INFLUENCE OF SOLUBLE FACTORS SECRETED BY DIFFERENT TYPES OF FIBROBLASTS ON DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS INTO RETINAL PIGMENT EPITHELIAL CELLS

Alexandra Mikhailova

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University of Tampere
Institute of Biomedical Technology

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TIIVISTELMÄ

Tutkielman tausta ja tavoitteet: Ihmisen alkion kantasolujen ainutlaatuisten ominaisuuksien ansiosta niitä voidaan hyödyntää monien sairauksien hoidossa. Esimerkiksi verkkokalvon pigmenttiepiteelisolujen rappeutumiseen liittyviä sairauksia voitaisiin hoitaa käyttämällä kantasoluihin perustuvia soluhoitoja. Kliinisiä sovelluksia varten tarvitaan vielä tehokkaampi erilaistusmenetelmä, jonka avulla voitaisiin tuottaa puhtaita pigmenttiepiteelisolupopulaatioita. Erilaistumistehokkuutta voidaan parantaa esimerkiksi tunnistamalla erilaisia kasvutekijöitä, jotka edistävät verkkokalvon pigmenttiepiteelisolujen erilaistumista kantasoluista. Tämän tutkimuksen tavoitteena oli vertailla eri fibroblastityyppien erittämien liukoisten tekijöiden vaikutuksia verkkokalvon pigmenttiepiteelisolujen erilaistumiseen. Tutkimuksessa mukana olivat hiiren alkion fibroblastit, ihmisen ihosta eristetyt fibroblastit, sekä ihmisen esinahasta eristetyt fibroblastit.

Materiaalit ja menetelmät: Erilaistumattomia kantasoluja kasvatettiin eri fibroblasteilla kyllästetyissä erilaistusliuoksissa sekä tavallisessa erilaistusliuoksessa. Koe koostui kahdesta vaiheesta: esierilaistuminen toteutettiin suspensiossa, ja sen jälkeen pigmentoituneet solut valikoitiin jatkokasvatukseen, jossa erilaistus jatkui alustaan kiinnittyneinä yksisolukerrosviljelminä. Ensimmäisen vaiheen aikana erilaistumista seurattiin vertaamalla solujen pigmentaatiota, sekä analysoimalla tiettyjen geenien ilmentymistasoja. Kypsymisvaiheen päätyttyä pigmenttiepiteelisolukerrokset karakterisoitiin todentamalla pigmenttiepiteelisoluille tyypillisten geenien ilmentymistä ja paikantamalla tiettyjä proteiineja. Kypsien solujen fagosytoosikyky analysoitiin käyttämällä *in vitro* fagosytoosimääritystä. Tämän lisäksi verrattiin neljän kasvutekijän (activin A, bFGF, TGF-β ja PEDF) eritystä käytetyissä fibroblastityypeissä.

Tulokset ja johtopäätökset: Tässä tutkimuksessa saadun tiedon perusteella ei voida yksiselitteisesti sanoa mikä tutkituista olosuhteista oli paras pigmenttiepiteelisolujen erilaistumiseen, sillä kaikissa olosuhteissa saatiin aikaan kypsiä ja toiminnallisia soluja. On kuitenkin selvää, että tavallinen erilaistusliuos oli fibroblasteilla kyllästettyjä erilaistusliuoksia heikompi. Kasvutekijäanalyysi osoitti, että ihmisen ihofibroblastit ja hiiren alkion fibroblastit tuottavat paljon enemmän activin A -kasvutekijää kuin ihmisen esinahan fibroblastit, jotka sen sijaan tuottavat eniten TGF-β -kasvutekijää. Nämä ja muut fibroblastien tuottamat liukoiset tekijät vaikuttavat pigmenttiepiteelisolujen erilaistumiseen ihmisen alkion kantasoluista monimutkaisella tavalla, ja sen ymmärtämiseen tarvitaan vielä lisää tutkimusta.

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ABSTRACT

Background and aims: Due to their unique properties of self-renewal and pluripotency, human embryonic stem cells (hESCs) have the potential to solve the problem of scarcity of tissue donors. Novel cell replacement therapies could be developed for the retinal diseases, which are linked to degeneration of retinal pigment epithelium (RPE). Before clinical applications can be developed, an efficient way of differentiating RPE cells from hESCs needs to be optimized. Identifying growth factors that enhance differentiation along the appropriate pathway is an important, yet time-consuming step in the process of optimization. The aim of this study was to compare the influence of soluble factors secreted by different types of fibroblasts on RPE differentiation. Mouse embryonic fibroblasts (mEFs), human dermal fibroblasts (hDFs) and human foreskin fibroblasts (hFFs) were chosen for this study.

Materials and methods: Undifferentiated hESCs were maintained using cell culture media conditioned by the different types of fibroblasts, as well as the unconditioned differentiation medium. The experiment consisted of two phases – the initial differentiation phase, carried out in suspension culture, after which pigmented cells were selected and used to create adherent cultures for the maturation phase. During the initial differentiation phase, extent of differentiation was evaluated visually by following pigmentation patterns, as well as by comparing expression levels of a series of key genes. After the maturation phase, mature monolayers of hESC-derived RPE cells were characterized with respect to expression of key genes and localization of RPE-specific proteins. Finally, an *in vitro* phagocytosis assay was carried out to assess the functionality of mature RPE cells. In addition, secretion of four growth factors (activin A, bFGF, TGF-β and PEDF) by each fibroblast type was analyzed.

Results and conclusions: Based on the data collected in this study, it is impossible to say whether one of the culture conditions was superior to others, because functional RPE cells with correct localization of key proteins were obtained in each of them. However, the unconditioned differentiation medium was clearly inferior to the media conditioned by fibroblasts. Analysis of growth factor secretion showed that hDFs and mEFs secrete far more activin A than hFFs, while secretion of TGF-β was highest in hFFs. Overall, these and other soluble factors secreted by fibroblasts influence RPE differentiation in a complex manner, and more research would be needed to fully understand it.

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ABBREVIATIONS

A2E	*N*-retinylidene-*N*-retinylethanolamine
ACTC	α-cardiac actin
AFP	α-fetoprotein
AMD	Age-related macular degeneration
BEST1	Bestrophin
BF	Brightfield
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
BVMD	Best vitelliform macular dystrophy
CHX10	Ceh-10 homeodomain containing homolog
CM	Conditioned medium
CRALBP	Cellular retinaldehyde-binding protein
CRX	Cone-rod homeobox
C_T	Cycle threshold
DAPI	4',6-diamidino-2-phenylindole
DPBS	Dulbecco's Phosphate Buffered Saline
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GMP	Good manufacturing practice
hDF	Human dermal fibroblast
hESC	Human embryonic stem cell
hFF	Human foreskin fibroblast
ICM	Inner cell mass
IMDM	Iscove's Modified Dulbecco's Medium
iPSC	Induced pluripotent stem cell

IVF	*In vitro* fertilization
KO-DMEM	Knock-Out Dulbecco's Modified Eagle Medium
KO-SR	Knock-Out Serum Replacement
mEF	Mouse embryonic fibroblast
MITF	Microphthalmia-associated transcription factor
NEAA	Non-essential Amino Acids
OCT3/4	Octamer-binding transcription factor 3/4
OTX2	Orthodenticle homeobox 2
PAX6	Paired box gene 6
PDGF	Platelet-derived growth factor
PEDF	Pigment epithelium-derived factor
PFA	Paraformaldehyde
PMEL17	Pre-melanosomal protein 17
POS	Photoreceptor outer segments
qPCR	Quantitative real-time polymerase chain reaction
RAX	Retina and anterior neural fold homeobox
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
RPE DM-	RPE differentiation medium
RPE65	Retinal pigment epithelium-specific 65 kDa protein
RT-PCR	Reverse transcription polymerase chain reaction
SOX10	Sex-determining region Y -box 10
SOX17	Sex-determining region Y -box 17
SSEA	Stage-specific embryonic antigens
T	Brachyury
TGF-β	Transforming growth factor beta
TYR	Tyrosinase
UD hESC	Undifferentiated hESC colonies
VEGF	Vascular endothelial growth factor
ZO1	Zona occludens -1

1. INTRODUCTION

The retina is a complex and layered structure lining the inner surface of the eye. Retinal pigment epithelium (RPE) is its outermost layer and its functions, which include light absorption, participation in the visual cycle and secretion of growth factors, make it essential for normal vision (Strauss, 2005). Dysfunction or deterioration of RPE is linked to degenerative retinal diseases, such as age-related macular degeneration (AMD), retinitis pigmentosa (RP) and Best's disease. Currently, treatment options for these disorders are far from ideal, especially because donor tissues are in short supply. However, continuous advances in stem cell research might provide a new source of tissue for cell replacement therapies, once the methods of differentiation are fully optimized.

Human embryonic stem cells (hESCs) are pluripotent, meaning that they are capable of unlimited proliferation without undergoing differentiation, while having the potential to differentiate into virtually any cell type (Reubinoff et al., 2000). To keep hESCs in their undifferentiated state they are cultured on layers of mitotically inactivated fibroblasts, commonly referred to as feeder cells (Hoffman & Carpenter, 2005). Feeder cell layers are also commonly utilized in differentiation culture. Traditionally, mouse embryonic fibroblasts (mEFs) were used for stem cell culture, and their use remains wide-spread to this day (Stacey et al., 2006). Recently, however, efforts have been put towards optimizing stem cell culture conditions free from animal-derived components, and so the use of fibroblasts of human origin is becoming more common. Fibroblasts secrete a large variety of soluble factors, most of which are currently unknown, and the array of secreted molecules differs depending on the origin of the fibroblast cells. The different soluble factors, in combination with direct cell contact, influence differentiation of hESCs by either promoting proliferation or inducing apoptosis of certain cell types (Schuldiner et al., 2000). Identification of factors that direct differentiation towards a certain cell lineage is an important step in optimizing differentiation culture. The aim of this study was to help narrow down the selection by comparing the ways in which media conditioned by three different types of fibroblasts affect differentiation of RPE cells from hESCs.

2. REVIEW OF THE LITERATURE

2.1 RETINAL PIGMENT EPITHELIUM

RPE is composed of a single layer of pigmented cuboidal epithelial cells situated in the retina, at the boundary between the eye and the bloodstream (Figure 2.1). The apical membrane of RPE, with its long microvilli, is in contact with photoreceptors, while the basolateral membrane faces Bruch's membrane – the layer, which separates RPE of the retina from the choriocapillaris of the choroid (Strauss, 2005).

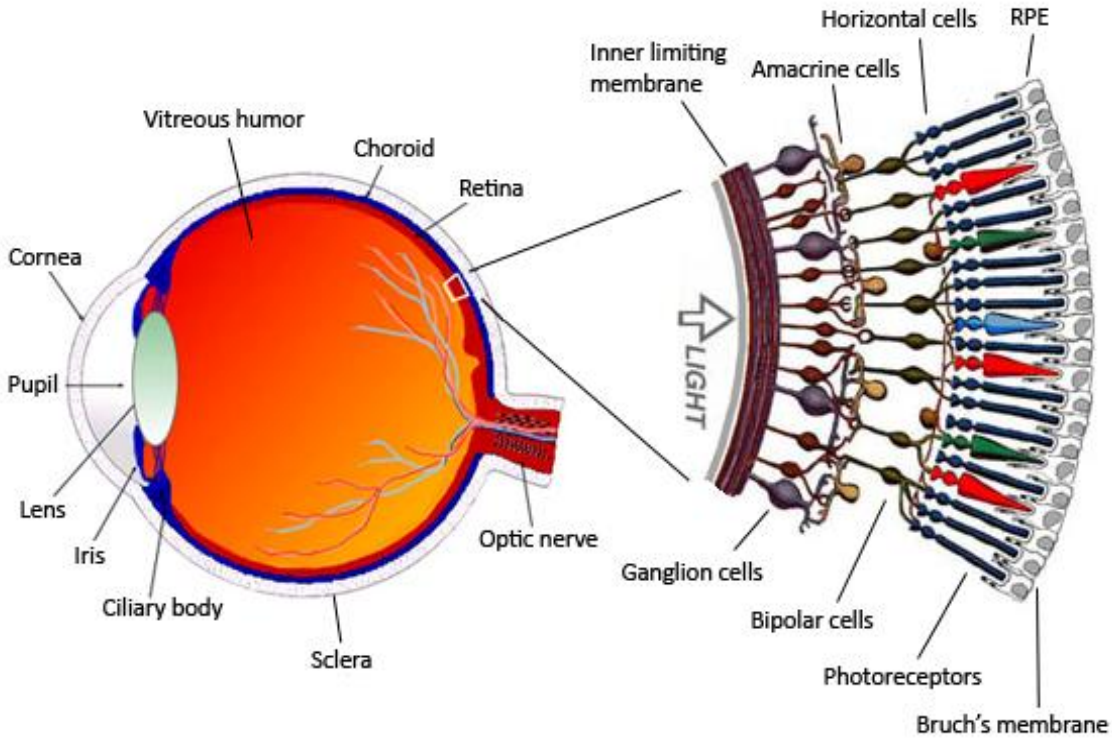

Figure 2.1 Schematic diagram of the human eye and retina
The retina lines the inner surface of the eye. It is multilayered and contains several cell types. RPE is the outermost layer of the retina, just outside the neurosensory layer and firmly attached to the underlying choroid via Bruch's membrane (modified from WebVision, 2003).

When viewed under a light microscope, RPE cells have distinct hexagonal morphology, resembling cobblestones, and their color varies from light brown to black due to the accumulations of melanin pigment (Maminishkis et al., 2006). RPE has many important functions, some of which are summarized in Figure 2.2, and it is essential for normal vision.

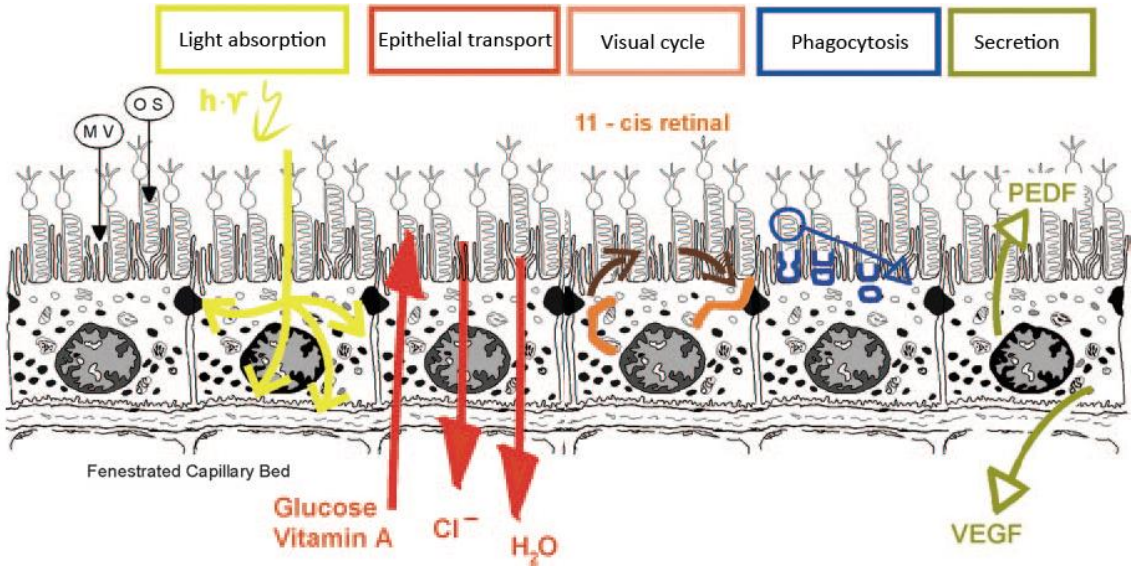

Figure 2.2 Functions of RPE
The most important functions of RPE are light absorption into melanin granules, which is how RPE acts as a protective barrier; epithelial transport of nutrients to photoreceptors; regeneration of visual pigment; phagocytosis of photoreceptor outer segments and secretion of growth factors. MV: microvilli, OS: outer segments (modified from Strauss, 2005).

Most importantly, RPE acts as a protective barrier, on account of the high levels of melanin pigment and antioxidants contained within each cell. The melanin filters light entering the retina, thereby significantly reducing photo-oxidation, while the antioxidants help remove or neutralize chemically reactive molecules (Kevany & Palczewski, 2010). In addition, RPE provides support and nourishment to the photoreceptor cells, forms the blood-retinal barrier, and has a crucial role in the visual cycle. The purpose of the visual cycle is to regenerate 11-*cis*-retinal from all-*trans*-retinal – a process that cannot take place in the photoreceptors and so requires molecule exchange between the photoreceptors and the RPE. Also, growth factors such as pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) are secreted by RPE, and serve to maintain structural integrity of surrounding cell types (Strauss, 2005). Another vital function of RPE is its ability to phagocytose outer segments shed daily by photoreceptors. Due to constant regeneration of outer segments, photoreceptors manage to maintain their length. By degrading photoreceptor outer segments (POS), RPE helps prevent the toxic effects of photo-oxidative products that accumulate in the photoreceptor cells. It is important that outer segments are degraded in the RPE as quickly as possible, because their excessive accumulation appears to be linked to degenerative retinal diseases, such as AMD (Kevany &

Palczewski, 2010). Phagocytic function of RPE relies on the polarized distribution of proteins such as αvβ5 integrin, which is the only integrin receptor localized to the apical side of the RPE cells. This receptor mediates retinal adhesion and, in response to the shedding of POS, promotes the cascade of phagocytic signaling in RPE cells (da Cruz et al., 2007). Na^+/K^+-ATPase, commonly known as the sodium pump, is also apically localized in these cells, which is the opposite of its localization in other types of epithelial cells (Marmorstein, 2001).

