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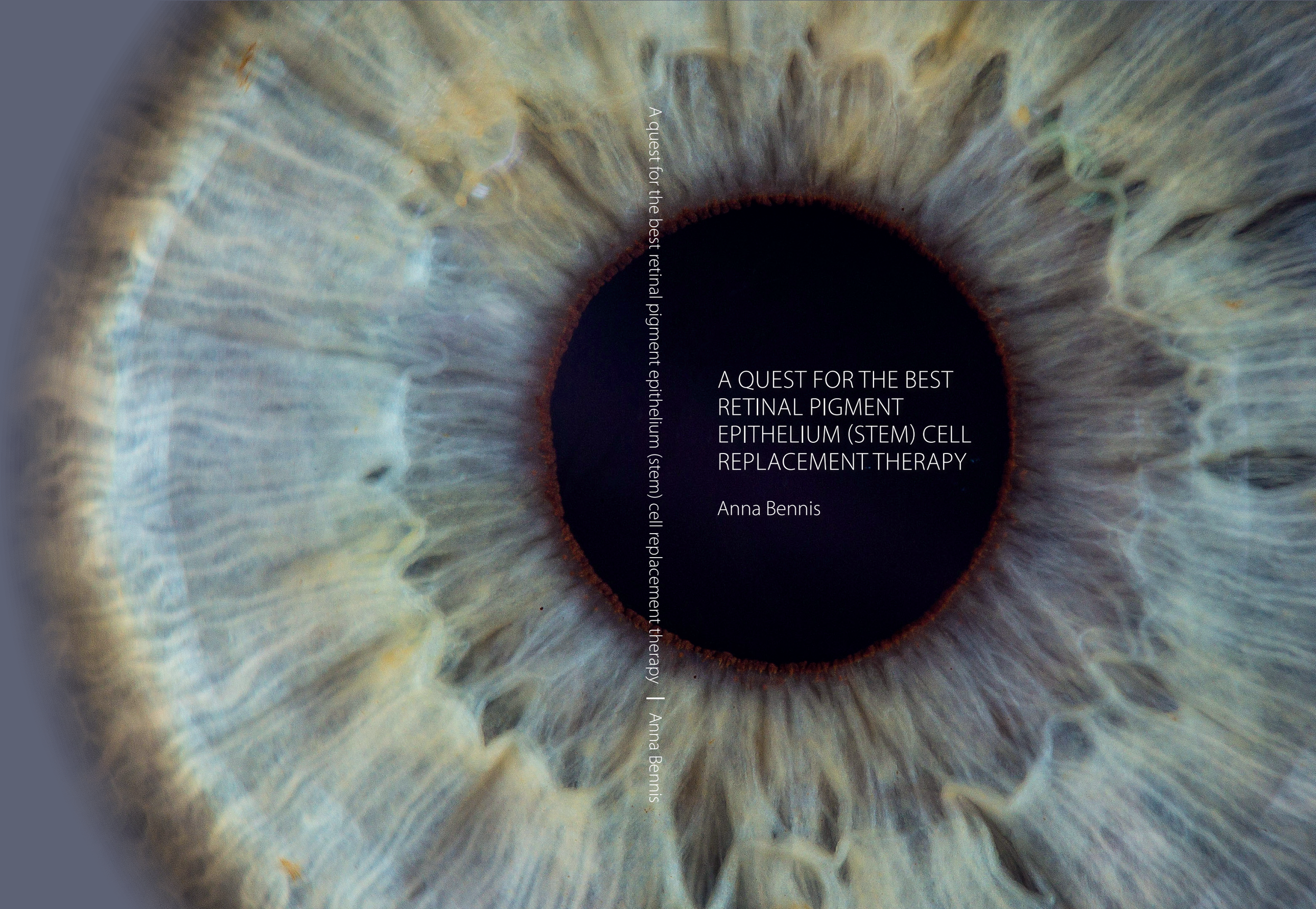

A QUEST FOR THE BEST RETINAL PIGMENT EPITHELIUM (STEM) CELL REPLACEMENT THERAPY

Anna Bennis

A quest for the best retinal pigment epithelium (stem) cell replacement therapy | Anna Bennis

A quest for the best retinal pigment epithelium (stem) cell replacement therapy

Anna Bennis

The work described in this thesis was performed at the Department of Clinical Genetics, Academic Medical Center Amsterdam, University of Amsterdam, The Netherlands; the Department of Clinical and Molecular Ophthalmogenetics, Netherlands Institute for Neuroscience (NIN-KNAW), an institute of the Royal Netherlands Academy of Arts and Sciences, Amsterdam, The Netherlands; Department of Pediatrics/Child Neurology, VU University Medical Center, Amsterdam, The Netherlands; Department of Complex Trait Genetics, Center for Neurogenomics and Cognitive Research, Amsterdam Campus, Vrije Universiteit, Amsterdam, The Netherlands.

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A quest for the best retinal pigment epithelium (stem) cell replacement therapy

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prof. dr. ir. K.I.J. Maex

ten overstaan van een door het College voor Promoties ingestelde commissie,

in het openbaar te verdedigen in de Agnietenkapel

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Faculteit der Geneeskunde

Congratulations!
Today is your day.
You're off to Great Places!
You're off and away!

You have brains in your head.
You have feet in your shoes.
You can steer yourself
any direction you choose.

- Dr Seuss

CONTENTS

Oh, the places you'll go!
There is fun to be done!
There are points to be scored.
There are games to be won.

- Dr Seuss

Introduction

The retinal pigment epithelium, its role in disease and potential remedies.

1

VISION

The processing of visual information starts in the eye. The first step is light perception: Light first passes the cornea, enters the pupil and is focused by the lens onto the retina. The retina lines the inner part of the eye and consists of two kinds of photoreceptors (PR), rods and cones. The PR are a specialized type of neurons, and part of the visual system, that are capable of converting the incoming light into electric and neurochemical signals to the brain. This information is used to build a representation of the surrounding environment. The PR are supported by the retinal pigment epithelium (RPE). The RPE is a monolayer of pigmented cells that lie underneath the PR. It forms an important part of the blood-retina barrier and faces the Bruch's membrane, which is located between the RPE and the fenestrated choroidal capillaries of the eye. See figure 1. Despite distinct functions of the RPE and PR they share their origin; they are both formed from neural crest derived epithelium.

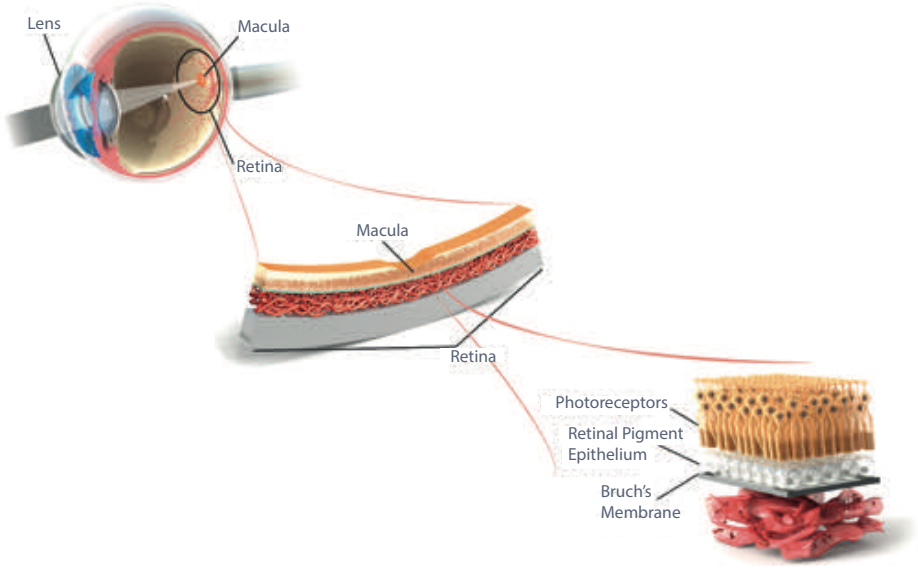

Figure 1. Schematic overview of the normal macular anatomy.

EMBRYOLOGY OF THE RPE

The development of the RPE and the photoreceptors is an interconnected process that reveals the strong relationship between the two. In the final stages of neural tube formation, the optic vesicles evaginate from the neuroepithelium of the ventral forebrain. Subsequently, the optic vesicles invaginate, leading to the formation of the bilayered

optic cup. From the two neuroepithelial cell layers the neural retina (inner layer), containing the photoreceptors, and the RPE (outer layer) develop (Figure 2).

Optic cup development occurs in a complex environment and is affected by many neighboring tissues. During early development the progenitor cells of the optic vesicle exhibit bi-potential competence and are able to adopt either the neural retina or RPE fate. The specific combination of key transcription factors, including OTX2, MITF, CRBP, CRABP, IRBP and RPE65, determine the RPE fate[1].

The RPE cells distinguish themselves morphologically from the remainder of the optic cup with the appearance of pigment granules and the formation of tight junctions between the cells. Stimulated by interaction with the neural retina, the RPE slowly stabilizes into an epithelial monolayer with apical-basal polarity and regulates transepithelial transport of substances from the subretinal space (the space between the PR and the RPE) to the choroid and vice versa[2]. The PR start to extend their outer segments and in response the RPE elongate its apical microvilli into the subretinal space. In this last maturation phase the RPE and PR interact to become a functional unit.

The coordinated differentiation and maturation of the RPE and PR causes the RPE cells to adjust to the functional properties of the PR. Due to the higher number of photoreceptors per RPE cell in the macula, the RPE cells in the macula are smaller and have a higher density. Additionally, the RPE cells in the macula have a higher melanin content for better light absorption and adapt to the higher turnover rate of the shed photoreceptor outer segments[3,4].

Besides the essential role in development of the optic cup, the RPE is also crucial for the proper functioning of adult photoreceptors.

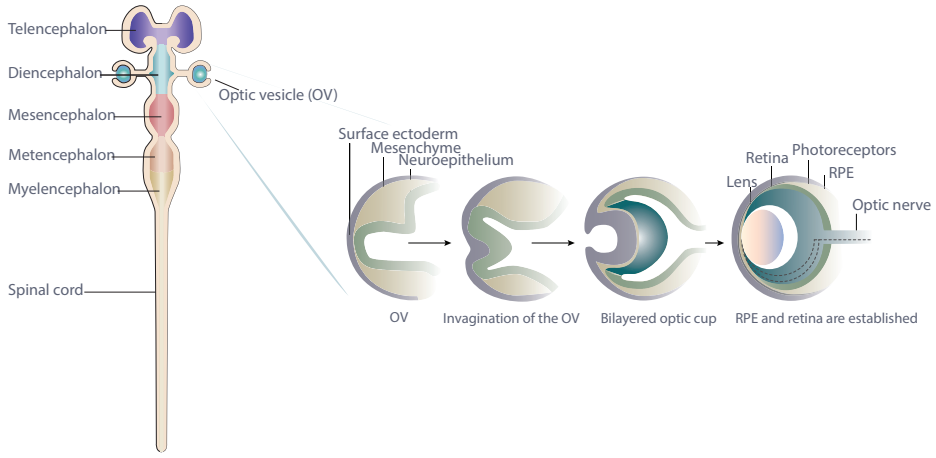

Figure 2. Schematic overview of the embryological development of the eye.

RPE FUNCTIONS

Below, I'll briefly discuss the various functionalities of the RPE (Figure 3).
First of all, the RPE has several mechanisms to counteract the high amount of photo-oxidative activity and subsequently oxidative damage. The light that is concentrated on the retina by the lens causes this, especially in the macular area. The pigmentation of the RPE is essential, since it absorbs excess light through the abundant presence of melanosomes that contain melanin. Melanin is an effective absorber of light and has a broad absorption spectrum. Another defense against oxidative stress is the high amount of enzymatic antioxidants such as superoxide dismutase and catalase, and carotenoids (lutein and zeaxanthin) as non-enzymatic antioxidants[5].

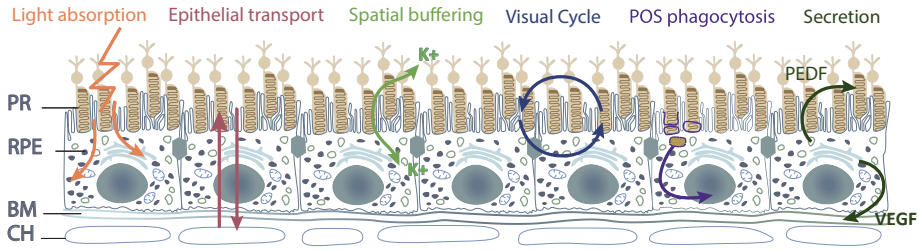

Figure 3. Schematic overview of various important functions of the RPE. It depicts the adjacent layers of the photoreceptors (PR), retinal pigment epithelium (RPE), Bruch's membrane (BM) and the choroid (CH). Figure adopted from Strauss et al (2005)[1].

Secondly, the RPE is part of the blood-retina barrier between the PR and the choroid. Therefore active trans-epithelial transport by the RPE is needed to supply the PR with nutrients such as glucose and vitamin A from the blood to the PR. Furthermore; the RPE eliminates metabolic end products from the PR and accumulated water from the subretinal space[6].
Thirdly, photoreceptor activity causes rapid occurring changes in ion composition in the subretinal space. The RPE is able to balance the homeostasis by spatial buffering function[7].
Fourthly, the PR convert light into an electrical signal that the brain can process as visual information, which is called the visual phototransduction cascade. The visual pigment in the PR absorbs the photon when it is hit by light. This 11-cis retinal is isomerized to all-trans retinal, and is no longer able to absorb photons. In the RPE it is reisomerized back to the functional form 11-cis-retinal before it is transported to the PR. The RPE maintains the visual cycle through this circular pathway[8].
Fifthly, the PR are exposed to intense levels of light, which causes oxidative stress and accumulation of photo damaged proteins and lipids. The tips of the PR, directed towards

the RPE, contain the highest concentration of these oxidative modified biomolecules. These photoreceptor outer segments are continuously renewed and shed from the PR to maintain excitability of the PR. The POS are phagocytized by the RPE, digested and some parts are recycled and transported back to the PR[9].
To conclude, the RPE produces and secretes growth factors and factors that are essential for the maintenance of the structural integrity of the retina and the choriocapillaris[10].

AGE RELATED MACULAR DEGENERATION

The variety of functions for the RPE, as described above, also illustrates the importance of the RPE for retinal health. Functional defects in the RPE may lead to physiological defects in the entire homeostatic unit of the retina and are the hallmark of retinal disease such as age-related macular degeneration (AMD). AMD is a late onset, degenerative and progressive disorder of the macula with a multifactorial etiology. It is the leading cause of severe visual impairment in the elderly in the western world. Since the proportion of people over 60 years is expanding faster than any other age group this is a growing problem[11].
Early AMD is characterized by the manifestation of pigmentary irregularities of the retina, basal laminar deposits (BLD) and presence of drusen in between the RPE and the Bruch's Membrane[12]. BLD and drusen are deposits of extracellular material that assemble below the RPE and are correlated with early AMD. This stage of AMD is clinically asymptomatic: There is little visual defect yet, but the presence of these deposits is a strong risk factor for further development of AMD. AMD is rarely diagnosed in the absence of drusen. Late AMD can manifest itself in two forms; the neovascular ("wet") form (nvAMD) or the geographic atrophy ("dry") form (GA AMD)[13] (Figure 4).
nvAMD is depicted by newly immature blood vessels that grow towards the retina from the underlying choroid, and can leak fluid and blood. As a consequence, the macula is rapidly and severely damaged. In GA AMD, the most prevalent form, there is a slow but progressive breakdown of the PR as a consequence of deteriorated RPE. The causes of degeneration are largely unknown.
GA AMD is currently untreatable. There is limited treatment available for nvAMD; the most common therapy is to inject anti angiogenic-drugs in the eye to block the growth of new vessels. For angiogenesis to occur signaling molecules that promote blood vessel growth must bind to the receptors on the surface of endothelial cells. When these promoting factors bind to the receptors on endothelial cells, it initiates growth and survival of new blood vessels. The anti-angiogenic-drugs interfere in this process in various ways. In addition, photodynamic therapy and laser surgery are also aimed at stopping the growth of

abnormal vessels, but are almost never used. Even though there is treatment for nvAMD, the condition may progress. Also, the injections are invasive and costly.

The primary site of AMD pathogenesis is unclear, but, increasingly, data suggest that it involves oxidative damage, inflammatory changes and gradual accumulation of indigestible material within and underneath the RPE cells[14]. A combination of these factors can lead to AMD and it can clinically present itself in several ways. But always, in every case, the RPE is at the core of the development of the disease.

AMD is a complex disease and both genetic and environmental aspects determine the development and progression. Environmental risk factors include age, smoking, gender, race, color of the iris, hypertension and diet. In addition, there are many genes that may be associated with the development of AMD. Lambert et al (2016) clustered these in functional related groups[15]. Retinal specific function, immune system related function, neovascularization, lipo-protein related function, and a group of the uncategorized genes. A complex interplay of genetic and environmental risk factors can lead to the development of AMD. This wide variety of involved factors makes it difficult to find a treatment that tackles the disease.

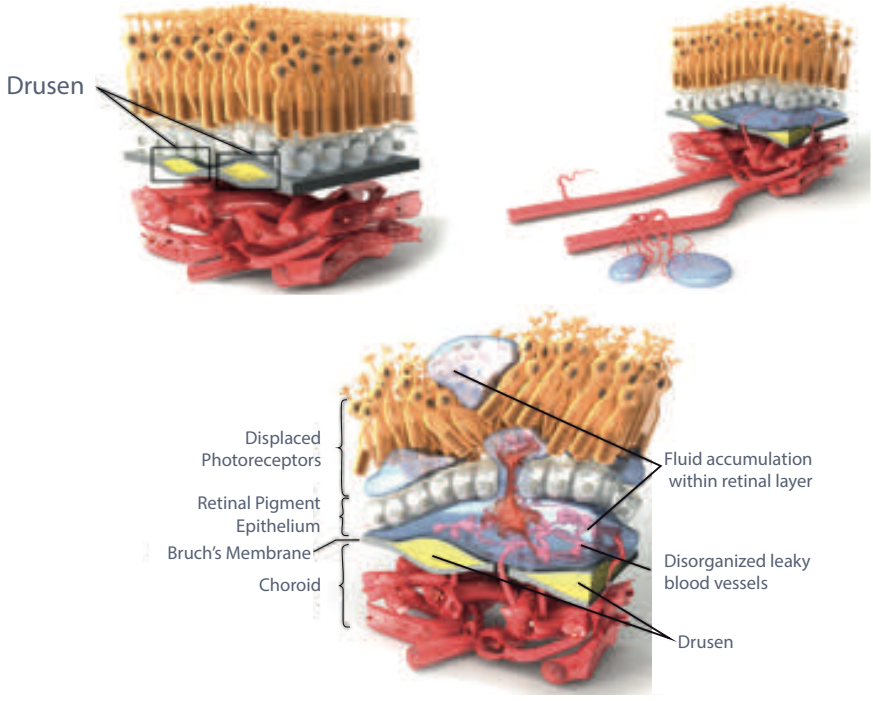

Figure 4. The manifestation of late AMD. The presence of drusen disturb the functional layers in the retina and can lead to leakage and subsequently damage to the retina and vision.

CELL REPLACEMENT THERAPY

The etiology of AMD is still largely unclear. Although the disease manifests itself in various ways, the performance of the RPE is at the heart of the disease. Contrary to the palliative pharmacological treatments, such as the anti-angiogenic therapy discussed above, replacement of the RPE has curative potential for AMD. Several cell sources are considered for this purpose.

Donor RPE and autologous RPE

Replacement of the degenerated tissue with donor material, or even the translocation of autologous RPE sheets from the periphery to the macula, have had limited success[16,17]. The drawback of these cell sources is that their use is technically challenging, as it is difficult to collect enough tissue, donor tissue may induce transplant rejection, plus the harvested cells are the same age as the cells they are meant to replace.

When human donor RPE cells are taken into culture, they undergo epithelial to mesenchymal transition (EMT). The cells loose lose some of their epithelial characteristics, like tight junction formation, polarized shape and gain mesenchymal features[18]. Also RPE cells from aged donors do not simply attach well to the BM and modification to the BM and the addition of extracellular matrix proteins must be considered for proper cell attachment and cell survival[19]. Recently, the adult RPE was reconsidered as a potential source for cell replacement therapy. A multipotent subpopulation of RPE cells can be activated and expanded *in vitro* to form a stable RPE monolayer[20,21]. When they are cultured on a polyester scaffold, meant as a BM substitute, and transplanted in a rabbit model, they survived for over a month and maintained their polarized structure[21]. Further investigations, using the primary RPE cultures for the development of cell transplantation are ongoing[22].

Pluripotent stem cells

Despite this renewed interest in primary RPE, most of the research in this field is focused on other cell sources, such as human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC). Pluripotent stem cells differ from all other cell types in the body, because of a few properties: they are able to self-renew and proliferate for long periods of time; they are in an undifferentiated state; they can differentiate into any cell type of the body.

To understand these unique characteristics of the stem cells I will shortly discuss the *in vivo* development of the cells. In a very early stage of the *in vivo* development (3-4 days post fertilization), the human embryo forms the morula. These cells arise only through cleavage of the zygote and all are found inside the pellucid zone, which cannot expand, thus no growth is seen. These cells are totipotent, meaning that the cells are able to divide

and produce all of the differentiated cell types in the human body. Around the fifth day post fertilization, the embryo liberates itself from the pellucid zone (layer surrounding the oocyte), and bulges out to form the blastocyst. At this stage the first differentiation has already taken place and the structure consists of extra embryonic tissue and the inner cell mass. The inner cell mass contains pluripotent stem cells: cells that can differentiate into any of the three germ layers (endo-, ecto-, mesoderm). These cells are commonly referred to as embryonic stem cells. From here the inner cell mass further proliferates and differentiates. The more the cell differentiates, the more they become specialized and less "potent". They go from pluri- to multipotent, to oligopotent, a progenitor stage where they have the ability to differentiate into just a few cell types.

Besides the embryonic stem cells that come from the blastocyst, the iPSC are a type of pluripotent stem cells. iPSC are made from somatic cells (e.g. skin fibroblasts), by the introduction of a set of reprogramming factors that can induce (re)expression of pluripotency-associated genes in the cell[23]. The advantage of using iPSC is that they are patient-specific thus decrease chances of tissue rejection after transplantation. Plus there is no need for the use of embryos for the derivation of pluripotent stem cells (Figure 5).

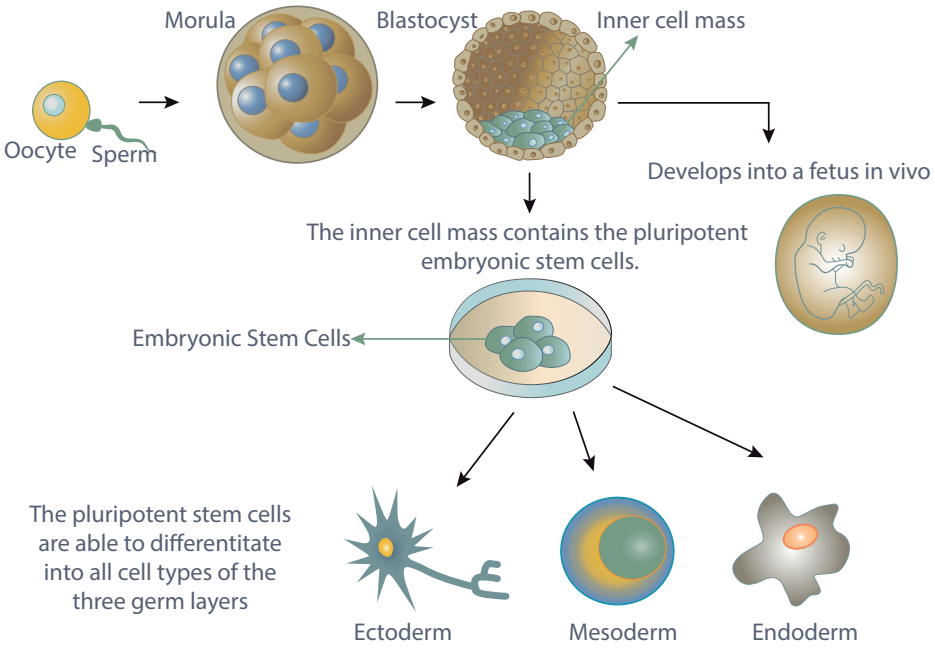

Figure 5. Embryonic stem cells originate from the inner cell mass within the blastocyst. These cells are pluripotent and able to differentiate into any tissue in the body.

RPE differentiation protocols

Clearly, pluripotent stem cells have great potential in regenerative medicine, as they can develop into any cell type that is needed. But it is a challenge to successfully control and regulate differentiation processes into the desired cell type. There are currently various protocols for the *in vitro* RPE differentiation. They can be classified as follows: spontaneous, directed and three-dimensional retinal differentiation[24,25].

The ***spontaneous differentiation protocols*** allow the pluripotent stem cells to freely differentiate. This is done as a continuous adherent culture or following an embryoid body method where the cells are cultured as free-floating aggregates. According to the adherent method, cells overgrow until pigmented foci start to appear. These cells are manually dissected out of the culture dish for enrichment[26–29]. In the suspension culture, cells are first cultured as embryoid bodies for a few weeks with subsequent adherent culturing[30,31]. Usually it takes about 5 to 8 weeks before pigmented foci become visible in these differentiations. Although both methods result in RPE cells, they are lengthy and inefficient.

Many ***directed RPE differentiation protocols*** have focused on steering RPE differentiation by the addition of small molecules and growth factors in order to mimic the *in vivo* development more closely. Directed differentiation protocols are quite diverse, using many different factors to speed up the process of RPE development[32–36]. The directed differentiation protocols can also be divided into adherent culture methods and suspension methods. There is substantial diversity among the protocols and their results. The protocols use between one and nine growth factors. Some protocols produce RPE cells after 14 days of differentiation, others after only 8-9 weeks, without a correlation between the amount of growth factors and the time that is needed for the differentiation.

To even further recapitulate the optic patterning events *in vitro*, ***three-dimensional culture methods*** were developed[37,38]. Cells start their differentiation three-dimensional and are transitioned at some point to adherent cultures. The emergence of RPE cells starts after about 4 weeks in differentiation. But, also here there is a clear variation in the efficiency of the differentiation, ranging from 25% to 95% of the culture being designated as RPE cells.

Transdifferentiated cells

Apart from the donor RPE cells and pluripotent stem cells, a third cell source that holds potential for regenerative medicine are mature somatic cells that are transformed into RPE cells. Transdifferentiation, or lineage reprogramming, is the process in which one somatic cell transforms into another mature somatic cells without undergoing an intermediate pluripotent state or progenitor cell type. The advantage of this method over the other two is that they are patient specific and do not have an intermittent plu-

ripotent state. This could reduce the risk of tumorigenicity after transplantation. Also, direct differentiation without a reprogramming step may shorten the *in vitro* stage of the therapy. A first study describes the conversion of human fibroblasts to Bestrophin1 expressing colonies with morphological and molecular features of RPE lineage[39]. In this study, fibroblasts were transduced with eight transcription factors to initiate the trans-differentiation process. This could be the start of a new therapeutic strategy for AMD.

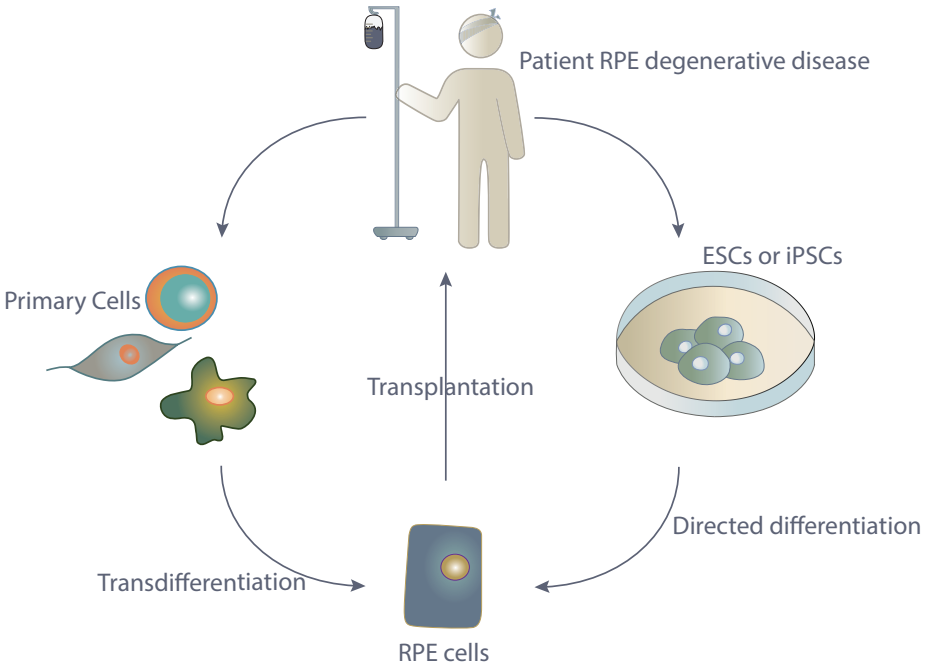

Figure 6. Different strategies for regenerative therapy for AMD. Both pluripotent stem cells and primary somatic cells can be derived from the patient and be used to differentiate towards RPE cells.

CONCLUSIONS

All together, we know that RPE cells are important for vision because they support the PR in maintaining their health and structural integrity. The development of a therapy for AMD would have great societal impact and there are many researchers focused on cell replacement therapy. Several cell sources are considered with each their (dis-) advantages. Currently there is no consensus on which is the best. Even though there are pre-clinical studies in which cells are transplanted into the eyes of patients, some hurdles need to be surmounted before an effective therapy can be developed.

AIM AND OUTLINE OF THIS THESIS

In this thesis I describe several studies that are related to the development of cell replacement therapy in RPE degenerative disorders. The focal point is extensive investigation of the molecular properties of the human RPE.
In these studies we used a microarray strategy for gene expression profiling to measure thousands of genes at once to give a global picture of cellular function. To understand the complex mechanisms underlying the gene expression data we used the knowledge database, Ingenuity's IPA. Here we derive biological meaning from the data. IPA describes biological processes, components or structures in which individual genes and proteins are known to be involved. It identifies genes that function in the same pathway. Identifying active pathways that differ can have more explanatory power than simply a list of genes. Using this high throughput screening allowed us to study a informed snapshot of the transcriptome by identifying co-regulated genes, pathways and systems.

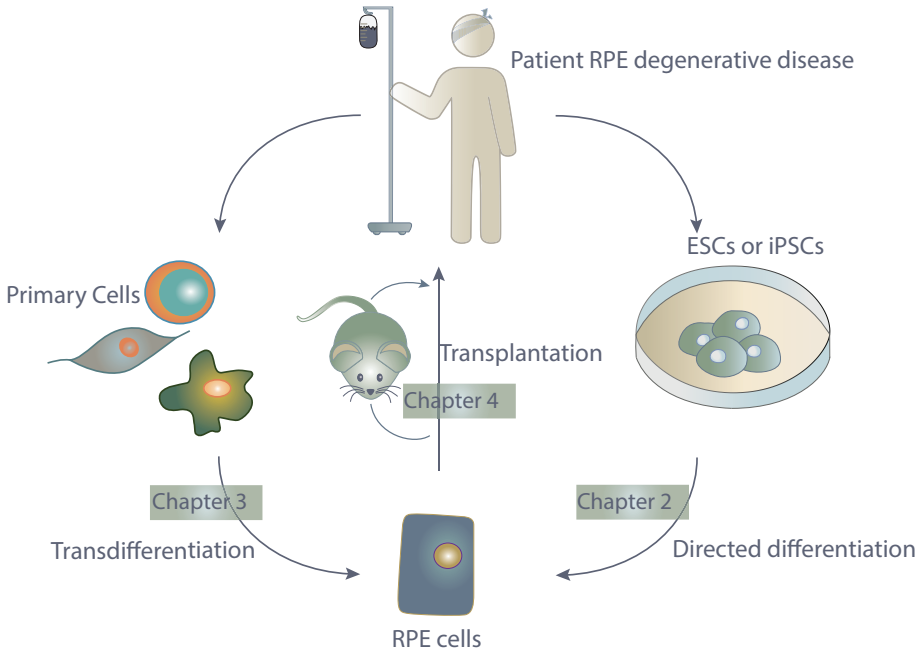

Figure 7. Schematic overview of the studies I describe in this thesis that are related to the development of cell replacement therapy in RPE degenerative disorders.

In **chapter 2,** we compared the human RPE with the mouse RPE. We determined the RPE signature genes for both species, plus the interspecies RPE signature genes. Also, we analyzed differences and similarities between their cellular functions based on

gene expression profiles. In **chapter 3,** we conducted an in depth analysis of the gene expression profiles of the *in vivo* IE and RPE to determine the potential of IE as a source for cell therapy in RPE degenerative disorders. We report pathways that are active in the IE that could allow the initial transition towards RPE and transplantation of the cells. We hypothesize that within certain limitations, the IE has good potential for RPE cell replacement. In **chapter 4,** I describe our study on the gene expression profiles of stem cell derived RPE cells. We compared SC-RPE cells that are early in development to late in development. Here we used the amount of pigmentation as a maturation marker. We also compare the SC-RPE to the *in vivo* RPE and determined the differences between the cell types. **Chapter 5** discusses the challenges and complexities that remain for the use of cell replacement therapy in RPE degenerative disorders.

