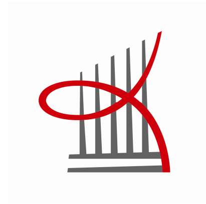

DIRECTED DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS INTO RETINAL PIGMENT EPITHELIUM

Comparison of Adherent and EB-like Culturing Methods

Jenna Tainio

Bachelor's Thesis
November 2014
Degree Programme in
Laboratory Sciences

TAMPEREEN AMMATTIKORKEAKOULU
Tampere University of Applied Sciences

ABSTRACT

Tampere University of Applied Sciences
Degree programme in Laboratory Sciences

TAINIO, JENNA:
Directed Differentiation of Human Pluripotent Stem Cells into Retinal Pigment Epithelium: Comparison of Adherent and EB-like Culture Methods

Bachelor's thesis 55 pages, appendices 1 page
November 2014

Retinal pigment epithelium (RPE) is an epithelial cell monolayer located in the retina region at the back of the eye. RPE has a significant function in the eyesight as it plays a key role in the supporting of photoreceptor functions. Disorders of RPE lead to degenerative eye diseases – such as macular degeneration and retinitis pigmentosa – that are the leading causes of blindness. In the future, these blinding retinal disorders could potentially be cured by utilizing regenerative medicine. Degenerated RPE could be replaced by tissue graft derived from human pluripotent stem cells (hPSC).

Currently, the differentiation of hPSCs to RPE cells is inefficient and results in low yields of functional cells. The yield of differentiated cells could be improved for example by chemical induction of the cells. The objective of the thesis was to further optimize a directed RPE differentiation method. In the future, more effective ways to produce hPSC-RPE could be used to develop tissue grafts that can be utilized in regenerative medicine.

The aim of the study was to compare adherent and embryoid body (EB)-like culture methods for directed differentiation of hPSCs into RPE. The comparison was done by evaluating the formation of melanin pigmentation between different culture methods and by analyzing the quality of the produced cells. The hPSCs used in this thesis were human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC). Differentiation was directed by a use of growth factor and small molecule induction.

Cells produced in this study were analyzed by morphology, transepithelial resistance (TER) and immunocytochemical stainings focusing on RPE markers. The characterization methods provided information about the differentiation status of the cells, including morphology, tightness of the cell junctions, protein expression and polarization of the cell monolayer.

The results suggest that for this protocol of directed differentiation of hPSCs into RPE, the adherent culture method gives significantly better yield than the EB-like culture method. The induction seemed to work with both hESCs and iPSCs, but the yield tended to differ between the stem cell types, favoring the differentiation of hESC-RPE cells. The cell cultures on porous Polyethylene Terephthalate (PET) membrane did not form a confluent cell monolayer, and further research is needed on the reasons behind this behavior. However, the immunostainings suggested that the quality of hESC-RPE was desirable despite the relatively young age of the differentiated cells.

Key words: human pluripotent stem cell, adherent cell culture, embryoid body, RPE, differentiation

TIIVISTELMÄ

Tampereen ammattikorkeakoulu
Laboratorioalan koulutusohjelma

JENNA TAINIO:
Ihmisen kaikkikykyisten kantasolujen suunnattu erilaistus verkkokalvon pigmenttiepiteelin soluiksi: Adherentin ja suspensioviljelymenetelmän vertailu

Opinnäytetyö 55 sivua, joista liitteitä 1 sivu
Marraskuu 2014

Verkkokalvon pigmenttiepiteeli (RPE) on retinassa sijaitseva yksikerroksinen solukerros, jolla on useita tehtäviä verkkokalvon valoaistinsolujen ylläpidossa. Monet sokeuteen johtavat sairaudet, kuten verkkokalvon ikärappeuma ja pigmenttidegeneraatio, ovat seurausta RPE-solujen vaurioitumisesta. Kantasolujen hyödyntämistä kudosteknologisissa sovelluksissa pidetään lupaavana tulevaisuuden hoitokeinona verkkokalvon vaurioiden hoidossa. Laboratorio-olosuhteissa voidaan tuottaa kaikkikykyisistä kantasoluista kasvatettuja siirteitä, joilla on mahdollista korvata vaurioituneita alueita.

Nykyisin yleisesti käytetyt RPE-solujen erilaistusmenetelmät ovat suhteellisen tehottomia, sillä erilaistuksella tuotettujen solujen saannot ovat pieniä. Opinnäytetyön tavoitteena oli selvittää adherentin ja suspensioviljelymenetelmien eroavaisuuksia erilaistettaessa kaikkikykyisistä kantasoluista RPE-soluja. Erilaistusmenetelmien kehittyessä kantasoluista voidaan tuottaa RPE-soluja, joita on mahdollista hyödyntää kudosteknologisissa sovelluksissa.

Opinnäytetyön tarkoituksena oli vertailla adherenttia ja suspensioviljelymenetelmää ihmisen kaikkikykyisten kantasolujen suunnatussa erilaistuksessa. Työn tutkittavina kaikkikykyisinä eli pluripotentteina kantasoluina käytettiin ihmisen alkion kantasoluja sekä indusoituja pluripotentteja kantasoluja. RPE-tyyppisten solujen erilaistumista seurattiin visuaalisesti solujen tuottaman melaniinipigmentin avulla. Erilaistusmenetelmiä vertailtiin tarkastelemalla tuotettujen RPE-solujen määrää ja laatua. Soluja karakterisoitiin transepiteelisen resistanssin ja immunosytokemiallisten värjäyksien perusteella. Karakterisointimenetelmät määrittivät epiteelin solujen konfluenssia, solujen välisten liitosten tiiviyttä sekä solujen morfologiaa, polarisoitumista ja erilaistumisastetta.

Erilaistusmenetelmistä adherentti soluviljelymenetelmä tuotti huomattavasti parempia tuloksia. Suunnattu erilaistus tuotti pigmentoituneita soluja sekä alkion kantasoluilla että indusoiduilla pluripotenteilla kantasoluilla, mutta vain alkion kantasoluista saatiin tuotettua tarpeeksi soluja jatkotutkimuksiin. Erilaistetut solut eivät muodostaneet kaikilla viljelyalustoilla RPE-soluille tyypillistä yksikerroksista epiteelisolukerrosta, ja syitä tämän käytöksen taustalla tulisikin tutkia tarkemmin. Solut olivat kuitenkin immunovärjäysten perusteella erilaistuksen kestoon nähden melko kypsiä RPE-tyyppisiä soluja.

Asiasanat: ihmisen kaikkikykyiset kantasolut, adherentti soluviljelmä, embryoid body, RPE, erilaistus

TABLE OF CONTENTS

1 INTRODUCTION 8
2 LITERATURE REVIEW 9
2.1 Stem cells 9
2.1.1 Adult stem cells 10
2.1.2 Human embryonic stem cells 10
2.1.3 Induced pluripotent stem cells 11
2.1.4 Stem cells in regenerative medicine 12
2.2 Stem cell culture and differentiation 13
2.2.1 Pluripotent stem cell culture 13
2.2.2 Differentiation in suspension culture 13
2.2.3 Differentiation in adherent cell culture 14
2.3 Structure of the eye 14
2.3.1 Neural retina 15
2.3.2 Retinal pigment epithelium 16
2.3.3 Retinal degeneration 18
2.4 Differentiation of human pluripotent stem cells into RPE cells 19
2.4.1 Spontaneous differentiation 20
2.4.2 Induction toward RPE-cell differentiation 21
2.5 RPE-cell characterization 22
2.5.1 Morphology 23
2.5.2 Transepithelial resistance 23
2.5.3 RPE specific proteins 24
2.5.4 Proteins indicating the polarity of the cells 25
2.5.5 Other characterization methods 26
3 AIMS OF THE STUDY 27
4 MATERIALS AND METHODS 28
4.1 Cell culture 29
4.1.1 Human pluripotent stem cells 29
4.1.2 Differentiation and culture media 30
4.1.3 RPE differentiation 30
4.1.4 Subculture 32
4.2 Reference material 32
4.3 Transepithelial resistance 34
4.4 Immunofluorescence staining 34

5 RESULTS 36
5.1 Appearance of pigmentated cells 36
5.2 Characterization 39
5.2.1 Morphology 39
5.2.2 Transepithelial electric resistance of the PET-membranes 40
5.2.3 Immunofluorescence staining 41
6 DISCUSSION 44
REFERENCES 48
APPENDICES 55
Appendix 1. M+/M- control cell culture immunostainings 55

ABBREVIATIONS

AMD	Age-related macular degeneration
bFGF or FGF	(Basic) fibroblast growth factor
BM	Bruch's membrane
BMP	Bone morphogenetic protein
BMSC	Bone marrow stromal cell
BSA	Bovine serum albumin
BVMD	Best vitelliform macular dystrophy (Best's disease)
c-Myc	Regulator gene that codes for a transcription factor.
CRALBP	Cellular retinaldehyde-binding protein
DAPI	4′, 6′ diamidino-2-phenylindole
DM-	Basic culturing medium based on DMEM
DM+	DM- medium with added growth factor and small molecule selective inhibitors
DPBS or PBS	(Dulbecco's) phosphate buffer saline
EB	Embryoid body
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
GLP	Good laboratory practice
hESC or ESC	(Human) embryonic stem cell
hiPSC or iPSC	(Human) induced pluripotent stem cell
hFF	Human foreskin fibroblasts
HSC	Hemopoietic st em cell
IF	Immunofluorescence
IVF	*in vitro* fertilization
IWP-2	N-(6-Methyl-2-benzothiazolyl)-2-[(3,4,6,7-tetrahydro-4-oxo-3-phenylthieno[3,2-d]pyrimidin-2-yl)thio]-acetamide
JAM	Junctional adhesion molecules
Klf4	Kruppel-like factor 4
KO-DMEM	Knockout Dulbecco's Modified Eagle Medium
KO-SR	Knockout Serum Replacement
MAPC	Multipotent adult progenitor cell

OCT4	Octamer-binding transcription factor
PET	Polyethylene Terephthalate
POS	Photoreceptor outer segment
RP	Retinitis pigmentosa
RT	Room temperature
RPE	Retinal pigment epithelium
RPE65	Retinal pigment epithelium-specific 65 kDa protein
SB-505124	2-(5-benzo[1,3]dioxol-5-yl-2-tert-butyl-3H-imidazol-4-yl)-6-methylpyridine hydrochloride
SCED	Single cell enzymatic dissociation
SCNT	Somatic cell nuclear transfer
STAP	Stimulus-triggered acquisition of pluripotency
OX2	Transcription factor orexin-2
TGF	Transforming growth factors
TER	Transepithelial electric resistance
ZO-1	Zonula occludens protein 1

1 INTRODUCTION

Retinal pigment epithelium (RPE) is an epithelial cell monolayer located in the retina region at the back of the eye. The RPE has many vital functions in the eye, including transepithelial transport and regulation of biomolecules, taking part in the visual cycle and phagocytosis of the photoreceptors shed outer segments. Because of this critical role of the RPE, the degeneration of RPE leads to eye diseases such as age-related macular degeneration or retinis pigmentosa. Damages caused by retinal degeneration are currently the leading causes of permanent blindness (Strauss 2005).

Currently, there are no effective widely accepted treatments to these drastic blinding diseases. It has been suggested that in the future, regenerative medicine could be the answer through RPE-cell tissue graft replacements. The functional RPE cells for these grafts can be differentiated from human pluripotent stem cells (hPSC). RPE-like cells can be derived from pluripotent stem cells spontaneously, but the yield could be improved through directed differentiation.

This bachelor’s thesis was carried out in the Ophthalmology research group in the Institute of Biomedical Technology at the University of Tampere. The group is led by Academy of Finland Research Fellow Heli Skottman, PhD, and this thesis was supervised by PhD Tanja Ilmarinen. The main research aim for the group is to develop stem cell based tissue engineering applications for the retinal and corneal repair through cell transplantation and ophthalmic *in vitro* tissue models.

This thesis took part in one of research group’s ongoing projects to develop more effective ways to differentiate RPE cells from hPSCs. The hPSCs used in this study were embryonic pluripotent stem cells (ESC) and induced pluripotent stem cells (iPSC). Objective was to derive hPSC-RPE cells with different differentiation conditions and to compare the differentiation efficiency and the quality of the produced cells. To enhance the differentiation efficiency, differentiation to RPE-like cells was directed by an induction method developed within the research group.

2 LITERATURE REVIEW

2.1 Stem cells

Stem cells are unspecialized cells capable of renewing themselves through cell division and they have potential to develop into many different cell types in the body. When a stem cell divides, each new cell can either remain as a stem cell or under certain physiologic or experimental conditions, they can be induced to become another type of tissue- or organ-specific cell with special functions (Kolios & Moodley 2013). Stem cells are classified into different categories depending on the differentiation capacity; totipotent, pluripotent, multipotent, oligopotent or unipotent stem cells.

Totipotent stem cells have the ability to differentiate to any type of cell in an organism. Zygotes or 2-3 days old embryos (morulas) are considered as totipotent cells. Pluripotent stem cells (PSC) have the ability to differentiate to any type of cell of the three germ layers, but cannot form even in favorable conditions a whole organ or individual since they lack the ability to form extraembryonic tissue. The embryonic stem cells (ESC) can be obtained from 4-7 day old embryos (blastocysts) or they can be induced from somatic cells (induced pluripotent stem cells, iPSC). (Kolios & Moodley 2013.)

Multipotent stem cells, or progenitor cells, are type of stem cells that are more differentiated and therefore less plastic. They can form different cell types within certain tissue type, and for example multipotent hematocyte can differentiate to platelets (thrombocytes) or red or white blood cells (erythrocytes and leukocytes, respectively.) Oligopotent cells are considered tissue-resident progenitor cells with the potency to form a limited number of terminally differentiated cells, and are similar in a way to unipotent stem cells, such as germ cells, that are capable to form only one type of cell (Kolios & Moodley 2013).

Given their unique regenerative abilities, stem cells offer new potentials for treating diseases which are caused by failure of certain tissue type, such as retinal degeneration, diabetes and heart diseases. Much work remains still to be done to understand how to use these cells for cell-based therapies to treat diseases.

2.1.1 Adult stem cells

Many tissues contain reservoirs of stem cells called adult stem cells. Compared with ESCs, adult stem cells cannot differentiate into multiple lineages. As multipotent stem cells, adult stem cells are usually capable of differentiating only into lineage-specific cells. Adult stem cells residing in specific tissues and organs are called tissue specific stem cells.

Bone marrow, for example, represents a unique reservoir of tissue specific stem cells. Bone marrow contains at least two other populations of stem cells; bone marrow stromal cells (BMSCs) and a heterogenous population of multipotent adult progenitor cells (MAPCs) such as mesenchymal stem cells, which appear to have broad developmental capabilities. MAPCs have the potential to differentiate to lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle, and marrow stroma. (Ross & Pawlina 2011.)

When injected into an early blastocyst *in vitro,* single MAPCs contribute to most, if not all, somatic cell types. On transplantation, MAPCs engraft and differentiate to the hematopoietic lineage, in addition to the epithelium of liver, lung and gut. As MAPCs proliferate extensively without obvious senescence or loss of differentiation potential, they could be one ideal cell source for therapy of inherited or degenerative diseases (Kolios & Moodley 2013.)

2.1.2 Human embryonic stem cells

Embryonic stem cells are undifferentiated pluripotent cells derived from the blastocyst stage embryos. Method to derive embryonic stem cells was first established with mouse embryonic stem cells (Evans & Kaufman, 1981) before the derivation of human embryonic stem cell (hESC) lines (Thomson et al. 1998). Embryonic stem cells form an ESC-line when they have been cultured under *in vitro* conditions and have been capable of dividing without differentiating for a prolonged period, usually months containing tens of passages (Kolios & Moodley 2013.)