2.1.1 Retinal degeneration

There are two late-stage manifestations of AMD – geographic atrophy and neovascularization – and they both cause severe loss of central vision. Geographic atrophy is caused by the atrophy of RPE and leads to vision loss in the center of the eye, whereas neovascular AMD is caused by abnormal blood vessel growth through Bruch's membrane, which results in leakage of blood and proteins below the macula. This leakage damages photoreceptors and quickly leads to loss of vision if left untreated (Chakravarthy et al., 2010). The underlying reason behind both forms of AMD is increased rigidity of the sclera, which leads to impaired choroidal perfusion and eventually accumulation of photoreceptor lipoproteins either in RPE or the Bruch's membrane (Friedman, 2008). This explains the epidemiologic associations of AMD, such as age, atherosclerosis, dietary lipid intake and smoking – all of these factors increase scleral rigidity. However, genetic factors, oxidative damage, angiogenic factors and inflammation also play an important causative role in the pathogenesis of AMD (Scholl et al., 2007; Ting et al., 2009). Currently, there is no medical or surgical way of treating geographic atrophy, but certain nutritional supplements are recommended, because they slow down the progression of the disease in some patients. The only effective way to treat neovascular AMD is with monoclonal antibodies, such as ranibizumab, that reduce blood vessel growth by inhibiting VEGF. The treatment is administered via injections into the vitreous humor of the eye – a painful procedure that needs to be repeated fairly often (Chakravarthy et al., 2010). Such treatments are only useful during early stages of AMD, because they prevent the progress of neovascularization, but are ineffective when the sensory retina and RPE are damaged (Jin et al., 2009).

Best vitelliform macular dystrophy (BVMD), also referred to as Best's disease, is a form of macular degeneration caused by a faulty *BEST1* gene. This gene codes for Bestrophin – a calcium-activated chloride channel (Marmorstein et al., 2009; Xiao et al., 2010). Both AMD and BVMD, along with several other degenerative disorders of the retina, are characterized by an abnormal accumulation of lipofuscin in RPE (Sparrow & Boulton, 2005). The key component of lipofuscin is A2E (*N*-retinylidene-*N*-retinylethanolamine) – a metabolite of retinal, which is a visual pigment found in photoreceptors. Accumulation of lipofuscin is most likely related to dysfunctions in phagocytosis of POS. It disrupts the lysosomal functions of RPE cells and promotes their apoptosis, which leads to retinopathies such as BVMD and AMD. It is unclear how dysfunction of *BEST1* is related to accumulation of lipofuscin (Xiao et al., 2010).

RP is a group of inherited retinal degenerative disorders and the leading cause of inherited blindness or visual impairment worldwide. This disorder is caused by the loss of rod photoreceptors and leads to night blindness and visual field defects (Jin et al., 2009). Atrophy of RPE and choriocapillaris are among the classic clinical findings in patients suffering from RP. Several modes of treatment aimed at slowing down the degenerative process or treating ocular complications are currently being investigated. They include gene therapy, injections of neurotrophic factors or anti-apoptotic agents and dietary supplementation (Musarella & MacDonald, 2011). The topic of the next section is retinal cell replacement, which appears to be the most promising mode of treatment for RP, as well as the aforementioned retinal disorders.

2.1.2 Cell replacement therapies

Over time, it has become easier to diagnose degenerative retinal diseases such as AMD, BVMD or RP, although therapeutic techniques are still limited and inadequate (Jin et al., 2009). A wide variety of strategies aimed at improving vision in patients suffering from retinal degeneration have been deployed. Some of these strategies were mentioned in the previous section. Cell replacement therapies have been developed to inhibit progressive visual loss by replacing damaged cells with healthy ones. In the case of degenerative disorders of the retina, two examples of such therapies are direct injections of RPE suspensions into the subretinal space, and transplantation of RPE tissues as grafts (Kubota et al., 2006). In both cases, transplantation can be either autologous or

homologous. The technique of autologous RPE transplantation has evolved overtime, and the most novel approach is to harvest a full-thickness patch graft from a healthy portion of the patient's retina and transplant it to the impaired site (MacLaren et al., 2007). Homologous RPE transplantation is a technique in which an RPE allograft is obtained in the form of sheets or microaggregates in suspension from either a human adult or fetus cadaver, and delivered into the patient's eye. Restoration of the damaged RPE with such techniques depends on how well donor cells attach to the Bruch's membrane and grow to form a healthy epithelial monolayer (da Cruz et al., 2007).

Unfortunately, these methods have many limitations. Treatment of retinal diseases using direct injections of RPE cell suspensions is relatively easy to perform, but involves many uncontrollable variables such as size and shape of injected cells (Kubota et al., 2006). Furthermore, the cells might not adhere to Bruch's membrane, acquire an incorrect apical-basal orientation, or fail to form a functional monolayer (da Cruz et al., 2007). Transplantation of cell monolayers capable of covering an area of an appropriate size would perhaps be more suitable for treating retinal disease (Kubota et al., 2006). However, this technique has its own disadvantages – following transplantation the monolayer sheets might fold and contract, and even migrate away from the implantation site. In addition, there is always the possibility of an RPE graft not surviving. In the case of autografts there has been evidence that even if the graft becomes well-integrated and vascularized, there may still be lack of visual improvement for the patient. This is mainly due to the fact that the transplanted cells, which appeared to be healthy at first, are actually damaged as well, and loss of vision progresses. Meanwhile, when dealing with allogenic RPE grafts, the most significant barrier to long-term survival of the graft is immune rejection (da Cruz et al., 2007). Finally, shortage of donors for homologous RPE transplantation is an obstacle that is not easy to overcome. Recent advances in stem cell research show great potential in developing cell replacement therapies, and may provide a solution to the aforementioned issues (Lee & MacLaren, 2010).

Using stem cells to create functional RPE monolayers is a technique that could prove to be an efficient way of treating diseases such as AMD and BVMD (Lund et al., 2006; Huang et al., 2010). When maintained in the appropriate way, in conditions conforming to good manufacturing practice (GMP), stem cell lines are pathogen-free with minimal variation among batches, and can be used to generate large numbers of RPE cells

(Klimanskaya, 2006). Currently, there are still many obstacles to wide-spread use of stem cells for cell replacement therapies. Some of the most important ones will be discussed in the following section.

2.2 STEM CELLS

Stem cells can be divided into categories based on the number of cell types they can form, in other words their potency. The fertilized oocyte and the cells after the first cleavage divisions are considered to be totipotent, meaning that they can develop into an entire organism. Cells of the inner cell mass (ICM) of the blastocyst are pluripotent – they have the ability to differentiate into all three embryonic germ layers but they cannot form extra-embryonic tissues, such as the umbilical cord and placenta (Jukes et al., 2008). These are the cells used to derive embryonic stem cells (ESCs), which are discussed in greater detail in the next section. Induced pluripotent stem cells (iPSCs) are a recent innovation – fully differentiated somatic cells are used to generate cells comparable to ESCs in their differentiation potential (Takahashi et al., 2007). Most stem cells present in the adult organism have the ability to form several cell types and are therefore referred to as multipotent stem cells. For example, mesenchymal stem cells are known to be multipotent, because they can differentiate into cells of the cartilage, bone, and fat. Finally, oligopotent (e.g. neural) and unipotent (e.g. spermatogonial) stem cells are very limited in their differentiation potential (Jukes et al., 2008).

It is possible to isolate adult stem cell lines from a number of tissues, such as umbilical cord blood, bone marrow, adipose tissue, and olfactory mucosa. Besides having a more limited differentiation potential than ESCs, another weakness of adult stem cells is that their ability to proliferate *in vitro* seems to be limited and declines with time, along with their ability to differentiate. The advantage of using adult stem cells is that it is possible to create patient-specific cell lines which would not elicit an immunological response. Furthermore, when it comes to the use of adult stem cells, there are far fewer ethical questions that need to be considered (Pouton & Haynes, 2005; Jukes et al., 2008).

2.2.1 Human embryonic stem cells

Because of their outstanding differentiation potential, ESCs are a fascinating subject of research. Mouse embryonic stem cells were derived first (Evans & Kaufman, 1981), but soon after, the first hESC lines were established (Thomson et al., 1998). Human ESCs are derived from the ICM of human embryos cultured to the morula or blastocyst stage. The embryos are acquired from *in vitro* fertilization (IVF) clinics – couples undergoing IVF treatment may choose to donate embryos to research either because they are left unused, or were identified as being of poor quality. Donors voluntarily consent to the donation of embryos for research and no financial compensation is made for the donation (Hasegawa et al., 2010). Cells of the ICM are placed on a layer of mitotically inactivated fibroblasts, commonly referred to as a feeder cell layer, and the resulting cell population is expanded *in vitro* by continuous subculture of undifferentiated areas (Hoffman & Carpenter, 2005). Expansion continues until a hESC line of sufficient quality is derived (Figure 2.3).

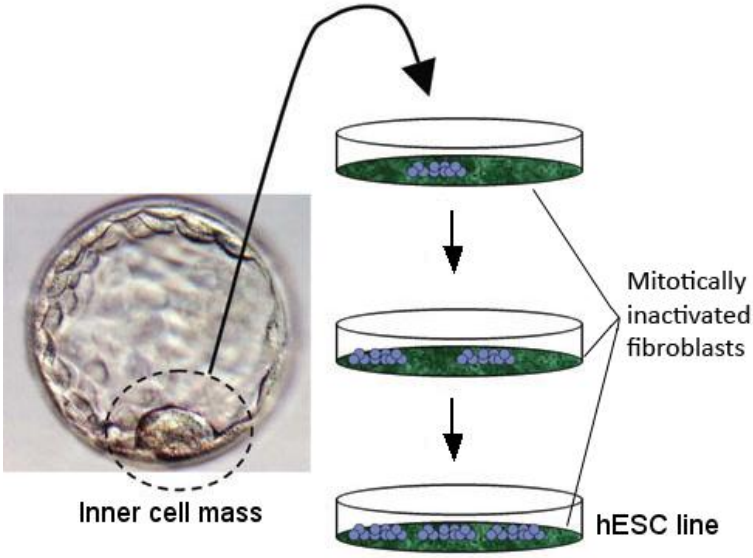

Figure 2.3 Derivation of a hESC line
ICM is isolated from an early-stage embryo and cultured and expanded on feeder cell layers until a hESC line is formed (modified from Hoffman & Carpenter, 2005).

Human ESCs are self-renewing and pluripotent: they are capable of unlimited proliferation without differentiating, and at the same time have the potential of forming derivatives of all three embryonic germ layers both *in vitro* (when cultured without factors that prevent differentiation) and *in vivo* (as teratomas in immunocompromised mice) (Hoffman & Carpenter, 2005). Other distinctive features of hESCs include a high

nucleus to cytoplasm ratio, prominent nucleoli, high levels of telomerase activity, which correlates with the length of their replicative life-span, and expression of specific cell surface markers, such as stage-specific embryonic antigens – SSEA (Thomson et al., 1998). Furthermore, a number of transcription factors that are involved in maintenance of hESC self-renewal have been identified. An example of such transcription factors is *OCT3/4* (Octamer-binding transcription factor 3/4) (Hoffman & Carpenter, 2005). Analysis of any combination of these features can be used to characterize undifferentiated hESCs.

Optimal culture conditions of hESCs depend entirely on what the cell line is to be used for. Generally, a defined matrix and defined media not containing supplements of animal origin should be used no matter what purpose the hESC line has. Also, phenotypic and karyotypic stability should always be maintained for prolonged time periods and hESCs should be capable of reproducible differentiation. Special attention should be paid to supporting pluripotency if the cell line is meant for study of early human development. Meanwhile, proliferative and differentiative capacity is of secondary importance when it comes to cell lines intended for therapeutic purposes, and it is far more important to focus on defined culture conditions in accordance to certain regulations, as well as reliable differentiation into appropriate cell populations (Bongso & Tan, 2005; Hoffman & Carpenter, 2005).

Despite the great promise and potential benefits of using hESCs in research, there are certain ethical and religious concerns that must be taken into account. Research using hESCs is limited in many countries and even forbidden in some. Lately, however, there has been a breakthrough, when it became possible to generate cells resembling hESCs by reprogramming somatic cells into the so-called iPSCs, which overcome the immunogenicity and ethical controversy of hESCs (Takahashi et al., 2007). Nevertheless, because of the use of retroviruses and a potentially tumorigenic gene *c-myc* in the induction process, further research is required before iPSCs can be used in clinical applications. Tumorigenicity is an important issue that needs to be considered when devising cell replacement therapies using pluripotent stem cells (Blum & Benvenisty, 2007). Teratomas (benign germ cell tumors), or even teratocarcinomas (malignant germ cell tumors) may form in the patient as a result of remnant undifferentiated cells present within the transplanted cell population. The molecular

basis of tumorigenicity depends on the properties of self-renewal and rapid proliferation of pluripotent cells, which make them rather similar to cancer cells (Kooreman & Wu, 2010). To avoid such adverse effects of cell replacement therapies, it is of vital importance to maximize the efficiency of stem cell differentiation, and purify cell populations prior transplantation. Furthermore, stem cell culture conditions are not fully defined yet, and the use of components derived from animal sources is still commonplace (Hoffman & Carpenter, 2005). Implementing GMP standards will ensure that hESCs are derived and maintained in a reproducible manner, putting the safety of the patient first, despite the fact that the resulting cells may not be of the highest possible quality for a certain application (Unger et al., 2008). These are only some of the main problems associated with the use of pluripotent stem cells, which need to be eliminated before their use in clinical applications can become wide-spread.

There are many valuable ways in which pluripotent stem cells could be used. For instance, they can provide a way to study developmental events that cannot be studied directly in the intact embryo (Thomson et al., 1998). This would offer insights into the process of normal human development as well as its possible abnormalities. Another immediate application of hESCs and iPSCs would be to use them as a source of human hepatocytes for *in vitro* experiments aimed at drug metabolism (Pouton & Haynes, 2005). Potentially teratogenic or toxic compounds, as well as target genes for new drugs could therefore be identified and studied. Most importantly, studying the mechanisms that control differentiation of hESCs could lead to development of novel therapies aimed at diseases that currently have no cure. For example, diseases that result from the lack of a certain cell type, such as type I diabetes and Parkinson's disease, could be cured by replacing the lacking cells with cells derived from hESCs (Thomson et al., 1998). Diseases linked to RPE degeneration could potentially be cured as well, by transplantation of hESC-derived RPE cell layers. In this case, hESCs would need to be directed to differentiate along RPE progenitor cell lines.

2.3 DIFFERENTIATION OF hESCs TOWARDS RPE CELLS

It has been noted that as long as no known inductive agents are introduced to the culture, ESCs choose a neural pathway for differentiation (Smukler et al., 2006). RPE, as well as the neural retina, is a derivative of the neuroectoderm, and is therefore a

product of this default differentiation pathway (Fuhrmann, 2010). The purpose of this section is to introduce some of the key features of the process through which cultures of hESC-derived RPE cells (hESC-RPE) can be established, as well as the most commonly used characterization techniques.

2.3.1 Cell culture conditions

It is quite easy to distinguish RPE cells from other cell types that may arise in differentiation culture, because they are pigmented and therefore visibly different from other cell types. Spontaneous differentiation of hESCs is triggered by removal of basic fibroblast growth factor (bFGF) from the culture medium and transfer of cells from adherent culture to suspension culture. Several studies have shown that bFGF is a key factor when maintaining pluripotency of hESCs, and addition of bFGF to the culture medium sustains their undifferentiated proliferation even in the absence of feeder cell layers (Levenstein et al., 2006; Xu et al., 2005). When hESCs are transferred to suspension culture lacking bFGF, they form three-dimensional cell aggregates, which undergo spontaneous differentiation into cell types of all three germ layers. As differentiation towards RPE cells progresses, the cell aggregates start to develop pigmented areas which can be used to create monolayer cultures (Klimanskaya, 2006). When cultured as a monolayer, the RPE cell population becomes more homogeneous and the maturity of cells is easier to evaluate than in suspension culture. Monolayer cultures cannot be established on untreated cell culture plastic, because hESC-RPE cells do not adhere well enough to it. This problem is easily overcome by coating the cell culture substrate with extracellular matrix (ECM) proteins such as collagen IV or laminin, both of which are important components of Bruch's membrane (Vugler et al., 2008). At first, when cell aggregates are dissociated and hESC-RPE cells are placed in adherent culture, they lose their pigmentation and acquire fibroblast-like morphology via epithelial-mesenchymal transition (EMT) – a process in which epithelial cells lose their differentiated phenotypes and transdifferentiate into mesenchymal-like cells (Tamiya et al., 2010). However, as soon as the transdifferentiated cells reach a certain degree of confluency, they redifferentiate and regain the morphology inherent to RPE cells (Klimanskaya, 2006). It appears that transdifferentiation to fibroblast-like cells is required for speedy cell proliferation, which is halted when the necessary amount of cell-cell contact is reached (Vugler et al., 2008).

There are many challenges and problems that must be overcome before differentiation of hESCs into RPE cells can be considered to be fully optimized. Most importantly, spontaneous differentiation always results in heterogeneous populations of cells, so the yield of hESC-RPE cells is typically fairly low. Each hESC line is different and some are more inclined to differentiate towards neural lineages than others, which affects the yield of hESC-RPE cells. Identifying inductive agents that would drive the differentiation of hESCs towards the RPE cell lineage would be a breakthrough in trying to create pure populations of hESC-RPE cells. Finally, there is evidence showing that ESC-derived ectoderm can give rise to cells of both neural and epidermal lineage (Watabe & Miyazono, 2009). This poses a problem when attempting to direct differentiation of hESCs towards RPE cells, because melanocytes are derivatives of the epidermis and may be mistaken for RPE cells due to the fact that they also produce melanin and are therefore pigmented. Overall, more research is required until a reliable and consistent differentiation protocol is developed.

2.3.2 Characterization of hESC-RPE cells

Following the degree of differentiation in hESC culture from an early stage is a good way of making sure that it is moving along the neural pathway. Typically, genes such as *RAX* (retina and anterior neural fold homeobox) and *PAX6* (paired box gene 6) should be up-regulated during the early stages of differentiation – they serve as regulators of early eye formation and early neural markers of RPE cells (Martinez-Morales et al., 2004; Fuhrmann, 2010). Expression levels of both *RAX* and *PAX6* should go down as RPE cells mature. Transcription factors *MITF* (microphthalmia-associated transcription factor), *OTX2* (orthodenticle homeobox 2), and *CHX10* (ceh-10 homeodomain containing homolog) are also present at a fairly early stage. *CHX10* is a gene typically expressed in precursor cells of the neural retina, so its expression should decrease with time, while *MITF*, being characteristic of RPE precursor cells, should remain up-regulated (Fuhrmann et al., 2000). The protein MITF is associated with the onset and maintenance of pigmentation and, together with OTX2, is required for RPE differentiation (Liao et al., 2010).