REFERENCES

1. Strauss O. The retinal pigment epithelium in visual function. Physiol Rev 2005;85(3):845–81.
2. Rizzolo LJ. Polarity and the development of the outer blood-retinal barrier. Histol Histopathol 1997;12(4):1057–67.
3. Van Soest SS, de Wit GMJ, Essing AHW, et al. Comparison of human retinal pigment epithelium gene expression in macula and periphery highlights potential topographic differences in Bruch's membrane. Mol Vis 2007;13:1608–17.
4. Boulton M, Dayhaw-Barker P. The role of the retinal pigment epithelium: topographical variation and ageing changes. Eye Lond Engl 2001;15(Pt 3):384–9.
5. Plafker SM, O'Mealey GB, Szweda LI. MECHANISMS FOR COUNTERING OXIDATIVE STRESS AND DAMAGE IN RETINAL PIGMENT EPITHELIUM. Int Rev Cell Mol Biol 2012;298:135–77.
6. Reichhart N, Strauß O. Ion channels and transporters of the retinal pigment epithelium. Exp Eye Res 2014;126:27–37.
7. Immel J, Steinberg RH. Spatial buffering of K+ by the retinal pigment epithelium in frog. J Neurosci Off J Soc Neurosci 1986;6(11):3197–204.
8. Saari JC. Vitamin A metabolism in rod and cone visual cycles. Annu Rev Nutr 2012;32:125–45.
9. Nguyen-Legros J, Hicks D. Renewal of photoreceptor outer segments and their phagocytosis by the retinal pigment epithelium. Int Rev Cytol 2000;196:245–313.
10. Kay P, Yang YC, Paraoan L. Directional protein secretion by the retinal pigment epithelium: roles in retinal health and the development of age-related macular degeneration. J Cell Mol Med 2013;17(7):833–43.
11. Wong WL, Su X, Li X, et al. Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. Lancet Glob Health 2014;2(2):e106–16.
12. Thompson RB, Reffatto V, Bundy JG, et al. Identification of hydroxyapatite spherules provides new insight into subretinal pigment epithelial deposit formation in the aging eye. Proc Natl Acad Sci U S A 2015;112(5):1565–70.
13. Zarbin MA, Casaroli-Marano RP, Rosenfeld PJ. Age-related macular degeneration: clinical findings, histopathology and imaging techniques. Dev Ophthalmol 2014;53:1–32.
14. Nazari H, Zhang L, Zhu D, et al. Stem cell based therapies for age-related macular degeneration: The promises and the challenges. Prog Retin Eye Res 2015;48:1–39.
15. Lambert NG, Singh MK, ElShelmani H, et al. Risk factors and biomarkers of age-related macular degeneration. Prog Retin Eye Res 2016;
16. van Meurs JC (last), Kirchhof B, MacLaren R, et al. Ryan's Retina 6th edition, Chapter 124: Retinal Pigment Epithelium and Choroid Translocation in Patients with Age-Related Macular Degeneration. Mosby; 2016.
17. Binder S, Stanzel BV, Krebs I, Glittenberg C. Transplantation of the RPE in AMD. Prog Retin Eye Res 2007;26(5):516–54.
18. Blenkinsop TA, Saini JS, Maminishkis A, et al. Human Adult Retinal Pigment Epithelial Stem Cell-Derived RPE Monolayers Exhibit Key Physiological Characteristics of Native Tissue. Invest Ophthalmol Vis Sci 2015;56(12):7085–99.
19. Tezel TH, Del Priore LV, Kaplan HJ. Reengineering of Aged Bruch's Membrane to Enhance Retinal Pigment Epithelium Repopulation. Investig Opthalmology Vis Sci 2004;45(9):3337.
20. Salero E, Blenkinsop TA, Corneo B, et al. Adult Human RPE Can Be Activated into a Multipotent Stem Cell that Produces Mesenchymal Derivatives. Cell Stem Cell 2012;10(1):88–95.

21. Stanzel BV, Liu Z, Somboonthanakij S, et al. Human RPE stem cells grown into polarized RPE monolayers on a polyester matrix are maintained after grafting into rabbit subretinal space. Stem Cell Rep 2014;2(1):64–77.
22. Davis RJ, Alam NM, Zhao C, et al. The Developmental Stage of Adult Human Stem Cell-Derived Retinal Pigment Epithelium Cells Influences Transplant Efficacy for Vision Rescue. Stem Cell Rep 2017;9(1):42–9.
23. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126(4):663–76.
24. Song MJ, Bharti K. Looking into the future: Using induced pluripotent stem cells to build two and three dimensional ocular tissue for cell therapy and disease modeling. Brain Res 2016;1638(Pt A):2–14.
25. Leach LL, Croze RH, Hu Q, et al. Induced Pluripotent Stem Cell-Derived Retinal Pigmented Epithelium: A Comparative Study Between Cell Lines and Differentiation Methods. J Ocul Pharmacol Ther Off J Assoc Ocul Pharmacol Ther 2016;
26. Liao J-L, Yu J, Huang K, et al. Molecular signature of primary retinal pigment epithelium and stem-cell-derived RPE cells. Hum Mol Genet 2010;19(21):4229–38.
27. Lund RD, Wang S, Klimanskaya I, et al. Human Embryonic Stem Cell–Derived Cells Rescue Visual Function in Dystrophic RCS Rats. Cloning Stem Cells 2006;8(3):189–99.
28. Carr A-J, Vugler A, Lawrence J, et al. Molecular characterization and functional analysis of phagocytosis by human embryonic stem cell-derived RPE cells using a novel human retinal assay. Mol Vis 2009;15:283–95.
29. Vugler A, Carr A-J, Lawrence J, et al. Elucidating the phenomenon of HESC-derived RPE: Anatomy of cell genesis, expansion and retinal transplantation. Exp Neurol 2008;214(2):347–61.
30. Lu B, Malcuit C, Wang S, et al. Long-term safety and function of RPE from human embryonic stem cells in preclinical models of macular degeneration. Stem Cells Dayt Ohio 2009;27(9):2126–35.
31. Klimanskaya I, Hipp J, Rezai KA, West M, Atala A, Lanza R. Derivation and Comparative Assessment of Retinal Pigment Epithelium from Human Embryonic Stem Cells Using Transcriptomics. Cloning Stem Cells 2004;6(3):217–45.
32. Buchholz DE, Pennington BO, Croze RH, Hinman CR, Coffey PJ, Clegg DO. Rapid and Efficient Directed Differentiation of Human Pluripotent Stem Cells Into Retinal Pigmented Epithelium. Stem Cells Transl Med 2013;2(5):384–93.
33. Idelson M, Alper R, Obolensky A, et al. Directed Differentiation of Human Embryonic Stem Cells into Functional Retinal Pigment Epithelium Cells. Cell Stem Cell 2009;5(4):396–408.
34. Bharti K, Miller SS, Arnheiter H. The new paradigm: retinal pigment epithelium cells generated from embryonic or induced pluripotent stem cells. Pigment Cell Melanoma Res 2011;24(1):21–34.
35. Osakada F, Ikeda H, Sasai Y, Takahashi M. Stepwise differentiation of pluripotent stem cells into retinal cells. Nat Protoc 2009;4(6):811–24.
36. Maruotti J, Wahlin K, Gorrell D, Bhutto I, Lutty G, Zack DJ. A Simple and Scalable Process for the Differentiation of Retinal Pigment Epithelium From Human Pluripotent Stem Cells. Stem Cells Transl Med 2013;2(5):341–54.
37. Nakano T, Ando S, Takata N, et al. Self-Formation of Optic Cups and Storable Stratified Neural Retina from Human ESCs. Cell Stem Cell 2012;10(6):771–85.
38. Zhu Y, Carido M, Meinhardt A, et al. Three-dimensional neuroepithelial culture from human embryonic stem cells and its use for quantitative conversion to retinal pigment epithelium. PloS One 2013;8(1):e54552.
39. Zhang K, Liu G-H, Yi F, et al. Direct conversion of human fibroblasts into retinal pigment epithelium-like cells by defined factors. Protein Cell 2014;5(1):48–58.

You will come to a place where the streets are not marked.
Some windows are lighted. But mostly they're darked.

A place you could sprain both your elbow and chin!
Do you dare to stay out? Do you dare to go in?
How much can you lose? How much can you win?

- Dr Seuss

Stem cell derived retinal pigment epithelium: the role of pigmentation as maturation marker and gene expression profile comparison with human endogenous retinal pigment epithelium.

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ABSTRACT

In age-related macular degeneration (AMD) the retinal pigment epithelium (RPE) deteriorates, leading to photoreceptor decay and severe vision loss. New therapeutic strategies aim at RPE replacement by transplantation of pluripotent stem cell (PSC)-derived RPE. Several protocols to generate RPE have been developed where appearance of pigmentation is commonly used as indicator of RPE differentiation and maturation. It is, however, unclear how different pigmentation stages reflect developmental stages and functionality of PSC-derived RPE cells.

We generated human embryonic stem cell-derived RPE (hESC-RPE) cells and investigated their gene expression profiles at early pigmentation (EP) and late pigmentation (LP) stages. In addition, we compared the hESC-RPE samples with human endogenous RPE. We used a common reference design microarray (44K).

Our analysis showed that maturing hESC-RPE, upon acquiring pigmentation, expresses markers specific for human RPE. Interestingly, our analysis revealed that EP and LP hESC-RPE do not differ much in gene expression. Our data further showed that pigmented hESC-RPE has a significant lower expression than human endogenous RPE in the visual cycle and oxidative stress pathways. In contrast, we observed a significantly higher expression of pathways related to the process adhesion-to-polarity model that is typical of developing epithelial cells.

We conclude that, *in vitro,* the first appearance of pigmentation hallmarks differentiated RPE. However, further increase in pigmentation does not result in much significant gene expression changes and does not add important RPE functionalities. Consequently, our results suggest that the time span for obtaining differentiated hESC-RPE cells, that are suitable for transplantation, may be greatly reduced.

INTRODUCTION

Regenerative medicine holds great promise for patients with degenerative diseases that are clinically characterized by tissue loss. Age-related macular degeneration (AMD) is a progressive degenerative disease and it is the leading cause of blindness in the elderly in the Western world. In people of 60 years of age or older, 4 percent is affected by a late severe stage of AMD[1]. AMD is classically characterized by the dysfunction and degeneration of the retinal pigment epithelium (RPE) in the macula, the part of the retina responsible for central vision. The RPE is a monolayer of cells in the back of the eye that plays an important role in the maintenance and health of the photoreceptors[2,3]. AMD presents itself in two forms: wet and dry. The more severe wet form accounts for 10-15% of the cases[4], and is characterized by neovascularization. This form can be treated by monthly intra-ocular injections of anti-angiogenic drugs. Even though frequently effective, this is a patient unfriendly, invasive and costly treatment. Dry AMD is more prevalent and is characterized by a slow buildup of yellowish deposits beneath the RPE, called drusen, which progresses to geographic loss of RPE and subsequently photoreceptor atrophy. There are several treatment options for dry AMD, including RPE transplantation, laser photocoagulation, photodynamic therapy, submacular surgery, transpupillary thermotherapy, and pharmacotherapy[5–8]. However, these approaches are not very effective, and thus there is much interest in the development of new therapies. AMD is a genetically complex disorder, and, at least in the classical view, the primary pathology is limited to a single cell type (the RPE). RPE transplantation may be the only AMD treatment that can restore the function of already degenerated cells, if this is performed in an early stage of AMD in order to prevent photoreceptor loss. However, replacement of degenerated tissue with donor material, or the translocation of autologous RPE sheets from the periphery to the macula, have had limited success so far[6,9]. This can partly be ascribed to the technical challenges involving the collection of sufficient tissue, transplant rejection, and the difficulties in controlling harvest and direct use of age- and genetically-matched cells.

The use of pluripotent stem cell derived-RPE cells (PSC-RPE) may circumvent some of these problems, as we have more and more control of generating specific neural subtypes, such as RPE, using HLA-matched PSC sources and scaling cell products to sufficiently high numbers.

Several groups recently optimized PSC differentiation protocols to generate RPE. Early protocols were based on so-called spontaneous differentiation by letting PSC freely differentiate using the adherent culture or floating embryoid body methods into pigmented RPE cells[10–12]. Although these protocols reliably produce pigmented cells, they are time-consuming and inefficient. Later protocols, so called the directed differentiation methods, showed improved efficiency. Directed differentiation methods use the

addition of growth factors to induce RPE differentiation, and either involve adherent, suspension or 3D cultures to resemble the *in vivo* development more closely (reviewed by Leach et al 2016[13]).

Although we are able to generate RPE (-like) cells *in vitro*, our knowledge about the most suitable differentiation state and corresponding function before and upon transplantation is limited. So far the emergence and increase of pigmentation is used as important hallmark for differentiation and further maturation of PSC-RPE. It is however unclear how the PSC-RPE changes during this increase in pigmentation, how PSC-RPE with little pigmentation compares to PSC-RPE with much pigmentation, and to what extent they represent stages in maturation towards the human endogenous RPE.

We adapted an established directed differentiation protocol to produce human embryonic stem cells derived-RPE cells (hESC-RPE)[14]. Subsequently, we compared the gene expression profiles of hESC-RPE samples that start to show pigmentation and that of samples that are almost fully pigmented. Finally, we compared the hESC-RPE samples to endogenous human RPE.

MATERIALS AND METHODS

Maintenance of hESC cells and RPE differentiation

hESC line H1 (WA01, WiCell Research Institute, Madison, USA) was cultured in Essential 8 medium (Thermo Fischer, Waltham, USA) on Geltrex LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix (Thermo Fischer, Waltham, USA) coated 6-well plates. The cells were passaged as clumps every 3 to 4 days using 0.5 mM UltraPure EDTA (Thermo Fischer, Waltham, USA) dissolved in DPBS without Calcium and Magnesium (Thermo Fischer, Waltham, USA). Morphologically distinguishable differentiated cells were mechanically removed at each passage. To improve cell survival during passaging, the Rho kinase inhibitor, Y-27632 (SelleckChem, Houston, USA), was added in the culture medium during the first 24 hours after plating.

To produce hESC-RPE cells, undifferentiated cell colonies were partially lifted by EDTA and scraped off with a cell scraper. The cell aggregates (150-250 um diameter) from one well of a six-well plate that was densely packed with colonies, were embedded in 150-250ul Matrigel (Corning, Corning, USA). The Matrigel containing the cells was plated 150ul per well on a six wells plate. They were plated as drops of Matrigel without touching the sidewalls of the wells. After gelling at 37°C for 10 minutes, neural induction medium N2B27 was added, prepared as described (Pollard, Benchoua and Lowell 2006[15]). After three days of differentiation, the cells were taken out of the Matrigel using Cell Recovery Solution (Corning, Corning, USA). To make single cells from the three-dimensional spheroids we treated it with TrypLE Express (Thermo Fischer, Waltham, USA), followed

by gentle trituration. The cells were resuspended in N2B27 medium, containing 10 uM Rho kinase inhibitor to promote cell survival and seeded onto growth factor reduced Matrigel (Corning, Corning, USA) coated 6.5mm Transwell inserts with 0.4uM pore polyester membrane (Corning, Corning, USA), at a density of 2-4x10^5 cells/insert. At day 4 the cells were washed with RPE medium (see Zhu et al 2013 for details[14]) and were kept in culture with RPE medium that contained human Activin A (100ng/ml) (Agrenvec, Madrid, Spain). RPE medium consists of DMEM/F-12; no glutamine supplemented with 20% KnockOut Serum Replacement; MEM Non-Essential Amino Acids Solution; GlutaMAX Supplement; 100U/ml Penicillin-Streptomycin and 0.1 mM 2-Mercaptoethanol (All from Thermo Fischer, Waltham, USA). Medium was changed every 2-3 days.

RNA isolation and (sq)RT-PCR

Total RNA was isolated using the RNeasy Micro Kit (Qiagen, Hilden, Germany). Subsequent reverse transcription to cDNA was performed with Superscript III reverse transcriptase (Life Technologies, Waltham, USA). The synthesized cDNA was amplified with transcript specific, intron-spanning primers (See Table S1 for the primer sequences). PCR was carried out with HOT FIREPol DNA Polymerase (Solis Biodyne, Tartu, Estonia) with an annealing temperature of 60°C and 33 cycles. For the sqRT-PCR's, we calculated the relative abundance of transcript expression by quantifying the gene expression in ImageJ and normalizing it to the housekeeping gene β-actin (*ACTB)*.

Immunocytochemistry

Cells were fixed with 2% paraformaldehyde for 20 minutes at room temperature, followed by blocking with 0.1% BSA, 0.3% Triton X-100, 5% normal goat serum, in 1x PBS. Incubation with the primary antibodies was performed in blocking buffer and done overnight at 4°C. The working solutions were as follows: rabbit anti-RLBP1 1:200 (PA5-29759, Thermo Fisher, Waltham, USA), rabbit anti-MITF 1:200 (PA5-38294, Thermo Fisher, Waltham, USA), rabbit anti-ZO1 1:100 (61-7300, Thermo Fisher, Waltham, USA), rabbit anti-BEST1 1:100 (ab14928, Abcam, Cambridge, UK). The immunoreactivity of the antibodies was confirmed by immunostainings on human retinal cryosections and ARPE19 cells as positive control (Figure S1). As a secondary antibody we used the Alexa Fluor 594 goat-anti-rabbit 1:1000 (A-111012, Thermo Fisher, Waltham, USA). Cell nuclei were counterstained with DAPI (Thermo Fisher, Waltham, USA). Cells were imaged using a Leica TCS SP8 X confocal microscope.

Microarray sample collection and preparation

We selected two microarray sample groups based on their pigmentation state during the hESC-RPE differentiation protocol. For six independent differentiation experiments we harvested cells, when the cells in the inserts started to show pigmentation (timepoint

"Early Pigmentation", EP) and when they were more than 80% pigmented (timepoint "Late Pigmentation", LP), measured in ImageJ. The average days in culture for the EP samples is 32 (*s*=8.6), and for the LP samples 62.5 (*s*=12.1). We used global (manual) thresholding to determine the percentage of pigmented area. Photographs of the inserts were made with an 8-megapixel phone camera. These were loaded into ImageJ and converted to 8-bit images in order to be able to segment the image. The membrane of the insert was selected to include the whole culture surface. By thresholding the area that contains pigmented cells was included in the percentage. Because of variation in lighting of the original photos, we determined the threshold independently for every sample.

RNA isolation, amplification and labelling procedures were carried out essentially as described elsewhere[16]. Quality of the total RNA was checked with a Bioanalyzer assay (RNA 6000 Pico Kit, Agilent Technologies, Amstelveen, The Netherlands). The average RIN value for the total RNA of both the EP and the LP samples was 9.7, indicating excellent quality. In our microarray study we used a common reference design. As a common reference we used RNA from human RPE/choroid that was used in previous and ongoing gene expression analyses in our lab[16,17]. In short, the common reference sample consists of RNA from a pool of RPE/choroid isolated from 10 donor eyes (mean age 60 years). It was prepared using the same methodology as our experimental samples, and labelled with Cy3 (Cy3 mono-reactive dye pack, GE Healthcare UK, Little Chalfont, Buckinghamshire, UK). See Janssen et al (2012)[16] for a more detailed description RNA processing and microarray procedures.

In addition, to make sure we compared hESC-RPE cells, we performed a RT-PCR experiment (Figure S2). We studied the expression of *RAX, VSX2, MITF, TYR, TRPM3, TJP1, RLBP1, RPE65, MERTK* in EP and LP samples. The results confirmed the RPE character of the cells.

Microarray data analysis

The microarray data were extracted using Agilent Feature Extraction Software (Agilent Technologies, version 9.5.3.1). Raw data were imported into R (version 2.14.0 for Windows, R Development Core Team, 2009) using the Bioconductor package LIMMA. Background correction was performed using the "normexp" method with an offset of 10 to adjust the foreground signal without introducing negative values. The resulting log-ratios were transformed using intensity-dependent loess normalization. We further normalized the average intensities across arrays using the Aquantile method[18].

The microarray data is available in the Gene Expression Omnibus database with the accession number GSE85907.

Genes that are differentially expressed between the EP and LP hESC-RPE, or between the hESC-RPE (EP and LP) and human endogenous RPE, were identified on the normalized log-ratios using a linear model. The data for the human endogenous RPE were derived from a previous study that used the exact same microarray strategy and

analysis (submitted). This dataset consists of 5 independent donor eyes that were enucleated and snap-frozen within 24 hours post mortem. The eyes were stored at -80°C until use. Donors were aged 49 to 73 at time of death. Donors were selected for not having any ophthalmic disorder and visual inspection examination showed no retinal pathology. To collect the RPE, a macular fragment of 16mm^2 with the fovea in its center was cut from the retina.12uM Sections from the macular area were used to isolate the RPE cells[19]. The sections were dehydrated with ethanol and air-dried before micro dissection. To minimize cellular cross-contamination in our procedure, we used the meticulous laser dissection microscope to cut the RPE monolayer specifically (PALM Carl Zeiss, MicroImaging GmbH, Munich, Germany).

Significant differences were determined using Bayes moderated paired t-statistics (package LIMMA in R). Resulting p-values were corrected for multiple testing using Benjamini-Hochberg False Discovery Rate adjustment. To identify specific differences between the EP hESC-RPE and the LP hESC-RPE, we used cutoff values of a fold change (FC) >2.5 and a p-value<0.05. We found 246 genes significantly higher expressed in the EP hESC-RPE and 65 genes significantly higher expressed in the LP hESC-RPE.

Subsequently, we statistically tested the differences between the hESC-RPE (EP and LP) and human endogenous RPE. We used the stringent cut off values of FC>5 and adjusted p value of p<0.001 because we were interested in the most significantly specific differences between the two groups. This resulted in 737 genes significantly higher expressed in the hESC-RPE (EP and LP) and 1022 genes significantly higher expressed in the human endogenous RPE.

To investigate the degree of equality between gene expression profiles of the various groups, we plotted the samples on a multidimensional scaling plot (two dimensions) in the LIMMA package in R. The purpose of this plot is to provide a visual representation of the pattern of proximities (i.e. similarities or distances) among a set of objects. Those objects that are perceived to be very similar to each other are placed near each other on the map, and the objects that are perceived as very different are placed far away from each other.

Functional annotation was done in IPA, Ingenuity (Ingenuity Systems, version 24718999, assessed at May 31st, 2016). To present the results as comprehensive as possible we highlighted only the Ingenuity canonical pathways because these depict the most simple and straightforward representation of our data and functionalities.

Confirmation of microarray results

We confirmed our microarray data with sqRT-PCR (Figure S3). sqRT-PCR was carried out using intron-spanning primers on cDNA from EP and LP, using 6 biological replicates. To minimize effects of RNA degradation artefacts, we generated primers near the 3'end of the gene. We quantified the gene expression in ImageJ.

RESULTS

Characterization of hESC-RPE differentiation

We differentiated hESC into RPE cells according to an adapted protocol previously described by Zhu et al 2013[14] (Figure 1A). We reduced the incubation time of the three-dimensional spheroids in the Matrigel from 5 to 3 days as in our hands the spheroids were already fully grown within 3 days. To confirm RPE development, we performed RT-PCR at different time points during hESC-RPE cell generation (Figure 1B). We measured gene expression of well-known RPE markers in our hESC-RPE cells at several time points (Figure 1C)[2]. Before pigmentation (time point 1 and 2), hESC-RPE expressed the early eye development markers *PAX6* and *OTX2*, which stay present till late differentiation stages (Figure 1C). By early onset of pigmentation (time point 2 and 3), most RPE-specific genes are turned on (*MITF, TYR, BEST1, TRPM3, RLBP1, MERTK, RPE65 and TJP1*). In our differentiation protocol, the early eye marker *RAX* is only clearly expressed at time points 3 and 4, but that does not seem to hinder the expression of other important RPE developmental genes (see previous sentence). RT-PCR analysis also confirmed the generation of the RPE by almost complete absence of *VSX2*, a marker for retinal progenitor cells. We see some VSX2 expression at time points 3 and 5, which disappears at later stages. This transient expression level of VSX2 may indicate the switching point between the development of photoreceptors or RPE[20]. In addition, as many PSC-derived protocols are challenged by high variability, we measured 50 independent samples, derived from 16 independent differentiation procedures, for a (semi-) quantification of the data after normalization of the expression to the housekeeping gene *ACTB* (Figure S4). We found a high amount of variation. Generation of RPE-like cells was further shown by light microscopy analysis, identifying typical epithelial cobblestone RPE-like appearance and the presence of pigment granules (Figure 1D), and by immunocytochemical analysis of RPE-specific markers ZO-1, MITF, RLBP1 and BEST1 (Figure 2). Additionally, hESC-RPE showed photoreceptor outer segment phagocytosis using a previously published protocol (Figure S5)[21,22].

Gene expression profile analysis of early and late-stage pigmentation of hESC-RPE

To investigate RPE maturity and functional properties of EP and LP hESC-RPE in more depth, we performed six independent experiments (see Materials and Methods for details). These samples were used for a microarray study.

After feature extraction, we performed a paired t test on the gene expression data of the two groups (EP and LP hESC-RPE) and made a selection using a Benjamini-Hochberg (B-H) corrected p value < 0.05 and fold change > 2.5. We found a total of 311 genes differentially expressed (Table S2). Even though the sample groups were determined by their pigmentation levels, there are no genes in this list that are well-known for the me-

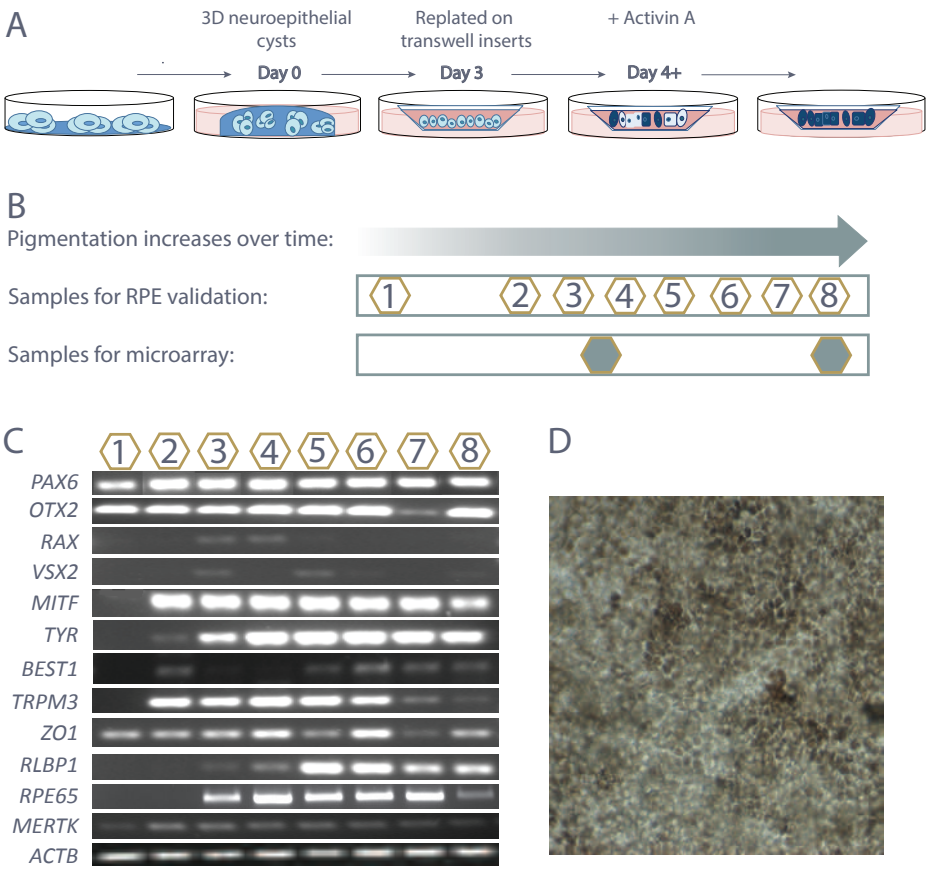

Figure 1. (A) Overview of the hESC-RPE differentiation protocol adapted from Zhu et al[14]. (B) Scheme shows the different time points for collection of samples for validation of hESC-RPE generation (1=3 days, 2=10-12 days, 3=20-25 days, 4=30-35 days, 5=40-45 days, 6=50-55 days, 7=60-63 days, 8= 70 days), by RT-PCR analysis. We also collected RNA when the cells started to show pigmentation (EP) and when more than 80% of the confluent culture was pigmented (LP). (C) RT-PCR analysis at time points 1-8 showed absence and expression of characteristic RPE genes. (D) The hESC-RPE cells started to show first pigmentation phenotypes and typical epithelial hexagonal morphology at timepoint 4.

lanogenesis in the RPE[23,24]. The expression levels of these melanogenesis genes (*PAX6, OTX2, TYR, TYRP1, DCT, MITF, SI, MLANA*) are comparable between EP and LP samples (for details see the normalized expression levels of the microarray at the Gene Expression Omnibus database, accession number GSE85907).

Subsequently, we used the IPA knowledge database to attribute a selection of over-represented pathways to the differences between EP and LP hESC-RPE cells. These functions are depicted in figure 3.

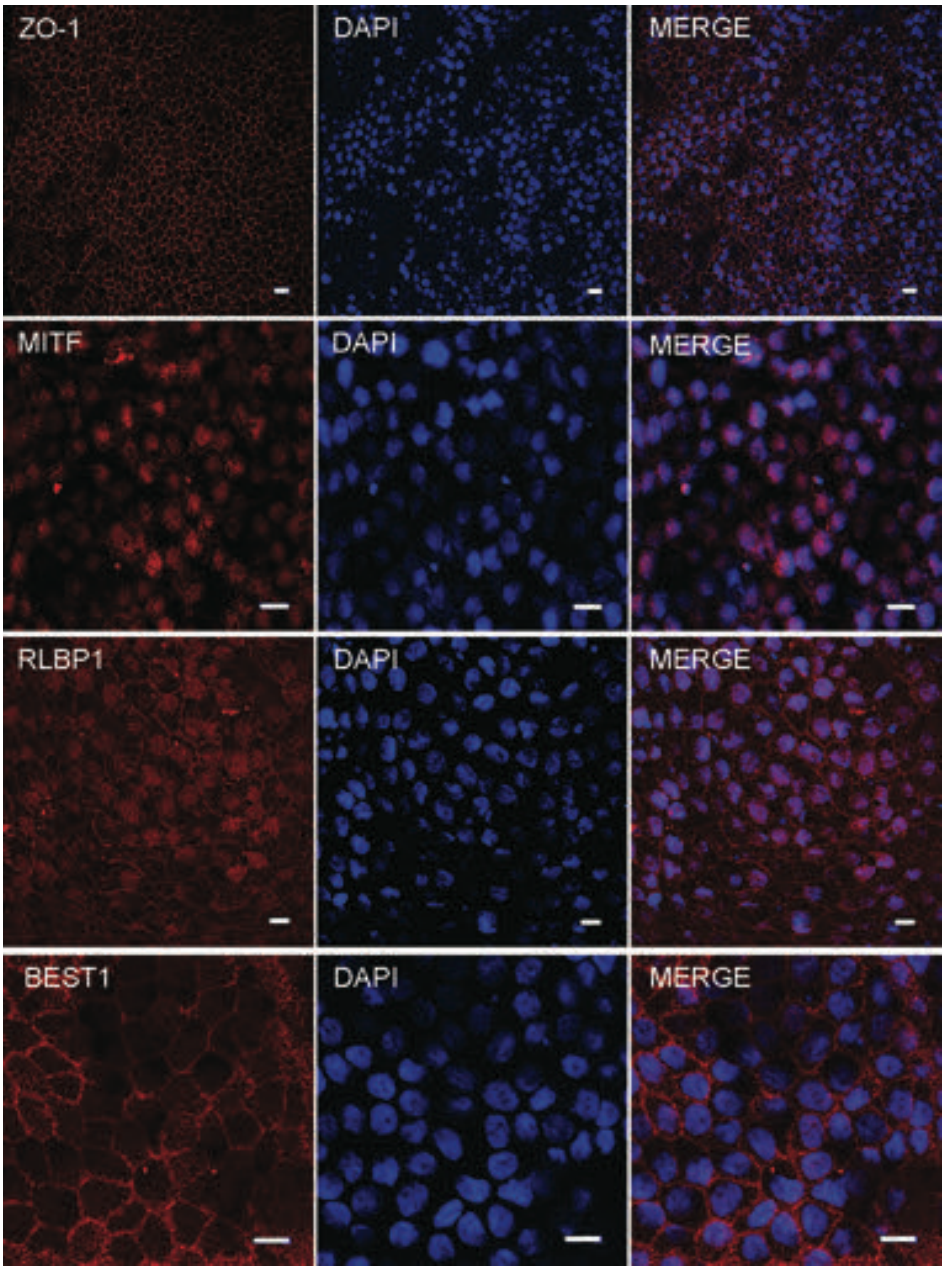

Figure 2. RPE generation was confirmed by immunocytochemistry for the tight junction protein ZO-1, transcription factor MITF, visual cycle related protein RLBP1 and the chloride channel BEST1 (scalebar = 10uM).

