Photoreceptor Cell Replacement Using Pluripotent Stem Cells: Current Knowledge and Remaining Questions

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Abstract:

Retinal degeneration is an increasing global burden without cure for the majority of patients. Once retinal cells have degenerated, vision is permanently lost. Different strategies have been developed in the recent years to prevent retinal degeneration or to restore sight (e.g., gene therapy, cell therapy and electronic implants). Herein, we present current treatment strategies with a focus on cell therapy for photoreceptor replacement using human pluripotent stem cells. We will describe the state of the art and discuss obstacles and limitations observed in preclinical animal models as well as future directions to improve graft integration and functionality.

Introduction

Vision is an essential sense used in almost every aspect of the everyday life, and, in particular in our modern societies with the increasing number of screens (smartphones, computers, TV…) and information transmitted through them. Thus, loss of vision has dramatic impacts in daily life and is a major economic burden for society (Wittenborn et al. 2013; Blindness et al. 2021a,b). In 2020, an estimated 596 million people had distance vision impairment worldwide, of whom 43 million were blind (Burton et al. 2021). Major causes of vision impairment are uncorrected refractive errors and cataracts for which effective treatments are available (Flaxman et al. 2017). On the other hand, retinal degenerative diseases are the leading cause of untreatable sight-loss in the industrialized world.

Retina is composed of several cell types including photoreceptors, which are a specialized type of neurons that convert light inputs into electrical signals. This signal is further processed and integrated by different other cell types of the retina until axons of ganglion cells (forming the optic nerve). In contact to photoreceptors, retinal pigment epithelial (RPE) cells form a specialized epithelium that provides a critical trophic support to these photoreceptors and maintain their homeostasis. As terminally differentiated cells without regenerative capabilities in human, damage and loss of photoreceptors result in diseases leading to permanent visual impairment, such as age-related macular degeneration (AMD) and Retinitis Pigmentosa (RP).

1. Retinal degenerative diseases

1.1. Age-related Macular Degeneration

AMD is the main cause of central vision loss in patients older than 55 years-old (Flaxman et al. 2017; Burton et al. 2021) and is caused by the degeneration of RPE cells. The main risk factor is ageing. Mechanisms causing RPE cell loss may involve at least in part inflammation, oxidative stress and complement system that alter surrounding cells and the supportive Bruch's membrane to which RPE cells are attached (Brown et al. 2018; Handa et al. 2019; Zarbin et al. 2019; Hussain et al. 2020).

Initially asymptomatic, the disease evolves to central vision distortion and finally central scotomas (Mitchell et al. 2018). At late stages, two forms are described: wet AMD, which is defined by the presence of macular choroidal neovascularization (CNV; 10-20% of cases), and dry AMD characterized by macular atrophy (corresponding to 80-90% of cases) (Mitchell et al. 2018; Al-Khersan et al. 2019). No treatment is available for the dry form of AMD. An anti-VEGF therapy is usually delivered to wet AMD patients in order to slow or prevent vision degradation. CNVs can also be removed by surgery.

1.2. Retinitis Pigmentosa

RP forms a heterogeneous group of inherited retinal dystrophies that affects 1.5 million of patients worldwide (Hartong et al. 2006; Verbakel et al. 2018; Pfeiffer et al. 2020). Symptoms are night blindness at early stages; later during disease progression, the visual field becomes more and more reduced starting from the periphery (tunnel vision) (Hartong et al. 2006). To date, more than 90 genes involved in RP have been described (https://sph.uth.edu/retnet/sum-dis.htm). Mutations affect photoreceptor functions and for about 5% of cases, RPE cell functions (Ben M'Barek et al. 2018). The other cell layers are initially well preserved but, after decades of degeneration, rewiring, glial hypertrophy and global cell death may occur (Pfeiffer et al. 2020). Only one approved treatment is available for patients with *RPE65* gene mutations (2% of RP cases). It consists of a gene therapy (Luxturna™ (voretigene neparvovec-rzyl)) that improves navigational abilities and light sensitivity (Maguire et al. 2019, 2021). Forty to fifty percent of RP patients may develop cataract that can be removed by surgery (Auffarth et al. 1997; Bayyoud et al. 2013).