Mature RPE cells acquire distinct cobblestone morphology and are dark brown in color due to melanin production (Figure 2.4A). These features are easily seen with a light

microscope, but in order to visualize cell polarization (microvilli at the apical side and a basement membrane underlying the epithelium), an electron microscope would need to be used (Figure 2.4B). Moreover, expression of genes specific to mature RPE cells, such as *BEST1*, *CRALBP* (cellular retinaldehyde-binding protein), *RPE65* (retinal pigment epithelium-specific 65kDa protein), *PEDF*, *PMEL17* (pre-melanosomal protein 17) and *TYR* (tyrosinase) should be detected. Expression of *CRALBP* and *RPE65* should be up-regulated in mature hESC-RPE cells, because proteins encoded by these genes are involved in the visual cycle (Liao et al., 2010). *TYR* and *PMEL17* are among the genes required for melanin synthesis and are therefore linked to pigmentation, and should be expressed in mature RPE cells (Harris et al., 2010). Expression levels of genes typical to other tissues, such as *T* (Brachyury, required for mesoderm formation), *SOX17* (Sex-determining region Y -box 17, involved in development of the endoderm), and *CRX* (cone-rod homeobox, late-stage marker of the neural retina) should be as low as possible. Expression levels of different genes can be analyzed with reverse transcription polymerase chain reaction (RT-PCR), or, if more quantitative results are needed – with quantitative real-time polymerase chain reaction (qPCR).

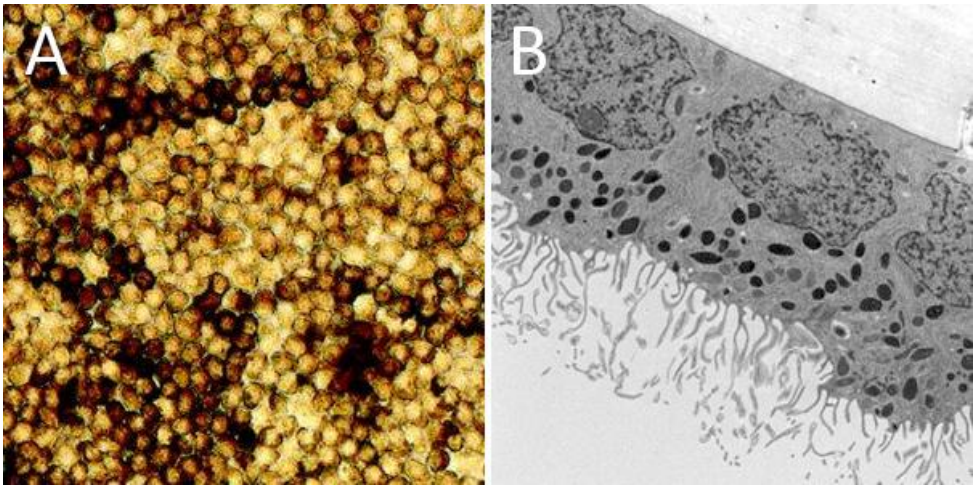

Figure 2.4 Microscope images of human fetal RPE cells
Human fetal RPE cells, with representative cobblestone cell morphology and varying degrees of pigmentation as seen with a light microscope at a 20x magnification (A) and an electron microscope at a 5000x magnification (B) (modified from Maminishkis et al., 2006).

In addition to analyses of gene expression, it is also important to verify the presence of several key proteins in mature hESC-RPE cells. The aforementioned genes *BEST1, CRALBP,* and *RPE65* code for the proteins Bestrophin, CRALBP and RPE65 respectively. Besides the proteins required for the main functions of RPE cells, it is

important to consider proteins associated with tight junctions, such as claudin, ZO1 (zona occludens-1), and occludin. Tight junctions regulate diffusion of substances across RPE monolayers and are therefore a key component of the blood-retinal barrier, which is why their presence in hESC-RPE needs to be confirmed (Carr et al., 2009). Immunocytochemical stainings are a good way of visualizing not only the presence, but also the localization of target proteins. Counter-staining cell nuclei provides further help in ensuring the localization of the target protein is correct.

Finally, an essential part of characterization of mature hESC-RPE cells is assessing their functionality. Double immunocytochemical stainings can be used for this purpose: in mature RPE cells, Bestrophin should be localized to the basolateral membrane – otherwise cells cannot be considered polarized and fully functional (Marmorstein et al., 2009). This can be tested by staining a protein commonly present at the apical membrane, such as Na^+/K^+-ATPase, in addition to Bestrophin. In functional hESC-RPE cells, the two proteins will not be co-localized. The presence of tight junction proteins is linked to functionality of RPE cells. Besides methods involving immunocytochemistry, described above, the presence of tight junctions can be assessed by measuring transepithelial resistance. It is calculated as the difference between the resistance of cells cultured on inserts specifically engineered for that purpose, and resistance of inserts without cells (Kubota et al., 2006). Finally, functionality of hESC-RPE can also be tested with an *in vitro* phagocytosis assay, using fluorescently-labeled POS isolated from either porcine or bovine eyes. Latex beads may also be used, although this technique is not as reliable, because *in vivo*, RPE cells will only phagocytose POS (Carr et al., 2009). Consequently, mature and functional hESC-RPE cells must be capable of binding and internalizing POS.

2.4 FEEDER CELL LAYERS AND hESCs

In order to keep pluripotent stem cells in their undifferentiated state, they are routinely co-cultured with a layer of mitotically inactivated fibroblasts, referred to as feeder cells (Stacey et al., 2006). Morphologically undifferentiated fragments of hESC colonies need to be passaged onto new feeder cell layers at regular intervals (e.g. weekly), because otherwise they begin to spontaneously differentiate (Hasegawa et al., 2010). Many fibroblast cell lines have been evaluated for hESC support, and it has been shown

that some are able to maintain hESCs in their undifferentiated state better than others (Richards et al., 2003). Feeder cell layers secrete a variety of soluble factors, most of which are currently unknown, and thus provide nourishment to the stem cells. Furthermore, the extracellular environment influences differentiation of stem cells along different cell lineages in the same way as during embryonic development. This was shown by Gong et al, who studied the effects of ECM and neighboring cells on induction of hESCs into RPE cells. For example, the basement membrane plays an important role in RPE differentiation and polarization during early eye development. Also, when co-cultured with human Bruch's membrane, hESC-derived neural progenitor cells begin expressing RPE-specific genes (Gong et al., 2008). Evidently, differentiation of hESCs towards a specific cell lineage largely depends on the extracellular environment, which includes direct contact between cells, interactions with the ECM, as well as soluble factors secreted by surrounding cells.

2.4.1 Mouse embryonic fibroblasts

Traditionally, pluripotent stem cells have been derived and maintained as co-cultures with feeder cell layers made up of mEFs, using cell culture media supplemented with fetal bovine serum (FBS) (Thomson et al., 1998; Reubinoff et al., 2000). These culture conditions have also been used in RPE differentiation culture, and the use of feeder cell layers of human origin for this purpose was only recently introduced (Buchholz et al., 2009; Idelson et al., 2009).

The use of mEFs as feeder cells in stem cell culture is still rather common, despite the fact that this way of maintaining human pluripotent stem cells has many shortcomings. Most importantly, stem cells that are derived and cultured on mEFs are exposed to various murine molecules and viruses that could be transmitted to the stem cells. This renders such stem cells unsuitable for clinical applications (Stacey et al., 2006). Furthermore, mEFs reach replicative senescence fairly quickly – only five to six passages after derivation. This means that new mEFs need to be derived continuously and the quality of different batches is subject to variation (Amit et al., 2003). The use of FBS is also disadvantageous because of its animal origin, although this problem can be overcome by optimizing serum-free culture conditions, or using serum replacement (Stacey et al., 2006). Serum replacement, despite offering more defined culture

conditions, is not ideal, as it contains AlbuMAX, a lipid-rich albumin fraction of bovine serum and other proteins of animal origin, such as bovine transferrin (Unger et al., 2008). Overall, when mEFs are used as feeder cell layers, defined culture conditions would not eliminate the issue of patient safety, which is a considerable setback to routine clinical use.

2.4.2 Fibroblasts of human origin

Currently, a lot of effort is being put towards optimizing xeno-free culture conditions for stem cells. One approach is to use fibroblasts of human origin as feeder cells. Different human fibroblast cell lines, such as neonatal foreskin fibroblasts, are available commercially, while adult human fibroblasts can also be derived in-house.

The morphology of hESC colonies seems to be dependent on the morphology of the feeder cells – colonies grown on human feeder cell layers are not as round as those grown on mEFs, but are rather angular, organized according to the direction of the feeder cell layer. Nevertheless, the morphology of individual hESCs remains the same, regardless of the fibroblast type used as a feeder cell layer (Amit et al., 2003).

In a study conducted by Richards et al, several different fibroblast cell lines of human origin were tested, and their qualities as feeder cells were evaluated and compared to those of mEFs. Results of this study show that fetal muscle, fetal skin and adult skin fibroblasts are the most supportive human feeder cells from the ones tested, and are superior to mEFs. Nevertheless, it is clear that not all human fibroblasts support hESC growth equally well, and several of the tested cell lines, such as neonatal foreskin and adult muscle fibroblasts, were inferior to mEFs (Richards et al., 2003). Results of a different study, conducted by Amit et al, demonstrate that hESCs cultured on human foreskin fibroblasts (hFFs) for prolonged periods of time possess the same developmental potential as hESCs cultured on mEFs and also maintain their characteristic features (Amit et al., 2003). Currently, the most commonly used type of human fibroblasts for derivation and culture of hESCs are the commercially available neonatal hFFs (Aguilar-Gallardo et al., 2010; Skottman, 2010; Ström et al., 2010). Skin is a more easily accessible tissue source than neonatal foreskin, and it is possible to derive human dermal fibroblasts (hDFs) with relative ease and reliability. Cell lines acquired in such a way have also been successfully used for derivation and maintenance

of hESCs (Richards et al., 2003; Tecirlioglu et al., 2010). The use of human fibroblasts as feeder cell layers is a step towards developing a methodology that would allow generation of clinical-grade hESCs in xeno-free conditions.

Naturally, there are problems arising from the use of fibroblasts of human origin as well. Most importantly, there is a risk of contamination by human infectious agents in the fibroblast donor that could be transmitted to the stem cells (Stacey et al., 2006). Careful safety testing would therefore be required prior clinical use to ensure that risks of contamination are minimal.

It is very likely that RPE development is influenced by the various soluble factors produced and secreted by neighboring cells. There is evidence gathered from primate pluripotent stem cells, which shows that the effects feeder cells have on stem cells are not reproduced by either conditioned media or ECM alone. This seems to imply that there are many different factors and ECM components acting synergistically, and some of them might be membrane-bound or delivered to neighboring cells through gap junctions, or simply highly unstable (Pera et al., 2000). This would mean that a feeder-free culture environment would not be as effective as one using feeder cell layers. However, a feeder-free environment has advantages that, depending on the application, may outweigh its shortcomings – it is xeno-free and defined. Furthermore, by coating cell culture substrate with an appropriate ECM component and adding growth factors to cell culture media, differentiation efficiency can be significantly improved.

2.5 SOLUBLE FACTORS AND RPE

Results of the study conducted by Schuldiner and co-authors suggest that different growth factors either promote proliferation or induce apoptosis of specific cell types, thereby influencing differentiation of hESCs. None of the eight growth factors tested in this study act as inducing agents of just one cell type, although certain growth factors seem to direct differentiation towards a specific germ layer (Schuldiner et al., 2000). There are numerous soluble factors that potentially affect differentiation of hESCs into RPE cells, and only a small fraction of them will be mentioned here.

2.5.1 Activin A, bFGF and TGF-β

As mentioned earlier, bFGF is often added to cell culture media to keep stem cells in their undifferentiated state due to its effects on self-renewal. This growth factor sustains undifferentiated proliferation of hESCs even in the absence of feeder cells or medium conditioned by them (Xu et al., 2005). Several members belonging to the transforming growth factor beta (TGF-β) superfamily of proteins, such as bone morphogenetic proteins (BMPs) and activins, are secreted by the extraocular mesenchyme. It appears as if these soluble factors direct the differentiation of the optic vesicle towards RPE. Activin A has been singled out as the growth factor that mimics the action of extraocular mesenchyme during early eye development (Fuhrmann et al., 2000). Nevertheless, there is also evidence suggesting that the TGF-β family as a whole plays an important role in maintaining self-renewal and pluripotency of ESCs. For instance, BMPs are key components of serum, and are often used as supplements in cell culture media due to their ability to block differentiation of pluripotent stem cells by inducing expression of certain transcription factors (Pouton & Haynes, 2005). Furthermore, it seems that TGF-β signaling inhibits the commitment of ESCs to neuroectodermal differentiation pathway, and boosts differentiation of mesodermal tissues (Watabe & Miyazono, 2009; Mignone et al., 2010).

Activin A, TGF-β and bFGF were all included in the study carried out by Schuldiner et al. Based on the results of the study, it seems that bFGF has an inductive effect on differentiation of the ectodermal and mesodermal cells, but not endodermal cells; TGF-β induces differentiation of stem cells into cells of mesodermal origin; and activin A has an inhibitory influence on differentiation of endodermal and ectodermal cells, but allows differentiation into mesodermal cells (Schuldiner et al., 2000). Based on this data, neither activin A nor TGF-β would be beneficial for generation of hESC-RPE cells or other derivatives of the ectoderm. However, there is evidence to the contrary, discussed below.

The RPE and the multilayered neural retina develop from common precursor cells and during the early stages of eye development become arranged so that the extraocular mesenchyme surrounds the RPE, while the neural retina is in close contact with the surface ectoderm. During early eye development, the ectoderm that faces the neural

retina expresses bFGF, which inhibits RPE formation and activates specification towards the neural retina (Fuhrmann et al., 2000). It appears that FGF signaling has an inductive effect on the expression of genes specific to the neural retina, such as *CHX10*, while down-regulating RPE-specific genes such as *MITF*, although the exact mechanisms are not clear (Martinez-Morales et al., 2004). Meanwhile, extraocular mesenchyme activates the expression of transcription factors essential for RPE development, most importantly *MITF*, and down-regulates the expression of *CHX10*. Activin A appears to be capable of eliciting similar effects on developing cells *in vitro*, and acts as an antagonist to the action of bFGF (Fuhrmann et al., 2000). In other words, by influencing expression of genes such as *MITF* and *CHX10*, bFGF directs differentiation of hESCs towards the cells of the neural retina, and activin A – towards cells of the RPE.

In a study conducted by Idelson et al, activin A was shown to further induce differentiation of hESCs towards RPE cells, but only after pre-treatment with nicotinamide. According to the authors, the reason for this was that expression of activin receptors was induced by nicotinamide, so in its absence, activin A had no inductive effect on differentiation (Idelson et al., 2009). There is evidence showing that mEFs secrete significantly higher amounts of activin A than several commercially available hFF cell lines (Eiselleova et al., 2008). It is possible to speculate that due to their high secretion of activin A, mEFs would induce RPE differentiation more strongly than hFFs.

It has also been suggested that bFGF induces RPE proliferation and transdifferentiation of RPE cells into cells bearing a fibroblast phenotype. This phenomenon can be used to accelerate formation of a confluent RPE monolayer – adding bFGF to the culture medium will cause transdifferentiation while speeding up cell proliferation, and once the monolayer is confluent, cells will reacquire the RPE morphology (Klimanskaya, 2006). It was mentioned earlier that hESC-RPE cells typically undergo EMT when transferred from suspension to adherent culture. TGF-β is involved in EMT in a variety of cell types, but it seems that in the case of RPE cells it only serves to enhance the transition to a myofibroblastic phenotype. EMT is initiated by loss of contacts between neighboring cells, and TGF-β alone is not enough in cultures where cell-cell contacts are intact (Tamiya et al., 2010).

Finally, Kubota and co-authors tested effects of bFGF, TGF-β2, ascorbic acid, and platelet-derived growth factor (PDGF) on cultured monolayers of RPE cells. Their results show that addition of bFGF to the culture medium caused the monolayers to become more fragile, so that it was impossible to harvest intact cell sheets. TGF-β2, on the other hand, strengthened the cell sheets and allowed for harvest of good-quality tear-resistant RPE monolayers. This effect can be attributed to the apparent increased deposition of ECM in RPE cells cultured in the presence of TGF-β2 (Kubota et al., 2006).

2.5.2 Other soluble factors

There are numerous other soluble factors that affect the rate of differentiation of hESCs and direct it towards different cell lineages. For example, it has been suggested that neurotrophins are the reason mEFs, or even media conditioned by mEFs, are good at maintaining undifferentiated hESC colonies (Pyle et al., 2006). PEDF is a multifunctional protein that belongs to the serpin (serine protease inhibitor) superfamily of proteins. PEDF has neurotrophic and neuroprotective properties, meaning that it induces cell differentiation and protects neurons in the brain. It also inhibits angiogenesis and allegedly has anti-tumor effects. Due to its neuroprotective and anti-angiogenic properties, PEDF might be useful as a therapeutic agent to treat ocular diseases such as AMD (Filleur et al., 2009). It is detected in RPE, photoreceptors and inner retinal cell types in the developing eye (Becerra, 2006). PEDF was also detected in cell culture medium conditioned by mEFs (Lim & Bodnar, 2002; Chin et al., 2007). Finally, according to a study conducted by Idelson et al, nicotinamide promotes neural differentiation of hESCs by preventing apoptosis of neural cells (Idelson et al., 2009). Addition of factors such as PEDF or nicotinamide to cell culture media used for differentiation of hESCs towards RPE cells would likely affect the process in a positive way, although reactions of cells to growth factors are not straightforward and cannot be accurately predicted.

3. AIMS OF THE STUDY

The purpose of this study was to compare the influence that different types of fibroblasts have on differentiation of RPE cells from hESCs. The main point of interest was the pool of soluble factors secreted by the different fibroblast cell lines, so the possible effects of direct contact between fibroblasts and differentiating hESCs were not taken into consideration. Two fibroblast cell lines of human origin – one neonatal, commercially available foreskin fibroblast cell line and one post-natal, in-house derived dermal fibroblast cell line – as well as a primary mEF cell line were chosen for this study. The hypothesis was that the pool of soluble factors secreted by mEFs is superior to that of the fibroblasts of human origin at inducing differentiation of RPE cells from hESCs. The evidence of mEFs secreting relatively high amounts of activin A, a soluble factor involved in RPE formation, served as the basis for this hypothesis.

4. MATERIALS AND METHODS

4.1 CELL CULTURE

All cells used in this study were handled in sterile conditions and cultured in humidified incubators (Thermo Electron Corp., Waltham, MA, USA) at 37°C and 5% CO_2. The study of human embryos at Regea - Institute for Regenerative Medicine, University of Tampere has been approved by National Authority for Medicolegal Affairs Finland (TEO) (Dnro 1426/32/300/05). Derivation of hESC lines from surplus human embryos, their culture and differentiation, as well as derivation of hDF cell lines from skin biopsies at Regea is supported by the Ethical Committee of Pirkanmaa Hospital District (R05116 and R05149, respectively).

