Induced Pluripotent Stem Cell Therapies for Degenerative Disease of the Outer Retina: Disease Modeling and Cell Replacement

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Abstract

Stem cell therapies are being explored as potential treatments for retinal disease. How to replace neurons in a degenerated retina presents a continued challenge for the regenerative medicine field that, if achieved, could restore sight. The major issues are: (i) the source and availability of donor cells for transplantation; (ii) the differentiation of stem cells into the required retinal cells; and (iii) the delivery, integration, functionality, and survival of new cells in the host neural network. This review considers the use of induced pluripotent stem cells (iPSC), currently under intense investigation, as a platform for cell transplantation therapy. Moreover, patient-specific iPSC are being developed for autologous cell transplantation and as a tool for modeling specific retinal diseases, testing gene therapies, and drug screening.

Introduction

INHERITED RETINAL DISEASES and age-related macular degeneration (AMD) leading to the loss of photoreceptor cells are a major cause of irreversible visual impairment worldwide. Unlike fish and amphibians,¹ mammals do not possess the ability to regenerate the damaged retina. Clinical treatments are needed to prevent the retinal degeneration that leads to blindness and to restore sight once the retina is damaged.

Retinal Degenerative Disease

The retina is a 0.5 mm thick neural sheet lining the posterior inner surface of the eye. It is organized in 3 layers of cell bodies, separated by 2 synaptic layers. Light stimuli are captured by the outer segments of the photoreceptor cells in the outer nuclear layer (ONL) and then converted to electrical impulses by a well characterized G-protein-coupled receptor signaling pathway (phototransduction), involving specific receptors (rhodopsin and cones opsins) and G proteins.² Rod photoreceptors can detect single photons of light and are important for dim light vision, while cone photoreceptors, concentrated in the central retina, are important for color vision and visual acuity. Horizontal, bipolar, and amacrine interneurons of the inner nuclear layer process signals from the photoreceptors, before transmitting them

via the retinal ganglion cells to the visual processing center in the brain, where sensory information is interpreted as vision.² Underlying the ONL there is a pigmented polarized monolayer of epithelial cells, the retinal pigmented epithelium (RPE), which performs a number of functions that are vital for the survival and function of the photoreceptor cells. RPE cells phagocytose photoreceptor cell outer segments, which are constantly renewed, and recycle the rhodopsin chromophore 11-*cis* retinal after absorption of each photon. RPE cells also form the blood barrier and transport metabolites between the retina and the blood supply of the underlying choriocapillaries.²

Retinal degenerative diseases causing outer retina pathology are a major cause of blindness and the most common neural degenerative disease.^{3,4} These diseases either exhibit Mendelian patterns of inheritance or, in the case of AMD, genetic factors, predispose to disease. The various inherited forms show different clinical presentation and age of onset, from birth, such as in Leber congenital amaurosis, or with juvenile or adult onset, such as in retinitis pigmentosa (RP), which may also occur in association with other nonocular conditions, such as the Usher syndrome. Photoreceptor cell degeneration can be primary, or in some cases a consequence of RPE dysfunction and cell loss. Either way, photoreceptor loss leads to progressive visual impairment; the rods, cones, or both can be affected first, with cone degeneration having the greatest impact on vision. Mutations

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in more than 200 different genes have been linked to inherited forms of retinal diseases.⁵ Even when the same gene is affected, the clinical features may differ. Many disease-causing mutations in different genes have been characterized, yet the genetic mechanisms that ultimately lead to photoreceptor cell death are not well understood. Many of the disease genes encode proteins acting within visual processes, such as phototransduction, retinol metabolism, or outer segment assembly and shedding, but others have more obscure roles.

Currently available treatments aim to slow down the disease progression, although they generally fail to arrest cell loss completely. A number of innovative treatments are being investigated to restore sight after the loss of photoreceptor cells; these include optogenetic approaches, endogenous retinal regeneration, neuroprotection, gene therapy,⁶ implanted visual prostheses, and cell transplantation.⁷ Neuroprotective strategies,⁸ targeted gene therapy,^{9,10} and visual prostheses are already in clinical trials.⁷ Nevertheless, it is currently not possible to repair the retina once photoreceptor cell loss has occurred. Over the last decade, human pluripotent stem cells have gained attention as future treatment options for currently untreatable and irreversible retinal diseases. The first embryonic stem cell (ESC) lines were derived from human blastocysts in 1998.¹¹ Subsequently, methods were discovered that derive human pluripotent stem cells (induced pluripotent stem cells; iPSC) not from an embryo, but from differentiated somatic cells instead. Pluripotent stem cells have wide ranging applications, since they are able to self-renew and give rise to all the body's cell lineages. Pluripotent stem cell-derived cells can be used to provide human cells in a number of key areas: (i) for cell replacement in the case of a specific cell type being destroyed by disease; (ii) to identify pathological molecular pathways for the targeted development of new drugs; and (iii) to test the effect of therapeutic drugs or viral vectors. In particular, iPSC technology provides a platform to model human diseases and the potential to develop patient-specific cell therapy.¹² This review will focus on evaluating cell replacement therapies and the use of iPSC lines to advance new treatments for outer retinal diseases. The retina holds a considerable advantage as a target for cell transplantation therapy because it is easily accessible compared to other parts of the central nervous system and it is immune privileged as the RPE forms a barrier for the vasculature. In addition, the retina is relatively small in size and therefore requires a small quantity of cells for transplantation. As it is isolated from other body tissues, there is a reduced risk of systemic effects and removal is possible, if necessary in the case of adverse outcomes. Moreover, there are highly developed noninvasive imaging techniques for monitoring progression of the therapy.

