowland et al., 2013; Sorkio et al., 2014), several biopolymers supported hPSC-RPE adherence and propagation in this study. The coated PI biomembrane supported the formation of polarized hPSC-RPE, which had several characteristics of authentic human RPE, such as gene and protein expression, polarization, the ability to phagocytize POS, high TER, and permeable properties. The non-selectivity in cell attachment indicated a variable expression of the cell adhesion proteins. Due to their simplicity and availability at a good manufacturing practice (GMP) grade, we concluded that human collagen IV and laminin, both from human placenta, are the most optimal coating material for hPSC-RPE culture on PI. Collagen IV and laminin are also the main components of the basal lamina of RPE (Booij et al., 2010). Further studies are needed to determine the suitability of GMP-grade forms of these proteins for this purpose.
PI is biostable, as are most of the cell carrier materials used in the proposed clinical trials (Bharti et al., 2014). A biostable scaffold would give constant support after transplantation and lack potentially harmful degradation products. However, a biostable scaffold may hamper interactions between the RPE and the choroid, which is essential for retinal health. Permeability is one of the most critical characteristics of an hPSC-RPE transplant (Stanzel et al., 2014). The epithelial permeability of 6-CF was shown to be higher in hPSC-RPE cells on PI than in bovine RPE and adult human RPE (Mannermaa et al., 2010; Pitkanen et al., 2005). The porosity of the PI studied here was 1 μm / 2.27x10^7 pores/cm². Paracellular permeability could be improved by increasing the pore size and density.

The thickness of studied PI membrane was 24 μm, which is significantly thicker than Bruch’s membrane (Ramrattan et al., 1994) and thus unsuitable for hPSC-RPE transplantations. However, we have later studied a thinner (7.6 μm) PI biomembrane and observed that it supported hPSC-RPE growth similarly to the thicker PI biomembrane studied here (Ilmarinen et al., unpublished results). Furthermore, in a previous study, a thinner (5 μm) PI biomembrane has been transplanted into the eye of a rat (Julien et al., 2011). For TER and permeability analyses, hPSC-RPE on PI was transferred from the cell culture to an Ussing chamber. Handling the transplant was straightforward, indicating that the mechanical properties of PI were sufficient for the further handling of the hPSC-RPE transplant. In addition, the yellow colour helps to determine which side of the biomembrane the hPSC-RPE is on; this eases the surgical procedure and enables the correct positioning of the cell sheet in the host. The yellow colour can also help post-transplantation observation of the transplant.

Coated PI is a promising cell carrier material for hPSC-RPE cell transplantations. To gain more data about the safety and function of hPSC-RPE combined with PI in cell transplantation, evaluation of the PI membrane has been continued in animals (unpublished results).

6.5 Future perspectives

The first hPSC cell line was derived in 1998 from a human embryo (Thomson et al., 1998). Six years later, darkly pigmented cells arising spontaneously from hPSC cultures were characterized as RPE cells (Klimanskaya et al., 2004). The first two human trials to treat retinal diseases using hPSC-RPE cells have now been launched and several others are proposed (Bharti et al., 2014; Schwartz et al., 2012).
The entire research field of regenerative medicine is impatiently awaiting to the outcome of these first human trials with hPSC-derived RPE cells. Currently, there are still many questions awaiting answers. Firstly, the safe use of these cells requires the avoidance of xeno-materials and non-invasive methods for hPSC-RPE purification. Although regulatory authorities have approved the use of undefined and xeno-materials in current human trials, to ensure standard quality and safety of cell therapy, we should aim for a directed differentiation protocol that avoids the use of undefined components and xeno-materials. Similarly, pluripotent cells present a high risk of tumorigenesis if they remain in the transplant, and hence it is broadly agreed that pluripotent cells must be excluded before transplantations. Therefore, the development of a non-invasive purification method to exclude non-RPE cells before transplantation is essential. The second issue to be resolved is related to the choice of cell type used. Since both hESCs and hiPSCs are included in proposed clinical trials, these trials will produce unique information about hPSC immunogenicity in humans. In our analyses, the differences between hESC and hiPS lines mainly concerned the RT-PCR profile. Otherwise, the differences were greater between distinctive hESC lines than between hESCs and hiPSCs. Since several groups have questioned the safety of hiPSC-RPE (Carr et al., 2009b; Okita and Yamanaka, 2011; Tanabe et al., 2014), it is very important to clarify the true nature of hiPSCs. In addition, it is important to generate safer and more effective methods to introduce reprogramming factors into hiPSCs. A generally agreed assay panel about the quality of transplantable RPE cells would improve the global quality of hPSC-RPE therapy. At a minimum, this panel should consist of the required characterization methods, functionality studies, and the methods for screening the presence of pathogens and possible genetic diseases (Buchholz et al., 2009). Indeed, some other cell type, such as adult RPE stem cells or RPE cells generated with direct conversion, may prove to be more suitable source of RPE (Salero et al., 2012; Zhang et al., 2014).

Finally, several issues related to transplantation – and the entire surgical procedure – must be clarified and standardized using large-eyed animals (Stanzel et al., 2012). This requires at least greater knowledge about transplantation method, such as whether the intact cell sheet improves cell integration to the retina better than an injected cell suspension, as could be supposed in a comparative study of these two transplantation methods (Diniz et al., 2013). It is estimated that the required cell quantity for macular defects is relatively low, in the range of 30,000–100,000, depending on the transplantation method (Buchholz et al., 2009; Kamao et al., 2014; Ramsden et al., 2013). However, if hPSC-RPE cell therapy becomes a
routine treatment for retinal diseases, an automated mass-produced culture system is required (Bharti et al., 2014; Terstegge et al., 2007). Some of these issues are difficult or even impossible to meet in vitro or in animal models, showing how far we are from harnessing hPSC-derived cells for the treatment of degenerative diseases. Hopefully, the proposed clinical trials will provide answers to the questions above.

Although stem cell research often focuses on the clinical use of these cells, the value of hPSC-derived cell and tissue models is significant (Inoue et al., 2014). Because hPSCs can generate all human tissues, they present an exceptional opportunity to model human development pathways or, as recently shown, even organogenesis (Nakano et al., 2012). This data is valuable, since only estimates can be drawn from animal studies (Vellonen et al., 2014). The wide characterization of hPSC-RPE cells has shown that hPSC-RPE cells have many advantages compared to immortalized cell lines; for instance, these cell can be used for modelling human eye diseases (da Cruz et al., 2007; Klimanskaya et al., 2004; Liao et al., 2010; Lu et al., 2009; Singh et al., 2013b). In addition, accurate human tissue models reduce the need for expensive and ethically questionable animal studies and drug industry costs (Vellonen et al., 2014). Utilization of hiPSC technology in personalized medicine may help many patients to get optimal treatment and also increase our understanding of disease pathogenesis (Tucker et al., 2014). In the future, hPSC-RPE cells will be combined with other retinal layers and even more complex structures (Eiraku et al., 2011; Phillips et al., 2012; Vellonen et al., 2014). Induced PSC technology provides a novel tool for personalized medicine; it provides unique information about the individual patient, e.g. drug responsiveness and pathogenesis, and thus helps us to find more effective treatments (Tucker et al., 2014). The steps taken in the hPSC-RPE research field during this dissertation work have been enormous and the speed of development is expected to increase in the future.
This dissertation investigated the potential of several human pluripotent stem cell (hPSC) lines maintained on human foreskin fibroblast (hFF) feeder cells to differentiate towards retinal pigment epithelial (RPE) cells. RPE differentiation was performed using an RPEregES xeno-free culture medium, which increases the safety of hPSC-RPE-related cell therapy. In addition, the RPE identity and functionality of the differentiated cells were evaluated. Novel information about efflux protein expression in hPSC-RPE was generated. Lastly, the suitability of a porous polyimide (PI) biomembrane as an hPSC-RPE cell carrier was investigated. Based on the studies performed, the following conclusions can be drawn:

1. The human PSC lines cultured on hFF feeder cells had a tendency to differentiate spontaneously towards RPE. However, the spontaneous generation of RPE cells from hPSCs was an inefficient and inconsistent process.

2. RPE differentiation was performed using RPEregES, a xeno-free differentiation medium.

3. Human PSC-RPE cells expressed several features of authentic RPE, such as phenotype, melanin granules, gene and protein expression, polarization, barrier properties, growth factor secretion, and the ability to phagocytize POS.

4. Efflux protein expression fluctuated during hPSC-RPE maturation, and the hPSC-RPE cells were shown to resemble their natural counterparts based on efflux protein expression.
   - This is the first study on efflux protein expression in hPSC-RPE. This knowledge can be exploited in pharmacokinetic studies of RPE.
5. The coated PI biomembrane supported the generation of functional and polarized hPSC-RPE, which indicates the functioning of PI as a proper hPSC-RPE carrier for future applications.
Acknowledgements

This study was conducted at the Institute of Biosciences and Medical Technology (BioMediTech), University of Tampere. I am grateful to the former and present directors of the Institute, Riitta Seppänen and Hannu Hanhijärvi, for providing and maintaining such an outstanding research environment. I would also like to sincerely thank the personnel of BioMediTech, especially the regenerative medicine groups and Tissue bank, for the exceptionally enjoyable working atmosphere and the many meaningful friendships I established there.

I also wish to warmly thank the Tampere Graduate Programme in Biomedicine and Biotechnology (TGPBB), the University of Tampere Foundation, the Evald and Hilda Nissi Foundation, the Orion-Farmos Research Foundation, the City of Tampere, the Hilda Kauhanen Foundation, the Finnish Eye and Tissue Bank Foundation, and Kyoto University for financially enabling my research.

My deepest gratitude goes to my supervisors: Docent Heli Skottman, PhD and Tanja Ilmarinen, PhD. I feel extremely privileged to have had you to guide me through this project. You gave me as much freedom and responsibility as I wanted, and at the same time all the support I needed. I am especially appreciative that you even scheduled your maternity leave – all five of them – in a way that didn’t leave me without supervision. I have many enduring memories from late nights in the office and in the lab with Tanja. I am grateful for all the scientific skills you have taught me and for all the times you looked out for me (like in Germany, where I probably would have lost myself without you). Heli, I thank you for the opportunity to work as part of your team and for making me feel that you would defend me, if ever needed, like a mother lion would defend her cub.

I owe my sincere thanks to the members of my follow-up group: Professors Arto Uurti, Hannu Uusitalo, and Riitta Seppänen. I am grateful that you found time for our annual meetings in your busy calendars, for sharing your scientific expertise, and for all the encouragement you gave during the project. Emerita professor Hanna Tähti and Professor Alexa Klettner are warmly thanked for the careful
revision of this dissertation and the constructive criticism that increased the quality of this work. I also wish to thank Docent Riička Lund for accepting the role of my opponent at my public defence. In addition, I must emphasize the importance of the co-authors: Astrid Subrizi, Soile Nymark, Kristiina Rajala, Susanna Narkilahti, Jari Hyttinen, Eliisa Mannermaa, Tuomas Ryhänen, Ka Kaarniranta, Marjo Yli-Peltula, Peter Dubruel, and Niina Onnela; without your contribution none of the papers would have seen the light of day. Astrid and Soile, you both have an extraordinary way of doing hard-core science with a smile on your face. Ulla Aapola, I thank you for organizing such educational seminars and for all the joy you spread around you. I thank Dr Goran Petrovski of the University of Szeged, Hungary, for providing the human adult RPE sample for the efflux protein study, and Professor Markku Mäki’s group for access to the Olympus microscope.

I am also grateful to all the past and present members of the Ophthalmology group. Kati Juuti-Uusitalo, I especially thank you for your input in the efflux protein study, for sharing your extensive scientific know-how, and for our long discussions in the office. The bright diamonds of our team, Anni Sorkio and Alexandra Mikhailova, I thank you for the friendship and the joyful atmosphere you create. I must also express my gratitude to our excellent laboratory staff, Hanna Pekkanen, Outi Melin, Elina Konsén, and Outi Heikkilä. Thank you for your friendship and for all the happy memories. Heidi Hongisto has greatly supported me in both career-related issues and in my personal life, and has become one of my dearest friends. Thank you, Heidi!

I am grateful to all my friends for helping me to forget about cells and science. Mari Elomäki, besides your loving friendship, I thank you for proofreading the Finnish parts of this book. I cannot emphasize enough the importance of my large but close family: my beloved little brother, Heikki; my cousins and grandparents; Jorma, Anja and my godparents. You all have had a significant influence on my life. Kati, Kai, Anni, Pekka, Vesa, Laura, Kai, Kitti, Simo, and Maija, I am very grateful for having you as my extended family. Maija, my amazing mother-in-law, thank you for taking care of Jaakko (and me!) while I was writing. Your help was priceless! Thank you Mom and Dad for always being there for me, for believing in me, and for encouraging me. Finally, a few words for those two men who hold my heart: Juho and Jaakko, there are no words to express my gratitude to and for you.


of ultrathin, biofunctionalized polyimide membranes into the subretinal space of rats. Biomaterials 32, 3890-3898.


**Supplemental Table 1.** Composition of house-made RegES concentrated developed by Rajala et al., 2010.

<table>
<thead>
<tr>
<th>Component</th>
<th>Conc. (mg/l)</th>
<th>Manufacturer</th>
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<tbody>
<tr>
<td>Glycine</td>
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<td>Sigma-Aldrich</td>
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Original publications
Toward the defined and xeno-free differentiation of functional human pluripotent stem cell–derived retinal pigment epithelial cells

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Purpose: The production of functional retinal pigment epithelium (RPE) cells from human embryonic (hESCs) and human induced pluripotent stem cells (hiPSCs) in defined and xeno-free conditions is highly desirable, especially for their use in cell therapy for retinal diseases. In addition, differentiated RPE cells provide an individualized disease model and drug discovery tool. In this study, we report the differentiation of functional RPE-like cells from several hESC lines and one hiPSC line in culture conditions, enabling easy translation to clinical quality cell production under Good Manufacturing Practice regulations.

Methods: Pluripotent stem cells were cultured on human fibroblast feeder cells in serum-free medium. The differentiation toward RPE was induced by removing basic fibroblast growth factor and feeder cells from the serum-free conditions. RPE differentiation was also achieved using xeno-free and defined culture conditions. The RPE cell morphology and pigmentation of the cells were analyzed and the expression of genes and proteins characteristic for RPE cells was evaluated. In vitro functionality of the cells was analyzed using ELISA measurements for pigment epithelium derived factor (PEDF) secretion and phagocytosis of photoreceptor outer segments (POS). The integrity of the generated RPE layers was analyzed using transepithelial electric resistance measurements.

Results: We generated putative RPE cells with typical pigmented cobblestone-like morphology. The expression of RPE-specific markers was confirmed at the gene and protein level. The differentiated cells were able to phagocytose POS and secrete PEDF characteristic of native RPE cells. In addition, cultured cells formed a polarized epithelium with high integrity and exhibited excellent transepithelial electric resistance values, indicating well established, tight junctions. Moreover, we introduced an improved method to generate functional putative RPE cells without xeno-components under defined conditions.