4.1.1 Stem cells

The hESC line Regea 06/040 (derived in-house) was maintained on mitotically inactivated hFFs using hESC culture medium, which consisted of Knock-Out Dulbecco's Modified Eagle Medium (KO-DMEM) supplemented with 20% Knock-Out Serum Replacement (KO-SR), 2 mM GlutaMax-I, 0.1 mM 2-mercaptoethanol (all from Life Technologies, Carlsbad, CA, USA), 1% Non-essential Amino Acids (NEAA), 50 U/ml Penicillin/Streptomycin (both from Lonza Group Ltd, Basel, Switzerland) and 8 ng/ml human bFGF (R&D Systems Inc., Minneapolis, MN, USA). The culture medium was changed six times a week and undifferentiated colonies were passaged weekly onto fresh feeder cell layers.

4.1.2 Feeder cells

Three fibroblast cell lines – hFF (CRL-2429™ ATCC, Manassas VA, USA), P-mEF (EmbryoMax®, Millipore, Billerica, MA, USA), and hDF003/06 (derived in-house) – were used in the study. The following culture media were used for their maintenance: Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS and 0.5% Penicillin/Streptomycin (Lonza Group Ltd) for hFFs; IMDM supplemented with 15% Human Serum (HS) and 0.5% Penicillin/Streptomycin for hDFs; and KO-DMEM supplemented with 10% FBS and 1% GlutaMax-I for mEFs. All basal media, as well as GlutaMax-I were from Life Technologies; and all sera were from PAA Laboratories

GmbH, Pasching, Austria. The hDF and mEF culture media were sterile filtered using 0.4 μm sterile filter tips. The cell culture flasks for mEFs were pre-coated with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO, USA). Coating was performed by adding 10 ml of 0.1% gelatin (in ddH_2O, sterilized by autoclaving) to each flask and following a 2 h incubation at room temperature, excess gelatin was removed and flasks were rinsed twice with Dulbecco's Phosphate Buffered Saline (DPBS) (Lonza Group Ltd).

4.1.3 Collection of conditioned media

When all fibroblast cultures reached confluence, they were detached using TrypLE Select (Life Technologies): culture media were aspirated and cells were rinsed twice with DPBS, 5 ml of pre-warmed TrypLE Select was added to each flask and allowed to take effect at 37°C for about 15 min. Once the cells had detached from the plastic, 5 ml of the appropriate, pre-warmed culture medium was added to each flask and the resulting single-cell suspensions were collected to 15 ml Falcon tubes. The single-cell suspensions were centrifuged at 1000 rpm for 3 min, after which the supernatants were aspirated and cell pellets resuspended in 1 ml of appropriate culture medium. Viable cells were counted with Trypan Blue exclusion as a 1:10 dilution (10 μl of each single-cell suspension combined with 90 μl of Trypan Blue) using a hematocytometer. Each single-cell suspension was treated with γ-radiation (40 Gy) in order to inactivate the mitotic activity of the cells. Irradiated cells were plated on 10-cm cell culture dishes pre-coated with 0.1% gelatin as previously described – two dishes per cell line, 2.8×10^6 cells per dish – and left overnight to adhere. The following day, adaptation to RPE differentiation medium (RPE DM-) began: in all culture dishes, the medium was changed to 50% RPE DM- (5 ml of appropriate maintenance medium and 5 ml of RPE DM-) and the next day to 100% RPE DM- (16 ml/dish). RPE DM- consisted of KO-DMEM supplemented in the same way as hESC culture medium, only without bFGF and using 15% KO-SR. Once the adaptation of fibroblasts to RPE DM- was completed, collection of conditioned media (CM) was initiated. For a period of 10 days, the media were collected from the culture dishes and replaced with 16 ml of fresh RPE DM-. To ensure that no cells are present in final CM, collected media were centrifuged at 1000 rpm for 4 min and transferred to new tubes. All CM were stored at -70°C until the collection period ended, then thawed and pooled according to fibroblast type to give a

total of about 320 ml of each medium. The pooled CM were divided into 12 ml aliquots and stored at -70°C for future use.

4.1.4 Differentiation culture

The undifferentiated colonies of hESCs were manually dissected into smaller pieces with a disposable sterile scalpel and, using a disposable sterile needle were transferred to a low cell bind 6-well plate (Nalge NUNC, Tokyo, Japan), where they would spontaneously form three-dimensional cell aggregates. Before dissection, hESC culture medium was aspirated and replaced by RPE DM- after being rinsed with DPBS, to make sure the amount of bFGF present in differentiation cultures is minimal. Differentiation in the 6-well plate was done using each type of CM (mEF-CM, hDF-CM and hFF-CM) and RPE DM-, which served as a control. The medium was changed six times a week. For medium replacement, the plates were tilted until cell clusters settled in the lower part of each well and roughly 80% of the medium was gently aspirated.

4.1.5 Maturation of hESC-RPE

After 45 days in suspension culture, the three-dimensional cell aggregates were used to create hESC-RPE monolayer cultures. From each medium type, pigmented cell aggregates were selected and washed with DPBS, after which each sample was treated with 100 µl of 1% Trypsin (Lonza Group Ltd). The treatment lasted for a total of about 20 min at 37°C, during which the cell aggregates were triturated several times until single-cell suspensions was obtained from each sample. Trypsin was inactivated with HS, 100 µl/sample. The cell suspensions were collected into sterile 1.5 ml microfuge tubes, centrifuged at 1000 rpm for 3 min and supernatants were aspirated. Cell pellets were resuspended in 200 µl of differentiation medium (RPE DM- or the appropriate type of CM) and 100 µl of each single-cell suspension was seeded onto a 24-well plate (Corning Cellbind, from Corning Inc., Corning, NY, USA) coated with 5 µg/cm^2 human placental collagen IV (Sigma-Aldrich) and containing 400 µl of medium, one well per sample. Coating was done by adding 250 µl of the collagen IV stock solution diluted in DPBS to each well and, following a 3 h incubation at 37°C aspirating excess solution and rinsing each well twice with DPBS. The remaining 100 µl of each cell suspension was seeded onto porous BD BioCoat™ cell culture inserts treated with mouse collagen IV (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing 150 µl of

differentiation medium, and placed inside the wells of a 24-well plate containing 500 µl of the same medium. The monolayer cultures were maintained using RPE DM- and each type of CM for four weeks. The media were changed three times a week. Aspiration of the medium was done under a stereomicroscope, so as to avoid cell detachment. The cultures were regularly observed under a light microscope (Nikon Corp., Tokyo, Japan) and changes in cell morphology and pigmentation were followed. Representative images of cells in each medium type were taken regularly.

4.2 EVALUATION OF DIFFERENTIATION

All cultures were observed daily under a light microscope and the appearance, as well as the progression, of pigmentation were followed. Efficiency of pigmentation was evaluated by counting the amount of pigmented areas in relation to the total number of cell aggregates in each of the culture conditions.

4.2.1 qPCR

At three time-points (7, 14, and 28 days in differentiation culture), 10-15 cell aggregates were collected from each medium type and lysed using a lysis buffer (RA1) supplemented with a reducing agent – tris(2-carboxyethyl)phosphine (TCEP). Both reagents were supplied in the NucleoSpin RNA XS kit (Macherey-Nagel, GmbH & Co, Düren, Germany). In addition, about 10-15 pieces of undifferentiated hESC colonies (UD hESC) were treated in the same way before differentiation cultures were set up. RNA was extracted from the lysates using the NucleoSpin RNA XS kit, following the manufacturer's protocol. RNA samples were eluted with 10 µl of nuclease-free H_2O. The RNA concentration of each sample was determined using NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). From each RNA sample, 200 ng were used to synthesize complementary DNA (cDNA) using the high-capacity cDNA RT kit (Applied Biosystems Inc., Foster City, CA, USA), according to the manufacturer's instructions. Each reaction mixture contained 2 µl RT Buffer (10x), 2 µl RT Random Primers (10x), 1 µl RNase Inhibitor (10 U/µl), 0.8 µl dNTP Mix (100 mM), 1 µl MultiScribe Reverse Transcriptase, and 200 ng RNA diluted with nuclease-free H_2O to the final volume of 20 µl. Synthesis of cDNA was carried out in PCR MasterCycler (Eppendorf AG, Hamburg, Germany): 10 min at 25°C, 120 min at 37°C,

5 min at 85°C and then cooled down to 4°C. The cDNA samples were analyzed with qPCR and the differences of expression of a number of genes representative of particular tissue types were evaluated. Sequence-specific 20x TaqMan Gene Expression Assays (Applied Biosystems Inc.) that were used for this purpose are presented in Table 4.1, along with the functions of the genes, assay numbers and time-points at which their expression was analyzed. *GAPDH* was used as an endogenous control, to correct the possible differences between the cDNA samples.

Table 4.1 TaqMan Gene Expression Assays
Abbreviations: *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase, *RAX*: retina and anterior neural fold homeobox, *PAX6*: paired box gene 6, *MITF*: microphthalmia-associated transcription factor, *SOX17*: sex-determining region Y-box 17, *T*: Brachyury, *RPE65*: retinal pigment epithelium-specific 65kDa protein, *BEST1*: bestrophin, *CHX10*: ceh-10 homeodomain containing homolog, *CRX*: cone-rod homeobox

Gene name	***Function***	***Assay Number***	***Time-point***
GAPDH	Housekeeping gene	Hs99999905_m1	d7, d14 and d28
RAX	Early eye marker	Hs00429459_m1	d7, d14 and d28
PAX6	Early neuroectodermal marker	Hs00240871_m1	d7, d14 and d28
MITF	RPE precursor marker	Hs01115553_m1	d7, d14 and d28
SOX17	Early endodermal marker	Hs00751752_s1	d7
T	Early mesodermal marker	Hs00610080_m1	d7
RPE65	Mature RPE marker	Hs01071462_m1	d28
BEST1	Mature RPE marker	Hs00959251_m1	d28
CHX10	Marker of the neural retina	Hs01584048_m1	d28
CRX	Photoreceptor marker	Hs01549313_m1	d28

Each reaction mixture consisted of 7.5 µl Taqman Universal PCR Master Mix (2x), 0.75 µl Gene Expression Assay (20x), 3 µl of cDNA (diluted 1:5 with sterile water) and sterile water to the final volume of 15 µl. All samples and controls were run as triplicate reactions in optical 96-well plates with the 7300 Real-time PCR system (Applied Biosystems Inc.) as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C, with the measurements being taken during the last step of each cycle. Data was analyzed using 7300 System SDS Software (Applied Biosystems Inc.). Based on the cycle threshold (C_T) values given by the software, the relative quantification of each gene was calculated by applying the $2^{-\Delta\Delta Ct}$ method (Livak &

Schmittgen, 2001). Mean fold changes were calculated in Microsoft Excel 2007 using the following equation:

$$Fold\,change = 2^{-\left[\left(C_{T,TARGET} - C_{T,GAPDH}\right) - \left(C_{T,CALIBRATOR}(avg) - C_{T,GAPDH}(avg)\right)\right]}$$

This way, the normalized gene expression of each target gene is compared to that of the calibrator. In this case, the data was analyzed using the UD hESC sample as the calibrator to visualize the extent of differentiation. Then, the difference between culture conditions was emphasized by using the RPE DM- sample as the calibrator. Fold regulations were determined for better visualization of down-regulation: for fold change values ≥ 1, fold regulation is equal to fold change, but for fold change values less than 1, fold regulation is the negative inverse of fold change, calculated as -1/(fold change). The difference in gene expression was considered to be significant when the difference in fold regulation was greater than 2. Results were plotted as bar charts using GraphPad Prism 5. There were no biological replicates in this study, because it was conducted only once and only one set of samples was collected at each time-point. Standard deviations were calculated for each set of technical replicates, and presented as error bars.

4.2.2 RT-PCR

After 30 days of maturation in the wells of the 24-well cell culture plate (a total of 75 days in differentiation culture), hESC-RPE monolayers were mechanically detached and transferred to nuclease-free 1.5 ml microfuge tubes. After being rinsed twice with DPBS, cells were lysed using RA1 lysis buffer supplemented with TCEP. RNA extraction and measurement of RNA concentration in each sample was done as described above, in section 4.2.1. From each RNA sample, 40 ng were used to synthesize cDNA using the high-capacity cDNA RT kit, according to the manufacturer's instructions, as described earlier for qPCR. Negative controls (-RT) were prepared for each sample by replacing MultiScribe Reverse Transcriptase with nuclease-free H_2O. Then, cDNA samples and -RT samples were used as templates for PCR, where each reaction consisted of 2.5 µl Taq Buffer with KCl (10x), 1.5 µl $MgCl_2$ (25 mM), 1.25 µl dNTP mix (2 mM), 1 µl forward primer (5 µM), 1 µl reverse primer (5 µM), 0.125 µl Taq Polymerase (5 U/µl), 1 µl cDNA sample, and nuclease-free H_2O to the final volume of 25 µl. All reagents from Fermentas GmbH (St. Leon-Rot,

Germany), and primers from Biomers.net GmbH (Söflinger, Germany). The primer sequences, along with their annealing temperature and product size are presented in Table 4.2. The PCR cycles were performed in PCR MasterCycler as follows: denaturation at 95°C for 3 min, followed by 38 cycles of denaturation at 95°C for 30 s, annealing at the correct temperature (Table 4.2) for 30 s and extension at 72°C for 1 min, after which the samples were cooled down to 10°C.

Table 4.2 Primers used in RT-PCR
Abbreviations: see Table 4.1, *ACTC*: α-cardiac actin, *AFP*: α-fetoprotein, *OCT3/4*: octamer-binding transcription factor 3/4, *OTX2*: orthodenticle homeobox 2, *PEDF*: pigment epithelium-derived factor, *PMEL17*: pre-melanosomal protein 17, *SOX10*: sex-determining region Y -box 10, *TYR*: tyrosinase.

Gene name	***Primer sequences***	***Annealing Temperature***	***Product size***
ACTC	F: GGA GTT ATG GTG GGT ATG GGT C	55°C	486 bp
	R: AGT GGT GAC AAA GGA GTA GCC A		
AFP	F: GCT GGA TTG TCT GCA GGA TGG GGA A	55°C	216 bp
	R: TCC CCT GAA GAA AAT TGG TTA AAA T		
BEST1	F: GAA TTT GCA GGT GTC CCT GT	55°C	214 bp
	R: ATC CTC CTC GTC CTC CTG AT		
GAPDH	F: GTT CGA CAG TCA GCC GCA TC	55°C	229 bp
	R: GGA ATT TGC CAT GGG TGG A		
MITF	F: AAG TCC TGA GCT TGC CAT GT	52°C	352 bp
	R: GGC AGA CCT TGG TTT CCA TA		
OCT3/4	F: CGT GAA GCT GGA GAA GGA GAA GCT G	62°C	245bp
	R: AAG GGC CGC AGC TTA CAC ATG TTC		
OTX2	F: GGG CCC TGG GCT TCT TGT CC	52°C	318 bp
	R: ATT GGC CAC TTG TTC CAC TC		
PAX6	F: AAC AGA CAC AGC CCT CAC AAA CA	60°C	274 bp
	R: CGG GAA CTT GAA CTG GAA CTG AC		
PEDF	F: AGC TCG CCA GGT CCA CAA AG	60°C	222 bp
	R: TGG GCA ATC TTG CAG CTG AG		
PMEL17	F: GTG GTC AGC ACC CAG CTT AT	52°C	233 bp
	R: GAG GAG GGG GCT ATT CTC AC		
RAX	F: CTG AAA GCC AAG GAG CAC ATC	55°C	409 bp
	R: CTC CTG GGA ATG GCC AAG TTT		
RPE65	F: TCC CCA ATA CAA CTG CCA CT	52°C	316 bp
	R: CAC CAC CAC ACT CAG AAC TA		
SOX10	F: AGC CCA GGT GAA GAC AGA GA	55°C	175 bp
	R: AGG AGA AGG CCG AGT AGA GG		
TYR	F: TGC CAA CGA TCC TAT CTT CC	52°C	316 bp
	R: GAC ACA GCA AGC TCA CAA GC		

Samples were stored at 4°C and resolved by agarose gel electrophoresis the following day. The 2% agarose gel was prepared by mixing 2 g of agarose (PEQLAB Biotechnologie GmbH, Erlangen, Germany) in 100 ml of 1x TBE buffer and heating in

a microwave oven until complete dissolution. The mixture was allowed to cool down to about 60°C, after which 5.5 µl of Ethidium bromide solution (10 mg/ml, Sigma-Aldrich) was added. Each sample and control was treated with 5 µl DNA Loading Dye (6x), and GeneRuler™ 50 bp DNA Ladder (0.5 µg/µl, Fermentas GmbH) was used as a molecular weight standard. The gels were run for 50 min at 90 V and the bands were visualized with Quantity one 4.5.2. Basic program (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

4.2.3 Immunocytochemistry

After 35 days of maturation on cell culture inserts (a total of 80 days in differentiation culture), hESC-RPE monolayers were rinsed twice with DPBS and fixed with 4% paraformaldehyde (PFA) for 20 min. After three washes with DPBS, cell membranes were permeabilized with 0.1% Triton X-100 (diluted in DPBS) for 15 min, and then washed three times with DPBS. Unspecific binding sites were blocked by treating cells for 1 h with 3% Bovine Serum Albumin (BSA, diluted in DPBS). All the aforementioned reagents (except DPBS) from Sigma-Aldrich. Then, the bottom of each cell culture insert was cut out and divided into four pieces. Each piece was treated for 1 h with one or two of the primary antibodies listed in Table 4.3 – anti-CRALBP and anti-MITF were used as a double-staining, and the rest of the antibodies as single stainings. All antibody dilutions were prepared using 0.5% BSA (in DPBS).