1.3. Current strategies under development

Current treatment strategies under development involves gene and cell therapies or electronic implants (Roska and Sahel 2018). These strategies address two challenges: preventing retinal degeneration before complete cell loss and restoring sight in late disease stages (**Figure 1**).

Gene therapy can be applied to RP patients, either by replacing the mutant gene with a healthy copy or by correcting the underlying mutations, prior to complete photoreceptor cell degeneration in order to achieve preservation of the remaining cells. However, a specific gene therapy should be developed to address each of the known RP-inducing genes. Such gene therapy approach is expensive regarding the very few number of RP patients affected by one specific gene (*e.g.* 425 000 $ per eye for LUXTURNA™ (Pennesi and Schlecther 2020)). Of note, gene mutations causing RP are not known in 30% of non-syndromic and 50% of autosomal-dominant RP patients (Hartong et al. 2006; Verbakel et al. 2018). Nevertheless, several gene therapies are in the pipeline of clinical development (Sahel et al. 2019). In the meantime, scientists are looking for alternative mutation-independent approaches. The discovery of the rod-derived cone viability factor (RdCVF) and its ability to convey neuroprotection and delay photoreceptor degeneration is an example of a neuroprotective strategy (Leveillard and Sahel 2010). Gene therapy with RdCVF is envisioned as a potential strategy to prevent oxidative stress and provide neuroprotection (Clerin et al. 2020).

The replacement of RPE cells that are not functional or degenerating in AMD and some forms of RP is also considered as a cell therapy strategy to protect photoreceptors. Our group has developed protocols to generate RPE cells, identified the formulation that conveys the most potent therapeutic effects and elaborated strategies to deliver the engineered retinal tissue in rodent and primates (Ben M'Barek et al. 2017, 2020; Ben M'Barek and Monville 2019). This work led in 2019 to an ongoing phase I/II clinical trial for RP patients (with known mutations affecting RPE cells) based on these technologies. Other groups developed similar approaches for AMD with already published results (Mandai et al. 2017b; da Cruz et al. 2018; Kashani et al. 2018; Qiu 2019).

Electronic implants aim to replace the function of dead photoreceptors through the conversion of images collected by camera into electrical signals that are transmitted to the retinal circuitry downstream photoreceptors. Current implants showed recently promising results but the technology is still in its infancy due to the very low-resolution vision (low number and density of electrodes) (Roska and Sahel 2018; Cehajic-Kapetanovic et al. 2022). Another approach of gene therapy is based on optogenetics. The overall strategy is to convert surviving retinal neurons (ganglion cells, bipolar cells, and dormant cones) into light-sensitive cells through transfer of gene coding for different microbial opsins. These modified retinal cells acquire the potential to convert light into electrical signals (artificial photoreceptors) (Busskamp et al. 2010; Klapper et al. 2016; Garita-Hernandez et al. 2018; Khabou et al. 2018). The first proof of concept of this strategy was recently obtained with the partial visual recovery in a late stage blind RP patient (Sahel et al. 2021). However, two main hurdles are associated to this strategy: the choice of the light-sensitivity of the selected protein (as it does not have the sensitivity and complexity of the different photoreceptor subtypes) and the loss of the sophisticated signal integration mediated by the different retinal cell subtypes when photoreceptors are bypassed. Finally, the replacement of dead photoreceptors by new exogenous photoreceptors through cell therapy will be specifically discussed in the following sections.

