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Molecular and Cellular Neuroscience

Human Pluripotent Stem Cells: a Toolbox to Understand And Treat Retinal Degeneration

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1. Introduction

Composed of different neural layers, the retina converts light inputs into electrical signals, which are then transmitted to the brain. Photoreceptors (PRs), cones and rods, are the primary cells involved in light detection. Rods are sensitive to dim light whereas cones detect specific wavelengths allowing color vision. The electrical signal generated by PRs is further processed by other neurons (bipolar, amacrine, horizontal cells) and transmitted to the brain through ganglion cells. A number of retinal diseases, such as Age-related Macular Degeneration and Retinitis Pigmentosa, leads to the degeneration of PRs which ultimately results in vision impairment and in some cases in blindness. Although highly informative, animal models are not able to recapitulate all the pathological hallmarks of these pathologies and human diseased tissues are difficult to obtain. **Development of differentiation protocols to generate retinal cells from human pluripotent stem cells (hPSCs) has considerably broaden the access to human cellular models of retinal degeneration.** They offer renewed opportunities to better understand the underlying pathological mechanisms and develop new therapeutics. In addition, retinal tissues derived from healthy hPSCs also represent a promising source for regenerative medicine. This review will discuss these different aspects of the use of hPSCs to understand and treat retinal disorders.

2. Retinal degenerative diseases

2.1. Age-related Macular Degeneration

After uncorrected refractive errors and cataracts, AMD is the third leading cause of moderate or severe vision loss worldwide [1]. Projections for 2040 are alarming with an estimated number of people affected with all stages of the disease of 288 million [2]. Interestingly, the prevalence of all stages of AMD is higher (12.3%) in people with European ancestry than Asian (7.4%) or African (7.5%) populations [2]. The geographically isolated population of Timor-Leste has an even lower prevalence for AMD - less than 0.5% - suggesting the influence of genetic context on disease appearance [3]. AMD is classified according to the stage of the disease based on ophthalmic examinations. Early or intermediate AMD is characterized by the presence in the macula of medium drusen (diameter between 63 and 125µm; early AMD) or large drusen (larger than 125µm) and changes in retinal pigmentation (intermediate AMD) [4]. Late AMD is defined by the presence of macular choroidal neovascularisation (wet AMD, corresponding to 10-20% of cases) or macular atrophy (dry AMD, corresponding to 80-90% of

cases) [4, 5]. Early AMD is usually asymptomatic [4]. Initial symptoms are distortion of central vision progressing towards scotomas in the central visual field. In wet AMD, new vessels develop in the macular area and are susceptible to leak and generate hemorrhages, damaging the macula [6]. Vision loss can be fast and severe in this context. Vision loss in dry AMD is more progressive with the degeneration of the retinal pigment epithelium (RPE) and subsequent atrophy of macular retina.

AMD is caused by a combination of genetic and environmental factors. The major risk factor is age. The prevalence of all stages of AMD is of 3.5% between the ages of 55 and 59 years but rises to 27.3% after 85 years in the European population [7]. Smoking is a critical modifiable risk factor in the development of late AMD [8]. Other environmental risks include sunlight exposition, diet, alcohol and hypertension. Among the genes associated with increased risk for AMD, complement factor H (*CFH*) and age-related maculopathy susceptibility 2/ high-temperature requirement factor A1 (*ARMS2/HTRA1*) polymorphisms are to date the most important [6]. Several mechanisms are suggested to play a role in AMD such as inflammation, defective regulation of the complement system, oxidative stress, metabolic dysfunctions, accumulation of lipids and extracellular matrix alterations [6, 9, 10]. However, the exact pathological mechanisms are still not fully understood. Accumulation of proteins and lipids in the Bruch's membrane is suspected to promote inflammation and affect the circulation of nutrients and liquids, damaging the RPE. In addition, RPE degeneration in dry AMD may precipitate the secondary loss of PRs.

No treatment is currently available for the dry form of AMD. The most effective clinical treatment for wet AMD patients is anti-VEGF therapy delivered at regular intervals to prevent worsening eyesight. Although response to therapy is not systematic, some patients experience a moderate improvement in visual acuity [5]. Patient compliance to treatment protocol could be one of the reasons for incomplete response as injections are frequent (once a month or every 2 months) and expensive. Diet supplementation with zinc and antioxidants in AMD patients has also been suggested to decrease the risk of progression to late AMD [11].

2.2. Retinitis Pigmentosa

RP is a heterogeneous group of inherited retinal dystrophies, which affects 1.5 million of patients worldwide [12]. The inheritance of RP is autosomal-recessive in 50-60% of all cases

and linked to chromosome X in 5-15% of patients [13, 14]. Syndromic forms of RP account for 20-30% of cases, including Usher's and Bardet-Biedl syndromes (the most frequent ones) [14]. The ophthalmic eye fundus examination reveals the classical clinical triad of RP: bone spicule pigmentation, which may correspond to melanin deposits from RPE cells that have migrated into the retina, attenuation of retinal vessels that could be secondary to PR loss and waxy pallor of the optic nerve likely due to the presence of glial cells [12]. While clinical manifestations vary according to the underlying genetic defect, RP patients typically experience night blindness - which may not be noticed by patients at early stages - followed by a progressive reduction of the visual field (tunnel vision). RP patients also report seeing flashes of light (photopsia) which worsen under stress [15, 16]. Central vision is usually preserved until the later stages of the disease. The median age of symptom appearance is 29-year old [17]. Of note, the age of onset as well as the clinical manifestation and progression of the disease vary depending on the mutated gene in addition to genetic background and environmental factors. Most patients with RP are legally blind by 40-year old due to severe visual field alterations [14].

The primary cellular defect is localized in rods or, for about 5% of cases, in the RPE [18]. At advanced stages of the disease, optical coherence tomography shows that the ganglion cell layer and inner nuclear layer are relatively well preserved while the PR layer is completely depleted. However, a retinal remodeling occurs following the complete loss of PRs leading after decades of degeneration to rewiring, glial hypertrophy and global cell death [13]. This important aspect of the pathology suggests that the retinal circuitry does not degenerate immediately after loss of PRs. RP patients could thus be eligible to PR cell transplantation during a specific time window. To date, a vast number of disease-causing variants in more than 80 genes for RP have been identified (<https://sph.uth.edu/retnet/sum-dis.htm>). Most of these genes encode proteins that play a role in the photo-transduction cascade, the visual cycle, ciliary structures and transport, RNA splicing as well as intracellular trafficking [12, 14]. However, disease-causing mutations are still unknown in 30% of non-syndromic and 50% of autosomal-dominant RP patients [12, 14].

Few therapeutic options are available to treat RP. The only approved treatment is a gene therapy for patients with *RPE65* gene mutations (2% of RP cases, Luxturna®) [19]. This therapy was shown to be safe and induced an improvement in navigational abilities and light

sensitivity. The effect was found maximal at 30-day post-injection and stable for at least 4 years [20]. Vitamin A palmitate as well as docosahexaenoic acid (DHA, an omega-3 fatty acid present in oily fish) supplementations are recommended by some clinicians. While still debated, a few studies reported that such nutritional treatments might delay vision degradation [14, 21, 22]. Approximately 40-50% of individuals with RP develop cataracts that further impair their visual acuity. These patients can benefit from cataract surgery with significant visual gain [23, 24]. Finally, night vision devices (ie. specific goggles) that amplify light can be used by patients to improve their mobility [25, 26].

3. Generation of retinal cells from human pluripotent stem cells

The development of physiologically relevant cellular models is a key step towards modeling the complexity of retinal diseases. Indeed, it is essential to accurately capture the biological complexity of the retina *in vitro* to better understand the underlying pathological mechanisms and ultimately find new therapeutics. The different cell sources to generate such models include early postnatal PR precursors from animal models, human fetal and adult primary cells or tissues, and more recently hPSCs - including both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) (reviewed in [19]). hPSCs have gained great interest from the research community owing to their unlimited self-renewal potential and their ability to differentiate into any cell type of the adult body. First derived in 1998, hESCs are isolated from the inner cell mass of human blastocyst-stage embryos [27]. Sharing the same key properties, hiPSCs are adult cells that have been genetically reprogrammed to an embryonic stem cell-like state by the forced expression of a cocktail of pluripotency factors [28]. Besides their use in disease modeling studies, they also represent a renewable cell source that enable the derivation of large numbers of functional retinal cells for regenerative medicine.

3.1. Eye development

Substantial progress has been made over the last 15 years in the development of robust protocols to differentiate both hESCs and hiPSCs into retinal cells by mimicking *in vitro* the developmental steps occurring in the human embryo. The vertebrate retina is derived from the neuroectoderm, initially through the concomitant inhibition of Activin/Nodal, bone morphogenetic protein (BMP) and Wnt/ β -catenin signaling [29-31]. Shortly after gastrulation, the anterior neural plate is specified into the eye field in which cells co-express a unique

combination of transcription factors called the eye-field transcription factors (EFTFs). Then the eye field evaginates from the diencephalon to form bilateral optic vesicles containing retinal stem cells that will give rise to the optic stalk, RPE and neural retina (NR) [32]. These retinal stem cells commit towards a specific cell lineage after invagination into the bi-layered optic cup depending on their initial regionalization: while the NR originates from the inner layer, the outer layer gives rise to the RPE [33]. Within the naive NR, retinal progenitor cells (RPCs) give rise in a temporal and conserved order to the different retinal cell types, including PR precursors, to form the mature retina with its characteristic laminar cytoarchitecture.