Table 4.3 Primary antibodies
Abbreviations: CRALBP: cellular retinaldehyde-binding protein, MITF: microphthalmia-associated transcription factor, RPE65: retinal pigment epithelium-specific 65kDa protein, ZO1: zona occludens-1

Antibody	***Host species***	***Dilution***	***Manufacturer***
anti-Bestrophin (IgG)	rabbit	1:500	Abcam PLC, Cambridge, UK
anti-CRALBP (IgG)	mouse	1:1000	
anti-MITF (IgG)	rabbit	1:350	
anti-RPE65 (IgG)	mouse	1:250	Millipore, Billerica, MA, USA
anti-ZO1 (IgG)	mouse	1:250	Invitrogen, Carlsbad, CA, USA

Primary antibody localization was performed by incubating the cells with a solution containing Alexa Fluor 488 donkey anti-mouse IgG and Alexa Fluor 568 goat anti-

rabbit IgG secondary antibodies (Molecular probes, Life Technologies, Paisley, UK), both diluted 1:1500 in 0.5% BSA, for 1 h, protected from light. VectaShield (Vector Laboratories Inc., Burlingame, CA, USA), a mounting medium containing 4',6-diamidino-2-phenylindole (DAPI), was used for the counter-staining of cell nuclei. All stainings were visualized with LSM 700 confocal microscope (Zeiss, Jena, Germany) using a 63x oil immersion objective. Brightfield (BF) images of each hESC-RPE monolayer were taken with Olympus BX60 confocal microscope (Olympus, Tokyo, Japan) using a 60x oil immersion objective. All images were edited using ZEN 2009 Light Edition (Zeiss), ImageJ, and Adobe Photoshop CS4.

4.2.4 In vitro phagocytosis assay

After 120 days in suspension culture, several cell aggregates from each medium type were tested for their ability to phagocytose porcine POS. For the duration of the assay, samples were protected from light. First, POS were stained with fluorescein isothiocyanate (FITC) as follows. Four POS samples (previously isolated by Vaajasaari & Konsén and stored in 2.5% saccharose buffer at -70°C) were thawed and centrifuged for 4 min at 7000 rpm, after which the saccharose buffer was removed, and POS resuspended in 100 µl of 0.1 M $NaHCO_3$. FITC stock solution (50 µg/µl) was diluted to 1 µg/µl with 0.1 M $NaHCO_3$, and 4 µl of the resulting dilution was added to each POS sample and incubated for 1 h. Each sample was then washed with DPBS three times (using centrifugations at 7000 rpm for 4 min) and finally POS pellets were resuspended in RPE DM- (100 µl/sample) and pooled together. Media from the wells in which the cell aggregates were maintained were aspirated so as to leave about 100 µl of medium in each well. Then, 100 µl of the POS solution was added to each well, and cells were incubated at 37°C and 5% CO_2 for 18 h. Cell aggregates were washed twice with DPBS and fixed with 4% PFA for 30 min at 37°C and 5% CO_2. After two DPBS washes, they were treated with 0.2% Trypan Blue for 10 min at room temperature – to quench the signal from unphagocytosed POS – and then washed with DPBS until clear. Permeabilization was done with 0.1% Triton X-100 for 10 min at room temperature, after which cell aggregates were washed twice with DPBS. Finally, cell aggregates were treated with phalloidin (Sigma-Aldrich) for 10 min at room temperature, washed three times with DPBS, transferred onto object glasses, treated with VectaShield (containing DAPI) and covered with cover slips. Results of the assay were visualized with LSM700

confocal microscope using a 63x oil immersion objective, and images acquired as z-stacks, which were then analyzed with Zen 2009 Light Edition.

4.3 ENZYME-LINKED IMMUNOSORBENT ASSAYS

Concentrations of TGF-β1, Activin A, bFGF, and PEDF in each CM, as well as RPE DM- and KO-SR, were determined using the following commercial kits: Human TGF-β1 Immunoassay, Human/Mouse/Rat Activin A Immunoassay, Human FGF basic Immunoassay (all from Quantikine®, R&D Systems, Minneapolis, MN, USA) and PEDF Sandwich ELISA (Millipore ChemiKine™, Billerica, MA, USA). In each of the assays, the manufacturer's instructions were followed. All standards (supplied in the kits), controls (RPE DM- and KO-SR) and samples (CM) were tested in duplicates. In the Activin A Immunoassay, each sample was diluted 1:5 and 1:25, using the diluent supplied in the kit. Each assay follows the same principle: the quantitative sandwich enzyme immunoassay technique. Each well of the 96-well plate is pre-coated with monoclonal antibodies specific for the target protein. When standards, controls and samples are added to the wells, the target protein is bound to the antibodies and any unbound substances are washed away. Then, an enzyme-linked polyclonal antibody specific for the target protein is added. The target proteins therefore become sandwiched between the two sets of antibodies. After the unbound substances are washed away, a substrate solution is added to each well. The color intensity is proportional to the amount of target protein bound to the antibody-coated wells. The color development is stopped with an acidic solution, and the optical density in each well is measured. In all assays, the optical densities were measured using Wallac Victor^{2}™ 1420 Multilabel counter (Perkin Elmer-Wallace, Norton, OH, USA). Using optical densities of the standard series, standard curves were created in Microsoft Excel 2007. Concentrations of the samples were calculated via the standard curves and presented in the form of bar charts. Standard deviations were calculated from the concentrations of the technical replicates of each tested sample, and were presented as error bars.

5. RESULTS

5.1 CELL MORPHOLOGY AND PIGMENTATION

Undifferentiated areas of hESC colonies were mechanically dissected and transferred to one of the four cell culture media (RPE DM-, mEF-CM, hDF-CM or hFF-CM). After 13 days in differentiation culture, first pigmented areas appeared in several cell aggregates cultured in mEF-CM and hDF-CM. In cultures maintained in hFF-CM and RPE DM-, pigment started appearing after 15 and 20 days of differentiation, respectively (Table 5.1). Pigmentation efficiency in each differentiation culture was counted at two time-points – d31 and d35. The ratio of pigmented areas to the total amount of cell aggregates was higher in hFF-CM than in other media (Table 5.1).

Table 5.1 Pigmentation in different culture conditions
Efficiency of pigmentation was counted after 31 and 35 days in differentiation culture, as a ratio of pigmented areas relative to the total amount of cell aggregates in each of the culture conditions.

Medium type	***First pigment***	***Pigmentation (d31)***	***Pigmentation (d35)***
RPE DM-	d20	22%	21%
mEF-CM	d13	26%	33%
hDF-CM	d13	29%	25%
hFF-CM	d15	39%	45%

While in suspension culture, cell aggregates maintained in RPE DM- and mEF-CM were large and tended to cluster with one another; while those in hDF-CM and hFF-CM were smaller in size and remained more separate (Figure 5.1). Also, aggregates cultured in hFF-CM had larger pigmented areas relative to their size, compared to the cell aggregates in other media.

After 45 days in suspension culture, pigmented areas of cell aggregates were isolated and seeded on substrate coated with collagen IV for maturation. Initially, pigmentation and RPE-specific cell morphology were lost (Figure 5.2A-D).

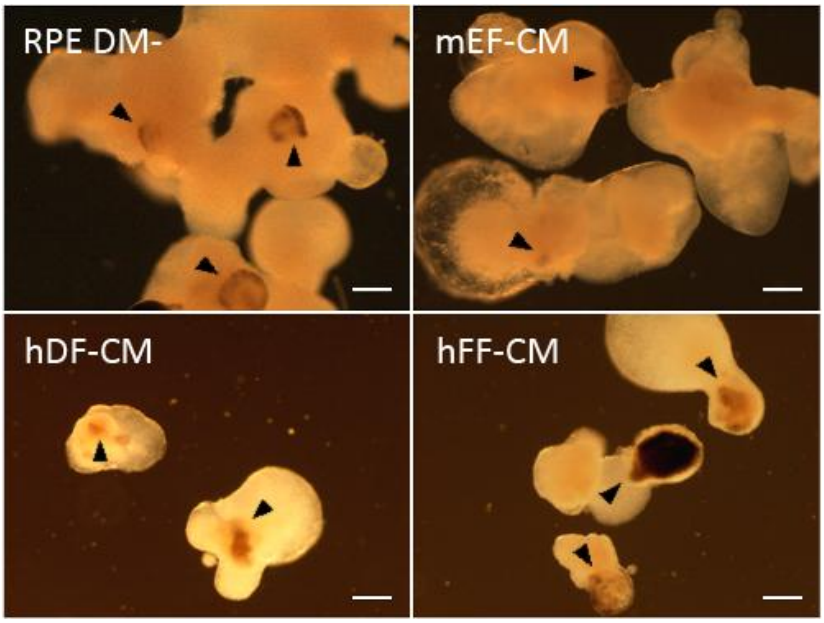

Figure 5.1 Suspension cultures
Cell aggregates after 30 days of differentiation in different culture conditions. Pigmented areas are indicated with arrowheads. Magnification 40x, scale bars 200 µm.

Cells acquired fibroblast-like morphology during the first five days in adherent culture, after which cobblestone morphology started appearing in the center of the cultures, where cells had the most contact with one another. In mEF-CM cobblestone morphology appeared earlier and spread faster than in other cultures, although pigmentation remained rather weak until the end of the experiment (Figure 5.2F). The monolayer in hFF-CM was for the most part heavily layered, which made evaluation of overall morphology and degree of pigmentation difficult. Nevertheless, areas that were not obscured by layering clearly possessed RPE morphology and were pigmented (Figure 5.2H). Pigment started to reappear in hDF-CM and hFF-CM first (around d10 in adherent culture) and then in RPE DM- and mEF-CM (around d14 in adherent culture). The most prominent and wide-spread pigmentation was observed in hDF-CM, starting from d17 onwards (Figure 5.2G). As a whole, the monolayer in RPE DM- was of the worst quality, and began detaching from the cell culture substrate after about 24 days of maturation. The detachment started along the edges and slowly progressed towards the center, although the monolayer did not detach fully within the duration of this study. Good cell morphology was observed at the center of the monolayer despite its weak attachment properties (Figure 5.2E).

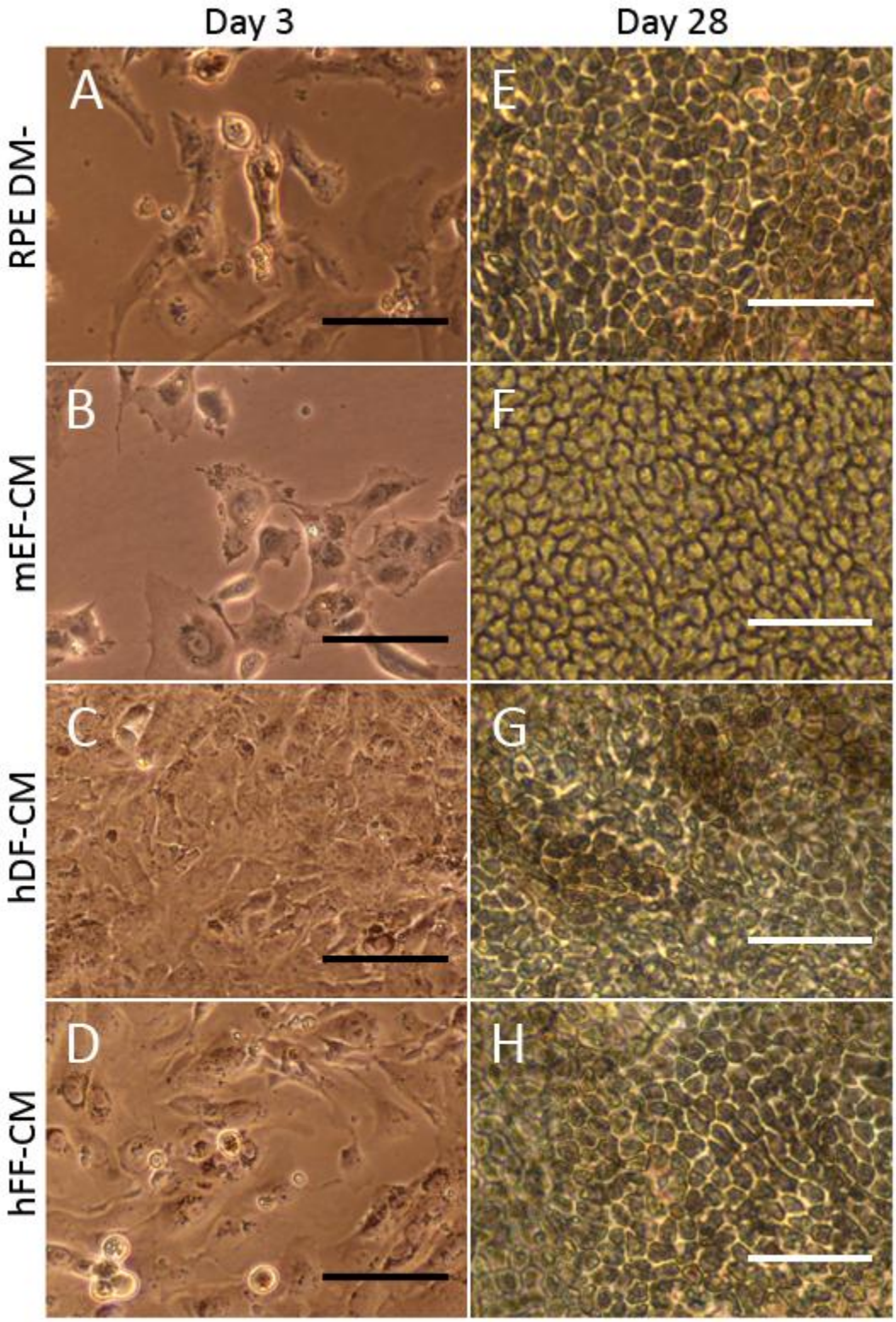

Figure 5.2 Maturation of hESC-RPE cells
Pigmented cells isolated from cell aggregates cultured on a 24-well plate coated with collagen IV in different culture conditions, after 3 days (A-D) and 28 days (E-H) in adherent culture.
Magnification 100x, black scale bars 100 µm, white scale bars 50 µm.

5.2 GENE EXPRESSION IN SUSPENSION CULTURE

In order to evaluate the effects different media had on early stages of differentiation of hESCs, expression levels of a series of markers representative of several key tissue types were analyzed using qPCR. Expression of precursor genes (*RAX*, *PAX6* and *MITF*) was analyzed at all three time-points (d7, d14 and d28), expression of endodermal (*SOX17*) and mesodermal (*T*) markers – at d7, and expression of markers specific to the RPE (*BEST1*, *RPE65*) and the neural retina (*CRX*, *CHX10*) – at d28. All data was normalized using expression levels of *GAPDH*, a commonly-used housekeeping gene. In the case of *RPE65*, *BEST1*, *SOX17* and *T,* expression levels were generally low, with C_T-values higher than 30, which means that fold regulations calculated based on these values are not as reliable as the others.

To better visualize progression of differentiation in different culture conditions, UD hESC sample was used as the calibrator. The data is grouped based on the tissue of origin: early neural markers (Figure 5.3), RPE precursor gene (Figure 5.4), markers of the mesoderm and the endoderm (Figure 5.5), markers of the neural retina (Figure 5.6), and RPE-specific markers (Figure 5.7).

Expression of neural precursor genes *RAX* and *PAX6* should increase at first, and begin declining as RPE cells mature (Fuhrmann, 2010). As seen in Figure 5.3, expression of *PAX6* continues increasing after 28 days in differentiation culture, while, at the last time-point, expression of *RAX* decreased in cells cultured in mEF-CM and hFF-CM. Expression of *MITF*, a gene typical of RPE precursor cells, should increase as differentiation progresses (Fuhrmann, 2010). This pattern is observed in Figure 5.4 – gene expression is slightly down-regulated after 7 days in differentiation culture, but reaches 10-20 fold increase after 28 days, irrespective of culture conditions.

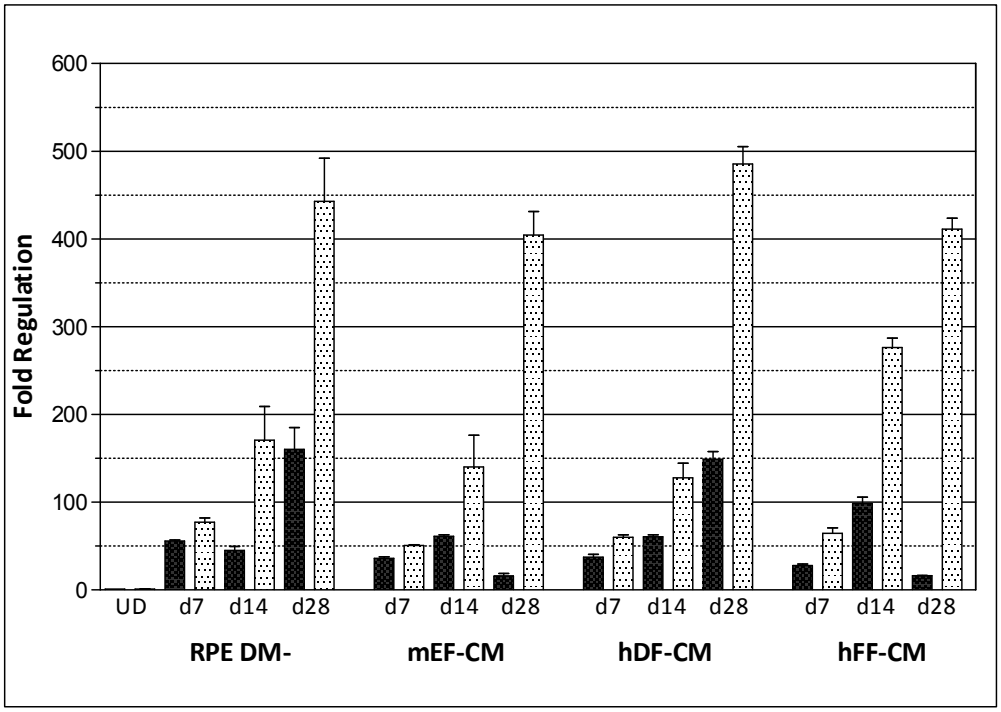

Figure 5.3 Expression of neural precursor genes
Gene expression levels of *RAX* (dark bars) and *PAX6* (light bars) in different culture conditions, measured after 7, 14 and 28 days in differentiation culture. Normalized to *GAPDH* expression, with UD hESC as the calibrator.