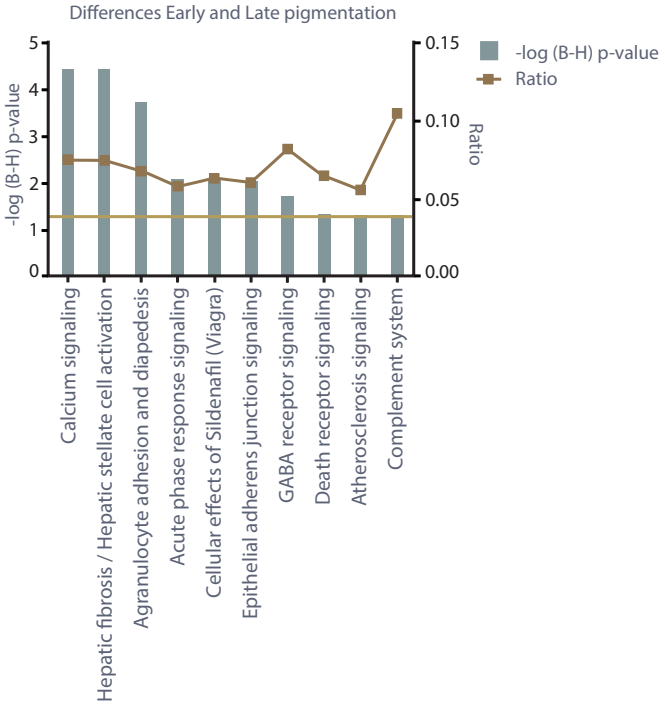

Figure 3. Canonical pathways identified by IPA for the genes that are significantly differentially expressed genes between EP and LP samples. The left y-axis displays the –log of the Benjamini-Hochberg corrected –value. The right axis displays the ratio of the number of genes derived from our dataset, divided by the total number of genes in the pathway. The bar graph represents the –log (B-H) p-value. The orange line indicates the threshold at a B-H corrected p-value < 0.05.

Because only a relatively small number of genes showed statistically significant differences (311 out of 19596 unique genes on array), we also analyzed the (dis)similarities of the *overall* expression of the individual samples. We plotted the normalized expression data (this includes the expression of all the entries that are measured on the array: 43376 entries per sample) in a multidimensional scaling plot to visualize the level of (dis)similarity (Figure 4). This plot showed no clear segregation between the EP and LP hESC-RPE groups.

Comparison of hESC-RPE and human endogenous RPE expression profiles

Next, we studied how similar the *in vitro* cultured hESC-RPE cells are to human endogenous RPE. EP and LP hESC-RPE did not show clear differences and we combined the data into one hESC-RPE group. We compared that group with human endogenous RPE gene expression data, previously generated from laser-dissected RPE from human donor eyes, using the same microarray platform and common reference design

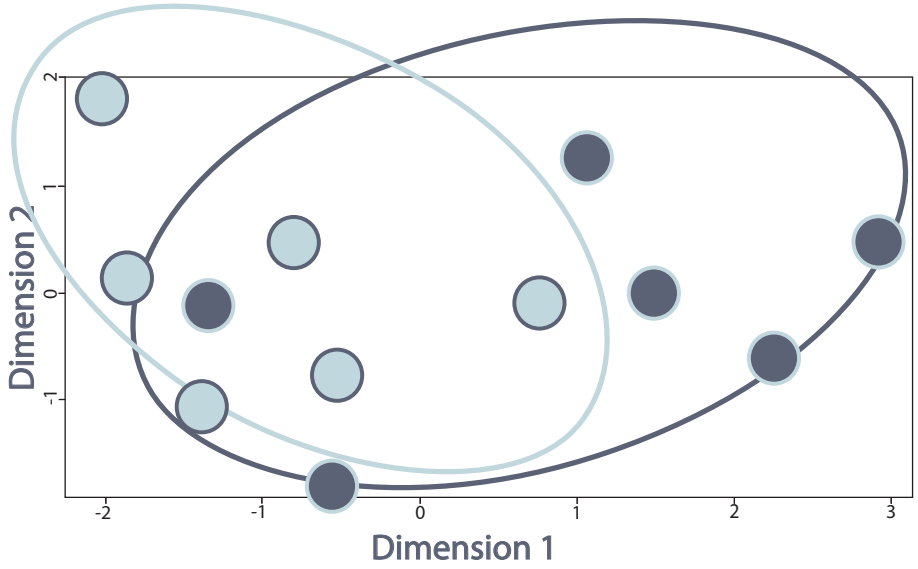

Figure 4. Multidimensional scaling plot to visually represent the (dis)similarities among the different hESC-RPE cell samples. The light blue dots represent the individual EP samples and the dark blue dots represent the LP samples. We used the LIMMA package in R, which is specific for the analysis of microarray data, and included all the normalized expression data of the individual samples: 43376 entries per sample.

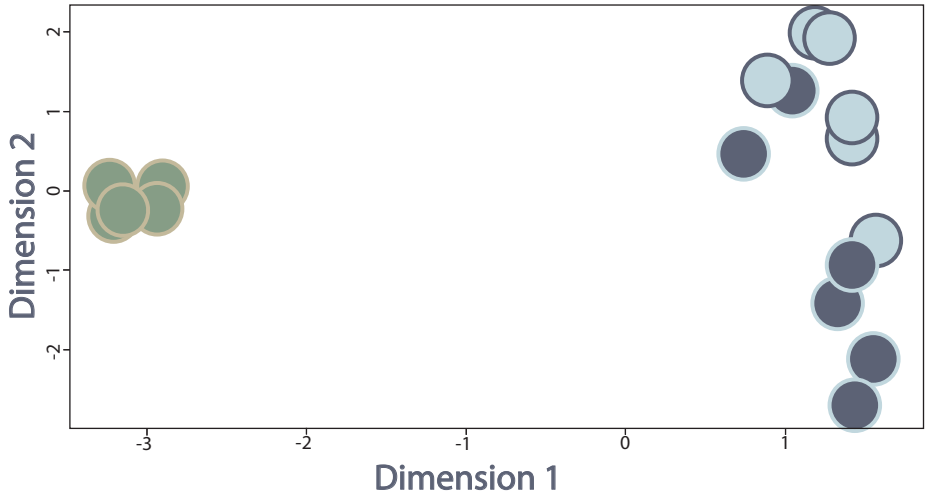

Figure 5. Multidimensional scaling plot to visually represent the (dis)similarities among the different hESC-RPE cells (blue dots) and human endogenous RPE (green dots). Also see figure 4.

(submitted). To begin, we analyzed the (dis) similarities of the *overall* expression of the individual sample using multidimensional scaling (Figure 5).

The multidimensional scaling plot shows that the overall expression profiles are very different between hESC-RPE and the human endogenous RPE sample groups. This analysis also shows that there is more variation within the hESC-RPE sample group than within the human endogenous RPE samples group.

To further compare the hESC-RPE and human endogenous RPE, we performed an unpaired t test. Here we considered genes significantly differentially expressed with a B-H adjusted p value < 0.001 and fold change > 5. We chose these stringent cutoff values in order to focus on the most prominent differences. We found 737 genes significantly higher expressed in the hESC-RPE (EP and LP) cells compared to the human endogenous RPE and 1022 genes significantly higher expressed in the human endogenous RPE compared to the hESC-RPE (Table S3). We conducted a functional annotation in IPA for the differentially expressed genes between the hESC-RPE (EP and LP) samples and the human endogenous RPE (Figure 6). This yielded 12 canonical pathways higher expressed in the hESC-RPE (EP and LP) cells, of which eight pathways are related to the so called adhesion-to-polarity model: Epithelial Adherens Junction Signaling, Actin Cytoskeleton Signaling, ILK Signaling, RhoGDI Signaling, Remodeling of Epithelial Adherens Junctions, Tec Kinase Signaling, Regulation of Actin-Based Motility by Rho, Signaling by Rho Family GTPases. The analysis in IPA resulted in 14 canonical pathways that are higher expressed in the human endogenous RPE. Most prominent was the appearance of pathways related to the visual system: Phototransduction Pathway and The Visual Cycle. Other pathways were relevant to oxidative stress handling: Protein Kinase A Signaling, cAMP-mediated Signaling, CREB Signaling in Neurons, Melatonin Signaling. And also maintenance of the blood-retina-barrier: Endothelin-1 Signaling and Thrombin Signaling.

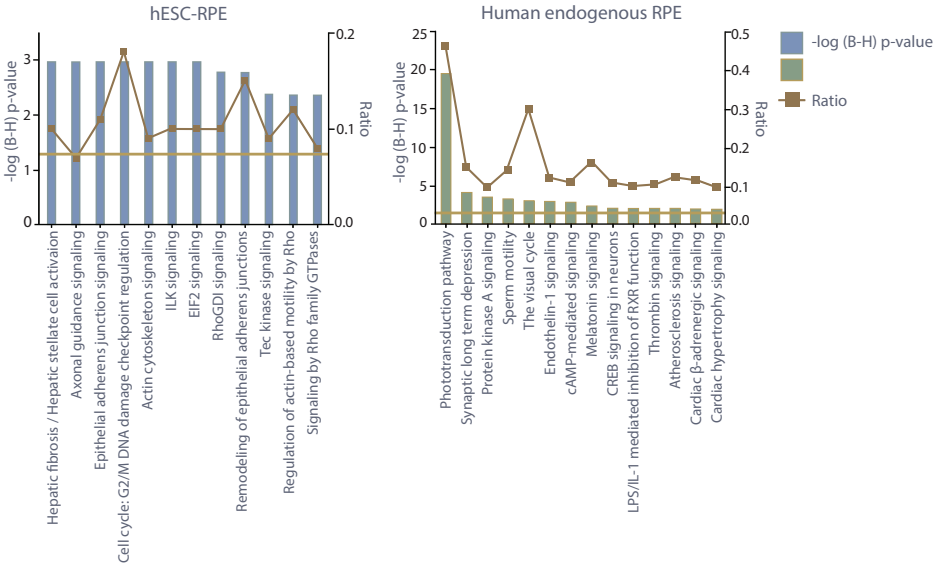

Figure 6. Canonical pathways identified by IPA for the genes that are significantly differentially expressed between the hESC-RPE cells and the human endogenous RPE. The left graph (blue) depicts the canonical pathways that relate to the genes specifically expressed in the hESC-RPE (this study). The right graph (green) depicts the canonical pathways that relate to the genes specifically expressed in the human endogenous RPE (submitted). In the graphs, the left y-axis displays the –log of the Benjamini-Hochberg corrected –value. The right axis displays the ratio of the number of genes derived from our dataset, divided by the total number of genes in the pathway. The bars show the –log (B-H) p-value. The orange line indicates the threshold at a B-H corrected p-value < 0.05.

DISCUSSION

In this study we expanded our knowledge on the development of hESC-RPE cells and generated expression profiles of EP and LP hESC-RPE samples, to investigate the suitability of pigmentation as a maturation marker in hESC-RPE differentiation. In addition, we compared the gene expression profiles of the hESC-RPE cells and the human endogenous RPE that it is supposed to replace.

We generated functional hESC-RPE cells using a well-established directed differentiation protocol. As many human stem cell-derived cultures are challenged by high amounts of variation, hESC-RPE cultures do not always mature with the same speed. Consequently, virtually all RPE differentiation studies use pigmentation as a maturation marker for the culture instead of time. This seems like a reliable benchmark and easy to use because it is clearly visible.

In attempt to answer the question whether increasing pigmentation indicates differentiation into more mature hESC-RPE cells, we performed a microarray study with the EP and LP hESC-RPE samples. In the comparison we found only a small amount of statistically significant differences. This implies that EP and LP hESC-RPE samples may be less different than generally accepted. Even though pigmentation seems to be a good biomarker for RPE development, the level of pigmentation does not reflect the maturation state of hESC-RPE. In terms of gene expression profile and functional annotation, cells seem to be at a similar developmental stage at EP and LP. Both the EP and LP cells show the expression of well-known RPE markers which is an important prerequisite for the transplantation of PSC-RPE cells[14,25–30]. This could mean that there is no need to wait for the cells to be fully pigmented because it does not make a substantial difference.

To be able to say more about how the hESC-RPE cells compare to human endogenous RPE, we subsequently compared the gene expression profiles of the hESC-RPE (EP and LP) samples and the gene expression profiles of human endogenous RPE samples.
In our analysis we found 12 canonical pathways highly expressed in the hESC-RPE (EP and LP) as compared to the human endogenous RPE. It is striking that eight of these are involved in the adhesion-to-polarity model that is typical for developing epithelial cells. The human endogenous RPE is a highly polarized cell type with distinct apical and basolateral plasma membrane domains. Cell polarity is initiated through a combination of spatial cues that depend on cell-cell interaction and cell-extracellular matrix interaction. Adherens junctions (AJs) and tight junctions (TJs) mediate the cell-cell contact of epithelial cells. Both types form extracellular adhesive contacts between cells and intracellular links to the actin cytoskeleton and signaling pathways, and they do this through different transmembrane proteins[31]. The ILK Signaling (integrin linked kinase) pathway may point to cell-extracellular matrix interaction that takes place during development of cell polarity. Since integrins do not exhibit intrinsic enzymatic activity, binding of integrins to the extracellular matrix proteins, results in recruitment of multiple intracellular proteins that activate signaling cascades and provide links to the actin cytoskeleton, including ILK[32]. ILK has been described to be an important modulator in cell-ECM interactions and the formation of AJs and TJs[33]. Several Rho signaling pathways have been connected to the hESC-RPE (EP and LP) specific dataset. Rho signaling has been implicated in the control of AJ integrity and the maintenance of the AJs[34]. These pathways, together with Actin Cytoskeleton Signaling and Tec Kinase Signaling (involved in actin cytoskeleton signaling), indicate that the hESC-RPE (EP and LP) cells are in the process of cellular remodeling to become a stable layer of epithelial cells.
Bear in mind that these pathways are highly expressed in hESC-RPE (EP and LP) compared to human endogenous RPE. Thus, the hESC-RPE (EP and LP) cells are in the

process of epithelial development, while the typical epithelial polarity is already well established in the collected human endogenous RPE.

The most noticeable pathways that are higher expressed in the human endogenous RPE compared to the hESC-RPE are Phototransduction Pathway and The Visual Cycle. *In vivo*, the phototransduction pathway is induced by photon-mediated activation and subsequent destabilization of rhodopsin in the photoreceptors. The adjacent RPE is essential for recycling opsin/all-transretinol back into 11-cis retinal in the coupled (visual) retinol cycle and thus the photoreceptors rely on the RPE for continuing visual phototransduction. It is likely that the *in vivo* laser-dissected RPE samples were contaminated with photoreceptor outer segments, as we observed and discussed extensively elsewhere[3,19], causing the overexpression of phototransduction genes.

To activate the retinol cycle in the hESC-RPE, physical interaction with the photoreceptor cells is critical. Thus, low expression of these pathways in the hESC-RPE (EP and LP) samples could be caused by the absence of this interactive microenvironment. However, this needs to be tested in future studies.

The human endogenous RPE shows expression of genes within Protein Kinase A (PKA) Signaling, cAMP-mediated Signaling and CREB Signaling in Neurons as shown by IPA. These pathways are intertwined, as CREB is a cellular transcription factor that can be activated by cAMP signaling through PKA. Furthermore, the cAMP-PKA-dependent phosphorylation of CREB affects the expression of Klotho (KL), a gene involved in aging, in RPE physiology and retinal health. KL has important functions in protecting against oxidative stress, in promoting POS phagocytosis by upregulating MERTK gene expression, and in regulating melanogenesis through the genes *MITF* and *TYR*[35]. Interestingly, melatonin levels are reduced in AMD patients and administration of melatonin has been shown to have a protective effect on RPE cells against oxidative stress[36–38]. Accordingly, the gene expression of Melatonin Signaling may also indicate oxidative stress[39]. So, the human endogenous RPE shows increased expression of genes involved in defense mechanisms against oxidative stress as compared to the hESC-RPE cells. This might reflect the age-related enhanced oxidative stress levels *in vivo*[40].

In summary, we show that the *in vitro* hESC-RPE cells are indeed RPE since they show RPE specific morphology and molecular characteristics. We did not find substantial differences in gene expression profiles between EP and LP hESC-RPE, but we did find a clear difference between the hESC-RPE cells and the human endogenous RPE. While they lack the human endogenous RPE expression related to photoreceptor cell presence and defense against oxidative stress, the hESC-RPE cells show expression of pathways that enable the cells to stabilize their epithelial morphology.

CONCLUSIONS

In our study we tried to elucidate to what extent increased pigmentation in hESC-RPE cells relates to differentiation and maturation towards human endogenous RPE. Our data suggest that even though pigmentation seems to be a good biomarker for RPE development, the level of pigmentation does not reflect the maturation state of hESC-RPE. In addition, the data suggest that the hESC-RPE and the human endogenous RPE are substantially different.

Future studies should show whether hESC-RPE cells adopt these functions after transplantation or after growing them on a supporting scaffold that mimics the Bruch's membrane. Importantly, hESC-RPE cells at early pigmentation stages already show an expression profile representative of differentiated RPE. This suggests that hESC-RPE differentiation procedures for RPE replacement therapies can be shortened significantly which has important implications for the development of new therapeutic strategies in AMD.

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Author contributions

AB conception and design, laboratory research, data analysis and interpretation, manuscript writing; JGJ provision of study material, laboratory research; LAEC laboratory research, data analysis and interpretation; CK laboratory research; ROS critical reading of the manuscript; JM critical reading of the manuscript; TGMFG conception and design, critical reading of the manuscript; JB provision of study material, laboratory research; PDM statistical analysis; VMH principal investigator, conception and design, data interpretation, final approval of manuscript; AAB principal investigator, conception and design, data interpretation, final approval of manuscript.

Supplementary material

The supplementary files can be found online on Stem Cell Reviews and Reports (doi: 10.1007/s12015-017-9754-0)

REFERENCES

1. Klein R, Klein BEK, Knudtson MD, Meuer SM, Swift M, Gangnon RE. Fifteen-year cumulative incidence of age-related macular degeneration: the Beaver Dam Eye Study. Ophthalmology 2007;114(2):253–62.
2. Strauss O. The retinal pigment epithelium in visual function. Physiol Rev 2005;85(3):845–81.
3. Booij JC, van Soest S, Swagemakers SMA, et al. Functional annotation of the human retinal pigment epithelium transcriptome. BMC Genomics 2009;10:164.
4. Jager RD, Mieler WF, Miller JW. Age-Related Macular Degeneration. N Engl J Med 2008;358(24):2606–17.
5. Zhou B, Wang B. Pegaptanib for the treatment of age-related macular degeneration. Exp Eye Res 2006;83(3):615–9.
6. van Meurs JC (last), Kirchhof B, MacLaren R, et al. Ryan's Retina 6th edition, Chapter 124: Retinal Pigment Epithelium and Choroid Translocation in Patients with Age-Related Macular Degeneration. Mosby; 2016.
7. Novais EA, Badaró E, Regatieri CVS, Duker J, de Oliveira Bonomo PP. Regression of drusen after combined treatment using photodynamic therapy with verteporfin and ranibizumab. Ophthalmic Surg Lasers Imaging Retina 2015;46(2):275–8.
8. Söderberg A-C, Algvere PV, Hengstler JC, Söderberg P, Seregard S, Kvanta A. Combination therapy with low-dose transpupillary thermotherapy and intravitreal ranibizumab for neovascular age-related macular degeneration: a 24-month prospective randomised clinical study. Br J Ophthalmol 2012;96(5):714–8.
9. Binder S, Stanzel BV, Krebs I, Glittenberg C. Transplantation of the RPE in AMD. Prog Retin Eye Res 2007;26(5):516–54.
10. Carr A-J, Vugler A, Lawrence J, et al. Molecular characterization and functional analysis of phagocytosis by human embryonic stem cell-derived RPE cells using a novel human retinal assay. Mol Vis 2009;15:283–95.
11. Klimanskaya I, Hipp J, Rezai KA, West M, Atala A, Lanza R. Derivation and Comparative Assessment of Retinal Pigment Epithelium from Human Embryonic Stem Cells Using Transcriptomics. Cloning Stem Cells 2004;6(3):217–45.
12. Liao J-L, Yu J, Huang K, et al. Molecular signature of primary retinal pigment epithelium and stem-cell-derived RPE cells. Hum Mol Genet 2010;19(21):4229–38.
13. Leach LL, Croze RH, Hu Q, et al. Induced Pluripotent Stem Cell-Derived Retinal Pigmented Epithelium: A Comparative Study Between Cell Lines and Differentiation Methods. J Ocul Pharmacol Ther Off J Assoc Ocul Pharmacol Ther 2016;
14. Zhu Y, Carido M, Meinhardt A, et al. Three-dimensional neuroepithelial culture from human embryonic stem cells and its use for quantitative conversion to retinal pigment epithelium. PloS One 2013;8(1):e54552.
15. Pollard SM, Benchoua A, Lowell S. Neural stem cells, neurons, and glia. Methods Enzymol 2006;418:151–69.
16. Janssen SF, Gorgels TGMF, Bossers K, et al. Gene expression and functional annotation of the human ciliary body epithelia. PloS One 2012;7(9):e44973.
17. Bennis A, Gorgels TGMF, Brink JB ten, et al. Comparison of Mouse and Human Retinal Pigment Epithelium Gene Expression Profiles: Potential Implications for Age-Related Macular Degeneration. PLOS ONE 2015;10(10):e0141597.
18. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004;3:Article3.

19. Van Soest SS, de Wit GMJ, Essing AHW, et al. Comparison of human retinal pigment epithelium gene expression in macula and periphery highlights potential topographic differences in Bruch's membrane. Mol Vis 2007;13:1608–17.
20. Horsford DJ, Nguyen M-TT, Sellar GC, Kothary R, Arnheiter H, McInnes RR. Chx10 repression of Mitf is required for the maintenance of mammalian neuroretinal identity. Dev Camb Engl 2005;132(1):177–87.
21. Westenskow PD, Moreno SK, Krohne TU, et al. Using Flow Cytometry to Compare the Dynamics of Photoreceptor Outer Segment Phagocytosis in iPS-Derived RPE Cells. Invest Ophthalmol Vis Sci 2012;53(10):6282–90.
22. Papermaster DS. Preparation of retinal rod outer segments. Methods Enzymol 1982;81:48–52.
23. Raviv S, Bharti K, Rencus-Lazar S, et al. PAX6 Regulates Melanogenesis in the Retinal Pigmented Epithelium through Feed-Forward Regulatory Interactions with MITF. PLOS Genet 2014;10(5):e1004360.
24. Reinisalo M, Putula J, Mannermaa E, Urtti A, Honkakoski P. Regulation of the human tyrosinase gene in retinal pigment epithelium cells: the significance of transcription factor orthodenticle homeobox 2 and its polymorphic binding site. Mol Vis 2012;18:38–54.
25. Plaza Reyes A, Petrus-Reurer S, Antonsson L, et al. Xeno-Free and Defined Human Embryonic Stem Cell-Derived Retinal Pigment Epithelial Cells Functionally Integrate in a Large-Eyed Preclinical Model. Stem Cell Rep 2015;6(1):9–17.
26. Schwartz SD, Tan G, Hosseini H, Nagiel A. Subretinal Transplantation of Embryonic Stem Cell-Derived Retinal Pigment Epithelium for the Treatment of Macular Degeneration: An Assessment at 4 Years. Invest Ophthalmol Vis Sci 2016;57(5):ORSFc1–9.
27. Carido M, Zhu Y, Postel K, et al. Characterization of a Mouse Model With Complete RPE Loss and Its Use for RPE Cell Transplantation. Investig Opthalmology Vis Sci 2014;55(8):5431.
28. Stanzel BV, Liu Z, Somboonthanakij S, et al. Human RPE stem cells grown into polarized RPE monolayers on a polyester matrix are maintained after grafting into rabbit subretinal space. Stem Cell Rep 2014;2(1):64–77.
29. Lund RD, Wang S, Klimanskaya I, et al. Human Embryonic Stem Cell–Derived Cells Rescue Visual Function in Dystrophic RCS Rats. Cloning Stem Cells 2006;8(3):189–99.
30. Idelson M, Alper R, Obolensky A, et al. Directed Differentiation of Human Embryonic Stem Cells into Functional Retinal Pigment Epithelium Cells. Cell Stem Cell 2009;5(4):396–408.
31. Niessen CM. Tight Junctions/Adherens Junctions: Basic Structure and Function. J Invest Dermatol 2007;127(11):2525–32.
32. Widmaier M, Rognoni E, Radovanac K, Azimifar SB, Fässler R. Integrin-linked kinase at a glance. J Cell Sci 2012;125(8):1839–43.
33. Vespa A, D'Souza SJA, Dagnino L. A novel role for integrin-linked kinase in epithelial sheet morphogenesis. Mol Biol Cell 2005;16(9):4084–95.
34. Menke A, Giehl K. Regulation of adherens junctions by Rho GTPases and p120-catenin. Arch Biochem Biophys 2012;524(1):48–55.
35. Kokkinaki M, Abu-Asab M, Gunawardena N, et al. Klotho Regulates Retinal Pigment Epithelial Functions and Protects Against Oxidative Stress. J Neurosci 2013;33(41):16346–59.
36. Rosen RB, Hu D-N, Chen M, McCormick SA, Walsh J, Roberts JE. Effects of melatonin and its receptor antagonist on retinal pigment epithelial cells against hydrogen peroxide damage. Mol Vis 2012;18:1640–8.
37. Liang F-Q, Green L, Wang C, Alssadi R, Godley BF. Melatonin protects human retinal pigment epithelial (RPE) cells against oxidative stress. Exp Eye Res 2004;78(6):1069–75.
38. Rastmanesh R. Potential of melatonin to treat or prevent age-related macular degeneration through stimulation of telomerase activity. Med Hypotheses 2011;76(1):79–85.

39. Özdemir G, Ergün Y, Bakariş S, Kılınç M, Durdu H, Ganiyusufoğlu E. Melatonin prevents retinal oxidative stress and vascular changes in diabetic rats. Eye 2014;28(8):1020–7.
40. Bonilha VL. Age and disease-related structural changes in the retinal pigment epithelium. Clin Ophthalmol Auckl NZ 2008;2(2):413–24.

And IF you go in, should you turn left or right...
or right-and-three-quarters? Or, maybe, not quite?
Or go around back and sneak in from behind?
Simple it's not, I'm afraid you will find,
for a mind-maker-upper to make up his mind.

- Dr Seuss

Comparative gene expression study and pathway analysis of the human iris- and the retinal pigment epithelium.

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Vivi M Heine, Arthur A Bergen

PLOS ONE (2017), accepted for publication

3

ABSTRACT

Background

The retinal pigment epithelium (RPE) is a neural monolayer lining the back of the eye. Degeneration of the RPE leads to severe vision loss in, so far incurable, diseases such as age-related macular degeneration and some forms of retinitis pigmentosa. A promising future replacement therapy may be autologous iris epithelial cell transdifferentiation into RPE *in vitro* and, subsequently, transplantation. In this study we compared the gene expression profiles of the iris epithelium (IE) and the RPE.

Methods

We collected both primary RPE- and IE cells from 5 freshly frozen human donor eyes, using respectively laser dissection microscopy and excision. We performed whole-genome expression profiling using 44k Agilent human microarrays. We investigated the gene expression profiles on both gene and functional network level, using R and the knowledge database Ingenuity.

Results

The major molecular pathways related to the RPE and IE were quite similar and yielded basic neuro-epithelial cell functions. Nonetheless, we also found major specific differences: For example, genes and molecular pathways, related to the visual cycle and retinol biosynthesis are significantly higher expressed in the RPE than in the IE. Interestingly, Wnt and aryl hydrocarbon receptor (AhR-) signaling pathways are much higher expressed in the IE than in the RPE, suggesting, respectively, a possible pluripotent and high detoxification state of the IE.

Conclusions

This study provides a valuation of the similarities and differences between the expression profiles of the RPE and IE. Our data combined with that of the literature, represent a most comprehensive perspective on transcriptional variation, which may support future research in the development of therapeutic transplantation of IE.

INTRODUCTION

In the vertebrate eye, the RPE is a monolayer of neural-crest derived cells located between the photoreceptors and the choroid. Dysfunctional RPE is involved in many retinal degenerative diseases such as age-related macular degeneration (AMD), Stargardt's disease, Best's disease and retinitis pigmentosa. For these disorders there is no (effective) treatment. One of the most promising future therapy options for RPE related disorders is cell replacement of the dysfunctional RPE.

Autologous intra-ocular RPE transplantation was previously carried out with limited success[1,2], since surgical variability and complications remained high. Therefore, many studies in the last decade focused on the development and use of induced pluripotent stem cells (iPSC) as a source for autologous cell replacement therapy. These iPSC can be differentiated *in vitro* towards RPE cells and used for experimental transplantation studies in animal models[3–5]. Recently, clinical stem cell/RPE replacement trials in patients with macula degeneration and patients with Stargardt's disease were started[6,7].

Alternative strategies for retinal cell replacement are currently also being explored[8]. One of them involves *transdifferentiation*, also called *direct conversion*, the process of transforming an adult somatic cell into another adult somatic cell. With the acquired knowledge on differentiation of pluripotent stem cells towards RPE, the field of transdifferentiation has gained renewed interest. Humans have a limited capacity to transdifferentiate cells *in vivo* or spontaneously regenerate and restore their tissues and organs[9,10]. However, several studies demonstrated that *in vitro* procedures could convert one cell into another cell type and thereby skipping the pluripotent state, using overexpression of cell-lineage specific genes[11–15]. Recent studies also presented new strategies, using criteria such as common cellular origin and developmental plasticity, to identify "the best possible" cell for transdifferentiation[16,17].

In the literature, iris epithelium (IE) cells have been considered as potential starting source for transdifferentiation into the RPE and cell replacement therapy for several reasons[1,8,18–20]. First of all, both RPE and IE are neuro-epithelia with a common embryological origin (neuroectoderm of the developing optic cup). Next, IE cells can be obtained relatively easily through iridectomy in patients. Therefore, IE cells are a potentially autologous cell source, reducing the chance of transplant rejection. Finally, the IE seems suitable because *in vitro* cultured IE cells display a number of functional RPE features, such as the presence of tight junctions and the phagocytosis of photoreceptor outer segments[21,22].

To improve our understanding of molecular and functional similarities and differences between the human IE and RPE, we conducted a new, in depth microarray study, comparing gene expression profiles and the functional annotations of these two tissues *in vivo*.

RESULTS

Similarities between the IE and the RPE transcriptomes

Following our previously published analyses strategies[23–25], we selected those genes with expression in the highest 10th percentile for the RPE and the IE, assuming these genes to have the highest biological relevance. Using these files, the knowledge database Ingenuity attributed similar statistically significant biological functions, canonical pathways, and molecular networks to the RPE and the IE.

The canonical pathways attributed to the highest percentile of the IE and RPE are quite similar (82.6% of these canonical pathways overlap). Many pathways underlie normal cellular physiology, which are similarly expressed in both cell types. Both the RPE and the IE show epithelial related canonical pathways such as Remodeling of Epithelial Adherens Junctions, Epithelial Adherens Junction Signaling, Integrin Signaling and Aldosterine Signaling in Epithelial Cells. The top 20 of these pathways are shown in Fig 1. Biological functions and molecular networks yielded similar functional annotations and can be found in the supplementary files (S1 Table).

We found considerable overlap between the canonical pathways expressed in the IE and the RPE. However, we observed significant differences as well. Here, we mainly focus on these differences.

Differences between the IE and the RPE transcriptomes

To focus on the most differentially expressed genes between the two epithelia, we used stringent selection criteria (B-H adjusted $p<0.001$ and $FC>5$). We report a set of 700 unique genes (3.6%) significantly more expressed in the RPE than in the IE. Vice versa, 488 (2.5%) genes were significantly higher expressed in the IE compared to the RPE. Tables 1 and 2 show the top 30 of these genes. For the complete lists of statistically differentially expressed genes see S2 Table.

Functional annotation of the genes that are enriched in the RPE

Functional annotation of the genes that are enriched in the RPE (significantly more expressed in the RPE compared to the IE) yielded 28 canonical pathways (Fig 2). Interestingly, at least 4 of these pathways are directly related to the expression of the visual cascade: Phototransduction Pathway, The Visual Cycle, Retinol Biosynthesis, Retinoate Biosynthesis. Examples of genes that these different pathways have in common are *LRAT, RGR, RBP1, RDH5, RDH8, RDH10, RDH11, RDH12, RPE65*. For the complete list of the involved genes see S3 Table.