Generation of iPSC

Mammalian embryogenesis follows specific developmental stages that are dictated mainly by epigenetic modifications of the zygotic DNA. These changes result in differentiation of cells, once considered an irreversible process. In 1997, the birth of the sheep Dolly at the Roslin Institute demonstrated for the first time that a new organism can develop from the DNA of a differentiated somatic cell after nuclear transfer into an oocyte,¹³ changing forever the

way we look at differentiated cells. After this important discovery, attention focused on the discovery of "reprogramming factors" to "dedifferentiate" somatic cells. Yamanaka and colleagues first successfully generated iPSC from mouse and human fibroblasts using retroviral transduction with 4 transcription factors (Oct3/4, Sox2, c-Myc, Klf4- then called the Yamanaka factors).^{14,15} Similar results were obtained using OCT4, SOX2, NANOG, and LIN28.¹⁶ The reprogrammed iPSC, like ESC, were able to give rise to tumors containing all 3 germ layer cells when injected subcutaneously in a nude mouse,¹⁴ fulfilling the definition of pluripotency and opening up the possibility of using these pluripotent cells for cell-based therapies. iPSC have been so far reprogrammed from several different somatic cells types, including fibroblasts,^{15,16} blood mononuclear cells,¹⁷ peripheral blood cells,¹⁸ human astrocytes,¹⁹ renal epithelial cells present in urine,²⁰ and different types of cells associated with the hair (keratinocytes),²¹ follicle dermal papilla,²² and as reviewed in Ref.²³ hiPSC are considered to have pluripotency properties equivalent to those of human ESC, derived from the inner cell mass of the blastocyst.²⁴ A number of criteria are used to assess pluripotency and reprogramming efficiency of new iPSC lines.¹²

Genome Stability of iPSC

One of the most crucial factors to take into account for reprogrammed cells is the maintenance of genome stability, due to the risk of tumor genesis or limitations to differentiation potential.^{25,26} Oncogenic properties of reprogramming factors such as c-myc and Klf4 must be considered, as failure to silence or inactivate these after reprogramming could lead to genome instability. Considerable effort has been made to generate pluripotent cells while preserving genome integrity, such as the use of Sendai RNA virus vector to deliver the reprogramming factors while avoiding integration in the host genome.²⁷ Regardless of the mode of derivation of iPSC, the accumulation of genomic aberrations needs to be minimized, and one of the main challenges for iPSC lines is to maintain pluripotency and genome integrity in long-term cultures.

Several commercially available human ESC lines have been widely used in research. Notably, to obtain photoreceptors *in vitro*, the WA09 line (otherwise called H9 cells), derived at the University of Wisconsin (USA), has been often used.^{28–31} These cells maintain proliferation capacity, express high levels of telomerase, and do not show karyotypic aberrations when cultured for as long as 14 months on irradiated mouse embryonic fibroblasts (MEF).^{11,32,33} Nevertheless, karyotypic changes have been reported in H9 cells in the same culture conditions, including frequent changes in chromosome 12 where the gene *NANOG* is located.³⁴ Analyses on different hESC lines revealed that single-cell enzymatic passage can be deleterious even at early passages due to the increase in DNA double-strand breaks.³⁵ In fact, labor-intensive mechanical passage on feeders appears most "protective" for the cells, even up to 100 passages.^{36–38} When directly compared, bulk enzymatic passaging methods of medium-size clumps of cells did not cause genomic aberration in the first 15 passages.³⁶ This is encouraging, as bulk passaging is crucial to scale up the number of cells necessary for clinical transplantations. It is also generally accepted that with existing technology, each

cell line reacts variably to different passaging.³⁸ In support of this, a collaborative effort between 38 laboratories worldwide under the International Stem Cell Initiative analyzed 125 human ESC lines and 11 iPSC (representing most major ethnic groups) at 1 early and 1 late passage and passaged using enzymes or mechanically.³⁹ Only two-thirds of all the lines remained karyotypically normal at late passages, and no differences were noted between the methodologies tested. In summary, it is imperative to find optimal culture conditions to balance between cell expansion and maintenance of the genomic stability of pluripotent cells. While MEF usage and manual passaging maintain the highest level of stability in long-term cultures, for short-term scale up for clinical applications, feeder free and enzymatic passaging is probably acceptable.

