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Factors Affecting Stem Cell–Based Regenerative Approaches in Retinal Degeneration

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Abstract

Inherited and age-associated vision loss is often associated with degeneration of the cells of the retina, the light-sensitive layer at the back of the eye. The mammalian retina, being a postmitotic neural tissue, does not have the capacity to repair itself through endogenous regeneration. There has been considerable excitement for the development of cell replacement approaches since the isolation and development of culture methods for human pluripotent stem cells, as well as the generation of induced pluripotent stem cells. This has now been combined with novel three-dimensional organoid culture systems that closely mimic human retinal development in vitro. In this review, we cover the current state of the field, with emphasis on the cell delivery challenges, role of the recipient immunological microenvironment, and challenges related to connectivity between transplanted cells and host circuitry both locally and centrally to the different areas of the brain.

INTRODUCTION

The retina is a complex tissue composed of multiple cell types that together orchestrate visual reception and processing. At the core of light sensing are the photoreceptors, of which there are two types—rods and cones. These photoreceptors are topographically organized, with cones, which are predominantly red and green sensitive, concentrated at the center of the visual axis, the macula. Photoreceptors conduct light signals through photo-isomerization of visual pigments within the disc membranes of their outer segments. These molecules, once isomerized, activate signaling cascades that can in turn activate intermediary interneurons and then ganglion cells, which then ultimately convey those signals to the brain via the optic nerves. Interneurons, including bipolar, horizontal, and amacrine cells, fine-tune many of the initial signals, while other cells, including retinal pigment epithelium (RPE) cells and Müller glia, carry out various roles in supporting the function of photoreceptors and maintaining the retinal microenvironment (McCannel 2020).

Unfortunately, owing to the interconnectedness, both physical and functional, among retinal cell types, damage to any one cell type can lead to progressive, significant deterioration in visual function. In Stargardt disease, for example, a deficiency in ABCA4—a transporter involved in retinal byproduct recycling—leads to accumulation of toxic byproducts within rod and cone discs. Upon photoreceptor renewal, these toxic byproducts are taken up by the RPE, inhibiting its normal metabolic function and further compromising photoreceptor activity (Tanna et al. 2017). In the same vein, diseases directly affecting the RPE, such as age-related macular degeneration (AMD), drive overlying photoreceptor dysfunction as a consequence of poor or absent support. Interestingly, in amphibians, the RPE serves as a source of cellular regeneration. Upon retinal cell loss or injury, the RPE differentiates, migrates, and subsequently proliferates as neurogenic progenitors capable of giving rise to all of the cells of the retina. In other species, such as the teleost fish, Müller glia act as the resident stem cell population, capable of regenerating all classes of retinal neurons; chick Müller glia, conversely, are far more fate restricted in their regenerative potential, preferentially generating amacrine cells (Todd & Reh 2022). Humans, in contrast, do not have regenerative capabilities and respond to retinal injury through inflammation and eventually scarring, as seen in the dry form of AMD. These scars are irreversible, typically affect the central part of the visual axis, and lead to a devastating decline in quality of life due to eventual or near blindness. Other diseases, such as glaucoma and related conditions that affect retinal ganglion cells (RGCs), progressively damage the nerve fiber layer in a similarly irreversible manner (Rein et al. 2022, Van Gelder et al. 2022).

This property of the postinjury mammalian retina has spurred numerous efforts to both promote endogenous regenerative activity and develop cell replacement therapies for damaged cells (Ludwig & Gamm 2021, Van Gelder et al. 2022). The landmark discovery of reprogramming adult cells back to a pluripotent state has completely revolutionized the way that the entire regenerative medicine field is thinking about restoring tissue function (Takahashi & Yamanaka 2006, Takahashi et al. 2007, Yu et al. 2007). These induced pluripotent stem cells (iPSCs), which do not prompt the same ethical qualms as human embryonic stem cells, have led to broader acceptance. Studies, too numerous to cite, have demonstrated at high efficiency the generation of all of the various retinal cell types (**Figure 1**). Cell replacement for damaged RPE in patients has reached the clinic. There are several clinical trials that have demonstrated the safety and engraftment of stem cell–derived RPE transplants in humans, with variable effects on visual acuity. Stem cell–derived photoreceptor transplants have also been performed, although primarily in preclinical models. Though some of this work has been complicated by questions of true donor cell engraftment versus material transfer, transplantation of primary human retinal progenitors has shown some efficacy in human trials. Ultimately, however, re-establishing the cell circuity—the connections between the newly added

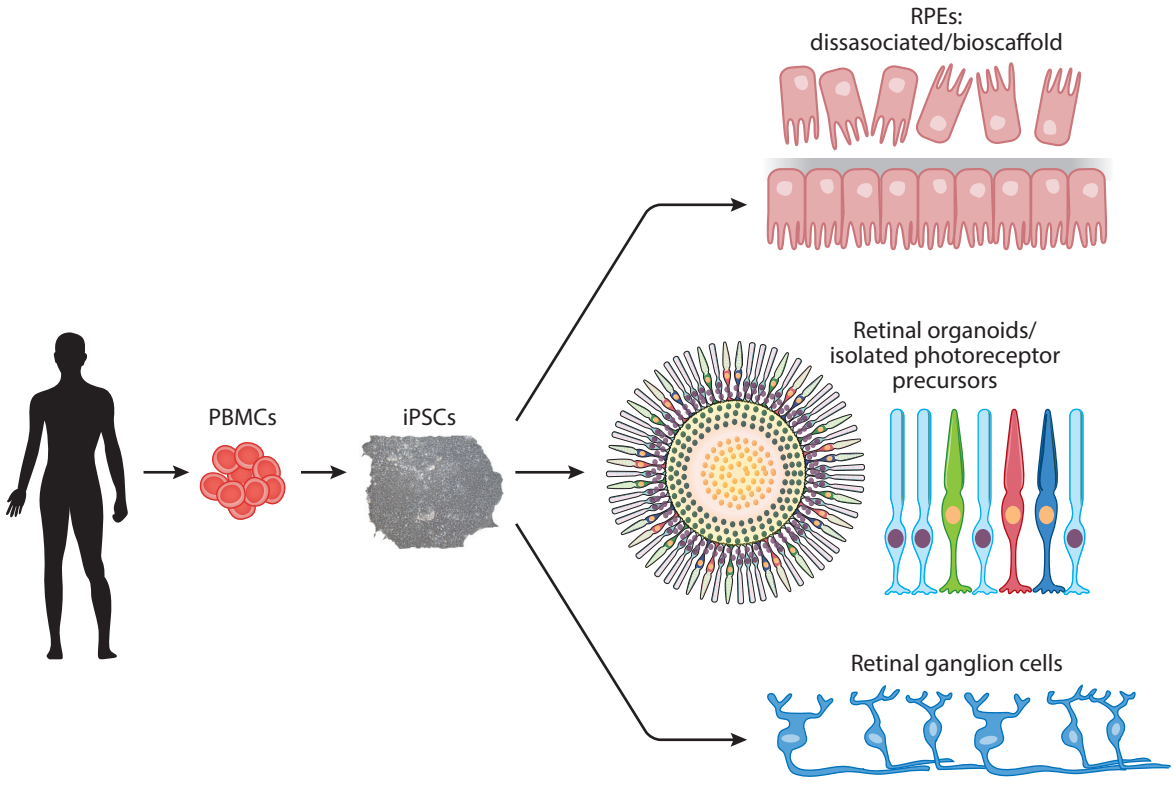

Figure 1

Schematic showing the potential of human induced pluripotent stem cells (iPSCs) derived from patient or normal peripheral blood mononuclear cells (PBMCs) to generate retinal pigment epithelium (RPE), photoreceptors, or ganglion cells to replace those damaged due to disease or injury of the retina. These cells could be delivered as dissociated cells, on natural or synthetic scaffolds, or in self-assembled organoids.

cells and the surrounding cellular microenvironment—is just as important as replacing deficient or defective cells. In this article, we review various aspects of cell replacement strategies, explore their current status, and discuss the challenges of functional integration. We also discuss barriers to transplantation, including the role of the immune system in both healthy and diseased retinas, as well as touching on complementary endogenous repair approaches.

