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SCALABLE, SAFE AND GMP-COMPATIBLE PRODUCTION OF EMBRYONIC STEM CELL DERIVED RETINAL PIGMENT EPITHELIAL CELLS

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To my family, who paved the path for me to get here or wherever I would like to be

ABSTRACT

Regenerative medicine is an exponentially growing field that aims to regenerate a lost function, cell type or tissue due to damage, ageing or disease. Currently, more than 30,000 gene- and cell-based therapies have been or are being tested in clinical trials. Since the eye benefits from accessibility and a supposed to be immune privilege, many groups are exploring different strategies to treat diseases affecting this organ. Age-related macular degeneration (AMD), the leading cause of blindness in people aged over 65 years old, could be one of the first diseases treated with human pluripotent stem cells (hPSC)- derived therapies. This thesis has been focused on the development of a scalable, robust, defined and xeno-free protocol to differentiate hPSC into RPE-like cells, ensuring the safety of the obtained product through genomic, tumorigenicity and biodistribution studies. Finally, the differentiation of an in-house derived GMP-grade hESC line using a completely GMP-compliant protocol, together with the validation of a set of in-process and Quality Control tests has allowed to engage in conversations with the regulatory authorities to bring these cells closer to near clinical trials, and ultimately to AMD patients.

LIST OF SCIENTIFIC PAPERS

I. **Sara Padrell Sánchez***, Sandra Petrus-Reurer*, Pankaj Kumar*, Monica Aronsson, Helder André, Hammurabi Bartuma, Alvaro Plaza Reyes, Emeline F. Nandrot, Anders Kvanta, Fredrik Lanner
Preclinical safety studies of human embryonic stem cell-derived retinal pigment epithelial cells for the treatment of age-related macular degeneration
Stem Cells Translational Medicine (under revision)
(*These authors contributed equally to this work)

II. Alvaro Plaza Reyes*, Sandra Petrus-Reurer*, **Sara Padrell Sánchez**, Pankaj Kumar, Iyadh Douagi, Hammurabi Bartuma, Monica Aronsson, Sofie Westman, Emma Lardner, Helder André, Anna Falk, Emeline F. Nandrot, Anders Kvanta, Fredrik Lanner
Identification of cell surface markers and establishment of monolayer differentiation to retinal pigment epithelial cells
Nature Communications (accepted)
(*These authors contributed equally to this work)

III. **Sara Padrell Sánchez**, Heather Main, Mona Hedenskog, Fredrik Lanner
Establishment of GMP-compatible production of hESC-derived RPE cells
Manuscript

IV. Marc Parrilla, María Cuartero, **Sara Padrell Sánchez**, Mina Rajabi, Niclas Roxhed, Frank Niklaus, and Gastón A. Crespo
Wearable All-Solid-State Potentiometric Microneedle Patch for Intradermal Potassium Detection
Analitical Chemistry, 2019, 91, 1578-1586

V. Rocío Cánovas, **Sara Padrell Sánchez**, Marc Parrilla, María Cuartero, and Gastón A. Crespo
Cytotoxicity Study of Ionophore-Based Membranes: Toward On-Body and in Vivo Ion Sensing
ACS Sens., 2019, 4, 2524-2535

CONTENTS

LIST OF ABBREVIATIONS

AMD	Age-related macular degeneration
BAM	Binary alignment map
BEST-1	Bestrophin1
bFGF	Basic fibroblast growth factor
CAR-T	Chimeric antigen receptor T-cells
CBS	Circular binary segmentation
cDNA	Complementary DNA
CNVs	Copy number variations
CRALBP	Cellular retinaldehyde-binding protein
CXCR4	C-X-C chemokine receptor type 4
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
EBs	Embryoid bodies
ELISA	Enzyme-linked immunosorbent assay
ERCC	External RNA controls consortium
ESC	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FS	Fixing solution
GA	Geographic atrophy
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gDNA	Genomic DNA

GMP	Good manufacture practices
gVCF	Genomic variant call format
GvHD	Graft versus host disease
HAMC	Hyaluronan-methylcellulose
HDFs	Human dermal fibroblasts
H-E	Haematoxylin-eosin
hESC	Human embryonic stem cells
hiPSC	Human induced pluripotent stem cells
HLA	Human leukocyte antigen
hrLN	Human recombinant laminin
iPSC	Induced pluripotent stem cells
MAP2	Microtubule associated protein 2
MITF	Microphthalmia-associated transcription factor
MT	Mitochondrial
NANOG	Nanog homeobox
NCAM1	Neural cell adhesion molecule 1
NuMA	Nuclear mitotic apparatus protein
OVs	Optic vesicles
PAX3	Paired box 3
PAX6	Paired box 6
PC	Principal component
PCA	Principal-component analysis
PD	Parkinson’s disease
PDGF	Platelet-derived growth factor
PDGFRB	Platelet-derived growth factor receptor beta
PEDF	Pigment epithelium-derived factor

PMEL	Premelanosome protein
POS	Photoreceptor outer segments
POU5F1	POU Class 5 Homeobox 1
PSC	Pluripotent stem cells
QC	Quality control
qPCR	Quantitative polymerase chain reaction
RCS	Royal college of surgeons
RNA	Ribonucleic acid
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
RPE65	Retinal pigment epithelium-specific protein 65kDa
RPLP0	Ribosomal protein lateral stalk subunit p0
SD-OCT	Spectral-domain optical coherence tomography
SEM	Scanning electron microscopy
SNPs	Single nucleotide polymorphisms
SNVs	Single nucleotide variants
SOX9	Sex-determining region Y-box 9 protein
TBB3	Tubulin beta 3 class III
TEER	Trans epithelial electrical resistance
TEM	Transmission electron microscopy
TGFbeta	Transforming growth factor beta
TMD	Tissue marking dye
t-SNE	T-distributed stochastic neighbour embedding
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick end labelling
TYR	Tyrosinase
UMIs	Unique molecular identifiers

VEGF Vascular endothelial growth factor

VQSR Variant quality score recalibration

ZO-1 Zona occludens-1

1 INTRODUCTION

1.1 REGENERATIVE MEDICINE

Regenerative medicine has the potential to recover or replace cells, tissues or organs damaged by age, disease or trauma. For many years, the only option for such replacement relied on the availability of donated organs or tissues. The limited amount of suitable and compatible donors encouraged the search for alternative sources. The stem cells' discovery, the advances in cell culture and differentiation, together with the refinement of the gene editing techniques have been crucial milestones to reach the broader spectrum of cell and gene therapies available nowadays.

Since many are the cases that could benefit from a cell or gene therapy, many efforts are being put in the development of this products. Up to now, almost 30,000 cell-based therapies and over 700 gene-based therapies have been or are being tested in clinical trials, being 16 the current number of products already approved by the Food and Drug Administration (FDA) [1].

1.1.1 GENE-BASED THERAPIES

The gene-based therapies' strategy is the introduction of genetic material into the cells to compensate abnormal genes or to express proteins that are beneficial for the treatment or prevention of a disease.

The first approved and main application of the gene therapy is the generation of chimeric antigen receptor T-cells (CAR-T) [2]. CAR-T cells are autologous T-cells that have been isolated and genetically modified to better recognise and kill haematopoietic cancerous cells once they are returned to the blood circulation. Nowadays, two CAR-T cell therapies are already in the market: Kymriah from Novartis to treat acute lymphoblastic leukaemia and B-cell lymphoma [3,4], and Yescarta from Kite Pharma/Gilead to treat large B-cell lymphoma [5].

Other gene therapies have been approved to treat prostate cancer [6], RPE65-mediated inherited retinal dystrophy [7], advanced melanoma [8] and coagulation disorders [9].

Gene-based therapies have proved to be a valuable tool for the treatment of genetic diseases caused by specific mutations, but in some cases, only a few patients share the same exact mutation, being necessary the development of multiple gene therapies to treat the same disease. For instance, over 4,000 different mutations have been reported to cause retinitis pigmentosa (RP) [10]. The extremely high cost of the development of these almost individualised therapies is not compatible with most of the companies' business models so other alternatives and novel reimbursement models are being studied at the moment.

1.1.2 CELL-BASED THERAPIES

On the other hand, cell-based therapies rely on the transplantation of healthy cells to replace the lost ones and/or to rescue the remaining ones by the secretion of growth factors and other beneficial molecules. Since the transplanted cells aim to recover the lost function without targeting a specific mutation, one single product would be sufficient to treat all the patients affected by the same disease even when presenting different mutations.

Cell-based therapies can be divided in two big groups: autologous, when the used cells belong to the patient [11–16], and allogeneic, when the cells come from a different donor [17–21]. In principle, an autologous therapy would be immune-tolerated by the patient, while an allogeneic one would require immunosuppression to avoid the rejection of the foreign transplanted cells. Unfortunately, in most of the cases, the patient's cells are not available or functional, being the allogeneic therapy the only option. In these cases, human leukocyte antigens (HLA)-matching will always be preferable. Currently, gene editing techniques are being used to remove and introduce some of the HLA genes that would be recognised by the immune system, thus developing a universal cell that would be compatible with everyone [22–28].

The discovery of the stem cells by James Till and Ernest McCulloch in 1961 [29,30] highly contributed to the evolution of this revolutionary field. Stem cells have the capacity to divide, proliferate and differentiate into multiple lineages and cell types. Adult stem cells can be found and extracted from adult tissues like bone marrow, skin, muscle, intestine, adipose tissue and blood, and they are usually multipotent, already committed to a limited number of mature cell types. Fetal stem cells come from aborted fetus and they are also multipotent. On the other hand, human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC) are pluripotent (**Fig. 1**). Although adult and fetal stem cells have been and are being used in several clinical trials [31–34] (clinicaltrials.gov numbers NCT02464436 and NCT03073733), they have some limitations, and the fact that hESC and hiPSC are an unlimited source able to differentiate into any cell type, makes them the true gem of the regenerative medicine.