2. Photoreceptor cell replacement

2.1. Historical perspective: transplantation using fetal or adult retinas

First grafting experiments of triturated rat embryonic or neonatal retinas into rat retinal lesion eyes in late 80s demonstrated the potential of a retinal graft to survive and continue its development into a host eye (Turner and Blair 1986; Blair and Turner 1987). Since then, a number of studies explored the potential of embryonic or neonatal retinal cells to engraft into host retinas leading to the first clinical trials (for review (Seiler and Aramant 2012; Ludwig and Gamm 2021)). For example, fetal embryonic

sheets composed of neural retinas and RPE cells where used for transplantation in clinical trials involving patients with late stage RP and AMD (Radtke et al. 2002, 2008). While the first study did not report visual improvements, the latter one showed positive visual outcomes measured using the ETDRS scale (early treatment of diabetic retinopathy study scale that quantifies visual acuity) in 7 of the 10 patients transplanted (Radtke et al. 2008). This improvement lasted for at least 6 years in one patient but it remains difficult to determine if the graft was integrated into the retinal circuitry or if it provided a trophic effect preserving remaining photoreceptors. No immunosuppression was used to prevent rejection. Nevertheless, this study suggested overall the safety of a retinal transplantation. Such retinal sheet transplantation has also been performed in sighted RP patients (Berger et al. 2003). Indeed, in a clinical trial, eight patients were transplanted with photoreceptor sheets obtained from cadavers (within 24 hours of death) without immunosuppression. Mean reading speed or visual acuity did not change during the 1-year follow up (no improvements or degradation).

These transplantation studies shed light onto several limitations associated with the graft origin that limit reproducibility of experiments, as well as large scale clinical trials and potential future use in clinical practice. Fetal human cells obtained from abortion are surrounded with ethical and legal constraints that are different from one country to another. The availability of this material is scarce and the age of fetuses may be variable. Adult retinas from cadavers may not be able to integrate and their procurement is also difficult. The emergence of human pluripotent stem cells (hPSCs) discovered in 1998 stimulated this research field through the promise of an easier access to human embryonic retinal cells. hPSCs including hESCs (human embryonic stem cells (Thomson et al. 1998)) and hiPSC (human induced pluripotent stem cells (Takahashi et al. 2007)) are characterized by a self-renewal potential and the ability to differentiate into any cell type of the adult body including retinal cells. Thus, providing the development of efficient protocols to differentiate hPSCs into photoreceptors, retinal cell material suitable for transplantation will not become anymore a limiting factor.

2.2. Photoreceptor differentiation from pluripotent stem cells

Differentiation of hPSCs into the various retinal cell types follows a sequence of events and cellular intermediate similar to what is observed *in vivo* (Ben M'Barek and Monville 2019; Gagliardi et al. 2019; O'Hara-Wright and Gonzalez-Cordero 2020). These steps are reproduced *in vitro* using a combination of chemical factors and cytokines added to the culture medium. The best results in producing photoreceptors are obtained through the generation of self-organized three-dimensional tissue cultures as retinal organoids, which can be compared to optic vesicles or optic cups observed *in vivo* (Nakano et

al. 2012; Reichman et al. 2014, 2017; Zhong et al. 2014; Kuwahara et al. 2015; Ohlemacher et al. 2015; Parfitt et al. 2016; Capowski et al. 2019). These organoids are laminated by a superposition of different retinal cell types similar to *in vivo* retina: photoreceptors located at the surface of organoids and at the opposite, ganglion cells located deep inside retinal organoids. Some degrees of photoreceptor maturation is observed after long culture duration (several months) with a timeline similar to human *in vivo* development (Cowan et al. 2020). Recently, Saha and colleagues (2022) observed light-evoked electrical responses in cones (35% of measured cones) from long-term retinal organoid cultures (240-270 days of differentiation). Thus, these retinal organoids represent a source of functional photoreceptors suitable for transplantation. However, photoreceptors may need to be isolated from other cells inside retinal organoids before transplantation. Different methods of cell isolation based on surface antigens were developed to either exclude contaminating cells or to specifically isolate photoreceptors (Lakowski et al. 2015, 2018; Santos-Ferreira et al. 2016b; Welby et al. 2017; Gagliardi et al. 2018). Finally, efforts were made to standardize organoid differentiation protocols across various hPSCs through the definition of three morphological stages of differentiation (Capowski et al. 2019).

2.3. Cell therapy formulation

2.3.1. Cell suspension

Once photoreceptors produced, different strategies were developed to formulate the final cell therapy product that will be transplanted. Initial studies grafted cells as suspension or small clumps of cells (when obtained from fetuses). Such formulation has the advantage of an easy manipulation and does not require sophisticated implantation tools.