CELL TRANSPLANTATION AND REPLACEMENT STRATEGIES

Retinal Pigment Epithelium

The RPE comprises a sheet of hexagonal pigmented cells that form the outer barrier between the retina and choroidal vasculature. Their tight junctions constitute the outer part of the retina–blood barrier, serving in some sense to immunologically protect overlying photoreceptor and support cells. Thus, they tightly regulate the passage of nutrients and metabolites from the underlying choroid, whose vasculature supplies the outer retina. One of the key functions of RPE cells is their role in visual pigment processing and recycling: Within photoreceptors, 11-*cis*-retinal is converted to all-*trans*-retinal, which is then transported to adjacent RPE and reisomerized to the 11-*cis* form. Additionally, RPE cells aid in renewal of photoreceptor outer segments, phagocytosing those that

are damaged or old. Damage to the RPE, or untethering of its close approximation to overlying photoreceptors, as in the wet form of AMD, can lead to significant visual compromise (McCannel 2020, Sharma et al. 2019a).

Early studies of autologous grafting laid the groundwork for RPE replacement strategies for the treatment of RPE-related retinal diseases, such as AMD. In these studies, peripheral RPE and choroid cells were transplanted into the region of the macula in patients with wet AMD (Binder et al. 2004, MacLaren et al. 2005, Van Meurs et al. 2004). Four-year follow-up demonstrated that grafts maintained function, and a best corrected visual acuity of >20/200 was achieved in approximately 15% of patients (the preoperative average was 20/250) (Van Zeeburg et al. 2012). The discovery of pluripotent stem cells (PSCs) and the realization that RPE could be differentiated from embryonic stem cells (ESCs) sparked interest in growing RPE for transplantation. Numerous protocols have since been developed to enhance the efficiency of differentiation and to more closely recapitulate native RPE properties, including formation of tight junctions. The use of various basement membrane scaffolds (discussed in detail below) has also helped in this regard (Sharma et al. 2019a).

Several clinical trials are underway or have been completed evaluating the safety and efficacy of PSC-derived RPE, delivered both as suspensions and as sheets with scaffolds. These trials have largely focused on patients with Stargardt's disease or AMD, with cells delivered into the subretinal space. It should be noted that these studies involved a small number of overall patients (Sharma et al. 2019a, Van Gelder et al. 2022); despite this, it is encouraging that none of these trials reported severe adverse effects with respect to teratoma formation or ectopic differentiation, although transplanted patients do require immunosuppression (Maeda et al. 2021). Visual acuity improvement has been variable, although up to a 21-letter improvement has been reported in some eyes; additionally, fixation was noted to move closer to the area of the graft, suggestive of possible improvement in functional activity of that area (da Cruz et al. 2018; Kashani et al. 2018; Mandai et al. 2017b; Schwartz et al. 2012, 2015). More recent preclinical and clinical trials have focused on delivering RPE cells as sheets (upon scaffolds) as opposed to cell suspensions (Ben M'Barek et al. 2017, da Cruz et al. 2018, Kashani et al. 2018, Mandai et al. 2017b, Sharma et al. 2019b) (**Figure 1**).

Retinal Precursors and Photoreceptors

While various cell sources have been tested to rescue photoreceptors, including fetal brain–derived neural stem cells, umbilical cord–derived cells, cord blood–derived stem cells, and long-term cultured retinal fetal tissue, this section focuses on direct replacement of photoreceptors. Early work in the 1980s established the feasibility of transplanting neonatal retinal tissue into sites of damage. Work from Turner, Blair, Cerro, and others demonstrated that neonatal rat tissue transplanted into the subretinal space of recipient adult rats could differentiate into various neuroretina cell types, including photoreceptors, and survive for at least 4 weeks (for a detailed review on the topic, see Ludwig & Gamm 2021). This tissue was transplanted as cell suspensions, microaggregates of retinal tissues, whole retinal or photoreceptor sheets, and photoreceptor–RPE cotransplants (Seiler & Aramant 2012). Subsequent work from other groups in the late 1980s demonstrated that the engraftment rate was contingent on the age of the donor tissue: Prenatal and early postnatal tissue more durably engrafted, with a gradual decline in engraftment as the postnatal age increased (Aramant et al. 1988). Similar results were observed with mouse dissociated cell transplants (Gust & Reh 2011). This study showed that graft failure with age was not necessarily due to poor integration of cells, but rather to a high rate of transplant failure, which the authors speculated was due to reduced tolerance to the isolation procedure itself.

These efforts led to the first human clinical trials using fetal retinal tissue. Two clinical trials have been carried out to test the safety of human retinal tissue in patients with either retinitis pigmentosa (RP) or AMD. The Del Priore lab carried out an adult retinal sheet transplant clinical study using human cadaveric tissue in the absence of any immunosuppression (Berger et al. 2003). While no functional improvements were reported, they did not observe any significant side effects or inflammation. Aramant and Seiler led a phase 2 clinical trial in patients using fetal retina and RPE sheets in 10 patients (Radtke et al. 2008). They reported transient improvements in visual recovery, showing the feasibility of such an approach. However, their visual outcome results were limited except in one patient, who reported persistent improvement to 20/200 from 20/800 preoperatively. The trial also did not use any immunosuppressants, although the tissue was type-matched for major histocompatibility complex (MHC) antigens, which likely may have affected survival.

A key paper by MacLaren et al. (2006) posited that transplantation of committed newborn photoreceptors, as opposed to retinal progenitor tissue, would increase the rate of integration. They genetically tagged postmitotic rod precursors expressing neural retina leucine zipper (*Nrl*) with GFP, isolated them from postnatal day 1 (P1) mouse retinas (corresponding to the peak of rod genesis), and transplanted them into the subretinal space of various mouse models of inherited retinal degeneration; these cells robustly integrated, and mice receiving P1 transplants developed a pupillary reflex, whereas sham-injected controls did not. As an alternative explanation for their results, the authors tested whether classical cell fusion was responsible for their observations—although dual color studies [transplantation of green fluorescent protein (GFP) donor cells into cyan fluorescent protein–expressing host tissue] did not show clear evidence of colocalization. Nonetheless, this work demonstrated the need for generating more mature photoreceptors for improved visual recovery.