Conclusions: We have developed a progressive differentiation protocol for the production of functional RPE-like cells from hESCs and hiPSCs. Our results demonstrate that putative hESC-RPE and hiPSC-RPE express genes and proteins characteristic for RPE cells, as well as being able to phagocytose POS and secrete PEDF. Furthermore, our results show that RPE-like cells can be differentiated in xeno-free and defined culture conditions, which is mandatory for Good Manufacturing Practice-production of these cells for clinical use.

Retinal pigment epithelium (RPE) is an epithelial cell monolayer located between the neural retina and choriocapillaris. RPE provides essential support for the long-term preservation of retinal integrity and visual functions by absorbing stray light, regenerating visual pigment, supplying nutrients, secreting growth factors, and phagocytosing the shed photoreceptor outer segments (POS) [1]. Dysfunctional RPE causes impairment and death of the photoreceptor cells, leading to deterioration or total loss of vision. These mechanisms play an important role in the pathogenesis of retinal diseases like age-related macular degeneration (AMD), which is the leading cause of blindness in the developed world [2]. Intravitreal vascular endothelial growth factor antagonism has been shown to prevent vision loss and even improve visual acuity in patients with neovascular AMD in the early course of the disease. However, in advanced cases of exudative AMD, as well as in the most common form of AMD, nonexudative AMD, there is no satisfactory cure. Even though vascular endothelial growth factor antagonists are effective, intravitreal injections are needed and this causes high costs for the health care system while exposing the patients to complications such as endophthalmitis, myocardial infarction, or stroke [3]. In the search for more a comprehensive therapy for AMD, tissue engineering and cell transplantation are among the most promising candidates. Several cell sources have been considered [4-9].

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The cellular origin of the retina is exclusively ectodermal. During development, the first morphological correlates of the eye are the optic pit and optic vesicle with the retinal progenitor cells, and eventually the optic cup with two distinct layers: the RPE originating from the outer layer, and the neural retina from the inner layer (Figure 1A). The organization of the vertebrate retina into well defined layers is a result of a complex series of developmental processes influenced by a variety of intrinsic and extrinsic factors. Retinal progenitor cells give rise to all retinal cell types such as RPE cells, photoreceptor cells (rods and cones), bipolar cells, ganglion cells, amacrine and horizontal cells, astrocytes, and Müller glial cells [10,11].

Human pluripotent stem cells may serve as an unlimited source of RPE cells for transplantation. Several groups have reported successful RPE differentiation originating from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs; summarized in Table 1). It is widely acknowledged that properties of the existing pluripotent cell lines vary depending on cell line and culture conditions [12-14]. Hence, it is necessary to test RPE differentiation methods and their capacities using several different cell lines. The hESC-RPE and hiPSC-RPE have been shown to delay photoreceptor loss and improve visual functions after subretinal transplantations in animal models. However, transplanted cells disappear within first months after transplantation [4,15-17]. Cell survival needs to be improved to maintain the total functionality of the retina. In addition, these cells have potential in disease modeling and drug discovery, where they are superior to widely used cell lines such as human retinal pigment epithelial cell line (ARPE-19), at least resembling more closely their native counterparts according to their gene expression [1].

Current differentiation methods for RPE cells mainly rely on spontaneous differentiation processes favoring neuroectodermal lineage, which is characteristic of hESCs [18-20]. RPE differentiation efficiency has been recently enhanced with a prolonged culture period and cell culture supplements. Studied supplements include: nicotinamide (NIC), Activin A, transforming growth factor beta (TGFβ) [16], Wnt signaling inhibitor casein kinase I inhibitor (CKI)-7, dickkopf-related protein-1 (Dkk-1), Lefty-A, fibroblast growth factor antagonist Y-27632, and nodal signaling inhibitor SB431542 [21,22]. Regardless of the improvement of the differentiation efficacy, reaching a sufficient amount of differentiated cells with RPE characteristics still demands long-term differentiation processes.

Xeno-products and undefined factors used in the differentiation processes pose further challenges, because animal-derived components may carry factors such as sialic acid or Neu5Gc, causing unwanted immunogenicity of the cells [23,24] or even animal pathogens. Fetal bovine serum (FBS) is widely used, at least in some stages of the culture of RPE cells (Table 1). KnockOut™ Serum Replacement (KO-SR), used to replace FBS in many laboratories, still contains BSA (BSA) and bovine transferrin [25]. In addition, most of the published differentiation methods utilize the culture environment produced by mouse embryonic fibroblast (MEF) cells widely used as feeder cells for hESCs and hiPSCs. It has been suggested that MEFs may even favor the spontaneous differentiation process of RPE cells [26]. Recently, Idelson and coworkers published RPE differentiation from hESCs cultured on human foreskin fibroblasts (hFFs) and by using KO-SR in differentiation medium [16], providing the first important steps toward a defined and xeno-free culture and differentiation process.

In this study, we demonstrate the differentiation potential toward RPE cells of four hESC lines and one hiPSC line, and further give the molecular and functional characterization of these cells. Human ESC- and hiPSC-derived pigmented cells show a typical RPE morphology and express genes and proteins that are characteristic for RPE. The monolayer of putative RPE cells demonstrates functional integrity analyzed by transepithelial electric resistance (TEER). Additionally, the cells phagocytose POS and secrete pigment epithelium-derived factor (PEDF), which is crucial for functional RPE. In the current study, we introduce a defined and xeno-free RPE cell differentiation method based on our previously developed culture system for undifferentiated hESCs and hiPSCs [27]. Such a culture system will be needed in the production of RPE cells following Good Manufacturing Practice for clinical applications.

METHODS

Cell lines: We evaluated the RPE differentiation capacity of four hESC lines: Regea 08/023 (46, XY), Regea 08/017 (46, XX), Regea 08/056 (46, XX), and Regea 08/013 (46, XY), which were previously derived in our laboratory, and one hiPSC line—FiPS 5–7—derived by Professor Otonkoski’s group at the University of Helsinki, Finland [27,28]. The hiPSC line FiPS 5–7 was generated from human fibroblasts using four factors: OCT3/4 (POU5F1), SOX2, nanog, and LIN28 [27]. Transgene silencing was confirmed with quantitative reverse transcription PCR (qRT–PCR) [29]. The cell lines were cultured at +37 °C in 5% CO2 at mitotically inactivated (γ-irradiated, 40 Gy) hFF (36,500 cells/cm²; CRL-2429; American Type Culture Collection, ATCC, Manassas, VA) with basic hESC culture medium. Basic hESC culture medium consisted of Knockout Dulbecco’s Modified Eagle Medium containing 20% KO-SR, 2 mM Glutamax, 0.1 mM 2-mercaptoethanol (all from Invitrogen, Carlsbad, CA), 1% Minimum Essential Medium nonessential amino acids, 50 U/ml penicillin/streptomycin (both from Cambrex Bio Science, Walkersville, MD), and 8 ng/ml human basic fibroblast growth factor (bFGF; R&D Systems Inc., Minneapolis, MN). The culture medium was changed six
Figure 1. Differentiation of human pluripotent stem cells toward retinal pigment epithelium cells. **A**: A schematic representation of retinal pigment epithelium (RPE) cell differentiation during retinal development. **B**: Reverse transcription (RT)–PCR analysis of typical genes for retinal development expressed during putative RPE differentiation of the human embryonic stem cell (hESC) line Regea 08/023 and human induced pluripotent stem cell (hiPSC) line FiPS 5–7 at sequential time points on D7–D72.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Number of cell lines</th>
<th>Feeder cells / matrix for undifferentiated cells</th>
<th>Serum / serum replacement for a) undifferentiated cells</th>
<th>Serum / serum replacement for b) differentiation</th>
<th>Differentiation matrix</th>
<th>Serum free</th>
<th>Xeno-free</th>
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<tr>
<td>[1]</td>
<td>hESC (11)</td>
<td>MEF</td>
<td>a) KO-SR, plasmanate</td>
<td>MEF, Gelatin; Feeder-free; EB</td>
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</tr>
<tr>
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<td>MEF</td>
<td>a) KO-SR, plasmanate</td>
<td>MEF, Gelatin</td>
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<td>-</td>
</tr>
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<td>[22]</td>
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<td>MEF</td>
<td>a) KO-SR</td>
<td>SFEB, Poly-D-Lysine-Laminin-Fibronectin</td>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>[50]</td>
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<td>MEF</td>
<td>b) KO-SR</td>
<td>Mouse PA6 cells, Human Bruch’s membrane, Matrigel™</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>[37]</td>
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<td>MEF</td>
<td>b) KO-SR</td>
<td>MEF, Matrigel™</td>
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<td>+</td>
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<td>a) KO-SR</td>
<td>MEF, Matrigel™</td>
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<td>MEF, SNL</td>
<td>b) KO-SR</td>
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<td>+</td>
<td>-</td>
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<tr>
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<td>hFF, MEF, Matrigel™</td>
<td>b) KO-SR</td>
<td>Gelatin</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>[16]</td>
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<td>hFF</td>
<td>b) KO-SR</td>
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<tr>
<td>[34]</td>
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<td>MEF</td>
<td>a) KO-SR</td>
<td>Free-floating aggregates, Laminin</td>
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<td>+</td>
<td>-</td>
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<tr>
<td>[33]</td>
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<td>MEF</td>
<td>b) KO-SR, NIM, RDM</td>
<td>MEF, Gelatin</td>
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<td>-</td>
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<tr>
<td>[53]</td>
<td>hESC (2)</td>
<td>Matrigel™</td>
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<td>Collagen-Laminin</td>
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1No human serum (HS), fetal bovine serum (FBS) or plasmanate used in any stage of pluripotent stem cell cultures or during differentiation. 2No animal cells, BSA (BSA)/FBS or other xeno components used in any stage of pluripotent stem cell cultures or during differentiation. Abbreviations: hESC represents human embryonic stem cell; hiPSC represents human induced pluripotent stem cell; MEF represents mouse embryonic fibroblast; KO-SR represents KnockOut Serum Replacement; EB represents embryoid body; SFEB represents serum-free floating culture of embryoid body-like aggregates; SNL represents a mouse fibroblast STO cell line; NIM represents chemically defined neural induction medium; MEF-CM represents mouse embryonic fibroblast conditioned medium.
times a week. Undifferentiated colonies were passaged manually once a week on the top of hFFs.

Retinal pigment epithelium differentiation in serum-free conditions: To induce spontaneous RPE differentiation, KO-SR concentration was reduced from 20% to 15% and bFGF was removed from the basic hESC culture medium (described above). This modified medium was called RPEbasic. The cells were cut manually between days 1 and 7 after initiation of differentiation onto low cell bind six-well plates (Nalge NUNC, Tokyo, Japan), where the cells formed floating aggregates. While the cells were cultured on hFFs, the culture medium was changed six times a week (2 ml/35×10 mm polystyrene dish), but for floating cell aggregates, the culture medium change was reduced to three times a week (3 ml/well in a six-well plate). Floating aggregates were passaged mechanically with a scalpel to make gas and nutrient exchange possible. To gain purer RPE populations, the areas with pigmented cells were isolated manually with a scalpel and subsequently dissociated with 1× Trypsin-EDTA (Lonza, Walkersville, MD). The cells were seeded either on Collagen IV (Sigma-Aldrich, St. Louis, MO)-coated 24 well plates for RT–PCR analyses or on permeable Collagen IV–coated 0.3 cm² BD Biocoat culture plate inserts (BD Biosciences, San Jose, CA). After the seeding, the cells were not subcultured.

Retinal pigment epithelium differentiation in xeno-free and defined conditions: RPE differentiation was studied using a previously developed xeno-free and defined culture medium formulation (RegES) [27] as base. For RPE differentiation, bFGF and retinol were excluded from the published medium formulation (RPEregES). Otherwise, the differentiation method was same as described above (RPEbasic). Analyses conducted in this study from cells differentiated using RPEregES methods are summarized in Figure 2.

Analyses of pigmentation rates: The appearance of the first pigmented cells was followed daily. Percentage of pigment containing cell aggregates from the total amount of aggregates was counted between days 21 and 30 after induction of the differentiation. In addition, the day of the first pigmented cell’s appearance was recorded.

Reverse transcription polymerase chain reaction: RNA samples were collected from cell cultures at five different time points, on days (D; D7, D28, D44, D52, D72) and, in addition, on D196, from selected RPE cells (RPEbasic). From cells differentiated using RPEregES, RNA samples were collected on D7 and D44. Total RNA was extracted using NucleoSpin XS-kit (Macherey-Nagel, GmbH & Co, Düren, Germany) according to the manufacturer’s instructions. Briefly, the cells were lysed in 102 µl of RA1-TEP mixture. Five µl of Carrier RNA working solution was added to the lysate. The sample was filtrated through the NucleoSpin® Filter Column. One hundred µl of 70% ethanol was added to the lysate and RNA bound to the NucleoSpin® RNA XS Column. The column was desalted using membrane desalting buffer. DNA was digested using DNase reaction mixture. rDNase was inactivated with RA2 buffer and silica membrane washed twice with RA3 buffer. Pure RNA was eluated in 10 µl H₂O. The RNA concentration and quality were assessed with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA (40 ng) was reverse-transcribed using MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions in the presence of RNase inhibitor. Briefly, 10 µl reaction consisted of: 2.0 µl 10× RT Buffer, 0.8 µl 100 mM dNTP Mix, 2 µl 10 µM gene specific primers, 1.0 µl MultiScribe™ Reverse Transcriptase, 1.0 µl RNase Inhibitor, 11.2 µl Nuclease-free H₂O, and 2 µl total RNA (20 ng/µl). The cDNA synthesis was performed as follows: 10 min at 25 °C, 120 min at 37 °C, 5 s at 85 °C. In addition, genomic control reactions excluding the enzyme (-RT) for each RNA sample were performed. CDNA was used as a template in a following PCR reaction, which was performed using 5 U/µl Taq DNA Polymerase (Fermentas, Thermo Fisher Scientific Inc., Leicestershire, UK) with 5 µM primers specific for particular genes (Biomers.net GmbH, Söflinger, Germany; Table 2).

PCR reactions were performed in PCR MasterCycler ep gradient (Eppendorf AG, Hamburg, Germany) as follows: after the hot start denaturation at 95 °C for 3 min and 38 cycles of denaturation at 95 °C for 30 s, 30 s annealing (annealing
Table 2. Reverse-transcriptase (RT)–PCR Primer sequences.