Patient-Specific iPSC

Animal models have been widely used to reveal disease mechanisms. However, limitations, such as differences in pathophysiology, need to be taken in consideration when compared to humans. Moreover, animal models are not always available for a specific disease, and most importantly for investigation of patient-specific mutations. iPSC technology provides the means to create pluripotent stem cells from somatic cells of a patient and then by differentiating these into the target tissue, in this case the retina, it enables

the study of a specific disease in a human cell environment (Fig. 1). Patient-specific cell lines provide a way to model the variable retinal phenotype found in patients, even those with the same genetic mutation, and to provide a human cell system to test molecular therapies. The only alternatives to obtain patient-specific retinal tissue are by postmortem/enucleation or by retinal biopsies during intraocular retinectomy. The quantity and quality of such harvested retinal tissue are usually inadequate for disease modeling and drug screening. The ability to reprogram adult somatic cells to iPSC in fact also overcomes several limitations of using allogeneic ESC in cell-based therapies, such as the use of human embryos to develop the ESC lines, and the possible host rejection following cell transplantation. These issues can be overcome by reprogramming directly the patient's own somatic cells to generate "patient-specific iPSC" for autologous therapy, or possibly by using human leukocyte antigen-matched banked iPSC lines.⁴⁰ To achieve such cell therapy, clinical grade iPSC need to be derived and maintained under specific safety rules dictated by the Good Manufacturing Practice (GMP) protocols defined by the European Medicines Agency and the Food and Drug Administration. These rules include (i) reprogramming methods that have to be guaranteed as exogenous DNA integration free, rapid, efficient, and cost effective and (ii) protocols for cell culturing, freezing, and expansion with the exclusion of non-native agents.^{41–43}

FIG. 1. iPSC applications. Patient-specific iPSC can be generated using skin biopsies (or other tissue biopsies) to obtain fibroblasts (or other somatic cells) that can be reprogrammed using specific "reprogramming transcription factors." Following iPSC expansion and differentiation into retinal cells (photoreceptors or RPE or laminated retinal tissue), these cells could be transplanted back into the patient to repair retinal structures and restore vision. This technology can be further used for various applications such as disease modeling, drug screening, and gene therapy. iPSC, induced pluripotent stem cell; RPE, retinal pigmented epithelium.

Retinal Differentiation of iPSC

In the last decade, various protocols have been established for differentiating ESC into retinal progeny and these have proved equally applicable to iPSC differentiation.^{12,44} Notably, some iPSC and ESC lines have shown a high propensity to differentiate into RPE. Production of RPE cells from ESC and iPSC has been established by a number of different laboratories that have developed protocols for generation of homogeneous polarized RPE monolayer cultures.^{12,45,46,47} iPSC-derived RPE cells can be isolated, expanded, differentiated, reseeded, and monitored both for morphology and functionality.⁴⁸

Also highly significant has been the discovery that iPSC/ESC are able to self-assemble into three-dimensional (3D) optic cup structures, which then form stratified neural retinal tissues. During embryogenesis, lateral evagination of the diencephalon forms the optic vesicle, the rudimentary retina. This neuroepithelial structure then invaginates to form the double-walled optic cup. Later in development, the external wall matures into the RPE and the internal wall gives rise to the neural retina.^{49,50} These steps are recapitulated in recently developed 3D retinal differentiation culture protocols. The Sasai laboratory reported the formation of optic cup structures using a serum-free suspension culture system of murine and human ESC,^{51,52} while the Gamm laboratory generated optic vesicles containing both photoreceptor-like cells and RPE-like cells.^{28,29} Last year human iPSC were differentiated to give rise to functional photoreceptors with outer segments able to respond to light stimuli.³⁰ The timing of retinal-specific gene expression in the mouse and human iPSC/ESC cultures reflects the timeline of mouse or human embryological development, respectively. The eye field markers PAX6 and RX are first expressed in these cultures, followed by the formation of optic vesicles by 20 days of differentiation.²⁹ At this stage, retinal progenitor cells that differentiate into RPE cells express microphthalmia associated transcription factor (MITF), whereas neural retinal cells downregulate MITF in response to increased expression of CHX10 (VSX2; marker of retinal progenitor cells).²⁹ It requires typically 40–50 days of differentiation to generate RPE from iPSC, compared with 70–100 days to generate photoreceptors (expression of opsins)²⁹ and development of a response to light after 200 days.³⁰