Organoid Technology for the Development of Mature Photoreceptors

The isolation of human embryonic stem cells and, later, the discovery of iPSCs further advanced the cell replacement field, as many of the ethical barriers to obtaining human fetal tissue for possible transplants could now be avoided. Seminal work from Gamm, Reh, Lamba, Sasai, and others showed that these PSCs could generate RPE, retinal progenitor cells, and photoreceptor precursors (Lamba et al. 2006, Meyer et al. 2011, Nakano et al. 2012, Zhong et al. 2014). Three-dimensional (3D) organoid culturing further improved upon the efficiency of the generation of mature photoreceptors while providing a platform to study ex vivo retinal development, genetic disease, and possible therapeutic interventions. Since the pioneering papers by Sasai's lab on the generation of organized 3D retinal organoids from PSCs (Eiraku et al. 2011, Nakano et al. 2012), several protocols have been developed (Bell et al. 2020), as well as modifications to induce or enrich particular cell types of interest (Chew et al. 2022). Notably, however, current organoids lack several cell types that constitute the retinal microenvironment, including microglia and other immune cells, vascular endothelium, and RPE. Several groups have begun coculturing organoids with RPE (Akhtar et al. 2019); interestingly, this leads to an enrichment of photoreceptor precursors and accelerates their maturation. Whether other support cells can be similarly cocultured and whether this may better recapitulate the human retina (both transcriptomically and organizationally) remain to be seen.

Retinal organoid transplantation studies have showed some success in degenerative photoreceptor mammalian models. The Goureau lab (Gagliardi et al. 2018) isolated Cd73+ photoreceptors and showed that they could survive and mature upon transplantation into a rat model of photoreceptor degeneration. Several other studies have shown similar success with retinal organoid transplantation in retinal degeneration models (Gonzalez-Cordero et al. 2017,

McLelland et al. 2018, Shao et al. 2017, Shirai et al. 2016, Singh et al. 2015), with some demonstrating visual improvement (McLelland et al. 2018, Shao et al. 2017). One of the other advantages of organoid technology is the opportunity to modify cell types. This feature could be exploited to enrich certain rare cell types or generate and incorporate cells that can secrete additional neurotrophic or anti-inflammatory factors. The main disadvantage of this technique, however, lies in the inability of the key cells, such as photoreceptors, to directly interface with the host bipolar cells. This would require them to disconnect their synaptic connections from the bipolar cells in the organoids prior to connecting to the ones in the host. However, the ultimate advantage of the approach is that we may be able to derive iPSCs from patients with inherited retinal disorders, correct the mutation with CRISPR/Cas9 technology (Chirco et al. 2021), and transplant genetically corrected organoids or enriched cells from organoids.

Ganglion Cells

Glaucoma is a progressive neurodegenerative disorder affecting the RGCs. While the exact pathogenesis on ganglion cells is still unclear, the damage leads to progressive loss and thinning of the retinal nerve fiber layer that forms the optic nerve and sends signals to the thalamic and visual processing centers. Replacement of RGCs is a key concern for treatment of glaucoma due to their lack of regenerative potential. Thus, strategies aimed at neuroprotection, enhancing or artificially promoting endogenous repair activity early in the disease, or cell transplant following cellular loss are important areas of exploration. Preclinical work is in its relative infancy (compared with work on RPE and retinal photoreceptors) in terms of transplanting RGCs into damaged retinas (Van Gelder et al. 2022). Developing a strategy for ganglion cell replacement is a significant challenge, as the new cells will need to first migrate to the appropriate space from the site of delivery (intravitreal or subretinal), then make appropriate dendritic connections to the surviving host bipolar cells along with amacrine interneurons and, most critically, project their axons along the optic nerve to make the right connections in the thalamus and visual cortex. Initial studies transplanting GFP+ mouse RGCs into host rats showed integration of the GFP+ cells into the host layers, as well as a recordable depolarization response to light flashes following whole-cell patch clamping (Venugopalan et al. 2016). Work from Wu et al. (2021) has shown that mouse ESC–derived RGCs, when transplanted into *N*-methyl-D-aspartate-induced RGC-depleted mice, can extend neurites into the inner plexiform layer of the host retina, although their axons did not extend into the optic nerves. Unfortunately, this work did not carry out any detailed analysis to rule out the potential of material transfer to host RGCs.

Material Transfer: Confounding Factor or Boon?

Recently, however, the photoreceptor cell replacement field has been temporarily paused due to the realization that many of the historical observations of donor tissue integration were in fact a consequence of material transfer between the transplanted cells and the host photoreceptors, rather than true integration (Pearson et al. 2016, Santos-Ferreira et al. 2016, Singh et al. 2016). Recent re-examination of cell transplantation studies of injecting *NRL*-driven GFP+ cells into mouse retinas has shown that what was originally interpreted as cell integration (via GFP+ labeling) was in fact a transfer of GFP to the host mouse photoreceptors. A paper by Waldron et al. (2018) utilizing similar dual-color strategies found that marker coexpression was a common occurrence following transplantation. They additionally found that numerous photoreceptor proteins could be transferred among cells, and that the likelihood of material transfer was contingent on the microenvironment, with retinas that were not fully degenerative (i.e., they contained some host photoreceptors) exhibiting higher levels of transfer. This and other related work has called into question whether true integration and synaptogenesis is indeed possible and understandably

raises the bar in terms of more thorough quantitative and functional assessment for future work. There is a significant effort in the field to come up with a set of tests to confirm real integration of transplanted cells. These include using nuclear-localized fluorescent tags, human-specific nuclear markers, ethynyl-deoxyuridine and bromo-deoxyuridine incorporation into cells prior to transplantation, cross-gender transplants, or cre-lox-based systems (Nickerson et al. 2018). Recent evidence suggests that this phenomenon is not restricted to photoreceptor cells and could play a role in ganglion cell replacement approaches as well (Zhang et al. 2022). Thus, detailed characterization of the ontology of the observed integrated cells is critical to restore confidence in cell replacement strategies for photoreceptor and perhaps ganglion cell therapies.

The exact mechanism of this material transfer phenomenon is still not clear. Protein transfer is not due to cell fusion between the transplanted photoreceptors, since none of the GFP+ cells transplanted into a female recipient retina from a male host could be detected by Y-chromosome fluorescent in-situ hybridization analysis. Some recent studies suggest that the transplanted and host photoreceptors connect with each other through nanotubes and transfer protein through actin-dependent transport mechanisms (Heisterkamp et al. 2022, Kalargyrou et al. 2021). It is possible that this mechanism could account for enough transfer of proteins or messenger RNA to restore function of a mutant protein in a host with inherited retinal degeneration to promote visual recovery. This has increased interest in identifying the mechanism of material transfer as a delivery method to diseased photoreceptors, with the goal of boosting transfer of proteins that hosts are lacking.

THE CHALLENGE OF CELL DELIVERY

One of the major challenges of cell replacement therapy is delivering the cells in a way that minimizes retinal disruption yet maximizes engraftment and donor cell functionality. Early transplantation studies utilized single-cell suspensions of RPE transplanted into the subretinal space (SRS) (Schwartz et al. 2015, Song et al. 2015). This mode of delivery has been associated with a high rate of cell death and improper localization. Additionally, efflux of cells during transvitreal injections is another concern, as it both reduces the cellular payload and introduces cells into the vitreous, with the latter potentially leading to proliferative vitreoretinopathy due to epithelial-to-mesenchymal transformation of RPE cells. RPE cells, in particular, do not form a polarized monolayer upon single-cell transplant, instead existing as rounded, nonpolarized cells of questionable functional potential (Sharma et al. 2019a). Indeed, initial studies have shown variable efficacy of RPE single-cell transplants with no correlation of visual acuity improvement with number of cells injected (Schwartz et al. 2015, Sharma et al. 2019a). It is likely that the damaged, thickened Bruch's membrane with drusen that exists in aged or dry AMD environments hinders cell adhesion and monolayer formation: Work from the Del Priore lab (Gullapalli et al. 2005) has shown that fetal human RPE cells do not adhere as well to and have altered morphology on unmodified submacular Bruch's membranes from donors >55 years of age. In that vein, some have speculated that single-cell transplants may be more effective at earlier stages of disease, when either Bruch's membrane is still intact and more amenable to engraftment or an RPE monolayer already exists.