<table>
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<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>RAX</td>
<td>CTGAAGACGAACTGACACACT</td>
<td>CTCTGGGAAATGGCCAAGTTT</td>
<td>55</td>
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<tr>
<td>MITF</td>
<td>AAGTCCTGAGTTCGATGC</td>
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<tr>
<td>RPE65</td>
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<tr>
<td>bestrophin</td>
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<td>ATCTCCTCTGTCATCTGT</td>
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<tr>
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<tr>
<td>PEDF</td>
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<td>TGGGCAATCTTGCAGCTGAG</td>
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<tr>
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<td>AAGGGCCGCAGCTTACATGCTG</td>
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Abbreviations: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), paired box gene 6 (PAX6), retina and anterior neural fold homeobox (RAX), microphthalmia-associated transcription factor (MITF), anti-retinal pigment epithelium-specific 65 kDa protein (RPE65), orthodenticle homebox 2 variant 1 (OTX2v1), premelanosome protein (PMEL), pigment epithelium-derived factor (PEDF), sex determining region Y-box 10 (SOX10).
and goat antirabbit IgG, both Alexa Fluor 488, goat antimouse IgG and goat anti-rabbit IgG, both Alexa Fluor 568 (all from Molecular Probes, Life Technologies, Paisley, UK). Secondary antibodies were diluted in 0.5% BSA-PBS and incubated 1.5 h at RT following repeated PBS washings. In addition, fluorescein isothiocyanate (FITC) phalloidin 1:50 (Invitrogen) was used to label filamentous actin. For clarity, FITC phalloidin is represented as red in Figure 3H, I, K, L and A568 is represented as green in Figure 3G, I, J, L. The nuclei were stained with 4′, 6′ diamidino-2-phenylidole (DAPI) included in the mounting media (Vector Laboratories Inc., Burlingame, CA). Images were taken either with an LSM 700 confocal microscope (Carl Zeiss, Jena, Germany) using a 63× oil immersion objective or Olympus BX60 microscope (Olympus, Tokyo, Japan) using a 60× oil immersion objective. 

In vitro phagocytosis assay: Porcine POS were isolated to study whether the cells have the ability to phagocytose POS [31]. Twenty-three porcine eyes received from a local abattoir were halved with scissors and the retinas were removed using tweezers in a dark room under red light. Retinas were homogenized with homogenizer in 0.73 M sucrose phosphate buffer. Homogenized retinas were filtered and cell types separated in sucrose gradient (0.75 M, 1.0 M, 1.25 M, 1.5 M, 1.75 M) using an ultracentrifuge (Optima ultracentrifuge, Beckman Coulter, Inc., Brea, CA) 60,000× g, 1 h. The pink POS layer was collected in phosphate buffer and centrifuged. For phagocytosis assay, sucrose-phosphate buffer was removed and POS were labeled with FITC (0.04 µg/µl; Sigma-Aldrich) in 0.1M NaHCO3 (pH 9) for 1 h at RT, following washing three times with PBS and resuspension in culture medium. Floating cell aggregates, which included pigmented and nonpigmented cells, were incubated with POS for 16 h in a cell culture incubator in culture medium. Subsequently, the cells were washed twice with PBS. Cells were fixed with 4% paraformaldehyde for 30 min at +37 °C following repeated PBS washings. Thereafter, 0.20% Trypan Blue was used to quench external fluorescence following PBS washing. Cells were made permeable using 0.1% Triton X-100 for 10 min at RT followed by repeated PBS washings. Filamentous actin was stained with 1:10 diluted phalloidin 0.02 µg/µl (Sigma-Aldrich) by incubating for 10 min at RT following several PBS washings. The nuclei were stained with DAPI that was in the mounting media (Vector Laboratories Inc.). The images of the RPE cells with intracellular POS fragments were taken using a confocal microscope (LSM 700, Carl Zeiss, 63× oil immersion objective).

Enzyme-linked immunosorbent assay: The functionality and maturity of putative RPE cells were evaluated by their PEDF secretion. RPEbasic and RPEregES media were conditioned with 10 strongly pigmented floating aggregates each. Secretion of nonpigmented aggregates was used as controls. PEDF secretion was evaluated in four time points; Regea 08/023 was differentiated in RPEbasic conditions for 49, 70, 97, and 230 days, and FiPS 5–7 for 49, 80, 97, and 250 days, respectively. From the RPEregES condition, analyses were conducted with both previously mentioned cell lines on days 49 and 98. Culture media were collected from each well every second or third day. PEDF concentration was determined using Chemikine PEDF Sandwich ELISA Kit according to the instructions of the manufacturer (Millipore). In brief, medium samples were treated with 8 M urea to gain total PEDF secreted in media and incubated on ice for an hour. Urea-treated samples were diluted 1:100 in assay diluent. One hundred µl of diluted samples were added per well of 96 well plate likewise PEDF standards. Incubated for hour at +37 °C following repeated washing with washing buffer. 1:500 diluted Biotinylated Mouse Anti-Human PEDF monoclonal antibody was added into the wells. The plate was incubated for an hour at +37 °C. After repeated washings, 100 µl 1:1000 diluted streptavidin peroxidase conjugate was added to the wells, following hour incubation at +37 °C. TMB/E reagent was added to the wells. Incubated at RT for 5–10 min before addition of Stop Solution, and absorbance was measured immediately using Wallac Victor™ 1420 Multilabel counter (Perkin Elmer-Wallace, Norton, OH).

Transepithelial electric resistance: Development of epithelia barrier properties and tight junction formation between the cells reflecting polarity was determined as duplicates from hESC-RPE and hiPSC-RPE cell monolayers cultured on permeable Collagen IV–coated 0.3 cm2 BD Biocoat culture plate inserts (BD Biosciences). Human ESC line Regea 08/023 and hiPSC line FiPS 5–7 were differentiated for 178 days and 197 days, respectively, before seeding on the inserts. TEER measurements were taken every time after replacing culture media during 90 day culture period of the monolayer with a Millicell-electrical resistance system volt-ohm meter (Millipore). TEER (Ωcm²) of the epithelia was obtained by subtracting the impact of the medium and similarly treated insert without cells from the result and multiplying this by the area of the filter membrane.

Ethical issues: Regea-Institute for Regenerative Medicine has the approval of the National Authority for Medicolegal Affairs Finland (TEO) to study human embryos (Dnr1426/32/300/05). Regea has the support of the Ethical Committee of the Pirkanmaa Hospital District to derive, culture, and differentiate hESC lines from surplus human embryos (R05116).

RESULTS

Comparison of the differentiation rate between cell lines: In this study, we successfully differentiated RPE-like cells from all five human pluripotent stem cell lines that were studied. First, the differentiation potentials of the hESC lines and the hiPSC line were compared by analyzing the appearance of the first pigmented cells emerging from each cell line. On average, pigmentation was observed as follows: Regea 08/013...
Figure 3. Immunofluorescence staining of human embryonic stem cell (hESC; Regea 08/023)- and human induced pluripotent stem cell (hiPSC; FiPS 5–7)-derived retinal pigment epithelium cells revealing maturation stage after 83 days of differentiation. Cellular retinaldehyde-binding protein (CRALBP) and microphthalmia-associated transcription factor (MITF) localization in A-C: manually selected hESC-RPE cells and D-F: hiPSC-retinal pigment epithelium (RPE) cells. G, I. RPE65 expression in hESC-RPE and J, L: hiPSC-RPE. H, K. For cell morphology, F-actins were stained using phalloidin. Tight junction protein anti-zonula occludens (ZO)-1 and proliferation marker Ki67 localization in M: hESC-RPE cells and N: hiPSC-RPE cells. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Images A-F were taken with an Olympus BX60 microscope (Olympus, Tokyo, Japan) using a 60× oil immersion objective, scale bar 20 μm. Images G-N were taken with an LSM 700 confocal microscope (Carl Zeiss) using a 63× oil immersion objective, scale bar 20 μm.
on D21, both Regea 08/017 and Regea 08/023 on D12, Regea 08/056 on D10, and FiPS 5–7 on D11 (Table 3). The percentage of pigmented cell clusters from each cell line was calculated between day 21 and 28 after the removal of bFGF (RPEbasic). Over 20% of the cell aggregates of the cell lines Regea 08/023, Regea 08/056, and FiPS 5–7 contained pigment cells, whereas less than 10% of the aggregates of the cell lines Regea 08/013 and Regea 08/017 contained pigment cells (Table 3).

**Eye-specific gene expression during differentiation:** Gene expression of the differentiating cells was analyzed using RT–PCR (Table 4). All of the hESC lines expressed the early eye lineage markers paired box gene 6 (PAX6) and RAX, and early RPE marker MITF seven days after removal of bFGF (RPEbasic). In contrast, the expression of RAX by the FiPS 5–7 cell line could not be consistently detected until D56. Thus, we further studied RAX expression with qRT–PCR. qRT–PCR analyses showed that RAX was expressed on D7 and the expression decreased during differentiation (Figure 4). The RPE cell specific markers RPE65 and Bestrophin were detected from all the cell lines on D7–D28 except for cell line Regea 08/013, from which the expression of RPE65 was detected for the first time on D44. Other RPE cell markers, orthodenticle homeobox 2 variant 1 (OTX2v1), premelanosome protein (PMEL), and PEDF were expressed relatively early during the differentiation process, with tyrosinase showing the latest appearance of the studied genes. Representative RT–PCR results from hESC line Regea 08/023 and hiPSC line FiPS 5–7 are presented in Figure 1B.

**Pigmented cells expressed retinal pigment epithelium–specific genes and proteins:** According to the pigmentation rate (Table 3), we chose the hESC line Regea 08/023 and

### Table 3. Pigmentation rate of studied cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Appearance of pigmentation± standard deviation (n)</th>
<th>Number of pigmented cell aggregates</th>
<th>Total number of cell aggregates</th>
<th>% Pigmented aggregates</th>
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<tr>
<td>08/013</td>
<td>d21±3.2 (5)</td>
<td>90</td>
<td>949</td>
<td>9.5</td>
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<tr>
<td>08/017</td>
<td>d12±7.4 (4)</td>
<td>50</td>
<td>775</td>
<td>6.5</td>
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<tr>
<td>08/023</td>
<td>d12±5.6 (6)</td>
<td>136</td>
<td>582</td>
<td>23.4</td>
</tr>
<tr>
<td>08/056</td>
<td>d10±2.1 (2)</td>
<td>55</td>
<td>249</td>
<td>22.1</td>
</tr>
<tr>
<td>FiPS 5–7</td>
<td>d11±5.3 (3)</td>
<td>83</td>
<td>333</td>
<td>24.9</td>
</tr>
</tbody>
</table>

n: number of independent experiments

### Table 4. Gene expression profiles during the differentiation.

<table>
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<th>Cell line</th>
<th>Day</th>
<th>GAPDH</th>
<th>PAX6</th>
<th>RAX</th>
<th>MITF</th>
<th>RPE65</th>
<th>bestrophin</th>
<th>OTX2v1</th>
<th>PMEL</th>
<th>PEDF</th>
<th>tyrosinase</th>
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<tbody>
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<td>08/013</td>
<td>d7</td>
<td>+</td>
<td>+ (+)</td>
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<tr>
<td>08/013</td>
<td>d28</td>
<td>+</td>
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<td>+ (+)</td>
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<td>d72</td>
<td>+</td>
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In the table + means positive expression, (+) means faint expression, - means negative expression. Abbreviations: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), paired box gene 6 (PAX6), retina and anterior neural fold homeobox (RAX), microphthalmia-associated transcription factor (MITF), anti-retinal pigment epithelium-specific 65 kDa protein (RPE65), orthodenticle homeobox 2 variant 1 (OTX2v1), premelanosome protein (PMEL), pigment epithelium-derived factor (PEDF).
hiPSC line FiPS 5–7 for more detailed evaluation. The isolated pigmented cells were matured on Collagen IV–coated well plates. Morphologically, cultured cells seemed to undergo an epithelial-mesenchymal transition (EMT) process; after attachment, cells had fibroblast-like morphology following transition to round cells and finally to morphologically typical cobblestone-like cells, putative RPE cells with pigment granules (Figure 5A). The isolated and long-term cultured (d196) hESC- and hiPSC-derived RPE-like cells expressed the typical RPE cell genes (MITF, RPE65, bestrophin, OTX2v1, PMEL, PEDF, and tyrosinase; Figure 5B). Of the studied genes, undifferentiated cells showed the expression of PMEL, PEDF, SOX10, alphacardiac actin, nanog, and OCT3/4. hFF used in undifferentiated cell cultures showed the expression of PMEL, PEDF, and SOX10 (Figure 5B). In addition, the cells expressed eye precursor gene PAX6; however, RAX showed only very faint expression. The endodermal marker alphafetoprotein was not expressed in long-term cultured cell population, but mesodermal marker alphacardiac actin was expressed by hiPSC-RPE cells and a marker of neural crest-derived melanocytes (SOX10) was expressed by both cell lines. In addition, the expression of the pluripotent markers nanog and OCT3/4 was detected from hiPSC-RPE but was absent in hESC-RPE cells (Figure 5B). However, qRT–PCR comparison between undifferentiated FiPS 5–7 cells and putative hiPSC-RPE cells showed that the expression level was very low on D196 of differentiation (Figure 5C).

To confirm that the manually isolated cells expressed proteins typical of functional RPE, the expression and localization of MITF, CRALBP, RPE65, and ZO-1 were analyzed with immunostaining (Figure 3). MITF localized in the nuclei and CRALBP both to the cytoplasm and cell membrane (Figure 3A,D), as expected. RPE65 was observed in cytoplasm. Phalloidin staining showed F-actin distribution adjacent to the cell membrane, resembling native RPE cells (Figure 3H,K). ZO-1 localized to the tight junctions on the cell membrane and K+-67 staining was negative, suggesting that cells were mature and did not proliferate at this stage (Figure 3M,N).

Functional characterization of differentiated cells: The functionality of putative hESC-RPE (Regea 08/023) and hiPSC-RPE (FiPS 5–7) was shown by POS phagocytosis, PEDF secretion, polarization of cells, and the integrity of the epithelial structure by TEER measurements. The results show that pigmented cells from both lines were able to phagocytose POS (Figure 6A,B), as opposed to nonpigmented cells, which were used as controls (data not shown).

Pigmented cell aggregates differentiated with RPEbasic method also secreted PEDF to the culture medium, whereas nonpigmented cells did not. On average, PEDF secretion after 49 days of differentiation was 1.7 ng/ml from putative hESC-RPE (Regea 08/023) and 13.8 ng/ml from putative hiPSC-RPE (FiPS 5–7). On D70, the secretion was increased to 77.5 ng/ml from putative hESC-RPE (Regea 08/023) cells and 44.8 ng/ml from putative hiPSC-RPE (FiPS 5–7) cells on D80, respectively. After 97 days of differentiation, PEDF secretion was 548.0 ng/ml (Regea 08/023) and 251.8 ng/ml (FiPS 5–7). After 230 days of differentiation, secretion was 434 ng/ml from hESC-RPE cells. From hiPSC-RPE-like cells, secretion was 15.5 ng/ml on D250. It must be noted that the exact cell amount was not the same between media collections. hFF cells, which were used as feeder cells for pluripotent cells, also showed PEDF expression at the gene level, but did not secrete a detectable amount of PEDF to the culture medium (data not shown).