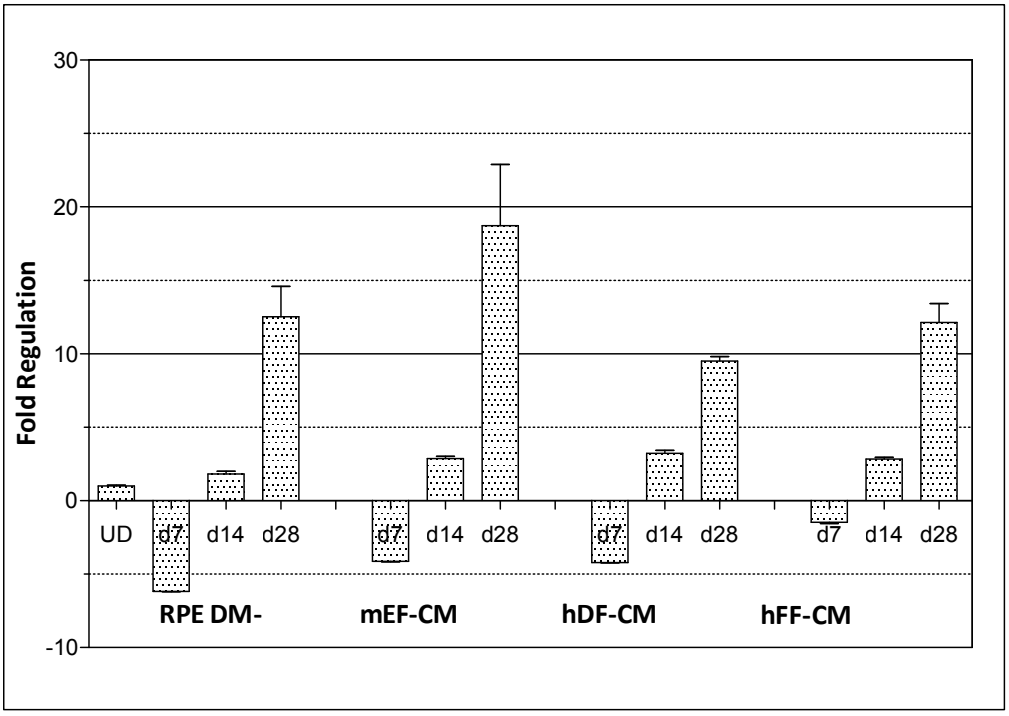

Figure 5.4 Expression of the RPE precursor gene
Gene expression levels of *MITF* in different culture conditions, measured after 7, 14 and 28 days in differentiation culture. Normalized to *GAPDH* expression, with UD hESC as the calibrator.

Expression levels of genes specific to tissues other than the RPE should be as low as possible. Differentiation towards mesodermal or endodermal tissues, analyzed using *T* and *SOX17* respectively, is undesired even as early as at d7. As seen in Figure 5.5, expression levels of both genes are low in cells cultured in RPE DM- or mEF-CM, whereas those cultured in hDF-CM express high levels of *SOX17*, but not *T*. Cells cultured in hFF-CM express rather high amounts of both genes after 7 days in differentiation culture.

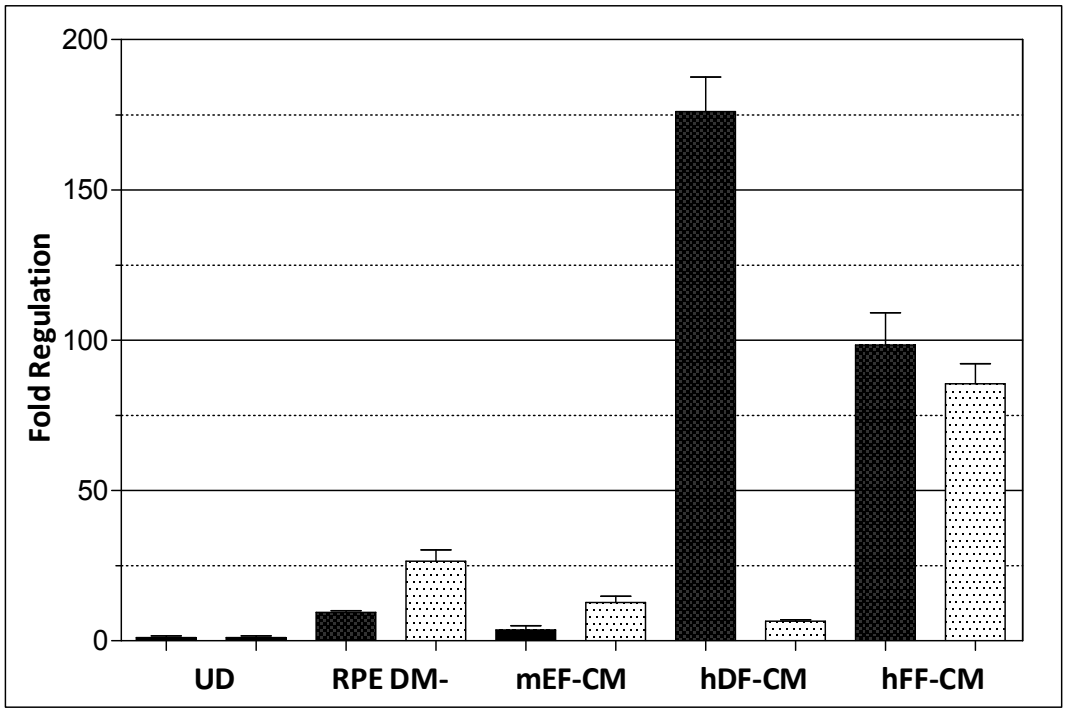

Figure 5.5 Expression of mesodermal and endodermal markers
Gene expression levels of *SOX17* (dark bars) and *T* (light bars) in different culture conditions, after 7 days in differentiation culture. Normalized to *GAPDH* expression, with UD hESC as the calibrator.

Both the RPE and the neural retina are derivatives of the neuroectoderm, which is why it is important to analyze expression levels of genes such as *CHX10* and *CRX* – the former being an early marker of the neural retina, and the latter a photoreceptor marker. After 28 days in differentiation culture, expression levels of *CHX10* were high compared to UD hESCs, and expression of *CRX* was 10-20 times greater than in UD hESCs, irrespective of culture conditions (Figure 5.6).

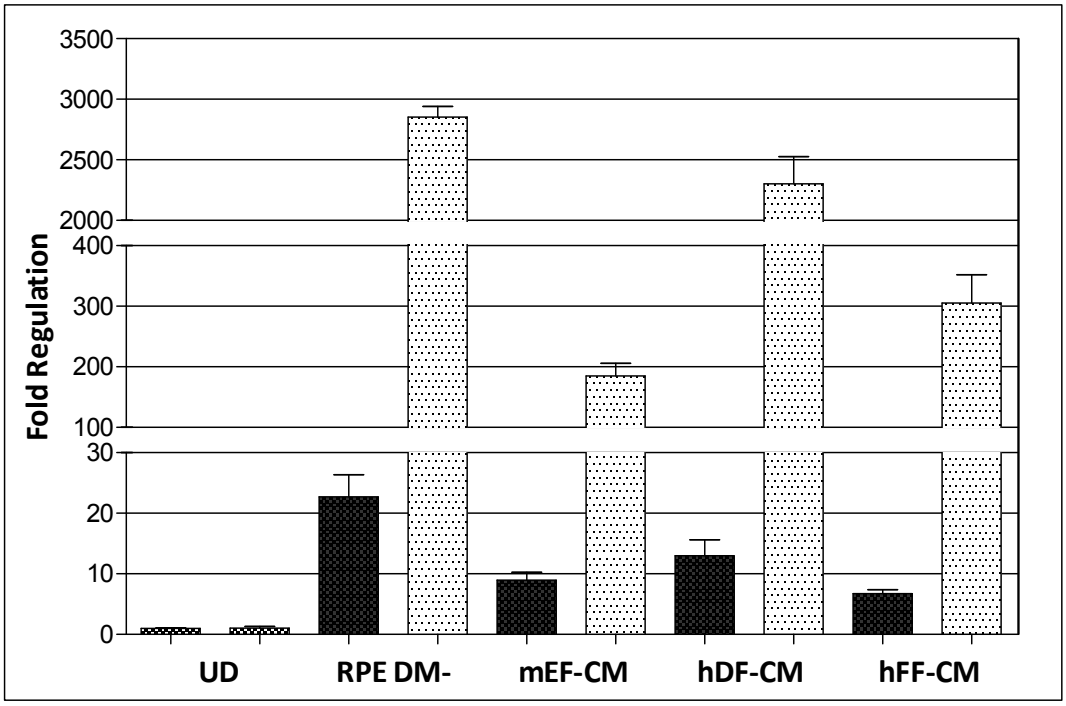

Figure 5.6 Expression of markers of the neural retina
Gene expression levels of *CRX* (dark bars) and *CHX10* (light bars) in different culture conditions, after 28 days in differentiation culture. Normalized to *GAPDH* expression, with UD hESC as the calibrator.

Finally, expression of RPE-specific markers should increase as the hESC-RPE cells mature. Expression levels of *RPE65* and *BEST1* after 28 days in differentiation culture are presented in Figure 5.7. Expression of *BEST1* remained fairly low in all cells, while *RPE65* was up-regulated in cells cultured in CM to a higher degree than in those cultured in the control medium.

Gene expression was also analyzed using the control medium, RPE DM-, as the calibrator. This way the influence of different culture conditions on differentiation is seen more clearly. Results of this analysis are grouped in accordance with the time-point at which representative samples were collected from each differentiation culture: d7 (Figure 5.8), d14 (Figure 5.9), and d28 (Figure 5.10).

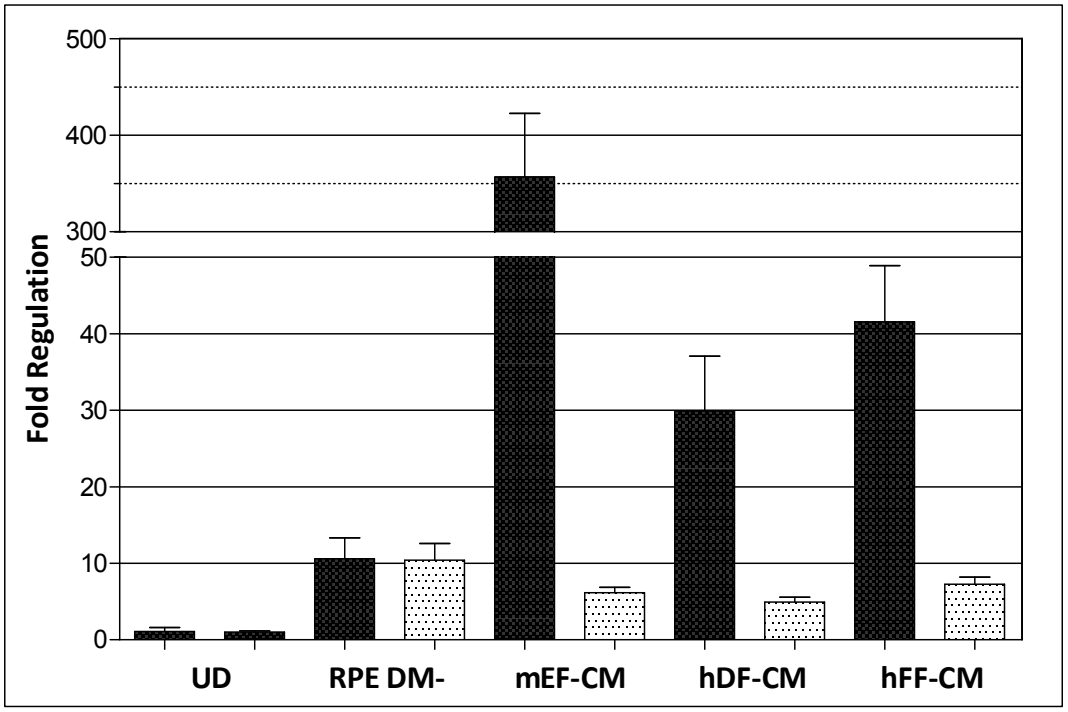

Figure 5.7 Expression of RPE-specific markers
Gene expression levels of *RPE65* (dark bars) and *BEST1* (light bars) in different culture conditions, after 28 days in differentiation culture. Normalized to *GAPDH* expression, with UD hESC as the calibrator.

After 7 days in differentiation culture (Figure 5.8), expression of *MITF* was about 4 times higher in cells maintained in hFF-CM, compared to cells cultured in RPE DM-. This was also the only difference of more than 2 fold regulations in the expression of *MITF* – at the other two time-points expression was more or less equal in every medium. Expression levels of *T* and *SOX17* were analyzed only at the d7 time-point, and for both genes it was higher in cells cultured in hFF-CM than in the control sample. In cells cultured in hDF-CM, *SOX17* was up-regulated, and *T* was down-regulated. Expression of both genes was slightly lower in cells cultured in mEF-CM than in the control sample. Expression levels of *RAX* and *PAX6* were lower in cells cultured in CM than in control medium.

After 14 days in suspension culture (Figure 5.9), the only difference in gene expression was in the case of *PAX6* – it was slightly lower in cells cultured in mEF-CM and hDF-CM, with fold regulations of just under -1.

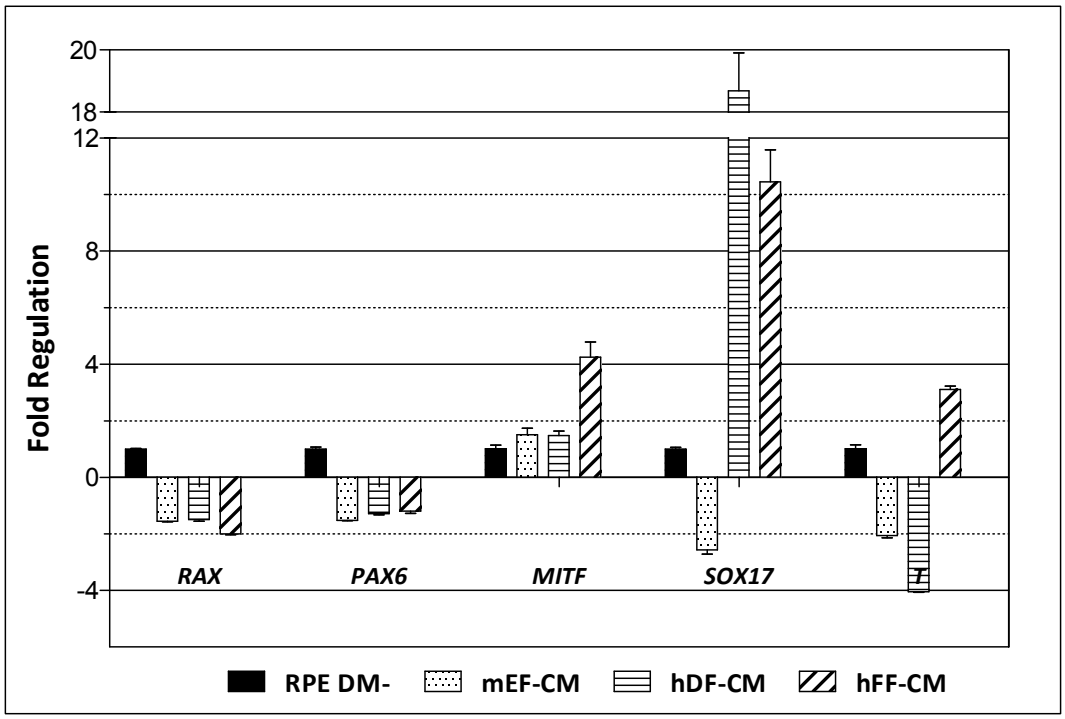

Figure 5.8 Gene expression at d7
Differences in expression levels of *RAX, PAX6, MITF, SOX17* and *T* after 7 days in different culture conditions. Normalized to *GAPDH* expression, with sample acquired from RPE DM- culture as the calibrator.

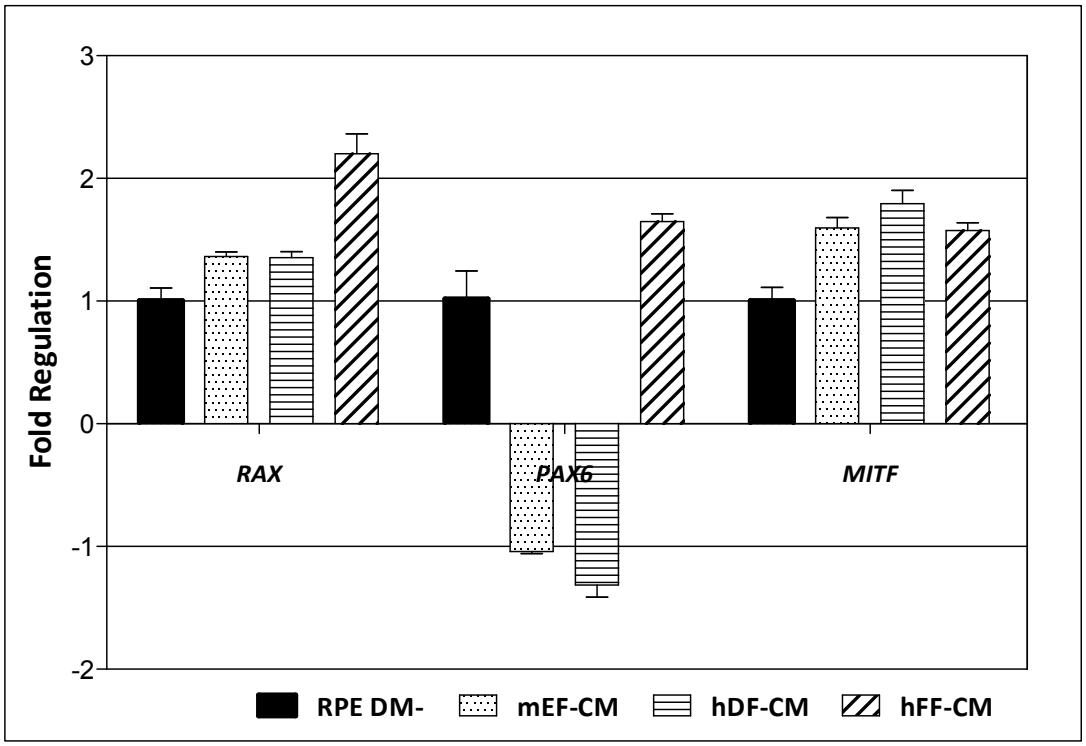

Figure 5.9 Gene expression at d14
Differences in expression levels of *RAX, PAX6* and *MITF* after 14 days in different culture conditions. Normalized to *GAPDH* expression, with sample acquired from RPE DM- culture as the calibrator.

Finally, after 28 days in suspension culture (Figure 5.10), several differences in levels of gene expression were observed.