iPSC Retinal Disease Modeling

Best vitelliform macular dystrophy is the first retinal disease affecting RPE to be modeled with patient-specific iPSC lines.⁵³ Defects in the RPE gene *BEST1* (encoding an anion channel located in the RPE basolateral plasma membrane) result in the accumulation of subretinal fluid and autofluorescent material, leading to loss of central vision due to photoreceptor death. When iPSC-derived RPE from affected and unaffected siblings was compared, the former exhibited a delay in the degradation of rhodopsin, indicating defects in fluid flow and outer segment protein degradation that were associated with altered calcium signaling and oxidative stress.⁵³ Adenovirus vectors have also been used to deliver *BEST1* mutants to iPSC-derived RPE; these studies have defined amino acids needed for channel activity and correct protein localization.⁵⁴ iPSC-derived RPE from patients with mutations in the *MERTK* gene, which causes RP, exhibits defective phagocytosis⁵⁵ and from patients with

mutations in *RP2* shows defects in IFT20 localization, Golgi cohesion, and protein trafficking, which could be corrected either by protein overexpression or by translational read through inducing drugs.⁵⁶ iPSC lines from AMD-affected individuals have also been used to derive RPE cells, to model the disease *in vitro* or for drug screening.^{57,58} Although AMD-iPSC are able to undergo normal RPE differentiation, they express lower levels of ZO-1 and RPE65, suggesting defective tight junction formation.⁵⁷ More importantly, they also show reduced antioxidant ability compared to iPSC controls,⁵⁷ which is, in part, due to impaired superoxide dismutase 2 (SOD2) stress response.⁵⁸ The increased susceptibility to oxidative damage is thought to contribute to AMD pathogenesis. Evaluation of RNA isolated from iPSC-derived RPE cells from a patient with Leber congenital amaurosis has been used to confirm that a cryptic splice site mutation disrupted transcription of the *RPE65* gene; importantly, demonstration of pathogenicity of the identified mutations allowed the patient to be enrolled in an RPE gene therapy trial.⁵⁹

The application of iPSC for modeling diseases affecting photoreceptors is also under development, although it has been limited by lack of robust generation of mature outer segments of the photoreceptors in culture. iPSC-derived photoreceptors have been used to study the effect of mutations in patients with RP (with distinct mutations in the *RHO*, *RP1*, *RP9*, *PRPH2*, and *USH2A* genes).^{60,61} A reduced number of iPSC-derived rod precursors were observed at differentiation day 120 and day 150 with *RP9* and *RP1* mutations.⁶⁰ With a similar approach, Tucker et al. modeled Usher syndrome.⁶¹ iPSC-derived photoreceptor precursor cells carrying an *USH2A* mutation showed increased ER stress due to misfolding of the protein.⁶¹ iPSC-derived photoreceptor precursors, this time from a patient with Leber congenital amaurosis caused by mutation in *CEP290*, have been used to test lentiviral vectors for gene replacement therapy⁶²; viral transduction improved the ciliogenesis defect in cells from affected individuals.

If the retinal structures obtained *in vitro* are to faithfully mimic the human retina for drug testing and/or to study human development and disease,^{46,61,63,64} the generation of optic cups with correctly stratified retinae is a crucial priority. Nevertheless, to date, juxtaposition of the ONL and RPE layer has not been shown *in vitro*. The 3D human ESC retinal protocol pioneered by Sasai and his group generated relatively low levels of CRX⁺ve optic structures (12%), and while stratified, these show few bipolar cells, and little evidence of photoreceptor synapse or outer segments at 126 days of differentiation.⁵¹ However, the formation of outer segment-bearing photoreceptors for the first time in highly stratified ESC-derived retinal tissues³⁰ provides new opportunities for modeling retinal disease. Three-dimensional tissue constructs have been attempted by coculturing pluripotent stem cell-derived retinal progenitor cells on a layer of RPE cells in an osmolarity gradient.⁶⁵ Ideally, development of a bilayered RPE-neural retina culture with outer segment disc phagocytosis and phototransduction will provide unprecedented opportunity for new drug development. It will enable evaluation of whether novel therapeutic interventions ameliorate human disease-associated cell phenotypes. To date, control and patient-derived iPSC lines were not genetically matched in comparative studies, which is an important limitation to take into account when iPSC are used to model

a disease. Future application of gene editing technologies (e.g., Crispr/Cas9) will enable assessment of the effect of specific mutations in congenic backgrounds.

RPE Cell Replacement

RPE cell transplantation is considered as a way of restoring impaired RPE function and and/or supporting photoreceptor cell survival. RPE cell transplants performed in various animal models showed varying degrees of cell survival.^{66,67} RPE transplantation in the Royal College of Surgeons (RCS) rat model of photoreceptor degeneration (caused by *MERTK* mutation) showed improved cell survival when the cells were transplanted at early stages of the disease.⁶⁸ Following xenografting in animal models, RPE sheets derived from fetal⁶⁹ and postmortem adult tissues⁷⁰ were tested in human patients and showed transplant survival. Dissociated RPE from the human fetus also survived for 3 months posttransplantation without immunosuppressants, but showed a weak immune response against the photoreceptor proteins, phosducin and rhodopsin.⁷¹ Later, when neural retina-RPE complex was transplanted from fetal donors, improvement in visual acuity was reported in 70% of the patients.⁷² However, in the majority of trials, long-term improvement in visual acuity was not reproduced, and fibrosis formed in the grafts due to immune rejection.⁷¹ Immunosuppression improved the outcome of transplantation,^{70,73} although in the long run these drugs lead to systematic side effects.⁷³ To avoid rejection, autologous RPE grafts were instead used.^{74,75} RPE, harvested from the nasal subretinal region, was dissociated and transplanted around the macular region. Significant improvement in multifocal electroretinography was reported after 1–3 months, despite lack of long-term visual acuity improvement.⁷⁵ The success of this procedure is dependent on the cells adhering to native Bruch's membrane and RPE forming a polarized layer. Due to the complications associated with the use of autologous RPE cell suspension, this has been replaced by RPE-choroid complex⁷⁶ and macular function was maintained for up to 7 years without major complications.^{77,78}

