Retinal degenerative diseases (RDDs) affecting photoreceptors (PRs) are one of the most prevalent sources of incurable blindness worldwide. Due to a lack of endogenous repair mechanisms, functional cell replacement of PRs and/or retinal pigmented epithelium (RPE) cells are among the most anticipated approaches for restoring vision in advanced RDD. Human pluripotent stem cell (hPSC) technologies have accelerated development of outer retinal cell therapies as they provide a theoretically unlimited source of donor cells. Human PSC-RPE replacement therapies have progressed rapidly, with several completed and ongoing clinical trials. Although potentially more promising, hPSC-PR replacement therapies are still in their infancy. A first-in-human trial of hPSC-derived neuroretinal transplantation has recently begun, but a number of questions regarding survival, reproducibility, functional integration, and mechanism of action remain. The discovery of biomaterial transfer between donor and PR cells has highlighted the need for rigorous safety and efficacy studies of PR replacement. In this review, we briefly discuss the history of neuroretinal and PR cell transplantation to identify remaining challenges and outline a stepwise approach to address specific pieces of the outer retinal cell replacement puzzle.

Introduction

The retina is a complex tissue whose anatomy and circuitry (Fig. 1A) is predicated on the function of rod and cone photoreceptors (PRs), highly specialized neurons (Fig. 1B) that have evolved over millions of years to optimally harness light for navigating diverse environments. In healthy retinas, PRs are the initiators of visual activity; they are defined by their ability to capture light entering the eye and generate an electrical signal through a cascade of biochemical activity known as phototransduction. Sparking this signal is not solely enough to confer vision—PRs must also success-fully relay light sensory information via synapses with retinal interneurons to begin a stepwise process of conveying visual stimuli to the brain along retinal ganglion cell (RGC) axons. The biochemical processes within PRs require extensive metabolic activity, largely mediated by their interactions with the retinal pigment epithelium (RPE). Together with Müller glia (MG), the RPE plays a crucial role in supporting PRs to maintain outer retinal structure, function, and homeostasis.

Like all retinal cells, both PRs and RPE arise from a common retinal progenitor cell (RPC) (Fig. 2); intrinsic and extrinsic factors work in concert to guide cells through distinct developmental stages to reach functional maturity. In outer retinal degenerative diseases (RDDs) the interdependent nature of PRs and RPE becomes a weakness; primary dysfunction in either population often causes secondary damage in the other. Regardless of the inciting cause, PR damage instigates a predictable cascade of degenerative changes within the retina, progressing from widespread PR malfunction to cell death, retinal remodeling, and—in the absence of successful intervention—inner retinal degeneration.
Figure 1. Organization and circuitry of the retina. (A) The retina contains three layers of cell bodies: the outer nuclear layer (ONL), in which rod and cone cell bodies reside; the inner nuclear layer (INL), containing horizontal cell (HC), bipolar cell (BC), amacrine cell (AC) and Müller glial (MG) cell bodies; and the ganglion cell layer (GCL) where retinal ganglion cell (RGC) somata and displaced ACs are found. PRs are supported by close apposition to the retinal pigment epithelium (RPE). The neural retina is bound apically by the outer limiting membrane (OLM) and basally by the inner limiting membrane (ILM), both formed by end-feet of the MG. PRs connect with BCs and HCs via synapses in the outer plexiform layer (OPL). The inner plexiform layer (IPL) contains signal-carrying synapses between BCs, ACs, and RGCs. (B) Rod and cone PRs display several distinct morphologic features. The outer segment (OS) contains discs of photosensitive opsins for light detection. The connecting cilium facilitates trafficking between outer and inner segments (IS), the latter of which are rich in mitochondria. Extending from the cell body are axons with synaptic terminals, which interact with inner retinal neurons at triad ribbon synapses.

neurodegeneration.\textsuperscript{13} Like most neurons, human PRs are nonregenerative, and these destructive processes ultimately lead to irreversible vision loss. Retinitis pigmentosa (RP) and age-related macular degeneration (AMD) are the most common inherited and acquired outer RDDs, respectively, and collectively affect millions of individuals worldwide. Blinding outer RDDs affect an increasing proportion of the global population,\textsuperscript{14–16} and beyond being a source of visual morbidity, can cause severe emotional distress in some individuals.\textsuperscript{17,18} The estimated global economic impact of potential

Figure 2. Phases of RPE and PR differentiation. Pluripotent stem cells pass through an anterior neuroectodermal stage to become multipotent retinal progenitor cells (RPCs), which are capable of producing all types of neuroretinal cells in addition to RPE. Neuroretinal progenitor cells (NRPCs) become further fate restricted over time and have the capacity to generate all neural retinal cell types, including photoreceptor precursors (PRP). Over time, PRP and RPE mature to express several characteristic morphologic features. Examples of key transcription factors and defining cell markers for each stage are listed below each stage. Human PSC technologies follow these developmental pathways to reproducibly generate a variety of donor cells for replacement therapies.
productivity lost due to moderate and severe visual impairment is a staggering US $411 billion annually.19
In response, the National Eye Institute launched the Audacious Goals Initiative (AGI) in 2015 to accelerate development and deployment of ocular stem cell-based therapies for incurable RDDs.20,21 Specifically, the AGI aims to restore “usable vision in humans through the regeneration of neurons and neural connections” through endogenous or exogenous replacement.20