When implanted as a cell suspension, the majority of photoreceptor precursors fail to properly integrate and complete their maturation. Visual outcomes following grafting in rodents with retinal degeneration were evaluated through electrophysiology (retinal electrical response to light) and/or visual behaviors (pupil light response or optokinetic reflex), and were usually moderately or not improved (Pearson et al. 2012; Barber et al. 2013; Singh et al. 2013; Gagliardi et al. 2018). In addition, these observed functional visual outcomes were mainly elicited by a phenomenon of cytoplasmic material transfer (Pearson et al. 2012, 2016; Santos-Ferreira et al. 2016a; Singh et al. 2016). This material transfer allows the exchange of cytoplasmic proteins and / or mRNAs (including those used to label donor photoreceptors) that are missing in the degenerated host retina. Host photoreceptors become functional and can elicit moderate visual functionality in rodent, thanks to the formation of nanotube-like process between donor and host

photoreceptors, facilitating the transfer of this material (Kalargyrou et al. 2021; Ortin-Martinez et al. 2021).

Some recent studies suggested that long-term follow-up in rodent models following implantation are required to observe an improved maturation. Degenerative context might also play a role: most studies focused on end-stage retinal degeneration models -where integration might be less favorable than conditions where photoreceptors, susceptible to provide support to the grafted cells- remain. Gasparini and collaborators recently evaluated this hypothesis. Indeed, this group used as grafting material cones isolated by cell sorting from Day 200 (D200) retinal organoids, thanks to a specific fluorescent reporter (Gasparini et al. 2022). These photoreceptors were injected into *Cpfl1* (cone photoreceptor function loss 1) deficient mice in which cones degenerate but rods remain preserved. After a follow-up of 6 months under local immune suppression, the authors observed an improved integration and maturation of donor photoreceptors with the presence of inner and outer segments. However, no proof of functionality *in vivo* was shown (only *ex vivo* electrophysiology). Ribeiro and collaborators suggested also to increase the number of grafted cells to improve integration / maturation in long-term studies (Ribeiro et al. 2021). To facilitate survival of transplanted human cells, they used the immunocompromised *rd1/Foxn1*^{nu} mice model that presents a complete loss of rods and central cones at 3 months of age. Cones were isolated by cell sorting from D120 retinal organoids previously transduced with AAV vector carrying GFP under the control of a cone promoter. Three months after subretinal injection of 500 000 cones, they observed modest maturation improvements (presence of nascent segment-like protrusions) in 20% of grafted cones and positive visual outcomes. More interestingly, they confirmed that the functionality observed was due to functional grafted cones as the grafting of nonfunctional cones (from mutant hiPSCs) did not elicit the same response (strongly suggesting that there was no material transfer or trophic effect involvements). Photoreceptors derived from D74-106 organoids were also subretinally delivered to photoreceptor-ablated retina in nonhuman primates, using vitrectomy as for human surgery (Aboualizadeh et al. 2020). Follow-up analysis showed that the transplanted photoreceptors did not reach full morphological maturity (presence of outer segments) even 41 weeks after transplantation. Of note, they reported that injected cells could reflux from the retinotomy or concentrate in the inferior area of the bleb due presumably to gravity. Thus, the authors suggested keeping the monkeys horizontal for 3-4 hours after the surgery. These are important points to consider for future clinical trials.