This differentiation process is tightly regulated by exogenous signals coming from adjacent tissues and cell-intrinsic factors, such as Wnt, fibroblast growth factor (FGF), BMP, Notch, sonic hedgehog (SHH), retinoic acid (RA) and activin A signalings [33]. Levering on developmental studies, the overall strategy is to expose hPSCs to signaling molecules in a time and dose dependent manner along the differentiation process to generate the retinal cell type of interest. For an in-depth description of differentiation protocols, we refer the reader to excellent review articles [34-36].

3.2. Derivation of RPE cells

Both hESCs and hiPSCs have the potency to differentiate into RPE cells that are equally effective in protecting the retina from degeneration following transplantation into the subretinal space of a RP rodent model [37]. **A summary of selected differentiation protocols to generate RPE cells from hPSCs is listed in Table S1.**

Initial studies demonstrated the spontaneous differentiation potential of hPSCs into RPE cells upon FGF2 withdrawal from the culture medium [38-41]. After a few weeks in culture, pigmented areas begin to emerge and cells acquire a distinctive cobblestone morphology. These pigmented patches are manually isolated and amplified to obtain a pure population of RPE cells (**Figure 1**). However, such “spontaneous” protocol is highly operator-dependent, time-consuming (2-3 months) and does not allow for the production of large cell banks. To overcome these limitations, studies have focused on directing RPE differentiation by adding signaling molecules that mimic developmental cues known to be important in RPE development and specification at critical time points.

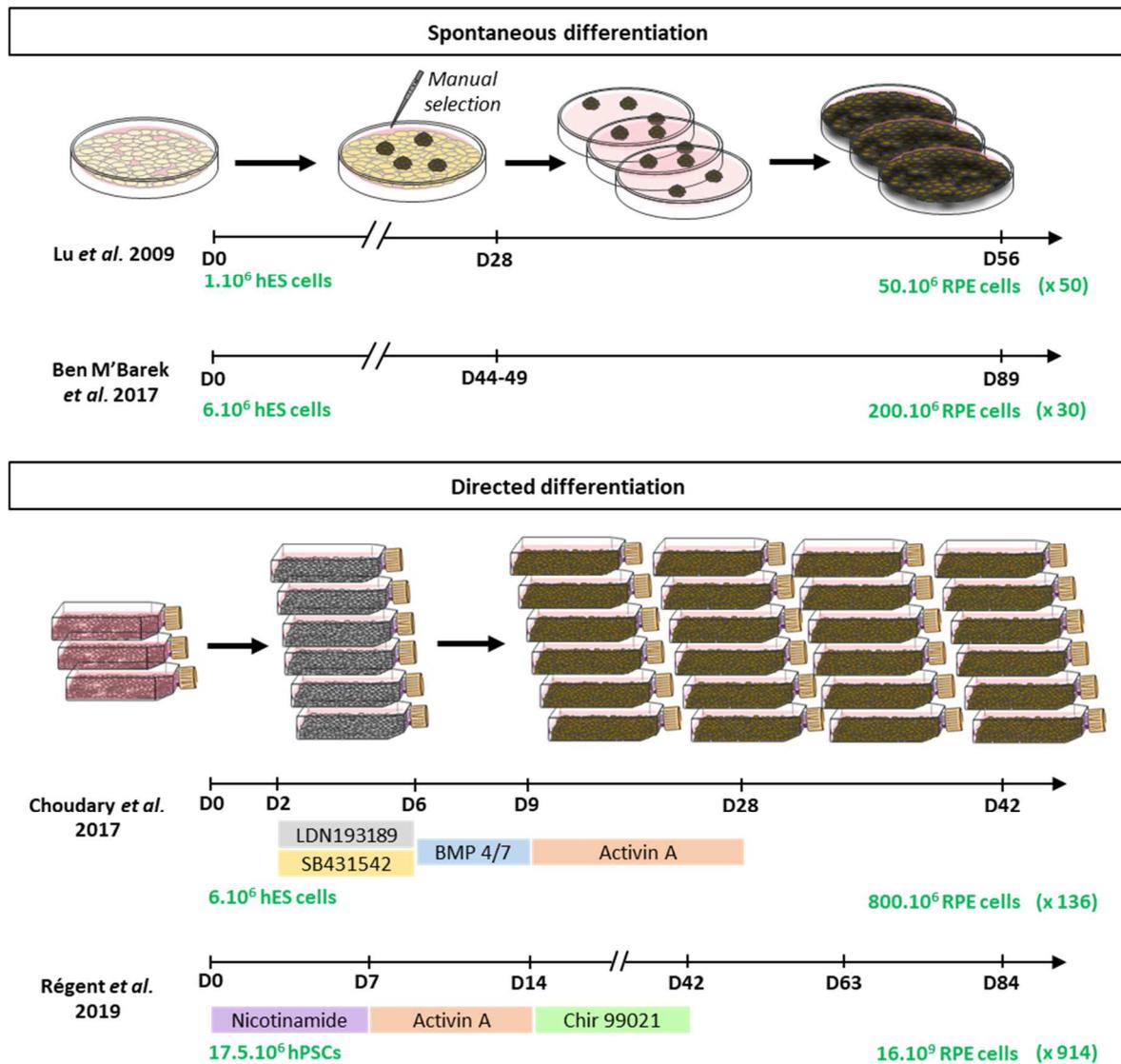


Figure 1: Schematic representation of spontaneous and directed differentiation methods to obtain RPE cells from hPSCs based on selected studies.

Early work by the group of Masayo Takahashi reported that hPSCs exposed to Wnt and Nodal antagonists using a SFEB (serum-free floating culture of embryoid body-like aggregates) system gave rise to 30-35% of cells positive for the RPE characteristic transcription factors PAX6 and MITF after 6 weeks [42, 43]. Two other studies showed that addition of nicotinamide and Activin A, a member of the TGF- β superfamily, promoted the differentiation of hPSCs to neural and subsequently to RPE fate with 80% of pigmented cells after 8 weeks in culture [44, 45]. Based on previous research [44, 46, 47], Buchholz *et al.* further combined use of retinal inducing factors (Noggin, DKK1, insulin-like growth factor-1 (IGF-1), FGF2) and known RPE differentiation factors (nicotinamide, Activin A, FGFR/VEGFR inhibitor SU5402, and vasoactive

intestinal peptide - VIP) [48]. Addition of these factors at specific times led to the conversion of approximately 80% of the cells to an RPE phenotype in only 14 days, as evidenced by the expression of pigmentation marker PMEL17 [48]. Although these directed protocols considerably increased the yield of RPE cells from hPSCs compared to that obtained with the spontaneous method, mechanical enrichment of pigmented cells during the course of the differentiation was still required to obtain a homogenous RPE cell population. The same team improved the protocol by addition of CHIR99021, an activator of the Wnt canonical pathway. It improved significantly the efficiency of RPE derivation from hESCs to 97%, bypassing the necessity of manual enrichment [49]. However, it is important to note that this protocol did not significantly reduce the differentiation duration from that of the “spontaneous” method as RPE cells were banked after more than 100 days in culture [49]. More recently, our group developed a simplified protocol in which hESCs were treated with nicotinamide, Activin A and CHIR99021 in a sequential manner to obtain highly enriched and functional RPE cells within 84 days [50].

As a step toward clinical use in regenerative medicine, differentiation protocols were optimized to obtain RPE cells: i) in conditions using a good manufacturing practice (GMP)-compliant production process and ii) in large numbers to treat the millions of patients affected by RPE-associated retinal degeneration (**Figure 1**). **Some studies addressed these bottlenecks by developing xeno-free/ feeder-free protocols [51-55]. The development of directed differentiation protocols has fostered the implementation of automated systems to upscale the production process and increase its robustness. The use of a modular platform was reported for the long-term maintenance and passaging of hiPSCs and suggested the potential to differentiate cells to retinal lineages, including RPE [56]. Matsumoto et al. further demonstrated the feasibility of the automated culture of hiPSC-derived RPE cell sheets in a closed automated cell-culture system, removing the need for operational skills and facilities [57]. We recently achieved a fully automated process allowing the large-scale production of RPE cells from hPSCs, considerably upscaling the production capacity as more than 16 billion of mature and functional RPE cells could now be produced within 12 weeks with only one round of production [50].**

3.3. Derivation of photoreceptors

A summary of selected differentiation protocols to generate PRs from hPSCs is presented in Table S2.