In Finland, the hESC lines are derived from surplus embryos from *in vitro* fertilization (IVF) clinics by the permission from the donors with no financial compensation made for the donation. (Skottman, 2010). Cells of the embryo’s inner cell mass are separated from the

outer cell mass and are placed on a layer of mitotically inactivated fibroblasts, commonly referred to as a feeder cell layer, or on a matrix which supports the pluripotency of the cells. The resulting cell population is expanded *in vitro* by subculturing the undifferentiated cells as colonies (Hoffman & Carpenter 2005; Kolios & Moodley 2013.)

2.1.3 Induced pluripotent stem cells

Induced pluripotent stem cells are stem cells that have been reprogrammed to pluripotent state from somatic cells. Currently, iPSCs can be generated with two notable methods; nuclear transfer from differentiated somatic cell to oocyte (i.e. egg cell) or by introducing a set of defined factors into somatic cells.

Nuclear transfer – or somatic cell nuclear transfer (SCNT) – to oocytes is used in both reproductive and therapeutic cloning, resulting a viable embryo. The first successful nuclear transfer was used when the first cloned mammal Dolly the Sheep was created. However, SCNT is considered as inefficient method, since the stress placed on both the oocyte and the introduced nucleus are exceptional (Lomax & Dewitt 2013).

Shinya Yamanaka and his research group introduced in 2007 a method to induce somatic cells to their pluripotent state by the introduction of four key transcription factors, OCT3/4, SOX2, Klf4, and c-Myc, which have since been referred as the Yamanaka factors. The first reprogramming of somatic cells was done with fibroblast from mouse (Takahashi 2006) and followed by generating iPSCs from human adult fibroblasts (Takahashi 2007). This research was remarkable in the field of stem cells, and Yamanaka and his fellow stem cell researcher John Gurdon were awarded the Nobel Prize for Physiology or Medicine in 2012 (The Nobel Assembly at Karolinska Institutet 2012.)

Other combinations of transcription factors have also been employed in different studies, including the use of 1-4 factors in most cases (Stadtfeld & Hochedlinger 2010.) Some viral vectors used in introduction of the transcription factors, such as retroviruses and plasmids, may cause a genomic alteration to the host cells. Fortunately, nonintegrative reprogramming alternatives, such as Sendai virus, directed mRNA and protein transduction, have been discovered (Carr et al. 2013).

2.1.4 Stem cells in regenerative medicine

Regenerative medicine and treatments based on stem cells seem to hold many promises for numerous applications in the future. The treatments are carried out with cells differentiated from the stem cells. Use of adult stem cells does not cause immune reaction when patients own autogeneic stem cells are used. However, since adult stem cells are already somewhat differentiated cells, they do not have the same differentiation potential as hPSCs. In the use of hPSCs, ethical issues are raised considering the use of hESCs, as derivation of hESC-lines results in destruction of living blastocysts. Using the iPSCs in regenerative medicine can possibly overcome the ethical controversy of hESCs. Treatments done with allogeneic iPSC could potentially also be conducted without immunogenecity issues (Takahashi et al. 2007; Yamanaka 2007).

However, in 2011 a research by Zhao et al. claimed that cells differentiated from ESCs derived from inbred mice line could efficiently form teratomas within the same line individuals without any evident immune rejection. A teratoma is a type of tumor that may contain several tissue derivatives from more than one germ layer. When used allogeneic ESCs in the differentiation, teratomas did not appear due to rapid rejection of the cells by recipients. The same study also suggested that when using iPSCs derived with both retroviral and novel episomal approach, not did the mice only form teratomas but they also triggered an immune response, a body’s immunologic reaction to an antigen, despite being derived from autonomous somatic cells. The results raised concerns about the safety of the future regenerative medicine treatments (Zhao et al. 2011).

The results provided by Zhao et al. (2011) have since been put on question, since it has been shown that in mouse (Araki et al. 2012,) rats (Kanemura et al. 2014) and monkey studies (Kamao et al. 2014) that cells differentiated from iPSCs were unlikely to cause tumors or immunogenicity. Since then, studies that utilize both ESC (Schwartz et al. 2014) and iPSC (Cyranoski 2014a) have advanced to clinical trials. For adult stem cells, currently only one widely used stem cell therapy treatment has been established. The treatment is provided with hematopoietic stem cells that are used mainly in bone-marrow transplants for cancer patients (Burt et al. 2008).

2.2 Stem cell culture and differentiation

2.2.1 Pluripotent stem cell culture

Native stem cells grow in complex environment and thus they tend to also have demanding *in vitro* culturing conditions. To keep the stem cell lines in undifferentiated state, some necessities have to be taken care of. Other type of cells, called feeder cells, can be co-cultured with stem cell colonies in the same culturing well or a petri dish (Stacey et al., 2006). The role of feeder cells is to secrete various growth factors and extracellular matrix (ECM) proteins that are beneficial by yet unclear ways for the growth and pluripotency of the stem cells (Hongisto et al. 2011.)

In order to keep the conditions free from animal-derived materials which could be harmful in clinical applications, use of human fibroblasts, like commercially available neonatal human foreskin fibroblasts (hFF), is to be considered (Skottman 2010; Vaajasaari 2011). As in any other cell cultures, aseptic work, sterile instruments and right conditions are important for stem cell culturing.

2.2.2 Differentiation in suspension culture

Methods for stem cell differentiation are diverse, and several protocols can be used for the differentiation of same target cells. When hPSCs colonies are dissected and transferred to a suspension culture with medium with low basic fibroblast growth factor (bFGF) concentrations, the cells tend to form three-dimensional cell aggregates, called embryoid bodies (EB). EB formation resembles embryonic development (Carr et al. 2013.) Cell aggregates are formed from multiple cells and EB-culturing is not to be confused with single cell suspension culturing.

EB-culturing makes the cells epithelioid tissue; similar to epithelium but they lack basement membrane where to adhere (Ross & Pawlina 2011.) In contrast to adherent cell cultures, EB cultures enable larger yields of cells, since free floating aggregates can distribute to the surrounding medium. Over time, the hPSCs cell aggregates start to spontaneously differentiate into cell types of all three germ layers (Klimanskaya 2006.)

2.2.3 Differentiation in adherent cell culture

In adherent cell culture the cells attach to a surface and adhere to it, and depending from cell type, can form a confluent layer of cells. Because of this, the maturity and differentiation stage of the cells is easier to evaluate in adherent cell cultures than in suspension culture.

Usually for cells to adhere, the surface has to be treated somehow, since in the native conditions cells adhere to extracellular matrix. ECM content differs from the origin of tissue, but constitutes mostly from specific proteins of proteoglycans, polysaccharides or fibers, such as collagen or laminin in basal lamina called Bruch's membrane (Ross & Pawlina 2011.)

2.3 Structure of the eye

The eye is a complex sphere-shaped sensory organ that has a lens system reminiscent of a camera. The act of seeing starts when the lens of the eye focuses light reflected from objects onto a light-sensitive membrane at the back of the eye, called the retina, where almost 70% of body's sense cells are located. These sense cells send the information to the brain along the optic nerve and the brain processes the information that is contained in the visible light (Haug et al. 1999.)

The wall of human eye consists of three separate layers, called sclera, choroid and retina, that all have different errands in the general functionality of the eye, presented in figure 1. The outer layer, sclera, serves as the eye's protective outer coat. Sclera is attached to the eye socket by six small muscles that enable the movement of eyeball. Sclera transforms from white, opaque tissue to transparent cornea in the frontal part of the eye. All the light that enters in the eye goes through refracting cornea and thus cornea's condition is highly important for maintaining a good vision. After passing through the cornea, light travels through ring-shaped pupil that manipulates the amount of light that enters in the retina. Pupil is attached to iris that is a colored, circular structure. Iris is responsible for controlling the diameter and size of the pupil depending of the intensity of the entering light. Behind the iris is located the lens that focuses the entering light in the retina by changing its shape (Ross & Pawlina 2011.)

The middle layer of the eye located next to the sclera is known as choroid. The choroid is a layer of blood vessels that is responsible for the nutrient transportation to retina. Between the iris and the choroid lays the ciliary body. Ciliary body contains smooth muscle fibers that change the shape of the lens and produces the transparent liquid (aqueous humor) that fills the front of the eye. Retina, the inner layer of the eye, consist of two main layers, the neural layer and retinal pigment epithelium (RPE) layer. The space between the lens and the retina is filled with gel called vitreous humor (Ross & Pawlina 2011.) Retina region is illustrated with the layers of the eye in the figure 1.

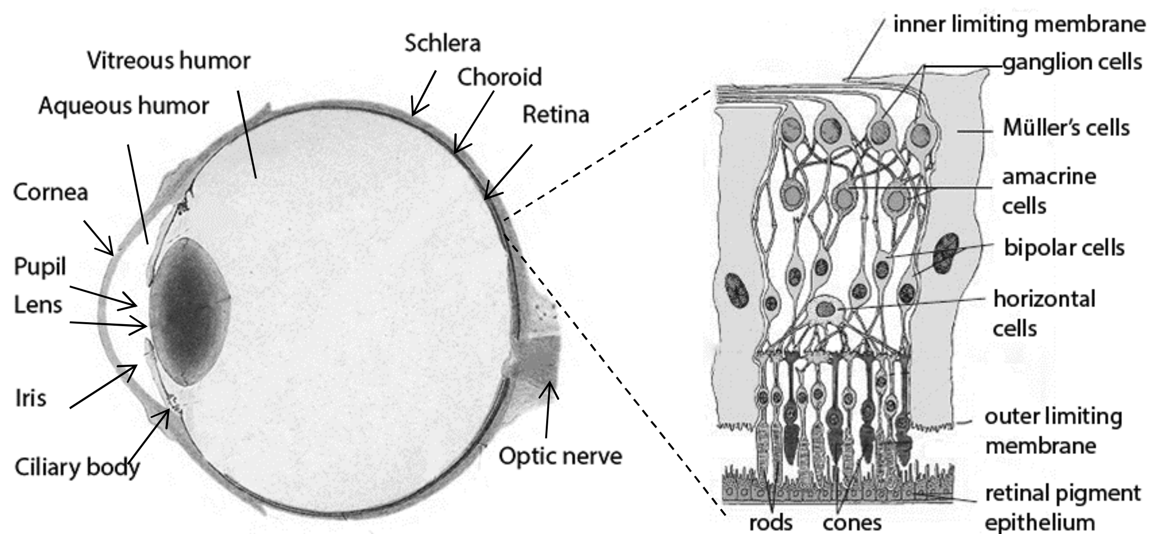

FIGURE 1. Gross anatomy of the eye and retina (modified from Ross & Pawlina, 2011)

2.3.1 Neural retina

Despite its location, the neural portion of the eye is actually part of the central nervous system. The retina, light sensitive layer of tissue, has a complex neural circuitry that converts the photoreceptors' electrical activity into action potentials that travel to the brain by axons in the optic nerve. (Purves et al. 2001) The retina consists of two main layers; neural retina and RPE. The RPE layer is described separately in more detail in following chapters.

Neural retina is the most inner part of retina and is formed of seven layers of different cells as seen in figure 1. There are five types of neurons in the retina: photoreceptors, horizontal

cells, bipolar cells, amacrine cells and ganglion cells. Two main types of light-sensitive pho-photoreceptors in the retina are called rods and cones. Rods provide black-and-white vision and function mainly in dim light, while cones function best in relatively bright light and are responsible for color vision (Purves et al. 2001.) Horizontal cells' function is to help integrate and regulate the input from multiple photoreceptor cells. Bipolar cells transmit the signals from either one type of photoreceptor or horizontal cells on to the ganglion cells directly or indirectly by amacrine cells and retinal ganglion cells collectively transmit signals onwards to several regions in the brain. In neural retina, there are located glial cells called Müller cells. Müller cells form architectural support structures that stretch radially across the thickness of the retina. The neural layer attaches to the RPE layer, which is the outermost layer of retina (Ross & Pawlina 2005).

2.3.2 Retinal pigment epithelium

Retinal pigment epithelium is an epithelial cell monolayer located in retina between neural retina and choriocapillaris (Strauss 2005). Like other epithelial cells, RPE is an avascular tissue where cells adhere to one another by means of specific cell-to-cell adhesion molecules that form specialized cell junctions. Epithelial cells are polarized so that they are associated with three distinct morphologic surface domains; apical, lateral and basal domain. Apical domain can be considered as the surface of the cells that is exposed to the environment, as later domain consists of the sides of the cells (cell-cell contacts) and basal domain is the surface epithelial by which the cell is attached to the extracellular matrix. The properties of each domain are determined by specific lipids and membrane proteins. The basal surface usually is attached to an underlying basement membrane, which for RPE is the Bruch's membrane. RPE is formed of hexagonal cells that are densely packed with melanin containing light-absorbing pigment granules called melanosomes that prevent reflection of the light and resultant glare (Ross & Pawlina 2011.)

RPE functions as a selective barrier that facilitates or inhibits the passage of specific substances between the exterior environment and the underlying tissue compartment. It secretes growth factors, regulates nutrient, ion and molecule transport between subretinal space and bloodstream by forming the blood-retina barrier (BRB) (Curcio & Johnson 2013, Ross & Pawlina 2011.) RPE has also many other crucial functions in the retina; it also

phagocytoses the shed membranous discs from photoreceptor outer segments (POS), regen-regenerates visual pigment and takes part in the visual cycle by the vitamin A metabolism. In vitamin A metabolism, regeneration of retinoids requires molecule exchange between the photoreceptors and the RPE. These functions have been illustrated in figure 2. Dysfunctional RPE can cause impairment and death of the photoreceptor cells, and may eventually to deterioration or total loss of vision. (Strauss 2005, Vaajasaari et al. 2011)

Adjacent RPE cells are connected by a junctional complex that forms the inner blood-retina barrier. On the basal side of the RPE layer lays the outer limit of retina, basal membrane known as Bruch's membrane (Strauss 2005, Ross & Pawlina 2011.) Bruch's membrane plays a crucial role together with RPE cells in maintaining photoreceptor viability as well as overall retinal health. Bruch's membrane anchors the RPE cells from their basal membrane via tight junctions and connects the RPE into the choroid. RPE forms in the other, outer BRB in collaboration with Bruch's membrane and the choriocapillaris (Rizzolo et al. 2014.) Basement membrane–forming collagens in the Bruch's membrane include type IV collagen, which is responsible for the collagen suprastructure in the basement membrane of epithelial cells (Ross & Pawlina 2011). The RPE's basal surface participates in metabolic exchanges with the blood vessels in the underlying choriocapillaris (Bonilha 2008).