2.3.2. Tissue formulation

Similar to RPE cell therapy, scientists evaluated other formulations for photoreceptor transplantation. Early studies indicated that neonatal retinal cells organized as aggregates had a better survival and organization that cell suspension ((Seiler and Aramant 2012) for review). Thus, sheet formulation was explored mainly through the direct use of pieces of retinal organoids for transplantation (Assawachananont et al. 2014; Shirai et al. 2016; Mandai et al. 2017a; Iraha et al. 2018; McLelland et al. 2018; Tu et al. 2019). Shirai and collaborators used as grafting material small pieces of 0.5 mm obtained from retinal organoids derived from hPSCs (Shirai et al. 2016). Different stages of organoid maturation (from D50 to D100) were evaluated following transplantation in an immunodeficient rat model of photoreceptor degeneration (*RhoS344^{ter}/Foxn1^{nu}*). The authors did not report any major differences in the thickness of the graft that reflects the number of photoreceptor nuclei, approximately 250 days post-transplantation. To reduce culture duration at minimum (and reduce associated manufacturing costs), D50-60 was selected as graft age for following experiments. These grafts developed after transplantation and formed outer and inner nuclear layer-like structure. These donor inner nuclear layers are susceptible to limit donor photoreceptor integration with host bipolar cells. In addition, donor photoreceptors formed rosettes that prevent interactions with host RPE cells. Outer segments are indeed located inside the lumen of these rosettes (Shirai et al. 2016; Mandai et al. 2017a; Iraha et al. 2018). The same approach was performed with hPSC-derived retinal organoids at D64-66 grafted in the immunocompromised *rd1/Foxn1^{nu}* mice model at 2 months of age (Iraha et al. 2018). Immunohistological analyses revealed a long-term survival of the graft (almost 6 months post-surgery) and some *in vivo* maturation of the grafted organoid sheet with the detection of both rods and cones presenting inner /outer segment-like structures (Iraha et al. 2018). Multi electrode array (MEA) recordings measuring light responses of *ex vivo* transplanted retinas indicated that some of them (3 out of 8 retinas) were responsive to light. However, *in vivo* full-field electroretinogram (ERG) recordings in response to light stimuli remained flat (no response). The number of integrated photoreceptors was suggested to be too low to elicit consistent responses (below 150 000 cells) (Iraha et al. 2018). When implanted in non-human primates, sheets of D59-63 retinal organoids also formed rosettes (Tu et al. 2019). Visual function (behavioral test) was modestly improved at 1.5 year in one monkey in the grafted laser-lesioned area compared to control retinal area, but no improvement was recorded by focal ERG. A recent study in dogs reported that retinal sheets obtained from D104-151 organoids survived up to 3-5 months in the subretinal space of normal dogs and can extend axons beyond the outer limiting membrane when transplanted in dogs with retinal degeneration (*rcd1/PDE6B* mutations) (Ripolles-Garcia et al. 2022).

Thus, all these experiments demonstrated that retinal organoids are able to continue their development following grafting into host retinas (even degenerated retinas) and to form *de novo* organized outer nuclear layers. However a number of challenges still need to be overcome in order to improve integration and functionality of grafts: (i) limit the presence of other retinal cell types into the graft, (ii) limit the formation of rosettes that prevent the interaction with RPE cells, and (iii) increase the proportion of cones to improve visual outcomes in humans.

2.4. Optimization of cell integration and functionality

To optimize functionality of grafted photoreceptors, different strategies are under investigation. These include refining the formulation of cell therapy products, combining with optogenetics or modifying the local microenvironment of the degenerative host retina to improve cell integration.

2.4.1. Selection of the cell types to be grafted

To improve survival and functional visual preservation, one recently proposed approach was to transplant, at the same time, both RPE cells and retinal progenitor cells (RPCs) that could give rise to photoreceptors. Injections of RPE cells or RPCs alone (D45-50 of differentiation in culture prior implantation) -both derived from hPSCs and labeled with GFP- were compared to injections of a combination of both cell types in RCS rats (presenting a RPE dysfunction) before photoreceptors degeneration (Salas et al. 2021). Combination of RPE cells and RPCs triggered a better graft survival at 12 weeks post-surgery as monitored by fluorescence fundus imaging to detect GFP in immunosuppressed animals. This survival was associated to a better preservation of host photoreceptors allowing a better restoration of visual functions (scotopic ERGs) compared to the same cell types injected alone (Salas et al. 2021). However, the integration was not formally evaluated and the immunosuppression strategy chosen was not optimal for long-term morphological and functional evaluation, *i.e.* difficulty of controlling the uptake of the immunosuppressive drug cyclosporine by the rodents. The visual outcomes observed in these experiments could be due to neuroprotective effects. Experiments with genetically immunocompromised models of retinal degeneration (Thomas et al. 2018) will better address the long-term integration of different cell types.