For later stages of degenerative disease, retinal sheets offer a distinct advantage. Transplantation of RPE sheets into the SRS is associated with higher rates of graft survival and cell integration, presumably since cells already exist within a monolayer. These observations have been extended to the development of RPE-seeded engineered scaffolds, which are then delivered into the subretinal space (White & Olabisi 2017). Various RPE scaffolds have been designed, including extracellular membrane (ECM)-based (collagen), plastic-based (polyethylene terephthalate and parylene), and biologic [poly-(lactic-coglycolic) acid (PLGA) and amniotic membrane grafts] scaffolds. These

are either biodegradable (ECM or PLGA based) or permanent (plastic based). Combination (natural and synthetic) scaffolds have also been developed that exploit the structural properties of the synthetic material and the biologic proteins and adhesion substrates of the natural component (White & Olabisi 2017). Xiang et al. (2014) designed a silk, polycaprolactone, and gelatin hybrid scaffold that promotes cell growth and RPE identity while minimizing local inflammation, which has been observed with some plastic-based scaffolds.

Similar to RPE transplants, transplants of whole retinal or photoreceptor sheets have shown functional integration in several preclinical studies, although early clinical trials have shown variable efficacy (Gasparini et al. 2019). One of the unique challenges to photoreceptor transplantation, however, is that the photoreceptor layer sits above the RPE and below the outer nuclear layer and outer plexiform layer. In several studies, transplants of whole retinal sheets have led to rosette formation (Assawachananont et al. 2014, Mandai et al. 2017a). Similar efforts to design photoreceptor scaffolds are underway; Lee et al. (2021) have developed an ice cube tray scaffold that maintains photoreceptor orientation and allows for high-density photoreceptor seeding. It remains to be seen whether such organization and cell polarity can be maintained in vivo.

Similarly, delivery of replacements for damaged or dying RGCs has turned out to be especially challenging. Several approaches have been tried, including delivery into the vitreous, intraretinal injections, and subretinal approaches (Oswald et al. 2021). While intravitreal delivery seems most attractive due to its close proximity to the host ganglion cell layer, the inner limiting membrane is a major obstacle to cell migration into the retina. Using chemicals to at least temporarily alter the porosity of the inner limiting membrane (ILM) may aid in cellular migration through ILM damage (Zhang et al. 2021). However, ILM manipulation in patients with epiretinal membranes has been associated with worse outcomes in patients with glaucoma (Tsuchiya et al. 2021).

THE ROLE OF THE RECIPIENT IMMUNOLOGIC MICROENVIRONMENT

The recipient milieu is another critical consideration for optimizing engraftment. Many of the diseases for which these therapies are being developed—including RP and AMD—are marked by inflammatory microenvironments that further inhibit any endogenous repair capacity and can lead to eventual gliosis. Additionally, as mentioned above, despite the subretinal space being classically thought of as immune privileged, in many retinal degenerative diseases, the RPE layer is disrupted or dysfunctional, and many of its immunosuppressive functions become compromised. The process of transplantation itself is likely also an inflammation-inciting event (Kramer et al. 2019, Petrash et al. 2021, Sugita et al. 2021).

Cell Rejection

Classically, the subretinal space is thought of as an immune-privileged site owing to both the barrier functions of the RPE and its immunosuppressive activity. RPE cells express TGF-β, PD-L1, CTLA-2a, IL1RA, and complement regulatory proteins that together function to quiet the innate and adaptive immune systems (Sugita et al. 2021). Interestingly, however, in an inflammatory milieu, RPE cells can in fact become immunogenic, expressing MHCII, functioning as antigen-presenting cells, and secreting IFN-γ-related chemokine and complement activators (Sugita et al. 2021). The balance of these two roles is a critical consideration in RPE transplantation approaches that further disrupt an already disrupted RPE barrier (due to existing neo-angiogenesis in the choroid or retina, retinal hemorrhages, uveitis, or retinal degeneration). Work from Sugita et al. (2021) has shown that the transplanted autologous RPE cells generate a robust direct and indirect immune response that can lead to graft failure from direct T-cell attack,

antibody-dependent cytotoxicity, or chronic inflammation. MHC matching of iPSC-derived RPE does attenuate the immune response, with reduced T-cell invasion and demonstrated survivability for at least 6 months without the need for immunosuppressants. Similar results were reported in allogenic transplant studies in rhesus macaque (McGill et al. 2018) and mini-pig (Sohn et al. 2015) models, where allogenic RPE cells in nonimmunosuppressed hosts led to rejection of the transplanted cells and an inflammatory response.

Significant work has been conducted examining the graft survivability upon autologous transplantation of cells (RPE and photoreceptor precursors) into diseased murine retinas. These studies have suggested that both the innate and adaptive immune systems are activated upon transplant, albeit at different times. Work from West et al. (2010), in particular, has shown that the majority of murine eyes transplanted with *Nrl*-GFP photoreceptor precursors exhibit a significant reduction of integrated cells at 4 months, a feature that was significantly reduced with immunosuppression with cyclosporine. In studies in our lab, we have tested the role of natural killer (NK) cells in particular in transplant cell rejection. Using mice lacking IL2r, which have an immature immune system but, in particular, lack NK cells, we transplanted human stem cell–derived photoreceptors (Zhu et al. 2017, 2018). We observed robust survival of true integrating photoreceptor cells bearing human-specific nuclear markers for extended periods of time (over 9 months) in both normal and degenerating hosts and showed the potential to restore visual function in a mouse model of congenital blindness (**Figure 2**). Various strategies have been developed to address these concerns, including the development of autologous, human leucocyte antigen (HLA)-matched iPSCs, in conjunction with broad-spectrum immunosuppression (steroids, cyclosporine) or local glucocorticoid injections (Sugita et al. 2021). We discuss these approaches below.

Cell rejection is triggered when the host's immune cells recognize the MHC on the surface of the transplanted cells. There are two main classes. MHCI molecules are expressed on all nucleated cells and are required for the activation of cytotoxic T cells. These include three major classes, HLA A, B, and C, and three minor classes, HLA E, F, and G. All of these require beta-2-microglobulin (B2M) in their complex. MHCII molecules are expressed on antigen-presenting cells and are required for activation of helper T cells. These molecules include HLA-DM, HLA-DOA, HLA-DOB, HLA-DP, HLA-DQ, and HLA-DR. MHC class II trans-activator (CIITA) is the master regulator of MHCII expression. One approach to circumvent immune rejection is to generate haplobanks of iPSCs of major HLA groups in a population. It is estimated that as few as 10 homozygous HLA iPSC lines can match over 41% of the Korean population (Lee et al. 2018). This is, however, more challenging in countries with higher diversity, such as the United States. A significant effort has been made to develop nonimmunogenic iPSC lines that can be used as universal donor cells. Initial efforts involved knockdown of MHCI HLAs (Xu et al. 2019) or B2M (Lu et al. 2013) using gene-editing approaches like TALEN and CRISPR/Cas. Similarly, to knock down MHCII expression, *CIITA* was edited out (Chen et al. 2015) or both *B2M* and *CIITA* were edited out in combination to knock down both classes (Mattapally et al. 2018). While this worked well in vitro, in vivo, cells lacking MHCI are recognized as foreign by NK cells and subsequently attacked. Some alternative approaches being considered involve preserving one MHCI class, such as HLA E, and matching donors based on the preserved class (Gornalusse et al. 2017, Wang et al. 2021). Another alternative idea that has shown promise is to upregulate CD47, which can prevent targeting by NK cells. When injected in immunocompetent humanized mice, iPSC lines lacking B2M and CIITA and upregulating CD47 were not rejected over the course of 50 days (Deuse et al. 2019).