Pioneering work from the group of Thomas Reh demonstrated the capacity of hESCs to differentiate *in vitro* into PRs (**Figure 2**) [47]. Using a combination of the eye field inducer IGF-1, BMP signaling inhibitor Noggin and Wnt/ β -catenin signaling pathway antagonist Dkk1, they were able to direct hESCs into 80% of PAX6+/VSX2+ retinal progenitors after 3 weeks in culture [31]. However, differentiation of these RPCs into PRs was infrequent with only 12% of CRX-positive PR precursors and less than 0.01% of S-Opsin- or Rhodopsin-positive mature PRs [31]. In two other studies, Osakada *et al.* cultured hPSCs as embryoid bodies using a SFEB system in presence of Wnt and Nodal antagonists for 20 days before switching them to adherent culture (3D/2D protocol) with the addition of factors known to be involved in rod genesis (RA, taurine, FGF2, Shh) [42, 58]. Twenty percent of CRX-positive cells were obtained, giving rise later to approximately 8.5% of Rhodopsin-positive mature PRs by day 200 [42, 58]. It was also the first demonstration that inhibition of Notch signaling *in vitro* with the γ -secretase inhibitor DAPT induced the cell cycle exit of proliferating RPCs and a concomitant increase in early born ganglion cells and CRX-positive PR precursors [42]. Using a similar 3D/2D approach, Meyer and collaborators showed that hPSCs could differentiate into early and late PR phenotypes without the need of exogenous factors in a sequence and time course highly reminiscent of normal retinal development [59]. This protocol was further refined into a 3D/2D/3D method to isolate neural rosette-containing cell colonies maintained as cellular aggregates in suspension [60]. Using this technique, a subpopulation of aggregates developed optic vesicle characteristics and differentiated toward the PR lineage with 56% of CRX-positive cells by day 80 [60]. Although these studies successfully recapitulated the main steps of retinal development *in vitro*, key structural and functional features of PRs, i.e. the presence of outer-segment discs and light sensitivity, were still lacking.

Considerable progress came with the first production of self-organized bi-layered optic cup structures from hESCs that then differentiated into a multilayered neural retina containing retinal cells, including cones and rods (**Figure 2**) [61]. Importantly, this study was the first to report the formation of PRs with reasonable development of inner segments and connecting cilia [61]. Based on the protocol developed by Meyer *et al.* [59, 60], the group of Valeria Canto-Soler more recently demonstrated that hiPSCs could also self-organize into retinal cups that

generate a fully laminated 3D retinal tissue in presence of serum, RA and taurine [62]. Noteworthy, it was the first evidence that PRs reached an advanced stage of maturation *in vitro* as evidenced by some level of photosensitivity in a limited number of cells and the presence of a minority of PRs with outer-segment discs after 27 weeks of differentiation [62]. Although introduction of 3D organoid technology has dramatically improved the generation of mature PRs, current protocols are still hindered by variability issues. A recent study by Capowski *et al.* generated retinal organoids from 16 hPSC lines and monitored their appearance and structural organization over time in an attempt to develop a staging system to reduce inconsistencies in cultures [63]. Three consistent morphological stages of hPSC-derived retinal organoid development were distinguished by light microscopy, each one corresponding to a specific cellular composition and lamination relative to the timeline of differentiation [63].

For future clinical use of hPSC-derived PRs, recent studies have focused on adapting these research-grade protocols to GMP-grade under feeder-free and xeno-free conditions [51, 52, 64]. While functional RPE cells are currently derived from hESCs with high yield and purity using GMP-compliant differentiation protocols, challenges remain for PR production. This is notably due to extremely long culturing times required to obtain mature PRs *in vitro* (>100 days), operator-dependent manual enrichment of neural retina / optic cup structures as well as heterogeneous cellular composition of organoids. Different selection strategies have been developed to enrich PRs from retinal cultures. A screen of human retinal samples and hPSC-derived retinal organoids against a large panel of human monoclonal antibodies using a high throughput flow cytometry approach identified PR-specific cell surface markers [65]. Double negative CD29/SSEA-1 selection increased the enrichment of CRX and Recoverin positive cells from 16.5% to 61% from day 200 retinal organoid cultures [65]. In addition, a cone biomarker panel combining CD markers for positive cone selection (CD26, CD147, CD133) and negative selection of undesirable cells (SSEA-1) successfully enriched L/S-opsin cones from fetal retina and 17-18 week-old hESC-derived retinal samples to 30% and 50% respectively [66]. **The group of Olivier Goureau also showed that CD73 targeting by magnetic-activated cell sorting (MACS), previously described for the enrichment of mouse PSC-derived PRs [67], was an effective strategy to isolate a transplantable population of PRs from hiPSC-derived retinal organoids at day 120 [68].**

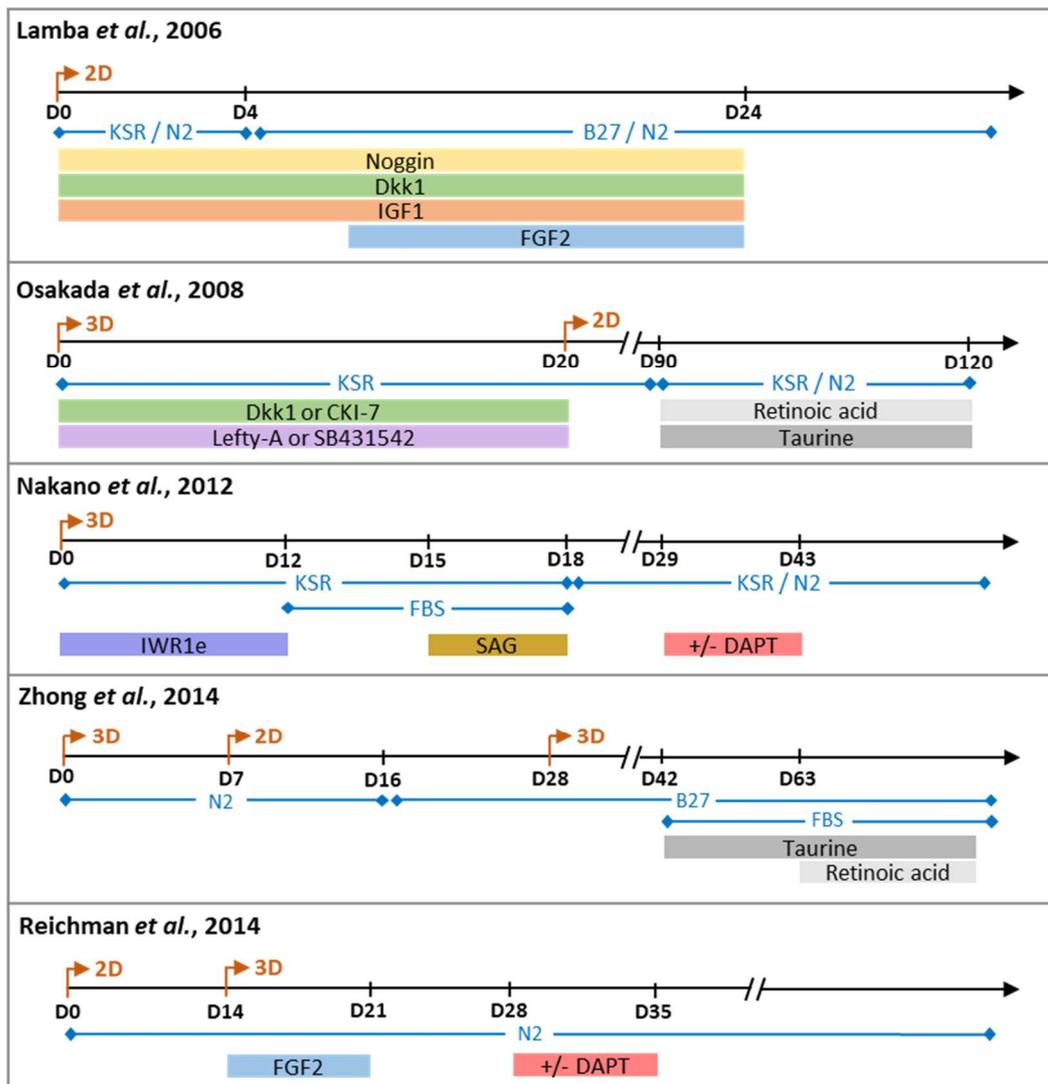


Figure 2: Main protocols for the differentiation of hPSCs into PRs *in vitro*.

4. Human pluripotent stem cells to model retinal degeneration

4.1. Cellular models

Disease modeling using hPSCs requires a precise experimental design in order to decipher the relevant pathological mechanism with minimal bias. Three parameters are key to define: (1) cell type(s) affected, (2) genetic and/or environmental nature of the disease and (3) appropriate controls.

First, the retinal cell type that is affected should be defined according to the selected disease. If the degeneration/dysfunction is a secondary cause of another cell type, co-cultures or complex assemblies such as organoids might be considered. Cultures of an isolated cell type helps to discriminate between molecular mechanisms that are cell

autonomous and those that are cell extrinsic. Therefore, these different approaches are complementary to capture the complexity of pathological mechanisms. The second aspect is related to the genetic nature of a disease. A causative gene (RP or other genetic disorders) are relatively easy to model. When susceptibility alleles are identified as suggested for AMD, the variability and inconsistency of the model could be difficult to predict. For this reason, appropriate controls should be defined early in experimental design. Pioneer studies used one hiPSC line to characterize a disease but this is not sufficient nowadays as troubles with reproducibility were frequently reported. Indeed, the genetic background of patient or control cell lines could interfere with the marker evaluated. Therefore, it is important to reproduce the experiments with other hPSC lines to capture the relevant phenotypes. In addition, CRISPR/Cas9 technology allows precise gene editing that could be used both to restore the corrected gene or protective allele in patient hPSCs or in the opposite case to generate the gene mutation or susceptibility allele in non-affected hPSCs. Such isogenic lines that neutralize the impact of different genetic backgrounds are powerful to isolate contribution of a gene mutation or an allele to a disease mechanism.