Gene therapies developed for individuals across the RDD spectrum have made remarkable progress in recent years22; however, with more than 200 distinct causative genes,16 curative interventions remain out of reach for most patients. Cell therapies—the delivery of live cells to treat or cure disease—have emerged as a promising alternative (or adjunct23) to gene therapy, offering a broad-spectrum and gene-independent strategy for restoring vision. There are currently no US Food and Drug Administration (FDA)-approved cell therapy applications for retinal disease, although several approaches have reached early phases of translational research (Box 1). Cell therapies come in many varieties, but are fundamentally characterized in the context of their source, their capacity to become other types of cells,24 and their purity (Table 1). Of the many donor cell sources proposed for use in RDDs, several autologous and allogeneic cell therapies have entered clinical trials.25,26 Human pluripotent stem cells (hPSCs) have proven to be an indispensable source of cells for such therapies as they can, theoretically, self-replicate indefinitely and form virtually any type of cell. Human PSC-based therapies advancing most rapidly toward clinical translation are aimed at supporting surviving host PRs, either through neuroprotective approaches (e.g. delivery of trophic factor-secreting stem/progenitor cells) or replacement of defective RPE.25,26 Exogenous replacement of PRs has progressed comparatively slowly, as it relies upon survival and integration of a sufficient number of these complex, nonreplicative, and highly specialized sensory neurons with establishment of functional synaptic connections to host interneurons.

Exogenous PR replacement currently appears best poised to reach the National Eye Institute’s (NEI’s) audacious goal first, although several key hurdles

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**Box 1. The Translational Research Continuum**

Translational research aims to maximize basic science discoveries for direct application in advancing human health (also referred to as “bench-to-bedside” research). The process of bringing a new discovery to clinical practice often takes decades, and retinal cell therapies are still in the early stages of this process. Translational research is typically classified in four phases—T1 through T4 (see Zarbin, 2020234 for further details):

- **T1** – scientific discovery and development from preclinical studies to phase I and II clinical trials
- **T2** – determination of efficacy in humans through phase III and IV clinical trials
- **T3** – dissemination and implementation of therapies beyond clinical trials
- **T4** – public health and policy-level assessment of established therapies

Each phase also represents a continuum of research activities. Retinal cell therapies—including RPE and PR replacement—are both currently in phase T1. RPE replacement is nearing phase T2 with several clinical trials underway, while PR replacement is largely still in preclinical development.

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**Table 1. Defining Characteristics of Donor Cell Populations**

| Source          | Autologous: patient-derived | Allogeneic: donor-derived (potentially HLA-matched and/or genetically engineered) |
|-----------------|-----------------------------|---------------------------------------------------------------------------------|
| Potential       | Pluripotent: capable of forming cells from all three germ layers (e.g. human ES or iP5 cell) | Multipotent: capable of forming a limited range of cell types from a common lineage (e.g. retinal progenitor cell) |
|                 | Unipotent: capable of forming one cell type or class (e.g. photoreceptor precursor) |
| Purity          | Heterogenous: the cell product consists of the target cell type intermixed with multiple off-target cell types | Enriched: the cell product is predominantly comprised of the target cell type |
|                 | Purified: the cell product is exclusively comprised of the target cell type |
remain as the field advances into clinical trials. With the initiation of a first-in-human safety study of hiPSC-derived neuroretinal sheets in advanced RP (RIKEN, JRCT ID jRCTa050200027),27 a critical appraisal of where the field currently stands with respect to PR replacement is warranted. This review summarizes relevant historic literature and highlights recent developments in exogenous hPSC-derived PR replacement therapies, identifying remaining challenges and emerging strategies. Therapies aimed at rescue of PRs have recently been reviewed elsewhere and, for the purposes of this review, are largely discussed in the context of principles relevant to PR replacement. Readers interested in PR rescue are directed to several excellent reviews of RPE replacement27,28 and other approaches including stem/progenitor trophic cell therapies.29 In addition, recent reviews of biomaterial transfer,30 outer retinal scaffolds,31,32 immune responses in subretinal cell therapies,33 PR replacement in rodents,34 and clinical trials of cell transplantation in RDDs25,26 may aid the reader in gaining a comprehensive understanding of the field.

The Rise of Photoreceptor Replacement Therapy

1950s to 1990s: Early Studies in Primary Cell Transplantation

The number of published studies aimed at retinal cell replacement has increased in recent years, but the field itself is far from new. Retinal regeneration stands on the shoulders of giants in many regards, building upon more than a century of research in stem cell biology and retinal development (Fig. 3). While intraocular delivery of retinal tissue began in the late 1950s,35,36 proof-of-principle for neuroretinal cell replacement (i.e. retina-into-retina transplantation) was established by a series of landmark studies by del Cerro, Turner, and Blair in the late 1980s.37–41 Turner and Blair were the first to transplant primary retinal tissue from neonatal rats into the subretinal space (SRS) of adult rats with outer retinal lesions,37 reporting survival and differentiation of grafts at 4 weeks post-transplant. These experiments documented some of the earliest evidence of “integration” between donor cells and host retinal tissue, a phenomenon that would eventually grow to become a source of significant debate in retinal regenerative therapies (see Supplementary Note S1).

Retinal developmental biology saw unprecedented progress throughout the 1980s and 1990s; the introduction of cell birth dating and advanced molecular biology techniques uncovered mechanisms of cell specification, organization, and neuronal maturation within the retina.6–8,42 As the developmental trajectory of PRs was defined (see Fig. 2), investigators continued to experiment with primary cell isolation to determine the optimal donor stage for cell replacement.43–45 Those studies led to the observation that donor cell suitability for transplantation decreased with age. That is, in both allografts (i.e. same-species transplants) and xenografts (i.e. cross-species trans-
plants), embryonic and early postnatal retinal grafts incorporated into lesioned retinas far more readily than their older counterparts.45,46 Given these findings, dissociated suspensions of multipotent neural47–52 or retinal progenitor cells53–56 were initially preferred by many. However, limited survival due to reflux and cell death (often less than 0.01% of the starting dose53,57) in addition to unpredictable differentiation led to low rates of PR engraftment, making translation to human therapies difficult.51