Immunosuppressive therapies have been commonly used for most large organ transplantation in humans. These have commonly included glucocorticoids or steroids, such as dexamethasone, that act by reducing inflammation; drugs that inhibit cytokine production, such as calcineurin

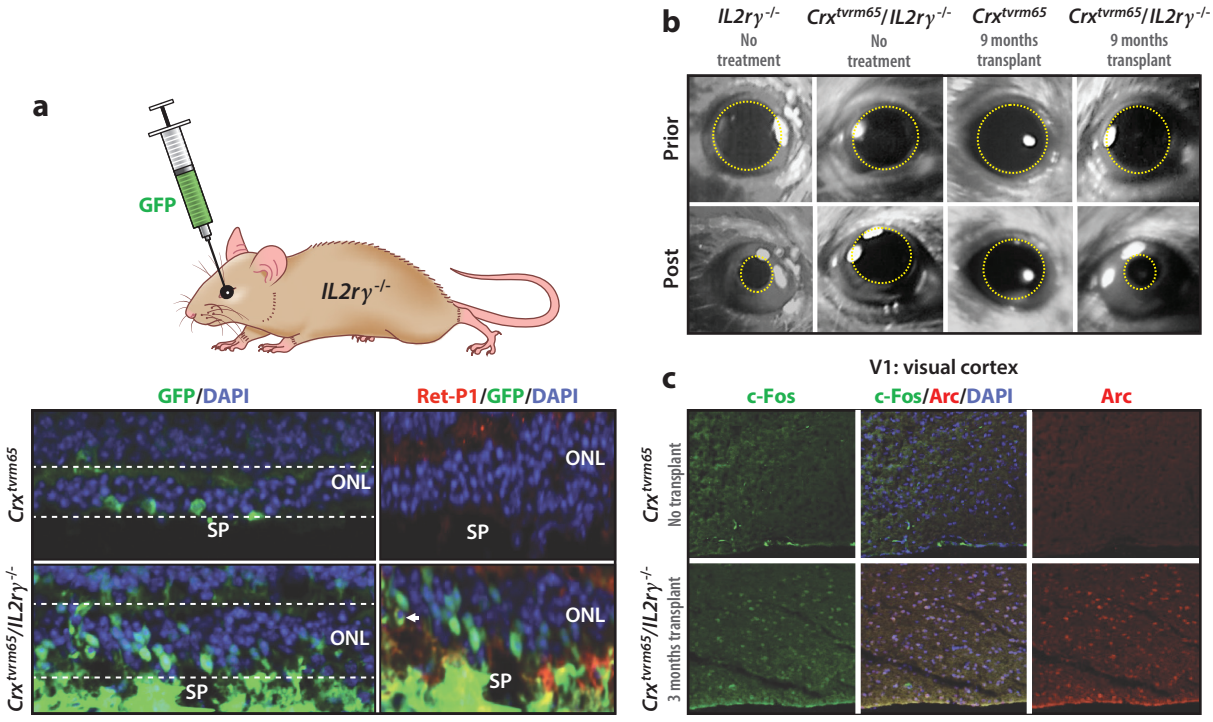

Figure 2

Immunosuppressed microenvironments promote enhanced long-term functional integration of transplanted photoreceptors. (*a*) GFP-expressing human stem cell–derived photoreceptors were transplanted into *IL2rγ*$^{-/-}$ mice, which lack NK cells, that were bred onto a *CRX* mutant mice model of retinal degeneration. Compared to control degenerating mice (*CRX*^{tvrm65}), mice on the IL2 mutant background have much higher survival of transplanted photoreceptors, which also expressed the rod phototransduction opsin, Rhodopsin (Ret-P1). DAPI in blue marks nuclei. (*b*) We performed a functional analysis by testing pupillary light reflex in the contralateral noninjected eye following brief light exposure to photoreceptor or sham injected eye and observed robust response only in the CRX mice on the IL background; this response occurred as late as nine months postintervention. (*c*) Similarly, these mice had immediate early gene expression (c-Fos) in the visual cortex following light exposure, which was absent in sham-injected mice. Abbreviations: CRX, Cone Rod Homeobox; DAPI, diamidino-phenylindole; GFP, green fluorescent protein; IL, interleukin; NK, natural killer; ONL, outer nuclear layer; SP, subretinal space.

inhibitors (tacrolimus or cyclosporine); or cytostatic agents such as methotrexate, which block T-cell proliferation. Such an approach was recently tested in human iPSC-derived retinal transplantation in a host dog model of RP with a *PDE6B* mutation. Without any immunosuppressive therapy, the cells were rapidly rejected within a few days. Using a combination of systemic immunosuppressive agents, which included oral prednisolone, cyclosporine A, and mycophenolate mofetil, the group observed preserved long-term survival of human photoreceptor progenitor–derived cells as far out as 22 weeks post-transplantation (Ripolles-Garcia et al. 2022). Thus, a combination of HLA-matching and immunosuppressive therapies may represent the ideal mix for promoting long-term survival of transplanted cells.

Inflammation

In addition to the immune response generation upon transplant of autologous cells, the existing inflammatory microenvironments that characterize many retinal diseases, including AMD, must also be addressed (**Figures 3*a*** and **4**). Injury to tissues often results in temporal polarization of