The biggest challenge in using autologous or postmortem RPE cells for grafting is the limited availability of RPE tissue. This limitation could be overcome by using iPSC for which supply is theoretically infinite. iPSC-derived RPE for transplantation must be fully characterized using the “4 P's system” to evaluate the desired cell properties—polygonal, pigmented, phagocytic, and polarized—to ensure that successful RPE cell differentiation is achieved.⁷⁹ Ensuring the purity of the final cell population by depletion of any residual pluripotent cell population is also crucial to avoid the risk of tumor formation.⁸⁰

Scaffold material, fabrication technique, and properties

Nonadherent transplanted RPE cells have limited survival and form aggregates that initiate an immune response. Correct attachment of the transplanted RPE to Bruch's membrane appears partly dependent on the age of the donor tissue.⁸¹ The use of scaffolds could solve these issues and provide structural support for donor- or iPSC-derived RPE cells to form and retain a monolayer structure for transplantation. Scaffolds potentially enable proper delivery of cells, direct their behavior, maintain

3D structures similar to native tissues,⁸² and could deliver bioactive molecules to the subretinal space. Scaffolds being tested range from natural to synthetic materials.⁷⁸ Human amniotic membrane,⁸³ lens capsule,⁸⁴ and Bruch's membrane⁸⁵ are some of the naturally occurring membranes that are considered for the subretinal transplantation.

Biocompatible naturally occurring polymers are being used such as collagen, hyaluronic acid, alginate, and fibrin. Among these, collagen is widely used as it is also the major component of Bruch's membrane.⁸⁶ Natural materials provide the advantage of directing cell behavior by closely imitating the native extracellular matrix (ECM) of the targeted tissue, although they raise concerns such as the purity of animal-derived scaffold, risk of infection, difficulties controlling consistency of the material, and mechanical instability.^{87,88} In contrast, synthetic scaffolds can be custom made, including incorporation of biocompatibility, mimicking native ECM, and biodegradability.⁸⁹ Poly lactic-co-glycolic acid is one of the most widely used synthetic polymers.⁹⁰ The disadvantages of the synthetic scaffolds are minimal cell adherence and lack of epitopes for cell attachment. There are various techniques used to manufacture scaffolds for the subretinal space, such as particulate leaching, electrospinning and freeze drying, microfabrication, and solvent casting, the last 2 being the most used.⁹¹

Regardless of their nature, scaffold size should range from 5 to 90 μm due to the limited subretinal compartment and be biocompatible, mechanically robust, flexible to be easily implantable, and porous to support formation of the polarized layer. Ideal scaffolds should (i) enable formation of a polarized RPE monolayer performing designated functions, (ii) have sufficient strength and flexibility to be transplanted at the back of the eye, (iii) support integration of RPE to Bruch's membrane, and lastly (iv) should be inert to the eye.

Cell delivery

Evaluation of human iPSC-derived retinal cell transplantation has been performed in animal models, whereby RPE cells are injected in the subretinal space of the retina (between the photoreceptors and the RPE) or implanted using scaffolds. In fact, human iPSC-derived RPE has been transplanted in the RCS rats with some visual benefits.⁴⁶ iPSC-derived RPE cells were able to integrate and have normal phagocytic function and preservation of visual function monitored by optokinetic head tracking in RCS rats.⁴⁶ In addition, human iPSC-derived RPE injected into the subretinal space of RPE65 mutant mice showed integration of cells in the host environment and some improvement of visual function.⁹² In contrast, various scaffold materials such as bovine collagen type 1, linear chains of poly (*N*-isopropylacrylamide),⁹³ methyl methacrylate, and polyethylene glycol methacrylate⁹⁴ are being used to deliver RPE cells into the subretinal space. Limited cytotoxic effects and minimal cell death were reported for H9-derived RPE cells using a mesh-supported submicron parylene-C membrane.⁹⁵ When RPE cells are cultured *in vitro* on this scaffold, they adhere, proliferate, and form tight junctions and an epithelial monolayer.⁹⁵