1990s to Early 2000s: Improving on Dissociated RPC Delivery

Two main approaches emerged to address the issue of poor engraftment. Studies building on the findings of del Cerro, Turner, and Blair suggested that human fetal RPC (fRPC) microaggregates (i.e. clusters of cells) and retinal sheets offered improved survival relative to dissociated cell transplants,58 likely due to enhanced structural support and maintenance of cell-cell contacts (reviewed by Seiler and Araman, 2012). Anoikis, the anchorage-dependent death of cells following loss of extracellular matrix (ECM) contacts, was thought to play a role in the poor survival of subretinally transplanted dissociated cells.59 Tissue-engineered scaffolds were introduced as a customizable approach for mimicking the native structure of retinal tissue to improve survival in RPC transplants.57,60–66 A variety of naturally occurring gelatinous matrices, hydrogels, and decellularized tissues were initially used; however, graft organization was limited and concerns regarding batch-to-batch variability restricted future clinical use.31,67–69 Among others, the Young laboratory developed criteria for an ideal neuroretinal scaffold: biodegradable and/or biocompatible, optically clear, porous, flexible yet strong, and thin enough for relatively easy subretinal delivery (<50 μm).57,60–66 Many synthetic biomaterials met these criteria, and a variety of polymers including poly(e-caprolactone) (PCL), poly(L-lactic acid) (PLLA), poly(lactic-co-glycolic acid) (PLGA), poly(methyl methacrylate) (PMMA), poly(dimethylsiloxane) (PDMS), and poly(glycerol sebacate) (PGS) were found to be well-tolerated in the SRS and supportive of improved RPC delivery in pigs and rodents.60,62–65,70,71 However, RPCs were not limited to producing PRs (see Fig. 2), and despite enhanced survival, the efficiency of PR engraftment following RPC scaffold delivery remained relatively low.57,64

The second approach—enrichment of committed PR precursors (PRPs)—was introduced by MacLaren et al. in 2006 with transplantation of green fluorescent protein (GFP)-labeled rod precursors (Nrl-gfp+/+) cells in mice.72 Characterized by a defined cell fate, PRPs offered substantial improvement in apparent PR engraftment in the retinas of wild type and rhodopsin-null mice. MacLaren and colleagues observed recovery of light sensitivity in rhodopsin-null mice, providing some of the earliest evidence of functional rescue following subretinal cell transplantation and sparking broad interest within scientific and lay communities alike. Although the study (and at least one subsequent report73) referenced fusion between donor and host cells as a potential alternative explanation for their results,72 it was not believed to occur to a significant degree in the retina at the time. Indeed, as a control, Nrl-gfp+/+ cells were transplanted into transgenic cyan fluorescent protein (CFP) reporter mice, and on the basis of qualitative data showing a lack of multinucleate or double-labeled GFP+/CFP+ cells, MacLaren et al. argued that cell fusion—at least in the classical sense—was unlikely.

Studies within the Ali, Wallace, and Ader laboratories (among others) replicated the findings of MacLaren et al. in the years that followed, primarily in rodent models with an intact or partially degenerated outer nuclear layer (ONL). Manipulation of the degenerative retinal environment by disrupting potential barriers to integration—including the outer limiting membrane (OLM),74,75 glial scarring,76,77 and chondroitin sulfate proteoglycan deposition76–80—was proposed to further enhance PRP incorporation. Fluorescence-activated cell sorting (FACS)72,81–83 and magnetic-activated cell sorting (MACS)84–86 were optimized to obtain relatively uniform batches of transplantable cells. The developmental window paradigm, which proposed that effective PRP cell replacement is best achieved through delivery of postmitotic cells at the precise peak of PRP genesis (E15 to P4 in mice), was introduced during this time.87 With mounting evidence of their success in cell replacement—including improvements in light-mediated activity76,82,88 even in advanced degeneration89—PRP became the preferred developmental stage for primary cell transplantation among most investigators.73,81–86,88–91 As in MacLaren et al.,72 integration was assumed to be the predominant mechanism in these studies; however, the MacLaren laboratory first raised concerns regarding PRs double-labeled with donor and host fluorescent reporters in 2014,92 suggesting that fusion between donor and host cells was indeed possible.

Limitations of Primary Cells

Both strategies—scaffolds and PRP enrichment—offered apparent improvements relative to dissociated RPC delivery, but primary cells presented major challenges to translation beyond animal studies. Phase
I and II clinical trials of human fetal-derived retinal cells,93 microaggregates,94 and retinal sheets with RPE95–97 in advanced RP and AMD began in the late 1990s, but ultimately reported mixed effects on visual outcomes in humans. A phase II study led by Radtke and colleagues reported transient visual acuity improvement in 7 of 10 subjects with long-term stabilization in a single subject,95 but the study did not distinguish trophic effects from functional integration, and interpretations of the underlying mechanism varied.26,58,98,99 These early studies (conducted without immunosuppression) provided evidence of safety for future cell therapies, demonstrating a clear path to clinical trials through careful preclinical study planning, but the primary cell approach to replacing PRs faced a difficult road to widespread application.

