Concise Review: Patient-Specific Stem Cells to Interrogate Inherited Eye Disease

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ABSTRACT

Whether we are driving to work or spending time with loved ones, we depend on our sense of vision to interact with the world around us. Therefore, it is understandable why blindness for many is feared above death itself. Heritable diseases of the retina, such as glaucoma, age-related macular degeneration, and retinitis pigmentosa, are major causes of blindness worldwide. The recent success of gene augmentation trials for the treatment of *RPE65*-associated Leber congenital amaurosis has underscored the need for model systems that accurately recapitulate disease. With the advent of patient-specific induced pluripotent stem cells (iPSCs), researchers are now able to obtain disease-specific cell types that would otherwise be unavailable for molecular analysis. In the present review, we discuss how the iPSC technology is being used to confirm the pathogenesis of novel genetic variants, interrogate the pathophysiology of disease, and accelerate the development of patient-centered treatments. STEM CELLS TRANSLATIONAL MEDICINE 2016;5:132–140

SIGNIFICANCE

Stem cell technology has created the opportunity to advance treatments for multiple forms of blindness. Researchers are now able to use a person's cells to generate tissues found in the eye. This technology can be used to elucidate the genetic causes of disease and develop treatment strategies. In the present review, how stem cell technology is being used to interrogate the pathophysiology of eye disease and accelerate the development of patient-centered treatments is discussed.

INTRODUCTION

The best introduction to the present review is a simple experiment. Readers should scan their environment and become familiar with their surroundings and then close their eyes and walk to the nearest restroom. The anxiety that everyone experiences in such an experiment stems, at one extreme from simply looking odd to strangers in the hall as one feels one's way along the wall, to a genuine fear of serious injury if moving equipment or open stairwells lie along the path to the goal. To relieve this artificially induced anxiety, a non-visually impaired person need only open their eyes. However, millions of people with inherited eye disease have lost the ability to see clearly enough to perform even the simplest daily activities.

From the anterior surface of the cornea to the posterior choroid and sclera, genetic defects have been linked to the dysfunction and death of the ocular cell types that are essential for normal vision. Many of these diseases are progressive and can eventually result in total blindness. For many, the fear of blindness is equal to the fear of death itself.

For most inherited eye diseases, no effective treatments exist. Although gene- and autologous cell-based treatment approaches have shown great promise in the laboratory, the speed with which gene discoveries have progressed to clinical trials has been disappointingly slow. The most common question asked by patients affected by an inherited eye disease is "how soon?" "How soon before you find my disease causing gene?" "How soon before I can receive gene replacement?" "How soon before you can put new cells into my eyes and restore my vision?" The physicians and scientists who care for such patients are constantly challenged to find ways to streamline the path from gene discovery to treatment. Patient-derived induced pluripotent stem cells can accelerate multiple steps along this path, including confirmation of the pathogenicity of disease-causing mutations,

elucidation of disease mechanisms, demonstration of the efficacy of novel treatments, and restoration of vision to those who have lost most of their photoreceptors before preventive therapies could be developed.

In the late 1980s and early 1990s, when the first ocular disease-causing genes were being discovered, large patient cohorts with numerous well-defined pedigrees were used to make convincing statistical arguments regarding the pathogenicity of the newly identified genetic variations [1–4]. Although this was a very successful strategy, especially when multiple different mutations could be identified in a single gene or when two seemingly null mutations could be identified in a single individual, it was sometimes difficult to be certain whether newly identified missense mutations were truly pathogenic or simply in linkage disequilibrium, with the true disease-causing variation yet to be identified [5, 6]. As new disease-causing genes and mutations have been identified during the past 25 years, the tendency has been for the newer findings to represent a smaller and smaller fraction of the total burden of genetic disease. It is now common for a newly discovered gene or class of variant within a gene to be responsible for the disease of only a few hundred individuals in the United States. Thus, the techniques that can rapidly investigate the pathophysiology of newly identified mutations are becoming increasingly valuable. Traditionally, knockout or transgenic animal models have been used for this purpose. However, this process is quite time consuming and expensive, and numerous examples exist in which the resultant animal models exhibited either no phenotype at all or a phenotype very different from that of the human disease it was intended to model. For example, in humans, mutations in *USH2A* are the leading cause of type 2 Usher syndrome and autosomal recessive retinitis pigmentosa. However, in mice, deletion of the *Ush2a* gene has no significant effect on retinal anatomy or visual function. This is likely because mouse photoreceptor cells do not have calyceal processes, structures that have been shown to be the predominant site of USH protein localization in humans [7].