Clinical trials

The first human trial using ESC-derived RPE started in 2011^{96,97} and various clinical trials are ongoing in different

countries, including the USA, UK, Israel, and Korea.⁹⁸ ESC-derived RPE cell suspension was transplanted in the sub-retinal space in patients at geographic atrophy stage of AMD and with Stargardt's disease. The first stem cell-derived RPE cell transplants indicated no serious adverse outcomes and encourage further investigation.⁹⁹ A human clinical trial in patients for treatment of AMD using iPSC-derived RPE cells started in 2013 at the RIKEN Center for Developmental Biology in Japan.¹⁰⁰ Previously, no immune response had been shown after transplantation of iPSC-derived RPE in monkey⁸⁶ or rodents.¹⁰¹ iPSC lines were generated from AMD patients' skin cells and differentiated into RPE cells that were then seeded onto collagen gel forming a monolayer without synthetic scaffold or matrices. Once confluence was achieved, the constructs were treated with collagenase, resulting in a sheet of RPE cells ready for transplantation (1.3 mm by 3.0 mm) into the damaged patient retina. While the first transplanted patient suffered no adverse effects, acquired mutations were discovered in a second patient's iPSC and the trial is currently on hold, pending consideration of the use of partially matched (allogeneic) donor iPSC rather than patient-specific ones. Although safety and feasibility of iPSC and ESC are still being established, in general, these trials hold promise for the future of regenerative medicine in retinal diseases. Retinal tissue engineering using appropriate scaffold materials and cell sources, combined with localized surgical delivery, may offer future potential for retinal reconstruction for treatment of end-stage retinal disease.

Photoreceptor Cell Replacement

Photoreceptor cell transplantation holds the potential to restore visual function by integrating new functional photoreceptors within the existing retinal circuitry of the patient. As the inner retina remains largely intact in patients with retinal degenerative disease, transplantation of cells into the subretinal space offers the opportunity for new cells to connect with host bipolar cells and transmit visual information to the brain. Different cell types have been evaluated as a source of retinal cells for transplantation.¹⁰² In this study, we focus on the use of pluripotent stem cells for photoreceptor cell replacement, highlighting the progress made so far and the limitations and future challenges when translating the results from animal models toward clinical trials.

Generating transplantation-competent photoreceptors: lessons from murine ESC

To ensure efficient cell integration, maturation, and function of transplanted photoreceptor cells in a host environment, the developmental stage of the transplanted donor cell population appears to be a critical determinant. The Ali group^{103,104} and others^{105,106} have demonstrated that cells isolated from the postnatal murine retina that were already committed to the photoreceptor fate are able to successfully integrate and give rise to correctly orientated rods in the mouse retina. When transplanted into mouse models of retinal disease, postnatal (neural retina leucine zipper [NRL^{+/ve}]) rod precursor cells are not only able to fully mature to light-sensitive rods with outer segments but also to restore rod-mediated vision^{102,104,107} and reconstitute a polarized ONL in

some cases.¹⁰⁸ Moreover, manipulation of the host environment by reducing gliosis and outer limiting membrane integrity increases integration and survival of transplanted cells.^{107,109} In contrast, transplanted embryonic (CRX^{+/ve}; cone rod homeobox gene) photoreceptor precursor cells were instead able to give rise to cones as well as rods.¹¹⁰

The importance of the differentiation status of donor cells at the time of transplantation has similarly been addressed through studies using mouse ESC (mESC).^{40,102,111} Neuralized mESC were able to integrate in almost all layers of the retina, acquire expression of some retinal markers, and enhance survival of the host photoreceptors, although these transplanted cells did not form outer segments.¹¹² The *in vitro* recapitulation of retinal development appears to be a crucial factor when generating donor cell populations for successful photoreceptor transplantation. This has been determined by comparing different protocols for mESC retinal differentiation. Some of the expression levels of photoreceptor markers are lower in adherent two-dimensional (2D) differentiation cultures compared to adult retinas^{113,114}; even though 60% of mESC-derived cells expressed CRX, these cells did not show any mature integrated photoreceptors after transplantation,¹¹⁴ suggesting they were not transplantation competent. The development of the new generation of 3D retinal differentiation protocols that mimic embryonic morphogenesis of the optic vesicle^{52,115} has provided the opportunity to test the importance of developmental stage to enhance transplantation competency. The Sasai group demonstrated that dissociated mESC reaggregate to form embryoid bodies in a 96-well low adhesion plate and form optic vesicles after addition of 2% Matrigel; when then cultured in suspension, they give rise to clearly recognizable retinal layers containing all the major retinal cell types as *in vivo*.⁵² Importantly, rod photoreceptor precursors obtained from mESC with this protocol were able to integrate and mature in the adult degenerate retina,^{116,117} contrary to what had been observed with a 2D retinal differentiation protocol.¹¹⁴ Using this new advanced 3D protocol, derived rod precursors transplanted in a degenerate adult retina successfully formed inner and outer segments, which were correctly orientated toward the RPE of the host retina.¹¹⁶ Transplanted cells also formed synapses and were functionally active.¹¹⁶ This study highlights the importance of developmentally correct derivation of photoreceptors as an indicator for efficient transplantation.