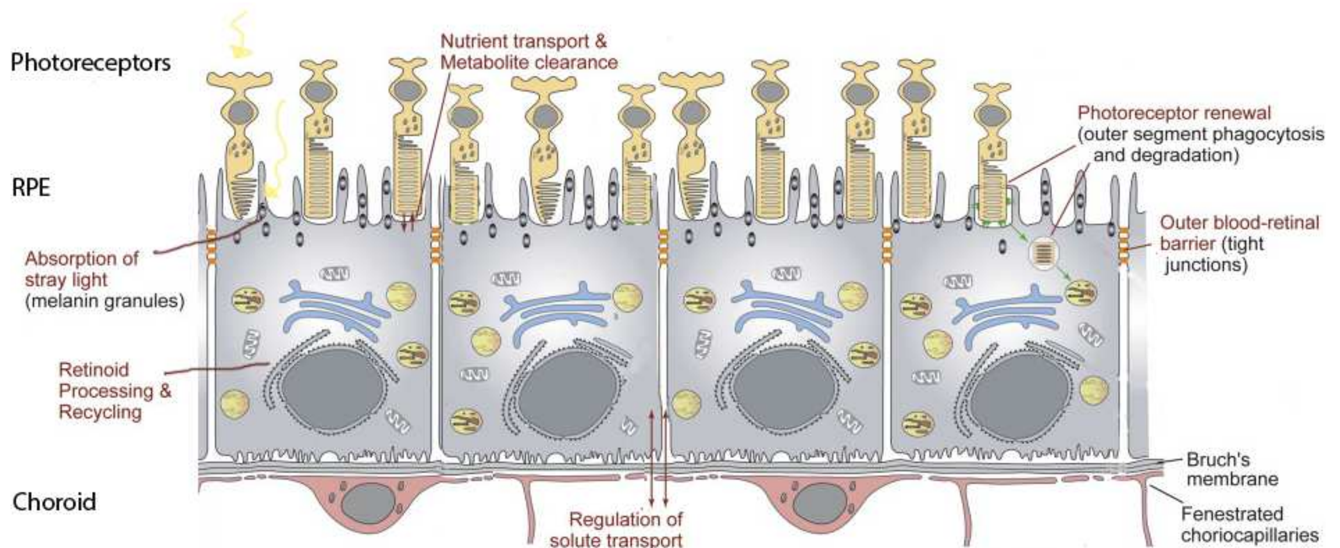

FIGURE 2. Major functions of the RPE (Modified from Lehmann et al. 2014)

2.3.3 Retinal degeneration

A collection of structural changes occur in aging eyes. Over time, overall thinning is apparent in the aged retina due to loss of neurons from all the neuronal cells and changes in photoreceptor cells. The RPE specifically is known to undergo several structural alterations. These alterations include changes in density and coactions with Bruch's membrane, loss of melanin granules, and even cell apoptosis (Bonilha 2008.) Dysfunction or deterioration of RPE is linked to degenerative retinal diseases, such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP) (Strauss 2005).

Age-related macular degeneration is a disease characterized by a breakdown of the macula. Macula is a relatively small area in the central retina that is responsible for the majority of useful photopic vision and almost 10 % of the entire visual field (Hageman 2014.) AMD occurs in two forms: neovascular or exudative (wet) and atrophic (dry) AMD. The rarer but more intense form of AMD, wet AMD, is characterized by abnormal growth of blood vessel from the choroid into the Bruch's membrane and RPE. Wet AMD covers approximately 20% of the AMD conditions. It results ultimately in severe, sudden visual loss by forming a scar in the macula. Dry AMD causes gradual loss of central vision due to the dysfunction or death of RPE and subsequent death of photoreceptor cells (Bonilha 2008.) AMD develops usually in later decades of life, but has been diagnosed in patients even in their forties. AMD causes loss of bilateral vision and is the leading cause of worldwide blindness in elder people (Chakravarthy et al. 2010.)

RPE degeneration is the most leading causes of blindness. Retinitis pigmentosa (RP) is the most common group of inherited eye disorders that are caused by damage in the retina. There are many variations of the disease, but common to all of them is retinal dystrophy caused by abnormalities of the photoreceptors or the RPE. This degeneration of retina is progressive and has no known cure (Jin et al. 2009.) Other typical retinal dystrophies include Stargardt's macular dystrophy, Best vitelliform macular dystrophy (BVMD,) also known as Best's disease, and diabetic retinopathy (Finnish Federation of the Visually Impaired, 2014.) High eye pressure as symptom of untreated glaucoma can cause blindness by damaging retinas sensor cells. Unlike many other retinal degeneration diseases, glaucoma can be effectively treated with medicine or surgery (Haug et al. 1999).

Since many diseases that cause retinal dystrophy are not currently treatable in other ways, cell replacement therapies are considered as a solution to these conditions. Treatment could be performed replacing damaged cells with healthy ones (Lund et al. 2006.) In RPE related diseases, clinical trials that utilize ESC-RPE cells in treatment of AMD are currently ongoing (Bharti et al. 2014) and so far the results have been promising (Schwartz et al. 2014). The cell replacement can be done by either direct injection of RPE cell suspension (Schwartz et al. 2012) or as transplant of RPE cell sheet (Cyranoski 2014a) into the subretinal space. Cell sheet grafts may be a better option, since with cell suspension injections involve many uncontrollable variables. When injected as suspension, the cells might fail to form a functional monolayer by not adhering to Bruch's membrane or by acquire an incorrect apical-basal orientation (da Cruz et al. 2007.)

In Japan, permission has been recently granted to use hiPSC-RPE in clinical trials (Cyranoski 2014a). The first treatment, executed after four days of the permission, was done by Masayo Takahashi, an ophthalmologist with years of study in the field of iPS-cells. Trial was performed with the use of hiPSC-derived RPE cells as a treatment of AMD (Cyranoski 2014b.)

2.4 Differentiation of human pluripotent stem cells into RPE cells

While hPSCs can differentiate to RPE cells, current differentiation protocols are inefficient and not optimal for clinical translation. The currently used protocols for RPE cell differentiation from hPSCs are mostly based on spontaneous differentiation that results in low yields (Klimayanska et al. 2004.) Phenomenon happens especially while deriving RPE cells from hiPSC lines (Rowland et al. 2013.) Effective culture methods and differentiation protocols are therefore needed and thus induction of differentiation can be used to guide differentiation towards defined cell type.

The differentiation of hPSC-RPE can be carried out in either adherent or EB-like culturing conditions. EB-like culturing is a widely used differentiating method (Idelson et al. 2009; Klimanskaya 2004; Klimanskaya 2006; Vaajasaari et al. 2011). As differentiation towards RPE cells progresses, pigmented areas start to develop on the EBs. These areas can then be selected and used to create monolayer cultures for further RPE enrichment and maturation

(Klimanskaya 2006.) Adherent differentiation can be used for generation of hPSC-RPE as well. Differentiation in adherent cell culture is usually carried out by removing the fibroblast growth factor (FGF) from the culturing medium, and stem cell colonies are let to overgrow and form a homogenous cell layer. Adherent cell culture could be suitable for hPSC-RPE differentiation, since RPE cell are epithelial cell that tend to grow in native conditions in monolayer (Vugler et al. 2008.) Adherent differentiation is used in hPSC-RPE culturing beside the EB-culturing (Carr et al. 2009, Klimanskaya 2006, Liao et al. 2010, Rowland et al. 2013, Vugler et al. 2008).

When subcultured, epithelial cells lose their cell-cell adhesion. These dissociated cells gain migratory and invasive properties through epithelial-mesenchymal transition (EMT) and temporary form a fibroblast-like morphology before they revert to their original RPE-like morphology. (Lee et al. 2010; Vugler et al. 2008). EMT is a process by which epithelial cells lose their differentiated phenotypes and transdifferentiate into motile mesenchymal-like cells (Tamiya et al. 2010). Propagated RPE cells also lose their pigmentation as melanocysomes are exocytoced or diluted by cell division. After RPE cells gain their polygonal uniformity, confluent cobblestone morphology, they start to gain back their melanin pigmentation (Klimanskaya 2006).

2.4.1 Spontaneous differentiation

Currently, the widely used derivation methods in development of RPE-cells from hPSCs favor spontaneous differentiation (Carr et al. 2013, Klimanskaya 2004 & 2006, Liao et al. 2010, Vaajasaari 2011, Vugler et al. 2008). When there are no known inductive agents in the culturing medium, ESCs tend to differentiate towards a neural pathway (Smukler et al. 2006). RPE being derivative of the neuroectoderm can be thus produced with this default differentiation pathway (Fuhrmann 2010).

When differentiating hPSC toward RPE cells, over time the RPE-like cells can be easily observed as they form distinguishable grey or black melanin pigmentation (Ross 2005). These areas on EB-like cell aggregates or adherent cultures can be then dissociated and placed in adherent culture to develop more homogeneous and mature cell cultures (Vaajasaari et al. 2011).

2.4.2 Induction toward RPE-cell differentiation

As the spontaneous differentiation protocols of hPSC-RPE result in low yields, new methods are needed to produce cells more efficiently for clinical applications (Klimayanska et al. 2006). The spontaneous differentiation tends to favor neuroectodermal differentiation, but it has also been shown that ESC-derived ectoderm can give rise to cells of both neural and epidermal lineage. Spontaneous differentiation results in heterogeneous cell populations, and since melanocytes are derivatives of the epidermis, they may be mistaken for RPE cells due to the fact that they also produce melanin and are therefore pigmented (Watabe & Miyazono 2009.) Effective culture methods and differentiation protocols are therefore needed and thus induction of differentiation can be used to guide differentiation towards defined cell type.

It has been suggested that the extracellular matrix can affect the cell differentiation, and therefore changes in ECM composition may improve the derivation of RPE-cells from pluripotent stem cells (PSC). In study published by Rowland et al. in 2013, several purified ECM proteins were tested for their ability to support iPSC-RPE differentiation and maintenance. The results showed that iPSCs differentiated on nearly all tested substrates developed melanin pigmented regions. However, although iPSC-RPEs cultured on the majority of the tested substrates expressed key RPE genes, only six substrates supported development of confluent monolayers with normal RPE morphology (Rowland et al. 2013.)

The differentiation can be directed also in other ways, where one method is a use of a specific culturing medium composition (Carret et al. 2013; Idelson et al. 2009). One method developed within the ophthalmology group was originally used in deriving corneal epithelial-like cells from hiPSCs (Mikhailova et al. 2014), but preliminary tests have also been conducted within the group in deriving hPSC-RPEs (data not published). Method replicates early developmental mechanisms in the eye by blocking the transforming growth factor β (TGF-β) and Wnt-signaling pathways with small molecule inhibitors while activating fibroblast growth factor signaling. This induction method is based on a use of basic fibroblast growth factor and two small molecules, SB-505124 and IWP-2. (Mikhailova et al. 2014.)

Basic fibroblast growth factor belongs to a family of polypeptide fibroblast growth factors that are involved in multiple functions. In normal tissue, basic fibroblast growth factor is

present in basement membranes and in the subendothelial extracellular matrix of blood ves-vessels (Ross & Pawlina, 2011.) Studies have shown that the bFGF may have a role for example in cell proliferation, survival, differentiation and motility (Watabe & Miyazono 2009). bFGF may also play a part in inducing of RPE proliferation and in the transdifferentiation of RPE cells into neural progenitor cells (Klimanskaya 2006). In the culture medium bFGF will cause EMT process while speeding up cell proliferation, providing in the end a formation of a confluent RPE monolayer (Tamiya et al. 2010).

Small molecule SB-505124 (2-(5-benzo[1,3]dioxol-5-yl-2-tert-butyl-3H-imidazol-4-yl)-6-methylpyridine hydrochloride) is a selective inhibitor of various transforming growth factor-β type I receptors. SB-505124 also blocks more complex endpoints of TGF-β action, as evidenced by its ability to abrogate cell death caused by TGF-β1 treatment (Byfield et al. 2003.) The TGF- β superfamily includes transforming growth factor (TGF), activins, and bone morphogenetic proteins (BMPs). They regulate a wide range of responses in the cell, including cell proliferation, differentiation, adhesion, migration, and apoptosis (Sporn & Roberts 1990, Byfield et al. 2003.)

IWP-2 (N-(6-Methyl-2-benzothiazolyl)-2-[(3,4,6,7-tetrahydro-4-oxo-3-phenylthieno[3,2-d]pyrimidin-2-yl)thio]-acetamide) is small molecule that acts as inactivator of Porcn function. IWP-2 also inhibits a signal transduction pathway called Wnt production, and blocks β-catenin accumulation. Wnt/β-catenin pathway affects the ability of stem cells to remain pluripotent and affects also the undifferentiated retinal progenitor by inducing the formation of the laminar structure of the retina (Mikhailova et al. 2014, Vugler et al. 2008.)

2.5 RPE-cell characterization

There are numerous different types of differentiated cells in human, and thus specific markers for certain cell type are needed to distinguish one cell from another. RPE-like cells can be distinguished by their specific morphology, expressed gene and protein markers and functionality.

2.5.1 Morphology

Most mammalian cells can be divided into three basic categories based on their morphology. These categories are fibroblast-, epithelial- and lymphoblast-like cells. Epithelial cells, like RPE, grow attached to a substrate in discrete patches and their shape is mostly polygonal with more regular dimensions (Life Technologies 2014.)

RPE-cells are bipolar cells which form a hexagonal cell monolayer. RPE is densely packed with pigment granules called melanocytes, which results in distinct brown or black color from melanin pigment. Hexagonal morphology of RPE resembles cobblestone or honeycomb type of formation (Maminishkis et al. 2006.) The RPE cells form tight, adherent and gap junctions between cells which create a blood-retinal-barrier between the retina and choriocapillaris (Strauss 2005).

2.5.2 Transepithelial resistance

Transepithelial resistance (TER) measures the epithelial layer's ability to resist current or diffusion of the ions across it. The measuring can be performed with either Ussing chamber or a voltohmmeter. TER depicts the development of epithelial barrier properties and tight junction formation between the cells (Carr et al. 2013.) Thus, TER can be used to analyze the formation of the RPE epithelial cell monolayer. Transepithelial resistance values measured from RPE-cells vary in different studies as shown in table 1. TER-values seem to be affected by the source of the cells, days of the culturing, measurement conditions and overall information of how the cells have attached to the surface and have been able to form resistant tight junctions. The selection of measurement system has also a great impact on the results.

Tight junctions, also known as zonula occludens, are found in the most apical component in the junctional complex between epithelial cells. Their function is to hold the cells tightly in close connection and also form a barrier that seals of the intracellular space. Zonula occludens strands correspond to the location of the rows of transmembrane proteins. Three major transmembrane groups found in zonula occludens are occludin, claudins are junctional adhesion molecules (JAM) (Ross & Pawlina 2011.)

TABLE 1. Transepithelial resistance of RPE cells in literature.

Reference	*Tissue*	*TER (Ω· cm^2)*
Frambach et al. 1990	Cultured human RPE	330
Quinn & Miller 1992	Human adult RPE	79±48
Quinn & Miller 1992	Human fetal RPE	206±151
Maminishkis et al. 2006	Human fetal RPE	501±138
Vaajasaari et al. 2011	putative hESC-RPE d30	6-32
Vaajasaari et al. 2011	putative hESC-RPE d60	145-188
Vaajasaari et al. 2011	putative hESC-RPE d90	311
Vaajasaari et al. 2011	putative hiPSC-RPE d30	6-10
Vaajasaari et al. 2011	putative hiPSC-RPE d60	23-38
Vaajasaari et al. 2011	putative hiPSC-RPE d90	74
Peng et al. 2014	Human fetal RPE	500 - 2000

2.5.3 RPE specific proteins

Immunofluorescence (IF) staining is a method that applies immunochemistry in a way to image specific substances. IF utilizes fluorophores and antibodies that are labeled with fluorescent dye (Wilson & Walker 2010.) Antibodies, or immunoglobulins, are proteins produced by plasma cells. They bind specifically to particular substances, referred as antigens (Ross & Pawlina 2011.) Antibodies can be used to visualize the distribution of a specific biomolecule with an antibody that recognizes the examined protein as antigen and binds to it. Fluorescent antibody complex can be formed by direct or indirect method (Wilson & Walker 2010.)

Direct method applies the antibody labeled with fluorescent dye directly to the antigen. Indirect method utilizes two different antibodies, where one recognizes the target protein and another contains the fluorescent label. In indirect method first the primary antibody that recognizes the antigen binds to the target biomolecule. Secondly, the secondary antibody with the fluorescent label binds to the primary antibody, forming this way a fluorescent complex. The fluorescence can be detected by fluorescent microscope after illuminating the sample with dye-specific excitatory wavelength (Wilson & Walker 2010.)

There are several known molecular markers of the RPE. Most distinct markers that RPE-like cells express are for example cellular retinaldehyde-binding protein (CRALBP), retinal pigment epithelium-specific 65 kDa protein (RPE65), bestrophin and pigment epithelium derived factor (PEDF) (Klimanskaya 2004.) Cultured RPE cells differ in gene expression

on different time points and hence functionality depends on cell passage and degree of epi-epithelial polarization (Mazzoni et al. 2014.) For example, in mature hESC-RPE cells, the expression of genes that are involved in visual cycle should be-up-regulated (Liao et al. 2010).