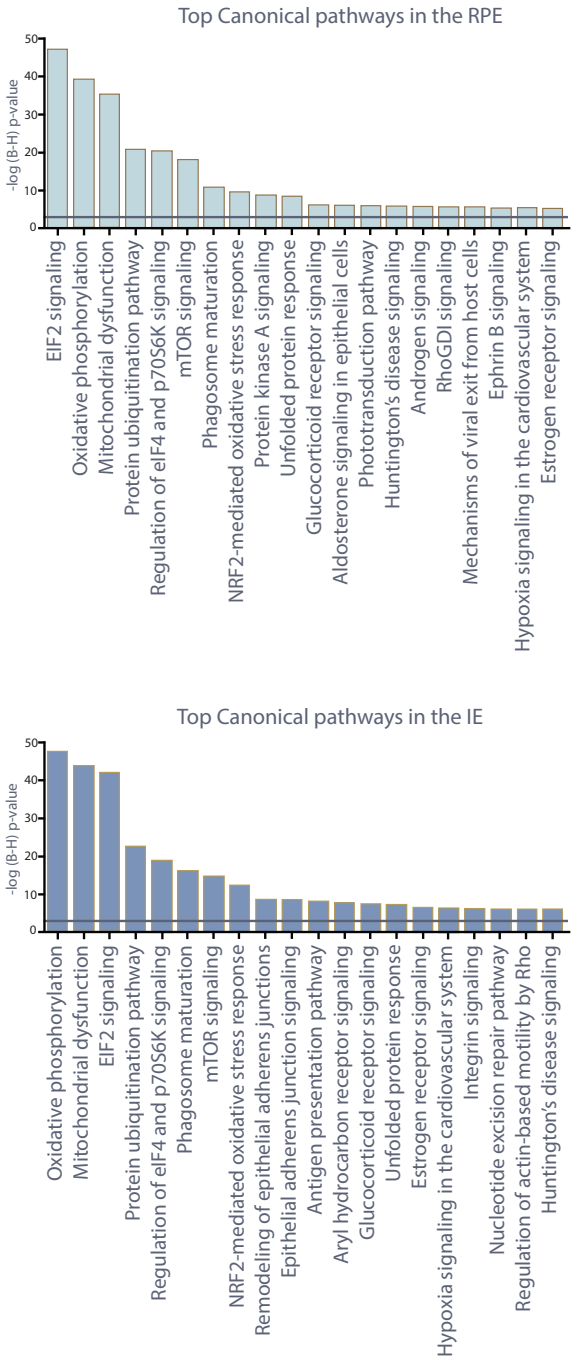

Figure 1. Top 20 significant canonical pathways of the core analysis in IPA (Ingenuity) of most highly expressed genes of the IE and the RPE. P-values indicate the significance of enrichment for the most highly expressed genes from our dataset. P-values were corrected for multiple testing using the Benjamini-Hochberg (B-H) false discovery rate. The upper graph (light blue bars) represents the –log (B-H) p-value of the RPE and the lower graph (dark blue bars) represents the –log(B-H) p-value of the IE. The orange line indicates the threshold of B-H corrected $p<0.001$.

Functional annotation of the genes that are enriched in the IE

Ingenuity assigned several canonical pathways to the genes in the IE specific dataset (significantly more expressed in the IE compared to the RPE) (Fig 3). Four of the five

Table 1. Top 30 genes significantly more highly expressed in the RPE compared to the IE. The genes that are in bold were shown to be enriched in the human RPE[26] . Asterisks mark the genes that might be present in our dataset by contamination of the mRNA on the photoreceptor-RPE interface or may be expressed to some extent in both adjacent cell layers (also see Materials and methods).

RPE			
GeneName	SystematicName	Adj p value	FC
RPE65	**NM_000329**	**6.9E-05**	**167.7**
PCAT4	AK056825	1.2E-04	146.1
SLCO1C1	**NM_017435**	**3.5E-05**	**138.1**
GNGT1	NM_021955	5.9E-04	136.3
CNGA1 *	NM_000087	5.4E-06	131.2
GUCA1B *	NM_002098	8.1E-06	116.1
KIRREL2	**NM_199180**	**7.5E-06**	**108.3**
MIR124-2HG	AK124256	6.1E-06	106.3
MAK	NM_005906	5.4E-06	98.5
LINC00982	AL833006	1.7E-05	96.8
OPCML	**NM_001012393**	**7.5E-06**	**94.8**
NEUROD1	NM_002500	7.8E-06	94.4
MPP4 *	NM_033066	7.5E-06	93.8
BEST1	**NM_004183**	**4.3E-05**	**92.9**
ITGB8	**NM_002214**	**4.7E-04**	**91.1**
DUSP6	**NM_001946**	**1.3E-04**	**89.5**
LRAT	**NM_004744**	**4.0E-05**	**81.9**
NRL *	NM_006177	2.4E-05	81.5
RRH	NM_006583	1.9E-05	78.3
RP1 *	NM_006269	6.1E-06	76.5
SAG *	NM_000541	8.5E-05	75.9
COL8A1	AL359062	2.5E-05	72.9
PDE6H	NM_006205	2.0E-05	72.4
AIPL1 *	NM_014336	2.0E-05	70.5
KIAA1189	NM_020711	1.7E-05	68.0
LOC100507521	BX101632	1.6E-05	67.6
SLC26A7	NM_052832	8.0E-05	66.2
TMEM16B	NM_020373	7.5E-06	65.5
GNAT2	ENST00000351050	4.2E-05	60.8
SERINC4	ENST00000319327	7.5E-06	60.3

significant canonical pathways: Basal Cell Carcinoma Signaling, Human Embryonic Stem Cell Pluripotency, Wnt/B-catenin Signaling, and PCP Pathway have large overlap in participating genes. These include multiple *WNT* genes (*4, 16, 10A, 2B, 5A, 7A, 7B*), *FZD10, NTF3, PDGFD, TGFB3. GLI3, GLIS1, PDGFD, ZIC3, INHBA, SOX1, CD44, SOX11, SFRP2, GJA1*.

Table 2. Top 30 genes significantly more highly expressed in the IE compared to the RPE.

IE			
GeneName	SystematicName	Adj p value	FC
DCT	ENST00000377028	1.4E-09	128.5
WNT16	NM_057168	5.8E-10	125.9
GLULD1	NM_016571	2.2E-06	117.5
PDE1A	NM_001003683	5.2E-09	104.4
KLF5	NM_001730	7.3E-07	86.2
AGXT2L1	NM_031279	3.4E-07	78.6
ZIC1	NM_003412	5.6E-07	71.2
CLCA2	NM_006536	6.0E-08	68.8
C8orf47	NM_173549	2.1E-09	61.5
SRD5A2	NM_000348	9.1E-09	60.5
GJB2	NM_004004	3.1E-08	57.5
MYOC	NM_000261	5.2E-07	55.5
CPAMD8	NM_015692	2.5E-08	52.7
GJA3	NM_021954	3.6E-08	52.4
CRYGS	NM_017541	9.2E-08	51.8
SFRP2	NM_003013	1.8E-09	49.9
F5	NM_000130	7.5E-08	40.6
FBP2	NM_003837	4.8E-08	39.5
SNCAIP	NM_005460	7.8E-10	37.6
NPFFR2	NM_053036	3.3E-07	37.4
OTX1	NM_014562	2.3E-08	36.4
WNT5A	NM_003392	2.1E-08	33.5
TFEC	NM_012252	3.4E-08	33.0
DSC1	NM_004948	3.1E-08	32.8
LINC00403	AK055145	7.1E-06	32.7
NTF3	NM_002527	8.5E-07	30.7
GBP7	NM_207398	7.7E-09	29.7
CRYBB2P1	BC047380	3.3E-06	29.3
INDO	NM_002164	1.1E-06	26.9
ADAMTS16	NM_139056	2.9E-09	26.9

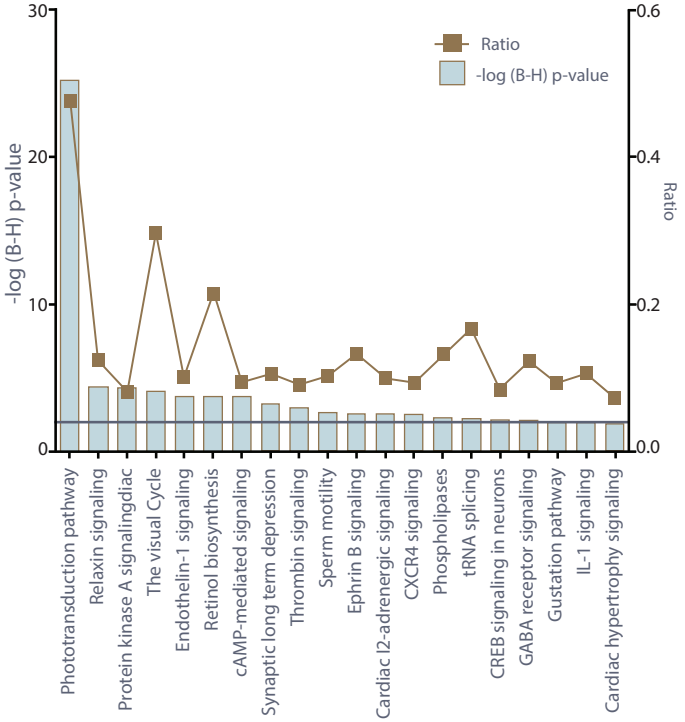

Figure 2. Canonical pathways identified by Ingenuity for the RPE enriched genes. The left y-axis displays the –log of the Benjamini-Hochberg corrected p-value. The right y-axis displays the ratio of number of genes derived from our dataset, divided by the total number of genes in the pathway. The bar graph represents the -log (B-H) p-value. The orange line indicates the threshold at a Benjamini-Hochberg corrected p<0.01.

The fifth pathway, Aryl Hydrocarbon Receptor Signaling is a more exclusive pathway, derived from the highly expressed *TGFB3, GSTA3, HSPB3, HSPB2, DCT, GSTM1, HSPB7, ALDH1L2, NR2F1, TYR, FAS, ALDH3A1* and *CYP1B1* genes. For the complete list of the involved genes see S4 Table.

Genes associated with established RPE functions in the IE and RPE

To evaluate the expression of genes involved in well-known RPE functions in both the RPE and IE of the individual samples, we compiled a list of most important RPE functions. We determined the categories according to what we derived from our dataset described here, and found in the literature[27]: "phagocytosis of photoreceptor outer segments"[28–30], "visual cycle"[31], "secretion of factors and signaling molecules"[23], "light absorption and pigmentation"[32–35] and "transepithelial transport and pH regulation"[36–39]. Subsequently, we selected the genes known to be involved in these functions and

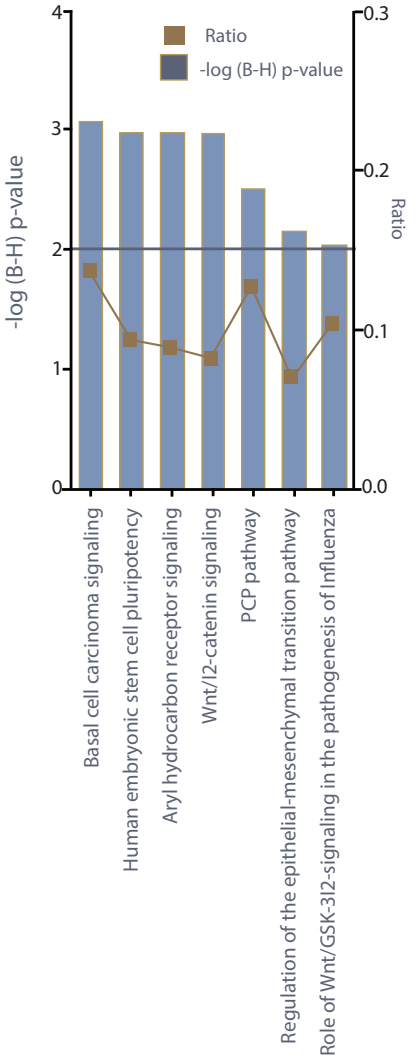

Figure 3. Canonical pathways identified by Ingenuity for the IE enriched genes. The left y-axis displays the –log of the Benjamini-Hochberg corrected p-value. The right y-axis displays the ratio of number of genes derived from our dataset, divided by the total number of genes in the pathway. The bar graph represents the -log (B-H) p-value. The orange line indicates the threshold at a Benjamini-Hochberg corrected $p<0.01$.

investigated the corresponding normalized IE and RPE gene expression levels in all samples, resulting in a heat map for these entries (Fig 4).

Interestingly, we found a very clear and consistent distinction in normalized expression levels across all samples between the RPE and IE. Especially for the expression of "visual cycle" and "phagocytosis photoreceptor outer segments" we found a pronounced difference. Also the normalized expression of genes in "transepithelial transport" and "light absorption and pigmentation" are undoubtedly tissue specific. Only the "secretion of growth factors" has some heterogeneity, however a hierarchical clustering tree shows that, overall, there is low intra-epithelial variability.

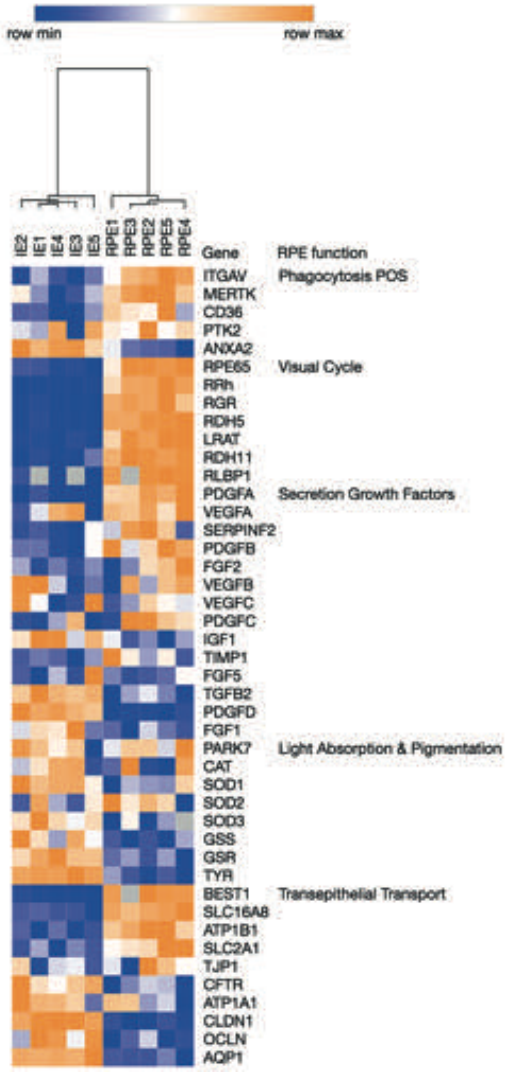

Figure 4. Heatmap for the expression of genes related to RPE specific functions. The normalized expression data are converted to heat map color using the mean and maximum values for each gene. The intensity scale of the standardized expression values ranges from dark blue (low expression) to dark orange (high expression). We added a hierarchical cluster tree that shows that the IE samples cluster together and the RPE samples cluster together.

In addition, we next composed a list of RPE expressed genes that were previously implicated in normal RPE function and/or retinal diseases[40], which we provide in the supplementary files for the interested reader (S1 Fig and S5 Table) .

DISCUSSION

There are a vast number of studies about the (promising) use of (pluripotent) stem cell derived RPE cells for transplantation use in degenerative disorders of the RPE[41].

However, instead of stem cells, several authors suggested to use IE cells for RPE replacement. Thumann et al[42] and Abe et al[18] argued that the IE and RPE have a common neuro-ectodermal origin and that an IE biopsy can be relatively easily obtained from the patient by iridectomy.
To investigate this idea further, we compared the gene expression profiles of the human RPE and the IE *in vivo*. We aimed to gain more insight into the (molecular) differences and similarities between these tissues.

Similarities between the IE and the RPE transcriptomes

The canonical pathways and corresponding statistically significantly enriched functions for the most highly expressed genes of the IE and the RPE were very similar.
However, there was also a set of statistically significantly differentially expressed genes. Out of 19596 unique genes, 700 (3.6%) were enriched in the RPE and 488 (2.5%) were enriched in the IE (S2 Table). It is important to note here that the cutoff values we chose in this study are relatively strict to make the study as comprehensible as possible. Obviously, more relaxed comparison parameters would yield more, but less significant differences between RPE and IE.

RPE enriched gene expression compared to the IE

Prominent features among the enriched RPE gene expression are those implicated in the phototransduction cascade. Obviously, *in vivo*, the expression of these genes is most likely invoked by the activation of rhodopsin and, subsequently, the entire phototransduction cascade in the adjacent photoreceptors.
An alternative explanation is the presence of some degree of photoreceptor contamination in our RPE samples, which is unavoidable even when we use meticulous laser dissection technology. This may have caused the enrichment of the phototransduction cascade in the RPE compared to the IE.

IE enriched gene expression compared to the RPE

Wnt signaling pathway is active in the IE, but not in the RPE

Ingenuity attributed specific canonical pathways to the IE that are related to the Wnt signaling pathway (Wnt SP): Basal Cell Carcinoma Signaling, Human Embryonic Stem Cell Pluripotency, Wnt/B-catenin Signaling and PCP Pathway. In general, the Wnt signaling pathway consists of a group of signal transduction pathways that regulate crucial aspects of cell fate determination, cell proliferation, cell polarization, neural patterning and organogenesis during embryonic development[43].
In general, Wnt SP expression maintains pluripotency and self-renewal in mouse and human embryonic stem cells[44,45]. The activation of Wnt SP improves the efficiency of reprogramming of somatic cells, including retinal neurons, into iPSCs, both *in vitro* and

in vivo[46–48]. This role is not only complex, but also time and dose dependent[49]. The Wnt SP is also crucial for the differentiation of pluripotent stem cells to RPE cells, but the impact on different stages of RPE differentiation from human embryonic stem cells is not yet well understood[50–52].

Nonetheless, the high expression of Wnt SP genes in the IE compared to the RPE suggests that the IE preserves (part of) its multipotent character during life. Indeed, a number of previous studies in chicken, rodents, pigs and humans also suggested the presence of multipotent neural progenitor cells in the IE[53–55]. The human IE can be cultured in neurosphere formation, displaying retinal stem/progenitor cell properties[19, 55] (and own unpublished observation). Finally, transduction and expression of only a few genes (*CRX, RX* and *NEUROD*) induced a functional photoreceptor like phenotype from rodents, primates and human iris cells[56]. Taken together, the available data suggest that IE cells retain, at least to some degree, developmental or functional plasticity, which may proof beneficial for potential therapeutic strategies for RPE replacement.

The aryl hydrocarbon signaling pathway is active in the IE but not in the (aged) RPE

Our Ingenuity analysis showed a high expression of the aryl hydrocarbon receptor (AhR) signaling pathway in the IE compared to the RPE. AhR is a ligand dependent transcription factor that regulates a cellular defense mechanism pathway against toxin overload in cells. Toxin overload in RPE cells may come from daily rhythmic phagocytosis of photoreceptor outer segments, oxidative stress and damaging light exposure. Several functional studies previously showed that the detoxifying AhR pathway is also active in human RPE, but that this activity decreases with age[57,58]. Indeed, our samples are derived from older donors, which might explain the relatively low expression of genes involved in AhR signaling in RPE samples. Interestingly, Esfandiary[59] et al found an association between detoxification genes, including AhR, and AMD. These data were supported by studies on a AhR-/- mice which presented features of AMD pathogenesis, including thick focal and diffuse sub-RPE deposits, regions of retinal hyper- and hypopigmentation as well as RPE degeneration[58,60,61].

Combining our data with those of the literature, older IE cells and relatively young RPE cells show high AhR related expression and detoxification functionalities, whereas older RPE cells do not. It is tempting to speculate here that older IE cells maintain specific detoxification capacities during life, whereas the corresponding RPE does not.

Well-known RPE functions

For further insight in the presence or absence of potential RPE functionalities in the IE we analyzed a number of well-known RPE specific functions.

For a limited number of RPE functions ("visual cycle", "phagocytosis of photoreceptor outer segments", "transepithelial transport" and "light absorption and pigmentation")

the underlying genes follow a unique and characteristic differential expression pattern in the RPE or in the IE across all donor eyes (Fig 4). One might thus argue that these functionalities are not present in the IE cells and may (have to) be invoked upon transformation of IE cell to RPE cell. Previous studies have shown that both human IE and RPE cells can be maintained and expanded *in vitro*[62–64], and are then able to phagocytize photoreceptor outer segments when provided in the medium[65]. In addition, cultured autologous IE cells were previously transplanted in monkeys, and they were able to phagocytize photoreceptor outer segments even 6 months after transplantation[66]. This support the flexibility of the IE cells to take on RPE functions, depending on microenvironmental factors.

For other RPE functions (secretion of growth factors, light absorption and pigmentation) gene expression is more heterogeneous across the IE and RPE samples, without disturbing the normal functions of the tissues in these individuals. Thus these functions may either be not fully specific or redundant in the IE or RPE.

Our IE-RPE microarray results compared to the literature

To our knowledge, only one other microarray study in the literature addressed potential gene expression similarities and differences between human IE and RPE: Cai and coworkers[67] concluded that there are major differences in gene expression profiles of IE and RPE, including lack of expression in IE of genes known to be critical for RPE function. Also they concluded that the native IE gene expression profile and corresponding functionalities may be a potential obstacle for successful subretinal transplantation. In our current study, we explored IE and RPE gene expression in a much larger dataset (we measured more than seven times the amount of gene probes), and we included extensive bioinformatics as well as functional annotation. For detailed technical and statistical differences between the study of Cai et al and our study, see S2 Fig. Our data and analysis partly support and extend the conclusions of Cai and coworkers.

Besides large similarities, we also find major differences in gene expression profiles between IE and RPE. We estimate these differences to affect at least 6.1% of the transcriptome. This appears not be a large difference, if we consider the findings of Van Soest et al[68], who concluded that the transcriptome differences between macular and peripheral RPE were 2-3 %. Nonetheless, such difference in transcriptomes and related functionalities may be an obstacle for direct transplantation.

A number of new findings and considerations from our study may be of interest:

(1) The IE and RPE show many similarities based on their gene expression profiles.

(2) The aryl hydrocarbon signaling pathway is active in the IE and young RPE, but not in the (aged) RPE[58]. This may represent a difference in detoxification capacity between the two tissues.

(3) The high activity of Wnt SP may reflect the multipotent character of IE cells. This could be of interest to studies that will further investigate IE's therapeutic potential.

SUMMARY AND CONCLUSIONS

In conclusion, our study provides in depth analysis of the gene expression profiles of the IE and the RPE. We analyzed these profiles to determine and report the differences and similarities between the two related tissues. Our data may be useful in the further exploration of IE as a potential source for regenerative medicine for RPE degeneration.

MATERIALS & METHODS

Ethics Statement

This study was performed in agreement with the declaration of Helsinki on the use of human material for research. The human donor eyes were obtained from the Netherlands Brain Bank (NBB) (Amsterdam, The Netherlands). The NBB obtained permission (informed consent) from the donors for enucleation of the eyes and to use the eyes for scientific purposes. All procedures of the NBB have been approved by the ethics Committee of VU University Medical Center (Amsterdam, The Netherlands) under the reference number 2009/148. All data were analyzed anonymously.

Tissue collecting and processing

We selected 5 donor eyes (3 male, 2 female). Donors were aged 49 to 73 at time of death. Donors were selected for not having any ophthalmic disorder or malignancy, ocular abnormalities on visual or histological inspection, drusen and poor morphology[23]. Globes were enucleated and snap-frozen between 10 and 22 hours post mortem. The eyes were stored at -80C until use. For full details see S6 Table. From each donor eye we collected both the IE and the RPE in order to reduce genetic variation in our study design.

To collect the RPE, a macular fragment of 16mm^2 with the fovea in its center was cut from the retina.12uM Sections from the macular area were used to isolate the RPE cells[68]. The sections were dehydrated with ethanol and air-dried before micro dissection. To minimize cellular cross-contamination in our procedure, we used the meticulous laser dissection microscope to cut the RPE monolayer specifically (PALM Carl Zeiss, MicroImaging GmbH, Munich, Germany). Nonetheless, considering the proximity and interactivity of the photoreceptors and the RPE, the chance of some contamination

of adjacent cell layers is very high. This has been previously observed and extensively discussed elsewhere[23,68].

To collect the IE, the anterior part of the eye was excised at the level of the ora serrata. This anterior part was snap frozen in isopentane in liquid nitrogen and stored at -80C. We removed the ciliary body from the anterior part to expose the iris. While keeping the eye frozen we scraped and collected the iris epithelium with forceps, detaching it from the stroma.

When we collected and select our samples, specificity of the tissue and integrity of the RNA are most important to ensure valid results. We used different techniques to collect the IE and the RPE, which is necessary for the specificity of the tissues.

To ensure that our findings are a reflection of a clear difference between IE and RPE and that the variance within sample groups is less than between, we conducted a principal component analysis (S3 Fig). The first component separates the IE samples from the RPE samples and explains 89% of the total variance in the data. In addition we made an overview of the measured expression levels of possible photoreceptor contaminating genes (S7 Table and S4 Fig).

RNA isolation, amplification and labelling procedures were carried out essentially as described elsewhere[24]. High quality RNA is challenging with postmortem ocular tissues, compared to isolating RNA from fresh cell cultures. Postmortem changes of the RNA can be determined by measuring its integrity. Given the lengthy procedures of sample selection, procedure and extensively quality controls, we included a limited number of the "very best samples" in our microarray analysis. To clarify: If RNA integrity was compromised in either the IE or RPE, no samples of this donor eye were used. We always used both IE and RPE from the same eye to minimize the variance. Quality of the total RNA was checked with a Bioanalyzer assay (RNA 6000 Pico Kit, Agilent Technologies, Amstelveen, The Netherlands). The RIN values of the tRNA ranged from 5.1 to 9 and the peak of the fragment length of the aRNA samples varied between 700 and 900nt (S5 Fig).

In our microarray study we used a common reference design. As a common reference we used RNA from human RPE/choroid that was used in previous and on-going gene expression analyses in our lab[24,25,69]. The common reference was prepared from human RPE/choroid RNA that was isolated, amplified using the same methodology as our experimental samples, and labelled with Cy3 (Cy3 mono-reactive dye pack, GE Healthcare UK, Little Chalfont, Buckinghamshire, UK). See Janssen et al[24] for a more detailed description of the laser dissection procedures, RNA processing and microarray procedures.

Microarray data analysis

The microarray data were extracted using Agilent Feature Extraction Software (Agilent Technologies, version 9.5.3.1), see S6 Fig. Raw data were imported into R (version 2.14.0 for Windows, R Development Core Team, 2009) using the Bioconductor package LIMMA. Background correction was performed using the "normexp" method with an offset of 10 to adjust the foreground signal without introducing negative values. The resulting log-ratios were transformed using intensity-dependent loess normalization. We further normalized the average intensities across arrays using the aquantile method[70]. The microarray data is available in the Gene Expression Omnibus database with the accession number GSE81058. We ranked the normalized intensities in the Cy5 channel corresponding to the experimental samples. Based on these ranks we divided the normalized intensities in bins corresponding to the highest 10 percentile, the 50^{th} – 90^{th} percentile, 10^{th}-50^{th} percentile and lowest 10^{th} percentile.

Genes that are differentially expressed between the RPE and the IE were identified on the normalized log-ratios using a linear model with patient as blocking factor. Significant differences were determined using Bayes moderated paired t-statistics (package LIMMA). Resulting p-values were corrected for multiple testing using the Benjamini-Hochberg False Discovery Rate adjustment. We used stringent statistical analysis and the paired t test to determine those IE-RPE differences that overcome the variation between the individual donors. To identify explicit differences between the IE and the RPE we used cutoff values of a fold change (FC) >5 and a p-value<0.001. We selected these stringent cutoff values because with the initial selection criteria of FC>2.5 and a p-value<0.001 we found 1277 genes enriched in the IE and 1581 genes in the RPE and we wanted to analyse the most significant differences between the IE and RPE, instead of less significant differences that are probably based on overlapping gene involvement in multiple functionalities. The genes derived from this analysis are referred to as "Significantly highly expressed in the IE" and "significantly highly expressed in the RPE".

Functional annotation was done in IPA, Ingenuity (Ingenuity® Systems, version 24718999, assessed at September 14^{th}, 2015). To present the results as comprehensive as possible we highlighted the Ingenuity canonical pathways only because they depict the most simple and straightforward representation of our data and functionalities. The associated biological functions and diseases are described in the supplementary files (S1 and S2 Table). To visualize the normalized expression data for the RPE specific functions we used the GENE-E software[71]. We made use of the hierarchical clustering function, using a Pearson correlation metric, to visualize the variation within our sample sets.

Confirmation of microarray results

We confirmed our microarray data with sqRT-PCR, see Fig 5 and S7 Fig for the photos of the gel electrophoresis. For a detailed description of the sqRT-PCR, see Janssen et

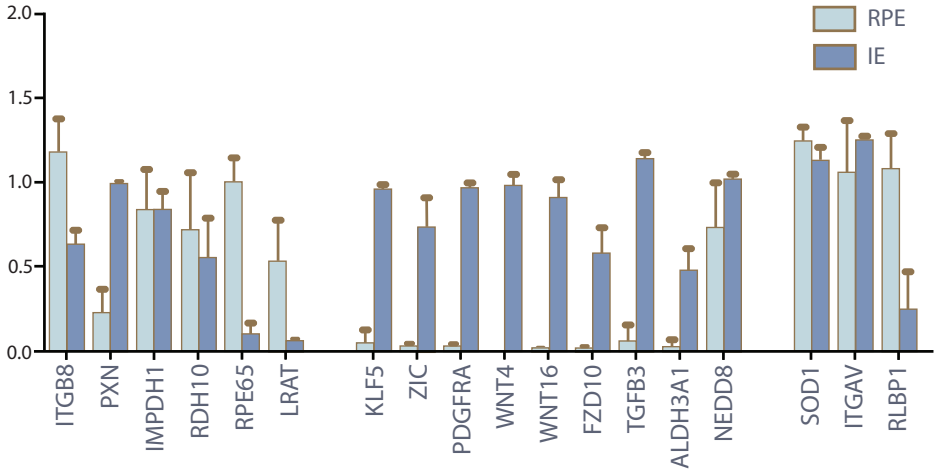

Figure 5. Confirmation of microarray results by sqRT-PCR. We used GAPDH as the housekeeping gene to normalize the gene expression of the IE and RPE samples. We depict the median and standard deviation for RPE samples in light blue, IE samples in dark blue. We selected genes that were highly expressed in the RPE (*ITGB8, PXN, IMPDH1, RDH10, RPE65, LRAT*), highly in the IE (*KLF5, ZIC, PDGFRA, WNT4, WNT16, FZD10, TGFB3, ALDH3A1, NEDD8*) and in both groups (*SOD1, ITGAV, RLBP1*). We find 89% (16 of the 18 genes) to be in agreement with the microarray results, only *PXN* and *RLBP1* give a different result.

al[24]. In short, sqRT-PCR was carried out using intron-spanning primers on cDNA from IE and RPE, using up to 5 biological replicates. To minimize effects of RNA degradation artefacts in the human post mortem samples, we generated primers near the 3'end of the gene. We quantified the gene expression in ImageJ and normalized expression by comparing it to the measured expression of housekeeping gene GAPDH.

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Author contribitions

AB conception and design, laboratory research, data analysis and interpretation, manuscript writing; JBtB provision of study material; PDM statistical analysis; VMH principal investigator, conception and design, final approval of manuscript; AAB principal investigator, conception and design, data interpretation, final approval of manuscript.

Supplementary material

The supplementary files can be found online on PLOS ONE.