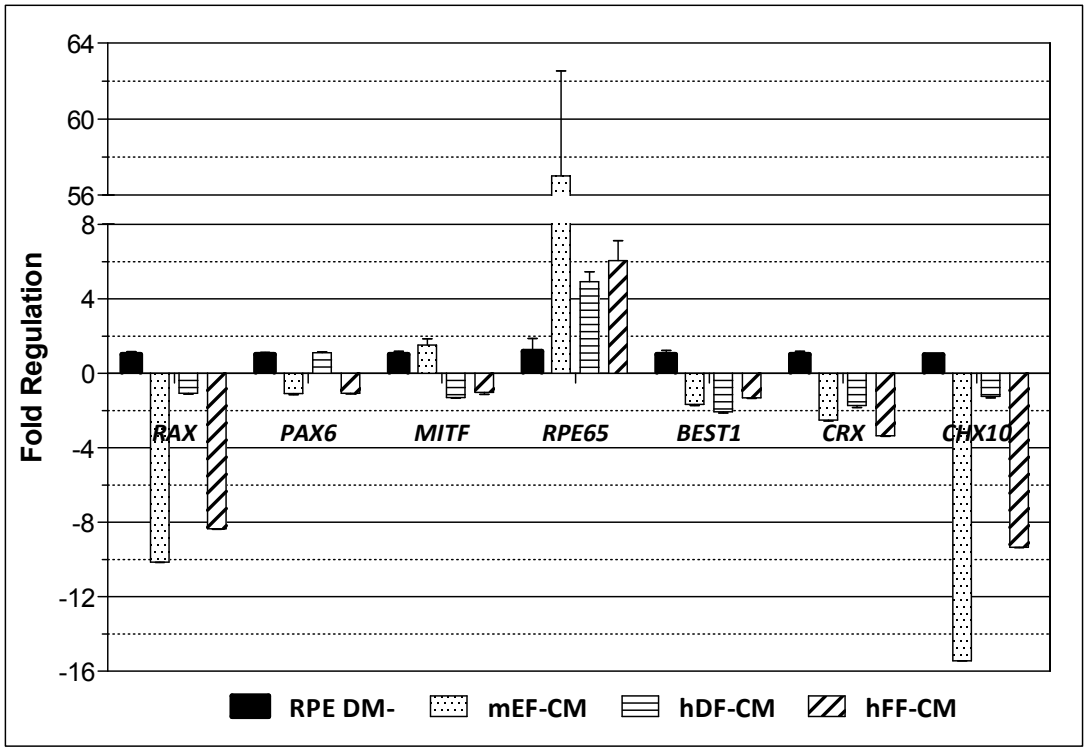

Figure 5.10 Gene expression at d28
Differences in expression levels of *RAX, PAX6, MITF, RPE65, BEST1, CRX* and *CHX10* after 28 days in different culture conditions. Normalized to *GAPDH* expression, with sample acquired from RPE DM- culture as the calibrator.

Expression levels of *RAX* and *CHX10* were lower in cells cultured in mEF-CM and hFF-CM than in the other two media, while expression levels of *BEST1* and *CRX* were lower in cells cultured in each CM compared to the control. Expression of *RPE65*, on the other hand, was higher in cells cultured in CM, especially mEF-CM. There was no difference that can be considered significant (change in fold regulation greater than 2) in expression levels of *PAX6* and *MITF*.

5.3 CHARACTERIZATION OF MATURE hESC-RPE

In this study, characterization of mature hESC-RPE cells was done by means of evaluating expression of a series of genes, visualizing localization of RPE-specific proteins and assessing functionality of mature hESC-RPE cells based on their ability to phagocytose POS *in vitro*.

5.3.1 Gene expression

After 30 days in adherent culture (a total of 75 days in differentiation culture), hESC-RPE cells were analyzed with RT-PCR to assess whether or not genes specific to RPE cells were expressed and those specific to other tissues undetected (Figure 5.11). *AFP,* an endodermal marker, and *ACTC,* a mesodermal marker, were undetected in all samples. *OCT3/4*, a pluripotent marker, as well as *RAX*, an early eye marker, were undetected in all except the RPE DM- sample. The rest of the markers (RPE precursor genes *MITF* and *OTX2*; RPE-specific markers *RPE65*, *BEST1*, *PMEL17*, *PEDF* and *TYR*; neural precursor gene *PAX6* and the marker for neural crest-derived cells *SOX10*) gave positive results in all samples, although some bands were faint in the hFF-CM sample.

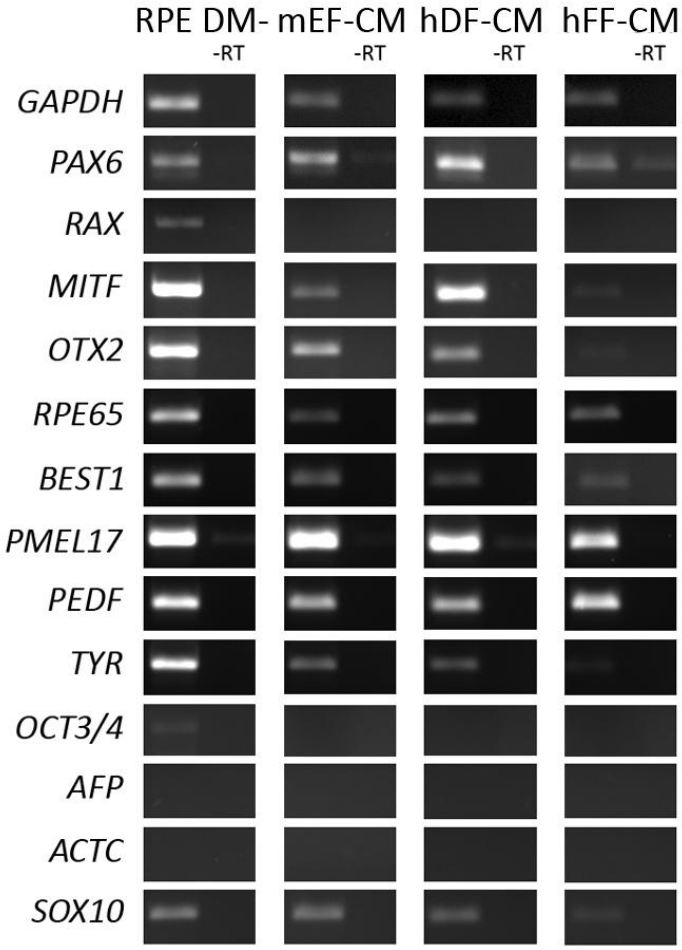

Figure 5.11 Gene expression in mature hESC-RPE cells
Gene expression of a series of genes specific for different tissues was assessed in each mature hESC-RPE monolayer. *GAPDH* was used as a positive control, and a negative control lacking the enzyme (-RT) was prepared for each reaction.

5.3.2 Protein localization

Following a 35-day maturation period on cell culture inserts, each hESC-RPE monolayer was analyzed by means of immunocytochemical stainings and visualized using LSM700 confocal microscope. BF images of each monolayer were acquired with Olympus BX60 confocal microscope. Representative images of each staining are presented in Figure 5.12.

Typical cobblestone morphology of hESC-RPE cells and overall degree of pigmentation is seen in BF images. As seen in Figure 5.2, pigmented cells appear as merely dark brown or black in color in images acquired with a light microscope at a low magnification. In images taken at a higher magnification, varying amounts of pigment granules are visible within each cell (Figure 5.12). The MITF, CRALBP, Bestrophin, and ZO1 stainings were successful and localization was appropriate for RPE cells – MITF in nuclei, and CRALBP and ZO1 in cell membranes. Bestrophin was not strictly in cell membranes, but also present inside the cells, which is most clearly seen in the image acquired from the monolayer cultured in hFF-CM. The staining of RPE65, normally present in the cytosol, was successful, although the cell membranes of hESC-RPE cultured in RPE DM- also stained positive for this antibody. Furthermore, this staining would have been easier to visualize with a counter-staining of the cell membranes. The images of the monolayer cultured in RPE DM- appear distorted due to the fact that it had detached from the cell culture insert. This is especially apparent in the case of the BF image and CRALBP/MITF fluorescence images. Immunocytochemical stainings of the hFF-CM sample were of the worst quality, although this is likely due to the heavy layering in the sample. The ZO1 staining was the least successful for this sample, as the fluorescent signal is not localized to the cell membranes as clearly as in the other samples. The most successful images were obtained from the hESC-RPE monolayers cultured in mEF-CM and hDF-CM – the BF images show regularly arranged hexagonal pigmented cells, while the fluorescence images demonstrate appropriate localization of RPE-specific proteins.

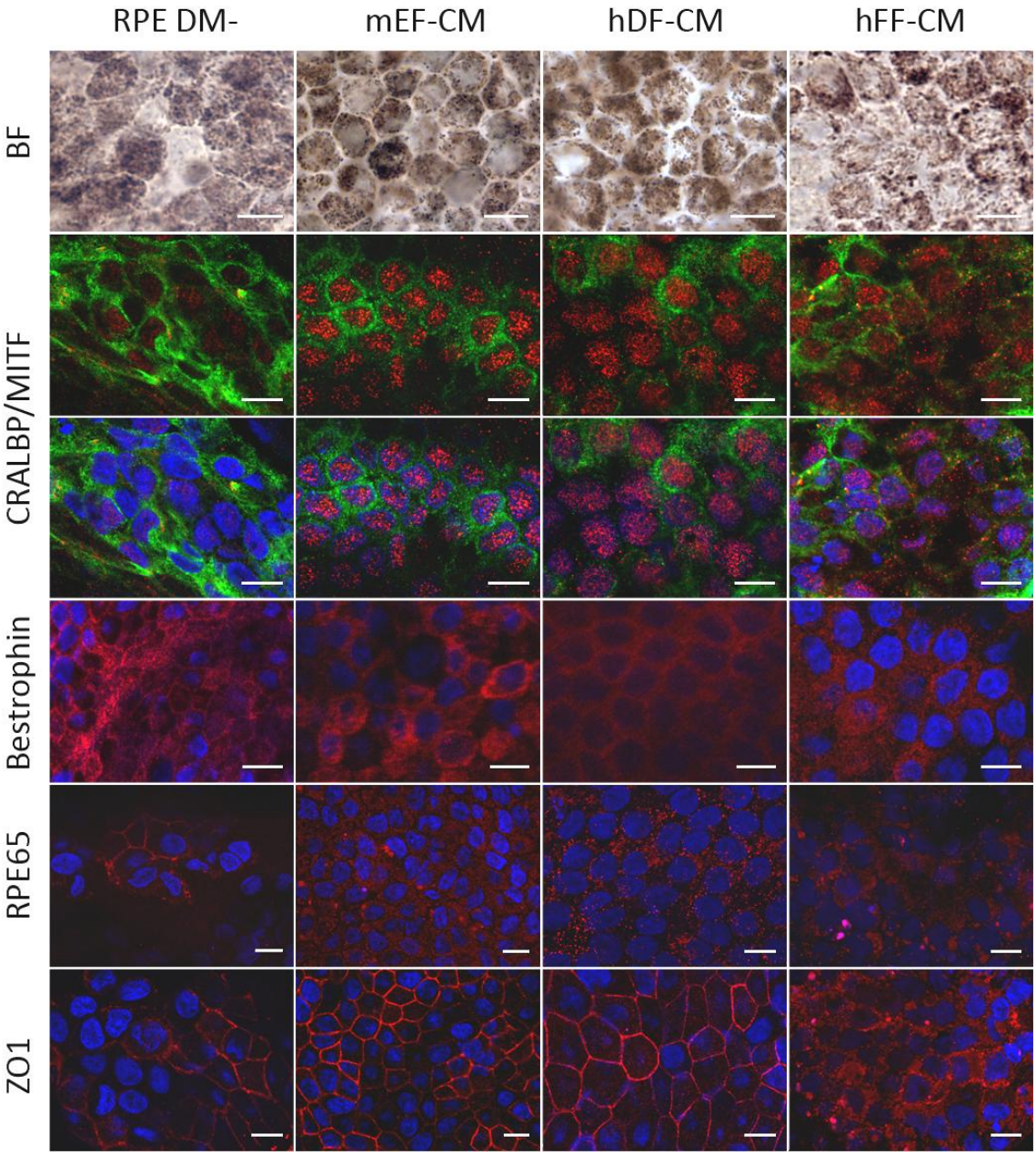

Figure 5.12 Immunocytochemical stainings of hESC-RPE monolayers
Bestrophin, RPE65 and ZO1 (all red) are presented as single stainings, with the nuclear counter-staining using DAPI (blue). The images for the CRALBP (green) / MITF (red) double staining are presented with and without DAPI. BF images were acquired with Olympus BX60 confocal microscope using a 60x oil objective. Fluorescence images were acquired with LSM700 confocal microscope using a 63x oil objective. Scale bars 10 µm.

5.3.3 Assessment of functionality

Finally, the ability of mature hESC-RPE cells to phagocytose POS was tested. The *in vitro* phagocytosis assay was conducted on three-dimensional cell aggregates that were kept in suspension culture for 120 days using RPE DM-, mEF-CM, hDF-CM or hFF-CM. A representative image, in which an internalized POS is visualized through the use of orthogonal sectioning, is presented in Figure 5.13. The image was obtained from hESC-RPE cells maintained in hDF-CM.

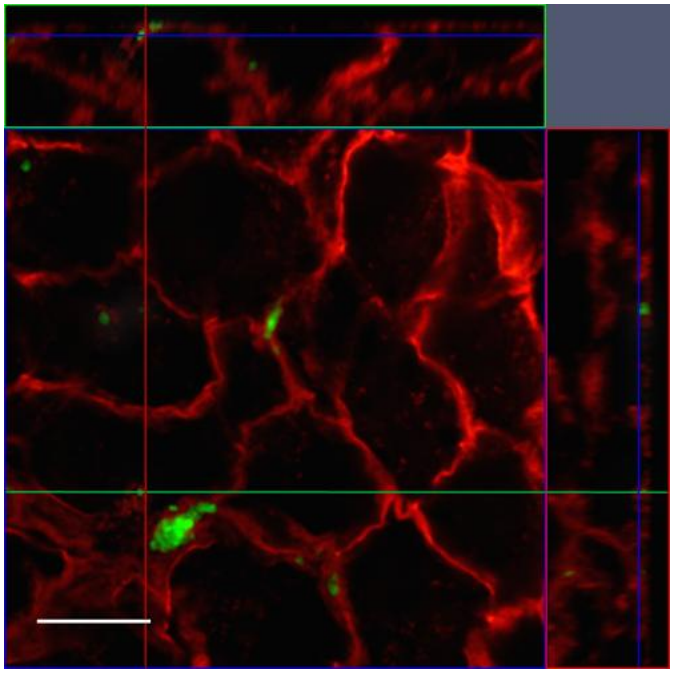

Figure 5.13 *In vitro* phagocytosis of POS
Mature hESC-RPE cells internalize FITC-labeled POS (green), which is demonstrated through the use of orthogonal section of the image. Actin filaments were stained with phalloidin (red) for better visualization of cell morphology. Image taken with LSM700 confocal microscope using a 63x oil objective. Scale bar 10 μm.

The assay was successful in the sense that some internalized POS were observed in each sample. However, the majority of POS were either outside the cells or in cell junctions (data not shown).

5.4 ANALYSIS OF KEY GROWTH FACTOR SECRETION

Concentrations of four soluble factors – bFGF, activin A, TGF-β, and PEDF – were measured in each CM using commercial ELISA kits. RPE DM- and KO-SR were used as controls. The data obtained from these assays is presented in Figure 5.14. Concentration of bFGF in KO-SR was about 24 pg/ml, but undetected in each CM, as

well as in RPE DM- (data not shown). When comparing CM, concentration of activin A was highest in hDF-CM and lowest in hFF-CM. High amounts of activin A were also detected in mEF-CM, although less than in hDF-CM. Activin A was undetected in RPE DM-, while KO-SR contained only about 17 pg/ml, meaning that practically all the activin A present in CM was secreted by the fibroblasts. The highest concentration of TGF-β was in hFF-CM, and the difference in concentration in mEF-CM and hDF-CM was negligible. RPE DM- and KO-SR contained 0.07 ng/ml and 0.3 ng/ml of TGF-β respectively. PEDF was undetected in all media, including RPE DM- and KO-SR (data not shown).

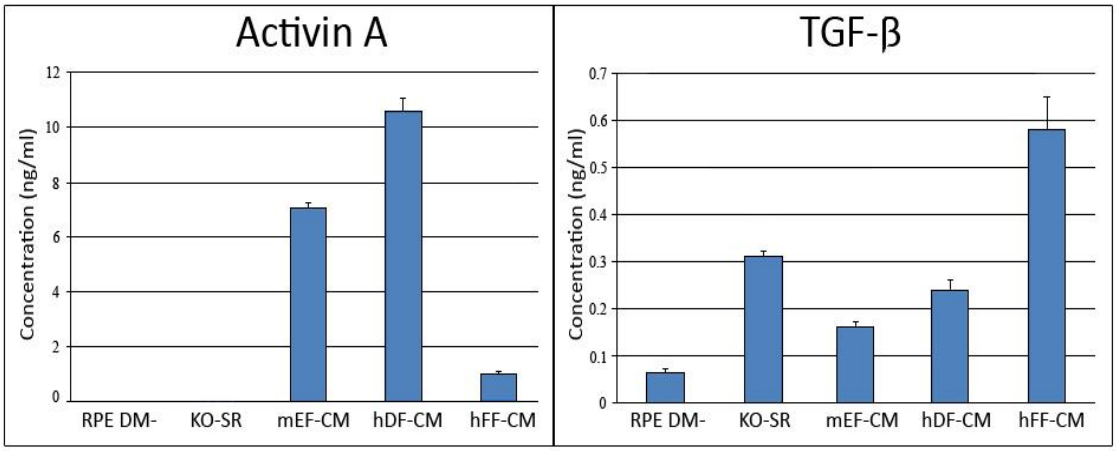

Figure 5.14 Analysis of growth factor secretion
Concentrations of activin A and TGF-β in the media conditioned by fibroblasts, as well as RPE DM- and KO-SR, as determined using ELISA.

Based on the results acquired with the use of ELISA, it was possible to calculate the amount of each soluble factor that was secreted by each fibroblast type (Table 5.2). This was done by determining the difference between the growth factor concentration in each CM and that in RPE DM-.

Table 5.2 Soluble factors secreted by fibroblasts
Amounts of each soluble factor secreted by each fibroblast type were calculated based on the results of immunosorbent assays.

	bFGF	***Activin A***	***TGF-β***	***PEDF***
mEF	-	7.1 ng/ml	0.1 ng/ml	-
hDF	-	10.6 ng/ml	0.2 ng/ml	-
hFF	-	1.0 ng/ml	0.5 ng/ml	-

Due to the small sample size and lack of biological replicates, it was not possible to evaluate the significance of these results using statistical testing.