The polarization of the cells was analyzed by localization of Na+/K+-ATPase and bestrophin proteins. Na+/K+-ATPase was localized on the apical cell membrane and Bestrophin on
Figure 5. Morphology and gene expression analysis of manually selected and long-term cultured human embryonic stem cell (hESC)-retinal pigment epithelium (RPE; Regea 08/023) and hiPSC-RPE (FiPS 5–7) cells. A: Bright-field micrograph of hESC-retinal pigment epithelium (RPE) and human induced pluripotent stem cell (hiPSC)-RPE cells cultured for 136 days on Collagen IV. The cells have acquired a cobblestone morphology and a high degree of pigmentation, which is typical of RPE cells. Low magnification images were captured with a Nikon Eclipse TE2000-S phase contrast microscope (Nikon Instruments Europe B.V. Amstelveen, The Netherlands) and higher magnification images with an Olympus BX60 microscope (Olympus, Tokyo, Japan) using a 60× oil immersion objective. Scale bar 20 μm. B: Reverse transcription (RT)–PCR analysis showing the expression of optic vesicle, optic cup, RPE, neural crest melanocyte, pluripotent stem cell, mesoderm and endoderm marker genes by undifferentiated cells (D0), human foreskin fibroblast (hFF) feeder cells, and putative RPE cells (D196) from Regea 08/023 and FiPS 5–7 cells. N/A=not analyzed. C: Relative OCT3/4 expression between undifferentiated FiPS 5–7 and putative hiPSC-RPE after 196 days of differentiation.
the basolateral side, demonstrating the polarization of analyzed cells (Figure 6C,D).

The development of the integrity of the epithelia was assessed weekly during a 90 day period after seeding the cells on porous culture inserts. Thirty days after plating the pigmented cells on the inserts, TEER values ranged between 6 and 32 Ωcm² and were 6–10 Ωcm² in hESC-RPE (Regea 08/023) and hiPSC-RPE (FiPSC 5–7), respectively. After 60 days on inserts, TEER values ranged between 145 and 188 Ωcm² for putative hESC-RPE and were 23–38 Ωcm² for

Figure 6. Phagocytosis of photoreceptor outer segments (POS) and cell membrane polarization of human embryonic stem cell (hESC; Regea 08/023) and human induced pluripotent stem cell (hiPSC; FiPSC 5–7)-derived retinal pigment epithelium (RPE) cells. A: Putative hESC-RPE and B: hiPSC-RPE internalize POS (green, arrowheads) for cell morphology; F-actins were stained using phalloidin (red). Vertical confocal sections showing apical localization of Na⁺/K⁺-ATPase (green) and basolateral localization of Bestrophin (red) in C: hESC-RPE and D: hiPSC-RPE. Images were taken with an LSM 700 confocal microscope (Carl Zeiss) using a 63× oil immersion objective, scale bar 20 μm.
putative hiPSC-RPE. After 90 days on the inserts, TEER values for hESC-RPE reached 311 Ωcm² for hiPSC-RPE, 74 Ωcm².

Xeno-free and defined conditions induce efficient retinal pigment epithelium differentiation: Finally, we wanted to evaluate RPE differentiation in defined and xeno-free conditions (RPEregES) and to compare this with our findings in basic conditions (RPEbasic). The results from a pilot study with the Regea 08/013 cell line, which was derived and maintained in RegES [27], indicated that hESCs derived and maintained in RegES are able to spontaneously differentiate into pigmented cells resembling RPE morphology when bFGF is excluded (data not shown). To compare the RPEregES method with RPEbasic, we implemented further studies with Regea 08/023 and FiPS 5–7. The first pigmented cells were observed in hESC line Regea 08/023 on D7 and in hiPSC line FiPS 5–7 on D6 in RPEregES conditions, compared to D12 and D11, respectively, in RPEbasic. The ratio of the pigmented cell clusters (n) was evaluated after 23–28 days of differentiation. The percentage of the pigmented clusters for FiPS 5–7 cells was 37% (n=129) in RPEregES conditions and 25% (n=457) in RPEbasic conditions, and for Regea 08/023 cells 43% (n=129) in RPEregES and 25% (n=645) in RPEbasic conditions.

RT–PCR and immunostainings were performed for the cells (Regea 08/023 and FiPS 5–7) differentiated in RPEregES medium. On D44, cells from both cell lines expressed all analyzed eye/RPE-specific markers (PAX6, RAX, MITF, RPE65, bestrophin, OTX2v1, PMEL, PEDF, tyrosinase; Regea 08/023, Figure 7A; FiPS 5–7, data not shown). The pigmented cells in RPEregES conditions were manually selected on D51. Their morphology and protein expression were analyzed on D83. The pigmented cells differentiated from both hESC and hiPSC lines had cobblestone-like morphology, which is typical for RPE cells. In addition, the cells were positive for RPE65, ZO-1, MITF, CRALBP, Bestrophin, and Na⁺/K-ATPase, which are important for the functionality of the RPE cell. There were some Ki67 positive cells, which were clearly more immature according to the cell morphology (Regea 08/023, Figure 7B-I; FiPS 5–7, data not shown). In addition to gene and protein expression, putative RPE cells differentiated with RPEregES secreted PEDF. On average, PEDF secretion after 49 days of differentiation was 16.2 ng/ml and 97 days was 182.4 ng/ml from hESC-RPE (Regea 08/023) cells. Corresponding values from hiPSC-RPE (FiPS 5–7) differentiated with the RPEregES method were 10.6 ng/ml and 463.15 ng/ml.

DISCUSSION

In this study, we successfully differentiated RPE-like cells from several human pluripotent stem cell lines without the use of animal cells or serum during the differentiation. In addition, we reported RPE differentiation in xeno-free and defined culture conditions. Putative RPE cells arise spontaneously from undifferentiated cells by the removal of bFGF from the culture conditions. The hESC lines used in this study have a similar background; all the cell lines were cultured on hFF feeder cells and in KO-SR containing culture medium without serum. The hiPSC line, FiPS 5–7, was cultured on top of hFF feeder cells from passage 26 onwards. Other published RPE differentiation methods have harnessed mainly hESC and hiPSC lines derived and cultured on MEFs or with FBS (Table 1). Only very recently, Idelson and cowokers published a protocol differentiating hESCs toward RPE cells from hESCs grown on hFF feeder cells and with KO-SR containing culture medium [16].

Our results demonstrate that the appearance of the first pigmented cells was relatively fast after the removal of bFGF, both in hESC and hiPSC lines, varying from 10 to 21 days. Most of the published data describe the appearance of the first pigmented cells usually around 2 to 8 weeks [1,32,33]. Thus, according to their pigmentation, hESCs and hiPSCs cultured on hFF seem to have equally good differentiation rate to those of other published cell lines cultured on MEFs. We analyzed the percentage of the pigment containing cell clusters to indicate the differentiation rate of each cell line on D21–D28 after initiation of the differentiation. Although calculations only give a rough estimate of the pigmentation rate, the results demonstrated differences between analyzed cell lines. Idelson and coworkers published comparable results with an equal amount of pigmented cell clusters (<15%) after four weeks of differentiation without NIC supplementation [16]. For comparison, Klimanskaya and coworkers reported the presence of pigmented islands in less than 1% of hESC-derived cell aggregates after 4–8 weeks of differentiation [1].

During the time course of differentiation, we already detected the expression of early eye lineage markers PAX6 and RAX, as well as early RPE marker MITF at D7 after removal of bFGF from all analyzed hESC lines. Although the expression of PAX6 was also detected during a later stage of the differentiation, our data demonstrated that the mature RPE cell specific markers, RPE65 and bestrophin, were detected from all but one of the hESC lines on D28 at the latest. In addition, other RPE cell markers—OTX2v1, PMEL, PEDF, and tyrosinase—were also expressed early in the differentiation process of hESC lines, with tyrosinase having the latest appearance of the studied genes. Surprisingly, the expression of RAX was not detected from hiPSC-RPE (FiPS 5–7) until D56. On the other hand, RPE cell specific markers RPE65, bestrophin, OTX2v1, PMEL, PEDF, and tyrosinase were expressed in hiPSC-RPE derived cells similarly as to hESC-RPE cells.

According to the pigmentation rate, we chose the most promising hESC line, Regea 08/023, and hiPSC line, FiPS 5–7, for more detailed evaluation. Manually selected and long-term cultured cells formed a monolayer of pigmented
cobblestone-like cells that had a very similar morphology to previously published hESC-RPE and hiPSC-RPE cells [1, 32,34]. After dissociation into single cells, the differentiated RPE-like cells started to proliferate and were observed to undergo morphological changes that are typical in EMT. EMT is related to normal development and tissue repair, but also pathological processes such as cancer and proliferative vitreoretinopathy. The EMT process has been recently described in isolated RPE cells [35]; thus it is a natural feature when RPE cells lose cell-cell contacts. Our results indicate that putative hESC-RPE and hiPSC-RPE cells also seem to undergo a similar process.

The gene expression analyses of hESC-RPE and hiPSC-RPE cells revealed that the cells expressed eye precursor genes *RAX* and *PAX6*, and RPE cell markers. The expression of *RAX* was not constantly detected from hiPSC-RPE (FiPS 5–7) until D56 with RT–PCR. However, more quantitative qRT–PCR analysis showed that *RAX* expression was similar to that of hESC-RPE cells. Both cell lines also expressed the marker of neural crest-derived melanocytes (*SOX10*), indicating the presence of other ectoderm derivatives. *SOX10* expression by selected hESC and hiPSC derived RPE-like cells is most probably the expression of other types of neural cells, which easily contaminate pluripotent human cell cultures [36]. Selected and long-term cultured hiPSC-RPE cells expressed mesodermal marker and pluripotent marker *OCT3/4* and nanog, indicating that the cell population contained undifferentiated cells. We showed that neither hESC-RPE nor hiPSC-RPE were homogenous, even after 196 days in culture, according to gene expression, although
morphologically the cells seemed to be of uniform quality. Thus, the manual selection, which we used in this study, is not sufficient to gain a pure population of putative RPE cells. Consequently, it is essential to develop more specific differentiation methods and more efficient purification and selection methods for these cells.

Microscopy of cells differentiated for 83 days showed that the cells were highly organized and pigmented. The immunocytochemical localization of proteins essential for mature and functional RPE cells was identical to previously described results [16,37]. Of note, the cells expressed RPE65, which is essential for the regeneration of the visual pigment required for both rod- and cone-mediated vision [38]. The expression of the RPE65 protein is typically lost in cultured RPE cells [39-41], and has been reported in only a few papers describing the differentiation of RPE cells from human ESCs [16,37]. The expression of RPE65 protein in a culture environment by human iPSC-RPE has been previously reported only by western blotting and was detected as late as after eight months of differentiation [32]. In the present study, both hESC-RPE and hiPSC-RPE cells showed RPE65 immunostaining at D83, suggesting that the differentiated cells are functional and closely resemble the native RPE cells.

One of the most important functions of the RPE cells is the phagocytosis of POS. In vivo, photoreceptor cells undergo a daily renewal process and RPE cells take care of the waste disposal by phagocytosing nonfunctional POS. Several groups have published the phagocytotic activities of hESC-RPE and hiPSC-RPE in vitro using latex beads [1] or POS isolated from animals [32,42]. It has been further suggested that the only proof for the specific phagocytosis activity of RPE cells is the capability for POS phagocytosis [42]. The hESC-RPE and hiPSC-RPE cells generated in our study possess the relevant molecular functions required for the phagocytosis of isolated POS, thus demonstrating their functionality in vitro.

In addition, the functionality of hESC-RPE and hiPSC-RPE cells was shown by PEDF secretion, with both differentiation methods described here. PEDF secreted by RPE cells is antiangiogenic and neuroprotective, protecting the retinal neurons from light damage, oxidative stress, and glutamate excitotoxicity [43]. According to our knowledge, the secretion of PEDF from putative hESC-RPE or hiPSC-RPE cells has not been previously published anyone other than Klimanskaya and coworkers [1]. Our data demonstrated that the differentiated pigmented cells were able to secrete high levels of PEDF into the culture medium, while nonpigmented cells did not secrete any detectable amounts of PEDF. There is no published data about the amount of PEDF secreted by putative hESC/hiPSC-RPE [1]. Tong and coworkers have reported that the ARPE19 line secretes PEDF at around the 5–35 ng/ml level to the culture medium [44], and Maminishkis and coworkers have stated that isolated human fetal RPE cells secrete PEDF at the 600 ng/ml level [45]. These levels have not been standardized to the cell number included in the experiment; thus, direct comparison of our results is not possible. However, the hESC-RPE and hiPSC-RPE cells in our study secrete reasonable levels (40 ng/ml- 430 ng/ml) of PEDF, demonstrating their functionality in relation to growth factor production in vitro.

Versatile determination of the maturation and polarization capacity of the derived cells in a specific culture protocol is important [32]. TEER is one of the assessments that can be used for this [46]. TEER values have been regularly assessed from human retinal explants [47] and primary and immortalized RPE cell lines reaching 206 Ωcm² and 100 Ωcm², respectively [48,49]. To our knowledge, the TEER of putative hESC-RPE or hiPSC-RPE cells has not yet been evaluated. In our culture, the putative hESC-RPE cells reached TEER values of 310 Ωcm² after 268 days of differentiation and the putative hiPSC-RPE cells reached TEER values of 74 Ωcm² after 287 days of differentiation. When the TEER values of hESC/hiPSC-RPE are compared to values derived from other RPE cell cultures, it appears that putative hESC-RPE cells are at least as good or even superior to immortalized RPE cell lines in forming a highly polarized monolayer. Further indications of a high degree of cellular polarization of differentiated cells are the separation of Na⁺/K⁺ATPase to the apical and bestrophin to the basal side of the cellular monolayer, and junctional localization of apical tight junction protein ZO-1.

We further demonstrated that xeno-free and defined differentiation conditions can be used for the induction and maturation of pluripotent stem cell derived RPE cells. With this method, we were able to differentiate RPE-like cells from hESCs and hiPSCs. The differentiated cells showed similar gene and protein expression profiles, as well as cellular polarization, to the RPE cells differentiated using RPEbasic conditions containing xenogeneic substances. Our results demonstrated that the appearance of the pigmented cells was slightly faster in RPEregES conditions as compared to the cells in RPEbasic conditions. In addition, the percentage of the pigmented clusters after 23 days of differentiation of both the hESC and hiPSC lines were higher in RPEregES conditions, with the hiPSC line having over 10% difference between the two conditions. These results suggested that our defined and xeno-free culture medium described previously [27] may even enhance the differentiation of RPE cells. This may be partly explained by the fact that the medium contains Activin A (5 ng/ml), a known inducer of RPE cell fate [26]. Our defined and xeno-free differentiation method is one step forward when optimizing proper culture conditions for clinically eligible RPE cells for transplantation. It remains to be studied whether supplements such as NIC [16] and Wnt and Nodal antagonists [21,22] increase RPE differentiation from hESCs and hiPSCs in our culture systems.
In conclusion, we have demonstrated a progressive differentiation protocol for the production of functional RPE-like cells from hESCs and hiPSCs. Our results demonstrate that putative hESC-RPE and hiPSC-RPE express genes and proteins characteristic for RPE cells, can phagocytose POS, are able to secrete PEDF and form highly polarized and tight epithelial structures in vitro. Our results show that highly mature RPE-like cells can be differentiated in xeno-free and defined culture systems that are easier to translate under the Good Manufacturing Practice production systems needed for clinical use. Furthermore, defined conditions will greatly elucidate the further development of more efficient differentiation protocols and the use of cells in drug screening and toxicology studies.