REFERENCES

1. Sheridan CM, Mason S, Pattwell DM, Kent D, Grierson I, Williams R. Replacement of the RPE monolayer. Eye 2009;23(10):1910–5.
2. van Meurs JC (last), Kirchhof B, MacLaren R, et al. Ryan's Retina 6th edition, Chapter 124: Retinal Pigment Epithelium and Choroid Translocation in Patients with Age-Related Macular Degeneration. Mosby; 2016.
3. Li Y, Tsai Y-T, Hsu C-W, et al. Long-term safety and efficacy of human-induced pluripotent stem cell (iPS) grafts in a preclinical model of retinitis pigmentosa. Mol Med 2012;18(9):1312–9.
4. Maeda T, Lee MJ, Palczewska G, et al. Retinal pigmented epithelial cells obtained from human induced pluripotent stem cells possess functional visual cycle enzymes in vitro and in vivo. J Biol Chem 2013;288(48):34484–93.
5. Stanzel BV, Liu Z, Somboonthanakij S, et al. Human RPE stem cells grown into polarized RPE monolayers on a polyester matrix are maintained after grafting into rabbit subretinal space. Stem Cell Rep 2014;2(1):64–77.
6. Schwartz SD, Tan G, Hosseini H, Nagiel A. Subretinal Transplantation of Embryonic Stem Cell-Derived Retinal Pigment Epithelium for the Treatment of Macular Degeneration: An Assessment at 4 Years. Invest Ophthalmol Vis Sci 2016;57(5):ORSFc1–9.
7. Wiley LA, Burnight ER, DeLuca AP, et al. cGMP production of patient-specific iPSCs and photoreceptor precursor cells to treat retinal degenerative blindness. Sci Rep 2016;6:srep30742.
8. Dhamodaran K, Subramani M, Ponnalagu M, Shetty R, Das D. Ocular stem cells: a status update! Stem Cell Res Ther 2014;5(2):56.
9. Singer AJ, Clark RAF. Cutaneous Wound Healing. N Engl J Med 1999;341(10):738–46.
10. Michalopoulos GK. Liver Regeneration. J Cell Physiol 2007;213(2):286–300.
11. Vierbuchen T. Direct conversion of fibroblasts to functional neurons by defined factors. Nature 2010;463:1035–41.
12. Yoo AS. MicroRNA-mediated conversion of human fibroblasts to neurons. Nature 2011;476:228–31.
13. Caiazzo M. Direct generation of functional dopaminergic neurons from mouse and human fibroblasts. Nature 2011;476:224–7.
14. Guo Z. In vivo direct reprogramming of reactive glial cells into functional neurons after brain injury and in an Alzheimer's disease model. Cell Stem Cell 2014;14:188–202.
15. Su Z, Niu W, Liu ML, Zou Y, Zhang CL. In vivo conversion of astrocytes to neurons in the injured adult spinal cord. Nat Commun 2014;5:3338.
16. Lanza R, Atala A. Essentials of Stem Cell Biology. Academic Press; 2013.
17. Heinrich C, Spagnoli FM, Berninger B. In vivo reprogramming for tissue repair. Nat Cell Biol 2015;17(3):204–11.
18. Abe T, Yoshida M, Yoshioka Y, et al. Iris pigment epithelial cell transplantation for degenerative retinal diseases. Prog Retin Eye Res 2007;26(3):302–21.
19. Thumann G, Stöcker M, Maltusch C, et al. High efficiency non-viral transfection of retinal and iris pigment epithelial cells with pigment epithelium-derived factor. Gene Ther 2010;17(2):181–9.
20. Jasty S, Srinivasan P, Pasricha G, Chatterjee N, Subramanian K. Gene expression profiles and retinal potential of stem/progenitor cells derived from human iris and ciliary pigment epithelium. Stem Cell Rev 2012;8(4):1163–77.
21. Rezai KA, Lappas A, Kohen L, Wiedemann P, Heimann K. Comparison of tight junction permeability for albumin in iris pigment epithelium and retinal pigment epithelium in vitro. Graefes Arch Clin Exp Ophthalmol Albrecht Von Graefes Arch Für Klin Exp Ophthalmol 1997;235(1):48–55.

22. Thumann G. Development and Cellular Functions of the Iris Pigment Epithelium. Surv Ophthalmol 2001;45(4):345–54.
23. Booij JC, van Soest S, Swagemakers SMA, et al. Functional annotation of the human retinal pigment epithelium transcriptome. BMC Genomics 2009;10:164.
24. Janssen SF, Gorgels TGMF, Bossers K, et al. Gene expression and functional annotation of the human ciliary body epithelia. PloS One 2012;7(9):e44973.
25. Janssen SF, van der Spek SJF, Ten Brink JB, et al. Gene expression and functional annotation of the human and mouse choroid plexus epithelium. PloS One 2013;8(12):e83345.
26. Bennis A, Gorgels TGMF, Brink JB ten, et al. Comparison of Mouse and Human Retinal Pigment Epithelium Gene Expression Profiles: Potential Implications for Age-Related Macular Degeneration. PLOS ONE 2015;10(10):e0141597.
27. Strauss O. The retinal pigment epithelium in visual function. Physiol Rev 2005;85(3):845–81.
28. Finnemann SC, Bonilha VL, Marmorstein AD, Rodriguez-Boulan E. Phagocytosis of rod outer segments by retinal pigment epithelial cells requires αvβ5 integrin for binding but not for internalization. Proc Natl Acad Sci U S A 1997;94(24):12932–7.
29. Finnemann SC, Nandrot EF. MERTK ACTIVATION DURING RPE PHAGOCYTOSIS IN VIVO REQUIRES αVβ5 INTEGRIN. Adv Exp Med Biol 2006;572:499–503.
30. Law A-L, Ling Q, Hajjar KA, et al. Annexin A2 regulates phagocytosis of photoreceptor outer segments in the mouse retina. Mol Biol Cell 2009;20(17):3896–904.
31. Thompson DA, Gal A. Vitamin A metabolism in the retinal pigment epithelium: genes, mutations, and diseases. Prog Retin Eye Res 2003;22(5):683–703.
32. Bazan NG. Survival signaling in retinal pigment epithelial cells in response to oxidative stress: significance in retinal degenerations. Adv Exp Med Biol 2006;572:531–40.
33. Plafker SM, O'Mealey GB, Szweda LI. MECHANISMS FOR COUNTERING OXIDATIVE STRESS AND DAMAGE IN RETINAL PIGMENT EPITHELIUM. Int Rev Cell Mol Biol 2012;298:135–77.
34. Shadrach KG, Rayborn ME, Hollyfield JG, Bonilha VL. DJ-1-Dependent Regulation of Oxidative Stress in the Retinal Pigment Epithelium (RPE). PLoS ONE 2013;8(7):e67983.
35. Boulton ME. Studying melanin and lipofuscin in RPE cell culture models. Exp Eye Res 2014;126:61–7.
36. Ruiz A, Bhat SP, Bok D. Characterization and quantification of full-length and truncated Na,K-ATPase alpha 1 and beta 1 RNA transcripts expressed in human retinal pigment epithelium. Gene 1995;155(2):179–84.
37. Philp NJ, Yoon H, Lombardi L. Mouse MCT3 gene is expressed preferentially in retinal pigment and choroid plexus epithelia. Am J Physiol Cell Physiol 2001;280(5):C1319–26.
38. Stamer WD, Bok D, Hu J, Jaffe GJ, McKay BS. Aquaporin-1 channels in human retinal pigment epithelium: role in transepithelial water movement. Invest Ophthalmol Vis Sci 2003;44(6):2803–8.
39. Simó R, Villarroel M, Corraliza L, et al. The Retinal Pigment Epithelium: Something More than a Constituent of the Blood-Retinal Barrier—Implications for the Pathogenesis of Diabetic Retinopathy, The Retinal Pigment Epithelium: Something More than a Constituent of the Blood-Retinal Barrier—Implications for the Pathogenesis of Diabetic Retinopathy. BioMed Res Int BioMed Res Int 2010;2010, 2010:e190724.
40. OMIM [Internet]. [cited 2016 May 12];Available from: http://www.omim.org/
41. Song MJ, Bharti K. Looking into the future: Using induced pluripotent stem cells to build two and three dimensional ocular tissue for cell therapy and disease modeling. Brain Res 2015;
42. Thumann G, Kirchhof B. [Transplantation of iris pigment epithelium]. Ophthalmol Z Dtsch Ophthalmol Ges 2004;101(9):882–5.

43. Kühl SJ, Kühl M. On the role of Wnt/β-catenin signaling in stem cells. Biochim Biophys Acta BBA - Gen Subj 2013;1830(2):2297–306.
44. Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat Med 2004;10(1):55–63.
45. Miki T, Yasuda S, Kahn M. Wnt/β-catenin signaling in embryonic stem cell self-renewal and somatic cell reprogramming. Stem Cell Rev 2011;7(4):836–46.
46. Marson A, Foreman R, Chevalier B, et al. Wnt signaling promotes reprogramming of somatic cells to pluripotency. Cell Stem Cell 2008;3(2):132–5.
47. Ross J, Busch J, Mintz E, et al. A rare human syndrome provides genetic evidence that WNT signaling is required for reprogramming of fibroblasts to induced pluripotent stem cells. Cell Rep 2014;9(5):1770–80.
48. Sanges D, Romo N, Simonte G, et al. Wnt/β-Catenin Signaling Triggers Neuron Reprogramming and Regeneration in the Mouse Retina. Cell Rep 2013;4(2):271–86.
49. Zhang P, Chang W-H, Fong B, et al. Regulation of induced pluripotent stem (iPS) cell induction by Wnt/β-catenin signaling. J Biol Chem 2014;289(13):9221–32.
50. Hägglund A-C, Berghard A, Carlsson L. Canonical Wnt/β-catenin signalling is essential for optic cup formation. PloS One 2013;8(12):e81158.
51. Leach LL, Buchholz DE, Nadar VP, Lowenstein SE, Clegg DO. Canonical/β-catenin Wnt pathway activation improves retinal pigmented epithelium derivation from human embryonic stem cells. Invest Ophthalmol Vis Sci 2015;56(2):1002–13.
52. Westenskow P, Piccolo S, Fuhrmann S. Beta-catenin controls differentiation of the retinal pigment epithelium in the mouse optic cup by regulating Mitf and Otx2 expression. Dev Camb Engl 2009;136(15):2505–10.
53. Arnhold S, Semkova I, Andressen C, et al. Iris pigment epithelial cells: a possible cell source for the future treatment of neurodegenerative diseases. Exp Neurol 2004;187(2):410–7.
54. Asami M, Sun G, Yamaguchi M, Kosaka M. Multipotent cells from mammalian iris pigment epithelium. Dev Biol 2007;304(1):433–46.
55. Sun G, Asami M, Ohta H, Kosaka J, Kosaka M. Retinal stem/progenitor properties of iris pigment epithelial cells. Dev Biol 2006;289(1):243–52.
56. Seko Y, Azuma N, Kaneda M, et al. Derivation of human differential photoreceptor-like cells from the iris by defined combinations of CRX, RX and NEUROD. PloS One 2012;7(4):e35611.
57. Dwyer MA, Kazmin D, Hu P, McDonnell DP, Malek G. Research Resource: Nuclear Receptor Atlas of Human Retinal Pigment Epithelial Cells: Potential Relevance to Age-Related Macular Degeneration. Mol Endocrinol 2011;25(2):360–72.
58. Hu P, Herrmann R, Bednar A, et al. Aryl hydrocarbon receptor deficiency causes dysregulated cellular matrix metabolism and age-related macular degeneration-like pathology. Proc Natl Acad Sci U S A 2013;110(43):E4069–78.
59. Esfandiary H, Chakravarthy U, Patterson C, Young I, Hughes AE. Association study of detoxification genes in age related macular degeneration. Br J Ophthalmol 2005;89(4):470–4.
60. Kim S-Y, Yang H-J, Chang Y-S, et al. Deletion of Aryl Hydrocarbon Receptor AHR in Mice Leads to Subretinal Accumulation of Microglia and RPE Atrophy. Invest Ophthalmol Vis Sci 2014;55(9):6031–40.
61. Choudhary M, Kazmin D, Hu P, Thomas RS, McDonnell DP, Malek G. Aryl hydrocarbon receptor knock-out exacerbates choroidal neovascularization via multiple pathogenic pathways. J Pathol 2015;235(1):101–12.

62. Hu DN, Ritch R, McCormick SA, Pelton-Henrion K. Isolation and cultivation of human iris pigment epithelium. Invest Ophthalmol Vis Sci 1992;33(8):2443–53.
63. Abe T, Tomita H, Ohashi T, et al. Characterization of iris pigment epithelial cell for auto cell transplantation. Cell Transplant 1999;8(5):501–10.
64. Mao Y, Finnemann SC. Analysis of photoreceptor outer segment phagocytosis by RPE cells in culture. Methods Mol Biol Clifton NJ 2013;935:285–95.
65. Thumann G, Bartz-Schmidt KU, Heimann K, Schraermeyer U. Phagocytosis of rod outer segments by human iris pigment epithelial cells in vitro. Graefes Arch Clin Exp Ophthalmol Albrecht Von Graefes Arch Für Klin Exp Ophthalmol 1998;236(10):753–7.
66. Abe T, Tomita H, Kano T, et al. Autologous iris pigment epithelial cell transplantation in monkey subretinal region. Curr Eye Res 2000;20(4):268–75.
67. Cai H, Shin MC, Tezel TH, Kaplan HJ, Del Priore LV. Use of iris pigment epithelium to replace retinal pigment epithelium in age-related macular degeneration: a gene expression analysis. Arch Ophthalmol Chic Ill 1960 2006;124(9):1276–85.
68. Van Soest SS, de Wit GMJ, Essing AHW, et al. Comparison of human retinal pigment epithelium gene expression in macula and periphery highlights potential topographic differences in Bruch's membrane. Mol Vis 2007;13:1608–17.
69. Booij JC, ten Brink JB, Swagemakers SMA, et al. A new strategy to identify and annotate human RPE-specific gene expression. PloS One 2010;5(5):e9341.
70. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004;3:Article3.
71. GENE-E. [Internet]. Available from: (http://www.broadinstitute.org/cancer/software/GENE-E/

Out there things can happen
and frequently do
to people as brainy
and footsy as you.
And when things start to happen, don't worry. Don't stew.
Just go right along. You'll start happening too.

- Dr Seuss

Comparison of Mouse and Human Retinal Pigment Epithelium Gene Expression Profiles: Potential Implications for Age-Related Macular Degeneration.

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ABSTRACT

The human retinal pigment epithelium (RPE) plays an important role in the pathogenesis of age related macular degeneration (AMD). AMD is the leading cause of blindness worldwide. There is currently no effective treatment available. Preclinical studies in AMD mouse models are essential to develop new therapeutics. This requires further in-depth knowledge of the similarities and differences between mouse and human RPE.

We performed a microarray study to identify and functionally annotate RPE specific gene expression in mouse and human RPE. We used a meticulous method to determine C57BL/6J mouse RPE signature genes, correcting for possible RNA contamination from its adjacent layers: the choroid and the photoreceptors.

We compared the signature genes, gene expression profiles and functional annotations of the mouse and human RPE. We defined sets of mouse (64), human (171) and mouse–human interspecies (22) RPE signature genes. Not unexpectedly, our gene expression analysis and comparative functional annotation suggested that, in general, the mouse and human RPE are very similar. For example, we found similarities for general features, like "organ development" and "disorders related to neurological tissue". However, detailed analysis of the molecular pathways and networks associated with RPE functions, suggested also multiple species-specific differences, some of which may be relevant for the development of AMD. For example, CFHR1, most likely the main complement regulator in AMD pathogenesis was highly expressed in human RPE, but almost absent in mouse RPE. Furthermore, functions assigned to mouse and human RPE expression profiles indicate (patho-) biological differences related to AMD, such as oxidative stress, Bruch's membrane, immune-regulation and outer blood retina barrier.

These differences may be important for the development of new therapeutic strategies and translational studies in age-related macular degeneration.

INTRODUCTION

Age related macular degeneration (AMD) is the leading cause of blindness worldwide. The disease affects 4 % of the population over age 60. With the increase of the aging population, AMD is becoming an even more important public health issue. The etiology of AMD remains largely unknown. The first clinical manifestations of the disease include the appearance of sub-retinal drusen and pigmentary or degenerative changes of the RPE. Ultimately, the disease affects the RPE, Bruch's membrane (BM), photoreceptors (PR) and choriocapillaries (CH). We focused this study on the RPE.

The RPE is a monolayer of pigmented neuro-epithelial cells, which forms part of the outer blood-retina barrier. It closely interacts with the PR to maintain visual function. The apical membrane of the RPE faces the photoreceptor outer segments and its basolateral membrane faces the BM. The BM separates the RPE from CH, which nourishes the RPE and outer layers of the retina[1]. In healthy eyes, BM functions as a structural support that is permeable to fluid and small molecules. Additionally it acts as a physical barrier, containing anti-angiogenic molecules, which protect the retina against neovascularization[2,3].

A healthy RPE is essential for visual function. It supplies the PR with nutrients, absorbs the excess light energy focused by the lens on the retina, recycles retinal from the PR, regulates the ion balance in the sub retinal space and maintains the function and survival of the PR by phagocytosis of the shed photoreceptor outer segments[1]. Failure of any of these functions can lead to degeneration of the retina, loss of visual function and, eventually, blindness in retinal diseases such as AMD or retinitis pigmentosa.

In AMD, RPE dysfunction or degeneration leads to a dystrophy of the PR and thereby vision loss[4]. The early stage of AMD is characterized by the presence of drusen and vision loss is relatively mild. Later stages of the disease involve two forms: the dry form (geographic atrophy) and the wet form (choroidal neovascularization). Both forms affect about half of the late stage AMD patients. AMD has a multifactorial etiology[5], and is caused by a variety of environmental and genetic risk factors[4]. There is evidence that positive life style changes (quit smoking; healthy food) and dietary supplements (Zn^{2+}) may postpone the onset or progression of the disease[6]. Patient-unfriendly, repeated intra-ocular injections with anti-VEGF may temporarily halt the progression of the wet form of AMD. However, it does not prevent the atrophy of RPE and PR[7,8]. Once vision is lost, a possible (future) cure for AMD may be cell replacement therapy. Pre-clinical experiments indicate that transplantation of stem cell derived RPE cells can successfully be used to rescue PR and vision[9–11]. However, these preclinical studies are predominantly performed in mice. To translate results and start clinical studies in man further knowledge of the similarities and differences between mouse and human RPE is essential.

In this study we compared the gene expression profiles and functional annotation of mouse and human RPE on a single microarray platform to further improve translational studies.

RESULTS

First, we determined the gene expression profiles of the mouse RPE, CH and PR (raw data available in the Gene Expression Omnibus database with the accession number GSE66916). We confirmed our microarray methodology by checking the expression of (well established) RPE genes using semi-quantitative RT-PCR (sqRT-PCR) (Fig.1 and S1 Fig.). Subsequently, we determined mouse and human RPE signature genes, we defined the functionalities of the gene expression profiles of mouse and man, and we analyzed the most extreme differences in RPE gene expression between the two species. Also these results were partly confirmed using sqRT-PCR (S2 Fig).

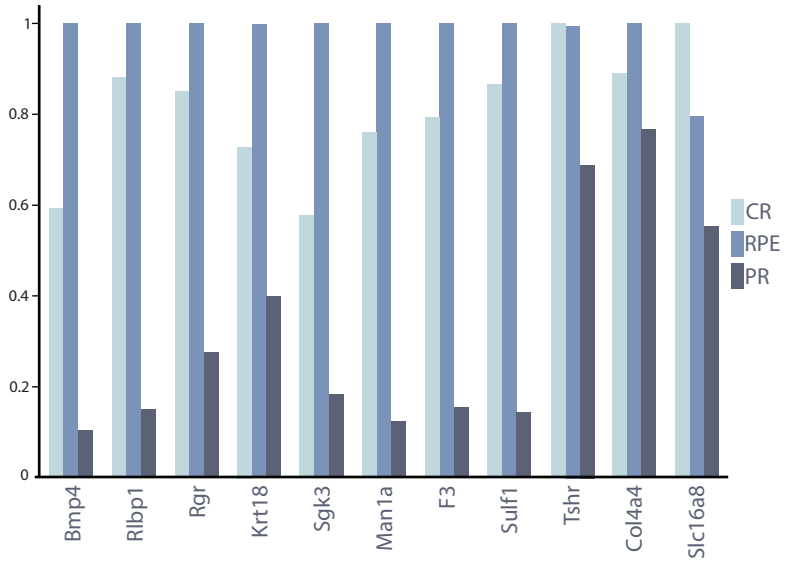

Figure. 1. Confirmation of microarray results by sqRT-PCR. Beta-actin (*Bact*), a housekeeping gene, was used to normalize gene expression in mouse CH, RPE and PR. The light blue bars indicate expression levels in CH, the blue bars expression levels in the RPE and the dark blue bars indicate expression levels in PR. Similar to the microarray data the expression level is highest in the RPE and lowest in the PR. The sqRT-PCR results confirm our findings; however *Tshr* and *Slc16a8* show expression lower in RPE compared to choroid. Overall, the sqRT-PCR confirmation rate in this, and in all our previous studies (combined), using exactly the same methodology and platform to investigate neuroepithelia from human donor eyes and brains was 87%[12–14].

Mouse, human and inter-species RPE signature genes

In our lab, we previously designed a new strategy to select RPE signature genes (Fig. 2). RPE specificity was determined by comparison of the gene expression levels between the RPE and its adjacent layers: the CH and PR[12]. In the current study, we applied this strategy to the mouse retina in order to select mouse RPE signature genes.

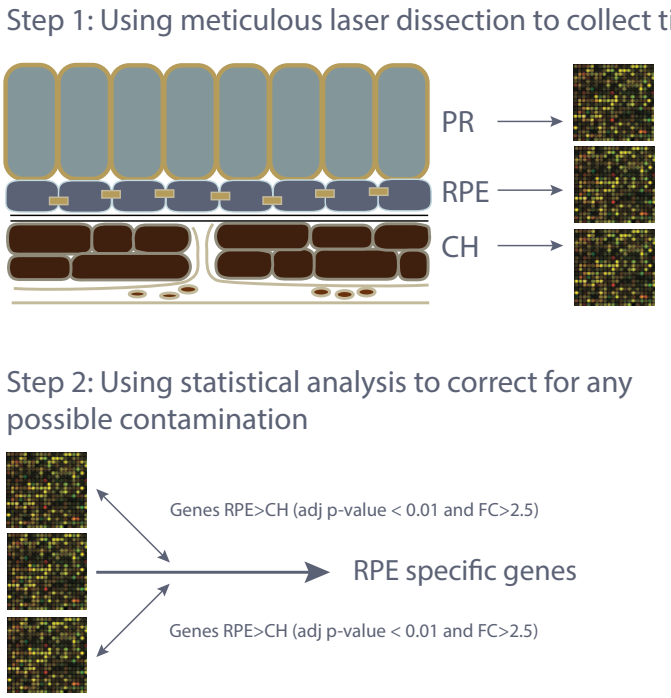

Figure. 2. Strategy to select RPE signature genes. In the first step of this strategy we laser dissect the RPE (and its adjacent layers, the CH and PR) for specific tissue collection. In the second step we statistically correct for possible contamination by adjacent layers.

We selected the genes that have a significant higher expression level in the mouse RPE compared to their expression in the CH and the PR, with a fold change (FC) higher than 2.5 and a B-H adjusted p-value<0.01. This resulted in a list of 64 genes that are specifically expressed in the RPE relative to both its adjacent layers; the CH and PR. We annotated this set the "Mouse RPE signature genes" dataset (see Table 1).

Using the same cut-off criteria; we determined a set of genes that is specific for the mouse CH compared to the RPE and a set of genes mouse PR specific when compared to the RPE (S1 Table).

We next defined a new "Human RPE signature genes" dataset. We carefully selected two previously published human RPE specific gene expression datasets for a comprehensive comparison between mouse and human RPE (Fig. 3)[12,15]. The first study was conducted in our lab using a similar methodology for determining RPE specific gene expression resulting in identification of 114 RPE specifically expressed genes[12]. The second microarray study included multiple RPE types but the investigators did not correct for possible contamination of adjacent tissues[15]. For the latter dataset, we removed possible CH and PR RNA contamination (see Methods), and generated a list of 86 human RPE specifically

Table 1. Our "Mouse RPE signature genes" dataset: 64 mouse RPE genes with an average expression of at least 2.5 fold higher in the mouse RPE than in both the PR and the CH with an adjusted p-value smaller than 0.01.

CH<RPE>PR genes		RPE compared to CH		RPE compared to PR	
GeneName	**SystematicName**	**adj.P.Val**	**FC RPE-CH**	**adj P value**	**FC RPE-PR**
Rgr	ENSMUST00000022338	5,93E-03	4,9	5,90E-06	306,1
LOC100045988	XM_001475309	6,03E-03	4,6	3,81E-03	3,5
Pon1	NM_011134	1,01E-03	4,2	2,10E-07	95,8
Rdh10	NM_133832	1,46E-03	4,1	9,38E-08	75,1
Arl6ip1	NM_019419	4,41E-03	3,2	2,73E-06	24,6
Rlbp1	NM_020599	5,78E-03	3,2	4,29E-07	42,4
Tbx5	NM_011537	2,65E-03	3,2	3,61E-04	3,5
Bmp4	NM_007554	3,03E-03	3,2	2,31E-05	47,9
F3	NM_010171	1,42E-03	3,1	4,09E-07	102,2
5730469M10Rik	NM_027464	2,28E-03	3,1	1,03E-06	14,0
Rrh	NM_009102	2,40E-03	3,1	8,94E-08	47,2
Man1a	NM_008548	2,07E-04	3,0	6,11E-08	10,3
Sema3c	NM_013657	5,52E-04	2,9	9,66E-08	504,3
Vldlr	NM_013703	1,35E-03	2,9	1,79E-05	4,6
Atp1b1	NM_009721	2,54E-03	2,9	1,29E-07	34,1
Ctsd	NM_009983	6,72E-03	2,9	6,81E-05	5,7
Cspg5	NM_001166273	5,34E-03	2,9	1,94E-06	15,1
Cldn2	NM_016675	6,79E-04	2,9	6,75E-07	7,8
Sulf1	NM_172294	4,05E-04	2,9	2,38E-07	22,9
BC048943	NM_001127685	1,48E-03	2,9	8,23E-05	3,4
Slc39a12	NM_001012305	6,97E-04	2,9	8,03E-08	123,3
Loxl4	NM_001164311	4,47E-04	2,8	7,52E-07	13,2
NAP114398-1	NAP114398-1	5,88E-04	2,8	2,70E-07	9,4
Slc1a1	NM_009199	6,31E-03	2,8	1,30E-07	27,2
Slc6a13	NM_144512	3,86E-03	2,8	9,05E-08	49,1
Car12	NM_178396	5,92E-03	2,8	2,86E-07	34,5
Iqgap2	NM_027711	3,55E-04	2,8	5,11E-08	13,4
Hist2h2aa1	NM_013549	2,53E-04	2,8	2,57E-07	5,7
Tgfa	NM_031199	1,07E-03	2,8	2,66E-07	11,9
Spon1	NM_145584	3,68E-04	2,7	2,54E-07	7,4
Flot2	NM_008028	4,72E-03	2,7	1,50E-05	7,1
Tmem27	NM_020626	1,64E-03	2,7	3,15E-05	108,8
Trhde	NM_146241	1,06E-03	2,7	7,15E-08	19,8
Hist2h4	NM_033596	8,85E-03	2,7	5,29E-05	7,0
Itgb8	NM_177290	2,57E-03	2,7	4,33E-07	14,9

Table 1. Our "Mouse RPE signature genes" dataset: 64 mouse RPE genes with an average expression of at least 2.5 fold higher in the mouse RPE than in both the PR and the CH with an adjusted p-value smaller than 0.01. (continued)

CH<RPE>PR genes		RPE compared to CH		RPE compared to PR	
Cbfa2t3	NM_001109873	6,18E-03	2,7	2,75E-06	11,8
Tcfl5	NM_178254	1,92E-03	2,7	4,74E-07	12,2
Adora2b	NM_007413	3,61E-04	2,7	1,30E-07	37,0
Spock1	NM_009262	2,29E-03	2,7	1,28E-06	9,3
Gpam	ENSMUST00000086868	8,06E-03	2,7	1,09E-03	3,0
Acsl6	NM_001033599	2,80E-03	2,7	2,96E-04	2,8
Lrp2	NM_001081088	6,73E-03	2,7	3,74E-06	10,7
Slc6a20a	NM_139142	5,91E-04	2,6	3,38E-07	7,6
Nt5dc2	NM_027289	1,30E-03	2,6	9,65E-07	7,7
Krt18	NM_010664	2,04E-03	2,6	1,73E-06	7,2
Slc16a8	NM_020516	1,87E-03	2,6	2,46E-07	14,9
Gabrb3	NM_008071	3,18E-04	2,6	4,83E-06	3,3
Mogat1	NM_026713	2,79E-03	2,6	5,42E-07	12,9
Hkdc1	NM_145419	2,81E-03	2,6	7,31E-06	5,9
Tmem56	NM_178936	4,12E-03	2,6	1,71E-08	148,2
Col4a4	NM_007735	2,96E-03	2,6	3,44E-06	4,0
Pebp4	NM_028560	4,39E-04	2,6	4,15E-08	13,5
Trpm3	NM_001035246	1,61E-04	2,6	5,35E-09	23,2
Hist1h4i	NM_175656	1,08E-03	2,6	4,09E-06	4,7
A2m	NM_175628	1,55E-03	2,5	8,00E-05	3,0
Bphl	NM_026512	8,30E-04	2,5	2,48E-06	3,1
Slc7a10	NM_017394	1,64E-03	2,5	3,27E-08	33,6
Tshr	NM_011648	4,22E-04	2,5	1,22E-08	58,3
Car14	NM_011797	3,58E-03	2,5	2,60E-07	18,4
Adra2c	NM_007418	4,16E-03	2,5	4,30E-08	54,8
Fam13a	NM_153574	5,08E-04	2,5	2,20E-08	19,6
Sgk3	NM_133220	3,38E-03	2,5	3,87E-06	7,0
Pde4b	NM_019840	5,03E-04	2,5	8,98E-07	5,0
Slco1a4	NM_030687	3,07E-04	2,5	6,65E-09	32,7

expressed genes. We subsequently merged the two human RPE specific gene expression datasets, resulting in 171 human RPE signature genes (S2 Table).

Finally, in order to facilitate comparative retinal studies between mouse and human, we aimed to develop a list of interspecies RPE signature genes. We determined the overlap between the mouse (64) and the human (171) RPE signature gene lists, resulting in an interspecies RPE signature gene list of 22 genes (Table 2).

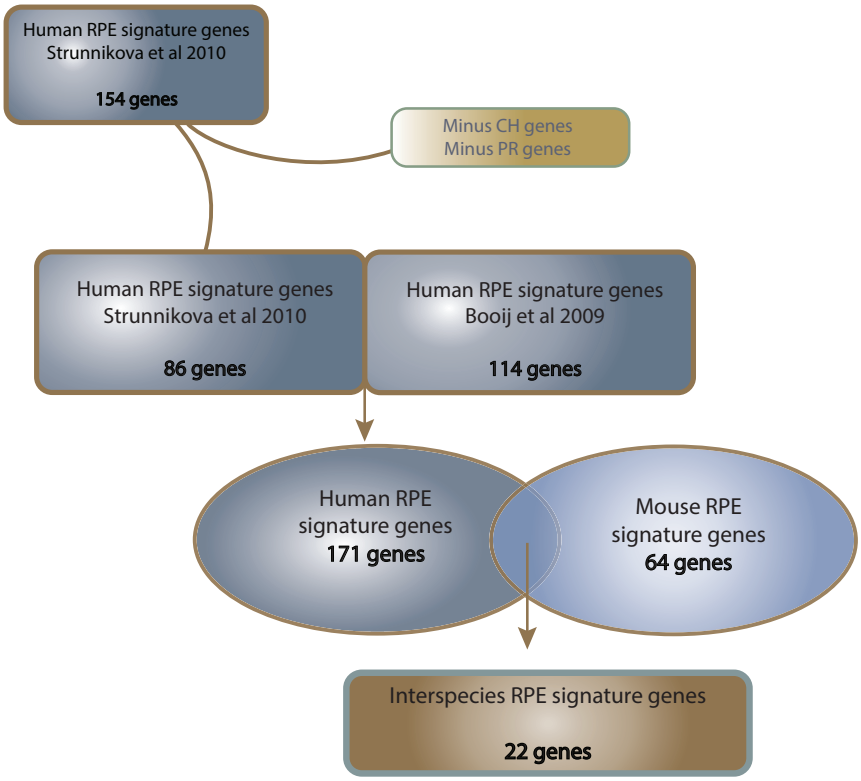

Figure 3. Strategy to determine "Interspecies RPE signature genes". Schematic overview of our comparison strategy: our "Mouse RPE signature genes" dataset and "Human RPE signature genes" dataset, which contains (a modification of) two human RPE transcriptome datasets [12,15]. This resulted in a new dataset, "Interspecies RPE signature genes".

Gene expression profiles and functions of the mouse and human RPE

Using our previously published methodology[12–14], we analyzed the highest expressed genes (highest 10th percentile: >90thP) of our mouse gene expression dataset (designated "Mouse high RPE gene expression") to determine the most important functionalities of the mouse RPE. Subsequently, we compared the gene expression pathways and functional annotations of the mouse and human RPE. The latter dataset was available from our previous studies ("Human high RPE specific gene expression dataset"[12]. We used the Ingenuity Knowledge Database to determine biological functions, canonical pathways and molecular networks specific for mouse and human RPE *in vivo*.

Functional annotation yielded statistically significant **biological functions** that were the same for mouse and human (Table 3). We also found that many important **canonical pathways** for mouse and human RPE were similar. A summary of these findings is presented in Fig. 4.

Table 2. The 22 signature genes that are specifically expressed in both RPE in mouse and in human. Derived from a comparison between our "Mouse RPE signature genes" dataset (this study) and two (modified) studies on the human RPE transcriptome [12,15]. We show the gene symbol, genbank ID for both species and the GO annotation of each gene.