Instead of combining RPE and RPCs in cell suspension, another approach is to combine pieces of retinal organoids with a preformed RPE sheet. Thomas and collaborators assembled RPE sheets on parylene with retinal organoid pieces using either gelatin, alginate or matrigel (Thomas et al. 2021). Alginate provided better performance as bio-adhesive and was selected to assemble the co-graft. These co-grafts

were implanted into immunodeficient RCS rats (*RCS-p+/Foxn1^{nu}* rats) at a stage of advanced retinal degeneration and graft survival was observed up to 7.7 months (maximum follow-up time). Optokinetic tests suggested a neuroprotective effect rather than functionality of co-grafted photoreceptors. Unfortunately, the co-graft was not compared to either RPE cells or retinal organoid sheets grafted alone. This multilayer strategy did not prevent the formation of rosettes when implanted *in vivo* due to the structure of the organoid and the RPE sheet was not entirely covered with the organoid (Thomas et al. 2021).

Finally, the last strategy is to genetically modify cells inside organoids to trigger selective depletion of cell populations not required for future transplantation. In this context, Yamasaki and collaborators generated hPSCs with the deletion of *ISLET-1* (*ISL1*) gene (Yamasaki et al. 2022). Differentiation of these *ISL1$^{-/-}$* hPSCs into retinal organoids leads to a reduction of bipolar cells while preserving photoreceptors, Müller glia and horizontal cells. Both wildtype and *ISL1$^{-/-}$* retinal organoids (stage D60) were cut into pieces and grafted into immunodeficient rat model of end-stage photoreceptor degeneration. Six months post transplantation, multiples rosettes presenting photoreceptor inner and outer segments in their lumen were observed with both grafts. Interestingly, better host-graft contacts between host bipolar cells and grafted photoreceptors were reported in *ISL1$^{-/-}$* grafts compared to wildtype grafts. At 8-10 months post-surgery, MEA recordings revealed a significant restauration of light responsiveness in host ganglion cells (Yamasaki et al. 2022). This response was improved with *ISL1$^{-/-}$* retinal organoids compared to wildtype grafts, suggesting that the selective depletion of bipolar cells inside organoids favors the contacts between grafted photoreceptors and host bipolar cells, leading to the formation of functional synapses even into this xenogeneic context.

2.4.2. Tissue engineering by design

To organize photoreceptors prior implantation, different approaches were envisioned such as decellularized retinas in which RPE cells and ocular progenitors differentiated from hPSCs can be seeded (Maqueda et al. 2021) or polymers (or mixtures of polymers) that are not structured by design (Lavik et al. 2005; Tucker et al. 2010; Singh et al. 2018). Beside such scaffolds, probably one of the most promising strategies is to design a specific 3D micro-structuration to guide the organization of photoreceptor precursors and to prevent the formation of rosettes. Indeed, different attempts were published using a variety of polymers (biodegradable or not) that can support this micro-structuration such as poly(dimethylsiloxane) (PDMS), poly(lactic-co-glycolic acid) (PLGA), poly (glycerol-sebacate) (PGS) or acrylated poly(caprolactone) (PCL) (Neeley et al. 2008; McUsic et al. 2012; Jung et al. 2018; Thompson et

al. 2019). The 3D micro-structuration design of the scaffold is critical to achieve both a sufficient density and a relevant polarized maturation. Thus, different micropore designs were proposed (Neeley et al. 2008; McUsic et al. 2012; Worthington et al. 2017; Jung et al. 2018; Lee et al. 2021).

Worthington and collaborators directly created micro-structured scaffolds with 2-photon lithography printing. This approach allowed the designing of highly complex 3D structures, composed of vertical micropores, which can theoretically be loaded with photoreceptors and horizontal micropores to favor exchange between cells (Worthington et al. 2017). This technical choice induced long writing steps to produce only one scaffold at a time despite their efforts to optimize printing steps. RPCs derived from retinal organoids at D30 plated into these scaffolds for 2 days survived and formed neuronal processes inside micropores. In addition, this group developed PCL biodegradable polymers (Thompson et al. 2019) as well as extracellular matrix based polymers (mixture of gelatin and hyaluronic acid) (Shrestha et al. 2020) for scaffolding. *In vivo* toxicity studies revealed that the PCL polymer was well tolerated, biocompatible and safe (Thompson et al. 2019). PCL scaffolds were loaded with RPCs derived from retinal organoids at D70 and transplanted into the eye of nude rats to evaluate the tolerance of the product (Han et al. 2022). The PCL scaffold loaded with cells was demonstrated to be safe but no functionality studies were conducted.