ACKNOWLEDGMENTS

The study was financially supported by two private donations, Academy of Finland (218050), the Competitive Research Funding of the Pirkannaa Hospital District (91118), Sokainen ystävät foundation, Päivikki and Sakari Sohlberg foundation, Evald and Hilda Nissin foundation, Silmä- and kudospankkki foundation, Finnish Cultural Foundation. We thank Samer Hussein, Jaan Palgi, Ras Trokovic, and Timo Otonkoski from Professor Timo Otonkoski’s group at University of Helsinki for the kind gift of the hiPSC line FiPS 5–7. We thank Elina Konsén, Hanna Koskenaho, Outi Melin, Alexandra Mikhailova, and Jaana Huuki for technical assistance and Prof Markku Mäki’s group at University of Tampere for the equipments. We want to acknowledge Professor Kai Kauraniranta group at University of Eastern Finland for technical support in POS isolation and Ms Maria Sundberg for designing SOX10 primer pairs. Part of the data was presented in EVER 2010 meeting Crete, Greece.

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Introduction

Age-related macular degeneration (AMD) is a complex eye disorder and is the leading cause of blindness in developed countries. AMD has a multifactorial etiology and leads to a progressive loss of central vision in the elderly. The number of AMD patients is projected to double over the next few decades, becoming a major public health issue in the near future [1]. AMD is characterized by the degeneration of retinal photoreceptors rod and cones, retinal pigment epithelium (RPE), and Bruch’s membrane, as well as detrimental alterations of the choroidal capillaries. One of the main functions of RPE cells is to nourish the neural cells, rod and cone cells. In senescent RPE cells, which are constantly exposed to oxidative stress, this ability is weakened, causing secondary adverse effects on the neural retina and ultimately leading to vision loss [2]. Thus, degeneration of the postmitotic RPE cells is one of the most important hallmarks of AMD.

The pathogenesis of AMD is complex and it has remained elusive. Therefore appropriate therapies have been difficult to establish. Only 20% of AMD patients, that have exudative form of disease, can be treated with intravitreal anti-VEGF injections. It is a huge challenge to develop new effective treatment alternatives for AMD. The most number of AMD patients are out of any treatments and exudative AMD cases load ophthalmological clinics by a new way that has created many problems to manage from injections in limited resources. One of the most interesting future treatment modality is certainly human pluripotent stem cell derived regenerative RPE cell therapy for AMD and other RPE -originated retinal diseases, such as retinitis pigmentosa [3,4]. In addition these cells provide a potential resource as biological tool for drug discovery, toxicity screening and targeted drug therapy.

The polarized RPE cells constitute a polygonal monolayer between the neurosensory retina rod and cones and the fenestrated capillaries of the choroid. The RPE has multiple functions: absorption of light energy, transport of metabolites and nutrients between photoreceptors and choriocapillaris, expression of growth factors for photoreceptors, regulation of homeostasis of the ionic environment, phagocytosis of the shed tips of photoreceptor outer segments (POS), regulation of visual cycle, and creation of the blood-retinal barrier (BRB) [5]. The BRB is composed of two components: the outer part comprises the RPE and the inner part comprises the endothelial cells of the retinal vessels [6,7]. Functionally, the RPE is very similar to the blood-brain barrier (BBB). Several membrane-associated transport proteins, such as P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs), breast cancer resistance protein (BCRP), and organic anion transporting polypeptides, have been characterized at the blood-brain barrier (BBB).
BRB and BBB and play a major role in regulating tissue bioavailability of several pharmacologic agents [8,9]. The nine MRPs (MRP1-MRP9) represent the majority of the 12 MRP subfamily members belonging to the 48 human ATP-binding cassette transporters [10,11]. Cloning, functional characterization, and cellular localization studies have identified most MRP subfamily members as ATP-dependent efflux pumps with high substrate specificity for the transport of endogenous and xenobiotic anionic substances. Efflux pumps both regulate drug transport and affect tissue pathology [10,11]. Our recent findings revealed that a similar efflux protein profile is shared between the human RPE cell line, ARPE-19, and bovine primary RPE cells [6].

The ARPE-19 cell line, however, does not fully resemble the human RPE; therefore, more relevant human-derived RPE cells are needed as better in vitro models for drug testing and screening [12]. RPE-like cells have been successfully differentiated from human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC) [12,13]. hESC and hiPSC-derived RPE cells (hiPSC-RPE) express genes and proteins corresponding to the human RPE [12,14,15]. In addition, strongly pigmented hESC-RPE cells are able to phagocytose photoreceptor outer segments, secrete RPE trophic factors, and form a tight epithelium with high resistance [15-17]. The hESC-RPE cells are suggested to be an excellent in vitro model of human RPE [12], but it is important to evaluate whether the properties of these in vitro-differentiated RPE cells are truly similar to those of human RPE. Here, we studied the expression and functionality of ATP-dependent efflux transporters in undifferentiated hESC and in hESC-derived pigmented RPE (hESC-RPE) cells at different maturation stages to evaluate whether hESC-RPE are useful for drug screening and toxicology studies.

Materials and Methods

Cell lines

We used the hESC line Regea08/017 previously derived in our laboratory [18] and the commercially available RPE cell line ARPE-19 as a control (American Type Culture Collection (ATCC), Manassas, VA). The hESC line Regea08/017 (46, XX), derived in our laboratory and characterized as previously described [18], was cultured on a mitotically inactivated (γ-irradiated, 40 Gy) human foreskin fibroblast (hFF) cell line (36 500 cells/cm²; CRL-2429, ATCC) at 37 °C in 5% CO₂ in hESC culture medium comprising Knock-Out Dulbecco’s Modified Eagle Medium (KO-DMEM), 20% Knock-Out serum replacement (KO-SR), 2 mM GlutaMax, 0.1 mM 2-mercaptoethanol (all from Life Technologies, Carlsbad, CA, USA), 1% Minimum Essential Medium non-essential amino acids, 8 ng/ml human basic fibroblast growth factor (bFGF) (R&D Systems Inc., Minneapolis, MN, USA), and 50 U/ml Penicillin/ Streptomycin (both from Cambrex Bio Science, Walkersville, MD, USA). The culture medium was replenished six times a week. The undifferentiated hESC (Fig. 1A) were passaged mechanically at 6 to 7-day intervals.

RPE cell differentiation was induced in floating cell aggregates by reducing the KO-SR concentration to 15% and removing the bFGF, as previously described [15]. The culture medium for the floating aggregates was changed three times a week.

Pigmented cells were manually dissected from the aggregates, and further dissociated with 1x Trypsin-EDTA before seeding on collagen IV- (5 μg/cm²; Sigma-Aldrich, St. Louis, MO, USA) coated wells of 24-well plates (NUNC, Thermofisher Scientific, Tokyo, Japan) or on BD Biocoat culture plate inserts (BD Biosciences, San Jose, CA). On adherent culture, pigmented cells underwent morphologic changes starting from a non-pigmented fusiform morphology (Fig. 1B) followed by rounding to more pigmented epithelial cells (Fig. 1C), and finally developed a typical RPE-like cobblestone morphology (Fig. 1D). We selected the samples for RNA and protein extraction, immunofluorescence labeling, and functional testing based on their morphologic appearance (Fig. 1B-D), rather than the culturing time (Fig. 1E).

The commercially available ARPE-19 cell line was grown in Dulbecco’s Modified Eagle Medium (DMEM-F12) (1:1) supplemented with 10% fetal bovine serum (PAA Laboratories, Coelle, Germany), 100 U/ml Streptomycin/Penicillin (both from Cambrex Bio Science). Cells were cultured in a 5% CO₂ atmosphere at 37 °C and subcultured on 25-cm² cell culture flasks until they reached 80% confluency. For the experiments, ARPE-19 cells were enzymatically dissociated and seeded similarly as the hESC-RPE cells. The medium was changed three times a week. The culture periods are shown in Figure 1. A spontaneously transformed RPE cell line (D407), Human Embryonic Kidney 293 cells (HEK293), and hFF were used as control materials for the polymerase chain reaction (PCR) analyses. The RNA samples from D407 and HEK293, were the same reference RNA samples used previously [6].

RNA isolation

Total RNA was isolated with Nucleospin XS-kit (Macherey-Nagel, GmbH & Co., Düren, Germany) according to the manufacturer’s instructions. The RNA concentration and the quality were assessed using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Reverse transcription- (RT) PCR

RNA (40 ng) was reverse-transcribed using MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions in the presence of an RNase inhibitor. In addition, genomic control reactions excluding the restriction enzyme for each RNA sample were performed. Complementary DNA was used as a template in a following PCR reaction, which was carried out using 5 μl Taq DNA Polymerase (Fermentas, Thermo Fisher Scientific Inc., Leicestershire, UK) with 5 μm primers specific for particular genes (Biomers.net GmbH, Sollingen, Germany; Table 1). The PCR reactions were carried out in PCR MasterCycler ep gradient (Eppendorf AG, Hamburg, Germany) as follows: 95 °C 3 min, 95 °C 30 s, annealing 30 s, 72 °C 1 min, 72 °C 5 min, for 38 cycles. Annealing temperatures and primer sequences are presented in Table 1. PCR products were analyzed on 2% agarose gels with a 50-bp DNA ladder (MassRulerTM DNA Ladder Mix, Fermentas). The bands were visualized with the Quantity one 4.5.2. Basic program (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Quantitative RT-PCR

Relative gene expression comparisons were performed using quantitative RT-PCR (qRT-PCR). FAM-labeled TaqMan Gene Expression Assays (Applied Biosystems) were used for the following genes: MRP1 (Hs00219905_m1), MRP2 (Hs00166123_m1), MRP3 (Hs00538656_m1), MRP4 (Hs00195260_m1), MRP5 (Hs00981071_m1), MRPE (Hs00184566_m1), P-gp (Hs00184500_m1), and BCRP (Hs01053790_m1). RNA (200 ng) was reverse transcribed to cDNA as described above. The synthesized cDNA was diluted 1:5 in Rnase-free water and 3.0 μl was added to the final reaction (total 15 μl). No template controls were prepared for any of the genes. Reactions were carried out according to the manufacturer’s instructions. The cDNAs were multiplied using Applied Biosystems 7300 Real-time Sequence Detection System;
Figure 1. Morphology and gene expression of hESC on different maturation stages. Brightfield micrographs of cell cultures showing the representative morphology of A) undifferentiated hESC (Regea08/017), B) fusiform hESC-RPE, C) epithelioid hESC-RPE, D) cobblestone hESC-RPE. Scale bars, 100 μm, E) Gene expression of 1: D407, 3: ARPE-19, 5: undifferentiated hESC, 7: fusiform hESC-RPE, 9: epithelioid hESC-RPE, 11: cobblestone hESC-RPE, 13: hFF. – RT- negative controls (i.e., samples not treated with reverse transcriptase) are placed adjacent to each sample in the same order: 2: D407, 4: ARPE-19, 6: undifferentiated hESC, 8: fusiform hESC-RPE, 10: epithelioid hESC-RPE, 12: cobblestone hESC-RPE, 14: hFF. F) Culture periods of the studied samples in all analyses. Cells were selected based on their morphology rather than the culture period. doi:10.1371/journal.pone.0030089.g001

Table 1. Reverse-transcriptase–PCR primer sequences and used annealing temperatures.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5’–3’)</th>
<th>T&lt;sub&gt;ann&lt;/sub&gt;</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>GTT CGA CAG TCA GCC GCA TC</td>
<td>55</td>
</tr>
<tr>
<td>POU5F1</td>
<td>CCGTGAAGCTGAGAAGGAGAAGCTG</td>
<td>62</td>
</tr>
<tr>
<td>nanog</td>
<td>TGAATGTCTTCGAGAGAT</td>
<td>55</td>
</tr>
<tr>
<td>PAX6</td>
<td>AAC AGA CAC AGG CCT AAA CA</td>
<td>60</td>
</tr>
<tr>
<td>RAX</td>
<td>CGT AAA GCC AAG GAG CAC ATC</td>
<td>55</td>
</tr>
<tr>
<td>MITF</td>
<td>AAG AGG TCA GGT TGG CAT GT</td>
<td>52</td>
</tr>
<tr>
<td>RPE65</td>
<td>TCC CCA ATA CAA CTG CCA CT</td>
<td>52</td>
</tr>
<tr>
<td>tyrosinase</td>
<td>TGC CAA CGA TCC TAT TCT CG</td>
<td>52</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0030089.t001
2 min at 50°C, 10 min at 95°C, and 40 cycles repeating denaturation 15 s at 95°C, and annealing for 1 min at 60°C. qPCR analyses were performed from three individual biologic experiments, each reaction prepared as technical triplicates. Threshold cycle (Ct) values were determined using 7300 System SDS Software (Applied Biosystems) and data were further analyzed with Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA). Relative gene expression was calculated using the 2^[-ΔΔCt] method [19]. An internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs99999905_m1), was used to normalize the data. The expression levels of either D407 or HEK293 were used as a reference depending on the gene studied. The technical replicate reactions were considered reliable if the standard deviation of the triplicate Ct values was less than 0.5.

Western blotting

The cell samples washed with phosphate buffered saline (PBS, Lonza Group Ltd., Basel, Switzerland) and cells were lysed in M-PER lysis buffer (Thermo Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. Protein concentrations of samples were analyzed using the Bradford method [20]. The amount of ARPE-19 and hESC samples was 10 µg. The samples were run in 7% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels and then wet-blotted to nitrocellulose membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Blocking was done with 3% fat-free dry milk in 0.3% Tween 20/PBS at room temperature (RT) for 1 h. Thereafter, membranes were incubated in primary antibody dilutions anti-MRP1 (1:2000, overnight at 4°C), anti-MRP4 (1:5000, 1 h at RT), or anti-MRP5 (1:2000, 1 h at RT) rat monoclonal MRP antibodies (all from Abcam, Cambridge, UK) or alpha-tubulin (1:4000, 30 min at RT, Sigma-Aldrich) that was used as a loading control. All primary antibodies were diluted in 0.5% bovine serum albumin (BSA) in 0.3% Tween 20/PBS. After 3×5 minutes washes with 0.3% Tween 20/PBS the membranes were incubated in horseradish peroxidase-conjugated anti-mouse IgG or antibody (GE Healthcare), diluted in 3% fat-free dry milk in 0.3% Tween 20/PBS (1:10 000 for MRP1, 1:10 000 for MRP4, and 1:2000 for MRP5) for 1 h at RT, and 30 min at RT for alpha-tubulin (1:10 000). Protein-antibody-complexes both in MRP and alpha-tubulin labeling were detected using an enhanced chemiluminescence method (Millipore, Billerica, MA, USA).