Gene Symbol	Genbank ID Mus Musculus	Genbank Homo Sapiens
ADORA2B	**NM_007413**	**NM_000676**
G-protein coupled adenosine receptor. This integral membrane protein stimulates adenylate cyclase activity in the presence of adenosine.		
BMP4	**NM_007554**	**NM_001202**
A member of the bone morphogenic protein family which is part of the transforming growth factor-beta superfamily. The superfamily includes large families of growth and differentiation factors.		
CA14	**NM_011797**	**NM_012113**
Carbonic anhydrases are a large family of zinc metalloenzymes that catalyze the reversible hydratyion of carbon dioxide.		
CSPG5	**NM_001166273**	**NM_001206942.1**
A proteoglycan that may function as a neural growth and differentiation factor.		
CTSD	**NM_009983**	**NM_001909.4**
An aspartic protease resident in endosomal and lysosomal compartments of all eukaryotic cells.		
GPAM	**ENSMUST00000086868**	
A mitochondrial enzyme which prefers saturated fatty acids as its substrate for the synthesis of glycerolipids.		
ITGB8	**NM_177290**	**NM_002214.2**
Cell surface adhesion receptor mediating cell-adhesion to extra cellular matrix or to other cells, through hetero dimerization and connecting to the cytoskeleton and various signaling molecules within cells.		
KRT18	**NM_010664**	**NM_000224**
Keratin 18 is a type I cytokeratin (this type constitutes the type I intermediate filaments of the intracytoplasmatic cytoskeleton, which is present in all mammalian epithelial cells), together with Krt8 is the most common found product of the intermediate filament gene family. They are expressed in single layer epithelial tissues of the body.		
RDH10	**NM_133832**	**[NM_172037**
A retinol dehydrogenase, which converts all-trans-retinol to all-trans-retinal, with preference for NADP as a cofactor.		
RGR	**ENSMUST00000022338**	**NM_002921**
A putative retinal G-protein coupled receptor and acts as a photoisomerase to catalyze the conversion of all-trans-retinal to 11-cis-retinal.		
RLBP1	**NM_020599**	**NM_000326**
Retinaldehyde binding protein 1. carries 11-cis-retinaldehyde or 11-cis-retinal as physiological ligands. It may be a functional component of the visual cycle.		
SEMA3C	**NM_013657**	**NM_006379.3**
Binds to plexin family members and plays an important role in the regulation of developmental processes.		
SLC16A8 [MCT3]	**NM_020516**	**NM_013356**
Belongs to a family of monocarboxylate transporters. It is expressed in the basolateral membrane of the RPE.		
SLC39A12	**NM_001012305**	**NM_152725**

Table 2. The 22 signature genes that are specifically expressed in both RPE in mouse and in human. Derived from a comparison between our "Mouse RPE signature genes" dataset (this study) and two (modified) studies on the human RPE transcriptome [12,15]. We show the gene symbol, genbank ID for both species and the GO annotation of each gene. (continued)

Gene Symbol	Genbank ID Mus Musculus	Genbank Homo Sapiens
Zinc transporter, which is a cofactor for hundreds of enzymes and therefore normal cell function.		
SLC6A13	**NM_144512**	**NM_016615**
Encodes a sodium- and chloride-dependent GABA transporter [GAT2]		
SLC6A20	**NM_139142**	**NM_020208**
Encodes an amino acid transmembrane transporter that mediates the transport of small hydrophilic substances across cell membranes.		
SLC7A10	**NM_017394**	**NM_019849**
Encodes an amino acid transmembrane transporter that mediates high-affinity transport of D-serine and several other neutral amino acids.		
SPOCK1	**NM_009262**	**NM_004598**
Encodes he protein core of a seminal plasma proteoglycan containing chondroitin- and heparin-sulfate chains.		
SULF1	**NM_172294**	**NM_015170**
Enzyme which can modulate the activity if heparan sulfate, thereby influencing the regulation of cell growth, proliferation, differentiation and migration.		
TMEM27	**NM_020626**	**NM_020665**
binds to amino acid transporters and regulates their expression on the plasma membrane		
TMEM56	**NM_178936**	**NM_152487**
function unknown		
TRPM3	**NM_001035246**	**NM_206948**
Belongs to the family of transient receptor potential channels. TRP channels are cation-selective channels important for cellular calcium signaling and homeostasis.		

In addition, we studied the **molecular networks** that were assigned to both the "Mouse" and "Human" "high RPE gene expression" datasets. Functions annotated to these datasets on a network level were more or less comparable (~75% overlap). The annotated functions included developmental disorders, hereditary disorders, small molecule/drug metabolism and cellular movement and maintenance. For an overview of the 10 most important networks for the "Mouse high RPE gene expression" dataset and the "Human high RPE gene expression" dataset, and to see which networks overlap, see S3 Table and S4 Table. For illustrative purpose we included an example of such a network (S3 Fig.). For additional support of our findings in Ingenuity we also included a functional enrichment pathway analysis (KEGG analysis) in Webgestalt[16]. This gives approximately the same results (S5 Table).

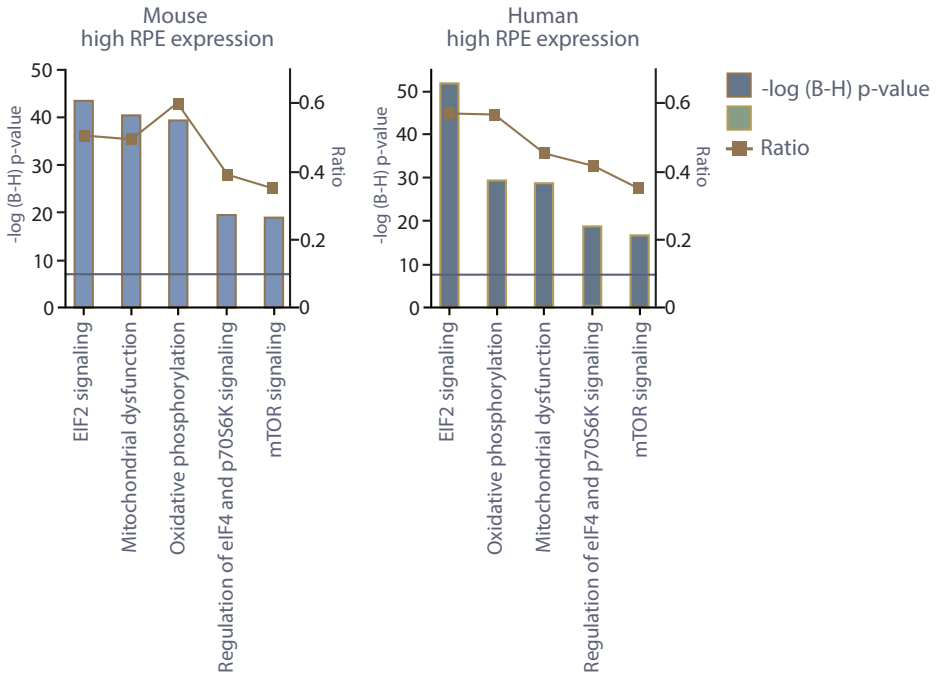

Figure 4. Most significant canonical pathways identified by Ingenuity for the "Mouse High RPE gene expression" and "Human High RPE expression gene expression" datasets. The left y-axis displays the –log of Benjamini-Hochberg corrected p-value. The right y-axis displays the ratio of the number of genes derived from our dataset, divided by the total number of genes in the pathway. The blue line indicates the threshold of the BH corrected p-value of 0.1.

Genes highly expressed in mouse RPE but hardly in human RPE

To investigate the largest gene expression and functional differences between the mouse and human RPE we subsequently compared the most extreme gene expression datasets of the two species, namely the very high (highest 10th percentile, >90thP; high expression) and very low (lowest 10th percentile <10thP; leaky expression) RPE expression datasets (GSE 66916).

Unexpectedly, the "Mouse high RPE gene expression" dataset (>90th P, 2663 genes) and the "Human very low RPE gene expression" dataset (0-10th P, 1770 genes) had 101 genes in common (S6 Table). Functional annotation of these genes yielded 31 **canonical pathways** in Ingenuity, whose activity or metabolic route may be differentially affected in mouse and human RPE. An overview is presented in Table 4.

The core analysis of the 101 differentially expressed genes resulted in 7 **molecular networks**. The associated representative functions include developmental disorders, connective tissue disorders, ophthalmic disease, neurological disease, drug metabolism and cancer. These networks are presented in S7 Table. For illustrative purpose we

Table 3. Overview of the major biological functions found in a functional annotation by Ingenuity of the "Mouse High RPE gene expression" and "Human High RPE gene expression" datasets. The p-value for these categories are indicated as a range because each category contains sub-functions that have their own p-value.

Mouse High RPE expression		**Human High RPE expression**	
Disease and Disorders	**p-value**	**Disease and Disorders**	**p-value**
Neurological Disease	7,31E-51-9,20E-05	Neurological Disease	7,54E-57-2,18E-05
Psychological Disorders	8,09E-44-9,20E-05	Psychological Disorders	2,04E-49-2,26E-07
Skeletal and Muscular Disorders	2,84E-41-2,62E-05	Skeletal and Muscular Disorders	5,84E-47-2,18E-05
Infectious Disease	5,89E-36-9,33E-05	Hereditary Disorder	1,77E-39-2,18E-05
Hereditary Disorder	6,10E-34-4,77E-05	Infectious Disease	2,29E-32-1,08E-05
Molecular and Cellular Functions		**Molecular and Cellular Functions**	
Cellular Growth and Proliferation	7,20E-39-8,57E-05	Cell Death and Survival	3,55E-42-2,06E-05
Cell Death and Survival	3,43E-38-5,57E-05	Cellular Growth and Proliferation	6,25E-37-1,64E-05
Cell Morphology	5,70E-25-9,33E-05	Protein Synthesis	1,77E-22-2,14E-05
Protein Synthesis	5,77E-22-9,28E-06	Cell Morphology	9,15E-21-1,66E-05
Cellular Development	3,25E-17-8,57E-05	Gene Expression	2,01E-20-2,15E-05
Physiological System Development & Function		**Physiological System Development & Function**	
Organismal Survival	7,97E-23-7,93E-06	Organismal Survival	3,18E-23-3,18E-23
Embryonic Development	9,01E-18-8,15E-05	Organismal Development	2,63E-16-2,01E-05
Organ Development	9,01E-18-8,15E-05	Nervous System Development and Function	3,17E-16-1,66E-05
Organ Morphology	9,01E-18-9,33E-05	Embryonic Development	2,01E-14-1,65E-05
Organismal Development	9,01E-18-8,15E-05	Organ Development	3,73E-13-1,45E-05

included an example of one of these networks, see S4 Fig. For additional support of our findings in Ingenuity we also included a functional enrichment pathway analysis (KEGG analysis) in Webgestalt. This gives approximately the same results (S8 Table).

Genes highly expressed in human RPE but hardly in mouse RPE

In order to identify additional differences between mouse and human RPE, we also compared the "Human high RPE gene expression" dataset (>90th P, 2399 genes) and the "Mouse very low RPE gene expression" dataset (10th P, 3374 genes). This analysis yielded 54 genes (S9 Table). We also functionally annotated this set of genes using the Ingenuity knowledge database. The significant **canonical pathways** assigned to this dataset included PXR/RXR activation, nicotine degradation and bupropion degradation. Ingenuity analysis yielded four networks. The functional annotations of these networks include drug metabolism, nucleic acid metabolism, small molecule biochemistry, cardiovascular disease and humoral immune response. The molecular pathways

Table 4. Overview of significant canonical pathways assigned by the Ingenuity knowledge database to the 101 genes that are the result of comparing the "Mouse high RPE gene expression" and the "Human very low RPE gene expression" datasets.

Endocrine Signaling & Metabolic Function
Ephrin Receptor Signaling
PEDF Signaling
Protein Kinase A Signaling
Gαq Signaling
FGF Signaling
Phospholipase C Signaling
NGF Signaling
GNRH Signaling
PXR/RXR Activation
Ephrin B Signaling
Immunological Function
iCOS-iCOSL Signaling in T Helper Cells
Role of NFAT in Regulation of the Immune Response
Dendritic Cell Maturation
B Cell Receptor Signaling
IL-8 Signaling
Thrombin Signaling
PKCθ Signaling in T Lymphocytes
CD28 Signaling in T Helper Cells
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis
GM-CSF Signaling
PI3K Signaling in B Lymphocytes
Basic pathways of cellular (dys)function
Prostate Cancer Signaling
Regulation of the Epithelial-Mesenchymal Transition Pathway
Wnt/Ca+ pathway
P2Y Purigenic Receptor Signaling Pathway
Estrogen-Dependent Breast Cancer Signaling
Colorectal Cancer Metastasis Signaling
Epithelial junctions
Tight Junction Signaling
Epithelial Adherens Junction Signaling
Vesicle mediated transport
Clathrin-mediated Endocytosis Signaling
Oxidative stress
Hypoxia Signaling in the Cardiovascular System

are presented in S10 Table. We also included an illustrative example of such a network, see S5 Fig.

Among the major **biological functions** and disease that came out of this analysis were hereditary hearing loss and Usher syndrome. Major differences in **molecular cellular functions** identified by Ingenuity were drug metabolism, nucleic acid metabolism, small molecule biochemistry and lipid metabolism. For additional support of our findings in Ingenuity we included a functional enrichment pathway analysis (KEGG analysis) in Webgestalt. This gives approximately the same results (see S11 Table).

DISCUSSION

In this study, we aimed to find similarities and differences between mouse and human RPE using RPE specific gene expression profiles and functional annotation on the same experimental platform. Our current data may be important for translational studies in age related macular degeneration, for creating and use of a representative AMD mouse model. Thus, we discuss here those aspects of our analyses of human and mouse RPE that are relevant for AMD.

Similarities and differences between mouse and human RPE transcriptomes in relation to AMD

Apart from the obvious similarities, there are a number of well-known differences between human and mouse RPE and adjacent tissues. These include the absence of a macula in the mouse, the difference in rod and cone number and distribution, and a thinner Bruch's membrane in the mouse. Mouse models are available for wet and dry AMD, mimicking several of the pathological features seen in AMD, but no model recreates all of the AMD characteristics[17–20].

We were interested in the potential usefulness of our entire comparative human and mouse gene expression dataset for the investigation of AMD (mouse models). Interestingly we did find similarities and differences in relation to a number of previously published (patho-) biological aspects related to AMD, namely oxidative stress, zinc homeostasis, presence of proteins of the complement system that are found in drusen, proteins in Bruch´s membrane, involvement in neovascularization and tight junctions. These differences and similarities are important to develop and use representative mouse models for AMD, and they may be partly responsible for (the observed) discrepancies between mouse model and human patients.

Age related macular degeneration: Oxidative Stress

The RPE suffers from chronic oxidative stress due to the exposure to light, relatively low oxygen levels, and daily phagocytosis and digestion of photoreceptor outer segments[21]. The mainstream hypothesis in AMD is that prolonged oxidative stress harms the vitality of the RPE and oxidatively modified drusen-bound fatty acids and proteins. These are subsequently recognized by the body as non-self, and invoke a chronic, complement mediated, immune response[22,23].

Oxidative stress in the RPE is, among others, mediated by the manganese superoxidase dismutase protein family, consisting of SOD1, SOD2 and SOD3; Respectively, these SODs exert their antioxidant effect in the cytosol, mitochondria and extracellular matrix[24,25]. We found similar expression of SOD1 (very high) and SOD3 (moderate) in human and mouse RPE. In contrast, we found that the SOD2 gene was highly expressed in the mouse RPE but only at low levels in the human RPE (Table 5). Reactive Oxygen species (ROS)-associated mitochondrial DNA damage was previously correlated with the progression of AMD[26–28]. But association studies between genetic variants in the SOD2 gene and AMD pathogenesis yielded conflicting results[27,29].

For both SOD1 and SOD2 mouse models were developed. *Sod1-/-* mice and *Sod2-/-* mice both show a thickened Bruch's membrane, photoreceptor atrophy and reduced electroretinographic response[30]. *Sod2-/-* mice lacked drusen like deposits but have RPE atrophy[31,32]. In the *Sod1-/-* mice, 10% of the older animals showed choroidal neovascularization and 86% showed drusen-like deposits that contained several markers of drusen[33].

The previous studies on SOD family members and the different expression we find between mouse and human, indicate that all three SOD family members may be critically involved in the local defense against oxidative stress, although the mitochondrial SOD2 may play a more important role in the mouse RPE than in the human RPE.

In addition to SODs, zinc has also been implicated in mediating oxidative stress. The retina and especially drusen contain high amounts of zinc[34]. There is an age related decrease in systemic and cellular zinc levels in human RPE cells, that correlates with several age related pathologies like AMD[35]. In 1988 the first clinical trial favoring zinc supplementation in AMD was published[36]. Since that time, multiple studies suggested that zinc reduces the oxidative burden on the retina although the underlying molecular mechanism(s) is unknown[7,37–39]. Zinc ions reach the retina by specific transporters. We determined which zinc transporters are highly expressed in mouse and human RPE. In the highest 10th percentile of the mouse RPE transcriptome we found expression of *Slc39a1*, *Slc39a4*, *Slc39a7* and *Slc39a12*. In the highest 10th percentile of the human RPE transcriptome we observed expression of *SLC39A8*, *SLC39A12* and *SLC39A13* (Table 6). Our data are largely in agreement with those of Leung and coworkers[40], who deter-

mined the expression of a large number of zinc transporters in cultured human RPE cells.

Interestingly, we found that *Slc39a4* was highly expressed in mouse RPE, but not in the human RPE. Indeed, Dufner-Beattie et al demonstrated the importance of this transporter in a *Slc39a4* knockout mouse, which develops severe abnormalities of the nervous system, such as anophthalmia, exencephaly and hydrocephalus[41]. Our finding (in older human donor eyes) may be explained by an age-related effect, since Leung et al[40] found that *Slc39a2* and *Slc39a4* expression and corresponding zinc uptake are reduced in RPE from older individuals. We determined the *Slc39a4* expression in RPE of five-month-old mice.

Table 5. SOD1, SOD2, SOD3 gene expression in human and mouse RPE. Sod1 and Sod3 are highly and moderately expressed respectively, in both species. Sod2 gene expression has a low expression in human RPE. In contrast it has a high expression in mouse RPE.

	Mouse		Human	
Isoenzyme	**Reporter**	**Percentile**	**Reporter**	**Percentile**
SOD1	NM_011434	High	NM_000454	High
SOD2	NM_013671	High	NM_000636, BM994509, AL050388	Low
SOD3	NM_011435	Intermediate	NM_003102	Intermediate

Table 6. Overview of zinc transporters that are highly expressed in human and mouse RPE.

Mouse		Human	
Zinc transporter	**reporter**	**Zinc transporter**	**reporter**
Slc39a1	NM_013901	*SLC39A8*	NM_022154
Slc39a4	NM_028064	*SLC39A12*	NM_152725
Slc39a7	NM_008202	*SLC39A13*	NM_152264
Slc39a12	NM_001012305		

Age-related macular degeneration: drusen and complement system

Chronic inflammatory and immune mediated events at the level of the Bruch's membrane and drusen play critical roles in AMD pathogenesis[42]. Initially, complement system related factors were immune-localized to drusen, a hallmark of AMD. Subsequently, genetic studies showed an association between polymorphisms of several complement pathway genes, such as *CFH, CFB, C3, CFHRs*, and AMD[2]. In our dataset we found high expression of several complement factors in the human and mouse RPE (Fig. 5), which may be of interest for studies of the complement system and AMD pathogenesis in a mouse model.

Interestingly, C1QTNF5 is highly expressed in the both mouse and human RPE. Mutations in C1QTNF5 have been associated with late-onset retinal degeneration. A *C1qtnf5 S163R* knock-in mouse model developed by Chavali et al showed many pathological features of AMD, such as RPE abnormalities, photoreceptor loss, retinal vascular leakage[43]. Contrary to this, Shu et al. developed a *C1qtnf5 Ser163Arg* knock-in mouse model that lacked any phenotypic abnormality[44]. The reason for this discrepancy is currently not clear.

We observed that the complement factor H related 1 gene (CFHR1) is highly expressed in human RPE, but not in mouse RPE. Our data corroborate, in part, the data of Luo et al (*2011*) who found absence of *Cfhr1* expression in mouse retina, RPE and choroid[45]. The regulation of the complement system (in AMD) is extremely complex, multiple regulators and feedback loops exist and the detailed mechanisms underlying the complement regulation at the RPE interface and macular area is not known. Nonetheless, several studies suggested that CFHR1, together with CFHR3, plays a central role in complement regulation of AMD. Several studies suggest that the absence of CFHR1 and/or its family member CFHR3 are highly protective against AMD in humans[46–48] .

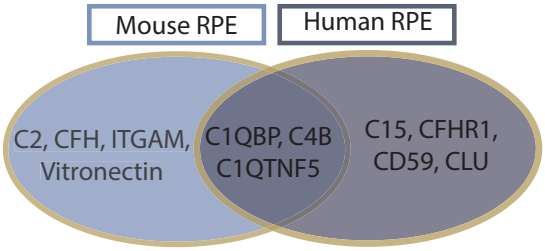

Figure 5. Overview of highly expressed complement factors in the human and mouse RPE. Complement factors in the overlay of the circles are highly expressed in RPE of both species.

Age-related macular degeneration: Bruch's membrane and neovascularization

Our data reveal differences in mouse and human RPE gene expression related to two other essential aspects of age-related macular degeneration: The build-up and turnover of Bruch's membrane and neovascularization. Bruch's membrane is a sheet of extracellular matrix that lies in between the RPE and the choroid. The extracellular matrix components that form the BM are made by RPE and CH. The BM has a major clinical significance because of its critical role in the pathogenesis of AMD[2].

We found that *Timp2* and *Col3a*, genes involved in extracellular matrix formation or turnover, are highly expressed in the mouse RPE, but not in human RPE. Vice versa, *COL16A1* is highly expressed in the human RPE, but not in mouse RPE.

We also found large inter-specifics gene expression differences annotated with the term "Angiogenesis". "Angiogenesis" refers to the process whereby new blood vessels are formed. In the context of our RPE/AMD analysis, choroidal blood vessels usually penetrate the BM and from new (leaky) vessels underneath the RPE. Our data specifically suggest expression and functional differences for the angiogenic factors *Fgf23* and *Fgfr1* (highly expressed in mouse RPE), as well as the prostaglandin synthase *PTGES* and *HS6ST1* (highly expressed in human RPE). In summary, our results suggest specific differences between mouse and man in terms of BM buildup or turnover, as well as related to neovascularization.

Age-related macular degeneration: tight junctions of the outer blood-retina barrier

The RPE constitutes the outer blood-retina barrier (oBRB). The tight junctions between neighboring RPE cells bind the monolayer and separates the outer layer of the neural retina from the choriocapillaris[49]. The RPE maintains the integrity of the oBRB through the tight junctions, which is important for control of fluid leakage, solute transport and immune reactions. oBRB supports the functional homeostasis of the retina. Disruptions of RPE cell junction and barrier integrity are associated with AMD[50,51]. We compared the tight junction gene expression of mouse and human RPE by investigating the distribution of these genes in four categories: high expression (>90th percentile), moderate (50-90th percentile), low (10-50th percentile) and very low (<10th percentile) (Fig. 6).
Overall, we find limited overlap of tight junction gene expression between mouse and human RPE. Our data suggest that the composition of the outer blood retina barrier differs between mouse and human. More investigation is necessary to determine the possible physiological or pathobiological effect of these differences.

CONCLUSIONS

In summary, in this study we determined 64 signature genes for mouse RPE, 171 signature genes for human RPE. We also deduced 22 mouse-human interspecies signature genes. We next analyzed the general mouse and human RPE gene expression profiles, and we found that (patho-) biological functions and canonical pathways assigned to the RPE of both species were highly similar. Nonetheless, more detailed studies, including analysis of specific molecular networks as well as extreme gene expression differences between mouse and human (expression of 155 genes), suggests substantial biological differences.
These similarities and differences may be important for the development of new therapeutic strategies and translational studies in age-related macular degeneration.

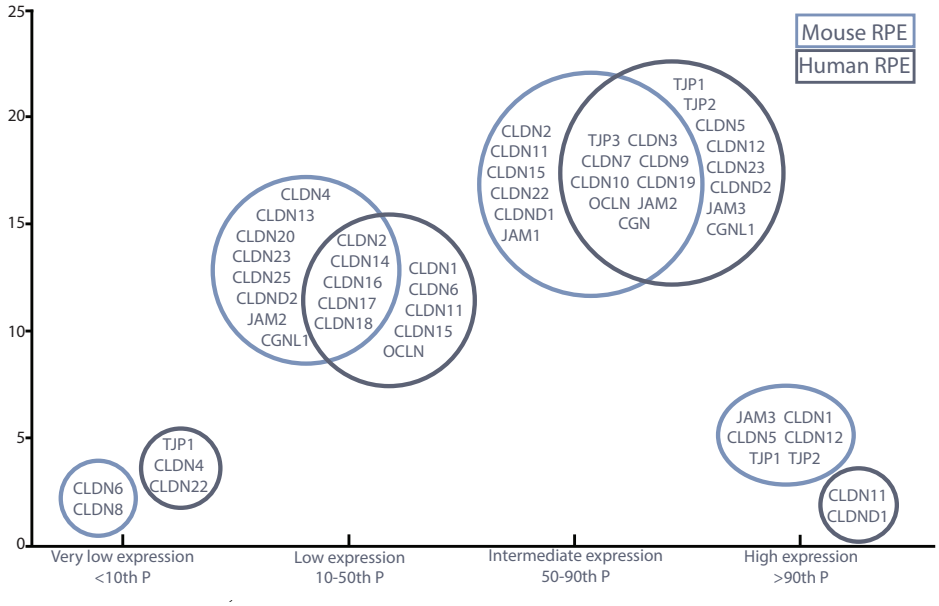

Figure. 6. This diagram depicts the tight junction gene expression of mouse and human RPE, divided in four categories: high expression (>90th percentile), moderate (50-90th percentile), low (10-50th percentile) and very low (<10th percentile). On the x-axis the four categories are displayed and on the y-axis the amount of genes found in a category is depicted. Light blue circles contain genes expressed in mouse RPE, Dark blue circles genes expressed in human RPE. Genes inside the overlapping parts of the circles are expressed in the RPE in both species in that category.

METHODS

Strengths and limitations of the study design

The technical and methodological strengths and limitations of this approach have been extensively discussed elsewhere[12,13]. Our lab has more than 10 years' experience in cellular microarray studies. In short, the strengths of this study include the use of selected healthy and freshly frozen samples with short post-mortem times. Sample preparation is characterized by minimal technical handling (such as mechanical or enzymatic dissociation, scraping, heating etc). In this way, the native "*in vivo*" gene expression profile is preserved. Next, we use laser dissection microscopy (LDM) of cryosections of the relevant cell-type. The use of the LDM ensures highly specific and homogeneous cell-type collection. After RNA isolation, we check RNA integrity and the quantity using the Agilent Bio-analyzer and Nanodrop during the procedure multiple times. Samples are labeled with Cy3 and Cy5 from the 3'-prime end to minimize effects from possible RNA degradation. RNA/samples that do not meet our quality criteria at any point in the

procedure are discarded of. We use a common reference design, which also serves as an internal technical control, and a large-scale 44k microarray.

There are also a number of limitations of our studies. Given the lengthy procedure of sample selection, procedure and extensive quality controls, we usually include a limited number of "the very best samples" in our final microarray analysis. The consequence is that we only can detect consistent similarities and differences (and not all, strongly variable or transient ones) in gene expression between samples.

Another limitation is that some degree of cellular contamination of adjacent cell layers in samples is unavoidable, even when we use meticulous laser dissection microscopy in the nicely structurally stacked retina. To overcome this problem we included the gene expression of the adjacent layers in our analysis: the so-called "double selection procedure" (Fig. 2)[12].

There are two limitations which are specific to this mouse-human study: The first is that there may be an oligo design difference for the comparative orthologous human and mouse genes on the Agilent whole Mouse and whole Human microarray. While, frequently, multiple different oligo's for a single gene and reference genes may be present on the micro-array, this may hamper direct comparison between mouse and human gene expression data. To overcome this problem, we ranked the gene expression data of each sample/species according to percentiles, and divided it into four expression groups: high, moderate, low and very low expression[13, 52]. By comparing the most extreme datasets, the high expressed genes with the very low expressed genes, between the two species; we could identify physiological relevant differences between the mouse and human RPE gene expressions, since these major differences could not be caused by different affinities alone. For further confirmation, we identified the genes that we described in our paper also by sqRT-PCR.

Finally, the mouse and human tissue used in this study had different post-mortem times: The mouse eyes were enucleated and embedded immediately after death, while for the donor eyes the post-mortem delay was between 16 and 22 hours. On the other hand, it has been shown that this has minimal effect on the RNA integrity of brain tissue. Up to 30 hours postmortem delay did not affect the mRNA[53]. During our experiments, we thoroughly checked the RNA integrity using BioAnalyzer, multiple times. In addition, since we designed labeling primers on the 3 prime end of the genes, potential starting degradation (first occurring at the 5 prime end) did not affect our gene expression results. A full description of the methodological (dis) advantages is beyond the scope of this paper. However, our approach enables us to determine highly specific RPE gene expression with a very limited amount of contamination, which is also corrected for in the analysis.

Mouse eyes, tissue processing and cell sampling

The study on mouse material was carried out in strict accordance with the recommendation in the Guide for the Care and Use of Laboratory Animals under the Dutch law, which is in accordance with the international declaration of Helsinki. The protocol was approved by the Committee on Ethical of Animal Experiments of the Netherlands Institute for Neuroscience (NIN), Royal Dutch Academy for Science (KNAW), the Netherlands (DEC protocol NIN 09.45). Mouse choroid, RPE and photoreceptors were obtained from eyes of healthy 5 months (-/+2 weeks) old C57BL/6 mice (J strain). We confirmed by sqRT-PCR that the mice of this sub strain (C57BL/6JOlaHsd) did not carry the rd8 mutation in the *Crb1* gene that has been found in C57BL/6N strains[54]. For each tissue we used 3 mouse eyes (and 3-6 selected human donor eyes). Mice were raised in a room with a temperature around 21˚C, on a 12:12-h light-dark cycle, and fed with standard pellet laboratory chow and water ad libitum. By the age of 5 months (-/+2 weeks) they were anesthetized with CO_2/O_2 and killed by cervical dislocation. The eyes were enucleated, embedded in OCT and snap frozen in liquid nitrogen. Until further use the eyes were stored in an -80˚C freezer. We selectively cut out the CH, RPE and PR with a laser dissection microscope (PALM Carl Zeiss, MicroImaging GmbH, Munich, Germany). For this the eyes were cut in 20uM cryosections for the photoreceptors and 12uM for the RPE and the choroid. For every sample one whole eye was used. We used Cresyl Violet staining to identify photoreceptor cells. Before the dissection of the choroid the RPE was removed to prevent as much contamination as possible. After processing the tissue and running the microarray, we determined the (low) variability of the samples using a multidimensional scaling plot (S6 Fig).

RNA isolation and amplification

RNA isolation, amplification and labelling procedures were carried out essentially as described elsewhere[14]. Quality of tRNA was checked with a Bioanalyzer assay (RNA 6000 Pico Kit, Agilent Technologies, Amstelveen, The Netherlands). RNA integrity numbers of tRNA of mouse CH ranged from 4.9 to 6.9, of the mouse RPE ranged from 4.9 to 7.2 and of mouse PR ranged from 5.5 to 7.6. In our microarray study we used a common reference design. The common reference was prepared from mouse RPE/choroid that was isolated, amplified using the same methodology as our experimental samples, and labelled with Cy3 (Cy3 mono-reactive dye pack, GE Healthcare UK, Little Chalfont, Buckinghamshire, UK).

See Janssen et al.[14] for a more detailed description of the laser dissection procedures, RNA processing and microarray procedures.

Microarray data analysis

The microarray files were analysed and processed using Agilent Feature Extraction Software (Agilent Technologies, version 9.5.3.1). We included examples of our strict quality control assessment of the hybridizations in the supplementary file S7 Fig and S8 Fig. Data were imported into R (version 2.14.0 for Windows, R Development Core Team, 2009) using LIMMA in the Bioconductor package. We studied the differences between the RPE and the photoreceptors making a statistical comparison using LIMMA for determining significantly changed genes (R package LIMMA, including Bayesian statistics). We did the same for RPE and the choroid:

Using a common reference design, in LIMMA we first estimated the difference between the sample (either CH / RPE / PR) and the common reference (hybridized against each other on a two channel array). Next, the differences between both RPE and CH or between RPE and PR were estimated. Subsequently, LIMMA fitted a linear model to the expression data for each gene. LIMMA uses empirical Bayes statistics to moderate the standard error of the estimated log-fold changes which results in more stable inference and improved power[55]. A fully detailed description of the script that was used in LIMMA is available upon request.

We selected the genes that had a positive fold change, meaning that they are higher expressed in the RPE than in either the photoreceptors or the choroid, of more than 2.5. Cut-off value for statistical significant difference was an adjusted p-value of less than 0.01 after Benjamini-Hochberg correction for multiple testing. Two data subsets were created that either contained all genes that show a significant higher expression in the RPE compared to the photoreceptors (RPE>PR), or a significant higher expression in the RPE compared to the choroid (RPE>CH). The volcano plots that visualize the symmetrical spread of the differentially expressed genes are included in S9 Fig. Next we compared these two subsets to determine the genes that are present in both lists using a comparison analysis in IPA (Ingenuity Systems). These represent RPE specifically expressed genes. To visualize these significant differences in gene expression levels we included a figure depicting the mean and (low) standard deviations (S10 Fig) and we included a figure of the mean and (the low) standard deviations of the genes mentioned in the discussions section (S11 Fig).