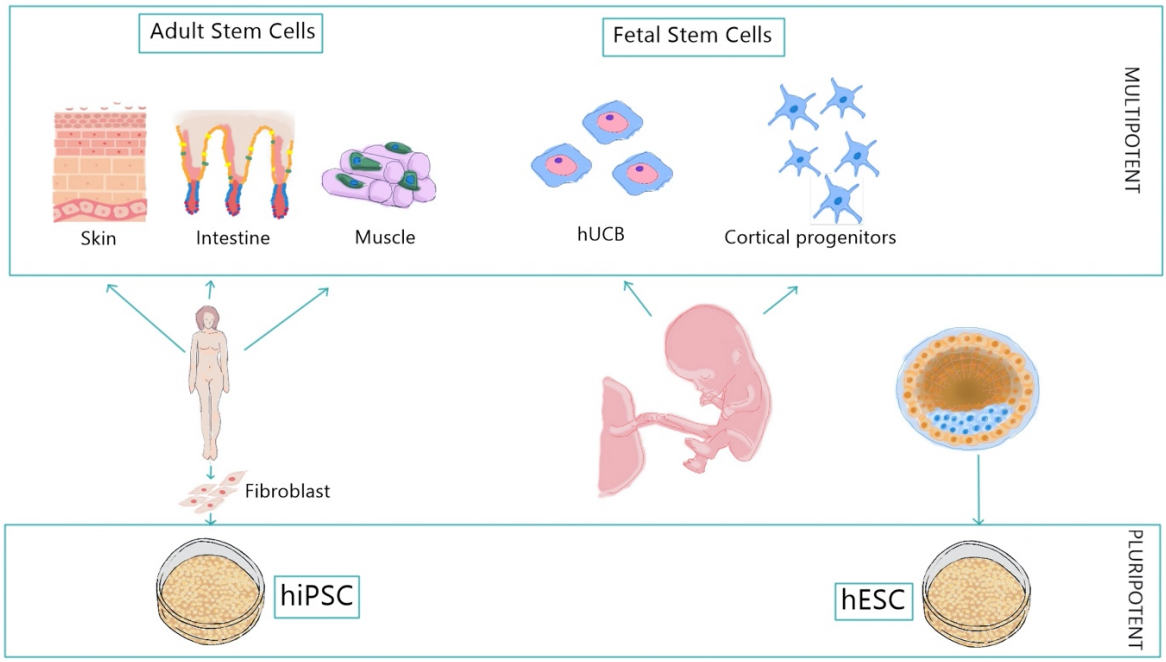

Figure 1. Schematic representation of main stem cell types used in regenerative medicine

Nowadays, multiple cell types are being tested in clinical trials, and several cell-based products are already in the market [15–17,19,20,35] to treat cartilage defects, wounds of the oral soft tissue, wrinkles appearance or hematopoietic disorders.

1.2 PLURIPOTENT STEM CELLS

Stem cells are a cell type that remains in an undifferentiated state. In most of adult tissues remains a small population of adult stem cells that keep dividing and differentiating to ensure the proper turnover and function of the tissue [36–38]. Nevertheless, these cells are unipotent or multipotent, meaning that they are already committed to generate one or few cell types of the tissue where they belong. Some therapies focus on the activation of these quiescent cells, already present in the tissue or organ [39,40], others on providing the right environment for these cells to repair and regenerate the damaged tissue [41].

On the other hand, PSC have two main features that makes them a unique source for cell-based products. PSC have the capacity to self-renew, meaning that when kept in the right conditions they can proliferate remaining undifferentiated and conforming an unlimited source of young cells, but when exposed to specific conditions they can also differentiate into any cell type in the body. Although in the recent years, several groups have been exploring new cell types that claim to be totipotent [42–46], hESC and hiPSC are the main sources used in cell therapies.

Even though most of the PSC-derived products are fully differentiated cells, one possible concern inherent to the nature of these cells is the possibility of remaining undifferentiated cells in the final product. Since the transplantation of proliferating PSC could result in a tumor formation among other complications, exhaustive tests have to performed to completely rule out the possible presence of these cells.

1.2.1 HUMAN EMBRYONIC STEM CELLS

When James A. Thomson published in 1998 the first successful derivation of hESC, a very promising new source for cell therapies was presented [47]. Since then, the immense potential of these cells has been greatly accepted by the scientific community and many are the groups that have been and still are exploring all their capabilities.

hESC are derived from the inner cell mass of surplus blastocysts. When they are cultured in the presence of basic fibroblast growth factor (bFGF) and transforming growth factor (TGFbeta) they maintain their stemness: proliferating and remaining undifferentiated [48]. The removal of one of these components or both from the media results in cell differentiation towards different lineages [49,50], and the addition of growth factors or small molecules are also being used to direct the differentiation towards specific cell types [51,52], promoting a faster and more efficient process.

Currently, multiple clinical trials are testing the potential of several hESC-derived products to treat diseases like age-related macular degeneration (AMD) [53–56], cardiac ischemia [57], type I diabetes (clinicaltrials.gov number NCT02239354, NCT03162926 and NCT03163511) or Parkinson's disease (PD) [58,59].

Although hESC lines are derived from in vitro fertilisation (IVF) surplus embryos that would have been discarded, the use of these lines has raised some ethical concerns in some society sectors.

The allogeneic nature of hESC-derived products could be considered as another handicap of these cells. The possible mismatch in the HLA complex would require the use of immunosuppressive therapies, not always avoiding the possible immune rejection of the transplanted cells. Nowadays, there are on-going initiatives to generate hESC banks that would comprise a broader spectrum of HLA types, and larger percentages of the population could benefit from HLA-matched hESC-derived cells [60].

These two main flaws of the hESC encouraged the search for an autologous alternative that would overcome rejection issues and possible ethical concerns.

1.2.2 INDUCED HUMAN PLURIPOTENT STEM CELLS

The discovery of iPSC by Shinya Yamanaka on 2006 represented a promising alternative to ESC: the introduction of few defined factors achieved the reprogramming of

somatic cells into an embryonic state-like [61]. This pluripotent cell type would provide an autologous source that would overcome ethical issues and, theoretically, immune rejection problems. Although it has been extensively assumed that an autologous transplant would be immune-tolerated, some studies have shown that iPSC-derived cells could have an abnormal gene expression that would induce an immune response [62]. The fact that hiPSC-derived cells would be individually generated for each patient makes this cell source highly expensive and time-consuming. Trying to overcome this drawback, as well as for hESC, hiPSC banks are being stablished [63–66]. Nevertheless, recent studies have shown that in the absence of immunosuppression, HLA-matching alone is insufficient to grant long-term survival of transplanted grafts [67].

Although hiPSC have had a shorter path than hESC, derivatives of these cells have also reached the patients to treat diseases like AMD [68], PD [69] and graft versus host disease (GvHD) (clinicaltrials.gov number NCT02923375).

1.3 THE EYE

The eye is the organ that provides the visual system. It is composed by many structures and its perimeter can be divided into three different layers (**Fig. 2**):

- Sclera and cornea: the external layer
- Iris, ciliary body and choroid: the intermediate layer
- Retina: the internal layer

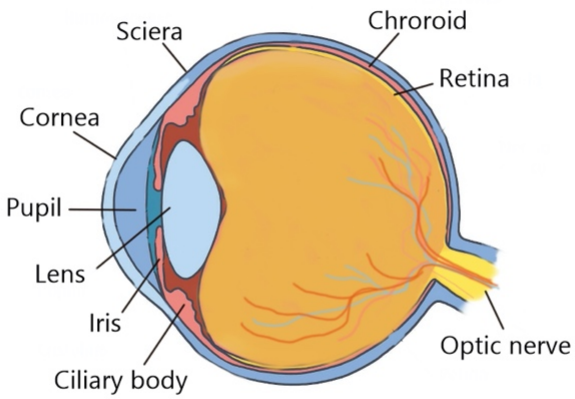

Figure 2. Schematic structure of the eye

The retina is a sensory layer that lines at the bottom of the eye, organized, at the same time, in several layers (**Fig. 3**). These layers contain different types of neurons that, after receiving the light stimuli focused by the cornea and the lens, will transduce chemical and electrical signals from the photoreceptors, situated at the bottom, all the way up to the ganglion cells, the optical nerve and to the brain, where the image will be finally generated.

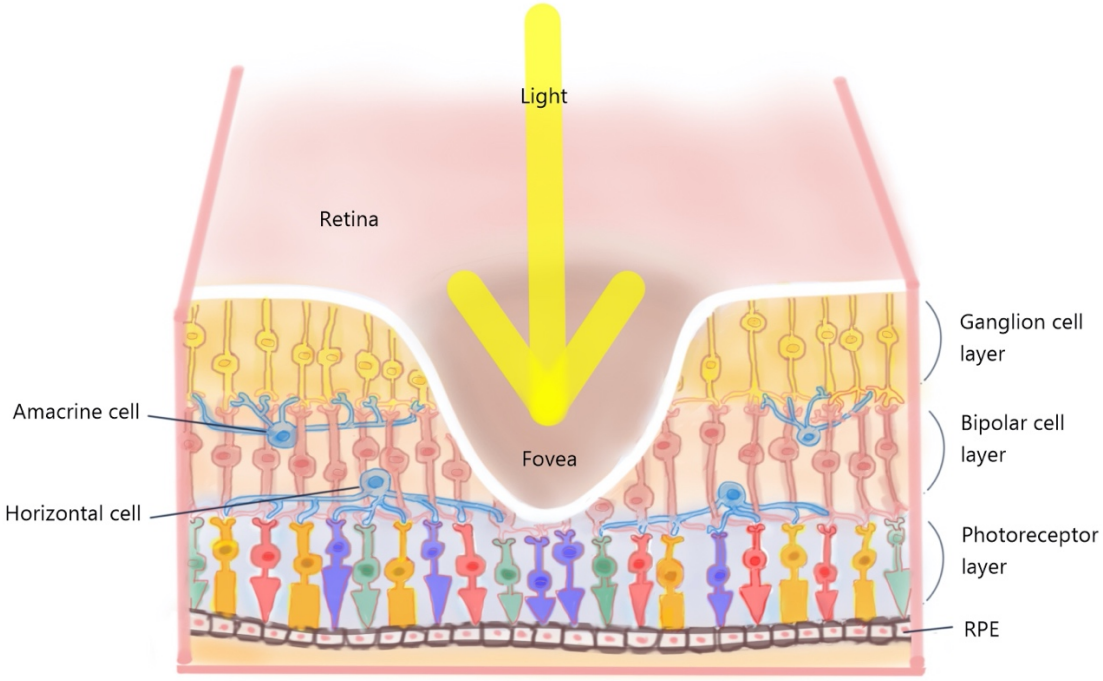

Figure 3. Representation of the different layers of the retina at the fovea

In the middle of the retina, there is a demarcated region called macula. This circular area is functionally and structurally different from the rest of the retina, and it is the presence of zeaxanthin and lutein what gives its characteristic "yellow" colour [70]. The main function of these pigments, together with other mechanisms, is to protect and preserve the most essential structure of the retina for human vision, the fovea. This small valley located in the middle of the macula shows the highest cones' density, providing colour discrimination and visual acuity (**Fig. 4**).

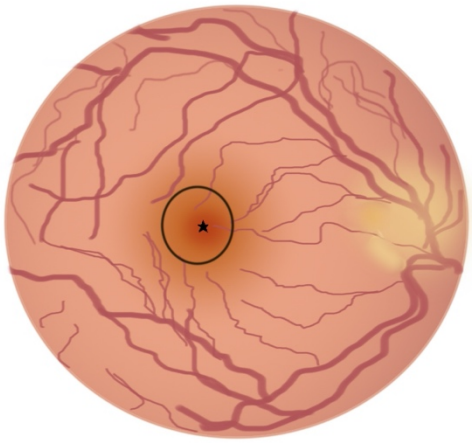

Figure 4. Representation of the fundus of the eye circling the macula with the fovea (*)

1.3.1 PHOTORECEPTORS

Cones and rods are two specialised types of neurons, globally known as photoreceptors, conforming the basal layer of the retina. These cells capture the light through structures called outer segments, posteriorly converted into chemical and electrical signals in the inner segments, and finally transduced to the rest of neuronal layers in the retina through synaptic connections, a phenomenon known as phototransduction [71].