4.1.1. Age-related macular degeneration

As a multifactorial and late onset disease, AMD is by nature difficult to model. The genetic contribution to the disease is well established with *CFH* and *ARMS2/HTRA1* variants being the most strongly associated [6]. In a recent genome-wide association study, 52 independent risk variants distributed across 34 loci were also identified [69]. However, their exact roles are still largely unknown. Thus, the global strategy is to evaluate the implication of the different risk alleles associated to AMD, focusing on the most affected cell type (ie. RPE cells). **Thereafter, we will only focus on AMD models linked to *CFH* and *ARMS2/HTRA1* variants.**

AMD hiPSC derived RPE (hiPSC-RPE) cells carrying the *ARMS2/HTRA1* high-risk genotype did not present an obvious diseased phenotype but were found similar to 1-year old wildtype monkey RPE [70]. As lipofuscin fluorophore A2E accumulate with age in normal RPE cells, hiPSC-RPE cells were treated with A2E for 10 days. In that context, hiPSC-RPE cells from AMD patients homozygous for the high-risk haplotype exhibited a reduced protein level and activity of superoxide dismutase 2 (SOD2), suggesting a compromised antioxidant capacity [70]. This was confirmed following exposure to blue light in order to generate oxidative stress [70]. The same results were also obtained by an exposure to H₂O₂ [71]. In addition, this last study

identified a dysfunction of the NAD-dependent deacetylase sirtuin1 (SIRT1) and peroxisome proliferator activated receptor gamma coactivator 1-alpha (PGC-1 α) pathway that is suggested to alter mitochondrial biogenesis and remodeling causing increased reactive oxygen species (ROS) production [71]. The group of Sally Temple further characterized the effect of the homozygous *ARMS2/HTRA1* risk genotype and evidenced a significantly higher expression of complement and inflammatory factors similarly to what is observed in AMD patients [72, 73] (Figure 3).

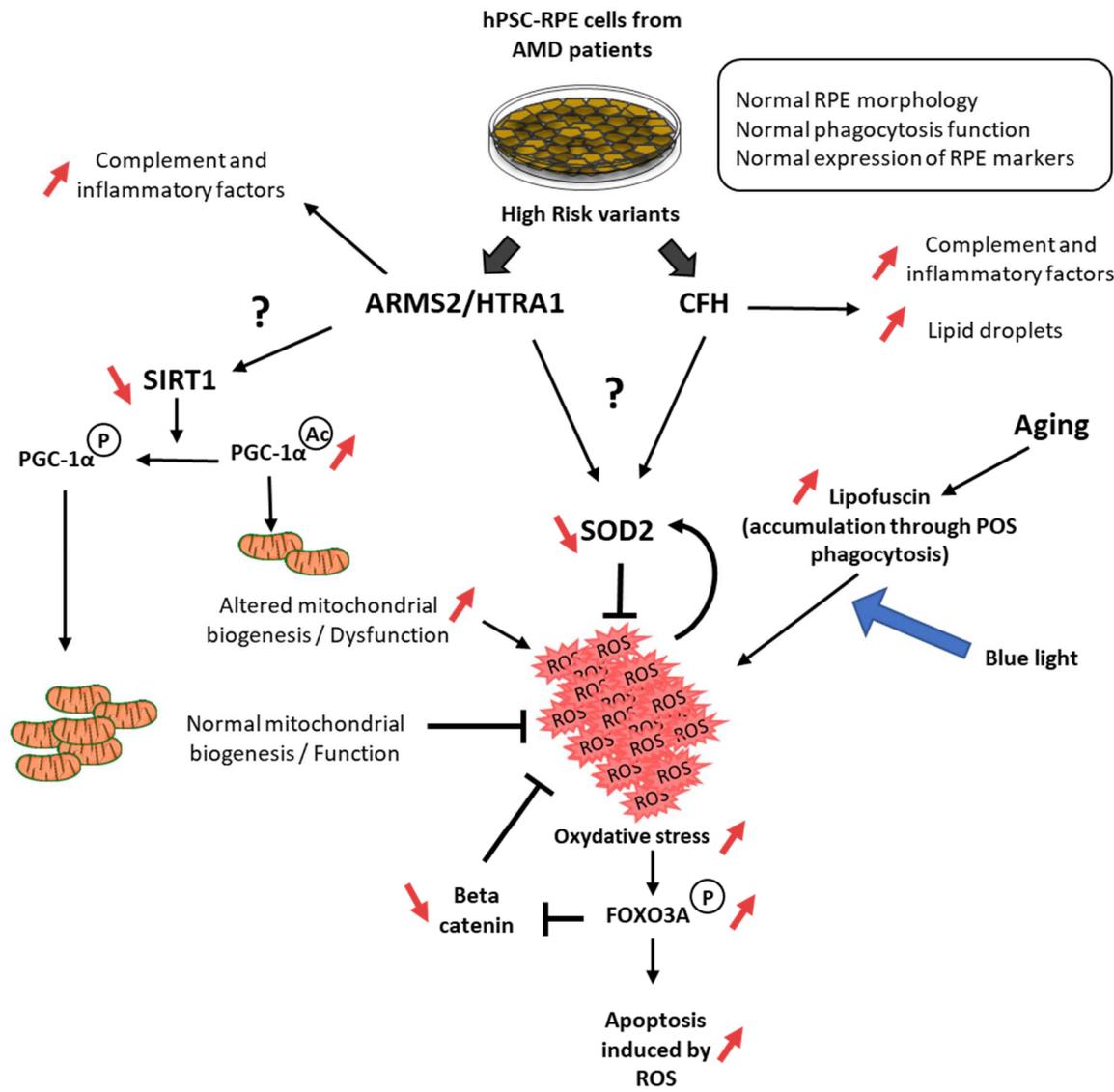


Figure 3: Current model of pathological mechanisms underlying AMD based on hiPSC-RPE cell studies. Red arrows indicate components that are modulated by the disease (increased or decreased function). P: phosphorylation; Ac: acetylation; RPE: retinal pigment epithelium; POS: photoreceptor outer segment; ROS: reactive oxygen species.

hiPSC-RPE cells carrying the high-risk Y402H polymorphism in *CFH* gene did not exhibit obvious morphological or functional defects (RPE markers, secretion of PEDF, transepithelial resistance, phagocytosis potential). A deeper characterization revealed signs reminiscent of AMD, including increased inflammation, cellular stress, accumulation of lipid droplets and deposition of drusen-like deposits [74] (**Figure 3**). *SOD2* gene expression level was, interestingly, also reduced in hiPSC-RPE cells carrying the high risk allele. **When *CFH* gene is overexpressed, hiPSC-RPE cells exposed to oxidized lipids are protected from cell death mediated by a caspase dependent apoptosis process [75]. Taken together, these results suggest that the *CFH* gene function in response to oxidative stress is altered by the high-risk Y402H polymorphism.**

4.1.2. Retinitis Pigmentosa

hPSC technology has also been used over the last decade as a model platform to study RP, the most common form of hereditary retinal disorder (**Table 1**). In the first report of an *in vitro* disease model, the group of Masayo Takahashi derived hiPSCs from RP patients carrying known causative mutations in the RP1 axonemal microtubule associated protein (*RP1*), Pim-1 kinase associated protein (*RP9*), peripherin 2 (*PRPH2*) or rhodopsin (*RHO*) gene [76, 77]. As observed in RP, patient-derived rod PRs from all mutations selectively degenerated *in vitro*. These cells expressed markers for endoplasmic reticulum stress (*RHO* mutation) and oxidative stress (*RP9* mutation) [76, 77]. hiPSCs derived from a RP patient harboring a different *RHO* mutation (E181K) in another study reproduced these findings [78]. This pathological phenotype was reverted to normal in PRs after correction of the expression of *RHO* using a helper-dependent adenoviral vector [78]. However, it should be noted that the gene correction was performed in hiPSCs, not in derived retinal cells. It is unclear whether this rescue could be also obtained in retinal cells and thus be relevant to gene therapy in RP patients.