Data analysis of two microarray studies on the human RPE transcriptome

To detect possible contamination of the choroid and the photoreceptors in the list of Strunnikova et al we used the expression data of the human choroid and the human photoreceptors as determined within our group[12]. We assumed that the main source of contamination of RPE sample(s) using this methodology comes primarily from the set of highest expressed genes in either the PR or CH. Consequently, we determined the highest 10th percentile of the average gene expression for both the photoreceptors and

the choroid in Microsoft Excel. We ran a comparison analysis in IPA (Ingenuity Systems) to subtract the genes in the highest 10th percentile of photoreceptors and choroid from the 154 genes determined as RPE specific by Strunnikova et al[15]. We merged the two human RPE specific gene expression lists in Ingenuity using a comparison analysis.

Interspecies RPE signature genes

We compared the human RPE signature gene expression list, that we determined using the *Booij et al* list and the corrected *Strunnikova et al* list (Fig. 3), with our new mouse RPE signature gene expression. We ran a comparison analysis in Ingenuity, which uses the Entrez Gene identifier, to investigate which genes are found in both datasets and thus are interspecies specific.

Confirmation of gene expression data by sqRT-PCR

We confirmed our microarray data with sqRT-PCR. For a detailed description of the sqRT-PCR, see Janssen et al. 2013[13]. In short, sqRT-PCR was carried out using intron-spanning primers on cDNA from laser dissection microscopy derived samples, using three biological replicates. To minimize effects of RNA degradation artefacts, we generated primers near the 3'end of the gene. We quantified the gene expression in ImageJ and normalized expression by comparing it to the measured expression of housekeeping gene *Bact*.

Previously, we confirmed the human gene microarrays[12]. In the current study study, we selected a total of 27 genes to confirm our mouse microarray data. First, we selected randomly 11 highly expressed genes from our "Mouse RPE signature genes" dataset (*Bmp4, Rlbp1, Rgr, Krt18, Sgk3, Man1a, F3, Sulf1, Thsr, Col4a4, SLc16a8*). For 9 out of the 11 "Mouse RPE signature genes" we found the highest expression levels in the mouse RPE and the lowest in the CH and PR (Fig. 1). Only *Thsr* and *Slc16a8* showed highest expression in CH.

We next selected 8 well-established RPE specifically expressed genes (*Mertk, Rrh, Tyr, Rpe65, Rdh5, Lrat, Tjp1 and Trpm3*). For 7 out of 8 of the well-established RPE specific genes, we found highest expression in the mouse RPE and lower in the CH and PR (S1 Fig). Only *Tyr* showed highest expression in CH in our RT-PCR.

We also included sqRT-PCR for genes that were mentioned in the Discussion section to further technically validate our microarray. We selected 8 genes, 6 genes that are highly expressed (found in the highest 10th percentile of the mouse RPE microarray; *Sod1, Sod2, Slc39a4, Timp2, Col3a, Cldn1*) and 2 genes that are low expressed (found in the lowest 10th percentile of the mouse RPE microarray; *Cldn8, Hs6st1*). We compared the expression levels of these genes with the expression level of *Bact*. For all genes we found the expected confirmatory result (S2 Fig.). Overall, in this study, we confirmed the expression levels for 24 out of 27 genes (89%), which is in line with the cumulative

RT-PCR confirmation rate (87%) of all previous microarray studies (using similar tissue and methodology).

Author contributions

Conceived and designed the experiments: AB TGMFG PJvdS KB VMH AAB. Performed the experiments: AB JBtB. Analyzed the data: AB JBtB KB AAB. Contributed reagents/materials/analysis tools: TGMFG PJvdS. Wrote the paper: AB TGMFG VMH AAB.

Supplementary material

The supplementary files can be found online on PLOS ONE (https://doi.org/10.1371/journal.pone.0141597).

REFERENCES

1. Strauss O. The retinal pigment epithelium in visual function. Physiol Rev 2005;85(3):845–81.
2. Booij JC, Baas DC, Beisekeeva J, Gorgels TGMF, Bergen A a. B. The dynamic nature of Bruch's membrane. Prog Retin Eye Res 2010;29(1):1–18.
3. Schlingemann RO. Role of growth factors and the wound healing response in age-related macular degeneration. Graefes Arch Clin Exp Ophthalmol Albrecht Von Graefes Arch Für Klin Exp Ophthalmol 2004;242(1):91–101.
4. De Jong PTVM. Age-related macular degeneration. N Engl J Med 2006;355(14):1474–85.
5. Hawkins BS, Bird A, Klein R, West SK. Epidemiology of age-related macular degeneration. Mol Vis 1999;5:26.
6. Age-Related Eye Disease Study 2 Research Group. Lutein + zeaxanthin and omega-3 fatty acids for age-related macular degeneration: the Age-Related Eye Disease Study 2 (AREDS2) randomized clinical trial. JAMA 2013;309(19):2005–15.
7. Schmidl D, Garhöfer G, Schmetterer L. Nutritional supplements in age-related macular degeneration. Acta Ophthalmol (Copenh) 2015;93(2):105–21.
8. Campa C, Harding SP. Anti-VEGF compounds in the treatment of neovascular age related macular degeneration. Curr Drug Targets 2011;12(2):173–81.
9. Stanzel BV, Liu Z, Somboonthanakij S, et al. Human RPE stem cells grown into polarized RPE monolayers on a polyester matrix are maintained after grafting into rabbit subretinal space. Stem Cell Rep 2014;2(1):64–77.
10. Maeda T, Lee MJ, Palczewska G, et al. Retinal pigmented epithelial cells obtained from human induced pluripotent stem cells possess functional visual cycle enzymes in vitro and in vivo. J Biol Chem 2013;288(48):34484–93.
11. Schwartz SD, Hubschman J-P, Heilwell G, et al. Embryonic stem cell trials for macular degeneration: a preliminary report. Lancet 2012;379(9817):713–20.
12. Booij JC, ten Brink JB, Swagemakers SMA, et al. A new strategy to identify and annotate human RPE-specific gene expression. PloS One 2010;5(5):e9341.
13. Janssen SF, van der Spek SJF, Brink JB Ten, et al. Gene expression and functional annotation of the human and mouse choroid plexus epithelium. PloS One 2013;8(12):e83345.
14. Janssen SF, Gorgels TGMF, Bossers K, et al. Gene expression and functional annotation of the human ciliary body epithelia. PloS One 2012;7(9):e44973.
15. Strunnikova NV, Maminishkis A, Barb JJ, et al. Transcriptome analysis and molecular signature of human retinal pigment epithelium. Hum Mol Genet 2010;19(12):2468–86.
16. Wang J, Duncan D, Shi Z, Zhang B. WEB-based GEne SeT AnaLysis Toolkit (WebGestalt): update 2013. Nucleic Acids Res 2013;41(Web Server issue):W77–83.
17. Coffey PJ, Gias C, McDermott CJ, et al. Complement factor H deficiency in aged mice causes retinal abnormalities and visual dysfunction. Proc Natl Acad Sci U S A 2007;104(42):16651–6.
18. Nozaki M, Raisler BJ, Sakurai E, et al. Drusen complement components C3a and C5a promote choroidal neovascularization. Proc Natl Acad Sci U S A 2006;103(7):2328–33.
19. Hollyfield JG, Bonilha VL, Rayborn ME, et al. Oxidative damage-induced inflammation initiates age-related macular degeneration. Nat Med 2008;14(2):194–8.
20. Ambati J, Anand A, Fernandez S, et al. An animal model of age-related macular degeneration in senescent Ccl-2- or Ccr-2-deficient mice. Nat Med 2003;9(11):1390–7.
21. Beatty S, Koh H-H, Phil M, Henson D, Boulton M. The Role of Oxidative Stress in the Pathogenesis of Age-Related Macular Degeneration. Surv Ophthalmol 2000;45(2):115–34.

22. Anderson DH, Mullins RF, Hageman GS, Johnson LV. A role for local inflammation in the formation of drusen in the aging eye. Am J Ophthalmol 2002;134(3):411–31.
23. Nita M, Grzybowski A, Ascaso FJ, Huerva V. Age-related macular degeneration in the aspect of chronic low-grade inflammation (pathophysiological parainflammation). Mediators Inflamm 2014;2014:930671.
24. Behndig A, Svensson B, Marklund SL, Karlsson K. Superoxide dismutase isoenzymes in the human eye. Invest Ophthalmol Vis Sci 1998;39(3):471–5.
25. Newsome DA, Dobard EP, Liles MR, Oliver PD. Human retinal pigment epithelium contains two distinct species of superoxide dismutase. Invest Ophthalmol Vis Sci 1990;31(12):2508–13.
26. Karunadharma PP, Nordgaard CL, Olsen TW, Ferrington DA. Mitochondrial DNA damage as a potential mechanism for age-related macular degeneration. Invest Ophthalmol Vis Sci 2010;51(11):5470–9.
27. Kondo N, Bessho H, Honda S, Negi A. SOD2 gene polymorphisms in neovascular age-related macular degeneration and polypoidal choroidal vasculopathy. Mol Vis 2009;15:1819–26.
28. Tokarz P, Kaarniranta K, Blasiak J. Role of antioxidant enzymes and small molecular weight antioxidants in the pathogenesis of age-related macular degeneration (AMD). Biogerontology 2013;14(5):461–82.
29. Kimura K, Isashiki Y, Sonoda S, Kakiuchi-Matsumoto T, Ohba N. Genetic association of manganese superoxide dismutase with exudative age-related macular degeneration. Am J Ophthalmol 2000;130(6):769–73.
30. Pennesi ME, Neuringer M, Courtney RJ. Animal models of age related macular degeneration. Mol Aspects Med 2012;33(4):487–509.
31. Justilien V, Pang J-J, Renganathan K, et al. SOD2 knockdown mouse model of early AMD. Invest Ophthalmol Vis Sci 2007;48(10):4407–20.
32. Seo S, Krebs MP, Mao H, Jones K, Conners M, Lewin AS. Pathological consequences of long-term mitochondrial oxidative stress in the mouse retinal pigment epithelium. Exp Eye Res 2012;101:60–71.
33. Imamura Y, Noda S, Hashizume K, et al. Drusen, choroidal neovascularization, and retinal pigment epithelium dysfunction in SOD1-deficient mice: a model of age-related macular degeneration. Proc Natl Acad Sci U S A 2006;103(30):11282–7.
34. Lengyel I, Flinn JM, Peto T, et al. High concentration of zinc in sub-retinal pigment epithelial deposits. Exp Eye Res 2007;84(4):772–80.
35. Flinn JM, Kakalec P, Tappero R, Jones B, Lengyel I. Correlations in distribution and concentration of calcium, copper and iron with zinc in isolated extracellular deposits associated with age-related macular degeneration. Met Integr Biometal Sci 2014;6(7):1223–8.
36. Newsome DA, Swartz M, Leone NC, Elston RC, Miller E. Oral zinc in macular degeneration. Arch Ophthalmol 1988;106(2):192–8.
37. Smailhodzic D, van Asten F, Blom AM, et al. Zinc supplementation inhibits complement activation in age-related macular degeneration. PloS One 2014;9(11):e112682.
38. Buschini E, Fea AM, Lavia CA, et al. Recent developments in the management of dry age-related macular degeneration. Clin Ophthalmol Auckl NZ 2015;9:563–74.
39. Chew EY, Clemons T, SanGiovanni JP, et al. The Age-related Eye Disease Study 2 (AREDS2): Study Design and Baseline Characteristics (AREDS2 Report Number 1). Ophthalmology 2012;119(11):2282–9.
40. Leung KW, Liu M, Xu X, Seiler MJ, Barnstable CJ, Tombran-Tink J. Expression of ZnT and ZIP Zinc Transporters in the Human RPE and Their Regulation by Neurotrophic Factors. Invest Ophthalmol Vis Sci 2008;49(3):1221–31.
41. Dufner-Beattie J, Weaver BP, Geiser J, et al. The mouse acrodermatitis enteropathica gene Slc39a4 (Zip4) is essential for early development and heterozygosity causes hypersensitivity to zinc deficiency. Hum Mol Genet 2007;16(12):1391–9.

42. Anderson DH, Radeke MJ, Gallo NB, et al. The Pivotal Role of the Complement System in Aging and Age-related Macular Degeneration: Hypothesis Re-visited. Prog Retin Eye Res 2010;29(2):95–112.
43. Chavali VRM, Khan NW, Cukras CA, Bartsch D-U, Jablonski MM, Ayyagari R. A CTRP5 gene S163R mutation knock-in mouse model for late-onset retinal degeneration. Hum Mol Genet 2011;20(10):2000–14.
44. Shu X, Luhmann UFO, Aleman TS, et al. Characterisation of a C1qtnf5 Ser163Arg Knock-In Mouse Model of Late-Onset Retinal Macular Degeneration. PLoS ONE 2011;6(11):e27433.
45. Luo C, Chen M, Xu H. Complement gene expression and regulation in mouse retina and retinal pigment epithelium/choroid. Mol Vis 2011;17:1588–97.
46. Hughes AE, Orr N, Esfandiary H, Diaz-Torres M, Goodship T, Chakravarthy U. A common CFH haplotype, with deletion of CFHR1 and CFHR3, is associated with lower risk of age-related macular degeneration. Nat Genet 2006;38(10):1173–7.
47. Hageman GS, Hancox LS, Taiber AJ, et al. Extended haplotypes in the complement factor H (CFH) and CFH-related (CFHR) family of genes protect against age-related macular degeneration: Characterization, ethnic distribution and evolutionary implications. Ann Med 2006;38(8):592–604.
48. Fritsche LG, Lauer N, Hartmann A, et al. An imbalance of human complement regulatory proteins CFHR1, CFHR3 and factor H influences risk for age-related macular degeneration (AMD). Hum Mol Genet 2010;19(23):4694–704.
49. Rizzolo LJ. Barrier properties of cultured retinal pigment epithelium. Exp Eye Res 2014;126:16–26.
50. Kaur C, Foulds WS, Ling EA. Blood-retinal barrier in hypoxic ischaemic conditions: basic concepts, clinical features and management. Prog Retin Eye Res 2008;27(6):622–47.
51. Bailey TA, Kanuga N, Romero IA, Greenwood J, Luthert PJ, Cheetham ME. Oxidative stress affects the junctional integrity of retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 2004;45(2):675–84.
52. Booij JC, van Soest S, Swagemakers SMA, et al. Functional annotation of the human retinal pigment epithelium transcriptome. BMC Genomics 2009;10:164.
53. Ervin JF, Heinzen EL, Cronin KD, et al. Postmortem delay has minimal effect on brain RNA integrity. J Neuropathol Exp Neurol 2007;66(12):1093–9.
54. Mattapallil MJ, Wawrousek EF, Chan C-C, et al. The Rd8 mutation of the Crb1 gene is present in vendor lines of C57BL/6N mice and embryonic stem cells, and confounds ocular induced mutant phenotypes. Invest Ophthalmol Vis Sci 2012;53(6):2921–7.
55. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004;3:Article3.

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General Discussion

Challenges and complexities of cell replacement therapies for retinal pigment epithelium degenerative disorders.

5

A PROMISING CLINICAL LANDSCAPE AND ITS CHALLENGES

The key role of the RPE in maintaining retinal functioning, and the involvement in several retinal degenerative disorders, such as AMD, Stargardt disease and some forms of retinitis pigmentosa, make it the main focus of many studies on cell replacement therapy.

At this time the precise etiology of AMD is unclear and we know that the disease is both phenotypically and genetically heterogeneous. Usually many risk factors, such as smoking, diet and variation in several genes, contribute to the onset and progression of the disease. Given this heterogeneity, it is difficult to target a single entity that may cure or postpone the disease effectively. We are currently not able to tackle the cause/origin of the disease but a restorative strategy through cell replacement has great potential. In contrast with pharmacological approaches or gene therapy, replacing dysfunctional retinal cells with healthy cells may not only lighten the symptoms but also possibly cure the disease. Despite the lack of fundamental knowledge clinical trials have started all over the world[1–3]. It is very exciting to have a stem cell therapy already this far in development. However, there is still a lot to be learned about the different cell sources that can be used, the methodology for transplantation and whether combination therapy provides better results than solely cell transplantation. In this chapter I will discuss these issues and contemplate what we could do to improve the road towards the clinic.

POSSIBLE CELL SOURCES FOR REPLACEMENT THERAPY

Many different cell sources are currently under investigation for RPE replacement therapy. A wonderful advantage of using pluripotent stem cells is their unique property of self-renewal and differentiation into many cell types, including the RPE. Two types of pluripotent stem cells, ESC and iPSC, share their potential to differentiate into any cell type derived from the three germ layers. They function as an unlimited source, compared to the scarcity of useful donor material.

Pluripotent stem cells

The ESC have paved the way for developing stem cell therapies. However, they have two major disadvantages. First, an ethical dilemma is raised by the use of a human embryo to derive the cells from. Second, the consideration of the host immune response to the transplanted cells, which could be a major problem in the clinic. The iPSC provide a solution to these problems. They appear to have the same properties and potential as ESC, but their generation is not dependent upon a source of embryos. Also, iPSC can be derived from autologous tissue, matching the human leukocyte antigens (HLA) profile

of the patient thereby preventing a (severe) graft rejection. On the other hand, there are several concerns for the use of iPSC at this point in time, such as that it is time consuming and costly, the variation between iPSC lines, the lack of a standardized strategy for reprogramming, the concern whether aged somatic cells have the capacity to rejuvenate sufficiently and their potential for uncontrolled cell proliferation and division.

iPSC banking

It would be ideal to produce autologous iPSC for treatment of each patient, but it is time-consuming to reprogram the cells, characterize them and test the cells for safety. This also makes it very expensive and thus difficult to use in the clinic. As an alternative an iPSC bank is considered. Here clinical grade iPSC lines should be available that are tested for functionality and safety with a known HLA genotype. But the high variability of HLA genotypes may pose a problem to developing such a bank[4,5]. Currently it there is no consensus on whether such an iPSC bank would be worth starting.

iPSC: Variation in reprogramming strategies

First, to use iPSC in the clinic we need well defined quality standards. At this time, there is a lot of variation in generation of iPSC: different combinations of reprogramming factors; vehicles for exogenous genes; and cell types used to generate the iPSC. The first reprogramming factors that were used are called the Yamanaka factors, a combination of *OCT3/4, SOX2, KLF4* and *C-MYC*[6]. After this first amazing discovery several new combinations of factors were studied, to improve reprogramming efficiency or to reduce tumorigenicity. Factors that were included in these variations are NANOG, LIN28, L-MYC, GLIS1[7–9]. Currently, it is still unclear what the best combination of reprogramming factors is and how the selection of factors influences the differentiation of the iPSC.

Also, there is a diversity of available gene delivery vehicles used in reprogramming[10]. Initially, viral vectors were used to deliver the reprogramming factors to somatic cells. Indeed, retro- and lenti-viruses yielded a good efficiency but their permanent genomic integration remains a problem for clinical use of iPSC because this could potentially invoke genome instability or disrupt functional gene expression. To overcome problems with genome integration and to improve reprogramming efficiency various other methods were developed. This includes the use of Sendai virus, episomal virus, piggyback transposon, RNA delivery, protein delivery and small molecules[11–21]. Currently many studies focus on determining the most efficient method to reprogram cells trying to avoid genomic integration.

Finally, we currently lack a standard cell source used for generating iPSC. The first iPSC were made from fibroblasts, since then various cell sources have been used. These include mesenchymal stem cells, peripheral blood cells, urine-derived cells, nasal epithelial cells, and more[22]. The choice of a somatic cell source can have a significant

impact on the potential of an iPSC-based therapy. Among other reasons, the proliferative capacity of cells seems to influence the reprogramming efficiency. It appears that generation of iPSC is easier when actively dividing cells are used.
If we want to take iPSC to the clinic for the treatment of AMD we need guidelines to select the most appropriate reprogramming strategy and donor source because these factors can affect the quality of the iPSC. This should be investigated specifically for the development of RPE cells because the reprogramming approach can influence the subsequent differentiation process.

Is age a problem for using iPSC?

Another factor to consider is the age of the cells that are used for reprogramming, as embryonic tissues are known to be more suitable for reprogramming than adult or aged tissues[23]. Cellular senescence, accumulated damage and shortened telomeres are associated with aging and may impair reprogramming efficiency when compared to younger cells. While many well-characterized iPSC lines are derived from young donor cells, iPSC therapy for age related diseases such as AMD (must) use somatic cells of aged patients. This may be a suboptimal starting point but iPSC lines have been generated from aged cells successfully according to multiple studies (for a nice overview see *Mahmoudi et al, 2012*[24]). It is critical that the cells are reprogrammed to a youthful state and that accumulated damage is cleared. When using aged cells, an important question is whether reprogramming can erase the characteristics associated with aging, characteristics like reduced telomere length, mitochondrial dysfunction, oxidative stress and epigenetic alterations.

Telomere Length

Often used as a hallmark for the aging of a cell is chromosomal telomere length. Telomeres are specialized repetitive DNA sequences at the end of the linear chromosomes that serve to maintain the integrity of the chromosomes. Telomerase activity maintains the telomere length. Without it the length progressively shortens which eventually limits the growth of cells and short telomere length and telomerase inactivity corresponds to aged somatic cells. Several studies reported that reprogramming somatic cells, including both young and aged cells, increases telomerase activity and telomere length to an ESC comparable state[25,26]. There is however, a lot of heterogeneity among the various iPSC cell lines. Interestingly, also the ESCs show variability in telomere length and it seems that for both pluripotent cell types the telomere length is correlated with pluripotency and proliferation efficiency[27]. At this point it is unclear what underlies this variation but it does not seem to be the age of donor cells. It might be the differences in reprogramming protocols and materials that are used or rather the variability between donors. This supports the need for standardized strategy to generate iPSC.

Epigenetic Memory

Another concern is the epigenetic memory of the iPSC. Each cell type has an individual epigenome: a certain set and pattern of posttranslational histone modifications and DNA methylation, and the presence of specific small non-coding RNAs. Epigenetic modifications play an important role in aging and age related pathologies[28]. There are studies reporting that reprogramming can leave an epigenetic memory of the tissue of origin that might influence the subsequent differentiation towards the wanted cell type[29]. Several groups have tested the effect of epigenetic memory on differentiation potential of iPSC derived from various donors and show that the variability of the donors exceeds the variability of tissue type[30,31]. One study showed this effect specifically for RPE differentiation from iPSC lines derived from both fetal and aged donor cell types[32]. Thus, though iPSC retain some traits of their initial somatic epigenomes, this is a negligible factor on their differentiation capability. This indicates that the epigenetic memory is probably not a problem for the use of iPSC for the treatment of age related diseases such as AMD.

Oxidative Stress

Another component of aging is the role of the mitochondria, which are the principle source of intracellular reactive oxygen species (ROS). In normal aging somatic cells have mitochondrial dysfunction and oxidative stress.

Compared to somatic cells, ESC exhibit low levels of ROS and mitochondrial activity. In multiple studies reprogramming changed the mitochondrial state to a state comparable to the ESC[33,34]. Interestingly the age of the donor cells does not influence this change after reprogramming. One study reported more mixed results and found that the iPSC are not completely identical to ESC but they do however cluster more closely to the ESCs than to somatic cells[35]. So even though the results are mixed, they all indicate a rejuvenating process for iPSC. This rejuvenating process could prove very beneficial for AMD, since the mitochondria of the RPE are severely damaged and the amount of mitochondria decreased, even more than seen with normal aging[36] and we know it is accompanied by enormous oxidative stress[37,38]. The damaged mitochondria in AMD are hypothesized to play a role in the pathogenesis and are a potential target for treatment[39].

Tumorigenicity

In addition, an important concern of the use of iPSC is the risk of uncontrolled cell growth, related to their praised characteristics of self-renewal and pluripotency[40]. This has been a hurdle for the introduction of iPSC-derived cell therapy in the clinic. The clinical trials that are currently ongoing therefore focus on the safety of the transplantation of pluripotent stem cells and the results have been positive. To extensively examine

the possibility of tumor development before transplantation a proper tumorigenicity test should be made[41].

Transdifferentiation

One possibility to overcome this difficulty of tumorigenicity is the use of lineage reprogramming of somatic cells. Here the pluripotent state is surpassed and cells are directly differentiated towards RPE. Thereby they could reduce the risk of tumorigenicity after transplantation that is related to the use of pluripotent stem cells. Transdifferentiation is a rapidly progressing field but the molecular mechanisms underlying such conversion need to be better understood. Interestingly, there already was a study conducted where human fibroblasts were directly converted towards *BEST* expressing cells that exhibit pigment and a cobblestone morphology[42,43]. Nonetheless, some major hurdles remain. The derived RPE seems to be at a progenitor stage thus maturation may be problematic. Also the cells haven not been tested on functionality. Not much is known about the influence of the epigenetic signature of the source cell type on the process of transdifferentiation, but currently it is thought that an embryological origin that is common to both the source and the desired cell type might facilitate the transdifferentiation. In **chapter 3** we describe the differences between the IE and RPE to uncover the differences and similarities. This may contribute to developing an optimal reprogramming strategy. At the same time it is important for therapeutic purposes to safely transdifferentiate cells and avoid genetic manipulations but use different strategies for the conversion. Even though lineage reprogramming is a very promising field it is still in its infancy.

Authentication of the RPE

Whatever cell source is selected for cell replacement therapy in RPE degenerative disorders, whether the RPE cells are derived from pluripotent stem cells or from iris epithelium, for a safe and functional treatment the cells need to be properly authenticated before transplantation. There is a lot of attention for the production and transplantation of RPE cells, but, surprisingly, less attention is given to extensively characterize these cells.

The characterization usually relies on the presence of pigmentation, cobblestone morphology, a handful of RPE specific markers and *in vitro* phagocytosis assays. This characterization does not include determining the presence of other characteristics of the RPE that are important for proper functioning in the retina. Functions like the spatial buffering of K+ and the secretion of (growth) factors that are important to provide structural integrity of the retina. As we discussed in **chapter 2**, markers such as morphology and pigmentation may not be very effective to determine the maturity of developing RPE cells. We differentiated hESC to hESC-RPE cells and validated their character based on

commonly used strategies (presence of pigmentation, the morphology, gene expression of RPE markers, immunocytochemistry of RPE markers and POS phagocytosis assay). When we compared the extensive gene expression profiles of hESC-RPE cells in an early and late stage of pigmentation we did not find clear differences. However, when we compared these hESC-RPE to endogenous RPE we found obvious differences. This suggests that even though cells look similar in important features of human RPE, they can still be very different. We do not yet know whether this difference is an obstacle for transplantation, or what is the optimal differentiation state of the cells.

Replacement of solely RPE or also other cell types?

Within the field of (stem) cell-based therapy for retinal diseases, the focus usually lies on the replacement of the RPE, and sometime the PR. But, the retina is a complex multilayered structure and secondary effects of AMD are most likely death of PR, disorganization of the deeper retinal layers and thinning of the choroid. Rejuvenating or replacing the RPE is a good place to start but when the secondary effects have made their entry, solely RPE cell transplantation is probably not enough. Dual cell replacement of RPE together with PR is most likely the best strategy in the advanced cases of AMD. A few studies describe the development of a layered optic cup structure *in vitro* from pluripotent stem cells[44–46]. This development may be a potential approach towards dual cell replacement therapy for patients with severe loss of RPE and PR.

Another promising development towards dual cell replacement is the possibility to generate PR from pluripotent stem cells. These are developed primarily for retinal degeneration of the PR in diseases such as Retinitis Pigmentosa and are able to integrate into the retina of a mouse model[47]. However, in the future it might be ideal to co-culture the *in vitro* developed RPE and PR cells before transplantation. This way the RPE and PR can be developed in a controlled manner and tested for their ability to make connections with other cells which is important for proper integration in the retina.

HOW DO WE INTRODUCE REGENERATION?

Transplantation of RPE has a long history that starts with the transplantation of autologous and donor RPE[48,49]. Two main strategies have been developed to deliver RPE cells in the subretinal space: Injecting dissociated RPE cells as a bolus and transplanting a RPE monolayer to the subretinal space.

Single cells or monolayer

Following the first strategy, the cells are injected as a bolus, in cell suspension. The advantages of delivery in cell suspension are that it confers minimal surgical trauma,

it is a relatively easy surgical procedure and has shown to preserve visual function in animal models[50,51]. A drawback is that the cells do not form a confluent monolayer, but they often clump together and can evoke an immune response that causes the cells to die[52,53].

One clinical trial for AMD with stem cell derived RPE delivered in suspension, reports that the patients tolerated the transplant and besides postoperative infectious endophthalmitis experienced no other negative effects[1,54]. Although the visual acuity of the treated patients did not improve, the results indicate that hESC-RPE derived cells could serve as potentially safe therapeutic strategy for AMD. This is an important first step in taking stem cells derived RPE cells to the clinic. However, the final aim is to enhance vision and therefore the second transplantation strategy may be more appropriate.

In the second approach the cells are transplanted as a monolayer, possibly supported by a scaffold. Cells delivered in suspension distribute unevenly and form clumps while the cell on scaffold most likely integrate as a confluent monolayer. Further, these scaffolds provide a substrate for the RPE cells which could be necessary since RPE cell suspensions may fail to survive on damaged Bruch's membrane[55,56]. A study comparing both transplantation strategy reports significant improvement of survival of polarized monolayers of hESC-RPE[53]. One more advantage of the monolayer transplantation is related to the immune response after surgery. The low immunogenicity is considered a major advantage of transplantation in the eye. But, the presence of an intact and healthy RPE maintains the blood retinal barrier and seems to be critical for this immune privilege[57]. Transplanting the RPE cells on a scaffold may not only improve the survivability but may also help maintain blood-retinal barrier integrity and thereby reduce the immune response after transplantation.

The attachment, integration, viability and function of the cells depend on many characteristics of the scaffold. Currently, there is a great amount of variation in the types of scaffolds ranging from natural to synthetic substrates (for an overview see *Jha and Bharti, 2015*[58]). Scaffolds made of natural polymers closely mimic the native extracellular matrix that surrounds the RPE in the healthy *in vivo* situation and is biocompatible. However, they are mostly not xeno-free and therefore not available for use in the clinic. The synthetic scaffolds are more easily available and can be adjusted to change certain properties, like thickness, surface topography, mechanical properties and degradation characteristics[59]. Many combinations between several sorts of scaffolds and stem cells derived RPE cells can be made and need to be investigated for efficacy and safety. I opt for developing a standardized strategy to bring cell replacement therapy into the clinic and thus we need to investigate which synthetic scaffold gives the optimal result.

Combined therapy

The treatments that are available now for AMD are ones that use preservation strategies to halt or slow down the progression of the disease and maintain the remaining visual function (see Table 1). Although these therapies may be of help in slowing down the progression of the disease, they do not restore already lost visual function.

The expectancy is that replacing the damaged tissue with rejuvenated cells will be enough to alleviate the symptoms of the disease. However, it could prove even more beneficial to combine cell replacement therapy with another therapeutic strategy. Also for the monogenic cases, such as Stargardt disease, and for AMD patients with a clear genetic risk factor, additional gene therapy should be considered as an overlapping approach. Of course, first we need to focus on the safety and efficacy of the stem cell treatment but in the future a patient specific and combined approach should be the aim.

Table 1. Overview of therapeutic strategies used to treat AMD.

Approach	Point of Action
Nutritional Supplements	According to the AREDS (Age-Related Eye Disease Study), a specific intake of a certain combination of nutrients can reduce the risk of developing advanced AMD.The AREDS supplementation was effective in high-risk patients, reducing the risk of AMD progression significantly.
Anti-inflammatory Drugs	Chronic inflammation is thought to be crucial in AMD pathogenesis ref. Several anti-inflammatory drugs are currently investigated for the treatment of AMD.
Choroidal blood flow restoration agents	Choroidal circulation plays an important role in the maintainence of a =healthy retina, by removing waste and providing nutrients from and to the RPE and other retinal layers. Several vasodilators are investigated with the rationale that the use may improve the blood flow and thus slow down the progression of AMD.
Visual cycle modification	A prominent feature of AMD is the accumulation of lipofuscin in the subretinal space due to a reduced uptake and elimination of this waste product by the RPE. Several visual cycle inhibitors are studied with the aim to reduce the accumulation of the toxic compounds that are produced during the visual cycle.
Neuroprotective agents	Neuroprotective drugs are aimed at the preservation of visual function by preventing apoptosis of RPE cells and photoreceptors.
Anti-angiogenic agents	Anti-angiogenic agents can be used to prevent abnormal growth of retinal blood vessels. These vessels may leak and cause visual loss. These agents can be applied by regular injections in the eye and gene therapy approaches are being investigated.

MODEL CONCEPTUALIZATION

Finding a suitable animal model

For the development of a therapy for AMD an accurate animal model is very useful. An ideal animal model is inexpensive, shows the histological and functional changes that are related to the disease, and evolves in a rapid time course. Because AMD is a multifactorial disease, developing a representative animal model for AMD is extremely challenging. It is important to specifically suit the animal model for the therapy that needs to be tested, which may mean that multiple models are needed that represent different aspects of the disease[60]. For (different aspects of) AMD models have been created in mice, rats, rabbits, pigs and non-human primates. The advantage of using rodents is the relatively low cost, the quick disease progression and the relative ease to perform genetic manipulation. But, a disadvantage is their lack of a macula. Nevertheless, the use of mice and rodents will give insight into the basic physiology and functioning of the RPE and there are many rodent models that each approach different aspects of AMD (for an overview see the review by *Pennesi et al, 2012*[60]).