Rods are greater in number, thinner and more sensitive to lower amounts of photons, procuring vision under dark-dim conditions at night. Cones are scarcer and require brighter light to be stimulated. Depending on the absorbed wavelength, we can discriminate between red cones (long wavelength), green cones (medium wavelength) and blue cones (short wavelength), and the combination of these three provides colour perception [72] (**Fig. 3**). Seeing the important role that these cells play on the visual system, it is not surprising that their dysfunction or deficiency leads to many vision problems and eye diseases. For instance, mitochondrial DNA deletions in foveal cones have been correlated to several maculopathies [73].

1.3.2 RETINAL PIGMENT EPITHELIUM

The Retinal Pigment Epithelium (RPE) is a compact monolayer of heavily pigmented hexagonal and polarized cells lying underneath the retina. The finger-like processes that emerge from its apical membrane are in direct contact with the photoreceptors, providing them nutrients like omega-3 fatty acids, amino acids and glucose, disposing their waste and recycling their outer segments through phagocytosis [74]. In order to sustain the photoreceptors, its apical part also secrete growth factors like pigment epithelium-derived factor (PEDF), TGFbeta and platelet-derived growth factor (PDGF) [75,76].

The RPE's location enables this structure to perform as a blood-retinal barrier, another important function that prevents nonspecific diffusion of material from the choroid but favours the transport of other substances like water, ions, metabolic end products and vascular endothelial growth factor (VEGF) from the subretinal space to the choroid [77]. It has been suggested that the secretion of immunosuppressive cytokines by the RPE cells would contribute to the immune privileged status of the eye [74,75,78], even though this statement is currently being reanalysed in the field.

The presence of melanosomes in its cytoplasm absorbs any excess of incoming light, playing an important role on the protection of the retina from light damage.

All these complex functions make the RPE a very essential structure to keep the homeostasis in the eye, and its disturbance can result in an abnormal visual function and develop into several eye diseases.

1.3.3 BRUCH'S MEMBRANE

The Bruch's membrane is a thin layer of connective tissue firmly attached to the basal site of the RPE, acting as a physical and biochemical barrier between the choroid and the RPE. This extracellular matrix rich in elastin, collagen and laminin [79,80] also provides physical support for RPE cell adhesion, migration and differentiation [81]. Any modification in its structure or composition, which depends on age, genetics, environmental factors, and disease state, could alter its diffusion properties, affecting RPE's and outer retina's function.

1.3.4 CHOROID

The choroid is the main source of blood supply to the retina, providing 65-85% of its flow, and it is crucial for the maintenance of the outer retina, especially the photoreceptors. The blood circulates through the choriocapillaris: fenestrated vessels adjacent to the Bruch's membrane with a polarized expression of VEGF receptor towards the retinal side [82]. Since they provide 90% of the oxygen consumed by the photoreceptors in darkness, any disruption in the choroidal blood flow would be detrimental to these cells [83].

1.4 RETINOPATHIES

There are several diseases affecting the retina called retinopathies. Macular degeneration, retinitis pigmentosa (RP), diabetic eye disease, retinal detachment, retinoblastoma, macular pucker, macular hole and floaters are the most common ones. Since the retina is a crucial structure for the vision, this sense is affected in different ways by these diseases, and in advanced stages they may even cause blindness.

1.4.1 RETINITIS PIGMENTOSA

RP is a group of rare inherited retinal diseases that affects 1 in 4000 people. Common symptoms comprise difficulty seeing at night and gradual loss of peripheral vision due to the progressive degeneration of photoreceptor cells. Over 4000 different mutations distributed along nearly 70 different genes have been reported in RP patients [10]. The heterogeneity presented by the disease adds another level of complexity to the development of a suitable therapy. Although some gene-replacement therapies are being explored for specific mutations, additional mutation-independent approaches are also being developed to slow down the retinal degeneration, embracing a broader percentage of affected people with one single product [84]. The viral-mediated expression of anti-apoptotic factors and secretion of retinal neurotrophic factors would diminish the photoreceptor cell death.

Cell-based therapies are also being explored at the moment in RP patients: autologous bone marrow-derived stem cells have shown promising results [85,86] (clinicaltrials.gov number NCT02280135), and retinal progenitor cells are currently being tested in two on-going

clinical trials, using two different approaches. While one relies on the activation and rescue of endogenous remaining photoreceptors by the intravitreous injection of the progenitors (clinicaltrials.gov number NCT03073733), the other one relies on the replacement of lost photoreceptors by the subretinal injection of the cells (clinicaltrials.gov number NCT02464436). Although this last clinical trial has not been completed yet, some results have already been shared: while three patients showed a considerable visual acuity improvement, five patients reported almost no gain, and two of them even experienced a decline. These inconsistent results suggest that optimal patient selection and surgical procedure standardisation for future studies design is essential [87].

1.4.2 STARGARDT'S DISEASE

Stargardt's disease is a genetic eye disorder and is one of the most common forms of macular degeneration in juvenile patients. The abnormal accumulation of the yellow pigment lipofuscin promotes photoreceptors' death and the progressive vision loss from the macula. Although there is no treatment for Stargardt's disease, several strategies are currently under study. One approach is to reduce the build-up of lipofuscin and other toxic byproducts in the retina by the administration of a synthetic form of vitamin A. Another approach would be the repair or replacement of *ABCA4* gene, one of the most common mutated genes in these patients, also aiming to reduce the amount of accumulated lipofuscin[88]. Finally, cell-based therapies to rescue or to replace the damaged cells are also being tested in clinical trials[55,56].

1.4.3 AGE-RELATED MACULAR DEGENERATION

AMD is the leading cause of blindness in industrialized countries in people over 65 years old [89]. Today, 8% of the world's population is affected by this disease, and the projected number for 2020 is around 196 million, reaching to 288 million by 2040 [90].

Even though the exact causes underlying the disease are still unclear, it is known that demographic, cardiovascular, dietary and medication factors play an important role on the development and progression of the disease [91]. For instance, the prevalence of AMD in female or white populations seems to be higher, although some studies revealed no sex differences in AMD risk [92] and the racial differences in the prevalence might be explained by factors other than pigmentation. On the other hand, smoking and hypertension would be lifestyle-related factors also associated with an increased risk of developing the disease [93].

Apart from environmental factors, the manifestation of AMD also has an important genetic component. Among others, several polymorphisms in genes encoding proteins involved in immune regulation, like the complement, have been related to the disease's development [94].

AMD is a degenerative disease that affects several structures in the eye like the outer

neural retina, the retinal pigment epithelium, the Bruch's membrane and the choroid, starting primarily in the macula generating a small black dot in the middle of the visual field, and eventually expanding through the fundus of the eye eradicating any residual vision (**Fig. 5**). Even though the disease presents defined hallmarks, depending on the presented pathogenesis, AMD has been extensively classified in two different types: exudative and non-exudative.

Figure 5. Scene looked through a healthy retina (left) and a diseased retina with AMD (right)

Exudative AMD (a.k.a. neovascular or wet) is characterised by the proliferation of new blood vessels from the choroid, through the Bruch's membrane and into the RPE layer, often causing subretinal haemorrhage (**Fig. 6**). It has been hypothesised that this vascular overgrowth could be a response of VEGF secretion from hypoxic RPE after a large vessel stenosis and choriocapillaris loss [95].

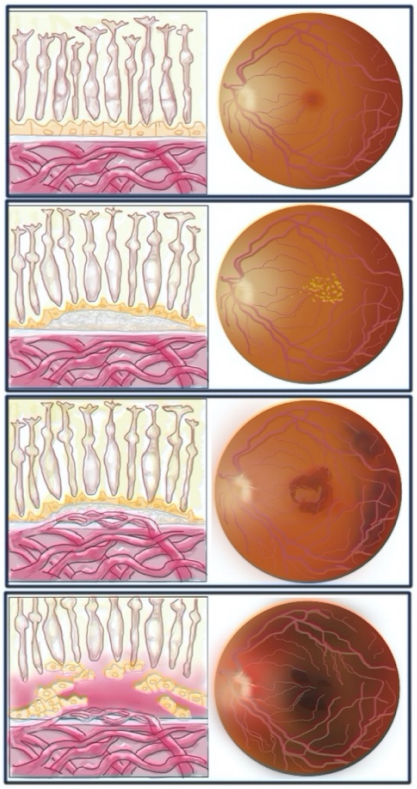

Figure 6. Schematic drawings and fundus representing healthy and diseased retinas

On the other hand, non-exudative AMD (a.k.a. non-neovascular or dry) is characterised by a complex sequence: accumulation of Drusen deposits, hyperpigmented dysfunctional RPE cells, Drusen resorption and RPE, photoreceptor and choriocapillaris depletion, evolving most of the times in confluent areas of RPE death and photoreceptor atrophy, also known as geographic atrophy (GA) [96]. It is this decrease in vessels' lumen and density what would give the "dry" appearance to the disease. Even though Drusen deposits are one of the hallmarks of the disease, the presence of these accumulations of lipoproteins, immunoglobulin and complement complexes situated between Bruch's membrane and RPE is not always correlated with AMD development [97–99].

Although this classification is strongly settled in the field, the fine line separating these two types might disappear in the future as it has been shown that both of them can evolve into the other in more advanced stages [100].

1.4.3.1 CURRENT APPROACHES TO TREAT AGE-RELATED MACULAR DEGENERATION

Currently, treatments like anti-VEGF ocular injections are being used to stop the progression, and in some cases to restore the vision, of exudative AMD [101], which would imply only 10-15% of the total AMD cases. On the other hand, there is no stablished treatment for non-exudative AMD patients. Administration of supplements like zeaxanthin and lutein [102], complement pathway inhibitors, visual cycle inhibitors, intravitreal neurotrophic factors and lipid metabolism modulators are some of the prevailing procedures [103], that far from curing the disease, would only delay the inevitable end point.

Seeing the importance of RPE cells in the visual function, it seems plausible that the replacement of the lost cells could stop the disease and even restore vision. This idea has brought many experts to study several sources for the restoration. The transplant of autologous RPE cells from peripheral areas would be the least immunogenic option; nevertheless, since the cells come from the patient himself, they would most probably suffer a similar fate than the lost ones [11,104]. Fetal RPE would be a healthier source but it is quite limited and its use would raise many ethical concerns [105]. An unlimited and free from ethical discussions source would be the use of RPE cell lines [106], but some of them might show modified properties, not resembling mature native RPE cells.

In the search for an unlimited, healthier, ethical and functional source, the discovery of hPSC opened many conceivable options [107]. The fact that these young cells can be differentiated into any cell type in the body makes them a very valuable tool to treat a wide spectrum of degenerative diseases, AMD being among them [108,109]. Nowadays, the use of hESC and hiPSC is being explored, both cell types presenting many advantages and drawbacks. Even though the use of autologous hiPSC, in principle, would not face rejection problems, it is still unclear the effect of the reprogramming mechanisms [110,111]. Furthermore, the development of individualised therapies with today´s technology would imply an

enormous cost, both money and time wise. On the other hand, these handicaps would be defeated with the use of hESC: any possible reprogramming effect would be avoided, the costs would be considerably reduced and, with the arrangement of an immune matching hESC bank, rejection would be minimised. Nevertheless, since hESC are derived from human embryos, their use might face some ethical concerns.