6. DISCUSSION

The aim of this study was to compare the effects soluble factors secreted by three different types of fibroblasts have on differentiation of hESCs into RPE cells. The reason mEFs were chosen is because they have traditionally been used as feeder cells in stem cell culture for many years. Moreover, up until recently, mEFs have been more commonly used as feeder cells in RPE differentiation culture (Klimanskaya et al., 2004; Vugler et al., 2008). The other fibroblast types used in this study were of human origin: a hFF cell line available commercially, and a hDF cell line, which was derived in-house. The experiment was conducted using media conditioned by each of the fibroblast cell lines as well as the unconditioned differentiation medium, which served as a control. The hypothesis was that the medium conditioned by mEFs would enhance differentiation and be superior to the other media used in the experiment. This hypothesis was based on the fact that there has been evidence of mEFs secreting relatively high amounts of activin A (Eiselleova, 2008). Moreover, it has been shown that activin A promotes RPE formation during early eye development (Fuhrmann et al., 2000). To my knowledge, there are currently no available publications discussing the effects soluble factors secreted by different types of fibroblasts might have on RPE differentiation. Furthermore, each fibroblast type secretes numerous growth factors and ECM proteins, not all of which are currently known. The ways in which certain growth factors, such as activin A, affect differentiation have been investigated to some extent, although in the case of this study, it is not the separate factors that are considered, but rather the whole array of secreted molecules as a whole.

6.1 EVALUATION OF DIFFERENTIATION

During the first stage of the study, hESCs were kept in suspension culture and the degree of differentiation was evaluated visually, by following the rate and amount of pigmentation, and also by comparing expression levels of a series of genes.

First pigment appeared in cell aggregates cultured in mEF-CM and hDF-CM two days earlier than in hFF-CM – a difference that is not necessarily due to the differences in media, but can be attributed to biological variation of hESCs. Appearance of the first pigment in control medium, on the other hand, can be considered as notably later than in

all three CM – a difference of five days from hFF-CM, and seven days from both mEF-CM and hDF-CM. At the end of suspension culture phase, the strongest pigmentation was in hFF-CM, especially in relation to the size of the aggregates: they were generally small and separate from one another, and either almost completely pigmented, or not pigmented at all. Cell aggregates cultured in hDF-CM were similar to those in hFF-CM, but more weakly pigmented. Cell aggregates in RPE DM- and mEF-CM, on the other hand, were large and heavily clustered, with only small specks of pigment. The same pattern of pigmentation was observed in previous studies conducted in adherent cell cultures – pigmentation appearing faster in cultures maintained in either mEF-CM or hDF-CM, but strongest pigmentation in hFF-CM at the end of experiment (Vaajasaari & Hongisto, unpublished data). The reason for this phenomenon is currently not understood. It is possible that the influence of soluble factors produced by each fibroblast type is targeted at cells at different stages of differentiation. The results of this study show that mEFs and hDFs secrete higher amounts of activin A than hFFs. Activin A is crucial for RPE development and induces RPE differentiation *in vitro* (Fuhrmann et al., 2000; Idelson et al., 2009). However, it is also a strong inducer of the mesoderm, and has been shown to promote cardiovascular differentiation (Schuldiner et al., 2000; Mignone et al., 2010). Therefore, assuming that the action of activin A is directed at the early stages of RPE development, initial differentiation in mEF-CM and hDF-CM would be enhanced, but less efficient overall due to the simultaneous differentiation towards the mesodermal cell lineage. The general effect of hFF-CM, on the other hand, may be focused on a different stage of RPE differentiation and result in slower but more constant differentiation. Finally, fibroblasts secrete a multitude of other soluble factors, which influence the rate and route of differentiation.

The cell aggregates agglomerated to a higher extent in RPE DM- and mEF-CM than in the other two media. The amount and size of cell aggregates in each medium was roughly equal at the beginning of the study, which means that cell density was not the issue in this case. It is possible that cell proliferation and division were faster in RPE DM- and mEF-CM, which would have caused rapid growth and therefore agglomeration of cell aggregates. This would then lead to inadequate diffusion of soluble factors and nutrients to the cells situated further from the surface of the aggregates. Cell growth, proliferation and differentiation would therefore be affected

negatively. Perhaps this phenomenon could have been minimized by distributing cell aggregates into several wells thereby lowering cell density in each of the culture conditions. Mechanical dissection of clusters on a regular basis would also have been helpful, although it would have had to be done to all cell aggregates equally. On the other hand, studies show that agglomeration of hESC aggregates is mediated by E-cadherin, a cell-cell adhesion molecule. Over time, as cells differentiate, expression of E-cadherin becomes down-regulated, and the rate of agglomeration between separate cell aggregates decreases (Dang et al., 2004). This suggests that hESCs cultured in hDF-CM and hFF-CM differentiated faster and therefore ceased to agglomerate earlier than the hESCs cultured in RPE DM- and mEF-CM.

During this phase of the study, cell aggregates were selected at random from each suspension culture at three time-points, and differences in expression levels of a series of genes were quantified. The problem with this method is that the cell population in differentiation culture is heterogeneous and gene expression levels are highly dependent on the cell aggregates that are selected. This problem could have been overcome by analyzing several biological replicates and averaging the results. Unfortunately, this was not possible during the timeframe of this study due to the fact that the amount of available hESCs of the same cell line and passage was limited. It is also important to remember that in the case of several of the analyzed genes, expression was so low that C_T-values obtained in the qPCR analysis were very high. In this case the C_T-values are likely to be outside of the linear phase of the amplification curve, and the resulting fold regulations are not necessarily as reliable as they could be.

Based on the results of the qPCR assays, mEF-CM appears to be somewhat better at promoting differentiation of hESCs towards RPE cells during the first few weeks in differentiation culture. Firstly, after 7 days of differentiation, expression levels of mesodermal and endodermal markers (*SOX17* and *T*) were lower than in cells maintained in RPE DM-, and not much higher than in UD hESCs. However, expression levels of these genes were not analyzed at the later time-points, so differentiation towards these lineages might have progressed with time. Secondly, expression of the early eye marker *RAX* began decreasing after 28 days in differentiation culture, which is expected to happen when early neural precursors progress towards RPE-specific precursors. This pattern of expression was not observed for *PAX6* though. Thirdly,

expression levels of *CHX10* and *CRX*, both of which are markers of the neural retina, were lower in cells cultured in mEF-CM than in those cultured in the control medium, even after 28 days of differentiation. These genes were only slightly up-regulated when compared with UD hESCs. And finally, expression of *RPE65* in cells cultured in mEF-CM was far superior to that in cells collected in all the other culture conditions after 28 days of differentiation. This suggests that cell aggregates differentiated in mEF-CM contained the highest amount of hESC-RPE cells after 28 days in suspension culture.

The pattern of gene expression in the samples collected from the hFF-CM culture were mostly similar to mEF-CM, however, both *SOX17* and *T* were up-regulated in relation to the control medium and expression levels of *RPE65* were not as high. Gene expression levels in samples collected from the hDF-CM culture did not differ greatly from those in the RPE DM- samples, with the exception of *SOX17* and *RPE65*, which were up-regulated, and *T*, which was down-regulated. Expression of genes specific to the neural retina, and also of *BEST1*, was highest in cells cultured in RPE DM-, while expression of *SOX17* and *T* in those cells was not much higher than in UD hESCs. Overall, it appears that differentiation of hESCs is the most uncontrolled in RPE DM-, with a certain degree of differentiation to a number of tissue types taking place simultaneously. According to the data discussed above, the most directed differentiation is observed in cells maintained in mEF-CM.

6.2 CHARACTERIZATION OF MATURE hESC-RPE

For the second phase of the study, pigmented areas were selected and transferred from suspension culture to adherent culture, where they were maintained using the same medium type as before. Cells were not counted before being seeded on the substrate coated with collagen IV – the pigmented single-cell suspensions were used in their entirety instead. This resulted in unequal amounts of cells in each culture, which is most likely the reason for the extensive layering observed in the monolayer cultured in hFF-CM. The layering made it difficult to compare the culture with the others, although some areas with the correct RPE-like morphology were visible regardless. However, if equal amounts of pigmented cells are to be used to create adherent cultures, the suspension culture with the weakest pigmentation serves as a limiting factor, and there is a risk that too little cells are used. Therefore, suspension culture phase should be

prolonged until there is no doubt that sufficient quantities of pigmented cells are present in all cell culture conditions used in the experiment. Unfortunately, this was not possible within the timeframe of this study.

The hESC-RPE monolayers in all culture conditions developed cell morphology inherent to mature RPE cells. In native tissue, pigmented cells are arranged in a regular hexagonal mosaic, often referred to as cobblestone morphology (Maminishkis et al., 2006; Tamiya et al., 2010). In this study, appropriate morphology was acquired quickest and spread fastest in the adherent culture maintained in mEF-CM. However, pigmentation in this culture was weaker than in other media. The strongest and most wide-spread pigmentation was observed in hDF-CM culture, and it remained as such until the endpoint of the experiment. Nonetheless, since hFF-CM suspension culture was strongly pigmented, it is possible to speculate that the rather weak pigmentation in the hFF-CM monolayer was attributable to the layering. The hESC-RPE monolayer in RPE DM- appeared to have the weakest attachment properties, as it began detaching from the substrate while the others remained attached until the end of the experiment. This could be due to the fact that CM are likely to contain ECM proteins secreted by fibroblasts, which strengthen the monolayers and help in adhering to the collagen IV-coated cell culture substrate. Such molecules were absent from the control medium, which seemingly resulted in weaker contacts between neighboring cells as well as between the cells and the substrate. Nonetheless, cell morphology specific to the RPE was observed at the center of the monolayer maintained in RPE DM-, where cell contacts were well-established and most abundant. Perhaps if a higher amount of pigmented cells was obtained from the cell aggregates differentiated in RPE DM-, a hESC-RPE monolayer of a better quality would have formed.

After about five weeks in adherent culture, expression of a series of genes was analyzed using RT-PCR. Here, the entire monolayers were used for the analysis, so there was no variation due to sample heterogeneity and the sensitivity of the method, as in the case of qPCR, described above. The sample collected from RPE DM- was the only one to still express the pluripotent marker *OCT3/4*, as well as the early eye marker *RAX*. Thus, it can be defined as inferior to CM. Nevertheless, all four samples expressed *SOX10* – a gene active in neural crest cells during embryonic development and essential for the establishment and normal function of melanocytes (Harris et al., 2010). This is

unfortunate, because it suggests that at least some of the cells in hESC-RPE monolayers might have been melanocytes, and not RPE cells. However, it is clear that hESC-RPE cells made up the majority of the monolayers, as it is proven by successful immunocytochemical stainings and *in vitro* phagocytosis assays, as discussed below.

Expression and localization of several RPE-specific proteins was evaluated using immunocytochemical stainings. In all likelihood, the differences in quality between the hESC-RPE monolayers of different culture conditions were due to technical problems, and not the culture conditions. For instance, the layering in the hFF-CM monolayer and the frailty of the RPE DM- monolayer resulted in stainings of lower quality. However, each hESC-RPE monolayer had some areas, where each staining was positive. Therefore, it is impossible to say with certainty that one of the cell culture media is superior to others, although the stainings help verify that the cells in question are mature RPE cells. Pigmentation affected the quality of immunocytochemical stainings, and, especially nuclear stainings – MITF and DAPI – appeared to be negatively influenced by pigment granules. The best confocal microscopy images were acquired from areas that were only slightly pigmented, yet possessed the correct cell morphology. The only way of overcoming this problem would be to remove all pigment from the cells prior cell fixation, but such treatment would be highly damaging to the cells, so the outcome of immunocytochemical stainings would be compromised regardless. Finally, although the stainings were successful in all samples it is possible that extended maturation of hESC-RPE monolayers in adherent culture would further improve their quality.

Functionality of hESC-RPE cells was evaluated using an *in vitro* phagocytosis assay. Internalized POS were found in each sample, suggesting that at least some of the cells were functional. However, the majority of POS were either outside of the cells or in cell junctions. There are several reasons that could have caused this. Firstly, it is possible that the staining of POS with FITC was not entirely successful, in which case unstained POS could have been present inside the cells, but were not visible. Secondly, aggregation of POS might have taken place and the aggregates would be too large to be internalized, meanwhile obstructing the means of entry for other POS. It is also possible that the binding and internalization of POS by hESC-RPE could be improved by varying the incubation time during which cells are allowed to interact with POS. This time might have been either too short, in which case the cells would not have had the

time to phagocytose POS, or too long, in which case the cells would have broken down the ingested POS and excreted the waste products. There is evidence according to which increasing numbers of POS are internalized by hESC-RPE over time, and the incubation time could be as long as 20 h (Carr et al., 2008). Moreover, the phagocytosis assay was performed using cell aggregates that had been in suspension culture for 120 days. It is possible that conducting the assay on cells in adherent culture would yield better results, which would be easier to visualize. Finally, further maturation of hESC-RPE cells would ensure higher amounts of functional cells.

6.3 SOLUBLE FACTORS SECRETED BY FIBROBLASTS

Concentrations of the four growth factors – activin A, bFGF, TGF-β and PEDF – were measured because prior studies show that each of them affects hESC differentiation in general, and RPE development in particular.

In a study conducted by Eiselleova et al, growth factor production of one mEF cell line and several hFF cell lines were tested using ELISA. Their results are more or less consistent with the results of this study: no bFGF could be detected in the media conditioned by the mEF cell line, and its concentration was low (about 5 pg/ml) in the medium containing KO-SR, conditioned by hFFs. Moreover, mEFs were found superior to hFFs in the production of activin A (Eiselleova et al., 2008). Both of these results were confirmed in this study. Finally, the differences in secretion of TGF-β between the cell lines were not large, whereas in this study TGF-β secretion of hFFs was five times higher than that of mEFs. This discrepancy is most likely attributable to the biological variation of different cell lines as well as the differences in study design. For example, mitotic inactivation was done by irradiation in this study, and with mitomycin C in the study carried out by Eiselleova et al. Treatment of feeder cells with mitomycin C is likely to affect growth factor production in a different way than γ-radiation does.

It has been shown that activin A acts as a substitute of the extraocular mesenchyme *in vitro*, and promotes expression of RPE-specific transcription factors while down-regulating expression of markers specific to the neural retina (Fuhrmann et al., 2000). The fact that activin A concentration was high in mEF-CM could explain the results obtained with qPCR – that is a gene expression profile suggesting enhanced RPE

differentiation during the first few weeks in culture. Nevertheless, if that were the case, hDF-CM would have been far superior to the other media, because it contained the highest amounts of activin A. Therefore, there must be other factors present in the media that interact with one another to elicit the observed response.

The fact that PEDF was undetected in all four media is inconsistent with the studies that found this growth factor in mEF-CM using proteomics (Lim & Bodnar, 2002; Chin et al., 2007). The commercial ELISA kit used in this study was human-specific, which could easily explain why PEDF was undetected in mEF-CM, assuming that the mouse and human homologs of this protein are not identical. Measurement of PEDF concentration could be utilized when characterizing mature hESC-RPE cells – they should secrete PEDF, and therefore its concentration would be high in hESC-RPE-CM. Hence, it would be possible to compare different culture conditions by comparing the levels of PEDF secretion.

The purpose of this small-scale growth factor analysis was to get a sense of just how different the secretion of soluble factors really is in different types of fibroblasts. Naturally, no individual growth factor is exclusively responsible for the way media conditioned by fibroblasts influence differentiation. Instead, it is the interplay of many soluble factors, as well as ECM proteins, which needs to be examined as a whole.

7. CONCLUSION

The effects that different soluble factors have on differentiation of hESCs are far from being fully understood. Furthermore, the possible interactions between soluble factors and ECM components influence the overall effect, and make it even more difficult to devise an optimal differentiation method. In the case of hESC-RPE cells, spontaneous differentiation in the absence of bFGF results in fairly high yields of cells with the correct phenotype, but the method could be improved further by directing the differentiation along the appropriate route. In this study, media conditioned by three types of fibroblasts, as well as an unconditioned medium, were used in differentiation of hESCs. In this way, the influence of soluble factors secreted by fibroblasts was studied.

Overall, the results of this study suggest that differentiation medium conditioned by mEFs served to enhance differentiation of hESCs in the direction of RPE cells during the first four weeks in culture. This was demonstrated by the results of the qPCR analysis – cells cultured in mEF-CM had the gene profile characteristic to RPE cells. Furthermore, cells maintained in mEF-CM were the fastest at acquiring RPE cell morphology when in adherent culture. Nevertheless, pigmentation in this medium type was weak during both phases of the experiment. With hFF-CM as the cell culture medium, overall pigmentation was superior to other conditions in suspension culture, but not in adherent culture, due to the extensive layering of the cells. Mature hESC-RPE monolayers were characterized with RT-PCR, which showed that cells cultured in the control medium express the pluripotency marker *OCT3/4* as well as the early eye marker *RAX*. Immunocytochemical stainings revealed slight differences in mature hESC-RPE monolayers cultured in different conditions, but each of them contained areas where RPE-specific proteins were expressed and localized in the correct way. Also, functional cells were produced in all four culture conditions, as proven by the *in vitro* phagocytosis assay. However, without quantification of the used methods, and a larger sample size with several biological replicates, it is impossible to say whether one of the culture conditions was significantly superior to others. When considering the results of this study as a whole though, it does appear that the control medium, RPE DM-, is inferior to the media conditioned by fibroblasts.

The analysis of key growth factor secretion revealed that hDFs and mEFs secrete far more activin A than hFFs. Meanwhile, TGF-β secretion was found to be the highest in hFFs. Both bFGF and PEDF were undetected in all media. The mechanisms through which different growth factors influence differentiation of hESCs are not straightforward. For instance, even though activin A has been shown to promote RPE differentiation, it is also likely to interact with other factors present in CM, thereby influencing the overall impact. Therefore, the results of this analysis alone are not sufficient to explain the effects that different media had on RPE differentiation, but they do illustrate that the secretion patterns of different fibroblast types are very different from one another. More research would be needed to identify the specific soluble factors that promote differentiation of hESCs towards RPE cells. Addition of such factors to the differentiation medium without feeder cell conditioning could help in developing xeno-free conditions for the production of high-quality hESC-RPE cells.

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