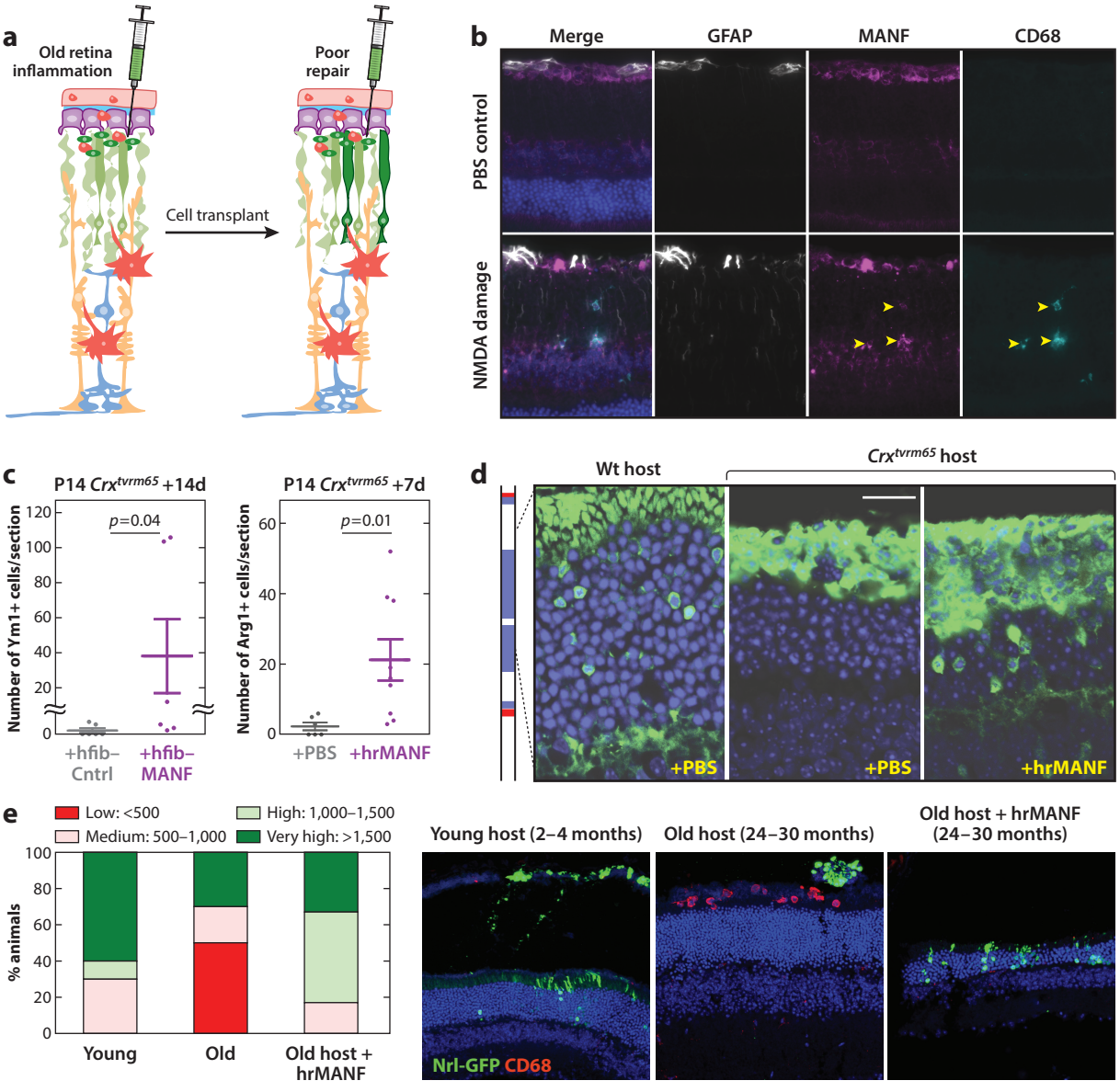

Figure 3

(*a*) Schematic showing that ageing retina and inflammation constitute significant roadblocks to the success of cell replacement therapies. (*b*) Representation fluorescence image showing changes associated with retinal damage using excitotoxin *N*-methyl-D-aspartate (NMDA). Compared to controls, NMDA damage leads to upregulation and change in localization of mesencephalic astrocyte-derived neurotrophic factor (MANF) (*purple*), especially in CD68+-activated microglia (*cyan*), along with Müller glial activation represented by GFAP expression in Müller glial processes. Yellow arrowheads mark CD68+ microglia expressing MANF. DAPI in blue marks nuclei. (*c*) Graphs showing that MANF supplementation drives alternative prorepair activation of microglia in the retina. Panel adapted with permission from Neves et al. (2016). (*d*) Representative image showing that human recombinant MANF (hrMANF) adjuvant therapy, along with photoreceptor replacement, significantly improves integration and likely causes some material transfer in *Crx* mutant mice. Panel adapted with permission from Neves et al. (2016). (*e*) Representative image and quantitation data showing that photoreceptor integration is significantly worse in old microenvironments, which are associated with infiltration of inflammatory (CD68+) microglia. This can be reversed upon MANF adjuvant therapy with photoreceptors. Low, medium, and high represent integration efficiency of transplanted cells in the graph. Panel adapted with permission from Neves et al. (2020).

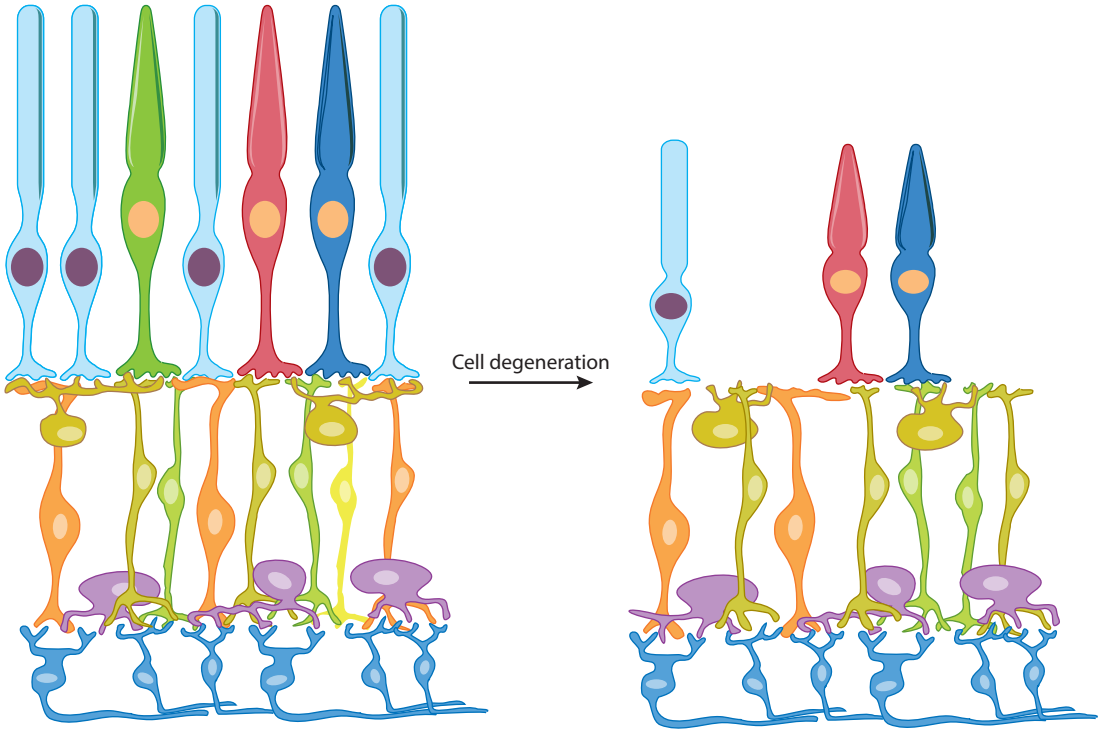

Figure 4

Schematic describing changes associated with photoreceptor or retinal ganglion cell degeneration. Apart from microglial activation, the loss of cells leads to remodeling of the bipolar, amacrine, and horizontal cell neurites. This often begins with abnormal extension as the neurite looks for new synaptic partners, but later in degeneration, it leads to eventual retraction.

tissue macrophages or microglia in the central nervous system (CNS), in which an initial proinflammatory activation of macrophages or microglia is followed by reparative anti-inflammatory activation to promote tissue healing in a tightly regulated balance between the two during normal repair. Unfortunately, in most chronic degenerative disorders, such as AMD and other age-related degenerative conditions, the tissue remains in a chronic inflammatory state. This involves dysregulated complement signaling and activation, secretion of proinflammatory cytokines (including TNF-α and IL1), macrophage polarization into the inflammatory phenotype, inflammasome activation, Müller glia hyperactivity, gliosis, and deposition of chrondroitin sulfate proteoglycan (Kauppinen et al. 2016).