Cellular retinaldehyde-binding protein (CRALBP) is a protein that takes part in the visual cycle by functioning as a substrate carrier protein that modulates interaction of retinoids (11-cis-retinaldehyde). CRALBP is expressed in RPE cells, but is present also in Müller cells in the retina (Saari & Crabb 2005.) Retinal pigment epithelium-specific 65 kDa protein (RPE65) is involved in the visual cycle by taking part in the conversion of all-trans retinol to 11-cis retinal during photo-transduction. RPE65 also regenerates the visual pigment in photoreceptor cells. (Cai et al. 2009)

Bestrophin, a product of the Best vitelliform macular dystrophy gene, is commonly used molecule marker in RPE cells (Klimanskaya et al. 2004, Burke et al. 2008). As one of the roles of RPE is to transport nutrients and ions between the choriocapillaris and photoreceptors, the bestrophin, as a type of calcium-activated anion channel, plays important role specifically in cloride conductance and calsium signalling in the RPE cells (Hartzell et al. 2008.)

2.5.4 Proteins indicating the polarity of the cells

RPE cells are polarized epithelial cells. Transmembrane proteins expressed in RPE cells include proteins such as sodium-potassium-adenosine triphosphatase (Na^+/K^+ -ATPase) and tight junction proteins zonula occludens 1 (ZO-1) and claudin-19 (Lehmann et al. 2014, Rizzolo 2014).

Sodium-potassium- adenosine triphosphatase (Na^+/K^+ -ATPase) is an ion pumping protein complex located in cell membrane. This pump is essential for the maintenance of Na^+ and K^+ ion concentrations across the membrane. Energy for transepithelial transport is provided by Na^+/K^+ -ATPase on the apical membrane of RPE cells (Lehmann et al. 2014.)

ZO-1 is one of the zonula occludens proteins. ZO-1 is an important link in transduction of signals from all transmembrane proteins (Ross & Pawlina 2011.) It is a transmembrane protein that is located on the apical (cytoplasmic) side of RPE-cell (Rizzolo 2014).

Claudins are transmembrane proteins that form the backbone of zonula occludens strands. They form and regulate aqueous channels used in paracellular diffusion (Ross & Pawlina 2011.) Claudin-19 is a protein that is an essential component of human RPE tight junctions since patients with genetic defect of claudin 19 suffer profound ocular deficits (Konrad et al. 2006.) It has been shown that claudin-19 is the dominant claudin expressed in cultured RPE-cells and it is the only claudin that was identified as RPE-specific when compared with photoreceptors and choroid (Peng et al. 2011).

2.5.5 Other characterization methods

Along with other vital functions in the retina, phagocytic activity is a core trait of RPE cells *in vivo* and in culture. Continuous renewal of the light-sensitive outer segment portions of photoreceptors is critical for vision and thus the phagocytosis by RPE cells is highly regulated and specific for spent outer segment fragments. Phagocytic processes consists of three distinct phases; recognition/binding, internalization, and digestion. (Mazzoni et al. 2014.) RPE cells are the most actively phagocytic cells in the human body. One RPE cell supports 30–50 photoreceptors, which shed daily ~5% of their outer segment mass (Bonilha 2008). *In situ* analysis of RPE phagosomes has identified essential proteins of the RPE phagocytic machinery. These phagosomes in the RPE may be identified by different microscopy techniques based on their sizes and location in the RPE (Mazzoni et al. 2014.)

Immunolabeling any outer segment protein may aid in identification of RPE phagosomes, but rhodopsin is the most ideal to immunolabel, since it is the most abundant protein in rod outer segments. The RPE cells do not express rhodopsin themselves and rhodopsin content of the RPE therefore will correlate directly with relative phagocytic load. (Mazzoni et al. 2014.) POS phagocytosis can be also tested with specific assays and quantification method that utilize for example latex beads (Klimanskaya 2006).

3 AIMS OF THE STUDY

The objective of this Bachelor's thesis was to further optimize a directed RPE differentiation method developed in the Skottman Ophthalmology group by providing knowledge on the differences between the efficiency of the adherent and EB-like culturing methods in the differentiation of RPE-like cells from human pluripotent stem cells. When more efficient ways to produce RPE-cells are discovered, these methods could be utilized in tissue engineering applications. In the future, disorders caused by retinal degeneration could be cured by applying regenerative medicine, where the damaged RPE would be replaced by tissue graft derived from hPSCs.

The aim of the study was to compare adherent and EB-like differentiation methods in directed differentiation of hPSCs into RPE. The main points of interest were to evaluate the formation of melanin pigment producing cells between the differentiation methods and to analyze of the quality of the produced cells. The cell lines chosen for this study were a hESC-line that is commonly used in differentiation of hESC-RPE within the research group, and one human iPSC-line for comparison.

4 MATERIALS AND METHODS

The experiment consisted of two separate phases. The initial differentiation phase consisted of chemical differentiation induction in EB cultures followed by comparison of the EB versus adherent culturing methods by the rate of melanin pigmented cells produced. In the following phase, the produced pigmented cells were selected for subculturing and characterized by TER and immunofluorescence stainings to determine their barrier junction properties, differentiation state, polarity and expression of RPE typical proteins. Course of the experiments are described in table 2. Experiments were performed following good laboratory practice (GLP) whenever possible.

TABLE 2. The course of the study

Method	*Number of days*	*Introduction of the method*
Induction of differentiation	d0	Stem cells colonies were mechanically cut and the pieces were placed in low attachment 6-well plate for differentiation in induction medium. The colonies form a three-dimensional aggregate, called EB (figure 3.)
	d4	EBs are divided into two groups of equal size. One group continues to differentiate as EBs in suspension culture, the other group is transferred to collagen IV coated adherent culturing 6-well plate. The induction medium is replaced with basic cell culturing medium.
Subculture	~d40	The differentiated, RPE-like pigmented areas of cultures are mechanically selected and dissociated to single cell suspension. The cells are counted with hemocytometer and seeded to collagen IV coated 24-well plates and permeable (Polyethylene Terephthalate) PET-membrane inserts.
TER measurements	d40 + 5-7 weeks	TER is measured from PET-membrane cultures.
Immunostainings	d 40 + 5-7 weeks	The PET-membrane cultures are stained with RPE-spesific antibodies and imaged with confocal microscopy.

4.1 Cell culture

4.1.1 Human pluripotent stem cells

Cell lines used in this study were hESC line Regea 08/017 previously derived in research group's laboratory (Skottman 2010) and hiPSC line UTA.04511.WTs. Two different batches of both cell lines were used to evaluate the pigmentation rate. The pluripotent cell lines were handled in sterile conditions and cultured in humidified incubators (Thermo Electron Corp., Waltham, MA, USA) at +37 °C in 5% CO2 on mitotically inactivated (by mitomycin C) hFF (36,500 cells/cm2; CRL-2429; American Type Culture Collection, ATCC, Manassas, VA) in serum-free conditions (Vaajasaari 2010) and passaged with single cell enzymatic dissociation (SCED) –method.

Research group gets the embryos for derivation of hESC lines as a donation from couples that have gone through fertility treatments and signed an informed consent form. The surplus embryos are unfit to be used in the treatments and would have otherwise been disposed of. Group leader Heli Skottman has the approval of the National Authority for Mediolegal Affairs Finland to study human embryos (Dnro1426/32/300/05) and has the support of the Ethical Committee of the Pirkanmaa Hospital District (R05116) to derive, culture and differentiate hESC lines from surplus embryos (Skottman 2010.)

Human iPSC line used in this study is a wildtype line inducted with Sendai-virus method. Line is mainly used in BioMediTech research group led by Katriina Aalto-Setälä. Aalto-Setälä's group does research in the field of cell - and tissue technology, focusing on heart cells. The hiPSC-line is used as a control cell line in the studies and it is formed within University of Tampere from patient skin biopsy (Haponen 2014.)

Basic hPSC culture medium consisted of Knockout Dulbecco's Modified Eagle Medium (KO-DMEM) containing 20% KnockOut Serum Replacement (KO-SR), 2 mM glutamine mixture (GlutaMax), 0.1 mM β-mercaptoethanol, 1% Minimum Essential Medium nonessential amino acids, (all from Invitrogen, Carlsbad, CA) 50 U/ml penicillin/streptomycin (Cambrex Bio Science, Walkersville, MD), and 8 ng/ml human basic fibroblast growth factor (bFGF; R&D Systems Inc., Minneapolis, MN) (Vaajasaari 2010).

4.1.2 Differentiation and culture media

The basic differentiation culture medium (called DM-) is used normally in spontaneous differentiation within the research group (Vaajasaari et al. 2010). DM- consists of basic growth medium KO-DMEM that is supplemented with 15% KO-SR, 50 U/ml penicillin/streptomycin, 1% non-essential aminoacids, 1% GlutaMax and β-mercaptoethanol.

The differentiation of the cells was induced by adding basic fibroblast growth factor (human FGF-basic 50 µg, PeproTech) and small molecule selective inhibitors SB505124 and IWP-2 (both from Sigma-Aldrich, Sigma Life Sciences) to DM- medium, that was thus being called as DM+ medium. The induction method is based on knowledge provided previously in the research group (Mikhailova et al. 2014; unpublished data.) DM+ medium used in induction was changed 2-4 times during the first four days of differentiation. After the induction, cells were transferred to the DM- medium. DM- medium was changed three times a week.

4.1.3 RPE differentiation

The human pluripotent stem cells were grown with supportive feeder cell layer of human foreskin fibroblasts. For differentiation, the stem cell colonies were mechanically cut and undifferentiated areas selected from the culturing wells and passaged to 6-well plate (Costar multiwell plate, 6-well Ultra-Low attachment surface, Corning Incorporated) with differentiation medium DM+. The passaged colonies formed floating cell aggregates spontaneously as they were placed on the medium. The method is demonstrated in figure 3.

Directed differentiation by small molecule induction took place for four first days of the differentiation. On d4 the medium was changed to basic culturing medium DM- and the formed EB-like cell aggregates were either kept in the EB suspension culture or transformed to adherent culture conditions. Adherent culturing took place on 6-well plate (Costar multiwell plate, 6-well Corning CellBind Surface, Corning Incorporated.) The wells were coated with Collagen IV (Sigma-Aldrich) 5 µg/cm^2. EB-like cell aggregates attached to the coated bottom of the well and started to form an adherent culture.

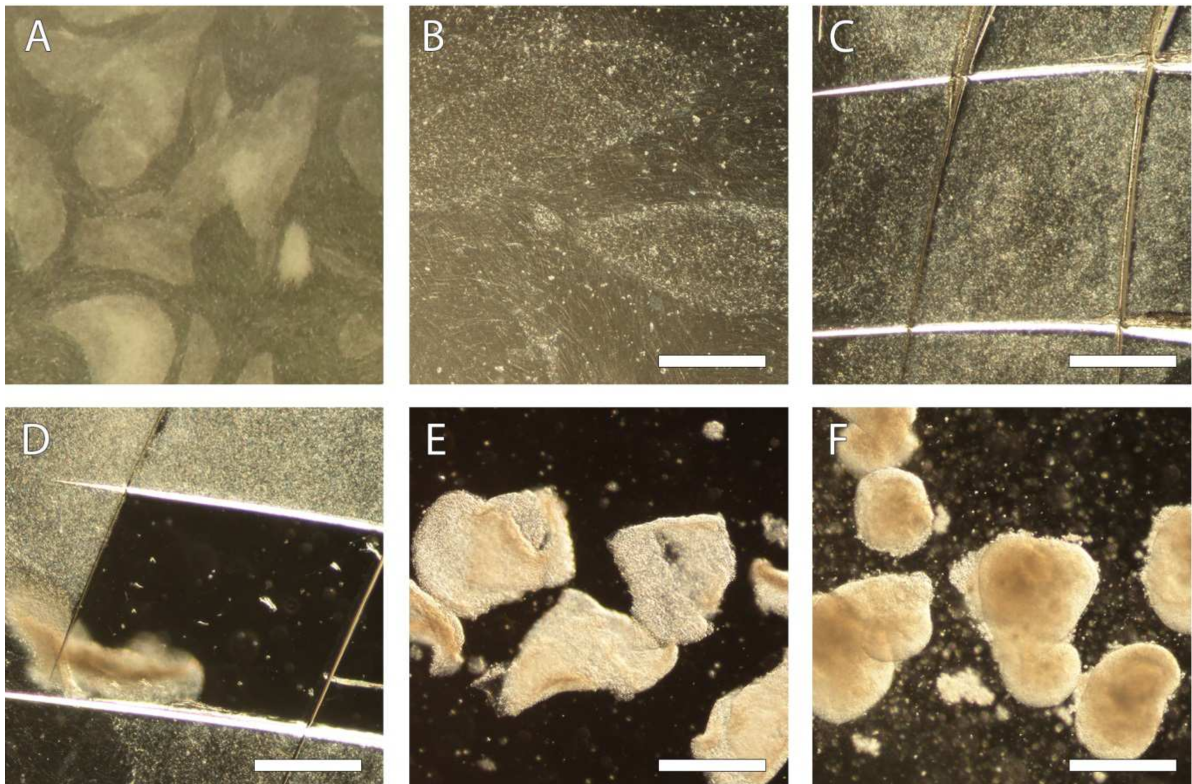

FIGURE 3. Overview of mechanical selection of stem cell colonies. (A) Stem cell colonies viewed by phase contrast microscope, picture taken through oculars. 1x magnification (B) Undifferentiated stem cell colonies on top of feeder cells. (C) & (D) Cutting was done by scalpel and colonies were unfastened from bottom of the well. (E) Colonies floating free in the medium (d0) (F) Colonies form EB-like cell aggregates within a day from the selection (d1) Pictures B-F magnification 4x, scale bar 200 μm.

The differentiation potentials of the adherent and EB-like culturing methods with hESC and hiPSC lines were analyzed by the appearance of the first pigmented cells emerging from each cell line. Observations were done five times a week with phase contrast light microscope (Nicon eclipse TE2000-5.) Pictures were taken in different time points to document the development of melanin pigmentation with camera attached to the microscope (Nikon Digital Sight DS-L5)

After 42 days of differentiation, pigmented areas were cut mechanically and passaged with SCED to Collagen IV coated 24-well plates and PET-membranes on culturing well inserts. The meaning of subculture was to gain more homogenous epithelial like RPE-cultures.

4.1.4 Subculture

For enrichment and maturation of the RPE-like cell produced, the areas with pigmented cells were chosen manually with a scalpel in sterile conditions and subsequently dissociated and passaged to a different well plate. Passaging was performed within d41-d42 of the initiation of differentiation. Usually longer differentiation develops better yield of pigmented cells, but in the limited timescale for the project, this step had to be performed with modest amounts of pigmented cell material.

After mechanical separation, cells were washed two times with Dulbecco's phosphate-buffered saline (DPBS.) A proteolytic enzyme with brand name TrypleSelect was used to dissociate the cells for 10-15 min at +37 C°. The cells were carefully triturated and put in DM- medium through 4.0 µm nylon cell strainer to gain a single cell suspension. Cell suspension was then centrifuged at 1500 rpm for 5 min. Medium containing TrypleSelect was removed and cells were resuspended to 1 ml of DM- and counted with hemocytometer. Reagents were manufactured by Invitrogen, Life Technologies.

The cells were plated either on collagen IV (5 µg/cm^2; Sigma-Aldrich)-coated 24 well plates (42 000 – 56 000 cells/cm^2; Costar multiwell plate, 24-well Corning CellBind Surface, Corning Incorporated) for pigmentation and morphology follow-up, or on permeable Collagen IV–coated PET-membrane inserts (100 000 – 200 000 cells/cm^2; Millicel 24-well hanging cell culture insert, 1,0 µm PET transparent, Millipore) for immunofluorescent stainings.