Toward clinical photoreceptor transplantation

To move toward clinical trials for human photoreceptor cell replacement, it will be necessary to (i) optimize differentiation protocols for photoreceptors *in vitro* using iPSC and/or ESC as an unlimited source of cells and (ii) define conditions to promote human donor cells to integrate and be functionally active in the recipient degenerate retina. Many efforts have been made in the last decade to achieve these goals. Some transplantation studies have tested the behavior of iPSC-derived retinal cells. Adherent 2D differentiation protocols generate cells expressing photoreceptor markers from human iPSC and ESC in only 3 weeks using small molecules (insulin-like growth factor, Dickkopf-Wnt signaling pathway inhibitor-1, and Noggin)^{105,118,119}; these cells were able to undergo some further maturation after injection into the degenerate mouse retina.¹¹⁸ ESC-derived

retinal cells migrated into the ONL after transplantation in the subretinal space of a CRX-deficient mouse model, although they did not form inner and outer segments.¹⁰⁵ The integration of these human cells in the mouse retina represents an important milestone for cell-based therapies. By learning from the previous work of transplanting mESC-derived rod photoreceptors, it is hoped that better survival and maturation of transplanted human cells may be achieved by improving differentiation protocols to recapitulate *in vivo* human development. Development of 3D protocols to generate photoreceptors from human ESC/iPSC^{29,51} opens up the possibility of applying similar transplantation approaches as developed in the mouse models to the human cases.

Clinical application will require a reproducible simple xeno-free culture system, capable of generating high yields of photoreceptors suitable for transplantation, preferably in a quick procedure, while limiting costs. These requirements will be discussed below, although it must be stressed that there is still much work to be done in parallel to investigate and optimize surgical parameters that best promote cell delivery, integration, and survival in the degenerate human retina.

Simplicity and speed

The method of formation of optic vesicle-like structures developed by the Gamm laboratory uses a simple inexpensive medium (containing B27 and Nutrient mix F12) and is based on manual separation of optic vesicle structures, which are then cultured in suspension.^{29,28,120} This protocol generated Recoverin+ functional cells, but the cell types obtained (bipolar, retinal ganglion cells, amacrine cells, and photoreceptors) are not highly organized in layered structures.^{28,29} By contrast, the more labor intensive protocol developed by Nakano et al. exhibits more organized retinal structures,⁵¹ but differentiation is induced by addition of small molecules, notably increasing the cost of the culture. Recent modifications of the Gamm protocols,³⁰ including the addition of retinoic acid, Taurine, and serum at different intervals for long-term cultures, generated light-responsive photoreceptors that show outer segments, 2/13 of which were also responsive to patch clamp techniques.³⁰ To avoid the presence of animal derivatives (animal-derived sera, or in most cases Matrigel), a chemically defined 3D retinal differentiation medium was recently reported, which only contains nicotinamide and 20 ng/mL of basic fibroblast growth factor.¹²¹ To date, efforts to speed up the differentiation protocols^{31,122,123} using molecules known to promote retinal differentiation^{31,118,123} or by passing the EB formation¹²² have generally reduced the survival of differentiated cells,^{31,123} and/or the stratification obtained.¹²² Whether correct lamination of pluripotent stem cell-derived donor retinal tissue is required for generation of transplantation-competent human photoreceptor cells remains to be resolved.

Yield and purity

High yields of photoreceptor cells will be needed for clinical application. Based on mouse studies, different steps during the 3D differentiation could be optimized. For example, enhanced photoreceptor yield may be achieved by controlling the size of the EB.^{114,124} Formation of a ciliary

margin stem cell niche in 3D optic vesicles was recently shown to expand the generation of the retinal progenitor cell population *in vitro*.¹²⁵ Further definition of conditions that expand progenitor populations committed to produce rod or cone photoreceptors would be very useful. A recent study demonstrated enrichment of ESC-derived cone photoreceptors using a new protocol inhibiting bone morphogenetic protein (BMP), transforming growth factor beta (TGF β), and Wnt signaling.¹²⁶