Several immune modulators have been uncovered, by our lab and others, that may serve to reprogram the dysregulated immune environment. One such immune modulator is mesencephalic astrocyte-derived neurotrophic factor (MANF), which we found to bias the inflammatory immune cells in degenerating mice (and *Drosophila*) toward an anti-inflammatory prorepair state (**Figure 3*c***). MANF, which is normally expressed at low levels in Müller glia and astrocytes, gets upregulated in inflamed microglia (CD68+ cells) following damage (**Figure 3*b***). Interestingly, MANF decreased with age both in circulation and in tissues, and its loss was correlated with increased accumulation of activated macrophages (Sousa-Victor et al. 2019). MANF increased the engraftment of transplanted photoreceptor precursors in a degenerating retinal microenvironment (Neves et al. 2016) (**Figure 3*d***). The engraftment was further enhanced in old (24–30-month-old) mice; at baseline, these mice exhibited reduced engraftment compared to

younger mice (Neves et al. 2020) (**Figure 3*e***), an observation that is likely generalizable to the human setting. Although the mechanism has not been fully elucidated, mainly due to the lack of an identifiable cell-surface receptor, it is clear that MANF has important effects in promoting anti-inflammatory macrophage activation that promotes repair and releases neuroprotective and trophic factors. It is also possible that some of the effects also enhance material transfer to the host photoreceptors as an alternate mechanism to improve visual recovery. Other similar immunemodulatory strategies include the modulation of the TGF-β/BMP superfamily, wherein TGF-β drives inflammatory activation, while BMP promotes repair in a conserved manner across species (Kramer et al. 2019). These pathways can be targeted with small molecules to create a more receptive microenvironment. Other growth and neurotrophic factors that have been shown to reduce inflammation include nerve growth factor, brain-derived neurotrophic factor, glial-derived ciliary neurotrophic factor, and leukemia inhibitory factor. Thus, a combined approach of pre- or peritransplant immune modulation and post-transplant immune suppression may be the most effective strategy to maximize engraftment.

SYNAPTIC CONNECTIVITY AND SIGNAL ENCODING

Beyond engraftment, ensuring that transplanted cells form functional synapses with nearby interneurons or ganglion cell central cortical targets to drive visual recovery represents one of the larger challenges of cell replacement therapies. Functional integration represents one of the holy grails of transplantation. Complicating this already sizable task is evidence of neural remodeling that occurs in a staged process during retinal degeneration (**Figure 4**). In other words, the existing neuronal circuity may be not only unplugged in certain areas, but also corrupted or miswired in others.

Photoreceptors

One of the biggest unknowns in the efforts to replace dying neurons in the CNS and peripheral nervous system, and especially in the retina, is how newly transplanted cells would integrate into the existing circuitry. Retinal circuitry is deeply exquisite. The photoreceptor cells communicate visual signals to downstream bipolar cells, which are of two broad categories—the ON cells, which depolarize in response to light, and the OFF cells, which hyperpolarize in response to light. Multiple photoreceptors connect to a single ON or OFF bipolar cell. There is an additional inhibitory interneuron that modulates signaling in that layer, the horizontal cell, of which there are two broad types, H1 and H2. The rod photoreceptor cells, which are the most numerous cells in the human retina (approximately 100 million), are synaptically connected to one main type of ON bipolar cell. In contrast, the cone photoreceptors (approximately 5 million) connect to both types of bipolar cells, the ON and OFF pathways. Typically, two horizontal cell dendrites connect with a single bipolar cell dendrite within the invaginating ribbon synapse of the photoreceptor cells modulating the response. Photoreceptors constantly release glutamate in the dark, which acts on mGluR6 receptors in ON bipolar cells, resulting in closure of TRPM1 channels and activation of AMPA receptors in horizontal cells and AMPA or kainate receptors in OFF bipolar cells. In the presence of light, light-evoked hyperpolarization of photoreceptors leads to reduction in glutamate release, thereby causing hyperpolarization of OFF bipolar and horizontal cells but a sign-inverting depolarization in ON bipolar cells. This overly simplistic map is further complicated by the existence of over 12 subtypes of bipolar cells. Additionally, precise connectivity and GABAergic inhibitory feedback from the horizontal cells directly onto cone photoreceptors provide a mechanism for generating center-surround receptive fields. Another important role played by horizontal cells is in color opponency. Wavelength information of light is also signal encoded

by comparing photoreceptors with different spectral sensitivities in the center versus the surround of a receptive field. H1 horizontal cells in humans and other primates do not contact with S cones, while H2 cells predominantly connect to blue cones and are critical sources of the red-green cone feedback signal and cone opponency in the S-cone receptive field (for more information on color vision circuits, see Thoreson & Dacey 2019). Thus, newly transplanted photoreceptors need to migrate from the subretinal space and make appropriate synaptic contacts such that rod photoreceptors connect to rod bipolar cells, while cones connect to cone ON and OFF bipolar cells, after which the horizontal cells can make appropriate connections with the transplanted photoreceptors. There is evidence that, on top of this, retinal degeneration leads to significant remodeling of the neurites from horizontal and bipolar cells (Lewis et al. 1998); this was validated in patient samples (Fariss et al. 2000). Interestingly, the parafoveal retinal circuit is much more simplistic, with individual cones connecting with one ON and one OFF midget-type bipolar cell, and these bipolar cells then connecting to individual midget ganglion cells. The retinal parafoveal region is expected to be the site for delivery of the photoreceptors to restore central vision. The somewhat simple circuit in this region should allow for better overall visual outcomes.

In our previous studies, our lab has assessed connectivity at the retina circuit level, as well as in central transmission of visual information (Zhu et al. 2017). Following transplantation in *CRX* mutant mice, which completely lack any functional photoreceptors, we analyzed the photoreceptor to bipolar cell connectivity by electroretinogram (ERG) analysis. We observed a restoration of the b-wave on the ERG that corresponds to the ON bipolar cell depolarization. We also observed the transmission of information to both the pretectal nuclei for pupillary light response in the contralateral eye (to the transplanted eye) and the visual cortex in response to light stimulation (**Figures 2*b*,*c***). Similarly, organoid transplant studies in rat models have shown some central visual responses (Lin et al. 2020). Lack of good models has to date prevented us from testing acuity post-transplantation.

Ganglion Cells

The RGCs receive visual information from the bipolar cells that is then modulated by inhibitory interneurons, the amacrine cells. Primate RGCs have been classified into several subtypes based on morphology, connectivity, and functional role, among others. There are >30 RGC types receiving varying types of inputs from different bipolar and amacrine cells. The predominant ganglion cell types in the human and primate retina are ON and OFF midget RGCs, together accounting for >80% of RGCs; they are critical for red-green color information. The next most numerous cells are ON and OFF parasol RGCs (approximately 10%), which are critical for movement information. The small bistratified cells are involved in transmission of blue-color information, and the remaining population of RGCs plays a role in edge-detection and ON-OFF direction information. The axons of the RGCs form the optic nerve, wherein approximately 60% of the nerve fibers cross over to the contralateral side. The axons project to the lateral geniculate nucleus of the thalamus, the superior colliculus, the pretectum, and the hypothalamus. Additionally, there are intrinsically photosensitive RGCs, which are critical for circadian rhythm due to their connection to the suprachiasmatic nucleus in the hypothalamus, as well as connections to the olivary pretectal nucleus in the midbrain, which controls the pupillary light reflex, and the ventrolateral preoptic nucleus, which is involved in sleep regulation. Recent studies using over 26 different mouse Cre lines marking different subtypes of RGCs have identified a rich variety of region-specific as well as lamina-specific projections from different types of RGCs (Martersteck et al. 2017). Thus, the diversity of information encoded in the retina is specifically targeted to various parts of the CNS.