At the moment, different strategies are being developed to differentiate hESC into RPE cells. While some protocols rely on the aggregation of hESC into embryoid bodies (EBs) maintained in suspension, others are focused on differentiating an adherent 2D monolayer. On the first scenario, after some time in culture, small dark protuberances called optical vesicles (OV) emerge from the EBs, containing the pigmented RPE cells, whereas on the second case the cells are kept in culture until the appearance and maturation of RPE colonies.

Using any of the previously mentioned strategies, it has been shown that a simple spontaneous differentiation is feasible removing bFGF from the culture media [112–114], although further purification processes would be required to enrich the obtained heterogeneous population, like manual dissection and enzymatic digestion of the OVs followed by its 2D culture, or manual selection and expansion of the RPE colonies. Aiming for a more efficient and directed differentiation towards RPE cells, some groups have developed other protocols that would demand the addition of several factors and the use of biological or engineered substrates; shortening the differentiation protocol and getting a more pure product [115–118].

After differentiation, the obtained hESC-RPE cells have to be fully characterised. Their identity can be confirmed by scrutinising several features: hexagonal morphology, presence of pigmentation, expression of specific RPE markers like retinal pigment epithelium-specific protein 65kDa (RPE65), bestrophin1 (BEST-1) and microphthalmia-associated transcription factor (MITF), and epithelial barrier integrity measured by trans epithelial electric resistance (TEER). Their functionality can be validated in vitro, by their ability to phagocyte photoreceptor outer segments and to differentially secrete VEGF and PEDF towards the basal and apical sides, respectively; and in vivo, by their capacity to integrate, survive and, in the best scenario, rescue visual function after their transplantation into animal models. Last but not least, before these cells can be considered as a therapeutic candidate, their purity has to be assessed using several tests, which will be explained in coming points.

Trying to meet the highest possible levels in purity and to produce clinically compliant cells, many efforts are being done to develop xeno-free and defined protocols [119–121]. Avoiding the use of non-human components and working only with chemically-defined substances would highly reduce possible contaminants on the final product, decrease the risk of rejection after transplantation, obtain greater homogeneity between batches, and meet the requirements to produce the cells under good manufacturing practice (GMP) conditions, something increasingly demanded by the regulatory authorities when it comes to cell

therapies.

Regarding to the therapy's delivery into the subretinal space, different methods are being considered, and the use of animal models is a very convenient tool to determine the best approach before moving forward to the patients. Nowadays, one can choose among a wide variety of retinal degeneration models [122], being the Royal College of Surgeons (RCS) rat one of the most used [114,123,124]. Nevertheless, the use of bigger animals like rabbits or primates would be preferable since it resembles more the human eye in several aspects [118,125].

Currently, the field is independently exploring two different techniques to identify which would fit better the diverse clinical scenarios: a) subretinal injections of a cell suspension [115,126,127] and b) transplant of 2D sheets on scaffolds of different nature [118,124,128]. The subretinal injection would be an easier procedure with minimal invasion covering bigger areas of cell loss but it relies on the inherent capacity of the cells to establish a monolayer on the subretinal space, whereas the transplant of the 2D sheet would require a more complex and invasive surgery, increasing the risk of damage on the retina's structure and subsequent complications. Nevertheless, since the 2D sheets are mounted on protein-based or polymer-derived scaffolds, their use might be more convenient in advanced stages of the disease where the Bruch's membrane's integrity is highly compromised.

After seeing the positive outcome of the first clinical trial with hESC-RPE cells delivered as a cell suspension [55,56], several laboratories started looking into the refinement of this technique. For instance, hyaluronan-methylcellulose (HAMC) hydrogel used as an injectable scaffold is showing promising results [129]. It would improve cell survival and quickly biodegrade after transplantation, overcoming some of the drawbacks observed with other substances like hyaluronic acid-based gels [130].

1.5 SAFETY OF STEM CELLS THERAPIES

In the development of any cell therapy that could be considered for transplantation to patients, reaching a product free from non-human components, containing only defined substances, is very important, but when it comes to cell therapies derived from stem cells it is also crucial to ensure their purity and safety.

Stem cells possess the inherited properties of pluripotency and proliferation at considerably high rates, so the presence of residual undifferentiated cells in the final product could lead to undesired consequences.

As the regenerative medicine field is moving forward, more groups are facing these challenges and, even though there are no standardised routines to follow, some authors have led the path suggesting several tests. The presence of undifferentiated cells could be checked in vitro, at a protein or RNA level by flow cytometry, immunostaining or qPCR, and in vivo by tumorigenicity and biodistribution tests [131,132]. According to the International Stem Cell Initiative, the best method to prove pluripotency is the tumour formation upon the injection of

stem cells under mice's skin [133,134]. Several groups have used this assertion to test the lack of migratory and/or undifferentiated cells in their derived products [55,123,135,136] following the most relevant guidelines for this type of application: "Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks" from the World Health Organization [137].

Apart from discarding the presence of proliferative cells in the product, it is also necessary to check the genetic stability of the cells. Several studies have shown that the reprogramming methods and/or keeping the cells in culture for long time could introduce mutations in the DNA [68,138–142]. Since some of the acquired mutations could be harmful for the patient, genomic analysis like karyotyping, genotyping or even whole genome sequencing could be required.

1.6 WEARABLE ANALYTICAL DEVICES

The regenerative medicine is an exponentially growing field that is generating many tools to treat diseases uncurable until now. As people's lifespan is expanding, there is an increasing interest to pursue a healthier lifestyle and reach certain ages with a better quality of life. Technological advances are allowing the expansion of another growing field like the wearable analytical devices. Such devices consist in the integration of different kind of sensors into conventional objects like sweat bands, contact lenses, epidermal patches, glasses or microneedle patches [143,144]. These sensors would allow a real-time monitoring of physiological parameters that could be used for rapid diagnosis, preventive medicine or even sport proficiency [145].

Currently, nanoscale devices like transdermal patches are being explored [146]. Most of the microneedle-based devices detect glucose through an amperometric readout, but only two published devices would detect ions using potentiometric sensors [147,148]. Since such devices would be in direct contact with skin cells and interstitial fluid, possible cytotoxic effects have to be tested previous to their use in humans.

2 AIMS

The general aim of this thesis has been to develop a scalable, safe and GMP-compatible hESC-derived RPE therapy to bring a treatment for AMD closer to the clinic.

The specific aims of the five projects have been:

I. To prove the safety of our hESC-derived RPE cells, in terms of genomic stability, and tumorigenicity and migration potential.

II. To develop a chemically defined, xeno- and manual selection-free protocol to differentiate hESC into RPE-like cells, and the discovery of cell-surface markers to validate the differentiation efficiency.

III. To optimize and translate the developed protocol to differentiate our GMP-grade hESC line using fully compliant GMP reagents and materials, defining a set of assays and thresholds to ensure a pure, safe and validated global clinical production.

IV. To evaluate the cytotoxicity of materials used in wearable microneedle patches for intradermal potentiometric detection of potassium in interstitial fluid.

V. To test possible cytotoxic effects of ionophore-based membranes used in on-body ion sensors.

3 MATERIALS AND METHODS

3.1 CELL CULTURE

3.1.1 hPSC CULTURE

hESC and hiPSC lines (with ethical permit from the Regional Ethics board of Stockholm, EPN 2011:745-31/3) were maintained by clonal propagation under xeno-free and defined conditions on human recombinant laminin (hrLN) 521-coated plates, in NutriStem hPSC XF medium and hypoxia conditions according to the previously described method [149].

3.1.2 hPSC-RPE DIFFERENTIATION (OLD PROTOCOL)

hESC and hiPSC were cultured to confluence on hrLN-521 and manually scraped to generate EBs according to the previously described method [120]. EBs were differentiated in suspension in NutriStem hPSC XF medium without bFGF and TGFbeta, and in normoxia conditions. After five-week differentiation, OVs were manually dissected from the EBs and enzymatically dissociated flushing through a 20G needle. Cells were strained and seeded onto freshly hrLN 521-coated dishes with NutriStem hESC XF medium without bFGF and TGFbeta, and maintained in 2D culture for 30 more days.

3.1.3 hPSC-RPE DIFFERENTIATION (NEW PROTOCOL)

hESC and hiPSC were seeded at different densities on hrLN 521 or 111 with NutriStem hPSC XF and rho-kinase inhibitor, and maintained in hypoxia conditions. 24 hours later, the cells were moved to normoxia conditions, and fed with NutriStem hPSC XF medium without bFGF and TGFbeta. From day 4,5, 6, 7 or 8 after plating, Activin A was added to the medium. Cells were fed three times a week and kept for 19 or 30 days, adding Activin A to the medium up to day 10, 15, 19, 25 or 30. Monolayers were collected for analysis or enzymatically dissociated into single cells for further differentiation. In this latter case, the cells were strained and seeded onto freshly hrLN 521-coated plates at different densities, kept for 19, 22 or 30 more days as monolayer with NutriStem hPSC XF medium without bFGF, TGFbeta and Activin A.

3.2 QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR)

Total RNA was isolated using the RNeasy Plus Mini Kit and treated with RNase-free DNase. Complementary DNA (cDNA) was synthesised using 1 µg of total RNA. TaqMan Real-Time PCR master Mix together with TaqMan probes for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), nanog homeobox (*NANOG*), POU Class 5 Homeobox 1 (*POU5F1*), sex-determining region Y-box 9 protein (*SOX9*), paired box 6 (*PAX6*), *BEST1*,

RPE65, premelanosome protein (*PMEL*), paired box 3 (*PAX3*), *MITF,* tyrosinase (*TYR*), platelet-derived growth factor receptor beta (*PDGFRB*), tubulin beta 3 class III (*TBB3*), and microtubule associated protein 2 (*MAP2*) were used. Samples were subjected to real-time PCR amplification protocol on a StepOne™ real-time PCR System. Biological triplicates were performed for every condition and technical duplicates were carried out for each reaction.

3.3 FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

hPSC-RPE samples were stained with BV421 Mouse Anti-Human CD140b, PE Mouse Anti-Human CD140b, BB515 Mouse Anti-Human CD56, Alexa Fluor 647 Mouse Anti-Human TRA-1-60, BV421 Mouse Anti-Human CD184, BV421 Mouse Anti-Human Disialoganglioside GD2, PECy7 Mouse Anti- Human CD184, BV605 Mouse Anti-Human Disialoganglioside GD2 and BV605 Mouse Anti-Human CD104 conjugated antibodies. Fluorescence minus one (FMO) controls were included for each condition to identify and gate negative and positive cells. Stained cells were analysed using a CytoFLEX flow cytometer equipped with 488 nm, 561 nm, 405 nm and 640 nm lasers. Analysis of the data was carried out using FlowJo v.10 software.

Cell sorting was performed on hPSC-RPE cultures after 21 days or 30 days of differentiation. Cells were incubated with the mentioned conjugated antibodies. FMO controls were included for each condition to identify and gate negative and positive cells. Stained cells were then sorted using a BD FACS Aria Fusion Cell Sorter.

Right after sorting, 70,000 cells were cytospinned onto glass slides. Slides were fixed with 4% methanol-free formaldehyde and stained by immunocytochemistry.