4.2 Reference material

To analyze the cells produced in this study, two hESC-RPE cell cultures were taken to the characterizations as reference material. The reference cells were produced within the group from the same hESC-line as the cells used in the study. Adherently differentiated hESC-RPE cells were compared to control cell cultures. Purpose of the reference material was to use them as control samples to see if there was a difference in the quality of the cells produced with induction and spontaneous differentiation methods. The controls were named based on their differentiation mediums DM- and DM+/DM- as M- and M+/M-, respectively.

The control cell M- cultures was produced with spontaneous differentiation process (method used previously in Mikhailova, 2011 and Vaajasaari et al., 2011) and the another control M+/M- represented a type of parallel sample by being derived with same directed differentiation method as the adherently differentiated hESC-RPE sample.

The comparison of the control cells and the adherently differentiated hESC-RPE cell sample is presented on table 3. The passage number is counted from each subculture of the originally differentiated RPE-like cells. Control M- cells were spontaneously differentiated in EB-form in DM- medium (passage +1), without small molecule or growth factor induction. After 58 days of differentiation, pigmented areas were cut mechanically (passage +2), and plated to collagen IV coated 24-well plates without SCED treatment as cell clusters. After RPE-like cells had gained confluence for 68 days on passage +2, they were passaged to collagen IV coated PET-membrane on culturing well inserts (passage +3).

The control cells called M+/M- were differentiated with same method as study inserts, first four days on DM+ and then DM- medium. Only difference is on that these cells were plated to inserts on their passage +3 after they had formed confluent RPE-like colonies on 24-well plate in passage +2. Differentiation in first passage took 47 days, and maturation on second passage 36 days.

TABLE 3. Comparison of the sample cells and the control cells

Type	*(name)*	*Diff. method*	*Medium*	*Diff. culturing condition*	*Passage on insert*
Control	M-	spontaneous	DM-	EB	+3
Control	M+/M-	directed	DM+/DM-	adherent	+3
Sample	hESC adherent	directed	DM+/DM-	adherent	+2

4.3 Transepithelial resistance

Transepithelial resistance was measured from the sample and control culture inserts. Measurement devise used in the study was Millicell Electrical resistance system 2 voltohmmeter. The measurement electrodes were disinfected by keeping them ~15 minutes (min) in 70% ethanol. Electrodes were let to dry in the room temperature (RT) before they were used in the measurements. For stabilization, the electrodes were rinsed in two different falcon tubes that contained PBS before every measurement. The PET-membranes were in the DM- medium during the measurements. Measuring was performed 2-3 times to each sample and a blank control. The resistance measurements were averaged and the value of the blank was subtracted from the sample-well resistance.

4.4 Immunofluorescence staining

Immunofluoresce stainings were performed to detect expression of commonly used RPE-spesific marker proteins by the cells. Imaging with confocal microscope enabled the analyses of the polarization of the marker proteins.

The cells were washed three times with PBS and fixed with 4% paraformaldehyde (pH 7.4; Sigma-Aldrich) for 10 min at RT following repeated washings with PBS. The cells were made permeable by incubating in 0.1% Triton X-100 in PBS (Sigma-Aldrich) at RT for 10 min. The unspecific binding sites were then blocked by treating cells with 3% BSA (BSA; Sigma–Aldrich) at RT for 1 hour (h) and the PET-membranes were cut to three or four pieces. Samples were incubated overnight at +4 °C with primary antibodies shown in table 4. Dilutions were prepared in 0.5% BSA in DPBS. Cells were washed three times with PBS and labeled with secondary antibodies shown in table 5. Secondary antibodies were diluted in 0.5% BSA-PBS and incubated 1.5 h at RT following repeated PBS washings. The nuclei were stained with 4´, 6´ diamidino-2-phenylidole (DAPI) included in the mounting media (Vector Laboratories Inc.)

TABLE 4. Primary antibodies

Primary antibodies	*Manufacturer / Product code*	*Dilution*
mouse anti-Na^+/K^+ -ATPase	Abcam / ab7671	1:200
rabbit anti-bestrophin	Abcam / ab14928	1:200
mouse anti-RPE-65	Millipore / MAB5428	1:200
rabbit anti-ZO-1	Life technologies / Novex 61-7300	1:200
mouse anti-CRALBP	Abcam / ab15051	1:500
mouse anti-claudin-19	Millipore / MAB6970	1:100

TABLE 5. Secondary antibodies

Secondary antibodies	*Manufacturer / Product code*	*Dilution*
donkey anti-mouse IgG A488	Life Technologies / Alexa Fluor A-21202	1:800
goat anti-rabbit IgG A568	Life Technologies / Alexa Fluor A-11011	1:800

The control cell cultures M- and M+/M- on PET-membranes were stained using all of the primary antibodies listed in table 4. Due to the poor outcome, the hESC-RPE cell cultures produced in this study were stained using only anti-Na^+/K^+ ATPase, anti-BEST, anti-CRALBP and antiZO-1 leaving out the RPE65, and the other tight junction protein, anti-claudin-19, which show usually only in well matured RPE-like cells.

At time of the immostainings, the cell cultures control DM-, control DM-/DM+ and hESC-RPE sample had been on the culturing inserts for 106 days, 91 days and 40 to 49 days, respectively. Images were taken with an LSM 700 confocal microscope (Carl Zeiss, Jena, Germany) using a 63×oil immersion objective.

5 RESULTS

The actual time points for each step of the experiment are shown up in the figure 4 and are discussed in detail in the following chapters.

	Induction		Pigmentation	Subculture	TER-measurements	IF stainings
Number of days	d0	d4		d41-d42	d (42 + 36)	d(42 + 40-49)
hESC (08/017)	EB	→ EB	d18 - d22	→ *		
		→ adherent	d17 - d19	→	→	→
hiPSC (04511.WTs)	EB	→ EB	d26 - d28			
		→ adherent	d17 - d19	→ *		
Medium	DM +	DM -				

FIGURE 4**.** Course of the practical work. *Some of the cells were passaged only on 24-well plates, but they did not manage to form a subculture.

5.1 Appearance of pigmentated cells

Appearance of pigmented cells was followed daily, excluding weekends. The formation of the pigmentation is presented in figure 5. The observations for each cell lines and culturing conditions are shown on table 6. The comparison of cell differentiation in different culture conditions can be seen in the images in figures 6 and 7. Figures 6 and 7 represents the differentiation of hESC line and hiPSC line on the 39th day of differentiation, respectively. The differentiation potentials of the culturing methods can be compared by the amount pigmented cells emerging from each cell line.

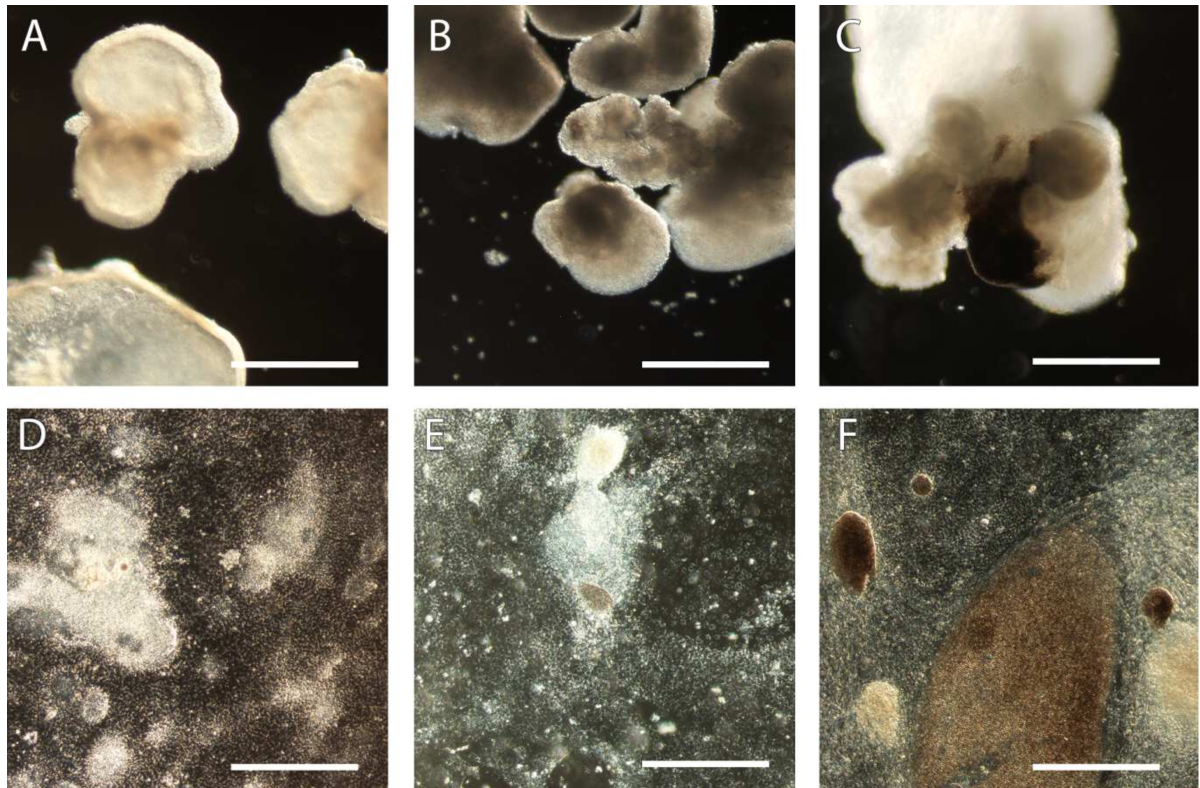

FIGURE 5. Melanin pigmentation development (A) Suspension culture, no pigmentation on EBs on (hESC, d11) (B) First sign of pigmentation on EB (hESC, d21) (C) Developed pigmentation on EB, (hESC, d35) (D) Adherent culture, no pigmentation (hESC, d6) (E) First signs of pigmentation on adherent culture (hESC, d21) (F) Well pigmented adherent culture (hESC, d35.) Magnification 4x, scale bar 200 µm.

TABLE 6. Summary of the pigmentation formation

Cell line / conditions	*Pigmentation first shown*
hESC adherent	d17-d21
hESC EB	d18-d22
hiPSC adherent	d17-d19
hiPSC EB	d26-d28

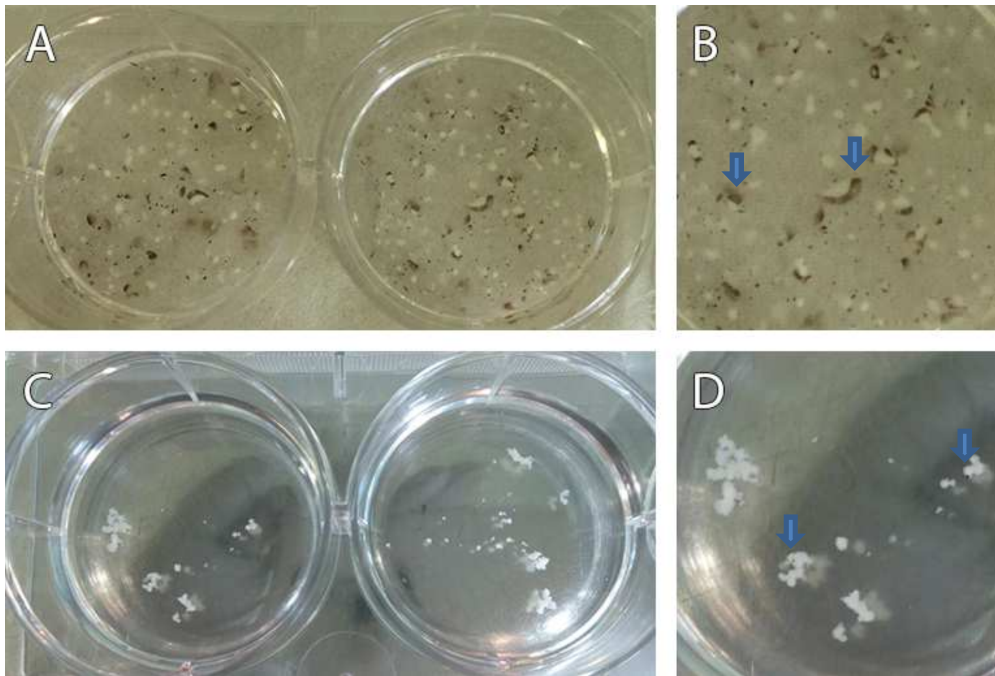

FIGURE 6 hESC line, day 39 of differentiation (A) adherent culturing conditions, overview. (B) adherent culturing conditions, close-up. (C) EB-like culturing conditions, overview. (D) EB-like culturing conditions, close-up. Some of the pigmented areas are marked with arrows.

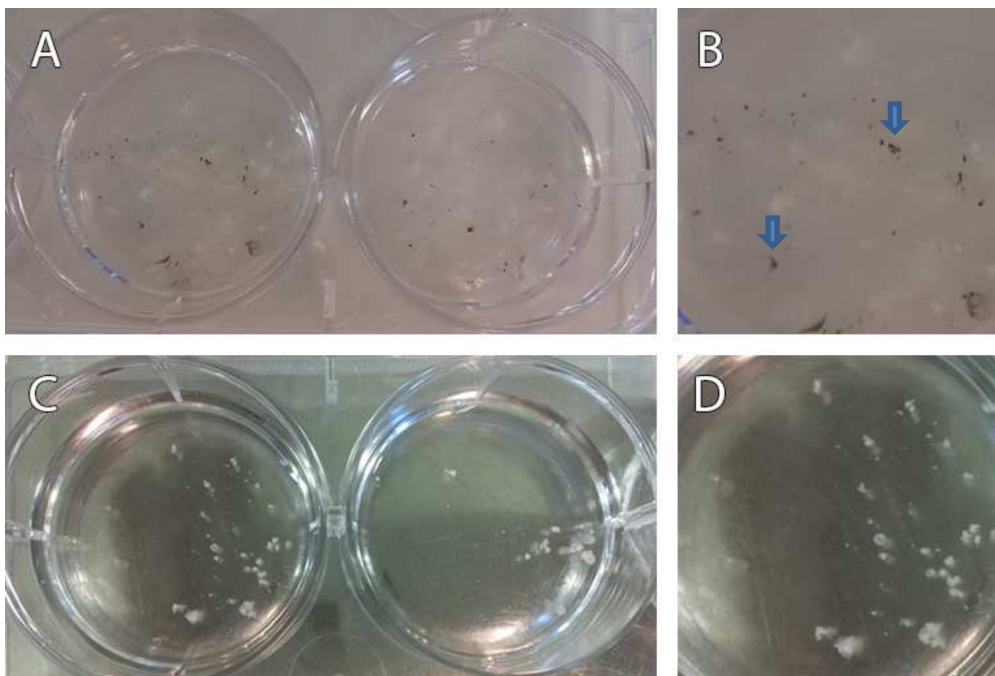

FIGURE 7. hiPSC line, day 39-40 of culturing. (A) adherent culturing conditions, overview. (B) adherent culturing conditions, close-up. (C) d40 EB-like culturing conditions, overview, (D) d40 EB-like culturing conditions, close-up. Some of the pigmented areas are marked with arrows.

5.2 Characterization

5.2.1 Morphology

Only hESC-RPEs that were differentiated with adherent culturing gave enough yield to be passaged successfully to 24-well plate and hanging culture inserts. EB-like cultured hESCs and adherent cultured hiPSCs were also plated to 24-well plate, but the passaged cell did not proliferate (data not shown.) After plating, the hESC subculture went through epithelial-mesenchymal transition (EMT) before reforming into RPE-like pigmentated cells of cobble-stone morphology (figure 8.)