Another important step for clinical cell therapy is the purification of the cell population of interest from the differentiated stem cell cultures, which typically contain multiple cell types.^{30,115,122} For RPE cell transplantation, it is possible to grow monolayers of RPE free from contaminating cells. For photoreceptor transplantation, photoreceptor cells suitable for transplantation might be best produced in synthetic 3D optic vesicles containing other retinal neurons and RPE. Enrichment of rod precursor cells from the developing retina can be achieved through magnetic or fluorescent-associated cell sorting for the cell surface marker cluster of differentiation 73 (CD73).^{127,128} Selection based on 2 surface markers (CD73 and CD24) increased the integration efficiency of transplanted cells 18-fold compared to unsorted cells.¹²⁸ This approach was recently extended to a panel of 5 cell surface markers that were used to sort rod precursor cells from mESC-3D retinal differentiation cultures.¹²⁹ At day 27 of differentiation, mESC were sorted for CD73+/CD24+/CD133+/CD47+/CD15- and transplanted in the adult murine retina; they were then able to integrate, acquire rod morphology, and form outer segments.¹²⁹ This unique combination of markers enriches for CRX+/Recoverin+/RHO+ cells transplantation-competent cells. Negative selection for CD15 allows removal of contaminating proliferating progenitor cells.^{129,130} These findings demonstrate that panels of surface markers are useful tools for the purification of transplantation-competent photoreceptors. The cell surface markers used to sort murine rod precursors show similar expression patterns in the developing human retina and so could help to enhance the photoreceptor yield from the iPSC retinal differentiations, as it did with the mESC.^{128,129} Human iPSC-derived photoreceptors are reported to express CD73 at day 42 of differentiation,¹²² and based on transcriptomic analysis, other potentially useful surface markers such as *Cacna2d4*, *Kcnv2*, and *Cnga1* have been identified.¹³¹

Delivery

Methods to optimize delivery of human photoreceptors to the diseased retina are in their infancy and present a considerable challenge. Lessons will be learned from the progress of clinical iPSC-derived RPE transplantations, particularly regarding safety and tolerance. However, the field of RPE transplantation is favoring the use of substrates to support delivery of a prefabricated RPE monolayer to a specific subretinal location. Studies to date in the mouse model have shown most success from transplantation of predifferentiated enriched photoreceptor precursor cells in dissociated suspensions. Whether it might be feasible to transplant iPSC-derived sheets of photoreceptors into the subretinal space, similar to RPE cell patches, is a tantalizing possibility as recently ESC-derived cones were shown to develop as polarized cell sheets *in vitro*.¹²⁶ Nevertheless,

substrates may prove useful to help support photoreceptor delivery to the central retina. Poly(L-lactic acid) (PLLA) and poly(lactic-co-glycolic acid) (PLGA) have been evaluated for delivery of retinal progenitor cells (RPC) where adverse reactions such as inflammation, fibrosis, and retinal detachment were reported.¹³² More recently, a combination of matrix metalloproteinase 2 (MMP2) and PLGA was used to deliver RPC to dystrophic retinas, which resulted in enhanced photoreceptor gene expression.¹³³ Scaffold materials are developed considering which type of cells will be utilized, as changing surface properties by wet chemical, plasma technique, and cell recognition peptides can enable better adherence to the scaffold surface.⁸⁹ Ultimately, such bioengineered surfaces may help replace complex retinal structure, such as both the RPE and the ONL, to create structures similar to the native tissue.¹³⁴

Conclusions and Future Challenges

The regenerative medicine field aims to develop cell therapy to replace RPE cells and/or photoreceptors in patients with incurable retinal degenerative disease. As discussed above, there are a number of requirements to take iPSC into cell therapy. These include finding the best non-invasive technique to harvest a patient's somatic cells and to reprogram cells into iPSC lines using safe techniques that maintain genomic integrity. Moreover, once iPSC lines have been established, they need to be maintained in a pluripotent state, expanded and stored accordingly to GMP regulations. Most importantly, the purification, storage, and delivery of iPSC-derived retinal cells for transplantation are vital to ensure the success of the procedure. In the case of autologous cell transplantation for patients with inherited retinal disease, combined approaches may require gene correction in iPSC before transplantation. Survival and function of the transplanted cells are similarly critical for success. New photoreceptors need to correctly integrate after delivery to the subretinal space, and to form connections with the neighboring cells. As discussed above, transplanted cells could be delivered with the support of scaffolds, which may improve survival and organization of cells.

iPSC technology also provides an innovative new platform to study human retinal development and disease, due to recently developed 3D differentiation protocols that are able to recapitulate retinogenesis and form laminated synthetic retina *in vitro*. There are still significant challenges for the application of iPSC in research and development, as it takes 4–6 months to generate retinal cells and maintenance is expensive. However, advances in cell culture automation and refinements in the reprogramming methods might help accelerate translation of iPSC-derived cell technology into personalized medicines. In fact, patient-derived cell lines using 3D culture protocols are already being developed to test the efficacy of drug-based therapies, to elucidate molecular pathogenic pathways of diseases, study phenotypes of unusual mutations identified in patients, and to optimize parameters for successful gene and pharmacological therapy, thus reducing the need for animal models, which are expensive and not always accurate.

Although clinical trials have been approved in the United States, United Kingdom, and Japan for RPE transplantation, implantation techniques, immune rejection, xeno-free techniques, and safety issues are still a matter of concern and

need attention for future regenerative medicine treatments. iPSC technology can be accelerated with a collaborative effort between research institutions and biotechnology industries for large-scale donor cell production. This will lead to the widespread application of iPSC-based technology in translational studies in the future.

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Author Disclosure Statement

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