Immunostaining

The cells were labeled as described previously [15]. Briefly, the cells were washed 3×5 minutes with PBS, fixed 10 min with 4% paraformaldehyde (pH 7.4; Sigma-Aldrich), and washed with PBS. Cells were permeabilized in 0.1% Triton X-100/PBS (Sigma-Aldrich), for 10 min and thereafter washed with PBS. Non-specific binding sites were blocked with 3% BSA (Sigma-Aldrich) in PBS at RT for 1 h. Primary antibody incubations were done in 0.5% BSA-PBS, with rat monoclonal anti-MRP-1 (1:100), anti-MRP-4 (1:100), and anti-MRP-5 (1:500), with rabbit anti-microphthalmia-associated transcription factor (MITF, 1:350), mouse anti-cellular retinaldehyde-binding protein (CRALBP, 1:1000), or mouse anti-Na+/K+ ATPase (1:50; all antibodies were from Abcam) for 1 h. Thereafter the cells were washed 3x with PBS. The secondary antibody incubations were done in 0.5% BSA-PBS with donkey anti-mouse IgG and goat anti-rabbit IgG (both Alexa fluor 488), goat anti-mouse IgG and goat anti-rabbit IgG (both Alexa fluor 568; all from Molecular Probes, Life Technologies, Paisley, UK) in a 1:1500 for 1 h, following repeated PBS washings. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole included in the mounting media (DAPI, Vector Laboratories Inc., Burlingame, CA). The entire labeling procedure was performed at RT. Confocal images were obtained with an LSM 700 confocal microscope (Carl Zeiss, Jena, Germany) using a 63× oil immersion objective and bright field images were obtained with an Olympus BX60 microscope (Olympus, Tokyo, Japan) with a 60× oil immersion objective with N.A. 1. Overlays and image processing of confocal images were done in ZEN-software (Carl Zeiss).

Efflux activity test with calcein-AM

Calcein-AM is a substrate both for P-gp and MRP1 proteins [21,22], thus MRP1 and P-gp efflux protein activity in the cells was assessed using the calcein-acetoxyethyl (AM) assay. Calcein-AM is cell permeable until it is metabolized by intracellular esterases to AM and calcein, a cell-impermeant fluorescent compound [9]. Efflux pump inhibitors either totally inhibit or slow the pumping rate, thus allowing the esterases more time to metabolize calcein-AM to calcein. The efficacy of inhibition is observed as an increase in intracellular fluorescence. The experiment was performed as previously described [6]. Briefly, the cells were pre-equilibrated with 25 mM HEPES-buffered Hank’s balanced salt solution (pH7.4) with or without one of the following inhibitors, 15 µM cyclosporine A (Calbiochem, La Jolla, CA, USA), 200 µM progesterone (Sigma-Aldrich), 500 µM verapamil (ICN Biomedicals, Irvine, CA), or 100 µM MK571 (Cayman Chemicals, Ann Arbor, MI, USA) for 20 min at 37°C. Thereafter, calcein-AM (Calbiochem) was added to a final concentration of 2 µM and incubation continued for an additional 20 min at 37°C. Test solutions were changed to ice-cold buffer and intracellular fluorescence was measured using a Victor 1420 Multilabel Counter (Wallack, Finland) with excitation wavelength of 480 nm and an emission wavelength of 535 nm.

Cell viability test

Cell viability was assessed from ARPE19 and hESC RPE cells simultaneously as the efflux pump activity test with the Live/Dead Viability/Cytotoxicity kit for Mammalian cells (Invitrogen). Briefly, the cells were rinsed with DPBS and incubated at RT for 40 min with a mixture of 0.25 µM Calcein AM (green fluorescence) and 0.5 µM Ethidium homodimer-1 (red fluorescence, EthD-1). A fluorescence microscope (Olympus IX) was used to image the viable cells (green fluorescence) and dead cells (red fluorescence) with 10x long working distance objective.

Statistical analyses

Statistical analysis of the qRT-PCR data was performed using analysis of variance (ANOVA) with Bonferroni’s correction for multiple comparisons, and the calcein-AM assay with a one-sample t-test, both with PASW Statistics, version 18. P-values of less than 0.05 were considered statistically significant and P values of less than 0.01 were considered highly significant.

Ethical issues

We have approval from the National Authority for Medicolegal Affairs Finland research with human embryos (Duro 1426/32/300/05) and a supportive statement was obtained from the local ethics committee of the Pirkkanmaa hospital district Finland to derive and expand hESC lines from surplus embryos not used in the treatment of infertility by the donating couples, and to use these lines for research purposes (R05116). No new cell lines were derived in this study.
Results

hESC-derived RPE cells express eye-specific genes

RT-PCR analysis was used to assess the cell maturation status. Pluripotency genes POU5F1 and nanog typically expressed by undifferentiated hESC were expressed only by undifferentiated hESC, as expected (Fig. 1E). PAX6, one of the first markers of the neuroectoderm and eye, and eye-specific genes RAX, MITF and RPE65 were expressed by both ARPE-19 and hESC-RPE cells at all maturation stages (i.e., fusiform, epithelioid, and cobblestone hESC-RPE). Undifferentiated hESC also expressed the eye-specific genes at very low levels. Tyrosinase, which is important for melanin synthesis, was expressed by hESC-RPE cells but not in ARPE-19 or D407 cells. None of the analyzed eye-specific genes, except for MITF, were detected in D407 cells. In addition, none of the studied genes, except for GAPDH, and faint expression of PAX6, were detected in hFF, which was analyzed as a possible source of background expression for the undifferentiated hESC samples.

Efflux protein gene expression during RPE cell differentiation

The relative expression of several ATP-dependent efflux transporters (MRP1, -2, -3, -4, -5, -6, p-gp, and BCRP) was examined with qRT-PCR. The mRNA expression levels in the spontaneously transformed cell line D407 were used as a reference sample for all other studied genes except the MRP6 gene, which has not been previously detected in D407 cells, and thus HEK293 cells were used as reference for MRP6 gene expression studies.

During all maturation stages, hESC-RPE cells expressed higher levels of MRP1 gene than D407 cells (Fig. 2). MRP2 gene expression levels were significantly lower in all studied cells than in D407 (see Supplementary Table S1). Fusiform and epithelioid hESC-RPE cells expressed equal amounts of the MRP5 as D407, while undifferentiated hESC, cobblestone hESC-RPE cells, and hFF expressed lower levels of the MRP5 gene than D407 cells (Fig. 2). Fusiform, epithelioid, and cobblestone hESC-RPE expressed higher levels of the MRP4 gene than D407 cells. MRP5 was expressed in hFF cells and ARPE-19 lower levels, and undifferentiated hESC expressed similar amounts of MRP5 gene as D407 cells. Conversely mature hESC-RPE cells expressed higher levels of the MRP5 gene than D407 cells. The MRP6 gene expression level was very low in D407 and ARPE-19 cells; therefore, HEK293 cells were used as a reference sample (Supplementary Table S1). Fusiform hESC-RPE expressed the MRP6 gene at the same level as HEK293. The MRP6 expression levels were much higher in epithelioid and cobblestone hESC-RPE cells than in HEK293 cells, and much lower in undifferentiated hESC, ARPE-19, and D407 cells than in HEK293 cells (Fig. 2). Fusiform and epithelioid hESC-RPE expressed significantly higher levels of the p-gp gene than D407 cells, whereas the p-gp gene was undetectable in ARPE-19 cells. In addition, the undifferentiated hESC, cobblestone hESC-RPE, and hFF expressed lower levels of the p-gp gene than D407 cells (Fig. 2) [Supplementary Table S1]. BCRP gene expression levels in all studied samples were significantly lower than those in D407 cells.

Efflux protein expression

To ensure that the gene transcripts were translated to proteins, we used Western blot to examine whether the ARPE-19 and fusiform, epithelioid and cobblestone hESC-RPE expressed MRP1, MRP4, and MRP5 protein. Both ARPE-19 and the hESC-RPE cells produced MRP1 (Fig. 3A), -4 (Fig. 3B), and -5 (Fig. 3C) proteins. The MRP1 protein expression slightly increased and MRP5 protein expression was extensively increased during the maturation process (Fig. 3A), whereas MRP4 expression remained stable during the maturation.

Efflux protein localization during RPE cell differentiation

The cellular localization of MRP1, -3, and -5 proteins was assessed in ARPE-19 cells and in fusiform, epithelioid, and cobblestone hESC-RPE (Fig. 4A–P). The overall labeling intensity with MRP antibodies was extremely low. None of the studied MRPs were detected in ARPE-19 cells (Fig. 4A, E, I). The fusiform hESC-RPE had low but still detectable amounts of MRP1 and MRP4, and a very low amount of MRP5 protein staining (Fig. 4B, F, J). The early cobblestone hESC-RPE had detectable amounts of subcellularly localized MRP1, -4, and -5 proteins (Fig. 4C, G, K). The cobblestone hESC-RPE cells had MRP1, -4, and -5 protein staining that coincided with apical Na+/K+ ATPase staining (Fig. 4D, H, L).

Functionality of MRP1 efflux pump during RPE cell differentiation

Efflux protein activity in the cells was assessed with Calcein-AM assay from ARPE-19 cells and in fusiform, epithelioid, and cobblestone hESC-RPE. ARPE-19 cells cultured for 7 days showed efflux activity, but the activity was lost when the cells were cultured for longer periods of time (Fig. 5A). On the other hand, fusiform hESC-RPE cells had higher MRP1 activity than cobblestone hESC-RPE. The undifferentiated hESC and hFF had no MRP1 efflux pump activity.

Cell viability

Microscopic observations revealed that after 7 days of culture, at the time of functionality tests, both ARPE19 (Fig. 5B and C) and fusiform hESC-RPE (Fig. 5D and E) cells were viable, and the number of dead cells was low.

Discussion

Currently there is no curative treatment for exudative AMD, therefore human pluripotent stem cell derived RPE cells are highly desirable source of cells for cell therapy in AMD [3,4]. Furthermore these cells offer a biological tool for drug discovery, toxicity screening and targeted drug therapy. For that purpose we have assessed the expression status and function of ATP-dependent efflux transporters in stem cell derived RPE cells.

Before examining the expression status of ATP-dependent efflux transporters, we assessed the maturation status of the samples using RT-PCR, which revealed that the spontaneously transformed retinal cell line, D407, previously used in efflux transporter studies [6,23], expressed no eye-specific genes other than MITF. Eye-specific gene expression was detected in ARPE-19, which is a cell line that is widely used for RPE drug transport studies [9,24], confirming that ARPE-19 cells are a good RPE standard. Human ESC-RPE cells at all maturation stages (fusiform, epithelioid, and cobblestone) expressed eye-specific genes, as expected. Furthermore, the expression of PAX6, RAX, RPE65, and tyrosinase increased from fusiform to epithelioid and from epithelioid to cobblestone hESC-RPE, confirming that classification according to morphology is valid.

MRP1 protein is predominantly expressed in human cells that form blood-tissue barriers [10,25]. Several xenobiotics, dietary, and synthetic flavonoids (e.g., fruit pigments) modulate the MRP1 pump [25]. MRP1 expression is detected in human RPE [26], primary RPE cells [27], and RPE cell lines [6,27]. The present
Figure 2. Expression of ATP-dependent efflux transporter genes. Relative expression of MRP1, MRP3, MRP4, MRP5, P-gp, and MRP6 genes. D407 used as reference sample for all genes except MRP-6, for which HEK-293 was used instead. Values that are significantly different from those of the reference sample are marked with an asterisk (*). For better visualization, fold-change is represented on a logarithmic scale. Standard deviations of fold-change from three separate experiments are presented as error bars. doi:10.1371/journal.pone.0030089.g002
MRP1 study showed for the first time that hESC-derived RPE cells also
expressed MRP1 at both the mRNA and protein levels. MRP1 mRNA expression clearly peaked in the early stages of differentiation in fusiform-shaped cells, and declined thereafter in fully mature cells with a cobblestone morphology. These results are consistent with those of previous studies [6,26,27], although this is the first study to confirm that MRP1 expression fluctuates depending on the maturation status. The fluctuation in expression was also observed in an efflux pump functional test with calcein-AM. In the functional test, epithelioid cells had higher activity than cobblestone cells, and undifferentiated hESC had no activity at all. Graff and coworkers [28] as well as Rao and coworkers [29] previously reported that MRP1 localizes on the apical side of the BBB. In the present study, the localization of MRP1 changed during hESC-RPE cell maturation; MRP1 expression was very low in undifferentiated hESC, but the expression was increased from fusiform hESC-RPE to epithelioid hESC-RPE and diminished again in cobblestone hESC-RPE.

MRP4 is an ATP-dependent organic anion transporter [10] that has a role, for example, in prostaglandin transport [31] in the eye. It also interacts with many drugs, such as 5′fluorouracil, zidovudine, ganciclovir, and vincristine that are used to treat retinal conditions [32–34]. In earlier studies MRP4 expression was detected in human retinal samples [26] and in ARPE-19 and D407 cell lines [6]. In the present study, MRP4 expression was low in undifferentiated hESC, but was induced when cells matured to fusiform hESC-RPE. MRP4 protein localizes either on the basolateral or apical membrane of the cells, depending on the cell type [35], nevertheless, the localization of MRP4 has not been previously studied in RPE cells. The microscopic examination revealed that the fusiform cells were weakly MRP4 positive, and the positivity was scattered within the cell. In highly pigmented cobblestone hESC-RPE cells, MRP4 protein was localized on the apical side near the Na+/K+ ATPase-expressing cells. The overall labeling intensity in immunofluorescence labeling was very low, and MRP4 labeling in ARPE-19 cells probably remained below the detection level.

MRP5 has a broad substrate and inhibitor specificity [10]. In eye diseases, MRP5 has an important role as it interacts with drugs, such as Etoposide, used for treatment of retinoblastoma [36]. MRP5 expression has also been linked to AMD development, and its expression decreases in RPE cells cultured on old Bruch’s membrane [37]. Both the MRP5 gene and protein are expressed in D407 and ARPE-19 cells [6,37]. Here, MRP5 expression increased both in mRNA and protein level during hESC-RPE cell maturation, which is consistent with a previous study [37]. MRP5 is apically localized in the BBB [38], but in the BRB the site of expression had not been previously determined. In immunofluorescence labeling, MRP5 was not detected in ARPE-19 cells and very few subcellular signals were observed in fusiform cells. In highly pigmented cobblestone hESC-RPE, the MRP5 protein localized to the apical side of the cells.

MRP6 gene expression has not been previously detected neither in native RPE [39] nor in ARPE-19 and D407 cell lines [6], although MRP6 gene ablation in mice increases the calcification of retina and Bruch’s membrane [40]. Interestingly, the hESC-RPE cells expressed MRP6 at similar level to HEK293, and the expression increased during maturation of the hESC-RPE cells, whereas very low expression was detected in ARPE-19 and D407 cells. The discrepancy in the MRP6 expression between hESC-RPE and cadaveric RPE might be due to the fact that hESC-RPE are in a different maturation state than native RPE or that MRP6 is expressed in very low levels in native RPE and thus remains undetected.

P-gp is expressed in human [41] and porcine [42] RPE, and at low levels in the h1RPE cell line derived from immortalized...
primary RPE cells [23] and in ARPE-19 cells [6,23]. In the present study, P-gp gene expression levels peaked in immature hESC-RPE. Thus, the expression pattern of p-gp was similar to that of MRP1 and MRP4. BCRP is expressed in human RPE [26] and in D407, but not in ARPE-19 cells [6]. BCRP expression was very low in ARPE-19 and hESC-RPE.