Another technical choice was based on the creation of a negative mold that will be reused a number of times to create numerous scaffolds (micromolding). Using this approach, Neeley and collaborators proposed a cylindrical micropore where retinal cells can grow and differentiate (Neeley et al. 2008; Redenti et al. 2009). 3D microstructured PLGA scaffolds using a similar cylindrical design containing retinal cells can also be co-cultured with RPE cells at least for 14 days (McUsic et al. 2012). However, this cylindrical design does not prevent cells from crossing the scaffold. Therefore, Jung and collaborators proposed an alternative “wineglass” design to retain cells on a reservoir. Thus, this design is composed of a cut shape reservoir where 1 to 3 photoreceptors can be loaded and a microchannel in which photoreceptors can grow extensions that can mature into inner/outer segment-like structures (Jung et al. 2018). Polymers used for this scaffold included PDMS or PGS, the last been biodegradable *in vivo*. Photoreceptor precursors isolated from retinal organoids at D80 continue to mature after 3 months *in vitro* by developing basal axon extensions on microchannels and expressing rod specific markers. Optimization of the scaffold by the same team lead to creation of an “ice cube tray” design (Lee et al. 2021). This evolution allowed to increase photoreceptor density and reduced the amount of synthetic biomaterial used to generate the scaffold (increase of the ratio cells *vs.* polymer). This design is

composed of a reservoir layer that receives a mean load of 18 photoreceptors and a base layer with pores (Lee et al. 2021). Interestingly, photoreceptors isolated from D120 retinal organoids seeded into these scaffolds seemed polarized after 5 days in cell culture, even though immunofluorescence staining did not clearly show a well-defined orientation of maturing photoreceptors.

Collectively, these studies started to explore a tissue engineering by design strategy that may improve outcomes following retinal implantation *in vivo*. Optimization of micropore designs as well as functionality of photoreceptors into these scaffolds is still required, as well as *in vivo* proof of concept.

2.4.3. Combination of optogenetic and cell transplantation

Functional integration of photoreceptors entails connecting to inner host retina but also development of light-sensitive outer segments, requiring interaction with the underlying RPE. An alternative strategy to tissue engineering is to combine stem cell-based therapy and optogenetics thereby conferring light sensitivity to donor cells (immature photoreceptors). This strategy allows overcoming the issue of maturation with formation of inner/outer segments into the host retina and the lack of contacts with endogenous RPE cells, by the production of functional photoreceptors thanks to the microbial opsin activity. Using specific AAV vectors, Garita-Hernandez and collaborators have been able to deliver a hyperpolarizing microbial opsin into photoreceptor precursors from D42 retinal organoids (Garita-Hernandez et al. 2019). Patch clamp recording on cell suspension from D70 retinal organoids demonstrated robust optogenetic light responses in these optogenetically-transformed photoreceptors lacking light sensitive outer segments. Furthermore, *in vivo* studies demonstrated that light-driven responses at the ganglion cell level can be observed into blind mice, after transplantation of these photoreceptors equipped with a microbial opsin but not recorded after transplantation of "control" photoreceptors (organoids transfected only with GFP). However, it remains unclear whether microbial opsin may elicit adverse immune responses in humans (Cehajic-Kapetanovic et al. 2022). Long-term follow-up using immunodeficient blind rodent models are also required to evaluate visual recovery after a longer period of maturation.