Procurement of human fetal retinal tissue for transplantation proved controversial from its introduction in the early 1980s,100 and debate around its use in biomedical research continues.101 The developmental window paradigm for PRP (E15-P4 in mice) coincided with the second trimester of pregnancy in humans, presenting an ethical minefield for translation to clinical use. Attempts to expand102,103 and immortalize104 human fetal retinal cells were largely unable to circumvent the issue as RPCs were by definition not restricted to the PR lineage (see Fig. 2) and generated few PRs. In most cases, human fRPCs lost neurogenic potential over time in culture105,106 and demonstrated poor long-term survival following transplantation.104 Improvement under low-oxygen culture conditions was reported in some cases,53,107–110 eventually resulting in the recent initiation of a phase I/IIa clinical trial of subretinal fRPC delivery in late-stage RP (ReNeuron, clinicaltrials.gov identifier NCT02464436). Although this trial is expected to yield valuable safety and efficacy data,26 results have not yet been published, and difficulty in distinguishing trophic support from functional PR replacement remains.26 Ethical constraints and ambiguous mechanisms aside, primary cells and their derivatives continued to present a yield dilemma: with millions of potential patients,14,16 reproducible manufacturing was expected to be a bottleneck for larger phase III clinical trials and beyond.26,58

Early 2000s to Late 2010s: Expanding Potential With Human Pluripotent Stem Cells

The isolation of human embryonic stem cells (hESCs) in 199811 ushered in a new era for retinal cell replacement. The first completely in vitro differentiation of RPE was achieved in relatively short order,112 but PR differentiation proved more challeng-
by Waldron et al., nearly all GFP+ cells (99%) found in wildtype host retinas and most GFP+ cells (>75%) in degenerative Nrl−/− and Prph2nd2/nd2 retinas were estimated to result from material transfer,157 calling the results of several previous rodent studies into question.72,76,82,88,145,158 Although the exact mechanism and longevity of this phenomenon remains to be determined, at present, several points are clear. First, material transfer is more likely to occur in degenerating retinas with remaining host PRs152–156 than in models of end-stage retinal degeneration.89,141,146,147,149,159–163 Second, a variety of PR-specific proteins (cone arrestin, opsins, and peripherin-2 [PRPH2]; see Fig. 2) as well as cytoplasmic reporters can be passed via material transfer in mice.34 Leading to a re-evaluation of how the field identifies and defines integration (see Supplementary Note S1 for further discussion). Third, PSC-derived PRP do not appear to be exempt from this phenomenon34,137,140,157; however, at least one study has suggested that the capacity for material transfer is lower in human-into-rodent xenografts than in allogeneic transplantation.144 Finally, many of the central tenets of successful cell replacement—including the developmental window paradigm, estimated donor cell survival rates, evidence of a dose response, integration, and synaptogenesis—require re-examination in the context of material transfer.

Lessons Learned From Historic Studies of PR Replacement

Collectively, early studies in retinal cell replacement identified several guiding principles for carrying PR cell therapies closer to the clinic. Multiple strategies, including the use of enriched donor cell populations and biomaterial-based scaffolds, have been shown to enhance cell survival in the face of low PR engraftment.34,70,72 The PRP stage of differentiation remains preferred for replacing PRs, although the window of transplant competence is likely not so narrow as previously estimated.34 Three-dimensional retinal organoids are the most often used source of authentic donor cells and tissue sheets, and have in some cases been associated with modest improvements in host retinal light sensitivity following transplantation.26,34 Finally, rodent PSC allografts and human-into-rodent PSC xenografts have established proof-of-concept for PR survival and anatomic engraftment following transplantation. Just as important, these studies have also identified remaining hurdles for the field to overcome. Surviving donor cells often remain disorganized within the subretinal space, and the mechanisms by which they affect host vision remain unclear. With many prior studies now known to result from material transfer rather than functional integration, there is substantial interest in the development and use of quantitative methods for assessing integration, organization, and synaptogenesis.

Current Status and Remaining Questions for Retinal Cell Therapies

As outlined above, cell replacement therapies in the retina have been studied for decades (see Fig. 3), and hPSC-RPE and hPSC-PRP cell products are now in the T1 translational research phase (which spans preclinical studies through phase II clinical trials). Thus far, the majority of clinical trials have used hESC- or hiPSC-RPE, inserted into the SRS either as dissociated cell suspensions or as monolayer sheets with or without scaffolds (see Uyama et al., 2021 for further discussion98). Early reports suggest that these therapies are feasible, safe, and well-tolerated in individuals with advanced retinal degenerative disease.26,98 However, functional outer retinal cell replacement—and more particularly PR replacement—remains a complex puzzle of cell manufacturing and preclinical testing challenges, some of which may not be fully surmountable ahead of human trials (Fig. 4). Even so, efforts to address each piece of this puzzle in a deliberate, stepwise manner would help build confidence in the potential for success. This section discusses these pieces in detail, comparing and contrasting major strategies and identifying areas where additional research is necessary to advance outer retinal cell therapeutics.

Clinical-Grade Production

Clinical hPSC-derived cell therapies must be sterile and free from infectious agents, impurities, residual pluripotent cells, unidentified cell types, and genomic instabilities.28 Such criteria must be met under strict Good Manufacturing Practice (GMP)-compliant conditions28,164–166 and also be scalable far beyond the capacity of an average laboratory setting to be feasible for clinical trials and commercialization. Although detailed discussions of stem cell source (ES or iPSC) and culture technique are beyond the scope of this review, proper induction and/or maintenance of PSCs is fundamental to any successful retinal differentiation program. Advantages and disadvantages of autologous and allogeneic cell replacement should be weighed early in product development to avoid the need for correction mid-program. Preclinical
Figure 4. The complex puzzle of therapeutic development for outer retinal cell therapies. As cell therapies transition from phase T1 to phase T2 studies and beyond, several interconnected factors related to donor cell manufacturing and preclinical testing must be addressed.

Safety studies for autologous products emphasize the manufacturing process for producing each cell line in addition to the final clinical product, whereas safety studies for allogeneic products focus on the latter. Thus, programs are effectively "locked in" very early to an autologous or allogeneic approach. Autologous therapies are subject to fewer infectious disease testing requirements and are theoretically less likely to result in immune rejection, although cost (an estimated $800,000/cell line for clinical-grade iPSC production) and reproducibility across patient-specific iPSC lines are often limiting factors. "Off-the-shelf" human leukocyte antigen (HLA)-matched or HLA knockout allogeneic products offer a scalable, more cost-effective production pipeline; however, immune rejection and long-term safety become a greater concern, and many dozens of HLA "super donor" cell lines would still be needed depending on the genetic diversity of the target population.