3.4 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

hPSC-RPE were cultured on Transwell membranes coated with hrLN 521. Supernatants from both the hPSC-RPE apical and basal sides (meaning upper and lower compartments of the transwell, respectively) were collected 60 hours after the medium was changed. PEDF secretion levels were measured in triplicates for each condition with commercially available human PEDF ELISA Kits after 60 days of culture. The optical density readings were measured using SpectraMax 250 Microplate Reader.

3.5 TRANSEPITHELIAL ELECTRICAL RESISTANCE (TEER)

TEER RPE cells plated on Transwell membranes was measured using the Millicell Electrical Resistance System volt-ohm meter. 60-day cultures were equilibrated outside the incubator at room temperature before the experiment. Measurements were performed in unchanged culture media in triplicate for each condition, at three different positions of each

well. Averages were used for further analysis. The background resistance was determined from a blank culture insert in the same media coated with the corresponding substrate but without cells, and subtracted from the respective experiment condition.

3.6 PHAGOCYTOSIS ASSAY

hPSC-RPE were cultured on Transwell membranes coated with hrLN 521 for 30 days after re-plating. Cells were incubated at 37°C or 4°C with fluorescein isothiocyanate (FITC)-labelled porcine photoreceptor outer segments (POS). After incubation, cells were quenched with Trypan Blue Solution, fixed with 4% methanol-free formaldehyde and permeabilized with 0.3% Triton X-100. Rhodamine-phalloidin staining was used to visualize cell boundaries. Nuclei were stained with Hoechst 33342.

Images were acquired with a Zeiss LSM710-NLO point scanning confocal microscope. Post-acquisition analysis of pictures was performed using IMARIS and POS quantifications were done with CellProfiler 2.1.1 software.

3.7 IMMUNOCYTOCHEMISTRY (ICC)

Protein expression of day 60 hPSC-RPE cells was assessed through immunofluorescence. Cells were fixed with 4% methanol-free formaldehyde, followed by permeabilization with 0.3% Triton X-100 and blocking with 4% fetal bovine serum (FBS) and 0.1% Tween-20. Primary antibodies against PAX6, NANOG, BEST-1, MITF, Zonula occludens-1 (ZO-1), cellular retinaldehyde-binding protein (CRALBP), PDGFRB (CD140b), C-X-C chemokine receptor type 4 (CXCR4 or CD184), Ganglioside GD2, Ki67 and caspase 3 (CASP3) were incubated overnight followed by incubation with secondary antibodies: Alexa Fluor 647 donkey anti-rabbit IgG, Alexa Fluor 488 donkey anti-mouse IgG, donkey anti-mouse IgG1 Alexa Fluor 568 and donkey anti- mouse IgG2a Alexa Fluor 488. Nuclei were stained with Hoechst 33342. Images were acquired with Zeiss LSM710-NLO point scanning confocal microscope. Post-acquisition analysis of the pictures was performed using IMARIS and/or Fiji/ImageJ.

3.8 KARYOTYPING

After EBs dissociation, hESC-RPE cells were plated on wells coated with hrLN 521. At day 7 (when cells were still proliferative), Karyomax colcemid was added to the medium for 28h. Cells were enzymatically dissociated. After centrifugation, the cell pellet was resuspended with the remaining solution after pouring off the supernatant, and 0.4% KCl was added. After centrifugation, 3:1 methanol:acetic acid fixative was added to the resuspended pellet. This action was repeated twice. Samples were analysed at Labmedicin Skåne, Genetiska Kliniken, Skånes Universitetssjukhus in Lund.

3.9 GENOTYPING

Genomic DNA (gDNA) was isolated using the QIAmp DNA Mini Kit and 250 ng gDNA were analysed for Copy Number Variations with Genome-Wide Human SNP Array 6.0 at Bioinformatics and Expression Analysis core facility (Karolinska Institute, Stockholm).

3.10 WHOLE-GENOME SEQUENCING ANALYSIS

gDNA was sequenced with Ilumina HiSeq X, 30X coverage. Whole-genome paired-end DNA sequencing reads of HS980 (p22), HS980 (p38) and hESC-RPE cells in biological triplicate experiments were aligned to the human reference genome using the Burrows-Wheeler Aligner. Aligned binary alignment map (BAM) files were sorted using Picard. "GATK Best Practice" guidelines were followed to generate analysis-ready BAM files which includes local realignments and base quality recalibration using GATK bundle "b37" files that include data sets from HapMap, Omni, Mills Indels and 1000 Genome Indels databases. Additionally, single nucleotide polymorphisms (SNPs) from NCBI-dbSNP were included in the analysis.

3.10.1 GERMLINE SINGLE NUCLEOTIDE VARIANTS

Analysis-ready BAM files of HS980 (p22) were processed using GATK 3.7 HaplotypeCaller walker in genomic variant call format (gVCF) mode with default parameters. Output gVCF files of individual HS980 (p22) replicates were used for raw single nucleotide variants (SNVs) identification using GenotypeGVCFs walker. Further, variant quality score recalibration (VQSR) was performed using VariantRecalibrator walker with default parameters followed by ApplyRecaliberation walker to select filter "PASS" variants separately for individual replicates. Finally, BCFtools "isec" utility was used to identify SNVs commonly present in all three replicates for further downstream analysis. As an additional control set for analysis, publicly available pre-processed germline SNVs from 11 participants from personal genome project: UK were downloaded and annotated for clinical significance.

3.10.2 SOMATIC SINGLE NUCLEOTIDE VARIANTS

Somatic SNVs calling was performed using GATK 3.7-MuTect2 in a pair-wise manner with default parameters. Brief comparisons were made between HS980 (p22) and hESC-RPE, followed by HS980 (p22) compared with HS980 (p38) to find somatic SNVs. All analyses were performed for the three independent replicates. dbSNP150 and COSMIC-v83 VCF files were considered as an argument for dbSNP and COSMIC, respectively. In addition, filter "PASS" somatic SNVs identified as a final outcome of MuTect2 pairwise

analysis were merged to create a non-redundant set of somatic SNVs for HS980 (p22) vs hESC-RPE and HS980 (p22) vs HS980 (p38).

3.10.3 COPY NUMBER VARIATIONS

In the copy number variations (CNVs) discovery, both advanced microarray- and next-generation sequencing platform-based approaches were used to identify potential copy number changes during HS980 (p22) to hESC-RPE and HS980 (p22) to HS980 (p38) differentiation processes. gDNA of all samples were hybridized with the Genome-wide Human SNP Array 6.0. Affymetrix CEL files were imported to the Partek®Genomic Suite 6.6 to perform pairwise CNVs analysis. Hybridization intensity signal for each hESC-RPE and HS980 (p38) samples were compared to HS980 (p22) control samples. The genomic segmentation algorithm (with the following parameters: minimum number of probes per segment = 10, *p*-value threshold ≤ 0.001, signal to noise ratio = 0.3 and diploid copy number range = 1.7 to 2.3) was used to identify loss and gain CNVs segments. Identified replicate-wise CNVs segments were merged to create non-redundant CNVs segments for hESC-RPE and HS980 (p38) samples.

Independently, BAM files were used to identify CNVs associated with hESC-RPE and HS980 (p38) compared to HS980 (p22) samples in a pairwise manner. The whole-genome sequencing pipeline of CNVkit 0.9.3 tool with default parameters in "batch" mode was used to compare individual hESC-RPE and HS980 (p38) samples with respective HS980 (p22) control samples. Copy number segments were identified using the circular binary segmentation (CBS) algorithm and annotated to genes using GRCh37 annotation from Ensembl-v75. Segments with log2 ratio ≥ 0.3 and ≤ 0.3 were classified as amplifications and deletions, respectively. Further, replicate-wise copy-number segments were merged to create non-redundant copy-number segments for hESC-RPE and HS980 (p38) samples. In-house Perl scripts were used to identify overlapping copy-number segments for hESC-RPE and HS980 (p38) samples.

3.10.4 CLINICAL INTERPRETATIONS

ANNOVAR utility tool integrated within UCSC Galaxy was used to functionally characterise all germline and somatic SNVs. To access clinical significance, clinically annotated SNVs from ClinVar databases and cancer specific coding mutations from COSMIC databases were downloaded. Further, overlapping study was performed with identified germline and somatic SNVs using BCFtools "isec" utility. Additionally, three separate lists of cancer-driver genes were prepared which include 723 genes from the COSMIC cancer gene census, 299 genes from Bailey MH *et al.*, and 242 genes from the Shibata list.

3.11 SINGLE-CELL RNA SEQUENCING

3.11.1 PROJECT I

Mature hESC-RPE cells cultured for 5 weeks after dissociation from OVs and hESC passage 14 were enzymatically dissociated and strained. Cells were further stained with live/dead marker 7-AAD and live single cells were sorted into a 384-well plate in lysis buffer using the SORP BD FACSAria Fusion instrument. hESC-RPE were sorted in 338 wells and hESC in 46 wells; 2 wells were left empty. A validation plate with 30 wells containing hESC-RPE (28 wells with single cells and 2 wells with 20 cells each) and two wells with lysis buffer only was run as control. Smart-Seq2 sequencing was carried out by the Eukaryotic Single Cell Genomics facility (ESCG, SciLifeLab, Stockholm, Sweden).

For sequencing analysis, single cell transcriptome sequencing reads were mapped to the human genome (hg19) using STAR aligner. The number of reads for each RefSeq and Ensemble annotated genes were calculated using featureCounts. Cells were quality-filtered based on the exclusion criterium: have total aligned reads (within transcriptomic boundaries) lesser than 10^3 and have showed expression of fewer than 2,000 unique genes. Read count matrix from quality-filtered cells was processed using R package Seurat. Gene expression measurement was performed using NormalizeData function in Seurat with scale factor 10,000 followed by log-transformation. RunPCA, JackStraw, FindClusters and RunTSNE functions were used to further process the data and obtain t-distributed stochastic neighbour embedding (t-SNE) cluster of cells.

3.11.2 PROJECT II

60 days hPSC-RPE cells were enzymatically dissociated and strained. Cells were transported to ESCG facility where a 3' cDNA library was prepared for single cell RNA sequencing with the 10X Genomics platform. Cell Ranger 2.1.1 pipeline was used to convert Illumina base call files to fastq format, align sequencing reads to the hg19 transcriptome using the STAR aligner, and generate feature-barcode matrices. Cell Ranger quality-control filtered cells were analysed in R, using Seurat suite. As a further quality-control measure, RPE cells with 17 uniquely expressed genes ($\geq$ 2,000 to $\leq$ 5,000), unique molecular identifiers (UMIs) ($\geq$ 10,000 to $\leq$ 30,000) and percentage of UMIs mapping to mitochondrial (MT)-genes ($\geq$ 0.025 to $\leq$ 0.10) were selected. Similarly, hESC cells with uniquely expressed genes ($\geq$ 2,000 to $\leq$ 8,000), UMIs ($\geq$ 10,000 to $\leq$ 80,000) and percentage of UMIs mapping to MT-genes ($\geq$ 0.025 to $\leq$ 0.10). This filtration step resulted in final dataset of 616, 725, 779 and 905 cells for CD140b+GD2-, CD140b+CD184-, re-plated 1:20 and hESC samples, respectively. Before, dimensionality reduction by principal-component analysis (PCA), cell-cell variation in gene expression driven by UMIs, mitochondrial gene expression and cell-cycle stages were regressed out during data scaling process. Variable genes within RPE samples were selected based on their normalized average expression and dispersion. For principal component (PC) selection, findings of PCHeatmap, jackStraw, PC standard deviations and Clustree analysis were assessed. The first 15 PCs were used for the t-SNE

projection and clustering analysis. Cell clusters were analysed by two approaches. Top differential genes were first identified for each cluster using Wilcoxon Rank Sum test. Secondary, signature gene expression (module scores) was computed for undifferentiated hESC and several cell types present in human retina. Cells expressing mesoderm markers were manually subdivided in a separate cluster using interactive plotting features of Seurat.