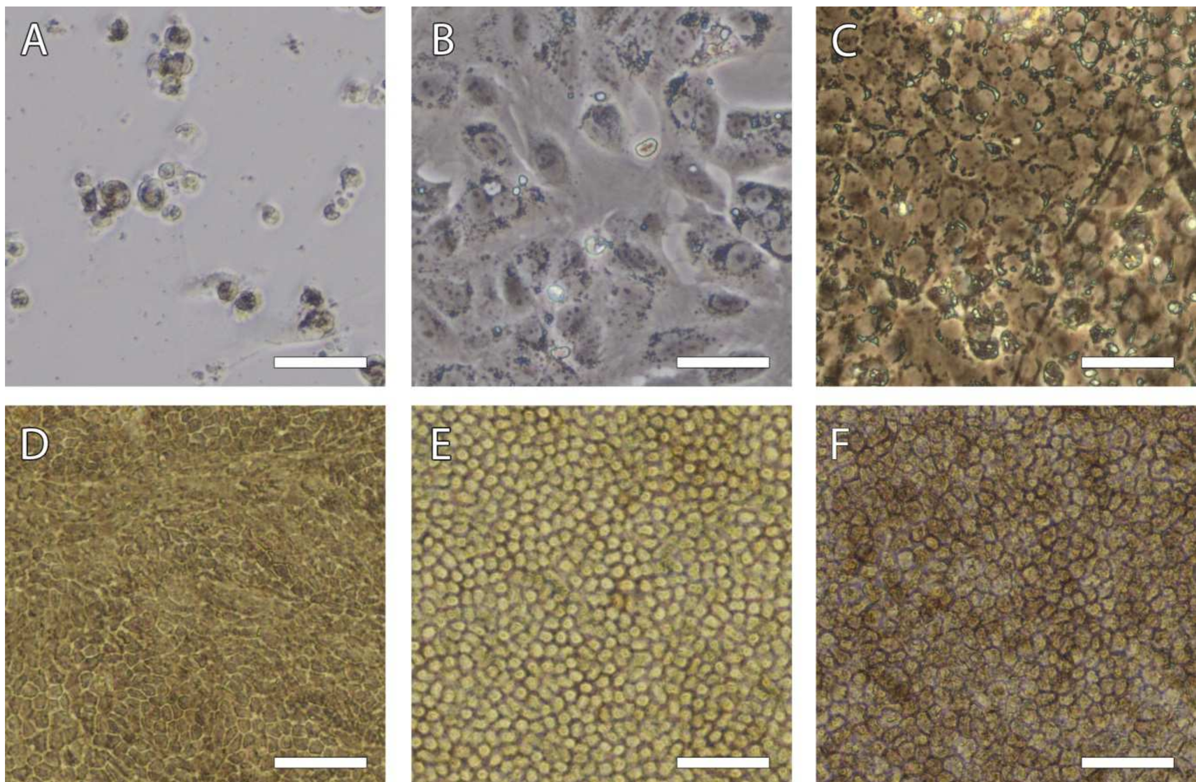

FIGURE 8. Plated adherent cultured hESC-RPE cells in different time points. (A) On d1 cells were attaching to the surface (B) on d6 cells were going through the EMT, fibroblast-like morphology (C) on d12 cells had proliferated and started to reach confluency (D) Cells started to form a cobblestone-like morphology during d18 but were not yet fully confluent (E) on d23 the cells had gained confluent morphology (F) d37 RPE-like cells had gained back their original appearance. Magnification 10x, scale bar 50 µm.

5.2.2 Transepithelial electric resistance of the PET-membranes

Transepitheliel electric resistance was measured from the PET-membrane inserts shown in figure 9 to analyze the barrier properties of the cell monolayer. Control insert without cells was used to reduce the background produced by the PET membrane. The measured TER was converted to unit area resistance with the membranes surface area. Results are shown in table 7.

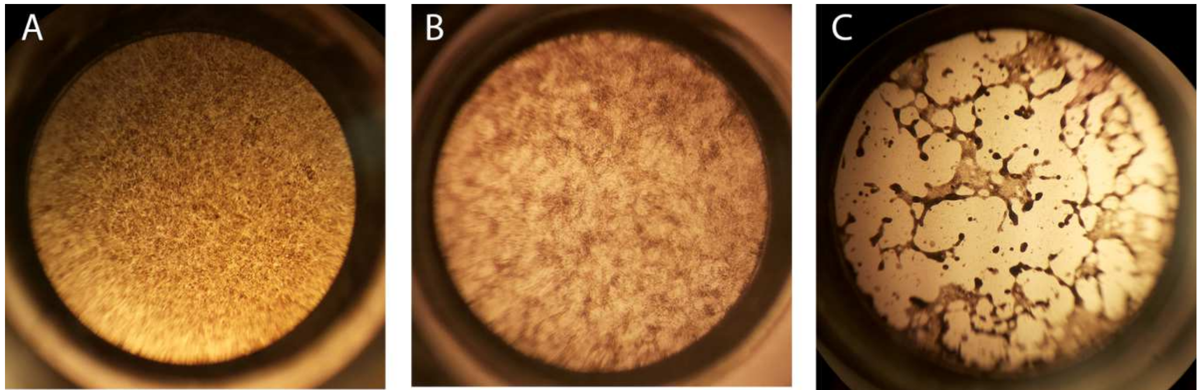

FIGURE 9. PET-membrane cultures before TER measurements. (A) M- control (d92 on insert) (B) M+/M- control (d78 on insert) (C) hESC adherent induction insert (d36 on insert.) Pictures taken through phase contrast microscopes oculars, magnification 2,5x. No scale bar provided.

TABLE 7 Results of the TER measurements

Cell sample	*Culturing days on insert*	*TER ($\Omega \cdot cm^2$)*
M- control	d92	420
M+/M- control	d78	115
hESC adherent	d36	2

5.2.3 Immunofluorescence staining

The immunofluorescence stainings were performed when the adherently differentiated hESC-RPE cells had been 40 to 49 days on the permeable PET-membrane inserts. Despite poor condition of the formed epithelial monolayer, there were nevertheless decent areas to analyze with immunofluorescense stainings. Nuclei stainings with DAPI were observed with microscope, but the observations did not show in the pictures since the dark melanin pigmentation concealed the DAPI in the cells. The immunostaining for CRALBP, ZO-1, Na^+-K^+-ATPase and bestrophin were positive for the hESC-RPEs developed in this study. The stainings for the control samples were not essential for this thesis and are not therefore discussed in detail in the results.

Figure 10 shows images taken from cells stained with CRALBP (A488, cyan green) and ZO-1 (A568, reddish orange) antibodies. Nuclei staining DAPI (cyan) cannot be recognized, due to the concealment by dense melanin pigmentation. The phenomena is relatively common with highly pigmented RPE-like cells. Blue dominating in the images is from background fluorescence from the PET-membrane. It should be noted that the images taken from the sample cover only a small area. The overview of the sample illustrated relatively high variation on different parts of the sample.

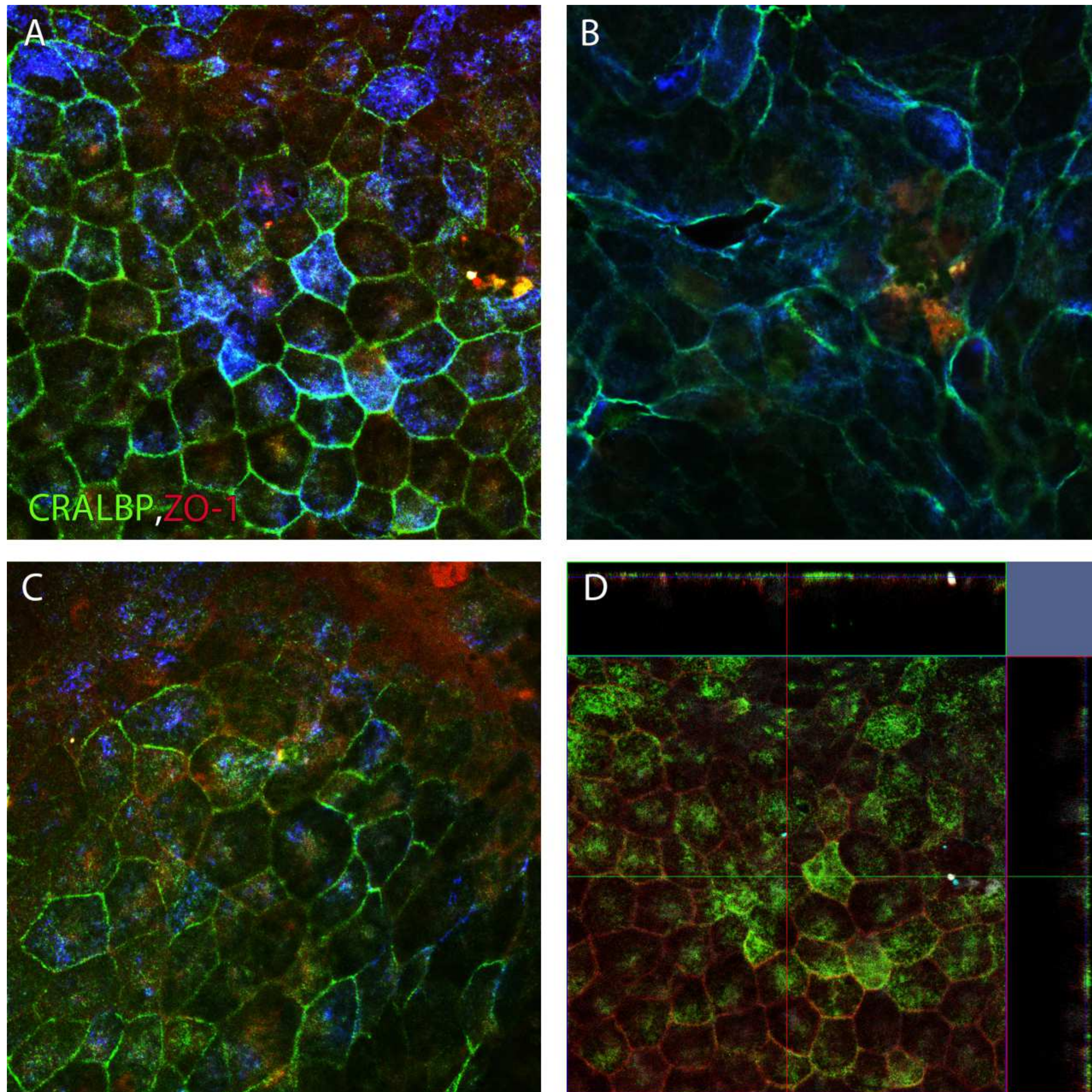

FIGURE 10. Images taken with confocal microscopy, magnification 63x (oil immersion) of CRALBP and ZO-1 immunostained hESC samples. Bluish/magenta color is mainly from the fluorescent PET-membrane, nuclei staining DAPI is colored with cyan (A) Parts of the sample that demonstrated a beautiful cobblestone morphology. (B) Demonstration from the distressed parts of the sample. (C) Variable cell morphology. (D) Confocal stack image that shows part of the sample where ZO-1 can be seen well on cell junctions and CRALPB in the cytoplasm.

Images in the figure 11 are from samples that were immunostained for Na^{+}/K^{+}-ATPase (A488, cyan green) and bestrophin (A568, reddish orange.) Images are formed with confocal image; image in the middle is the most describing overview of the sample, and the slides on top and left are cross-sections formed from stack of images taken from the different depths of the sample. The DAPI-stained nuclei can be seen as cyan colored with some cells on the area on the left image 11B.

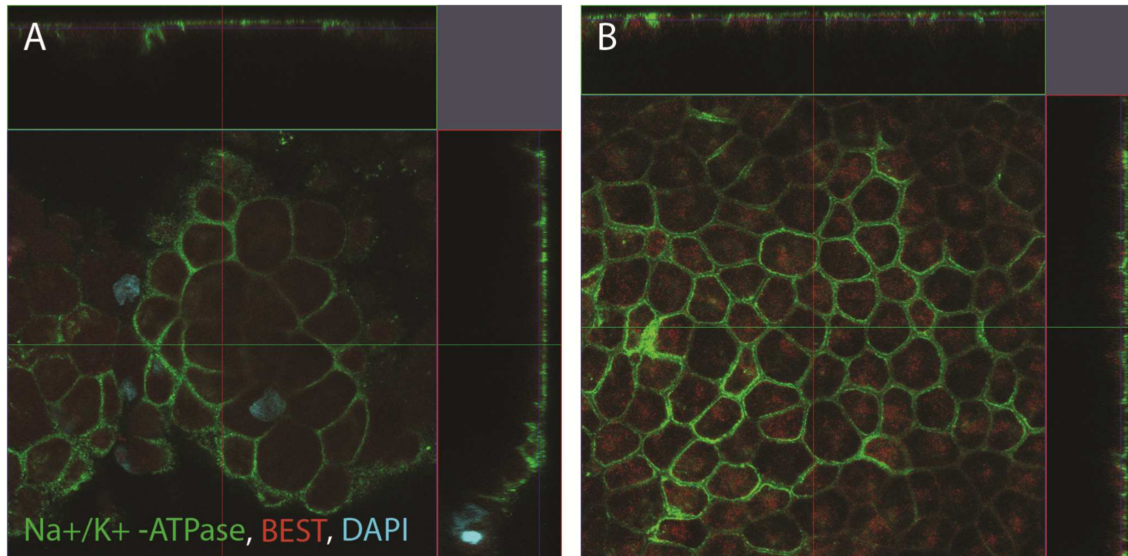

FIGURE 11. Confocal stack images from immunostained hESC-RPE samples, magnification 63x (oil immersion.) Na^+/K^+-ATPase can be seen as cyan green (A488) and bestrophin as reddish orange (A568.) DAPI stained the cell nuclei as cyan blue. Na^+/K^+ is polarized on the apical cell membrane. A) Stratified part of the sample, image taken first batch of the hESCs, (7 weeks on inserts) B) Cells formed RPE-like monolayer, part found on the second batch of hESCs patches (5 weeks on inserts.)

The immunocytochemical analyses of the control sample M- showed that the cells were stressed probably duo to cellular senescence which often occurs in prolonged cell culture. Beside these results, other comparable immunofluorescence stainings from cells with same differentiation method can be found in the research of Vaajasaari et al. 2011. Some of the data collected from control M+/M- can be seen in the appendix 1. Condition of the observed areas indicated that the cells had possibly been detached during the stainings. The results of the stainings were in conformity with the outcome of the sample hESC-RPE. The control M+/M- had two additional stainings with antibodies for RPE65 and claudin-19. These are included in appendix 1. Since RPE65 was almost nonexistent, the staining was concluded as negative. Immunostaining for claudin-19 was positive, with higher intensity on proximity of certain individual cells.

6 DISCUSSION

The objective of the thesis was to further optimize a directed RPE differentiation method by comparing adherent and EB-like culturing methods. Differentiation of hPSCs into RPE-like cells was first determined by observation of melanin pigmentation development. In hESC line the pigmentation was detected a day apart in both adherent and EB-like cultures, but in the end, only adherent culture developed enough pigmented cells for subculture. With differentiating hiPSCs, the EB-like cultures had delayed formation of pigmentation compared to adherent cultures. Differentiation in EB-like suspension cultures did not show improvement even when cultured further from the first timepoint of around d40 (data not shown.)

The adherent hESC-RPE cultures were selected and passaged successfully forward to 24-well plates and PET-membrane inserts, and after a 40 to 49-day maturation period, analyzed by means of immunofluorescent stainings. Based on the results provided in this study, adherent differentiation conditions may turn out to be more preferable for hPSC-RPE when the differentiation has been directed with induction. Also, in the native environment epithelial RPE monolayer grows adherently attached to Bruch's membrane.

Induction of differentiation did not seem work well for EB-like aggregates, as can be approximated from figures 6 and 7. The first pigmentation of cells in adherent hESC cultures (d17-d22) seemed to appear just slightly faster than in the spontaneous EB-like cultures developed for another studies in the same laboratory; these spontaneously differentiated hESC of the same particular cell line started to develop pigmentation on average on day 23 (the mean results with five different hESC passages, data not shown.)