Lessons learned from existing Investigational New Drug (IND)-enabling studies, particularly those from investigators with experience in cell therapies and Biologics License Applications with the FDA, should be considered early in product development to mitigate additional “known unknown” risks for scaling regenerative therapies (for further discussion of quality control for clinical-grade hPSC retinal cell production, see Wright et al., 2014 and Sharma et al., 2020). Current methods for generating hPSC retinal organoids are both time and labor-intensive, limiting their utility in clinical production pipelines. The use of bioreactors, microfluidics and automated culture systems are all promising approaches currently under investigation for scaling clinical-grade organoid-based technologies.

Donor Cell Enrichment

Robust methods for purifying or enriching PRP from stem cell-derived retinal organoids represent a critical bottleneck in the regenerative medicine pipeline for PR degenerative diseases. MACS and FACS-based enrichment strategies originally developed in mouse models have not yet translated into consistent success for enrichment of hPSC-PRP for transplantation, possibly due to species- or maturation stage-specific differences in PR cell surface markers. Although some groups have reported successful development of hPSC-PRP enrichment protocols, most have not been widely adopted outside individual laboratories, possibly due to low yield (<1 million cells) and/or suboptimal purity (40–70%) across various differentiation protocols. A fully homogeneous cell product is not necessarily a prerequisite for clinical trial initiation because the FDA allows study sponsors to set their own release criteria for product purity, but a highly enriched PRP product (>80%) would be desirable. Some groups have proceeded with unsorted cell populations or retinal sheets in the absence of reproducible sorting methods, but residual proliferating cells (e.g. immature RPE, RPCs, etc.) often remain. Unsorted populations thus contain cells that may continue to divide, leading to PRs being outnumbered by off-target cell types or to the development of disorganized, rosetted grafts following tissue sheet transplants.
Human PSC reporter lines\textsuperscript{135,177,180–183} and viral labeling constructs\textsuperscript{144} have been generated as an alternative approach to enable rapid PRP enrichment via FACS. Although precedent exists for FDA allowance of biologics expressing fluorescent proteins in clinical trials\textsuperscript{184} (GenSight Biologics’ optogenetic GS030 [clinicaltrials.gov identifier NCT03326336] encodes a tdTomato-linked fusion protein), nontherapeutic inclusions add safety and regulatory hurdles to an already complex approval process. Sorting via cell surface markers\textsuperscript{162,176} (e.g. MACS) or label-free microfluidics\textsuperscript{175} offers a more favorable approach to obtaining enriched PRP for cell replacement. Given the current lack of consensus on optimal PRP sorting strategies, particularly for cones,\textsuperscript{34} further investigations of human PRP-specific cell surface markers and enrichment approaches are warranted.

**Functional Validation of Donor Cells**

The advancement of hPSC-RPE therapies has been accelerated in part by the relative ease with which RPE donor cell identity and function can be validated.\textsuperscript{28} A battery of biomarkers and assays, including cobblestone morphology, pigmentation, transepithelial resistance (TER), photoreceptor outer segment (POS) phagocytosis, electrophysiology, apical-basal polarization, tight junction marker expression, and microvilli formation, are all indicative of healthy, maturing hPSC-RPE.\textsuperscript{28,31,98,185,186} Standardized methods for PRP validation have proven less straightforward, partly due to cell heterogeneity (rods; short-, medium-, and long-wavelength sensitive cones) and complexity, and partly due to the range in donor cell maturation used across studies. Neuronal age is a simple and widely used metric for estimating maturity in hPSC culture systems,\textsuperscript{187} and functional maturation is associated with age in hPSC-derived retinal neurons\textsuperscript{114,117,122} regardless of the differentiation protocol used. However, the rate of maturation is often asynchronous across RO differentiation protocols, cell lines, and even differentiation batches.\textsuperscript{129} Age can serve as a rough surrogate marker of maturation, but this metric provides a somewhat false sense of assurance for consistency between lines or differentiations.\textsuperscript{129,188} A recent study by Capowski et al. demonstrated the utility of morphology for assessing maturation, introducing a light microscopic staging system for classifying ROs.\textsuperscript{129} Stage 1 ROs contain RPCs, early-born inner retinal neurons, and an outer neuroblastic layer, whereas stage 2 ROs are characterized by differentiation of an abundance of PRs and inner retinal neurons. The hallmarks of stage 3 ROs are the development of PR outer segments and increased outer neuroretinal organization along with production and maturation of Müller glia and ongoing deterioration of the innermost retinal layers.\textsuperscript{129}

Because the characteristic light-sensitive component of PRs, outer segments, often appear months past the peak of PRP genesis in ROs (stage 2, approximately day 80–120 of differentiation), development of in vitro potency assays for validating hPSC-PRP remains a high priority. One intriguing approach to this conundrum is the use of optogenetically engineered hiPSC-PRP, which have recently been shown to generate modest responses to bright light in vitro and in vivo.\textsuperscript{163,189} However, such a genetic modification presumes that hPSC-derived PRs cannot innately respond to light and also introduces aforementioned regulatory hurdles. In the absence of genetic modification of hPSC-PRP, other potency assays may prove useful for authenticating batches of transplantable hPSC-PRP, including examinations of cell polarity, synaptic marker expression,\textsuperscript{190} PR marker expression, axon outgrowth, and membrane electrophysiology.\textsuperscript{135}