In the new era of precision medicine, robust model systems that are reflective of an individual patient's disease will be needed in some cases. Patient-specific induced pluripotent stem cells are well suited for this purpose.

INDUCED PLURIPOTENT STEM CELLS

The advent of patient-specific induced pluripotent stem cells (iPSCs) [8, 9] has allowed scientists access to living diseased tissues that would otherwise be unavailable for molecular analysis. This new technology has opened up three major areas of investigation, each of which are pertinent to the treatment of inherited blindness: (a) investigation of specific mutations and their associated pathophysiologic mechanisms; (b) the evaluation of novel gene augmentation, gene silencing, and small molecule therapies; and (c) the restoration of function through transplantation of manufactured cells and tissues.

Unlike medical disciplines, such as hematology and dermatology, in which diseased cells and tissues are readily accessible for laboratory investigation, most inherited eye diseases affect cell types that cannot be sampled in living individuals without inflicting clinically significant and irreversible damage. For example, it would be unreasonable to take a biopsy of the retina solely to determine whether and how specific genetic variants are affecting

cellular function and viability. The ability to create otherwise inaccessible cell types using patient-specific iPSCs has made it possible to determine whether novel variants that have been identified in an individual patient are truly pathogenic. Unlike linkage analysis and genotype-phenotype correlation analysis, this approach is not dependent on disease prevalence or the fortuitous discovery of one or more large families with multiple affected individuals. One can use gene augmentation or genome editing to add or subtract specific genetic variants and thereby discover whether and how the specific mutations identified in a single patient have caused their disease. This technology will undoubtedly be a common feature of the ophthalmic division of precision medicine.

In addition to their utility for determining whether and how genetic mutations cause disease, another valuable application of iPSC technology is the evaluation of novel therapeutics. For both large-scale drug screening and disease-specific gene-based treatments, patient-derived iPSCs provide scientists with a relatively inexpensive and often more authentic alternative to animal models. Normal animals can be used to establish the safety of the therapy after patient-derived iPSCs have been used to demonstrate efficacy at the cellular and molecular levels.

Perhaps the ultimate use for patient derived iPSCs is the restoration of vision in the patient from whom the cells were obtained. When paired with genome editing, one can create genetically corrected, immunologically matched cells suitable for replacement of any posterior eye tissues from the bipolar cells to the choriocapillaris. By using the patient's own cells, one obviates the need for lifelong immunosuppression, which is very expensive, progressively harmful to vital organs such as the heart, liver, and kidneys, and associated with a significantly increased risk of infection and malignancy. The remainder of the present review focuses on the utility of iPSCs for investigation of disease pathophysiology. In-depth reviews of the other medical applications of iPSCs have recently been published [10–12].

LIMITATIONS OF INDUCED PLURIPOTENT STEM CELLS

Before discussing the extraordinary potential of iPSCs for modeling retinal development and interrogation of disease pathophysiology, it is important to briefly point out some of the shortcomings of this technology. First, unlike many standard cell culture systems, which are routinely performed by countless laboratories around the world, the generation, maintenance, and differentiation of iPSCs is time consuming and requires specialized equipment and expertise. This is especially true when attempting to model late-onset disease such as age-related macular degeneration (AMD), which takes decades to develop, or when attempting to model diseases that require the development of fully mature cellular structures such as photoreceptor outer segments, which require months of differentiation [13].