One of the two hiPSC batches yielded notably small amount of differentiated, melanin pigmented cells (data not shown.) The better success with the second batch indicated that the problem was more likely with the quantity of the cells than the quality, since in the beginning of differentiation the first hiPSC batch had significantly less stem cell colonies to even begin to differentiate. However, with these particular cell lines the hiPSCs gave poorer yield of RPE-like cells than the hESCs. The hiPSC-RPE cells did not live through of the subculturing and therefore, there are no follow-up results for the cell line.

The differentiated melanin pigmented cells were characterized to identify if they resembled RPE-like cells. Since in the time limits of the study only the adherent differentiation for hESC provided sufficient yields, the quality of the hPSC-RPE cells was thus determined only for these cell samples. The cell seedings on the porous PET-insert did not form an epithelial-like monolayer, but instead a network-like formation of stratified cells. This is not expected behavior with RPE-like cells, but the phenomenon has been noted also in another study (Rowland et al. 2013.) Despite these problems, the immunostainings turned out to be successful and gave informative results.

TER interprets the confluence and barrier properties of a cell monolayer. Some of the reference values for RPE monolayer presented in literature can be found on table 1 on page 24. The TER measurements for control sample M- showed of a formation of a proper monolayer, while the values were slightly lower for the control M+/M-. As for the hESC-RPE sample, it can be seen from measured TER values and even from figure 9 on page 40, that the cells produced in this study did not form a monolayer that could have had any kind of barrier properties. However, regarding the immunofluorescence stainings, the quality of the hESC-RPE cells was still relatively good.

The immunofluorescence staining provided information about how the cell expressed known markers for RPE-like cells. The existence and location of the RPE markers depicted the maturity and development of the RPE-like cells. The sample cells that were differentiated with induction method were rather matured, despite their short differentiation and culturing period (Carr et al. 2014.) Early maturation could have been resulted with the induction used in the beginning of the differentiation process.

RPE marker protein CRALBP was found in the cytoplasm and in close proximate of cell membrane (10D, confocal image.) The faintly showing junction protein ZO-1 was found mainly in the nucleus of the cells. In some parts of the sample, ZO-1 could be also seen in the cell-cell junctions suggesting formation of tight junctions. ZO-1 was positive on all over the sample, but higher intensity of fluorescence could be observed between some certain cells shown outside of the area in figure 10. The cells show a cobblestone-like morphology. Some stretching of the cells can be seen on images 10B and 10C. Most of the images were taken from the prominent parts of the sample that formed epithelial-like monolayer.

In confocal stacks on figure 11A and 11B, Na^+/K^+-ATPase was located on the apical side of the cells membrane. Normally bestrophin should be found on the basal membrane on matured RPE-cells. However, as seen more properly on the image 11B, bestrophin can also be found elsewhere, in nucleus and cytoplasm, when the cells are still relatively unmatured. Morphology of the cells is relatively good, but the undesirable formation of stratification can be seen especially on figure 11A.

The cell monolayer was relatively well formed in the control culture M+/M-, but after the immunostainings, microscope-examination revealed that the monolayer was destroyed on some areas and cells were detached from most parts of the membrane. This suggests that the cell were not attached properly to the basal membrane during the culturing period. The same phenomena could have appeared in the hESC-RPE sample in the formation of the stratified, network-like growth.

Reasons for the problematic development of the cell monolayer could be caused by many different variables. One difference between the hESC-RPE sample and M+/M- control was that the sample cells were plated straight from the differentiation stage. Thus, the sample hESC-RPE cells did not have opportunity to form a subculture of homogenous colonies, which could have yielded purer RPE-like populations (Vaajasaari et al. 2011.) More likely, the problems could be with the insert culturing on a PET-membrane, since the seeding to 24-well plate turned out fine as can be seen on figure 8 on page 39.

The control culture M- with spontaneous induction developed the finest monolayer of the characterized cells, which proposes influence of the executed induction. It should also be noted that in the time of the study, collagen IV coatings gave varied development of monolayer in other cell cultures developed in the laboratory (data not shown.) It has been shown that the coating substance has an impact in the formation of the confluent monolayer (Rowland et al. 2013.)

In conclusion, the results found in this study suggested that TER is not by itself a reliable measurement of the development state of the RPE-like cells. Immunostainings revealed that the cells were polarized resembling RPE cells by expressing the specific molecular markers. The marker proteins were not in all cases yet located where they are normally expressed in mature native RPE-cells.

The results provided in this study suggest that for specific type of directed differentiation of hPSCs into RPE, the adherent culture method gives significantly better yield than the EB-like culture method. The induction seems to work with both hESCs and iPSCs, but the yield tends to differ. It has been shown in other studies that iPSCs and hESCs show variability in their propensity to generate viable RPE cells (Buchholz et al. 2009; Rowland et al. 2013; Vaajasaari et al. 2011).

To determine if this culturing method combined with directed differentiation could be promising way to derive hPSC-RPE cells, further research is needed on the subject with different cell lines and bigger cell amounts to compare more closely on the yields and efficiency with the spontaneous culturing method. Results from the immunostainings suggest that the differentiation method used in this provided RPE-like cells that were yet completely mature in the given time, but promisingly well developed. Functionality of the RPE-like cells could be shown with for example by testing the cell's ability of phagocytosis of POS.

The problems with monolayer development on inserts and thus effects in the TER values suggest a problem in the attachment of the cells on the surface. The main causes of this could possibly be the coating of the surfaces by collagen IV, the quality of PET-membranes or the passage of the differentiated cells. It is necessary to determinate the main source of problematic formation of monolayer for the development of cell cultures in the future. It should be kept in mind that hPSCs and their differentiation are very delicate biological processes, and even minor variables, such as subtle changes in culturing media or basal membrane, could possibly affect in drastic measurements.

Research aims were achieved to the extent that there were found notable difference in the yield between the cell culturing methods. Based on the immunostaining results, adherent culturing method with directed differentiation produced RPE-like cells in relatively short time period and could be a promising new method to develop RPE-cells for future applications.

REFERENCES

Araki, R., Uda, M., Hoki, Y., Sunayama, M., Nakamura, M., Ando, S., Sugiura, M., Ideno, H., Shimada, A., Nifuji, A. & Abe, M. 2012. Negligible immunogenicity of terminally differentiated cells derived from induced pluripotent or embryonic stem cells. Nature. Vol. 494, pp. 100–104. 02/2013.

Bharti, K. , Rao, M., Hull, S.C., Stroncek, D., Brooks, B.P., Feigal, E., van Meurs, J., Huang, C.A. & Miller, S.S. 2014. Developing cellular therapies for retinal degenerative diseases. Investigative Ophthalmology and Visual Science. Vol. 55(2), pp. 1191-120. 02/2014.

Bonilha, V.L. 2008. Age and disease-related structural changes in the retinal pigment epithelium. Clinical Ophthalmology. Vol. 2(2), pp. 413–424. 06/2008.

Booij, J.C., Baas, D.C., Beisekeeva, J., Gorgels, T.S. & Bergen, A.A. 2010. The dynamic nature of Bruch's membrane. Progressive Retininal Eye Research. 01/2010.

Buchholz, D.E., Hikita, S.T. & Rowland, T.J.. 2009; Derivation of functional retinal pigmented epithelium from induced pluripotent stem cells. Stem Cells. Vol 27(10), pp. 2427–2434. 08/2009.

Burt, R., Loh, Y., Pearce, W., Beohar, N., Barr, W., Craig, R., Wen, Y., Rapp, J. & Kessler J. 2008. Clinical Applications of Blood-Derived and Marrow-Derived Stem Cells for Nonmalignant Diseases. Clinical review. The Journal of the American Medical Association Vol. 299(8), pp. 925-936. 02/2008.

Cai, X., Conley, S.M. & Naash, M.I. 2009. RPE65: role in the visual cycle, human retinal disease, and gene therapy. Opthalmic Genetics. Vol. 30(2)m pp. 57-62. 06/2009.

Carr, A.J., Smart, M., Ramsden, C., Powner, M., da Cruz, L. & Coffreu P. 2013. Development of human embryonic stem cell therapies for age-related macular degeneration. Review. Trends in Neurosciences. Vol. 36(7), pp. 385-395. 07/2013.

Carr, A.J., Vugler, A.A., Hikita, S.T., Lawrence, J.M., Gias, C., Chen, L.L., Buchholz, D.E., Ahmado, A., Semo, M., Smart, M.J., Hasan, S., da Cruz, L., Johnson, L.V., Clegg, D.O. & Coffey, P.J.. 2009. Protective effects of human iPS-derived retinal pigment epithelium cell transplantation in the retinal dystrophic rat. PLoS One. Vol. 4(12). 12/2009.

Chakravarthy, U., Evans, J. & Rosenfeld, P.J. 2010. Age related macular degeneration. BMJ. Vol.340, pp. 526-530. 02/2010.

Chen, B., Dodge, M.E., Tang, W., Lu, J., Ma, Z., Fan, C.W., Wei, S., Hao, W., Kilgore, J., Williams, N.S., Roth, M.G., Amatruda, J.F., Chen, C. & Lum, L. 2009. Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. Nature chemical biology. Vol. 5(2) , pp. 100-107. 02/2009.

Curcio, C.A. & Johnson, M. 2013. Structure, Function and Pathology of Bruch's Membrane. Retina, 5th Edition. Saunders, Philadelphia. pp. 465 – 481. http://www.bme.northwestern.edu/docs/markj/Curcio2013.pdf

Cyranoski, D. 2014a. Next-generation stem cells cleared for human trial. Nature News, 10 September 2014, updated 12 September 2014. http://www.nature.com/news/next-generation-stem-cells-cleared-for-human-trial-1.15897. Cited 13.09.14.

Cyranoski, D. 2014b. Japanese woman is first recipient of next-generation stem cells. Nature News, 12 September 2014. http://www.nature.com/news/japanese-woman-is-first-recipient-of-next-generation-stem-cells-1.15915?WT.mc_id=FBK_NatureNews. Cited 13.09.14.

DaCosta Byfield, S., Major, C., Laping, N.J. & Roberts, A.B. 2004. SB-505124 is a selective inhibitor of transforming growth factor-beta type I receptors ALK4, ALK5, and ALK7. Molecular Pharmacology. Vol. 65(3), pp. 744-752. 03/2004.

da Cruz, L., Chen, F.K., Ahmado, A., Greenwood, J. & Coffey, P. 2007. RPE transplantation and its role in retinal disease. Progressive Retinal Eye Research. Vol. 26(6), pp. 598-635. 11/2007.

Evans, M.J. & Kaufman, M.H. 1981. Establishment in culture of pluripotential cells from mouse embryos. Nature. Vol. 292(5819), pp. 154-156. 07/1981.

Finnish Federation of the Visually Impaired. Yleiset silmäsairaudet. www.nkl.fi/fi/etusivu/nakeminen/yleiset_silmasairaudet. Cited 29.7.2014.

Frambach, D.A., Fain, G.L., Farber, D.B. & Bok, D. 1990. Beta adrenergic receptors on cultured human retinal pigment epithelium. Investigative Ophthalmology & Visual Science. Vol. 31, pp. 1767. 10/1990.

Fuhrmann, S. 2010. Eye morphogenesis and patterning of the optic vesicle. Current Topics in Developmental Biology Vol. 93, pp.61-84. 08/2010.

Hageman, G.S. Age-Related Macular Degeneration (AMD.) 2014. Webvision, The Organization of the Retina and Visual System. http://webvision.med.utah.edu/book/part-xii-cell-biology-of-retinal-degenerations/age-related-macular-degeneration-amd/ Cited 6.9.2014.

Haponen, M. 2014. Bachelor of Laboratory Services. Personal notification via email. markus.haponen@uta.fi. Received 05.09.2014.

Hartzell, H.C., Qu, Z., Yu, K., Xiao, Q. & Chien, L.T. 2008. Molecular physiology of bestrophins: multifunctional membrane proteins linked to best disease and other retinopathies. Physiological Reviews. Vol.88(2), pp. 639–672. 04/2008.

Hasegawa, K., Pomeroy, J.E. & Pera, M.F. 2010. Current technology for the derivation of pluripotent stem cell lines from human embryos. Cell Stem Cell. Vol. 6(6), pp. 521-531. 06/2010.

Haug, E., Sand, O., Sjaadstad, Ö. & Toverud, K. 1999. Ihmisen fysiologia. 1-2. edition. Porvoo 1999, WSOY. p. 536.

Hoffman, L.M. & Carpenter, M.K. 2005. Characterization and culture of human embryonic stem cells. Nature Biotechnology. Vol 23(6), pp. 699-708. 06/2005.

Idelson, M., Alper, R, Obolensky, A., Ben-Shushan, E., Hemo, I., Yachimovich-Cohen, N., Khaner, H., Smith, Y., Wiser, O., Gropp, M., Cohen, M.A., Even-Ram, S., Berman-Zaken, Y., Matzrafi, L., Rechavi, G., Banin, E., Reubinoff, B. 2009. Directed Differentiation of Human Embryonic Stem Cells into Functional Retinal Pigment Epithelium Cells. Cell Stem Cell,.Volume (4), pp. 396-408 09/2009.

Jiang, Y., Jahagirdar, B.N., Reinhardt, R.L., Schwartz, R.E., Keene, C.D., Ortiz-Gonzalez, X.R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W.C., Lergaespada, D.A. &Verfaillie, C.M. 2002. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. Vol. 418(6893), pp. 41-49. 07/2002.

Jin, Z.B., Okamoto, S., Mandai, M. & Takahashi, M. 2009. Induced pluripotent stem cells for retinal degenerative diseases: a new perspective on the challenges. Journal of Genetics. Vol 88(4), pp. 417- 424. 12/2009.

Kamao, H.,Mandai, M., Okamoto, S., Sakai, N., Suga, A., Sugita, S., Kiryu, J. & Takahashi, M. 2014. Characterization of Human Induced Pluripotent Stem Cell-Derived Retinal Pigment Epithelium Cell Sheets Aiming for Clinical Application. Stem Cell Reports. Vol. 2(2), pp. 205-218. 02/2014.

Kanemura, H., Go, M.J., Shikamura, M., Nishishita, N., Sakai, N., Kamao, H., Mandai, M., Morinaga, C., Takahashi, M. & Kawamata, S. 2014. Tumorigenicity studies of induced pluripotent stem cell (iPSC)-derived retinal pigment epithelium (RPE) for the treatment of age-related macular degeneration. PLoS One. Vol 9(1). 01/2014.

Klimanskaya, I. 2006. Retinal Pigment Epithelium. Methods in Enzymology. Vol. 418, pp. 169-194. 11/2006.

Klimanskaya, I., Hipp, J., Rezai, K.A., West, M., Atala, A. & Lanza, R. 2004. Derivation and comparative assessment of retinal pigment epithelium from human embryonic stem cells using transcriptomics. Cloning and Stem Cells. Vol. 6(3), pp. 217-245. 09/2004.

Kolb, H., Nelson, R., Fernandez, E. & Jones, B. 2014. Webvision. The organization of the Retina and Visual System. University of Utah. http://webvision.med.utah.edu/

Kolios, G. & Moodley, Y. 2013. Introduction to stem cells and regenerative medicine. Review.Respiration Vol. 85(1) pp. 3-10. 12/2012.

Konrad M., Schaller A., Seelow D., Padley AV., Waldegger S., Lesslauer A., Vitzthum H., Suzuki Y., Luk JM, Becker, C., Schlingmann KP., Schmid M., Rodriguez-Soriano J., Ariceto G., Cano F., Enriques R., Jüppner H., Bakkaloglu S. A., Hediger M. A., Gallati S., Neuhauss S.C.F., Nürnber P. & Weber, S. 2006. Mutations in the Tight-Junction Gene Claudin 19 (CLDN19) Are Associated with Renal Magnesium Wasting, Renal Failure, and Severe Ocular Involvement. The American Journal of Human Genetics. Vol. 79(5), pp. 949–957. 11/2006.