**Cell Preparation, Delivery, and Organization**

A number of recent xenograft studies have demonstrated proof-of-concept for PR survival and anatomic engraftment (see Supplementary Note S1) following transplantation of dissociated hPSC-derived cell suspensions or retinal sheets in rodents\textsuperscript{146,149,161–163,179,189} and non-human primates (NHPs),\textsuperscript{147,160} using controls for biomaterial transfer. Dissociated cell injections have the advantage of being relatively simple, cost-effective, rapid, and minimally invasive, although graft organization and cell survival is often suboptimal.\textsuperscript{34} Retinal sheet delivery can afford a striking degree of self-organization relative to dissociated cells.\textsuperscript{34,98,146,147,149,179} but the surgical technique requires specialized instrumentation and is more complex and invasive than simple subretinal injections. Furthermore, rosetted PRs, formation of ectopic inner retinal laminae, and lack of apposition to host RPE remain limitations to this approach.\textsuperscript{26}

PRs and RPE are both highly specialized cells for which apical-basal polarity plays a crucial role in function; there is thus substantial interest in cell delivery strategies supportive of donor cell organization. Polymeric retinal patches or scaffolds are among the most promising solutions for improving cell retention and 3D distribution\textsuperscript{191} and maintaining cell orientation.\textsuperscript{192–194} Added benefits also include a defined dose, targeted delivery to a discrete region, and potential for customization of scaffold size, shape, and material. Scaffold-based hPSC-RPE delivery has thus
far been well-tolerated in clinical trials, and despite more complex surgical procedures, there is evidence to suggest that scaffold delivery may be achievable in an outpatient setting. Neuroretinal scaffold approaches are still in their infancy but appear to be similarly advantageous for PRP replacement. Current hPSC approaches include two-photon polymerized PCL scaffolds seeded with clinical-grade RPCs and micromolded PGS scaffolds seeded with hPSC-PRPs. Both scaffolds are sterilizable, biodegradable, and have a desirable elastic and/or compressive modulus, which play a critical role in ease of surgical handling. Extensive in vivo safety testing has been performed for the former, although the use of RPCs rather than PRPs was a limiting factor in determining capacity for PR delivery. Micromolded scaffolds are capable of pre-organizing polarized PRPs even at high cell densities, although it remains to be seen whether such organization can be retained in vivo. Optimization of scaffold delivery requires the use of clinically relevant large animal models to simulate targeted subretinal scaffold implantation in the human macula. In addition to delivery of PRP-only scaffolds, envisaged applications include co-delivery of hPSC-RPE and PRPs, as replacement of both cell types will likely be necessary for individuals suffering from late-stage AMD or inherited maculopathies, such as Stargardt and Best disease.

Assessment of Donor Cell Survival

Dissociated cell survival in RPC or allogeneic PSC-PRP transplants in rodents is extremely low (1–4%), and because these studies predate the discovery of material transfer, may be overestimated. Given the widespread use of percentages rather than discrete cell counts in published datasets, it is often difficult to obtain a true approximation of cell survival relative to the starting dose. Standardized methods for counting cells or regions of interest, like the QUANTOS workflow developed for synapse identification, will be critical for rigorously studying such outcomes. Where possible (and with appropriate controls), unbiased stereology and automated image analysis can also provide a less subjective approach to histologic analyses. Several studies have highlighted the importance of standardized cell quantification in biological research to increase reproducibility and aid comparisons between studies or across research groups. Greater adoption of such methods for assessing donor cell survival in PR replacement would serve the field well.

Functional Integration and Synaptogenesis

The presence of new synaptic connections following transplantation is often inferred by pre- and postsynaptic protein co-immunolabeling or electron microscopic evidence of synaptic ribbons near donor cells. However, immunocytochemical evidence of synaptic marker expression does not establish a definitive causal link to observed changes in retinal function or visual behavior. Further evidence in favor of functional synaptogenesis includes electrophysiologic, reflexive, and behavioral assessments of light responsivity, although most of these readouts measure processes several synapses downstream from presumptive donor-host contacts. High levels of donor cell disorganization and relatively mild degrees of light-induced response recovery observed in most hPSC-PRP transplants also suggest that synapse formation likely occurs at lower rates than previously predicted. It is currently difficult to fully distinguish bona fide synaptic connections from existing ones—however rare they may be—in the absence of direct and effective methods for studying synaptic contacts of donor cells. A recent study by Cowan et al. suggests that PRP are capable of forming functional synapses within retinal organoids as evidenced by calcium imaging. However, no study to date has definitively shown that hPSC-PRPs can form new functional synapses after being isolated from retinal organoids. Evidence of functional post-transplant synaptogenesis currently includes modest light responses recorded with multielectrode array (MEA) and micro-electroretinography (mERG), and often does not conclusively distinguish light-induced donor cell responses from possible neuroprotective effects on residual host retinal circuitry. Reproducible, well-controlled approaches for assessing de novo synaptogenesis at the level of individual donor hPSC-PRPs (via calcium imaging or viral monosynaptic circuit tracing), particularly in the context of xenogeneic transplantation, will be necessary to further clarify mechanisms of functional recovery. The efficiency of synaptogenesis in xenografts is currently unknown, however, by increasing PRP survival, alignment, and organization, it may be possible to increase the likelihood of synapse formation between donor and host cells. Strategies to directly measure hPSC-PRP synaptic contacts via trans-synaptic tracing or patch-clamp recordings have been highlighted as crucial, but have not yet come to fruition.
Animal Model Selection for Safety and Efficacy Studies

Rodents have historically been the preferred model system for retinal cell replacement studies due to cost, ease of genetic manipulation, and widespread availability. Several reports have shown that transplanted hPSC-PRP can survive and be associated with varying degrees of light-evoked behavior and/or electrophysiologic activity in degenerating rodent retinas, but there is not yet direct evidence of causation. The well-documented neuroprotection caused by virtually any subretinally transplanted material (including control vehicles) in the Royal College of Surgeons (RCS) rat makes it highly difficult to fully control for alternate mechanisms in this model. To address the confounding variables of neuroprotection and biomaterial transfer, many investigators have instead opted for models with near-complete PR loss. Models with severe PR loss are currently considered most appropriate for studying functional integration, although even these models are not free of confounding variables, because residual cones remain in severely atrophic models like the rd1 mouse and S334ter-3 rat.