2.4.4. Modification of the microenvironment

An aspect yet underestimated in the context of photoreceptor transplantation is related to the retinal microenvironment. Indeed, most research groups focused on improving the transplant to ameliorate visual outcomes but few considered the retinal degenerative environment. Indeed, during the process of retinal degeneration, a number of signaling pathways deregulating gene expression are involved (inflammation, endoplasmic reticulum stress, oxidative stress, aberrant autophagy, calcium homeostasis

dysfunctions) (Gorbatyuk et al. 2020). Therefore, a “reconfiguration” of this host microenvironment might improve integration and functionality of donor photoreceptors.

Several studies showed that injection of purified solutions of extracellular vesicles (EVs; secreted by almost all cells and containing a cargo composed of DNA, RNA, proteins and lipids) are susceptible to limit inflammation (Mead and Tomarev 2020; Jarrige et al. 2021). For example, following subretinal injection in a RP rat model, EVs derived from neural progenitor cells were mostly internalized by microglia which was therefore inhibited and induced the downregulation of pro-inflammatory cytokines (Peng et al. 2014; Bian et al. 2020). We could envision a strategy of injecting EVs prior or concomitantly to cell transplantation to modulate inflammation and favor integration / maturation of grafted cells (Jarrige et al. 2021). Other groups suggested a role of inhibitory extracellular matrix such as CD44 and neurocan, which accumulate due to glial hypertrophy during retinal degeneration (Tucker et al. 2010; Yao et al. 2011). Matrix metalloproteinase 2 (MMP2) incorporated into PLGA beads were proposed as co-transplants with RPCs in order to favor integration of donor cells (Yao et al. 2011).

Concluding remarks

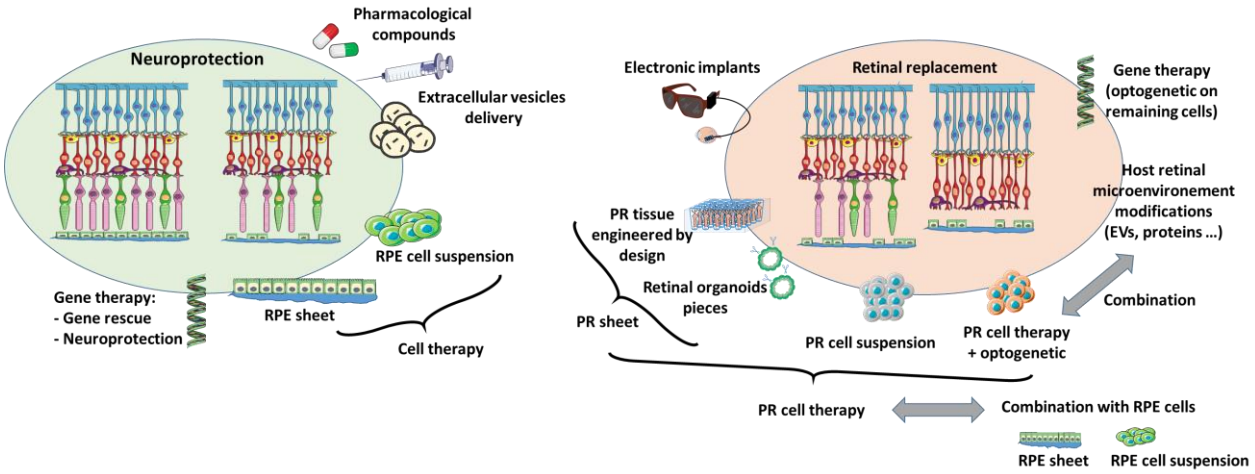
The field have made significant progresses these last years to improve our knowledge related to photoreceptor cell grafting, in particular understanding material transfer mechanisms and developing more complex cell therapies. It remains a long way to develop efficient cell therapy for photoreceptor replacement perhaps structured as engineered tissues. Nevertheless, first patients with advanced RP were already implanted with hPSC-derived neuroretinal sheets (RIKEN, JRCT ID jRCTa050200027) (Maeda et al. 2022). We hope this first-in-man study will pave the way for clinical trials exploring other types of photoreceptor cell therapy products.

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Figure legend

Figure 1: Scheme describing different therapeutic options envisioned to prevent degeneration or restore vision of patients with retinal degenerative disorders. PR =photoreceptors; EVs = extracellular vesicles.



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