Kubota, A., Nishida, K., Yamato, M., Yang, J., Kikuchi, A., Okano, T. & Tano Y. 2006. Transplantable retinal pigment epithelial cell sheets for tissue engineering. Biomaterials Vol. 27(19), pp. 3639-3644. 06/2006.

Kurosawa, H. 2007. Methods for inducing embryoid body formation: in vitro differentiation system of embryonic stem cells. Journal of Bioscience and Bioengineering. Vol. 103(5), pp. 389-398. 05/2007.

Lee, E. & MacLaren, R.E. 2010. Sources of retinal pigment epithelium (RPE) for replacement therapy. British Journal of Ophthalmology. Vol. 95(4), pp. 445-449. 07/2010.

Lehmann, G., Benedicto, I., Philp, N. & Rodriguez-Boulan, E. 2014. Plasma membrane protein polarity and trafficking in RPE cells: Past, present and future. Review. Experimental Eye Research. Vol. 126, pp. 5-15. 9/2014.

Levenstein, M.E., Ludwig, T.E., Xu, R.H., Llanas, R.A., Van Den Heuvel – Kramer, K., Manning, D. & Thomson, J.A. 2006. Basic fibroblast growth factor support of human embryonic stem cell self-renewal. Stem Cells. Vol. 24(3), pp. 568-574. 03/2006.

Liao, J.L., Yu, J., Huang, K., Hu, J., Diemer, T., Ma, Z., Dvash, T., Yang, X.J., Travis, G.H., Williams, D.S., Bok, D. & Fan G. 2010. Molecular signature of primary retinal pigment epithelium and stem-cell-derived RPE cells. Human Molecular Genetics Vol. 19(21). pp. 4229-4238. 08/2010.

Life Technologies. 2014. Cell culture basics - Handbook. http://www.vanderbilt.edu/viibre/CellCultureBasicsEU.pdf. Cited 05.08.2014.

Lund, R.D., Wang, S., Klimanskaya, I., Holmes, T., Ramos-Kelsey, R., Lu, B., Girman, S., Bischoff, N., Sauvé, Y. & Lanza, R. 2006. Human embryonic stem cell-derived cells rescue visual function in dystrophic RCS rats. Cloning and Stem Cells. Vol. 8(3), pp. 189-199. 09/2006.

Maminishkis, A., Chen, S., Jalickee, S., Banzon, T., Shi, G., Wang, F.E., Ehalt, T., Hammer, J.A. & Miller, S.S. 2006. Confluent monolayers of cultured human fetal retinal pigment epithelium exhibit morphology and physiology of native tissue. Investigative Ophthalmology & Visual Science. Vol. 47(8), pp. 3612-3624. 08/2006.

Mazzoni, F., Safa, H. & Finnemann, S.C. 2014. Understanding photoreceptor outer segment phagocytosis: Use and utility of RPE cells in culture. Review. Experimental Eye Research. Vol.126, pp. 51-60. 09/2014.

Mikhailova, A. 2011. Influence of soluble factors secreted by different types of fibroblasts on differentiation of human embryonic stem cells into retinal pigment epithelial cells. Master's Thesis, University of Tampere, Institute of Biomedical Technology. 02/2011.

Mikhailova, A., Ilmarinen, T., Uusitalo, H., Skottman, H. Small-Molecule Induction Promotes Corneal Epithelial Cell Differentiation from Human Induced Pluripotent Stem Cells. Stem Cell Reports Vol. 2. pp. 219–231. 02/2014.

Nicholas J. & Smith A. 2009. Naive and Primed Pluripotent States. Cell Stem Cell Vol. 4(6), pp. 487-492. 06/2009.

The Nobel Assembly at Karolinska Institutet. 2012. The Nobel Prize in Physiology Or Medicine 2012. Press release 8.10.2012.
http://www.nobelprize.org/nobel_prizes/medicine/laureates/2012/press.html

Peng, S., Rao, V., Adelman, R.A. & Rizzolo, L.J. 2011. Claudin-19 and the barrier properties of the human retinal pigment epithelium. Investigative Ophthalmology & Visual Science. Vol. 52(3), pp. 1392-1403. 03/2011.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S. & Marshak, D.R. 1999. Multilineage potential of adult human mesenchymal stem cells. Science. Vol. 284(5411), pp. 143-147. 04/1999.

Purves, D., Augustine, G.J., Fitzpatrick, D., et al., editors. The Retina. Neuroscience. 2nd edition. Sunderland (MA): Sinauer Associates; 2001. http://www.ncbi.nlm.nih.gov/books/NBK10885/

Quinn R.H. & Miller S.S. 1992. Ion transport mechanisms in native human retinal pigment epithelium. Investigative Ophthalmology & Visual Science. Vol 33., pp. 3513-3527. 12/1992.

Rizzolo, L. 2014. Barrier properties of cultured retinal pigment epithelium. Review. Experimental Eye Research. Vol. 126, pp. 16-26. 09/2014.

Ross, M. & Pawlina, W. 2011. Histology. A Text and Atlas, with Correlated Cell and Molecular Biology. 6th edition. Baltimore: Lippincott Williams & Wilkins.

Rowland, T.J , Blaschke, A.J., Buchholz, D.E., Hikita, S.T.abc, Johnson, L.V. & Clegg, D.O. 2013. Differentiation of human pluripotent stem cells to retinal pigmented epithelium in defined conditions using purified extracellular matrix proteins. Journal of Tissue Engineering and Regenerative Medicine. Vol. 7(8), pp. 642-653. 08/2013.

Rowland, T.J., Buchholz, D.E. & Clegg, D.O. 2012. Pluripotent human stem cells for the treatment of retinal disease. Journal of Cell Physiology. Vol. 227, pp. 457–466. 02/2012.

Saari, J.C. & Crabb, J.W. 2005. Focus on molecules: cellular retinaldehyde-binding protein (CRALBP). Experimental Eye Research. Vol 81(3), pp. 245-246. 09/2005.

Saito, W., Yamamoto, S., Hayashi, M. & Ogata K. 2003. Morphological and functional analyses of adult onset vitelliform macular dystrophy. British Journal of Ophthalmology. Vol. 87(6), pp. 758-762. 10/2003.

Schwartz, S., Hubschman, J.P., Heilwell, G., Franco-Cardenas, V., Pan, C.K., Ostrick, R.M., Mickunas, E., Gay, R., Klimanskaya, I. & Lanza, R. 2012. Embryonic stem cell trials for macular degeneration: a preliminary report. The Lancet. Vol 379(9817), pp. 713-720. 02/2012.

Schwartz, S., Regillo, C., Lam, B., Eliott, D., Rosenfeld, P., Gregori, N., Hubschman, J.P., Davis, J., Heilwell, G., Spirn, M., Maguire, J., Bateman, J., Ostrick, R., Morris, D.,Vincent, M., Anglade, E., Del Priore, L.V. & Lanza, R. 2014. Human embryonic stem cell-derived retinal pigment epithelium in patients with age- related maculardegeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. http://download.thelancet.com/flatcontentassets/pdfs/S0140673614613763.pdf
Published online 15.10.2014. Smukler, S.R., Runciman, S.B., Xu, S. & van der Kooy, D. 2006. Embryonic stem cells assume a primitive neural stem cell fate in the absence of extrinsic influences. Journal of Cell Biology. Vol.172(1), pp. 79-90. 01/2006.

Skottman, H. 2010. Derivation and characterization of three new human pluripotent stem cell lines in Finland. In Vitro Cellular & Developmental Biology- Animal. Vol 46(3-4), pp.206-209. 04/2010.

Stacey, G.N., Cobo, F., Nieto, A., Talavera, P., Healy, L., Concha, A. 2006. The development of 'feeder' cells for the preparation of clinical grade hES cell lines: challenges and solutions. Journal of Biotechnology. Vol. 125(4), pp. 583-588. 10/2006.

Stadtfeld, M. & Hochedlinger, K. 2010. Induced pluripotency: history, mechanisms, and applications. Genes & Development. Vol. 20, pp. 2239-2273. 10/2010.

Strauss, O. 2005. The retinal pigment epithelium in visual function. Physiological Reviews. Vol. 85(3), pp. 845-881. 07/2005.

Takahashi, K., Yamanaka, S. 2006. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell. Volume 126(4), pp. 663-676. 08/2006.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. & Yamanaka, S. 2007. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell. Vol. 131(5), pp. 861–872. 11/2007.

Tamiya, S., Liu, L. & Kaplan. H,J. 2010. Epithelial-mesenchymal transition and proliferation of retinal pigment epithelial cells initiated upon loss of cell-cell contact. Investigative Ophthalmology & Visual Science. Vol. 51(5), pp. 2755-2763. 05/2010.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S. & Jones, J.M. 1998. Embryonic stem cell lines derived from human blastocysts. Science. Vol. 282(5391), pp. 1145-1147. 12/1998.

Tecirlioglu, R.T., Nguyen, L., Koh, K., Trounson, A.O. & Michalska, A.E. 2010. Derivation and maintenance of human embryonic stem cell line on human adult skin fibroblast feeder cells in serum replacement medium. In Vitro Cellular & Developmental Biology – Animal. Vol. 46(3-4), pp. 231- 235. 04/2010.

Unger, C., Skottman, H., Blomberg, P., Dilber, M.S. & Hovatta O.2008. Good manufacturing practice and clinical-grade human embryonic stem cell lines. Human Molecular Genetics. Vol. 17(1), pp. 48-53. 03/2008.

Vaajasaari H., Ilmarinen T., Juuti-Uusitalo K., Rajala K., Onnela N., Narkilahti S., Suuronen R., Hyttinen J., Uusitalo H. & Skottman H. 2011. Toward the defined and xeno-free differentiation of functional human pluripotent stem cell-derived retinal pigment epithelial cells. Molecular Vision. Vol. 17, pp. 558-575. 02/2011.

Vugler, A., Carr, A.J., Lawrence, J., Chen, L.L., Burrell, K., Wright, A., Lundh, P., Semo, M., Ahmado, A., Gias, C., da Cruz, L., Moore, H., Andrews, P., Walsh, J. & Coffey, P. 2008. Elucidating the phenomenon of HESC-derived RPE: anatomy of cell genesis, expansion and retinal transplantation. Experimental Neurology. Vol. 214(2), pp. 347-361. 12/2008.

Watabe ,T. & Miyazono, K. 2009 . Roles of TGF-beta family signaling in stem cell renewal and differentiation. Cell Research. Vol. 19(1), pp. 103-115. 01/2009.
Wilson, K. & Walker, J. (ed.) 2010. Principles and Techniques of Biochemistry and Molecular Biology. 7th edition. U.S.A.: Cambridge University Press.

Xiao, Q., Hartzell, H.C. & Yu, K. Bestrophins and retinopathies. 2010. Pflügers Archiv - European Journal of Physiology. Vol.460(2), pp. 559-569. 03/2010.

Xu, R.H., Peck, R.M., Li, D.S., Feng, X., Ludwig, T. & Thomson, J.A. 2005. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nature Methods. Vol. 2(3), pp. 185-190. 03/2005.

Yamanaka S. 2007. Strategies and new developments in the generation of patient-specific pluripotent stem cells. Cell Stem Cell. Vol. 1(1), pp. 39-49. 06/2007.

Yeoh, J.S. & de Haan, G. 2007. Fibroblast growth factors as regulators of stem cell self-renewal and aging. Mechanisms of Ageing and Development. Vol. 128(1), pp.17-24. 01/2007.

Zhao, T., Zhang, Z.N., Rong, Z. & Xu, Y. 2011. Immunogenicity of induced pluripotent stem cells. Nature. Vol. 474, pp. 212–215. 06/2011.

APPENDICES

Appendix 1. M+/M- control cell culture immunostainings

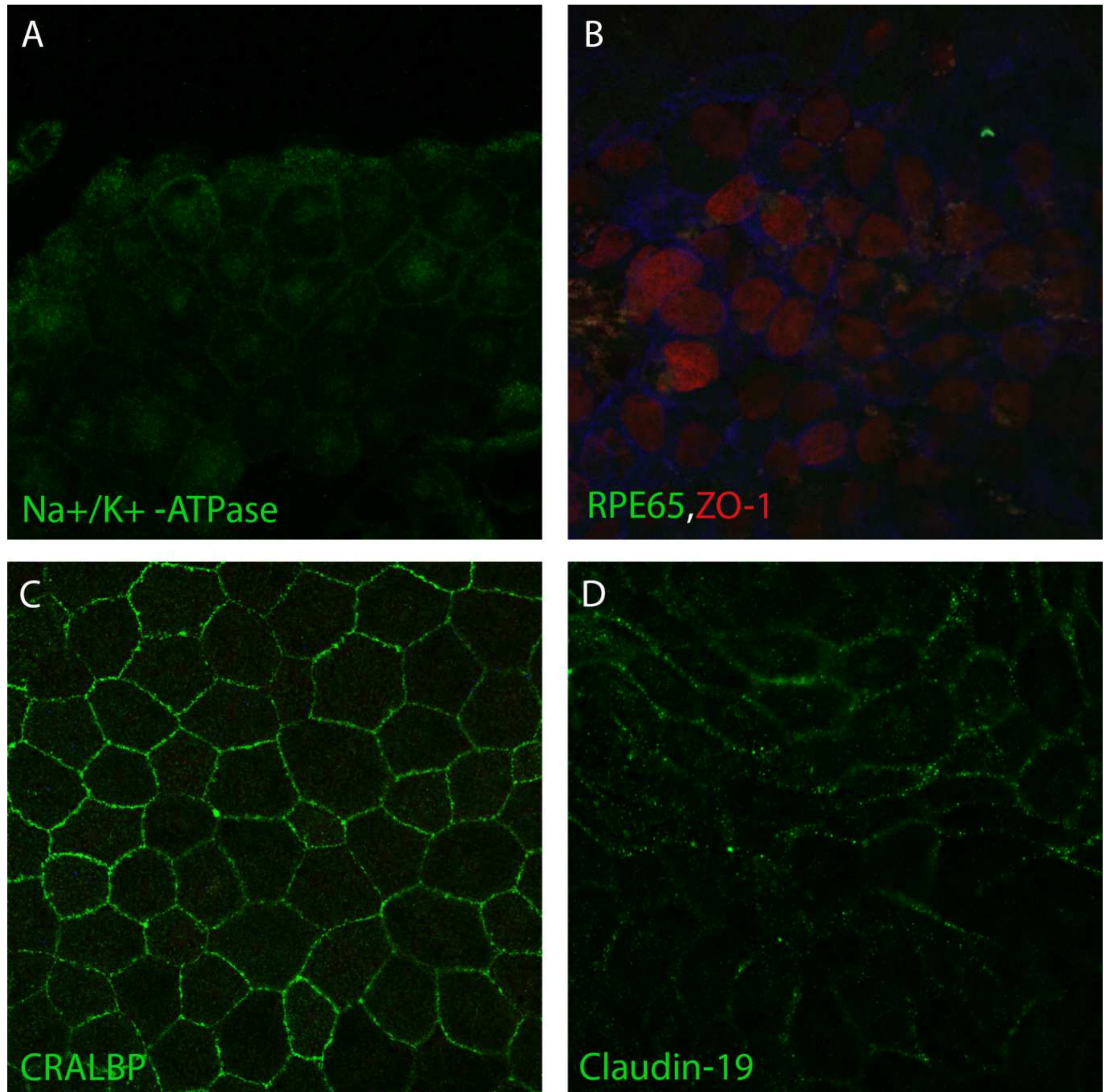

FIGURE 12 Control cell culture M+/M- immunostainings. A) Na+/K+-ATPase A488, B) RPE65 A488 (negative), ZO-1 A568 C) CRALBP A488 D) Claudin 19 A488. Confocal microscope, 63x oil immersion.