To date, most available data regarding cell survival and effects on vision are skewed toward rod-dominant rodent models, although there is some evidence to suggest similar responses are possible in NHPs. Given notable species-specific differences in PR development and synaptic architecture, the degree to which these observations will directly translate to human allogeneic or autologous transplants remains to be seen. The introduction of scaffolds and more complex tissue constructs, which necessarily includes more complicated surgeries, will require a shift toward larger animal models with ocular anatomy more akin to that of humans to provide meaningful assessments of such approaches. Development of translation-enabling models that faithfully recapitulate aspects of human RDDs is an explicit aim of the NEI AGI, and these models will be a valuable resource for advancing retinal cell therapies. There is substantial interest in allogeneic transplantation of same-species PSC-derived retinal cells in parallel with xenogeneic studies, as this approach can potentially provide extrapolatable insight into the degree of functional restoration that may be reasonably expected in human clinical trials. Continued observance of field standards for defining integration (see Supplementary Note S1 for further discussion) and development of protocols to generate retinal organoids from additional laboratory model species will be essential to such activities.

Noninvasive Imaging to Assess Therapeutic Efficacy

Although a variety of reflexive and behavioral assays are available for assessing visual function, even electrophysiologic methods ultimately may not be sensitive enough to directly assay PR transplant-driven responses. Several such studies have documented light-evoked electrophysiologic responses and visual behavior in degenerating retinas even when surviving donor PRs are nearly absent. Adequately powered studies controlling for alternate explanations of restored function—including material transfer to host interneurons, aberrant firing of intrinsically photosensitive RGCs (ipRGCs), and neuroprotection of remaining host retinal cells—will be challenging, but necessary, for definitively establishing a causal link between anatomic integration and vision rescue.

There is no single animal model that is perfect for each cell replacement application. Rather, a variety of factors, including ocular anatomy, nocturnal versus diurnal activity (i.e. rod versus cone-dominance), and genetic causation should be taken into consideration when designing preclinical IND-enabling studies for cell therapies (summarized in Table 2; also see Winkler et al. 2020 for a discussion of RDD animal models). A recent study by Laver and Matsubara also suggests that the lack of robust responses observed in human-to-rat xenografts may be due in part to synapse incompatibilities between donor PRP and host retinal interneurons. The degree of divergence in synaptic proteins between humans and non-human model organisms is just one of many factors to consider when selecting preclinical models for testing functional effects of hPSC-PRP therapeutics.

Noninvasive Imaging to Assess Therapeutic Efficacy

The retina is a highly organized, laminated structure that has evolved to maximally harness light entering the eye. Recent advances in noninvasive retinal imaging have capitalized on these features to provide increasingly detailed pictures of in vivo retinal architecture. Both the NEI AGI and the Monaciano Consortium have highlighted a relative lack of rigorous, reproducible ocular imaging as a potential bottleneck in advancing clinical trials. Several recent studies have demonstrated the utility of noninvasive imaging for comprehensively studying integration and therapeutic efficacy in hPSC-PRP cell therapies. The Singh laboratory at Johns Hopkins identified quantifiable biomarkers for tracking fluorescent mouse cells after transplantation, developing a scoring system for multimodal confocal scanning laser ophthalmoscopy (cSLO) imaging. Several
### Table 2. Animal RDD Models and Factors Affecting Suitability for Preclinical Retinal Cell Replacement

| Species         | Ocular Anatomy: Similarity to Human | Features                                      | Predicted TRS Compatibility with Human | Options for Immune Suppression | Selected RDD Models |
|-----------------|-------------------------------------|-----------------------------------------------|----------------------------------------|-------------------------------|---------------------|
| Mouse           | +                                   | - Small globe with large lens - Rod-dominant retina | 89%                                   | Genetically modified Pharmacologic | Rd1, Rd10, many others |
| Rat             | +                                   | - Small globe with large lens - Rod-dominant retina | 88%                                   | Genetically modified Pharmacologic | RCS, S334ter, P23H   |
| Ground squirrel | ++                                  | - Small globe with small lens - Cone-dominant retina | 44%                                   | Pharmacologic                | Retinal detachment   |
| Rabbit          | ++                                  | - Medium-sized globe with small lens - Visual streak | 86%                                   | Pharmacologic                | RHO (P347L)          |
| Cat             | +++                                 | - Medium-sized globe with small lens - Area centralis | 92%                                   | Pharmacologic                | RDHS, CEP290, AIPL1  |
| Dog             | +++                                 | - Moderately large globe with small lens - Area centralis | 81%                                   | Pharmacologic                | RHO, RPE65, PDE6A, PDE6B, SAG, ABCA4 |
| Pig             | ++++                                | - Large globe with small lens - Visual streak | 85%                                   | Pharmacologic | Genetic models Surgical-induced | Laser damage         |
| Macaque         | ++++++                               | - Large globe with small lens - Macula | 98%                                   | Pharmacologic | PDE6C, BBS7             | Laser damage         |

*a A summary of findings from: Stanzel et al., 2019 (ocular anatomy, RDD models), Laver and Matsubara, 2017 (xenograft compatibility), and Winkler et al., 2020 (RDD models).

*b Photoreceptor triad ribbon synapse.