Disease	Mutation	Cell type	Phenotype	Reference
XLRP	RPGR	3D retinal organoids hiPSC-RPE	Mutations perturb RPGR/gelsolin interaction, compromising gelsolin activation (gelsolin controls actin disassembly in connecting cilium, thereby facilitating rhodopsin transport to POS). Mutations affect cilia length and PR number.	Megaw et al. 2017 [79] Deng <i>et al.</i> 2018 [80]

XLRP	RP2	hiPSC-RPE	Complete lack of RP2 protein. IFT20 localization, Golgi cohesion and Gb1 trafficking altered.	Schwarz <i>et al.</i> 2015 [81]
XLRP	RP2	3D retinal organoids	Complete lack of RP2 protein. Reduced levels of Kif7 at cilia tips (involved in stabilizing cilia tips).	Schwarz <i>et al.</i> 2017 [82]
RP	RP1	Rod photoreceptors	Decreased number of patient-derived rods <i>in vitro</i> .	Jin <i>et al.</i> 2011 [76]
RP	RP9	Rod photoreceptors	Decreased number of patient-derived rods <i>in vitro</i> . Markers for oxidation or endoplasmic reticulum stress. Oxidation is involved in the photoreceptor degeneration.	Jin <i>et al.</i> 2011 [76]
RP	PRPH2	Rod photoreceptors	Decreased number of patient-derived rods <i>in vitro</i> .	Jin <i>et al.</i> 2011 [76]
RP	RHO	Rod photoreceptors hiPSC-RPE	Decreased number of patient-derived rods <i>in vitro</i> . Markers for oxidation or endoplasmic reticulum stress. Mislocalization of RHO in cytoplasm.	Jin <i>et al.</i> 2011 [76]; Jin <i>et al.</i> 2012 [77] ; Yoshida <i>et al.</i> 2014 [78]
RP	MAK	Retinal progenitor cells	MAK is expressed in the inner segments, cell bodies, and axons of photoreceptors. Loss of retina-specific isoform in mutated cells.	Tucker <i>et al.</i> 2011 [83]
RP	USH2A	3D retinal organoids hiPSC-RPE	Upregulation of GRP78 and GRP94. Protein misfolding and ER stress. Organoids :Morphological defects, reduced laminin, defect in retinal progenitor cell development, layer formation RPE: abnormal morphology, lack of pigment foci, and reduced expression of RPE markers	Tucker <i>et al.</i> 2013 [84] Guo <i>et al.</i> 2019 [85]
RP	TRNT1	3D retinal organoids	Autophagy defect. Accumulation of LC3-II. Elevated level of oxydative stress.	Sharma <i>et al.</i> 2017 [86]
RP	MFRP	hiPSC-RPE	Disorganized actin, loss of RPE cellular morphology and apical microvilli.	Li <i>et al.</i> 2014 [87]
RP	MERTK	hiPSC-RPE	Phagocytosis defects.	Ramsden <i>et al.</i> 2017 [88]
RP	PRPF31	3D retinal organoids and hiPSC-RPE	RPE: disrupted epithelium (polarity, TER) Phagocytosis and cilia defects PR: cilia defects, progressive degeneration and cellular stress RPE defects restored by AAV-PRPF31 gen augmentation	Buskin <i>et al.</i> 2018 [89] Brydon <i>et al.</i> 2019 [90]
RP	PRPF8	hiPSC-RPE	Near normal phenotype, no difference regarding phagocytosis	Foltz <i>et al.</i> 2018 [91]
RP	NRL	3D retinal organoids	Retinal organoids with S-opsin dominant PR population	Kallman <i>et al.</i> 2020 [92]
late onset RP	PDE6B	3D retinal organoids	At D230 modified expression of genes regulating cGMP hydrolysis leading to elevated cGMP levels in patient cells.	Gao <i>et al.</i> 2020 [93]

Table 1: hiPSC based models of RP and associated characteristic phenotypes.

In an innovative approach, the exome sequencing of an individual with sporadic RP led to the identification of a homozygous Alu insertion in exon 9 of male germ cell-associated kinase (*MAK*), a protein normally expressed in the inner segments, cell bodies, and axons of rods and cones [83]. hiPSCs were then generated from a RP patient homozygous for the Alu insertion and a non-*MAK*-associated RP patient as control. Retinal cells derived from the hiPSCs of the RP patient with the *MAK* Alu insertion presented a loss of a tissue-specific *MAK* splice variant normally found in retinal precursors [83]. The same group later used a similar strategy to identify disease-causing *USH2A* mutations in an adult patient with autosomal recessive RP [84]. *USH2A* mutations were associated to an upregulation of GRP78 and GRP94 indicative of protein misfolding and subsequent ER stress in hiPSC-derived PR precursors [84]. When grafted in mice's subretinal space, these patient's cells were able to develop and mature as PRs. This suggests that the mutation may not interfere with the normal development of the retina but rather with the survival of PRs later in life. Such patients may therefore be amenable to preventive therapeutic strategies before the onset of degeneration. *TRNT1*, a CCA-adding enzyme which belongs to the tRNA nucleotidyltransferase/poly(A) polymerase family functions by adding CCA to the 3' end of all tRNA precursors. A mutation in this gene was expected to cause protein misfolding and ER stress. However, it was not the case in hiPSC-derived retinal organoids from RP patients with mutations in *TRNT1* [86]. The cells rather exhibited a defect in autophagy as evidenced by an accumulation of the microtubule-associated protein LC3-II and elevated levels of oxidative stress [86].

Mutations in the Retinitis Pigmentosa GTPase Regulator (*RPGR*) and *RP2* gene account respectively for 70–90% and 10-20% of X-linked RP (XLRP) leading to rapid vision loss in boys and young men [94-96]. Using a 3D retinal differentiation protocol, Megaw *et al.* generated PRs from hiPSCs carrying a *RPGR* mutation to investigate its role in PR maintenance and its molecular pathogenesis mechanisms [79]. This group identified an increased actin polymerization in the PR connecting cilia, resulting in rhodopsin mislocalisation to the inner segment and ultimately in cell stress and degeneration [79]. CRISPR/Cas9-mediated hiPSC gene editing correction of the mutation rescued both PR structure and electrophysiological properties in retinal organoids [80]. Mutations in the *RP2* gene is the second most common cause of XLRP. In accordance with early studies hinting at the central role of *RP2* in vesicle

trafficking and cilia function, Schwarz *et al.* characterized the phenotype of hiPSC-RPE cells from an *RP2* patient that lost the RP2 protein expression. These cells displayed mislocalization of the intraflagellar transport protein IFT20 and a disrupted Golgi cohesion [81]. They later showed in hiPSC-derived 3D optic cups that the RP2 protein, along with the small GTPase ARL3, were key regulators of the trafficking of ciliary tip kinesins [82]. Interestingly, the use of translational read-through inducing drugs caused an increase in functional RP2 protein levels, reversing the observed disease phenotypes in hiPSC derived retinal cells [81, 82].

While the primary cellular defect in RP patients is in most cases localized in PRs, abnormalities in the RPE are also reported in about 5% of cases [18]. For instance, mutations in Membrane Frizzled-related Protein (*MFRP*), a RPE-specific type II transmembrane protein similar to WNT-binding frizzled proteins, cause autosomal recessive RP. To investigate disease mechanisms, hiPSC-RPE cells were derived from two patients with *MFRP* mutations. These cells had altered actin polymerization, abnormal morphology with less pigments, mislocalized pigment distribution, as well as loss of clear cellular boundaries and cell-to-cell contacts [87]. Furthermore, reintroducing normal *MFRP* expression with AAV vector therapy directly in hiPSC-RPE cells reversed the pathological phenotype [87]. This result suggests that gene therapy could be used to correct disease-causing mutations in RPE cells.

One of the most important functions of RPE cells is their ability to phagocytose shed PR outer segments (POS), a process vital for proper retinal function. Indeed, defects in RPE phagocytosis lead to an accumulation of cellular debris in the subretinal space ultimately resulting in progressive degeneration of PRs, as seen in the RCS rat, a widely used model for recessively inherited retinal degeneration [97]. Individuals with mutations in MER receptor tyrosine kinase (*MERTK*), the human orthologue of the RCS rat retinal dystrophy gene, are affected with severe and progressive RP [98]. *MERTK*-signaling in the retinal pigment epithelium was later shown to be essential for efficient phagocytosis of POS by RPE cells [99]. In accordance with these observations, hiPSC-RPE cells from individuals harboring mutations in the *MERTK* gene were unable to mediate the engulfment of POS [88, 100]. The use of translational read-through inducing drugs were able to restore *MERTK* expression and phagocytosis function [88].