3.12 HISTOLOGICAL ANALYSIS

Mice teratomas were excised, fixed with 4% methanol-free formaldehyde and paraffin embedded. 4 μm tissue sections were processed further for haematoxylin-eosin (H-E) staining.

Immediately after euthanasia, the rabbit eyes were enucleated and the bleb injection area was marked with green Tissue Marking Dye (TMD). An intravitreal injection of fixing solution (FS) and embedding in paraffin was performed. 4 μm serial sections were produced through the TMD-labelled area and stained with H-E.

For immunostaining, slides were deparaffinised and put through antigen retrieval. Slides were blocked and incubated with primary antibodies against human nuclear mitotic apparatus protein (NuMA), BEST-1, CD140b/PDGFRB and CD56/neural cell adhesion molecule 1 (NCAM1), and secondary antibodies (Alexa Fluor 555 donkey anti-rabbit IgG and Alexa Fluor 647 donkey anti- mouse IgG). Sections were mounted with vector vectashield with DAPI mounting medium. For immunohistochemistry (IHC), slides were deparaffinised followed by antigen retrieval and stained for CD140b/PDGFRB and CD56/NCAM1. Images were taken with Olympus IX81 fluorescence inverted microscope. Post-acquisition analysis of the pictures was performed using ImageJ software.

3.13 TUNEL ASSAY

Apoptotic markers were analysed on tissue sections by Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay. Images were taken with an Olympus IX81 inverted epifluorescence microscope. Post-acquisition analysis of the pictures was performed using the ImageJ software.

3.14 ANIMALS

After approval by the Northern Stockholm Animal Experimental Ethics Committee (DNR N56/15 and DNR N25/14), New Zealand white albino rabbits, aged 5 months and weighing 3.5 to 4.0 kg were used in these studies. All experiments were conducted in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research. After approval by the Southern Stockholm Animal Experimental Ethics Committee (DNR S14/15), CIEA NOG mice aged 4 weeks were used in these studies.

3.15 TUMORIGENICITY AND BIODISTRIBUTION STUDIES

3.15.1 MICE

hESC, EBs and hESC-RPE monolayers were enzymatically dissociated into single cell suspensions. Cells were counted, resuspended in medium to reach different concentrations and mixed with Matrigel Matrix. Matrigel cell suspensions were injected subcutaneously in NOG mice neck. A total of 90 NOG mice were injected, divided into 9 groups of 10 mice each (6 groups with 10, 100, $1x10^3$, $1x10^4$, $1x10^5$ or $1x10^6$ hESC, 2 groups with $1x10^7$ of 3- or 5-week EBs and 1 group with $1x10^7$ hESC-RPE cells. Teratomas' growth was monitored weekly up to 4 weeks or 7 months. 7 months after subcutaneous injection of 10 million hESC-RPE cells, the mice were euthanised, and the organs (lung, liver, spleen, kidneys, heart and gonads) and transplanted cells were independently collected. Each organ was homogenised and 3 aliquots were processed for RNA isolation.

For hESC and hESC-RPE cell spiking, serial dilutions of cells (ranging from 1 to $1x10^6$ cells) were mixed with mouse tissue.

Isolated RNA from all samples was analysed by qPCR using the SYBR green protocol and human ribosomal protein lateral stalk subunit p0 (*RPLPO)* primers. Calculation of the equation relating log (cell/mg tissue) with Ct value allowed the inference of the amount of cells/mg present in each of the analysed organs based on the obtained Ct values.

3.15.2 RABBITS

hESC-RPE monolayers were enzymatically dissociated into single cell suspensions. Cells were counted and resuspended in Dulbecco's phosphate-buffered saline (DPBS). Animals were anesthetized and the pupils were dilated. Microsurgeries were performed on both eyes using a 2-port 25G transvitreal pars plana technique. Without infusion or prior vitrectomy, the cell suspension (equivalent to 50,000 cells) was subretinally injected with a syringe connected to a cannula through the upper temporal trocar. After instrument removal, a light pressure was applied to the self-sealing suture-less sclerotomies. Local immunosuppression with intravitreal triamcinolone was administered one week prior to the surgery. In animals kept for long-term evaluation, intravitreal triamcinolone was re-administered every 3 months.

After confirming absence of immune rejection and integration of the transplanted cells through spectral-domain optical coherence tomography (SD-OCT) at 1, 4, 12 weeks and 12 months, the rabbits were euthanised and the organs (lung, liver, spleen, kidneys, heart, optic nerve and vitreous) were independently collected. Each organ was homogenised and 3 aliquots were processed for RNA isolation.

For hESC and hESC-RPE cell spiking, serial dilutions of cells (ranging from 10 to $1x10^6$ cells) were mixed with rabbit tissue.

3.16 SCANNING ELECTGRON MICROSCOPY (SEM)

hPSC-RPE cells were cultured on Transwell membranes coated with hrLN 521 for 60 days and fixed. The membranes were cut out, ethanol-dehydrated and critical-point-dried using carbon dioxide. Inserts were mounted on specimen stubs using carbon adhesive tabs and sputter coated with a thin layer of platinum. SEM images were acquired using an Ultra 55 field emission scanning electron microscope at 3 kV and the SE2 detector.

3.17 TRANSMISSION ELECTRON MICROSCOPY (TEM)

hPSC-RPE cells were cultured on Transwell membranes coated with hrLN 521 for 60 days and fixed. The membranes were cut out, put into thin strips and post-fixated in osmium tetroxide. The membrane strips were ethanol-dehydrated and finally flat embedded in LX-112. Ultrathin sections (~50–60 nm) were prepared using a Leica EM UC7 and contrasted with uranyl acetate followed by lead citrate. TEM imaging was done on a Hitachi HT7700 transmission electron microscope operated at 80 kV and digital images were acquired using a Veleta CCD camera.

3.18 STATISTICAL ANALYSIS

For statistical analyses, two-way ANOVA and posthoc multiple comparisons using Tukey test were performed.

3.19 VIABILITY TESTS

To test the possible cytotoxicity of the membranes used in ion-sensing devices 35,000 human dermal fibroblasts (HDFs) were cultured in different wells for 72h before adding the different membranes and components to the wells, either directly floating in the medium or on top of a Transwell. After 96h of incubation with the membranes, the cells were counted with MOXI automated cell counter.

3.20 PROLIFERATION TESTS

35,000 HDFs were cultured in different plates for 72h before adding the different membranes and components to the wells. After 6, 24, 36, 48, 72 and 96h of incubation with the membranes, the cells were counted with MOXI automated cell counter.

3.21 ADHESION TESTS

The membranes were drop-casted into the empty wells, followed by the seeding of 35,000 HDFs per well cultured for 48h approximately. Then, the cells were fixed with 4% methanol-free formaldehyde to continue with the immunocytochemistry.

4 RESULTS AND DISCUSSION

One of the main challenges that gene- and cell-based therapies are facing is the scalability of the process. There are many good protocols nowadays that unfortunately are only feasible at a research level, in some cases being able to generate enough product to cover the clinical trials' demand. Some products that have proven efficacy in this stage, and have been even approved, have encountered problems when scaling-up the production to reach commercial amounts. For this reason, it is crucial to design a scalable manufacturing process from the beginning of the protocol's development.

In our lab, a protocol to differentiate hESC into RPE like-cells had been developed [120]. This protocol, as well as many others [150,151] relies on the spontaneous differentiation of the cells through EB's formation, cultured in suspension. Five weeks later, pigmented OVs that have emerged from the EB's are manually dissected and dissociated into single cells, that are plated and cultured on a monolayer for 30 more days until the cells reach maturation (**Fig. 7**). Although this protocol generates a highly pure RPE population, it presents several drawbacks: feeding EB's in suspension and manually dissecting OVs is highly time consuming, the spontaneous differentiation results in batch-to-batch dependent efficiency, and the low ratio between the obtained RPE-like cells and the starting hESC could be highly improved.

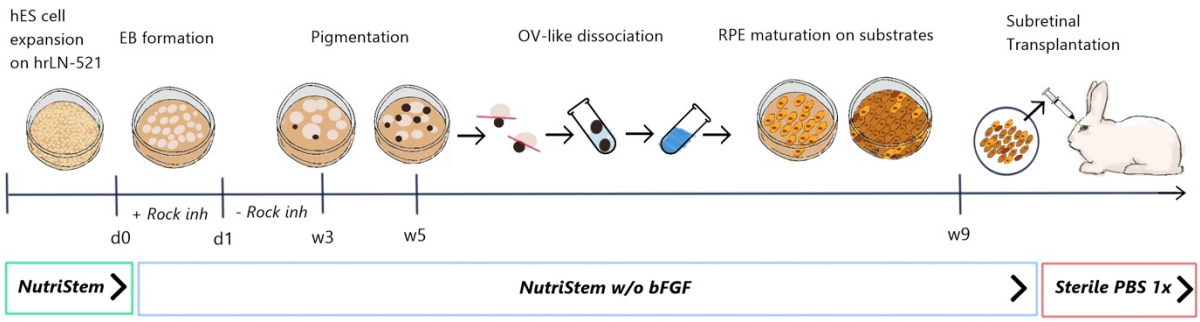

Figure 7. Graphical summary of the old protocol to differentiate hESC into RPE-like cells

In order to overcome the mentioned flaws, we have introduced several modifications developing a defined, scalable, robust, xeno- and manual selection-free protocol: instead of EB's suspension, plated hESC are maintained on a 2D monolayer culture on hrLN 521 with Activin A addition from day 6. 30 days later, Activin A is discontinued, and the cells are re-plated on fresh hrLN 521 and kept for 30 more days to reach maturation (**Fig. 8**). The fact that the cells are cultured from the beginning as a monolayer and that no manual dissection is needed generates a more streamlined and amenable to automatization process. Since at day 30 the cells are re-plated in 1:20 dilution, there is a ~1,300-fold increase in the yield, allowing a huge expansion in the production. Activin A addition, as shown previously [115,152–154],

promotes a neuroectoderm fate, increasing the efficiency of the differentiation and reducing batch-to-batch variation. In fact, this new protocol has proven to efficiently differentiate several hESC and hiPSC lines, showing high reproducibility and robustness.