In conclusion, the findings of the present study clearly demonstrated that expression of genes for the ATP-dependent efflux transporters MRP1, -3, -4, -5, and P-gp fluctuates in undifferentiated hESC and hESC-RPE at different maturation stages. In addition, based on the gene expression profile, hESC-RPE cells more closely resemble ARPE-19 cells than D407 cells, suggesting that hESC-RPE cells have important RPE cell-like properties, which make these cells an excellent in vitro cell model for drug transportation studies for AMD drug testing and development.
Figure 5. Functional testing of ATP-dependent efflux transporter proteins and viability of cultured cells. A) Calcein retention in ARPE-19, undifferentiated hESC, fusiform, and cobblestone hESC-RPE, and hFF cells in the presence or absence (= control) of efflux protein inhibitors. Retention is expressed as a percentage of fluorescence relative to control (control = 100%). The studies were repeated at least three times for ARPE-19 and fusiform hESC-RPE, and once each for undifferentiated hESC, cobblestone hESC-RPE, and hFF. Data are expressed as mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001. B-E) Representative images of viable (green fluorescence) and dead (red fluorescence) ARPE19 (B,C) and fusiform hESC RPE cells (D,E). The scale bar 100 µM. doi:10.1371/journal.pone.0030089.g005
Supporting Information

Table S1  Gene expression data with standard deviations (SD) and calculations of statistical significance (p).

Acknowledgments

We thank Outi Melin, Hanna Koskenaho, Elina Konsen, Alexandra Mikhailova and Anne Kontkanen for their technical assistance, and Heini Huhtala and Heidi Hounioti for assistance with the statistical analysis.

References


Author Contributions

Conceived and designed the experiments: KJ-U HV KK KS. Performed the experiments: KJ-U HV TR. Analyzed the data: KJ-U HV.

Supported by: Heini Huhtala and Heidi Hounioti for assistance with the statistical analysis. And calculations of statistical significance (p).
Generation of hESC-derived retinal pigment epithelium on biopolymer coated polyimide membranes

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A R T I C L E   I N F O

Article history:
Received 2 July 2012
Accepted 15 July 2012
Available online 11 August 2012

Keywords:
Ophthalmology; Retina; Transplantation; Stem cell; In vitro test;
Membrane

A B S T R A C T

The in vitro generation of a functional retinal pigment epithelium (RPE) for therapeutic applications requires a limitless source of RPE cells and a supporting scaffold, which improves cell survival and promotes the acquisition of the RPE phenotype. We successfully differentiated human embryonic stem cells (hESCs) toward RPE on a transplantable, biopolymer coated polyimide (PI) membrane. We studied various membrane coatings of which several lead to the generation of a tight and highly polarized epithelium having typical characteristics and functions of human RPE. The cells established a distinctive hexagonal, cobblestone morphology with strong pigmentation, expressed RPE specific genes and proteins, and phagocytosed photoreceptor outer segments (POS) after co-culture with rat retinal explants. The barrier function of hESC-derived RPE (hESC-RPE) monolayers was confirmed by trans-epithelial electrical resistance and permeability measurements. In conclusion, we show that the PI biomembrane is a suitable scaffold for hESC-RPE tissue engineering.

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1. Introduction

The retinal pigment epithelium (RPE) is a monolayer of postmitotic, pigmented cells forming the outer blood-retinal barrier (BRB). The apical side of the RPE faces the photoreceptor outer segments (POS), and its basolateral surface rests on the Bruch’s membrane, a pentalaminar, 1–4 μm thick structure overlaying the fenestrated chorioidal capillaries of the eye. The RPE exerts several essential functions of maintenance and homeostasis in the neural retina, including phagocytosis of POS disks and antioxidant defense; hence, it is not surprising that the RPE plays a central role in the pathogenesis of several degenerative retinal disorders that lead to irreversible vision loss [1]. Degeneration of RPE cells and disruption of the RPE-photoreceptor interface are the cause of loss of macular function in age-related macular degeneration (AMD), a common blinding disorder and major public health problem in the industrialized world. The lack of effective RPE regeneration results in a marked degradation in quality of life; for this reason tissue engineering approaches have been attempted in order to reconstruct a functional RPE monolayer in vitro. Moreover, the potential medical applications of an in vitro generated RPE tissue unit go beyond cell therapy; these constructs may also provide a unique platform from which to study disease, identify new drugs, and screen for their toxicity and permeation across the outer BRB. Human embryonic stem cells (hESCs) are an attractive source of replacement cells for RPE tissue engineering because they are readily available in limitless supply. Schraermeyer and colleagues [2] were the first to demonstrate that subretinal injection of mouse ESCs in the Royal College of Surgeons (RCS) rat delay photoreceptor degeneration, thus suggesting that RPE-like properties may have been acquired by the transplanted ESCs. Further studies [3–5] indicated that the transplantation of hESC-derived...
RPE (hESC-RPE) provides long-term rescue without loss of RPE phenotype or signs of tumor formation. In addition, the first clinical trial has assessed the safety and tolerability of hESC-RPE cell therapy in humans [6]. In all the above mentioned studies, the hESC-RPE cells were injected subretinally as a suspension. Several investigators [7–10], however, have shown that freshly harvested or cultured RPE cell suspensions fail to survive and function appropriately on aged or damaged Bruch’s membrane. Moreover, when cells are injected into the subretinal space, the cellular arrangement cannot anymore be controlled. For these reasons, the transplantation of a polarized RPE monolayer as an intact epithelial arrangement cannot anymore be controlled. For these reasons, the paper, we report on a thin (24 μm, 2.2 × 107 pores/cm2) PI biomembrane, that, when coated with Matrigel® is the only material that has been studied together with Matrigel® on aged or damaged Bruch’s membrane. Moreover, the transplanted cells. Scaffolds fabricated appropriately on aged or damaged Bruch’s membrane from a variety of natural [11–13] and synthetic [14–17] materials have been widely investigated. To our knowledge, however, parylene-C is the only material that has been studied together with parylene-C with Matrigel®.

2. Materials and methods

2.1. Human ESC culture and RPE differentiation

The pluripotent hESC lines, Regea06/040 (46,XX) and Regea08/017 (46,XX), were derived from blastocyst stage embryos as described previously [22]. Both hESC lines were maintained on inactivated human foreskin fibroblast (hFF) (CRL-2429, ATCC, Manassas, VA, USA) in basic hESC culture medium, consisting in Knock-Out Dulbecco’s Modified Eagle Medium (KO-DMEM) with 20% KnockOut™ Serum Replacement (KO-SR), 2 mM Glutamax, 0.1 mM 2-mercaptoethanol (all from Invi- trogen, Carlsbad, CA, USA), 1% Minimum Essential Medium non-essential amino acids, 50 μM Penicillin/Streptomycin (both from Cambrex Bio Science, Walkersville, MD, USA), and 8 ng/ml human basic fibroblast growth factor (bFGF) (PeproTech, NJ, USA). Fresh medium was changed three times a week. Cells were cultured at 37 °C, 5% CO2.

The differentiation of RPE from pluripotent hESCs was performed as floating aggregates using the RPE-basic differentiation method previously described [23]. This method is based on the normal neural model where ESCs choose neural fate in absence of external factors [24] and a proportion of these cells will choose RPE fate [23,25]. Pigmented areas were manually dissected from the cell aggregates, and the cell clumps were further dissociated with 1× Trypsin-EDTA (Lonza Walkersville, MD, USA). Cells were seeded through a cell strainer (ø 40 μm, Ref. 352340, BD Biosciences) on commercially available track etched PI biomembranes (CELLCULTURE™) (thickness 24 μm, pore diameter 1 μm, 2.27 × 107 pores/cm2, Ref 300MS2/721M103/25, iproph s.a.; Seneffe, Belgium). The membrane was made from Kapton-like type H PI films. Proprietary hydrophilic treatment was done by the manufacturer, the contact angle value after the treatment is 47°. For technical properties of the membrane, see Supplementary Table 1. PI biomembranes were either uncoated or coated with different adhesive molecules. Coating procedures and concentrations are presented in Table 1. For cell culture PI membranes were stretched and held in position by Sclafides CellCrown™ inserts (Sclafides Oy, Tampere, Finland). CellCrown™ 48, 24, and 12 were used depending on the analyses performed. Cell growth and pigmentation were followed weekly by Nikon Eclipse TE2000-S phase contrast microscope (Nikon Instruments Europe B.V, Amstelveen, The Netherlands).

2.2. Reverse transcription polymerase chain reaction (RT-PCR)

PCR analysis was conducted on pigmented cells differentiated from Regea06/040 cell line, cultured on coated PI biomembrane for 98 days. The total RNA was isolated using NucleoSpin XS-kit (Macherey–Nagel, GmbH & Co, Duren, Germany) according to the manufacturer’s instructions. RNA quality and concentration were assessed with NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The complementary DNA was synthesized from 40 ng RNA using MultiScribe Reverse Transcriptase (High capacity CDNA, Applied Biosystems, Foster city, CA, USA) in the presence of an RNase inhibitor. The cDNA was used as a template in the PCR reaction, which was carried out using 5 U Taq DNA Polymerase (Fermentas, Thermo Fisher Scientific Inc., Leicesterhers, UK) with 5 μM primers specific for representative genes (Biomers.net GmbH, Söllingen, Germany). Primer sequences and annealing temperatures are shown in Table 2. PCR reactions were carried out on a PCR MasterCycler ep gradient (Eppendorf AG, Hamburg, Germany) as follows: 95 °C for 3 min, 95 °C for 30 s, annealing for 30 s, 72 °C for 1 min, +72 °C for 5 min, 38 cycles. The PCR products were analyzed on 2.0% agarose gels with a 50 bp DNA ladder (MassRulerTM DNA Ladder Mix, Fermentas). The bands were visualized with Quantity One 4.5.2. Basic program (Bio-Rad Laboratories, Inc, Hercules, CA, USA).

2.3. Immunocytochemistry

The protein expression of hESC-RPE monolayers was studied with immunocytochemistry. At first, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature (RT), followed by permeabilization with 0.1% Triton X-100 in Dulbecco’s Phosphate Buffered Saline (DPBS), for 10 min, and blocking with 3% bovine serum albumin for 1 h. The cells were incubated for 1 h at RT with primary antibodies against following proteins: microphthalmia associated transcription factor (MITF) 1:350 (ab59232), cellular retinaldehyde-binding protein (CRALBP) 1:1000 (ab15051), sodium/potassium-dependent ATPase (NaKATPase) 1:50 (ab7671), Bafilomycin A1 1:1000 (ab14928) (all from Abcam, Cambridge, UK), Zonula occludens-1 (ZO-1) 1:250 (33-9100, Gibco-Invitrogen), Ki-67 1:300 (AB9260, Abnova). The incubation with primary antibodies was followed by 90 min incubation at RT with secondary antibodies: goat anti-rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor 568, donkey anti-mouse Alexa Fluor 488, and goat anti-mouse Alexa Fluor 488 (all from Molecular Probes, Life Technologies, Paisley, UK) diluted 1:800 in 0.5% BSA in DPBS. Secondary controls

---

Table 1

<table>
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<th>Adhesive molecules and coating procedures</th>
<th>Source</th>
<th>Manufacturer</th>
<th>Ref</th>
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<th>Coating procedure</th>
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<td>Sigma–Aldrich</td>
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<td>1 h, +37 °C</td>
<td>Y</td>
</tr>
<tr>
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<td>Glycosan Biosystems</td>
<td>GS313</td>
<td>1% (w/v) solution</td>
<td>20 min at RT</td>
<td>N</td>
</tr>
</tbody>
</table>

Abbreviations: room temperature – RT, overnight – o/n, extracellular matrix – ECM, hour – h, yes – Y, no – N.
were prepared without primary antibody to detect possible unspecific binding of secondary antibodies. The nuclei were stained with 4’,6-diamidino-2-phenylidole (DAPI) included in the mounting medium (Vector Laboratories Inc., Burlingame, CA). Images were captured with Zeiss LSM 700 confocal microscope (Carl Zeiss Ltd., Jena, Germany).

The protein absorption on PI membranes after coating with collagen types I and IV, human laminin, and MaxGel™ was studied with immunofluorescence. The coatings were prepared as described on Table 1. In addition, one membrane was treated only with DPBS. The same immunostaining protocol was used as described above. Primary antibodies were: anti-human laminin 1:200 (ab11575, Abcam), anti-collagen I 1:250 (ab6308), anti-collagen IV 1:100 (Ms-747-S1, Thermo Scientific), and used secondary antibodies were donkey anti-mouse Alexa Fluor 488 and donkey anti-rabbit Alexa Fluor 568 (both from Molecular Probes, Life Technologies) diluted 1:800 in 0.5% BSA in DPBS. The images were captured on a Zeiss LSM 700 confocal microscope (Carl Zeiss Ltd.). The autofluorescence of PI was used to visualize the membrane.

2.4. In vitro diffusion apparatus

Human ESC-RPE monolayers grown on PI biomembranes were carefully removed from Scaffdex CellCrown™ inserts and fitted into vertical Ussing/diffusion chambers (Harvard Apparatus, MA, USA) equipped with silicone adapters with a circular aperture of 6 mm. Both sides of the chambers were filled with 5 ml pre-warmed balanced salt solution (BSS Plus) (Alcon Laboratories, TX, USA) containing 10 mM Hepes (pH 7.4). The chambers were maintained at 37 °C by using a heating block and a circulating water bath (Haake DC10-W26/B, Thermo Scientific, MA, USA).

2.5. Permeability study

BSS Plus containing glutathione, glucose, bicarbonate, and electrolytes, supplemented with 10 mM Hepes (pH 7.4), was used as the diffusion medium. At the beginning of the experiment, 500 µl diffusion medium were removed from the apical side of the monolayer and replaced with an equal volume of 6-carboxyfluorescein (6-CF) stock solution. The initial concentration of 6-CF in the donor chamber was 0.0377 mg/ml (100 µM). Volumes of 100 µl were sampled from the receptor chamber at 15, 30, 45, 60, 75, 90, 105, 120, 150, and 180 min and replaced with fresh diffusion medium. Samples from the donor chamber were also collected at the end of the experiment. All samples were protected from light by covering the whole diffusion apparatus with foil. The samples were analyzed immediately with a 96-well fluorescence plate reader (Varioskan Flash, Thermo Scientific, MA, USA) at 485 nm excitation and 530 nm emission wavelength settings. The standard curves of fluorescence versus concentration were obtained by serial dilution of 6-CF in diffusion medium. The concentration of the samples was determined by linear regression analysis within the linear portion of the standard curve. The diffusion of 6-CF across the hESC-RPE monolayers was characterized by calculating the apparent permeability coefficient (Papp, cm/s) as Papp = dC/dt/(DCa×A), where dC/dt is the slope of the linear portion of the permeability curve (nmol/min), DC is the initial concentration in the donor chamber (nmol/ml), and A is the exposed surface area of the RPE monolayer (0.28 cm²).