Replacement strategies to date have not focused either on the diversity of cells generated in vitro or on the ability of the transplanted cells to target to the right areas. The targeting issue is particularly critical for cell replacement strategies, as the cues involved in identifying the axonal target of an RGC, as well as those involved in its axonal migration, are present during embryonic development based on a variety of signaling factors such as Netrins, Semaphorins, and Ephrins (Oster & Sretavan 2003). It is unclear if these cues are present in adults, especially in inflammatory, degenerative states. Several studies in optic nerve crush models have provided clues to the inflammatory processes involved in the damage response. Inflammatory preconditioning has been shown to play a critical role in axonal regeneration following crush injury. Driving ocular inflammation via intravitreal Zymosan resulted in massive upregulation in ocular Oncomodulin (Ocm) through Ocm-expressing infiltrating neutrophils, which in turn promotes axonal growth (Kurimoto et al. 2013). Ocm drives up cAMP levels in RGCs, which has been exploited as an alternative approach to promote axonal regeneration via cell-permeable cAMP analogs that can potentially be used as adjuvant therapy along with stem cells (Cui et al. 2003). Alternative strategies have included either knockdown of the tumor-suppressor gene *PTEN* or inhibition of the Jak-Stat pathway with SOCS3 in RGCs, both of which also lead to robust axonal growth (Smith et al. 2009). Other factors such as IGF-1 and Osteopontin have more restricted effects on only specific subtypes of RGCs (αRGCs) (Duan et al. 2015). For a detailed review of RGC axonal regeneration, the reader is referred to Wong & Benowitz (2022). Finally, the precise connectivity during development does involve activity-dependent refinement, which may help axons find the right targets so that the right brain areas receive information regarding color, motion, and illumination from the matching parts of the retina (D'Orazi et al. 2014). The above studies and future studies on this topic will be essential for progress toward realization of RGC replacement therapies for patients with end-stage glaucoma.

ENDOGENOUS REGENERATION AS AN ALTERNATIVE TO CELL REPLACEMENT

An alternative strategy for cell replacement is to activate latent endogenous regenerative pathways. These pathways are active within amphibians, fish, birds, and other vertebrate species: Upon retinal injury, various retinal support cells (primarily Müller glia or RPE) are reprogrammed toward a retinal progenitor-like state, proliferate, and then reconstitute any cellular deficiencies to varying degrees. Moreover, to an extent, they can regenerate a functional circuit with meaningful vision restoration: Fish treated with retinal injections of oubain (which leads to destruction of RGCs) regain certain visual behaviors, albeit with reduced amplitude on ERG testing (Sherpa et al. 2008). Restoration of proper circuity and visual function may also depend on the degree of injury: Work from Yoshimatsu et al. (2016) has shown that, following loss of photoreceptors, the cues directing the correct synaptic partners persist for a very short time, and loss of these cues for interneurons can drive the formation of abnormal circuitry. Nevertheless, the native regenerative responses of these species offer insight into gene regulatory programs that may be exploited for human contexts—single-cell transcriptomic and epigenomic analysis has revealed key regulators of the dedifferentiation process, including the transcription factors Ascl1 and Atoh7 (Todd & Reh 2022, Van Gelder et al. 2022).

One of the challenges in humans and other mammals is that, in injury settings, Müller glia upregulate inflammatory cytokines and profibrotic proteins that ultimately lead to chronic inflammation and scarring. Recent work has tried to ectopically express genes that are involved in the regenerative responses of Müller glia in other species. Work from Xiao et al. (2019) found that expressing Math7/Atoh7 together with Brn3b/Pou4f2 reprogrammed mouse Müller glia

into RGCs; these RGCs demonstrated extension into the optic nerves with synapsing at the lateral geniculate nucleus, superior colliculus, and other structures. Another group (Todd et al. 2022) demonstrated similar reprogramming in an injury model with Ascl1 expression, histone deacetylase inhibition, and the overexpression of RGC factors Pou4f2 and Islet1. Interestingly, such transdifferentiation potential is not limited to Müller glia. Indeed, amacrine cells can also be coaxed to produce RGCs in the setting of RGC factor overexpression (Atoh7, Brn3b, Sox4, Sox11, and Isl1; Wei et al. 2020).

CONCLUDING REMARKS

Cell replacement therapy represents a viable strategy for the treatment and potential cure of various retinal degenerative conditions. The hope is that, with the discovery of iPSCs and, more recently, the development of 3D organoid technology, we may be able to create an endless supply of replacement cells that more faithfully mimic our own retinal cellular constituents. These approaches are limited by several hurdles that must be addressed, including immunologic and delivery considerations. Moreover, how these new cells coexist within the recipient retinal microenvironment and whether donor cells can integrate within existing synaptic networks remain critical questions. As we develop a better understanding of retinal architecture and rewiring, especially in the context of disease, and as our technologies and model systems continue to improve, it is likely that we will be able to address these questions in the near future on the quest for better and more effective cell replacement approaches for the retina.

SUMMARY POINTS

1. Cell replacement therapies represent a viable solution for treating both inherited and age-related retinal degenerative diseases.
2. Retinal pigment epithelium transplants have been performed in humans and have been shown to be well-tolerated with variable effects on visual outcome.
3. Scaffolds represent a novel way to provide structural support and organization to transplanted donor cells.
4. Three-dimensional organoid technology has allowed for more faithful recapitulation of retinal organization and for improved photoreceptor maturation, as well as providing a platform to study development and disease and correct inherited disorders with gene-editing technology.
5. Immune modulators, including mesencephalic astrocyte-derived neurotrophic factor, have been shown to improve the receptivity of the recipient microenvironment, underlining the importance of modulating the local immune environment toward enhanced graft survivability and long-term function.

FUTURE ISSUES

1. While much progress has been made on generating various cell types needed for replacement therapies, there is a major lack of understanding of dynamic mechanisms associated with retinal tissue reorganization from early in disease states to end-stage disease to identify an appropriate opportunity window to deliver replacement cells.

2. A degenerative host environment is not very permissive to new cells. Thus, efforts to create a receptive microenvironment to improve engraftment and prevent immunologic rejection of transplants will be critical to the success of such therapies.
3. Cues involved in the axonal targeting from the eye to the central visual processing centers are present in early development, and the same is true for local retinal circuitry. It is not clear how well these cues are preserved in adults and especially in disease states. In the future, studies looking into adult axonal retargeting following damage or replacement of retinal ganglion cells and local synaptic targeting in case of photoreceptor replacement will provide necessary insight into expected visual outcomes.
4. A major subject of discussion in the field of cell replacement is the degree of passive transfer of material between the transplanted cells and host tissue. Studies focused on detailed characterization of transplanted cell ontology are required to separate the outcomes on visual recovery due to true newly integrated neurons from those occurring secondary to material transfer to recipient cells.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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