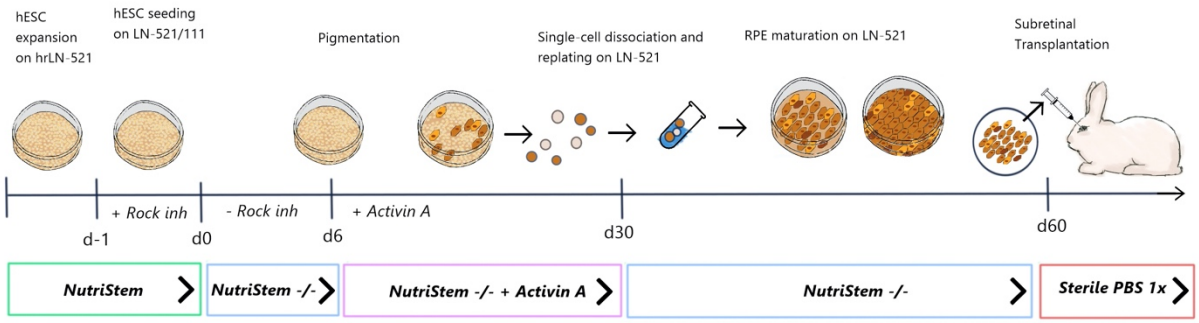

Figure 8. Graphical summary of the new protocol to differentiate hESC into RPE-like cells

Unlike other protocols, where multiple molecules like nicotinamide, taurine or triiodo-thyronin are added to induce an RPE fate [115,116,123,154], our protocol only relies on the addition of Activin A, which has shown to improve RPE differentiation efficiency from 40% to 90%. The fact that Activin A is the only added compound, makes the translation to the future GMP production much easier.

Since our lab was stablished by Professor Outi Hovatta, a pioneer in the derivation of hESC in Europe, the extensive knowledge and know-how on these cells is the reason behind choosing this source for our therapy. Nevertheless, other cells types have been and are being explored to treat AMD. Algvere et al. transplanted fetal RPE cells to dry and wet AMD patients without immunosuppression, concluding that the integrity of the blood-retinal barrier is essential to avoid the rejection of an allogenic graft [155]. Binder et al. and MacLaren et al. transplanted autologous temporal, nasal or extrafoveal RPE cells to treat foveal neovascularisation [11,156,157] resulting in transient improved visual acuity in some of the treated eyes, without recurrence of the neovascularisation. Autologous transplants of adult cells present less chances to be rejected, nevertheless, these cells could still manifest the underlaying disease following the same degenerative fate. hiPSC-RPE would generate another autologous source, and since these cells would be “younger”, could still be functional for many years [68,154].

Currently, several clinical trials are testing different conformations of the transplanted cells. The first-in-human iPSC-derived therapy to reach clinical trials was the transplant of an RPE sheet to treat wet AMD [68]. The advantage of transplanting the cells as a monolayer is that the cell-to-cell junctions and interactions are already stablished, which improves the survival rate of the transplanted cells as well as the maintenance of their polarity, a crucial feature in RPE function [128].

Kashani et al. and da Cruz et al. also transplanted an already stablished monolayer, but in both cases, the RPE cells lay on a synthetic scaffold, made of parylene and polyester, respectively [128,151,158]. Another option is the strategy used by Sharma et al., with a biodegradable substrate [154]. In advanced stages of the disease, when the Bruch’s membrane

is highly compromised, the support of a platform could offer great benefits. Nevertheless, it is important to keep in mind that sheet transplantations require a purpose-built delivery tool and more complicated surgery procedures that can carry postoperative complications.

On the other hand, cell suspension injections, like our approach and others [55,123,150,159], involves a less invasive procedure, minimizing possible adverse events. Schwartz et al. were the first ones to prove the safety of hESC-RPE carrying out the first-in-human clinical trial involving hESC-derived transplant tissue [55,56]. Their positive results on AMD and Stargardts's disease encouraged the multiple clinical trials running nowadays.

Another advantage of cell suspension is the feasibility to cryopreserve the cells, banking them and having a ready "off-the-shelf" product. Although it is under development, RPE sheets do not tolerate well the current freeze/thaw methods.

Cell suspensions also allow sorting the desired cells right before the transplantation. A positive/negative selection using RPE/hPSC (or any undesired cell type) markers could be implemented. In fact, we have validated an RPE cell-surface marker that could be used for that purpose: PDGFRbeta (a.k.a CD140b). The fact that RPE progenitors, and not hPSC, start expressing this protein, being kept by mature cells, allows its use to enrich the product in an automated manner, either in the middle of the protocol or at the end. Furthermore, the marker has also proved to be useful on the quantitative analysis of RPE purity, a very convenient application when developing in-process and QC tests for GMP-manufacturing. Although Choudhary et al. presented CD59 as another RPE marker that could be used in the same manner, our data show that CD59 is also expressed by hPSC, not being able to discriminate between differentiated and undifferentiated cells [160].

Exploring a positive/negative selection, we also identified two other markers that could be used to eliminate alternative lineages that appear during the differentiation process: CD184 (a.k.a. CXCR4) and GD2. Nevertheless, our single cell RNA sequencing data has shown that that this strategy would not be required to achieve a highly pure product since no undifferentiated cells have been found in the non-sorted samples, and it would only remove a small existing mesoderm contaminant (1.2% present without selection) and a portion of eye-field progenitors (from 11.3% to 3%), two lineages without apparent harmful effects.

As shown by many clinical trials, stem cell-derived therapies, like the one studied in this thesis, have a great potential to rescue and/or to regenerate a lost function, cell type or tissue, in the best cases being able to cure diseases that could not be treated otherwise. Nevertheless, they also present some risks that cannot be underestimated and have to be minimised.

Due to the inherent properties of hPSC, the source used in this kind of therapies, together with the culture and differentiation processes that these cells are exposed to, three undesired events have to be scrutinised in the final product: the presence of lingering undifferentiated and proliferative cells, the possible insertion of harmful mutations, and the migration to locations different than the intended ones.

Up to date, several groups with hPSC-RPE cells already in clinical studies have performed some tests to prove the safety of their products [68,135,154,158,161,162]. Their leadership, together with the World Health Organisation's suggestions [137] have served us to put together

a broad panel of assays to evaluate the safety of our cells. The exhaustive genomic analyses, single cell RNA sequencing, tumorigenicity studies and biodistribution tests performed have enabled to address all the events mentioned above (**Fig. 9**).

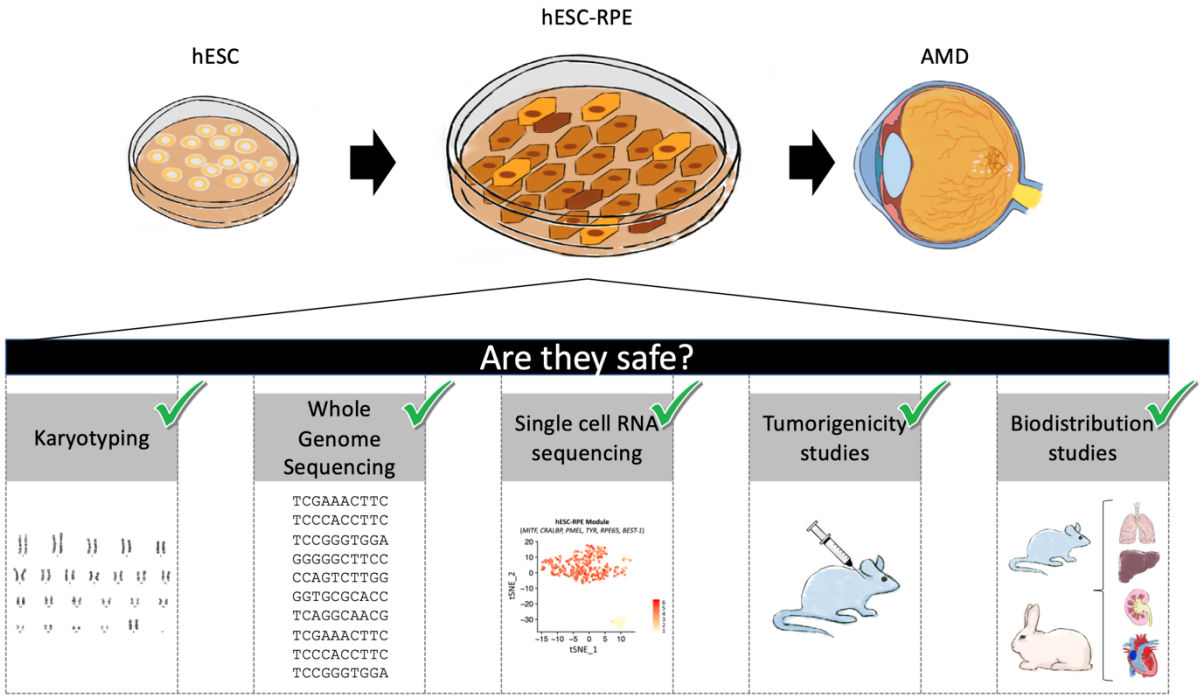

Figure 9. Graphical summary of safety studies addressed in Paper I

Some studies have shown that cells exposed to extended culture conditions or differentiation processes may acquire mutations [140,163]. Since the developed protocol entails 60 days in culture, the acquisition of variations cannot be underestimated. To further assess if the possible mutations would be induced by the differentiation itself or by the time that the cells have been in culture, whole genome sequencing has been performed on the source cells (hESC p22), the differentiated cells (hESC-RPE) and undifferentiated cells that have been maintained in culture for a similar period of time (hESC p38).

Interestingly, ~1,500 somatic SNVs have been found in both samples, hESC p38 and hESC-RPE, when compared to the source hESC p22. Also, when looking at CNVs or larger structural changes, similar numbers (~290 and ~20, respectively) have been found in both samples, with 70% of overlap. These findings suggest that the acquired mutations are mainly a consequence of the time in culture rather than the differentiation procedure, which also emphasise the general need for shorter protocols.

A major concern is the possible harmful effect of the found variations. For instance, after treating one patient, the first-in-human trial using RPE cells derived from hiPSC was suspended due to the finding of three SNVs and three CNVs that where not present in the

patient's fibroblasts [164]. Although the mutated genes were not driver genes for tumor formation, one of the SNVs was listed in a database of cancer somatic mutations. Aligned with this approach, we have matched the found variations with several cancer-related mutations databases like COSMIC or ClinVar. Remarkably, from the mentioned ~1,500 somatic SNVs, only 8 have been reported in COSMIC, and even more importantly, none of them or the larger structural changes are found in cancer-driver genes.

Apart from the acquired mutations, it is also very important to pay attention to the already present variations on the source cells. When we have compared our hESC p22 with the reference genome, more than 4,300,000 germline SNVs have been identified. Fortunately, only between 18 and 35 of these SNVs have been reported in COSMIC, ClinVar or the Shibata list, being all of them common variants, and similar numbers have been found when analysing the genome of 11 people from Personal Genome Project UK. The fact that the existing germline variants load is higher than the acquired through in vitro culture or differentiation, and that our source cells and normal participants show comparable load of clinically relevant germline SNVs emphasise the importance to examine the genome integrity at a deeper level than just karyotype and the challenge to find mutations-free starting material.

Although matching the variations with cancer-related databases is a legit strategy, the clinical relevance of the found mutations has to be analysed with further and more informative functional assays.