2.6. Bioelectric measurements

The Ussing/diffusion chamber was equipped with electrode caps to measure the transepithelial electrical potential difference (TEP) and transepithelial electrical resistance (TER) with glass barrel Ag/AgCl electrodes (World Precision Instruments, FL, USA) and a voltage—current clamp (VCC MC 6, Physiologic Instruments, CA, USA). The measurements were conducted under open-circuit conditions. Current pulses of ±5 μA were applied for 2 s across the monolayer, and the change in TEP was monitored to calculate TER. The asymmetry voltage between the voltage measuring electrodes and the value of the resistance due to the diffusion medium were compensated for before each experiment. The TER value of the PI biomembrane without cells was subtracted from TER values of hESC-RPE monolayers.

2.7. Phagocytosis of POS from rat retinal explants

We studied the phagocytic properties of hESC-RPE monolayers using rat retinal explants. For the phagocytosis assay, hESC-RPE cells were cultured on PI biomembrane (CellCrown™, surface area 0.4 cm²) until they acquired RPE morphology, pigmentation, and integrity of the cell layer. The use and handling of the animals was conducted according to the Finland Animal Welfare Act of 1986, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the guidelines of the Animal Experimentation Committee. Non-dystrophic RCS rats at ages 3, 6, and 10 weeks were euthanized using carbon dioxide inhalation following cervical dislocation. The eyes were enucleated and immersed in ice-cold DPBS, and the retinas were detached from hemisected eyes under dim red light. Isolated rat retinas were placed with the photoreceptors facing the hESC-RPE monolayers in B27/N2 medium, consisting of Neurobasal A, 1% N2, 2% B27, 2 mM Glutamax (all from Gibco, Life Technologies), and 100 U/ml Penicillin/Streptomycin (Cambrex) [26]. A small piece of lens paper was used to transfer the retina on top of the hESC-RPE monolayers, and it was left on top of the retinas to hold them flat and steady over the hESC-RPE monolayers. Human ESC-RPE monolayers were cultured together with rat retinal explants for two days at +37 °C, 5% CO2. Half of the medium was changed daily during the co-culture. On the second day, retinas were gently removed and hESC-RPE cells were analyzed by immunocytochemistry. The internalization of rat rhodopsin by hESC-RPE cells was visualized using rat anti-Opsin antibody (O48869; Sigma–Aldrich), while filamentous actin was detected with 0.02 µg/ml phalloidin-TRITC (P1951; Sigma–Aldrich). Donkey anti-rabbit Alexa 488 (Molecular Probes, Life Technologies) diluted 1:800 was used as secondary antibody. Otherwise staining was performed as described above. The experiment was repeated twice, and until paraformaldehyde fixing, it was performed in dim red light conditions. Images of the retinas were captured with Zeiss LSM 700 confocal microscope using sectional scanning.

3. Results

3.1. Human ESC-RPE growth on PI membranes

Cell attachment and growth was poor on uncoated PI membrane (Fig. 1A and B), for this reason we coated the membranes with different adhesive molecules (Table 1). Synthetic laminin peptide, heparin sulphate (HS), and hyaluronic acid (HA) did not improve cell attachment, growth, or pigmentation (Fig. 1C and D). Laminins both from mouse and human placenta, collagens type I and type IV, CELLStart™, MaxGel™, and HyStem™ supported cell growth and the cells acquired RPE morphology and pigmentation (Fig. 1E and F). Although cell morphology and pigmentation were good on HyStem™ coated PI, the cell sheet easily detached from PI upon handling. Mouse laminin was also studied in mixture with both collagens type I and IV, and HyStem™. No advantages, however, were gained using these mixtures compared to individual coatings. Experiments with the most promising coatings were repeated 4–6 times. Human ESC-RPE cells reproducibly gained pigmented and cilia and morphology on the best coatings.

Immunofluorescence analyses of protein absorption showed that the adhesive molecules formed an even layer on PI. MaxGel™ did not stain against collagen types I and IV but was strongly

| Table 2
<table>
<thead>
<tr>
<th>Primer sequences and annealing temperatures</th>
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glyceraldehyde 3-phosphate dehydrogenase (GAPDH), octamer-binding transcription factor (OCT4), paired box gene 6 (PAX6), retina and anterior neural fold homeobox (RAX), microphthalmal-association transcription factor (MITF), retinal pigment epithelium-specific 65 kDa protein (RPE65), bestrophin-1 (BEST), pre-melanosome protein 17 (PMEL17), pigment epithelium-derived factor protein (PEDF), tyrosinase (TYR).
stained when treated with human laminin antibody. Human laminin coated PI treated with anti-laminin is presented in Fig. 2 to illustrate coating of PI.

3.2. Gene and protein expression of hESC-RPE cells

Human ESC-RPE cells cultured on PI membranes coated with collagens type I and IV, or human and mouse laminin did not express the pluripotency marker octamer-binding transcription factor 4 (OCT4, POU5F1) (Fig. 3). Important genes for early eye-field development, paired box protein-6 (PAX6) and retina and anterior neural fold homeobox (RAX) were faintly expressed. Melanogenesis related genes: MITF, pre-melanosome protein (PMEL), and TYR as well as retinal pigment epithelium-specific protein 65 kDa (RPE65), involved in visual pigment regeneration, were strongly expressed by hESC-RPE cells. In addition, BEST and pigment epithelium derived factor (PEDF) which are both known to be expressed by human RPE cells, were also expressed (Fig. 3). There were no differences in gene expression between hESC-RPE cells cultured on PI coated with collagens type I and IV, or human and mouse laminins (data not shown).

The maturation of hESC-RPE cells cultured on coated PI biomembranes was studied by analyzing the expression and...
localization of several proteins that are distinctive for RPE cells. MITF and CRALBP were co-expressed in mature cells. MITF was localized in the nuclei and CRALBP in the cytoplasm and near the cell membrane (Fig. 4A). Besides CRALBP and MITF, RPE-specific protein BEST was also expressed by hESC-RPE (Fig. 4B). ZO1 expression on the apical cell membrane illustrates the formation of tight junctions (Fig. 4C). Apical expression of NaKATPase (Fig. 4D) as well as MERTK (Fig. 4E) is required for phagocytosis of POS, and also indicates polarization of the epithelium. The cellular proliferation marker, Ki67, was found not to be expressed by hESC-RPE monolayers when confluence was reached (data not shown).

3.3. Functionality of hESC-RPE cells cultured on PI membrane

Rat rhodopsin was internalized by hESC-RPE monolayers during rat retina explants co-culture (Fig. 4F). The paracellular permeability
of 6-CF and the TER were measured in order to establish the quality of barrier function and the integrity of hESC-RPE monolayers. The retina-to-choroid permeability of 6-CF was similar between the selected membranes and ranged between 1.3 and 1.5 \( \frac{\mu g}{cm^2 \cdot m/s} \). The TER of monolayers varied among the selected coatings and was 40–80 \( \mu \Omega \cdot cm^2 \), the highest values were obtained with collagen type I and type IV.

4. Discussion

Generation of a functional, human stem cell-based and tissue engineered RPE may provide a unique platform for \textit{in vitro} drug screening, cell replacement therapy and disease model development. A cell culture model of the outer BRB may be used for several purposes including the development of drugs and delivery systems with favorable pharmacokinetic and pharmacodynamic properties, the \textit{in vitro} study of barrier function and regulation, transport processes and metabolism of xenobiotics and endogenous compounds [27]. A validated RPE cell culture model may also be able to replace widely criticized animal experiments, which currently are still required in ocular pharmacokinetic, pharmacodynamic and toxicity studies. Predictions of human ocular drug exposure derived from such models may be more accurate than those registered from animal eyes. For example, the albino rabbit eye, a commonly used animal model, lacks melanin pigment, which is known to bind drugs and act as a depot prolonging their activity [28]. The development of pigmented RPE monolayers will take into consideration the delayed elimination of the test compounds due to melanin binding and produce therefore more precise pharmacological predictions. Another important medical application of tissue engineered RPE cells is replacement therapy for the treatment of retinal degenerations. A valid therapeutic option in AMD is the sub-retinal transplantation of healthy RPE cells, which may rescue the remaining photoreceptors by re-establishing retinal homeostasis and a functional outer BRB.

In this study, we have explored the use of biopolymer coated PI membranes as RPE substrates, with the aim to promote differentiation and maturation of hESC toward RPE phenotype. PI meets several requirements needed for retinal scaffolds: the polymer is approved by regulatory agencies for intraocular use, it is biocompatible [29] and well tolerated in the subretinal space [21], mechanically robust and flexible (for surgical manipulation), and its pores will allow the transport of oxygen and nutrients between the choroid and the outer retina. In this study, our foremost criterion for the PI biomembranes was the development and maintenance of a functional RPE phenotype \textit{in vitro}. The importance of the substrate in hESC differentiation is well known; however the choice of extracellular matrix molecules and carrier material to direct growth and maturation of hESC toward RPE is still poorly studied. Also, direct comparison to studies carried out with immortalized RPE cell lines should be done carefully, because hESC-RPE have been shown to differ, i.e. in pigmentation and gene expression [25]. We tested several coatings and found that hESC-RPE matured into highly pigmented RPE monolayers when cultured on laminin, collagen type I and IV, CELLstart\textsuperscript{TM}, MaxGel\textsuperscript{TM}, and HyStem\textsuperscript{TM} hydrogel coated PI membranes. The suitability of laminin and collagen type IV is not surprising, since both proteins are major constituents of the RPE basal lamina [30], which serves as the anchoring surface for the RPE. Collagen type I is found in the inner and outer collagen layers of Bruch’s membrane [31]. MaxGel\textsuperscript{TM} coating also promoted RPE-like monolayer development; however, due to its
undefined content and batch to batch variation, we chose to discard it. CELLstart™ was discarded because it did not have any advantage over individual protein coatings. HyStem™ hydrogel was also discontinued because the hESC-RPE monolayers easily detached from the PI biomembrane upon handling. Among the selected coatings, human laminin is available at GMP grade (BioLamina AB), meeting the strict standards required for clinical applications; for this reason it is a very good candidate for future use in cell replacement therapy. For *in vitro* drug screening purposes, where the requirements are not so rigorous, animal-derived products may also be suitable. To summarize the coating selection, we wish to point out that successful maturation of hESC-RPE monolayers was not restricted to one single bioadhesive molecule, but instead it was achieved with several coatings, whereas naked PI membrane did not support growth of these cells. Moreover, the selected coatings promoted maturation of both tested hESC lines (Regea06/040 and Regea08/017), indicating a potential robustness of our coated membrane approach. This aspect is very important when considering the intended use of coated PI membranes as cell culture model substrate or in tissue engineering applications.

Human ESC-RPE grown on the selected PI biomembranes formed a monolayer and acquired typical and homogenous RPE phenotype (pigmented, hexagonal, cobblestone like, closely packed cells). Furthermore, hESC-RPE cells exhibited polarity with apical localization of NaKATPase and MERTK. Confluent hESC-RPE monolayers developed differentiated properties as evidenced by the expression pattern of RPE-specific marker proteins and, once confluence was reached, cells stopped dividing. The inactivation of cell growth is essential, because in the tissue in situ, RPE cells are growth quiescent and proliferation only occurs in disease.

The RPE forms the outer BRB and plays an essential role in maintaining the viability and function of the neural retina. For this reason, emphasis was put in engineering hESC-RPE monolayers with proper barrier function. The tightness of the monolayers was studied by 6-CF paracellular permeability and by TER measurements. The apical to basolateral permeability of the low molecular weight, hydrophilic probe (6-CF) was between 1.3 and 1.5 ± 0.6 × 10⁻⁵ cm/s; these values are only slightly higher than published bovine RPE-choroid data of 2.33 ± 1.06 × 10⁻⁶ cm/s [32], and human RPE cell line ARPE-19 (7.3 ± 2.02 × 10⁻⁶ cm/s) [33]. TER of hESC-RPE monolayers grown on PI biomembranes were 40–80 ± 12 Ω cm², depending on the cell line and membrane coating, indicating that the tight junctions were properly formed. In general, published TER data of RPE monolayers vary greatly depending on the developmental stage, whether primary or secondary cultures were used, from which species they were derived, the culture conditions, and the instrument used for the measurements. Reported TER values of human adult and fetal RPE-choroid specimen are 79 ± 48 Ω cm² and 206 ± 151 Ω cm², respectively [34]. Resistances of 30–100 Ω cm² have been described for ARPE-19 monolayers [35,36], while in general, published TER data of RPE monolayers vary greatly depending on the developmental stage, whether primary or secondary cultures were used, from which species they were derived, the culture conditions, and the instrument used for the measurements. Reported TER values of human adult and fetal RPE-choroid specimen are 79 ± 48 Ω cm² and 206 ± 151 Ω cm², respectively [34].

Of particular relevance to the pathogenesis of macular degeneration is the ability of RPE to daily engulf and degrade the distal 10% of each POS [41]. Therefore it is crucial for engineered hESC-RPE monolayers to exhibit phagocytic activity in vitro. Human ESC-RPE monolayers grown on PI biomembranes expressed MERTK, a protein involved in POS phagocytosis that induces engulfment of shed outer segments [42,43]. In addition, we have shown that hESC-RPE monolayers cultured with rat retina explants were capable of binding and internalizing shed rat outer segments.

Several studies have elucidated human pluripotent stem cells (including hESC and human induced pluripotent stem cells) as an inexhaustible source of RPE for clinical applications. Moreover, the first on-going clinical trials are assessing the safety and tolerability of hESC-RPE cell therapy in humans. The suitability of the coated PI membrane for RPE replacement therapy will naturally need further *in vivo* investigations but we consider non-biodegradability of PI beneficial for transplantation as it should effectively protect the hESC-RPE monolayer from the hostile environment of deteriorated Bruch’s membrane in the diseased eye. In addition, engineered human RPE constructs with PI biomembrane may also provide a unique platform for drug discovery and toxicology.

### 5. Conclusions

The PI biomembrane presented in this study was shown to promote growth and maturation of hESC-RPE monolayers. These *in vitro* grown epithelia acquired RPE-like properties, including characteristic RPE phenotype, expression of RPE markers, barrier and phagocytic function. Based on our results, we suggest the suitability of our PI biomembrane as scaffold for hESC-RPE tissue engineering.

### Ethical issues

We have approval from the National Authority for Medicolegal Affairs Finland to do research with human embryos (Dnro 1426/32/300/05) and a supportive statement from the local ethics committee of the Pirkkanmaa hospital district Finland to derive and expand hESC lines from surplus embryos not used in the treatment of infertility by the donating couples, and to use these lines for research purposes (R05116). No new cell lines were derived for this study.

### Acknowledgment

This study was financially supported by two private donations, Academy of Finland (218050, 133879), TEKES (886/31/09), Tampere Graduate Program in Biomedicine and Biotechnology (TGPBB), PolExGene STREP project (EU-FP6 contract number 019114), and Hilda Kauhanen foundation. We would like to express our thanks to Hanna Koskenoaho, Ouli Meltin, Enea Konsén and Ouli Kokkonen for technical assistance. We also want to thank the whole PolExGene consortium, Veli-Pekka Ranta for help with bioelectric measurements, Giedrius Kalesnykas and Jinghun Wang for advice and help with phagocytosis study and Professor Robin Ali for non-dystrophic RCS rats.

### Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at [http://dx.doi.org/10.1016/j.biomaterials.2012.07.033](http://dx.doi.org/10.1016/j.biomaterials.2012.07.033).

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