Although mice and rat models have proven indispensable to preclinical studies for AMD treatment, for some studies it may be useful to have bigger eyes. A rabbit model is very suitable in that case, for example to explore surgical techniques[61]. But also the rabbit eye does not contain a structure like the macula. In humans the macula is severely affected by AMD, thus for some studies it may be necessary to have this structure present in the animal model as well. Pig eyes are very similar to human eyes in size and also present a macula-like structure[62]. Non-human primates offer the best resemblance to the anatomy and functioning of humans, but apart from ethical considerations, they are very costly, difficult to genetically manipulate, have a long life span, a slow disease progression and little offspring.

Overall, the use of rodent models has many advantages over other animals because it enables testing various aspects of the complex disease AMD. They are very important for the development of new therapeutic strategies for AMD, because they are able to accurately recreate most histological and pathological feature of the disease and serve as a great platform to test new therapeutic strategies. Nonetheless, it is important to pay attention to the differences and similarities between the species before developing an animal model. An approach is to study the transcriptomes and functional annotations of animal RPE and human RPE, as we discussed in **chapter 4** for the mouse. We studied multiple characteristics of the RPE that are involved in the pathogenesis of AMD and this may provide important for the development of translational studies and mouse models for AMD.

CONCLUSIONS

In conclusion, even though the use of stem cell derived RPE cells and transdifferentiated cells have a unique potential for regenerative therapy in AMD, there is a lack of deep knowledge to systematically test their therapeutic potential. Currently the first clinical trials are ongoing. These studies are pioneering work in the move towards clinical application. But to ensure reproducibility and consistency, we need standardized protocols. Also for optimal effect we need more knowledge of the functionalities of the *in vitro* produced RPE cells at various differentiation stages.

REFERENCES

1. Schwartz SD, Hubschman J-P, Heilwell G, et al. Embryonic stem cell trials for macular degeneration: a preliminary report. The Lancet 2012;379(9817):713–20.
2. Takahashi M. [Retinal Cell Therapy Using iPS Cells]. Nippon Ganka Gakkai Zasshi 2016;120(3):210–24; discussion 225.
3. Shim SH, Kim G, Lee DR, Lee JE, Kwon HJ, Song WK. Survival of Transplanted Human Embryonic Stem Cell–Derived Retinal Pigment Epithelial Cells in a Human Recipient for 22 Months. JAMA Ophthalmol 2017;135(3):287–90.
4. Barry J, Hyllner J, Stacey G, Taylor CJ, Turner M. Setting Up a Haplobank: Issues and Solutions. Curr Stem Cell Rep 2015;1(2):110–7.
5. De Rham C, Villard J. Potential and Limitation of HLA-Based Banking of Human Pluripotent Stem Cells for Cell Therapy [Internet]. J. Immunol. Res. 2014 [cited 2017 Aug 19];Available from: https://www.hindawi.com/journals/jir/2014/518135/
6. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126(4):663–76.
7. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007;318(5858):1917–20.
8. Yoshioka N, Gros E, Li H-R, et al. Efficient generation of human iPSCs by a synthetic self-replicative RNA. Cell Stem Cell 2013;13(2):246–54.
9. Nakagawa M, Takizawa N, Narita M, Ichisaka T, Yamanaka S. Promotion of direct reprogramming by transformation-deficient Myc. Proc Natl Acad Sci U S A 2010;107(32):14152–7.
10. Revilla A, González C, Iriondo A, et al. Current advances in the generation of human iPS cells: implications in cell-based regenerative medicine. J Tissue Eng Regen Med 2016;10(11):893–907.
11. Rim YA, Nam Y, Ju JH. Induced Pluripotent Stem Cell Generation from Blood Cells Using Sendai Virus and Centrifugation. J Vis Exp JoVE 2016;(118).
12. Seki T, Yuasa S, Oda M, et al. Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. Cell Stem Cell 2010;7(1):11–4.
13. Yu J, Hu K, Smuga-Otto K, et al. Human induced pluripotent stem cells free of vector and transgene sequences. Science 2009;324(5928):797–801.
14. Chen G, Gulbranson DR, Hou Z, et al. Chemically defined conditions for human iPSC derivation and culture. Nat Methods 2011;8(5):424–9.
15. Drozd AM, Walczak MP, Piaskowski S, Stoczynska-Fidelus E, Rieske P, Grzela DP. Generation of human iPSCs from cells of fibroblastic and epithelial origin by means of the oriP/EBNA-1 episomal reprogramming system. Stem Cell Res Ther 2015;6:122.
16. Woltjen K, Hämäläinen R, Kibschull M, Mileikovsky M, Nagy A. Transgene-free production of pluripotent stem cells using piggyBac transposons. Methods Mol Biol Clifton NJ 2011;767:87–103.
17. Anokye-Danso F, Trivedi CM, Juhr D, et al. Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. Cell Stem Cell 2011;8(4):376–88.
18. Bao X, Zhu X, Liao B, et al. MicroRNAs in somatic cell reprogramming. Curr Opin Cell Biol 2013;25(2):208–14.
19. Cho H-J, Lee C-S, Kwon Y-W, et al. Induction of pluripotent stem cells from adult somatic cells by protein-based reprogramming without genetic manipulation. Blood 2010;116(3):386–95.
20. Lin T, Ambasudhan R, Yuan X, et al. A Chemical Platform for Improved Induction of Human iPS Cells. Nat Methods 2009;6(11):805–8.

21. Hou P, Li Y, Zhang X, et al. Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. Science 2013;341(6146):651–4.
22. Raab S, Klingenstein M, Liebau S, Linta L. A Comparative View on Human Somatic Cell Sources for iPSC Generation. Stem Cells Int [Internet] 2014;2014. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4241335/
23. Rohani L, Johnson AA, Arnold A, Stolzing A. The aging signature: a hallmark of induced pluripotent stem cells? Aging Cell 2014;13(1):2–7.
24. Mahmoudi S, Brunet A. Aging and reprogramming: a two-way street. Curr Opin Cell Biol 2012;24(6):744–56.
25. Marion RM, Strati K, Li H, et al. Telomeres acquire embryonic stem cell characteristics in induced pluripotent stem cells. Cell Stem Cell 2009;4(2):141–54.
26. Suhr ST, Chang EA, Rodriguez RM, et al. Telomere dynamics in human cells reprogrammed to pluripotency. PloS One 2009;4(12):e8124.
27. Huang J, Wang F, Okuka M, et al. Association of telomere length with authentic pluripotency of ES/iPS cells. Cell Res 2011;21(5):779–92.
28. Brunet A, Berger SL. Epigenetics of Aging and Aging-related Disease. J Gerontol A Biol Sci Med Sci 2014;69(Suppl 1):S17–20.
29. Kim K, Doi A, Wen B, et al. Epigenetic memory in induced pluripotent stem cells. Nature 2010;467(7313):285–90.
30. Nasu A, Ikeya M, Yamamoto T, et al. Genetically Matched Human iPS Cells Reveal that Propensity for Cartilage and Bone Differentiation Differs with Clones, not Cell Type of Origin. PLoS ONE [Internet] 2013;8(1). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3561398/
31. Hartjes KA, Li X, Martinez-Fernandez A, et al. Selection via pluripotency-related transcriptional screen minimizes the influence of somatic origin on iPSC differentiation propensity. Stem Cells Dayt Ohio 2014;32(9):2350–9.
32. Miyagishima KJ, Wan Q, Corneo B, et al. In Pursuit of Authenticity: Induced Pluripotent Stem Cell-Derived Retinal Pigment Epithelium for Clinical Applications. Stem Cells Transl Med 2016;5(11):1562–74.
33. Prigione A, Fauler B, Lurz R, Lehrach H, Adjaye J. The senescence-related mitochondrial/oxidative stress pathway is repressed in human induced pluripotent stem cells. Stem Cells Dayt Ohio 2010;28(4):721–33.
34. Suhr ST, Chang EA, Tjong J, et al. Mitochondrial Rejuvenation After Induced Pluripotency. PLoS ONE [Internet] 2010;5(11). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2991355/
35. Varum S, Rodrigues AS, Moura MB, et al. Energy Metabolism in Human Pluripotent Stem Cells and Their Differentiated Counterparts. PLoS ONE [Internet] 2011;6(6). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3117868/
36. Feher J, Kovacs I, Artico M, Cavallotti C, Papale A, Gabrieli CB. Mitochondrial alterations of retinal pigment epithelium in age-related macular degeneration. Neurobiol Aging 2006;27(7):983–93.
37. Datta S, Cano M, Ebrahimi K, Wang L, Handa JT. The impact of oxidative stress and inflammation on RPE degeneration in non-neovascular AMD. Prog Retin Eye Res 2017;
38. Fanjul-Moles ML, López-Riquelme GO. Relationship between Oxidative Stress, Circadian Rhythms, and AMD. Oxid Med Cell Longev 2016;2016:7420637.
39. Terluk MR, Kapphahn RJ, Soukup LM, et al. Investigating mitochondria as a target for treating age-related macular degeneration. J Neurosci Off J Soc Neurosci 2015;35(18):7304–11.
40. Knoepfler PS. Deconstructing stem cell tumorigenicity: a roadmap to safe regenerative medicine. Stem Cells Dayt Ohio 2009;27(5):1050–6.

41. Kawamata S, Kanemura H, Sakai N, Takahashi M, Go MJ. Design of a Tumorigenicity Test for Induced Pluripotent Stem Cell (iPSC)-Derived Cell Products. J Clin Med 2015;4(1):159–71.
42. Heinrich C, Spagnoli FM, Berninger B. In vivo reprogramming for tissue repair. Nat Cell Biol 2015;17(3):204–11.
43. Zhang K, Liu G-H, Yi F, et al. Direct conversion of human fibroblasts into retinal pigment epithelium-like cells by defined factors. Protein Cell 2014;5(1):48–58.
44. Nistor G, Seiler MJ, Yan F, Ferguson D, Keirstead HS. Three-dimensional early retinal progenitor 3D tissue constructs derived from human embryonic stem cells. J Neurosci Methods 2010;190(1):63–70.
45. Nakano T, Ando S, Takata N, et al. Self-Formation of Optic Cups and Storable Stratified Neural Retina from Human ESCs. Cell Stem Cell 2012;10(6):771–85.
46. Sasai Y, Eiraku M, Suga H. In vitro organogenesis in three dimensions: self-organising stem cells. Dev Camb Engl 2012;139(22):4111–21.
47. Barnea-Cramer AO, Wang W, Lu S-J, et al. Function of human pluripotent stem cell-derived photoreceptor progenitors in blind mice. Sci Rep 2016;6:srep29784.
48. Peyman GA, Blinder KJ, Paris CL, Alturki W, Nelson NC, Desai U. A technique for retinal pigment epithelium transplantation for age-related macular degeneration secondary to extensive subfoveal scarring. Ophthalmic Surg 1991;22(2):102–8.
49. van Meurs JC (last), Kirchhof B, MacLaren R, et al. Ryan's Retina 6th edition, Chapter 124: Retinal Pigment Epithelium and Choroid Translocation in Patients with Age-Related Macular Degeneration. Mosby; 2016.
50. Lund RD, Wang S, Klimanskaya I, et al. Human Embryonic Stem Cell–Derived Cells Rescue Visual Function in Dystrophic RCS Rats. Cloning Stem Cells 2006;8(3):189–99.
51. Lu B, Malcuit C, Wang S, et al. Long-term safety and function of RPE from human embryonic stem cells in preclinical models of macular degeneration. Stem Cells Dayt Ohio 2009;27(9):2126–35.
52. Carr A-J, Vugler A, Lawrence J, et al. Molecular characterization and functional analysis of phagocytosis by human embryonic stem cell-derived RPE cells using a novel human retinal assay. Mol Vis 2009;15:283–95.
53. Diniz B, Thomas P, Thomas B, et al. Subretinal Implantation of Retinal Pigment Epithelial Cells Derived From Human Embryonic Stem Cells: Improved Survival When Implanted as a Monolayer. Invest Ophthalmol Vis Sci 2013;54(7):5087–96.
54. Schwartz SD, Tan G, Hosseini H, Nagiel A. Subretinal Transplantation of Embryonic Stem Cell-Derived Retinal Pigment Epithelium for the Treatment of Macular Degeneration: An Assessment at 4 Years. Invest Ophthalmol Vis Sci 2016;57(5):ORSFc1–9.
55. Booij JC, Baas DC, Beisekeeva J, Gorgels TGMF, Bergen A a. B. The dynamic nature of Bruch's membrane. Prog Retin Eye Res 2010;29(1):1–18.
56. Feeney-Burns L, Ellersieck MR. Age-related changes in the ultrastructure of Bruch's membrane. Am J Ophthalmol 1985;100(5):686–97.
57. Xian B, Huang B. The immune response of stem cells in subretinal transplantation. Stem Cell Res Ther [Internet] 2015;6. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4568575/
58. Jha BS, Bharti K. Regenerating Retinal Pigment Epithelial Cells to Cure Blindness: A Road Towards Personalized Artificial Tissue. Curr Stem Cell Rep 2015;1(2):79–91.
59. Kador KE, Goldberg JL. Scaffolds and stem cells: delivery of cell transplants for retinal degenerations. Expert Rev Ophthalmol 2012;7(5):459–70.
60. Pennesi ME, Neuringer M, Courtney RJ. Animal models of age related macular degeneration. Mol Aspects Med 2012;33(4):487–509.

61. Al-Nawaiseh S, Thieltges F, Liu Z, et al. A Step by Step Protocol for Subretinal Surgery in Rabbits. J Vis Exp JoVE [Internet] 2016;(115). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5092014/
62. Sanchez I, Martin R, Ussa F, Fernandez-Bueno I. The parameters of the porcine eyeball. Graefes Arch Clin Exp Ophthalmol Albrecht Von Graefes Arch Klin Exp Ophthalmol 2011;249(4):475–82.

It's opener there
in the wide open air.

- Dr Seuss

Summary

Samenvatting

6

A quest for the best retinal pigment epithelium (stem) cell replacement therapy

In this thesis the focus of study lies on the retinal pigment epithelium (RPE), a monolayer of pigmented cells that lie underneath the photoreceptors (PR). The PR are specialized type of neurons that are capable of converting the incoming light into electric and neurochemical signals to the brain. This information is used to build a representation of the surrounding environment. The RPE performs various specialized functions that maintain the PR healthy and consequently the RPE is important for retinal health and vision. Functional defects of the RPE lead to physiological defects in the entire homeostatic unit of the retina and are the hallmark of retinal disease such as age-related macular degeneration (AMD) and some forms of retinitis pigmentosa (RP). AMD is a late onset, degenerative and progressive disorder of the macula with a multifactorial etiology. Cell replacement therapy is considered an important strategy in AMD treatment and stem cells are an interesting cells source to use for this purpose. We performed studies that are related to the development of cell replacement therapy for RPE degenerative disorders such as AMD with the focal point on the molecular properties of the human RPE. We used microarray for gene expression profiling to measure thousands of genes at once to give a global picture of molecular and cellular RPE function. We extracted biological meaning from the data using Ingenuity's IPA and used this to compare the human RPE to stem cell derived RPE, the human iris epithelium and mouse RPE.

Here, I will summarize and discuss our findings, elaborate on the opportunities for cell replacement therapy and consider ideas for future studies.

Chapter 1 gives a brief introduction into the embryology, anatomy and function of the RPE. The RPE and the PR evolve from the same ectodermal tissue in the optic cup early in development. The RPE matures into an epithelial monolayer with apical-basal polarity that sits in between the layer of photoreceptors and the choroid. From there it plays a very important role in maintenance of proper functioning of the retina and thus in vision. When functioning is distorted it can lead to disorders such as AMD and some forms of RP.
For cell replacement therapy for AMD, several cell sources are considered. These include donor RPE; pluripotent stem cell derived RPE; and transdifferentiated cells. Currently there is no consensus on which is the best; each carry their (dis-) advantages. Even though there are pre clinical studies in which cells are already transplanted into the eyes of patients, some hurdles still need to be taken before we have a ready available therapy. We aimed to deepen the knowledge of the character of RPE cells in comparison to cells that can possibly replace them, which we describe in the following chapters.

In **Chapter 2** we used a well-established directed differentiation protocol to develop RPE cells from human embryonic stem cells (hESC-RPE). A clearly visible hallmark of RPE development is the appearance of pigmentation and this is commonly used as an indicator of RPE differentiation and maturation. It is however unclear how different pigmentation stages reflect developmental stages and functionality of pluripotent stem cell derived RPE cells. We first studied the gene expression profiles of our hESC-RPE cells at early pigmentation (EP) and late pigmentation (LP) stages. Interestingly we found that the EP and LP hESC-RPE cells do not differ much in gene expression. This implies that they may be less different than generally accepted and both sample types show the expression of well-known RPE markers. This could mean that there is no need to wait for the cells to be fully pigmented to assume maturity, because it does not make a substantial difference. The hESC-RPE cells at early pigmentation stages already show an expression profile representative of differentiated RPE. This suggests that hESC-RPE differentiation procedures for RPE replacement therapies can be shortened significantly which has important implications for the development of new therapeutic strategies in AMD.

Chapter 3 describes an alternative cell source for cell replacement in AMD, namely autologous iris epithelium (IE). The interest for such an alternative cell source stems from the potential of direct conversion: the process of transforming an adult somatic cell into another adult somatic cell. With the acquired knowledge on differentiation of pluripotent stem cells towards RPE, the field of this so called *transdifferentiation* has gained renewed interest. Humans have a limited capacity to transdifferentiate cells *in vivo* or spontaneously regenerate and restore their tissues and organs. However, several studies demonstrated that *in vitro* procedures could convert one cell into another cell type and thereby skipping the pluripotent state, using overexpression of cell-lineage specific genes. Reasons that IE cells are a potential starting source is the common embryological origin of the RPE and IE; IE cells can be obtain relatively easily through iridectomy from patients; and the IE cells display a number of functional RPE features such as the presence of tight junctions and phagocytosis of POS. To improve our understanding of molecular and functional similarities and differences between the human IE and RPE, we conducted an in-depth microarray study, comparing gene expression profiles and the functional annotations of these two tissues *in vivo*.
Overall, the canonical pathways and corresponding statistically significantly enriched functions for the most highly expressed genes of the IE and the RPE were very similar. However, there was also a set of statistically significantly differentially expressed genes. Prominent features among the enriched RPE gene expression are those implicated in the phototransduction cascade. On the other hand, Ingenuity attributed specific canonical pathways to the IE that are related to the Wnt signaling pathway (Wnt SP).

The high expression of Wnt SP genes in the IE compared to the RPE suggests that the IE preserves (part of) its multipotent character during life. Also, our Ingenuity analysis showed a high expression of the aryl hydrocarbon receptor (AhR) signaling pathway in the IE compared to the RPE. AhR is a ligand dependent transcription factor that regulates a cellular defense mechanism pathway against toxin overload in cells.
Our study provides in depth analysis of the gene expression profiles of the IE and the RPE. Our data may be useful in the further exploration of IE as a potential source for regenerative medicine for RPE degeneration.

In **Chapter 4** we explored the similarities and differences between mouse and human RPE. These could be important for translational studies that are performed on mouse for the development of a therapy for RPE related diseases.
Apart from the obvious similarities, there are a number of well-known differences between human and mouse RPE and adjacent tissues, such as the absence of a macula in the mouse, the difference in rod and cone number and distribution and a thinner Bruch's membrane in the mouse.
We were interested in the potential usefulness of our entire comparative human and mouse gene expression dataset for the investigation of AMD mouse models.
First, we determined 64 signature genes for mouse RPE, 171 signature genes for human RPE. From these two sets of genes we deduced 22 mouse-human interspecies signature genes.
Next, we analyzed the mouse and human RPE gene expression profiles, and we found that (patho-) biological functions and canonical pathways assigned to the RPE of both species were highly similar. Nonetheless, more detailed studies, including analysis of specific molecular networks as well as extreme gene expression differences between mouse and human suggests substantial biological differences.
Interestingly we did find similarities and differences in relation to a number of previously published (patho-) biological aspects related to AMD, namely oxidative stress, zinc homeostasis, presence of proteins of the complement system that are found in drusen, proteins in Bruch´s membrane, involvement in neovascularization and tight junctions. These differences and similarities are important to develop and use representative mouse models for AMD, and they may be partly responsible for (the observed) discrepancies between mouse model and human patients.

Chapter 5 discusses the potential and challenges of getting cell replacement therapy for AMD working in the clinic. There are many studies focused on this, there are even several clinical trials ongoing, but we lack standardized protocols, a consensus on what cell types are optimal and the best transplantation method. So, even though a lot of progress has been made over the last few decades, we should face the important

challenges to determine the most optimal therapeutic strategy for RPE degenerative disorders.

Een zoektocht naar de beste (stam) cel therapie voor het retinaal pigment epitheel

De focus van dit proefschrift ligt op het retinaal pigment epitheel (RPE), een enkele laag van gepigmenteerde cellen in het oog. Deze cellen liggen onder de staafjes en de kegeltjes, de fotoreceptoren (FR). De FR vertalen het inkomende licht in een elektrisch signaal dat de hersenen kunnen verwerken tot een beeld. Het RPE vervult verschillende functies in het onderhoud van de FR, zorgt er voor dat de FR gezond blijven, en is daarom belangrijk voor het gezichtsvermogen. Verstoringen in de werking van het RPE leiden tot fysiologische defecten in het oog en zijn een belangrijke factor in de ontwikkeling van oogaandoeningen, zoals macula degeneratie (MD) en sommige vormen van retinitis pigmentosa (RP). MD is een degeneratieve en progressieve aandoening van de macula (het deel van het oog verantwoordelijk voor het centrale zicht), met een grote verscheidenheid aan risicofactoren. Celtransplantatie wordt gezien als een belangrijke (toekomstige) methode om MD te behandelen en stamcellen zijn daarvoor een interessante bron van cellen. Wij hebben onderzoek gedaan naar de ontwikkeling van cel transplantatie therapie voor RPE degeneratieve aandoeningen zoals MD, waarbij we ons gericht hebben op de moleculaire en cellulaire eigenschappen van het menselijke RPE. Wij gebruikten hiervoor microarrays voor gen expressie profilering, waarbij wij duizenden genen tegelijkertijd konden meten en een overzicht konden maken van de functies van de cel. Om aan deze data biologische betekenis te geven, gebruikten wij Ingenuity's IPA. Deze aanpak hebben wij gebruikt om het humaan RPE te vergelijken met stam cel afkomstige RPE, humaan iris epitheel en muis RPE.
In dit hoofdstuk bespreek ik in het kort onze bevindingen, zal ik uitweiden over de mogelijkheden voor cel transplantatie therapie en mogelijkheden in de toekomst beschouwen.

Hoofdstuk 1 bevat een introductie van de embryologie, anatomie en functies van het RPE. Het RPE en de FR stammen af van dezelfde voorlopercel wanneer zij zich ontwikkelen in de oogbekers, vroeg in de embryonale ontwikkeling. Het RPE vormt zich tot een gepolariseerde enkele epitheel laag die zich tussen de FR en het vaatvlies bevindt. Het RPE verzorgt de FR laag door het van voedingsstoffen en zuurstof te voorzien en afval af te voeren. Het RPE speelt derhalve een belangrijke rol in het gezichtsvermogen. Wanneer RPE niet goed functioneert leidt dat uiteindelijk tot aandoeningen zoals MD en RP. Voor celtherapie voor MD worden verschillende bronnen van cellen overwogen. Onder andere RPE van donorogen; RPE gemaakt van pluripotente stamcellen; en getransdifferentieerde cellen. Op dit moment is er geen consensus welke bron het "beste" is; elk celtype heeft zijn voor- en nadelen. Ondanks dat zijn er al klinische studies bezig en worden er al verschillende celtypes getransplanteerd in de ogen van patiënten. Er

zijn echter nog tal van hindernissen die overwonnen moeten worden voordat er een gestandaardiseerde en effectieve behandeling is voor in de kliniek. Ons streven is om een bijdrage te leveren aan de ontwikkeling hiervan door de kennis te vergroten over de cellen die gebruikt zouden kunnen worden voor (stam) cel therapie.

In **Hoofdstuk 2** gebruiken we een erkende en ontwikkelde methode om RPE cellen te maken van humane embryonale stamcellen (hESC-RPE). Een zeer duidelijk kenmerk van de ontwikkeling van RPE cellen is de aanwezigheid van pigmentatie en dit wordt dan ook vaak gebruikt als eenvoudige indicatie van RPE ontwikkeling. Het is echter onduidelijk in hoeverre de mate van pigmentatie het ontwikkelingsstadium weergeeft of een representatie is van de functionaliteit van de RPE cellen. Daarom hebben wij eerst de genexpressie profielen van hESC-RPE met klein beetje pigmentatie (EP) en die met veel pigmentatie (LP) met elkaar vergeleken. We vonden dat deze twee groepen niet veel van elkaar verschilden. Dit impliceert dat ze wellicht niet zoveel van elkaar verschillen als vaak gedacht wordt aangezien beiden ook wel bekende RPE kenmerken vertonen. Dit zou kunnen betekenen dat het niet nodig is om de cellen volledig te laten pigmenteren voordat ze gebruikt kunnen worden voor volgende experimenten of voor transplantatie aangezien ze voor de belangrijkste functionele *pathways* op hetzelfde ontwikkelingsniveau zitten. Het zou kunnen betekenen dat de ontwikkelingsprotocollen zoals ze nu zijn, verkort kunnen worden, wat belangrijk is voor implementatie in de kliniek.

Hoofdstuk 3 beschrijft een alternatief celtype voor transplantatie therapie voor MD, namelijk het iris epitheel (IE) van de patiënt zelf. Interesse voor zo'n alternatief komt door de mogelijkheid sommige cellen direct te kunnen omzetten naar een ander celtype (ook wel transdifferentiatie genoemd). Hierbij gaat het om cellen in een volwassen, somatisch stadium direct om te zetten naar andere volwassen somatische cellen. Doordat we steeds meer te weten komen over de verschillende methodes om pluripotente stamcellen om te zetten in andere celtypen, heeft het vakgebied van de transdifferentie opnieuw de aandacht getrokken. Mensen kunnen uit zichzelf nauwelijks cellen transdifferentiëren of zichzelf regenereren. Maar er zijn verschillende studies die laten zien dat het mogelijk is om van het ene celtype naar het andere te gaan zonder een pluripotent stadium te doorlopen. Voor RPE transplantatie is het IE interessant omdat deze zich ontwikkelt uit hetzelfde weefsel; omdat het relatief makkelijk is om het van de patiënt af te nemen; en omdat het een aantal functies uitvoert vergelijkbaar met het RPE. Vanwege deze redenen hebben wij onderzoek gedaan naar de moleculaire en functionele overeenkomsten en verschillen. De functionaliteiten die toegeschreven worden aan de twee weefsel komen grotendeels overeen. Echter, er is ook een groep genen gevonden die significant anders zijn.

Een belangrijke eigenschap van het RPE is de fototransductie, de cascade waarbij licht in het oog wordt omgezet in elektrisch signaal voor de hersenen. Niet verbazingwekkend, aangezien de bijbehorende genen geactiveerd worden door factoren die niet in de iris zitten.
Daarentegen werd in het IE specifieke activiteit gevonden van de zogeheten Wnt signaal transductie route (Wnt SP). De hoge expressie van Wnt SP genen in het IE in vergelijking met het RPE kan betekenen dat het IE een deel van zijn multipotente karakter behoudt gedurende het leven. Dit zou gunstig kunnen zijn voor het gebruik van IE cellen om RPE te vervangen. Daarnaast zagen wij specifiek een hoge expressie van het Aryl koolwaterstof Receptor (AhR) netwerk in het IE in tegenstelling tot het RPE. AhR reguleert het afweersysteem van cellen en beschermd tegen de ophoop van gifstoffen. Uit ons onderzoek en de literatuur blijkt dat oudere IE cellen en jonge RPE cellen een actief AhR netwerk hebben en ontgiftingswerking, maar oudere RPE cellen niet. Het is verleidelijk om te speculeren dat in onze ogen IE cellen ontgiftingsactiviteiten behouden gedurende het leven terwijl het RPE dat niet kan/doet.
Ons onderzoek resulteerde in een diepgaande analyse over de genexpressie profielen en daarvan afgeleide functionaliteiten van het IE en RPE van het menselijk oog. Onze data zijn wellicht van nut voor verder onderzoek naar IE cellen als mogelijke bron van regeneratieve behandeling van RPE degeneratie.

In **Hoofdstuk 4** onderzochten wij de overeenkomsten en verschillen tussen het muis en humaan RPE. Dit zou van belang kunnen zijn voor translationeel onderzoek naar RPE gerelateerde aandoeningen in muizen.
Naast de overduidelijk overeenkomsten, zijn er een aantal bekende verschillen tussen mens en muis, zoals het missen van een macula in de muis; het verschil in de verdeling en hoeveelheid van staafjes en kegeltjes; en de dikte van het Bruch's membraan waarop het RPE ligt.

Wij wilden in dit onderzoek de gen expressie profielen van de muis en mens met elkaar vergelijken omdat dit van belang zou kunnen zijn voor het gebruik van muis modellen. Allereerst hebben we 64 genen geselecteerd die specifiek zijn voor het muis RPE en 171 gene specifiek voor humaan RPE. Van deze twee collecties genen hebben we 22 muis-mens specifieke RPE genen afgeleid.
Vervolgens hebben we de muis en humaan RPE gen expressie profielen onderzocht en gevonden dat de biologische functionaliteiten van de twee soorten zeer op elkaar lijken. Desalniettemin blijken er na meer gedetailleerd onderzoek wel degelijk een aantal interessante verschillen te zijn. Deze hebben ook te maken met functies die betrokken zijn bij MD, namelijk oxidatieve stress, zinc homeostase, complement systeem eiwitten die in drusen zitten, Bruch's membraan eiwitten, bloedvatgroei in het oog en tight junc-

tions. Deze verschillen en overeenkomsten zijn van belang voor het ontwikkelen van een representatief muis model voor MD en zou deels de oorzaak kunnen zijn van de afwijking van een muismodel van de patiënt.

In **Hoofdstuk 5** behandel ik de mogelijkheden en (technische) moeilijkheden van het gebruik van (stam) cel therapie voor MD in de kliniek. Er wordt veel onderzoek naar gedaan en er zijn zelfs al verschillende klinische onderzoeken bezig, maar we missen tot dusver gestandaardiseerde protocollen, een consensus over welk celtype het best gebruikt kan worden en wat de beste transplantatie techniek is. Ondanks dat er een enorme vooruitgang is geboekt de afgelopen decennia zijn er nog belangrijk uitdagingen die aangepakt moeten worden voordat er een optimale behandeling ontwikkeld is voor degeneratieve aandoeningen van het RPE.

You'll get mixed up, of course,
as you already know.
You'll get mixed up
with many strange birds as you go.

- Dr Seuss

A

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PORTFOLIO

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COURSES/MASTERCLASSES

	Year	Workload(hrs)
Neuropsychopharmacology	2012	56
Imaging life at the molecular level	2013	50
Practical biostatistics	2013	40
Laboratory animal science (art.9)	2014	100
Degenerative diseases of the nervous system	2014	40
Functional neuroanatomy	2014	40
Masterclass Magdalena Götz	2012	3
Masterclass Oliver Brüstle	2014	3

TEACHING

	Year	Workload
Supervising internships		
Master's student	2014	6 months
Bachelor's student	2015	3 months
Master's student	2015	6 months
Master's student	2016	7 months
Other		
Lecturing Master's student about PhD experiences, University of Amsterdam	2013-2014	2 times
Lecturing Molecular Cell Biology, University of Sierra Leone	2017	3 months

COMMITTEES

	Year
ONWAR PhD Annual Retreat	2013-2014
ONWAR Career Event	2015

CONFERENCES, SEMINARS AND MASTER CLASSES

	Year	Presentation
International Society of Differentiation Conference, Amsterdam	2012	poster
ARVO-NED Research Course in Ophthalmology, Amsterdam	2012	oral
Neuroscience Campus Annual Meeting, Amsterdam	2012	poster
Retinadag Patientenvereniging Retina, Rhenen	2013	oral
ONWAR Phd retreat, Zeist	2013	poster
Neuroscience Campus Annual Meeting, Amsterdam	2013	poster
International Society for Stem Cell Research	2013	poster
Phd Seminar, Center for Neurogenomics and Cognitive	2014	oral
Dutch Neuroscience Meeting	2014	oral
VUmc Science Exchange Day, Amsterdam	2014	poster
ONWAR Phd retreat, Zeist	2014	poster
The Biology of Regenerative Medicines, Hinxton, UK	2015	poster
Phd Seminar, Center for Neurogenomics and Cognitive Research VU, Amsterdam	2015	oral
ONWAR Phd retreat, Zeist	2015	oral
Refereeravond Ophthalmogenetica, Amsterdam	2016	oral

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