4.2. Screening strategies for drug discovery

Besides enabling in-depth mechanistic studies of disease phenotypes *in vitro*, the development of robust differentiation protocols of hPSCs into retinal cells has proven instrumental in the development of high-throughput drug screening strategies to identify novel therapeutic agents. Drug discovery has relied for a long time on molecular target-based approaches, also called “reverse pharmacology” [101]. Such hypothesis-driven strategy relies on the identification of a molecular target whose activity or expression is directly associated to a disease pathogenesis. Compounds are then screened for their ability to bind and modulate the target. On the contrary, a phenotypic approach does not require prior understanding of the molecular mechanism of action but rather focuses on identifying compounds capable of reversing a phenotype thought to be representative of the disease. The contribution of phenotypic screening to the discovery of new first-in-class small molecule drugs approved by the US Food and Drug Administration (FDA) between 1999 and 2008 largely exceeded that of target-based approaches [102]. A critical efficiency variable of this approach is the use of physiologically relevant cellular models that accurately capture the biological complexity of the disease state *in vitro* [103]. **With the advent of PSC technology, hPSC-derived cellular models have gained great interest from the scientific community as an alternative platform for drug screening [104].**

The group of Masayo Takahashi first evaluated the potential of candidate drugs in hiPSC-derived PRs [76]. As rod PRs from RP patients underwent degeneration *in vitro* similarly to what is observed *in vivo*, they assessed effects of antioxidant vitamins α -tocopherol, ascorbic acid, and β -carotene on this cellular model. Interestingly, α -tocopherol treatment significantly increased the number of rhodopsin-positive cells harboring *RP9* mutation, providing proof-of-principle for drug screening based on the phenotype of hiPSC-derived retinal cells [45, 76]. Similarly, Yoshida *et al.* screened for agents able to protect rods from the accelerated cell loss induced by the rhodopsin mutation [78]. hiPSC-derived PRs were treated with molecules that could modify ER stress-related pathways. In such context, rhodopsin-related cell loss was suppressed, with a concomitant reduction of ER stress and apoptosis markers, after treatment with mTOR inhibition (rapamycin, PP242), AMP kinase activation (AICAR), apoptosis signal-regulating kinase 1 [ASK1] inhibition (NQDI-1), or suppression of protein synthesis (salubrinal) [78]. A proof-of-principle example of a target-based screening approach was also shown in hiPSC-RPE cells from a RP patient with a *MERTK* nonsense mutation [88]. Two translational

read-through inducing drugs, G418 and PTC124 demonstrated their ability to restore *MERTK* gene expression and full-length protein level. In addition, PTC124 functionally rescued 12% of the phagocytic function of RP hiPSC-RPE cells [88].

hiPSC-based models of AMD were also successful in identifying disease-modulating compounds. Following the differentiation of AMD hiPSC into RPE cells, Chang *et al.* found that these cells displayed an abnormal accumulation of ROS. Therefore, to attenuate oxidative damage, they screened compounds to find an effective scavenger of ROS [105]. Among several dietary supplements for retinal protection and natural antioxidant compounds, the team demonstrated that curcumin effectively protected AMD hiPSC-RPE cells from H₂O₂-induced cell death through upregulation of several oxidative stress regulatory proteins and significant reduction of ROS production [105]. More recently, the group of Sally Temple used a panel of AMD biomarkers combined with transcriptome analysis to demonstrate that nicotinamide treatment improved AMD-related phenotypes. They suggested that this effect was mediated by the inhibition of the production of drusen proteins and VEGF, as well as by decreasing inflammatory and complement factors [72]. **Recent machine learning approaches were developed to predict RPE cell functionality (transepithelial resistance, polarized VEGF secretion) [106]. Broaden use of deep neural networks applied to drug screening would be a potent alternative to identify new compounds.**

Bruch's membrane (BM), the extracellular matrix in which RPE cells are attached plays a critical function in AMD progression. Therefore, several strategies were developed to reproduce the interaction between BM and RPE cells [107]. In particular, non-enzymatic nitration of the extracellular matrix mimics the damages accumulated in aged BM [108]. BM is constantly attacked by matrix metalloproteinase and renewed by RPE and choroid [109]. In a similar manner, an immortalized RPE cell line (ARPE-19) was used in vitro to produce a BM-like extracellular matrix [108]. Alternatively, BM could be obtained from aged human cadavers [110]. First examples of AMD modeling demonstrated that AMD-like extracellular matrix perturbed functions of normal hiPSC-RPE cells (increased VEGF release, complement activation) [108]. Moreover, AMD hiPSC-RPE cells had reduced ability to attach and survive on nitrite modified extracellular matrix [111]. While not yet implemented in drug screening, future studies should take into account RPE matrix modifications on disease modeling as BM could affect both normal and AMD RPE cells.

Although it is now possible to synthesize and test increasingly large compound libraries, theoretically increasing the probability of identifying novel lead compounds, more than 85% of drug development programs across all indications fail to progress from phase I clinical trials to approval [112]. This gap between lead compound identification and success in the clinic might be attributed, at least in part, to the fact that the vast majority of high-throughput screening strategies relies on assays carried out on two-dimensional (2D) cell culture models that do not adequately recapitulate human pathophysiology. Indeed, while 2D models are well suited to study cell intrinsic deficits, they ultimately fail to recapitulate the cellular microenvironment, in particular endogenous signaling and cell-cell as well as cell-extracellular matrix interactions, limiting the extent to which diseases can be modeled. To overcome these shortcomings, three-dimensional (3D) culture systems that more closely resemble the native tissue architecture are becoming part of the drug discovery toolbox. At the forefront of this effort is the generation of retinal organoids that, as discussed before, more faithfully recapitulate some aspects of the histoarchitecture and cellular composition of the developing human retina both spatially and temporally. **Although highly informative, it is important to note that retinal organoids might not necessarily be suited for RPE screens as they contain rather low number of RPE cells.**

While retinal organoids hold promising advantages over other culture systems, several critical technical limitations still exist. The first concern is that organoids contain immature cells corresponding to a fetal stage, where PRs develop a partially mature organization and neurons display an abnormal synaptic connectivity [113, 114]. The addition of an extracellular matrix might improve connectivity and responsiveness to light [114]. Another concern is the cell death that occurs in organoid centers. Cells self-organize into 3D structures, develop a well-organized initial lamination and correct temporal production of the diverse cell types. However, cells localized in the center of the organoid will disorganize and undergo progressively cell death: this is particularly the case for retinal ganglion cells and inner cell layers [63, 113]. Another hurdle to overcome is that existing retinal organoid protocols are not able to generate a specific area of the retina such as the fovea or the macula, which is obviously a limitation when addressing macular degenerative diseases. The last limitation is related to the absence of RPE adjacent to PR. Indeed the microenvironment provided by RPE cells (nutrients, matrix, debris phagocytosis...) might be important

particularly for the long culture duration required for PRs as suggested recently [115, 116]. Thus, organoids with current protocols are only a step forward that improve existing 2D models.

Knowing these shortcomings, the use of retinal organoids for drug screening remains the object of intense research to overcome technical constraints. Indeed, incorporating 3D culture systems into miniaturized 384- or 1536-well formats in an automated and cost-effective screening setup is still in infancy [117]. Two of the biggest challenges are the visualization of 3D structures with automated imaging systems as well as the general lack of robust quantitative technologies to analyze organoids on a large scale [117, 118]. To address this need, Vergara *et al.* developed a screening platform that enabled accurate quantification of fluorescent reporters in complex hiPSC-derived retinal organoids, allowing for quantitative analysis of dynamics of developmental processes and cellular physiological states [118]. Of importance, technologies and readout assays that have been developed and successfully implemented in low- to high-throughput strategies in other 3D systems, for example cancer-like spheroids, could be adapted to retinal organoids [119, 120].

Adding another level of complexity, recent studies have focused on increasing the physiological relevance of hPSC-derived retinal models by juxtaposing a polarized RPE sheet to the retinal structure [115, 116]. Achberger *et al.* developed a microfluidic platform to create a complex multi-layer structure from hiPSCs that includes all cell types and layers present in retinal organoids in direct interplay with an RPE layer, recapitulating the complex *in vivo* anatomy of the human retina *in vitro* [115, 121]. Combining the two promising technologies, organoids and organ-on-a-chip, they provided a controllable vasculature-like perfusion to the 3D culture system and recapitulated the interaction of mature PR segments with RPE cells. This microfluidic retina-on-a-chip was validated for compound screening and toxicological studies by exposing the system to two molecules whose pathological side effects on the retina had been previously described, the anti-malaria drug chloroquine and the antibiotic gentamicin [115, 121]. While much work remains before complex retina-like 3D *in vitro* systems become standards for toxicity and drug screenings, the rapid development in recent years of organoids, biomaterials and microfluidic technologies are bringing this prospect closer to realization.

5. Human pluripotent stem cells for cell therapy

5.1. Strategies for tissue restoration

Depending on the retinal degenerative disease and its specific stage of progression, different cell types are lost. In AMD, RPE cells stop performing their support functions and degenerate, which then triggers PR death. While the primary cellular defect in RP patients is mostly localized in PRs, dysfunctional RPE cells are also reported in about 5% of cases. Cell therapy products for AMP and RP patients should be designed accordingly to replace or supplement the remaining RPE cells and/or PRs (**Figure 4**). The other major aspect of tissue restoration is to determine precisely the injection site as the grafted material cannot cover the totality of the retina. Even if RP affects the peripheral retina first, the macula is ultimately damaged. Thus, in AMD and RP, the macula is the targeted site of implantation to maximize visual recovery as visual acuity mostly rely upon this area.