To address the potential risks of the mutations, tumorigenicity and biodistribution studies have been performed. Fortunately, 7 months after the injection of 10 million hESC-RPE cells in the neck of 10 mice, which supposes 100 times what a patient would receive, no tumor has been found. In the same line, when analysing the organs, no human cDNA has been found either, suggesting the lack of tumorigenic and migratory potential of the product. The rabbit experiments showed similar results, although small levels of human cDNA could be detected in the optic nerve and vitreous samples, most probably due to the sampling procedure or cells that refluxed into the vitreous after the transplantation.

The fact that the injection of 1.000 hESC or less has not been able to generate any tumor in the mice suggests that a residing small amount of undifferentiated cells among the RPE-like cells would most probably not suppose a harm for the patient. Nevertheless, since there is no certainty on this matter, it is very important to ensure the purity of the final product. Our analysis of the single-cell RNA sequencing generated two completely separated clusters, characterised by the expression of hESC and RPE markers, respectively. And most importantly, all cells in the RPE samples showed high levels of RPE markers, while none of them expressed any undifferentiated ones.

Another feature that has raised some concerns regarding the use of hESC as a source for regenerative therapies is the possibility of yolk sac's formation. Historically, this structure has been related to malignancy properties of the cells. In the teratomas formed by the

injection of our undifferentiated hESC, derivatives from the three germ layers have been found, but some of them also showed yolk sac formations. After doing some research in the literature, we have found that these structures are not a rare event [134,165,166], and the fact that the mature product is not tumorigenic suggests that this assumption could be reanalysed.

Until now, all the groups embarked on clinical studies with hPSC-derived products have had to figure out the required pre-clinical studies together with the pertinent authorities. Although there are some available guidelines [137,167], a thorough standardisation of the informative studies is crucial to ensure the safety and success of stem cells derived therapies.

With the ultimate goal of this thesis in mind, which is to bring hESC-RPE cells closer to the clinic, having proved the safety of the product and developed a scalable and robust protocol, its translation into a GMP-compliant process is the natural next step.

Testing suitable GMP-compliant reagents and materials to efficiently differentiate the cells, as well as arranging a set of in-process and quality control (QC) tests with defined thresholds to ensure the potency and purity of the final product (**Fig. 10**) has constituted the last part of this endeavour.

As mentioned, many protocols around the world have been developed to differentiate hPSC into RPE cells, but only a few of them fully defined, xeno-free and meet all GMP requirements.

One of the main advantages of our protocol, when compared to other available ones, is the reduced number of reagents and growth factors that are used, making a short list to be replaced. Our defined protocol mainly relies on the use of NutriStem hPSC XF medium without bFGF and TGFbeta to start a spontaneous differentiation, on hrLN 521 to support cell adhesion, and on Activin A to promote RPE fate. After arduous discussions between the medium manufacturer and the GMP facility, NutriStem hPSC XF medium has been approved, but the research-grade hrLN 521 and Activin A needed to be replaced. Fortunately, both GMP-friendly Activin A from R&D systems and hrLN 521 from Biolamina have reproduced results with similar efficiencies and purities on the differentiation of our GMP-grade hESC line (KARO1). These achievements, combined with the scalable manual selection-free monolayer protocol, are a great value for the future clinical production.

Trying to reduce some time and cost of goods on the manufacturing, different exposure windows to Activin A and different lengths of each part the protocol were tested. Interestingly, the optimal exposure time to Activin A seems to be line dependent, and re-plating the cells at least 30 days after starting the differentiation seems to be necessary, most probably related to the exposure time to Activin A. Since this molecule has showed to maintain pluripotency [48,168–170], the optimization of Activin A exposure is not only required to increase the RPE yield but also to minimise the lingering undifferentiated cells.

As mentioned, currently there are several groups running clinical trials with hPSC-derived RPE. The increasing diversity in protocols, manufacturing sites and starting materials

raises the need for a unified criterion capable of ensuring a constant and border-cross product's quality. Having a combination of molecular and functional tests with defined thresholds that fully characterise intermediate and differentiated cells from three hESC lines will enable a robust and validated global production, ensuring the cells' potency and minimising batch-to-batch variation (**Fig. 10**).

The previously mentioned identification of CD140b as an RPE marker has been an extremely valuable addition to this set of tests as it is able to quantitatively evaluate the differentiation efficiency at the middle and the end of the protocol.

Aiming for cheaper, safer and more convenient "off-the-shelf" product, the viability of the cells in the presence of the cryopreservant has also been tested, and again, the effect seems to be line dependent. It is still unclear which degree of cell maturation is the best in terms of transplantation, survival and integration, so the fact that the freeze/thaw step is well tolerated, by both the intermediate and more mature stages, is indeed encouraging.

In-Process tests		QC tests	
qPCR	Fold change	**qPCR**	Fold change
RPE65	> 5	*RPE65*	> 800
BEST1	> 3	*BEST1*	> 1500
MITF	> 80	*MITF*	> 300
SOX9	1 > x < 20	*SOX9*	4 > x < 30
TYR	> 50.000	*TYR*	> 150.000
PMEL	> 15	*PMEL*	> 50
TBB3	< 5	*TBB3*	< 2
NANOG	< 3	*NANOG*	< 1
Flow Cytometry		**Flow Cytometry**	
CD140b	> 50%	CD140b	> 80%
TRA-1-60	< 30%	TRA-1-60	< 1%
		PEDF secretion	
		Apical	> 350ng
		Basal	< 50ng
		TEER	> 10Ω*cm2
		Viability in Cryopreservant	
		0'	> 80%
		90'	> 70%
		4h	> 60%
		Viability after thawing	> 50%

Figure 10. Proposed threshold values for in-process and Quality Control tests for GMP-production of hPSC-derived RPE cells at day 30 and 60 of differentiation

As important as it is to prove the safety of stem cell-derived therapies, it is also important to prove the safety of any device intended to be on-the body. With the evolution of the health care system, there is an increasing demand for wearable sensors with different applications [171], from ion-detection on the sweat for a personalised recovery to disease prevention. Some of these sensors are synthetic devices that lay on the skin [172–174], sometimes even penetrating several layers and being in direct contact with the cells and interstitial fluids

[175–179]. For instance, ion-selective electrodes, a type of sensor that has attracted increasing attention over the past years [180–182], might comprise an ion-selective membrane composed by polymers and plasticisers, an ion exchanger and an ionophore. A major concern is the possible cytotoxic effect of any of these compounds that could lead to multiple adverse effects.

After doing some research on the available literature, we have realised that there is a lack of studies addressing the cytocompatibility of such compounds, especially with fibroblasts, one of the main cell types of the skin. Thus, the performed viability, proliferation and adhesion tests provide valuable information for the design and fabrication of future devices.

The culture of HDFs in the presence of different membranes with different compositions for a specific period of time has allowed us to conclude that only potassium ionophore I (a.k.a. valinomycin) and ammonium ionophore I (a.k.a nonactin) are able to leach from the membrane to the media, having a cytotoxic effect, with a ~55% of cell viability compared to control conditions. Nevertheless, it cannot be discharged that other ionophores may also present cytotoxicity with longer exposures (> 96h)

The time-course assays have suggested that, since the reduction in cell numbers starts from 36h, the leaching of the ionophores occurs between 24 and 36h of incubation. Furthermore, the leaching also seems to be dependent on the conformation of the membranes, being the membrane typically used in inner-filling solution electrode the one with the worst outcome, most probably due to the higher content of valinomycin. Although it has been hypothesised that different plasticisers could also have an effect on the leaching of the ionophores, our results have not shown differences big enough to stablish a general conclusion.

Up to now, all the observed cytotoxic effects have been a result of the ionophores' leaching into the media. Trying to mimic a more real scenario with the cells in direct contact with the membranes, adhesion tests have been performed. These tests have been able to evaluate two different possible events: the cytotoxicity due to the direct contact with compounds and the cells' capacity to adhere and grow on the membranes. For instance, Miller et al. reported the need for a cell-resistant coating to inhibit macrophage adhesion to their developed microneedle [183], which could interfere in the electroanalytical performance. While no preferential growth on the membranes has been observed, the plasticiser FNDPE, apart from the already described toxic ionophores, has also shown a cytotoxic effect after 36h of exposure.

In order to evaluate the possible mechanisms behind the reduction in cell numbers by the different conditions, immunostaining for Ki67 (proliferation marker) and CASP3 (apoptosis marker) has been performed. The reduced number in Ki67+ cells and the lack of CASP3+ cells on the valinomycin and nonactin membranes suggests that these compounds act inhibiting the fibroblasts' proliferation, emphasising their possible adverse effects on the

skin’s turnover. Further experiments would be required to rule out mechanisms of action for cell death different than apoptosis.

Since mutacin, another potassium ionophore, presents similar potentiometric performances and does not present toxicity, it could be considered as a biocompatible alternative to valinomycin.

All these results encourage a prompt cytotoxic evaluation of the available compounds on the early stages of any sensor design and development with an intended biomedical application.

5 CONCLUSIONS

With the increasing numbers of stem cells-derived therapies reaching clinical studies, there is a striking need for robust and scalable protocols that are able to produce large amounts of fully characterised and safe cells.

The work developed during this thesis has contributed to the development of a defined, scalable, robust, xeno- and manual selection-free protocol that has proven to efficiently differentiate several hPSC lines into highly pure RPE-like cells. The identification of the RPE marker CD140b has provided a very practical tool to enrich the final product in an automated manner if desired, and to quantitatively assess the differentiation efficiency.

An exhaustive panel of genomic and functional assays have proved the safety of our derived RPE-like cells, characterised by a considerable genomic stability as well as the lack of tumorigenic and migratory potential. The compiled tests could be a reference for the needed standardisation of safety studies on the development of stem cells-derived products.

The replacement of some reagents and materials has allowed to efficiently differentiate an in-house derived GMP-grade hESC into RPE-like cells, following a fully GMP-compliant protocol. This achievement combined with the validation of a set of in-process and QC tests with defined thresholds is bringing these cells closer to the GMP-production and the near clinical studies.

The findings on the cytotoxicity assays performed on the components used on ion-sensing devices increases the awareness about the need for implementing this kind of studies during the very first steps of any sensor development with an intended on-body application.

6 FUTURE PERSPECTIVES

At a global level, many efforts are being done around the world to keep developing new gene- and cell-based therapies to cover unmet needs. Currently, 16 products have been already approved by the FDA, and with over 30,000 new compounds in clinical trials, many more approvals are expected. These coming novel therapies present many new safety, manufacturing and regulation challenges that will have to be quickly addressed. Furthermore, in order to reach the patients, since these products carry a very high production cost, alternative reimbursement models will have to be found.

When it comes to AMD's treatment, different approaches have been and are being tested currently in several clinical trials. The coming results will put some light on which is the best path to follow.

At a closer level, our lab has already stablished a hESC line under GMP conditions, and it has proved to efficiently differentiate it towards RPE-like cells following a GMP-compliant protocol. Since a list of in-process and QC tests has been already validated, the next step is to start manufacturing the cells in Vecura, a GMP facility part of the Karolinska Cell Therapy Center (Stockholm). In order to safely bring these cells to clinical trials, early conversations with the pertinent regulatory authorities have already been stablished.

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