*c Based on the Pikachurin sequence similarity (percentage) between humans and the listed species.

*d Laver and Matsubara broadly refer to squirrels; this may not directly reflect TRS compatibility of specific models (e.g., 13-lined ground squirrels).

Properties, including fluorescence size and intensity, graft placement, lamination, and peri-retinal proliferation, were scored longitudinally, facilitating long-term tracking of individual grafts over time. Similarly, Abualizadeh et al. recently used fluorescence adaptive optics scanning light ophthalmoscopy (FAOSLO) to follow individual PRs in vivo in a laser-damage NHP model of PR loss.160 These types of correlative studies augment histologic assessment of efficacy, although further research is necessary to determine how to translate these imaging techniques to clinical trials and commercial products and how to distinguish donor cells from host biomaterial transfer in vivo. As mentioned earlier, fluorescent reporters are not necessarily prohibited in clinical trials, but development of high-resolution, label-free, noninvasive methods for tracking migration and integration of donor cells is preferable.

### Modulating Retinal Microenvironment and Immune Response

The ideal cell replacement toolbox will likely include approaches for priming the degenerative host retina for enhanced integration. Although there is evidence of some efficacy following hPSC-PRP
delivery even in end-stage retinal degeneration—suggesting that host inner retinal circuitry remains viable for a time—the exact window of opportunity for effective cell replacement is currently unknown.34,219 Treatments being studied seek to modulate a variety of naturally occurring processes that may act as barriers to donor PR integration in the degenerate outer retina, including glial scarring,220 interneuron plasticity,21 and neurite outgrowth,222 which may in turn help create a more donor cell–receptive environment. Basic discovery research to better understand the molecular mechanisms involved in retinal circuit assembly, disassembly, and re-assembly will also be essential to address host-centered barriers to neuronal replacement.20,223 While the eye is historically considered to be immune-privileged, current evidence suggests that this privilege is relative rather than absolute, and is perhaps lost in the course of disease.33 Preclinical xenografts require immunodeficient hosts,146,150,224 or immunosuppressive regimens,147,160,225 but allograft studies and clinical trials to date report conflicting evidence regarding the degree of immune suppression necessary for long-term donor cell survival. Recent studies suggest that the immunogenicity of hPSC-derived retinal tissues may actually be relatively low, and hPSC-derived retinal cells might even confer a degree of local immune suppression.226 As methods for assessing graft survival improve, further research regarding the role of the immune system in xenografts, allografts, and autografts will be necessary to predict best practices. Reports from hPSC-RPE clinical trials, which use a variety of immune suppressive regimens, will be highly informative for designing future clinical trials aimed at outer retinal cell replacement.26,33

Conclusions: A Shared Responsibility

Exogenous RPE and PR transplantation efforts—bolstered by decades of research in regenerative medicine and retina developmental biology—have overcome significant hurdles in recent years and are now being tested in clinical trials. Although hPSC-RPE therapies are further along, remaining challenges to clinical translation for hPSC-PRP include scaling clinical-grade cell production, creating organized grafts, addressing synapse formation and functional integration, and optimizing safety and efficacy outcomes in relevant model systems. Singh et al. recently observed that, as these challenges are met and retinal cell therapies reach early phase clinical trials, peer-reviewed interim reports may have unintended ripple effects on patients and lay audiences.26 Eye-catching headlines rarely reflect the nuance of underlying research findings and further fuel unrestrained public desire for stem cell–based therapies. Preclinical research can often inadvertently elicit similar responses when reports of vision restoration in animal models are picked up by the media. The current landscape of milestone-oriented funding and open-source science necessitates timely publication of results, but Singh et al. stress the importance of appropriately powered, long-term follow-up to mitigate “scientifically unfounded over-optimism” within the non-scientific community. Recent case reports have underscored the grave impacts227–229 of clinics prematurely capitalizing on this enthusiasm and preying on patient hope230 with unregulated stem cell treatments.

A recent Lancet commission on regenerative medicine argues that the shift from “small-scale bespoke experimental interventions” to bona fide clinical application of hPSC-based therapies will require “substantial rethinking of the social contract that supports such research and clinical practice in the public arena.” The commission contends that improving four areas—science, funding models, governance, and public/patient engagement—can prevent erosion of public trust and bridge the gap between patient expectations and currently available therapies.231 While it is clear that tremendous scientific progress has been made toward outer retinal cell replacement, transitioning from bench to bedside will require substantial engagement from a variety of stakeholders regarding economic burden,21 international governance,27 and public/patient interaction.26

The challenges that lie ahead for outer retinal cell therapies can be overcome, and the recent advances highlighted in this review suggest that the future for retinal regenerative medicine is bright. However, translation to clinical application will require considerable investment of time and scientific effort from public and private entities alike. Moreover, the necessary focus on safety in early phase research means that efficacy in human subjects, who will necessarily be at the severe end of the disease spectrum, will likely be modest at first. In short, the reality we collectively face is that translating cell therapies to effective clinical practice will take time, and for families currently battling vision loss, it will rarely feel like progress comes fast enough. In the interim, scientists and clinicians will continue to play a crucial role in right-sizing public expectations and encouraging patients to make informed decisions regarding stem cell treatments. To this end, several organizations have developed educational materials geared toward a lay audience that are freely available to share with individuals considering stem cell therapies. Materials from the International Society for Stem Cell Research, including the Patient Handbook on Stem Cell...
Therapies232 (available in 12 languages) and disease-specific fact sheets,233 as well as the McPherson Eye Research Institute’s similarly themed “10 Things to Know Before You Fall Victim to a Retinal Stem Cell Scam” (see Supplementary Note S2) can help patients navigate stem cell claims while researchers around the world continue to work toward solving the complex puzzle of outer retinal cell replacement.

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