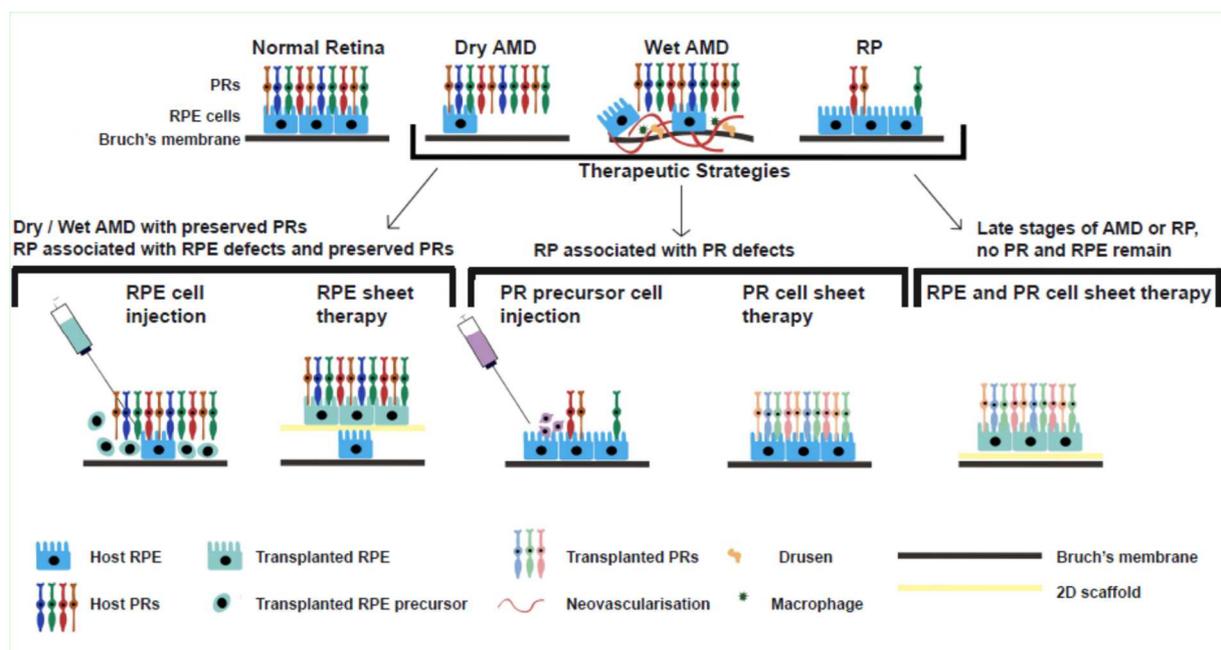


Figure 4: Schematic representation of retinal degeneration in AMD/RP patients and associated cell therapy strategies. PR: photoreceptor; RPE: retinal pigment epithelium; AMD: Age-Related Macular Degeneration; RP: Retinitis Pigmentosa.

5.1.1. RPE cell therapy

RPE cells can either be delivered as a cell suspension or as an organized monolayer. The cell suspension formulation offers the advantage of a simplified logistic: cells are sent frozen to the hospital where they can be stored and thawed when desired. In addition, little manipulation is required before transplantation in the subretinal space and a simple syringe

is used for eye injection. Delivery of hPSC-RPE cells as a cell suspension into the subretinal space of the RCS rat model slowed down PR degeneration and preserved visual functions [41, 44, 122-124]. The first hPSC-RPE clinical trials based on this strategy targeted AMD and Stargardt's disease. The safety profiles were reassuring as no adverse event related to the use of hPSCs was observed [125, 126]. Moreover, preliminary signs of efficacy were reported in some patients for up to 4 years [127].

The second formulation approach is to deliver an already formed hPSC-RPE epithelium. This strategy drastically complicates the transport, storage and subretinal delivery. Indeed, as a living monolayer of cells, the graft can only be kept for a limited period of time in a controlled environment [128, 129]. A supporting scaffold made of polymers or of biological composition is usually needed *in vitro* during epithelial reformation of hPSC-RPE cells to ensure that cells can be removed from the culture dish [18, 130-133]. For implantation, specific devices have to be developed in order to deliver the hPSC-RPE sheet into the subretinal space [41, 129, 134-136]. The rationale leading to the development of this strategy is that RPE cells are functional only when organized as an epithelium with the secretion of cytokines [137]. In addition, the diseased environment with oxidative stress and altered Bruch's membrane could limit *in vivo* epithelial reformation of hPSC-RPE cells grafted as a cell suspension. Indeed, hESC-RPE are more resistant to oxidative stress, which is present in AMD and RP, when organized as monolayers [138]. Compared to cell suspension injections, the viability of hPSC-RPE cells grafted in the subretinal space of Nude rat was higher when delivered as a sheet [131]. Our group, as well as others, demonstrated that hPSC-RPE sheets improved the therapeutic visual outcomes of RCS rats compared to the same cells injected as a cell suspension [41, 132]. Preliminary results of three human clinical trials using a RPE sheet formulation to treat AMD were published recently [133, 135, 139]. The graft monolayer was cultured over polyester [135], parylene [133] or without scaffold [139]. None of these studies reported a serious adverse event related to the grafted hPSC-RPE cells. While these studies were not designed to evaluate efficacy, first data are encouraging. **In particular, Prof. Coffey and collaborators treated two severe exudative AMD patients with a hESC-RPE sheet on a polyester scaffold [135]. Such patients had lost dramatically their visual acuity due to retinal dysfunction [140]. If untreated, their retinas will degenerate and their vision permanently lost. Existing treatments consist of CNV removal and/or anti-VEGF medication that might improve the**

visual outcomes but the incidence of RPE rips is more frequent (17%) than without treatment (10%) [141]. These RPE rips expose Bruch's membrane and choroids and have a poor visual prognosis. The CNV removal itself does not improve visual acuity but reduce the risk of severe visual loss [142]. In Coffey's study, the CNV might have been removed unintentionally [143] and the outcomes should be taken with caution (few treated patients, no control untreated eyes or anti-VEGF treated eyes for comparison). Nevertheless, one of the two patients improved enough his visual acuity that he was able to read again [135].

5.1.2. PR cell therapy

For successful cell replacement, PR precursors need to integrate into the pre-existing neuronal network of the host retina and mature with the formation of outer segments sensitive to light. Similarly, to RPE cells, they can either be delivered as a cell suspension or as an organized tissue.

Cell suspension injection

Pioneer studies provided evidence that stage-specific post-mitotic PR precursors from mice could integrate into the host outer nuclear layer (ONL - corresponding to the layer of PR nuclei), differentiate to acquire the morphological characteristics of mature PRs and improve moderately the vision in murine models of retinal degeneration [144-147]. Altogether, these data led researchers to the conclusion that migration and integration of donor PR cells was the underlying mechanism of some visual function recovery.

Five independent back-to-back studies published starting from late 2016 called for a re-evaluation of this paradigm [148-152]. Indeed, the vast majority of transplanted PR precursors did not integrate into the host retina but rather remained in the subretinal space where they exchanged cytoplasmic material with host PRs. Following GFP positive cell grafting into the subretinal space of mice ubiquitously expressing a red fluorescent protein, cells coexpressing both reporters were found in the ONL, which indicates exchange of material [148-150]. In complementary experiments, Nrl-GFP mice were injected with the thymidine analogue EdU during development to independently label the nucleus and cytoplasm [149, 152]. While the majority of GFP+ donor cells in the subretinal space was EdU+, no EdU labelling was observed in GFP+ cells located in the host ONL. Thus, donor and host cells only exchanged their cytoplasmic content, not their nuclei. Gender-mismatched transplants confirmed this

observation as the nuclei of GFP+ cells in the host ONL contained only the X- or Y-chromosome present in the host [148-150]. No polynucleated cells were observed. Such result suggests that GFP protein and/or mRNA was transmitted between donor/recipient cells. In light of these recent findings, we should reevaluate previous reports that had concluded that donor cells could integrate in the host ONL where they expressed donor proteins that were otherwise missing in the endogenous PRs of the recipient (such as rod α -Transducin, Peripherin-2 and Rhodopsin) [145, 146, 153, 154]. It can be inferred that these vision-related proteins may in fact be transferred from donor to host PRs through material exchange.

The Cre/Lox system gave direct evidence for bidirectional exchange of intracellular material between donor and host cells [148-150]. Transplantation of donor PRs isolated from floxed reporter mice into PR-specific Cre mice showed expression of the reporter on transplanted cells in the subretinal space, revealing transfer of Cre-recombinase from host PRs to donor cells - and vice versa. Together these results demonstrate that while donor PR migration and integration do occur in rare events, the majority of transplanted cells engages in a process of material transfer through molecular mechanisms that remain to be determined. As a result, material transfer of functional proteins from murine donor cells to remaining host PRs after transplantation, rather than their structural integration, could explain partial recovery of visual function in retinal degeneration models. This material exchange appears to be specific between donor PR precursors/ host PRs. Indeed, no exchange was observed when GFP proteins were injected directly in the subretinal space or when GFP retinal progenitor cells as well as fibroblasts were transplanted [148]. This transfer is also limited to the ONL. Only one report, not confirmed by other studies, suggests that other cell types might be involved, including bipolars and Mullers [152]. **The mechanisms involved in this material exchange are not elucidated but a vector is needed to mediate this intercellular communication. Several options are suggested including a fusion of donor/host plasma membranes or through the exchange of extracellular vesicles (like exosomes or microvesicles) [155]. This last proposition is attractive as such vesicles allow the transfer of mRNA / miRNA /proteins / lipids from one cell type to another with specificity [156].**

It is important to note that all the above-mentioned studies were conducted with mouse donor cells. Recent transplantation experiments using human cells showed that material exchange was rather limited within the rodent eye as the vast majority of donor cells remained

in the subretinal space and only few cells integrated into the host ONL [68, 157, 158]. Therefore, cell suspension transplantation into primates are required to evaluate the level of material transfer, the integration of human donor cells into the ONL and their capacity to form outer segments *in vivo*.

When reevaluating only studies where donor PRs were transplanted in completely degenerated rodent to avoid cytoplasmic exchanges, visual gain remains unclear. For example, Rd1 mice with a degenerated ONL and zero functionality were transplanted with a GFP photoreceptor cell suspension at 10-12 weeks in 2013 [159]. The authors suggested an anatomical connection between donor rod and host bipolar cells (rod specific synaptic proteins, GFP labelling). This was correlated with pupil light responses. However, when reevaluating these results in light of material transfer, only separate analysis of cone and rod ERG responses could address each contribution to functionality. Indeed, in this model, cones could remain, even nonfunctional for a long time and it is not excluded that cones could be functionally rescued following rod transplantation and elicit a pupil light response [155]. When grafted in degenerated Rd1 mice, hESC derived photoreceptors differentiated into cones and appeared to make connection with bipolar cells as evidenced by histology, but without evaluation of visual functions [160]. Thus, in atrophic mice, donor PRs are able to make connection with host neurons but without a clear demonstration of visual functionality.

Retinal sheet transplantation

An alternative to the injection of PRs as a cell suspension is the transplantation of retinal sheets. This approach, based on the dissection of retinal organoids in small sections, was evaluated after transplantation in rodents, primates and cats [161-163]. In such tissue, PRs are transplanted with other cell types [162-164] that might limit the connection with second order neurons of the recipient retina. In addition, implanted retinal sheets tend to form rosettes *in vivo* with outer segments inside, leading to a separation with RPE cells [161, 162, 164].

Despite these limitations, synaptic connections with host retinas and some visual recovery were observed in rodent models of retinal degeneration, as evidenced by multi-electrode array recordings and assessment of visual behavior [162, 164-166]. Similar results were

reported in primates (only one animal at 1.5 year post-surgery) by measuring visually-guided saccades [166]. While the advantage of this strategy is to engraft an already formed and organized layer of PRs, additional work is needed to limit the presence of neurons other than PRs in the graft and to improve the product delivery into the eye in order to reduce rosette formation.

5.2. Future of retinal cell therapy

Both preclinical and clinical studies hint that the use of hPSC derivatives is a safe and viable therapeutic option for the treatment of retinal degenerative diseases. However, a number of important questions must be addressed before this strategy can be part of the arsenal of clinical tools, among which the challenge of transplant immune rejection, the development of more complex retinal grafts through tissue engineering and the potential combined use of optogenetic tools.

Graft rejection: immunological considerations

The success of cell replacement therapies does not rely solely on the quality and maturation of the grafted cell product but also on our understanding of the host immunological responses following transplantation. As ocular immune privilege provides the eye with immune protection, it was initially postulated that transplanted grafts into the eye would largely be protected from immune rejection. However, it is difficult to address the graft immunogenicity as hPSC derived retinal cells are usually grafted in a xenogeneic context and therefore under heavy immune suppression [41, 68, 129, 163, 164, 167-169]. In addition, the diseased ocular microenvironment is likely to increase the risk of immune rejection. For example, progressive loss of RPE cells as well as alteration of Bruch's membrane in AMD ultimately compromise the blood-retinal barrier, further degrading the immune privilege of the eye. Besides, the injection procedure itself could also favor transplant rejection by allowing immune cells to enter the retina. The result of this uncertainty is that the best immunosuppression strategy is still debated in ongoing and planned human clinical trials, with current strategies ranging from local to high systemic immunosuppression regimen [125, 133, 135].

One way to evaluate the immune response in an allogeneic context is to transplant monkey iPS-RPE allografts into the non-immune-suppressed subretinal space of monkeys. In that context, retinal tissue damage was observed in major histocompatibility complex (MHC)-

mismatched monkeys but not in MHC-matched animals [170-172]. *In vivo* imaging and histological analysis evidenced a strong inflammatory response to MHC-mismatched allografts in a highly localized and aggressive manner by 4 days post-transplantation, continued through 3 weeks, ultimately resulting in the rejection of iPS-RPE grafts [171, 172].

Autologous hPSC-derived cell products could overcome the rejection risk. However, such strategy is not achievable for wide-scale clinical practice. An alternative is to establish hPSC banks from “super-donors” homozygous for conserved human leucocyte antigen (HLA) haplotypes that are representative of a specific population to enable HLA matching and therefore minimize the risk of allograft rejection [173]. Current estimates predict that a bank of hiPSCs from 100 to 150 highly selected donors would meet the needs of 50-90% of the recipient population in the UK [174, 175]. However, the cost of such banks remains high.

Another promising strategy is the engineering of allogenic hPSCs to be hypoimmunogenic to both adaptive and innate immune responses using CRISPR-Cas9 gene editing. In a recent approach, hypoimmunogenic hESCs were generated after deletion of the highly polymorphic MHC class I and class II molecules and expression of immunomodulatory factors PD-L1, HLA-G, and CD47 [176]. Accordingly, engineered vascular smooth muscle cells derived from these hESCs elicited significantly less T cell- and natural killer cell-mediated immune responses with minimal engulfment by macrophages [176]. CD47 overexpression and ablation of MHC class I and II were later found sufficient to generate hypoimmunogenic hiPSCs [177]. Cardiomyocytes, endothelial cells as well as smooth muscle cells derived from these cells reliably escaped immune rejection in MHC-mismatched allogenic recipients and survived long-term without the use of immunosuppression [177]. Thus, the generation of hypoimmunogenic hPSCs holds the promise of universal cell grafts from a unique cell bank.

Looking forward: scaffold-based tissue engineering

The final formulation of the cell therapy product has major impact on correct integration and polarization of the grafted material in the host cellular environment [19]. Attempts at delivering single cell suspensions of hPSC-derived PRs and RPE cells have led to poor integration rate and limited visual recovery compared to their transplantation as cell sheets [41, 129, 148-150, 163-165]. Importantly, PRs transplanted as cell sheets from retinal organoids formed synaptic connections and developed polarized segments [163-165].

However, the formation of rosettes was also observed, within which outer segments of PRs were separated from the support of endogenous RPE cells [163, 164]. Levering on the clinical success of RPE sheet transplants grown on biocompatible substrates, the addition of supportive scaffolds is currently explored to deliver PRs in a more physiologically structured way. This may improve implantation and facilitate proper apical-basal polarization of the graft [178-180]. Of importance, Jung *et al.* recently developed a 3D biodegradable micro-structured scaffold designed to capture PRs in cup-shaped wells that support the correct orientation of axonal processes [180]. While the long-term biocompatibility and integration of such system within the host retina remains to be investigated *in vivo*, this scaffold-based tissue engineering approach provides an innovative way forward for the development of more complex retinal grafts. In addition, the design of bio-engineering tissues composed of a combination of layers of organized RPE cells and PRs might be a suitable graft for advanced stage of AMD and RP where RPE cells are defective and PRs lost.

Combining stem cell therapy and optogenetics

Optogenetic uses light to control cells genetically modified to express an optogene encoding light-sensitive membrane proteins known as opsins [181]. The overall strategy is to convert surviving retinal neurons into light-sensitive cells and thereby functionally turn them into artificial PRs able to convert light into electrical signal to restore vision [182]. Importantly, the use of optogenes is a mutation-independent approach that can be used in a wide range of retinal degenerative conditions. Targeted electrical stimulation has been reported in dormant cone PRs lacking their light-sensitive outer segments [181, 183, 184] as well as in downstream retinal neurons such as RGCs [185-188] and bipolar cells [189-191]. These studies further hinted that the restored light information was transmitted to the visual cortex of formerly blind mice as they exhibited an improvement in visually guided behavioral responses. So far, two clinical trials based on this technology (targeting RGCs) are underway in patients affected with RP (clinicaltrials.gov identifiers: NCT03326336 and NCT02556736). However, rendering RGCs sensitive to light engender the loss of the sophisticated processing that occurs through the retina (from PRs to RGCs) while a small number of dormant cones remains. As significant progress is being made with the generation of more specific and improved opsins, the use of optogenetic tools could be broadened by combining stem cell therapy and optogenetics to counteract the difficulty to obtain functional PRs from hPSCs with light-sensitive outer

segments [36]. This was achieved recently in mice suggesting that this strategy may be viable [158].

6. Conclusion

As highlighted in this review, hPSCs have become valuable tools to investigate the different stages of retinal degeneration and help tailor therapeutic strategies for the future. Before the complete loss of PRs, drug compounds identified through high throughput screening on RP/AMD hPSC models could be delivered to patients. **When the degeneration is at an advanced stage, hPSCs differentiated into retinal cells could be transplanted into the eye to replace lost cells or to support remaining PRs through cytoplasmic exchanges.** While significant roadblocks need to be addressed, the fast development of more physiologically relevant cellular models that accurately capture the biological complexity of the retina *in vitro* bring these expectations closer to reality. There is still a long way to go before first treatments reach patients but many phase 1/2 clinical trials have already started.

Conflicts of Interest

CM and KB are inventors of a patent (FR3078712) related to medical devices for the preparation of retinal tissues for regenerative medicine. LM, CM and KB are inventors of a pending patent related to the automated differentiation of hPSC into RPE cells.

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