In addition, it is important to note that unlike inbred model systems, which have fixed genetic backgrounds, when designing experiments using iPSC technology, one must consider the genetic variability between the patients and controls. This could obscure the interpretation of a disease-related phenotype [14]. A solution to this issue is to increase the sample size or to use genome editing-based techniques to correct and directly compare the genetically corrected and uncorrected cells from the same patient. However, this solution also requires expertise and reagents that, for many laboratories, might not be readily available. With

these drawbacks in mind—as will be apparent in our review—when the experiments are properly designed, iPSC technology holds tremendous possibility.

Production of Ocular Disease-Specific Cell Types

To use iPSCs to investigate the pathophysiology of disease, one must be able to create each of the specific cell types that are affected. Fortunately, for most inherited eye diseases, very effective cellular differentiation protocols have already been developed for the primary cells of interest. In the following sections, the differentiation protocols relevant to diseases that affect tissues of the anterior and posterior globe are reviewed.

Anterior Globe (Cornea, Lens, and Trabecular Meshwork)

Three structures contained within the anterior portion of the eye most relevant to our review are the cornea, lens, and trabecular meshwork. The cornea is an avascular laminated structure composed of the surface epithelium, Bowman's layer, the collagenous stroma (which accounts for approximately 90% of the corneal volume), Descemet's membrane, and the corneal endothelium (Fig. 1A1). The corneal epithelium is a stratified epithelial cell layer essential for maintaining the surface tear film and barrier function [15]. At the peripheral rim of the cornea, a specialized population of limbal epithelial stem cells, which function to maintain the corneal epithelial cell layer, exist [16, 17]. The corneal "endothelium" consists of a single layer of hexagonal epithelial cells, which are essential for maintaining corneal clarity [15]. Because most inherited cornea dystrophies result in defects in the surface epithelial cells and corneal endothelial cells (e.g., Meesmann corneal dystrophy and Fuchs endothelial dystrophy), the iPSC differentiation protocols developed to date have been largely focused on generating these two cell types. A recent study by Sareen et al. demonstrated how cell surface substrates that closely mimic the native limbal stem cell niche could be used to induce an iPSC to limbal stem cell fate [18]. As previously demonstrated by Hayashi et al., iPSCs generated from limbal epithelial cells had a greater propensity to adopt a corneal epithelial cell fate than those generated from dermal fibroblasts, indicating a possible role of epigenetic memory in cellular differentiation [18, 19]. Chen et al., in a recent study, treated iPSCs in suspension culture with all-trans retinoic acid to promote sphere formation and neural crest cell differentiation [20]. Spheres subsequently cultured under adherent conditions and fed with either lens-conditioned or primary corneal epithelia cell-conditioned media differentiated into corneal endothelial-like cells positive for the corneal endothelial cell markers AQP1, ZO-1, Na$^+$-K$^+$-ATPase, N-cadherin, VE-cadherin, and vimentin [20].

Like the cornea, the lens is an avascular structure that is capable of refracting light. The lens changes shape in response to the contraction or relaxation of the ciliary body and thereby adjusts the eye's focus for nearer and farther targets, respectively (Fig. 1A2). Structurally, the lens consists of two types of cells, lens epithelial cells and lens fiber cells. The epithelial cells form a single cuboidal layer around the anterior portion of the lens. These cells terminally differentiate at the lens equator to form fiber cells, resulting in lens growth [21, 22]. Mitosis of the epithelial cells occurs in the germinative zone adjacent to the equator, and growth factors within the vitreous humor have been shown to drive differentiation toward the fiber cell fate

[21–24]. Recently, Qiu et al. developed a method to drive lens-specific differentiation [25]. The induction procedure consisted of three steps: (a) administration of noggin from days 0 to 5; (b) administration of bFGF, BMP4, and BMP7 from days 5 to 15; and (c) administration of FGF2 and Wnt-3a from days 15 to 30. Lens progenitor cells expressed the lens-specific markers PAX6, SOX2, SIX3, CRYAB, CRYAA, BFSP1, and MIP [25]. When using a protocol designed to induce differentiation of iPSCs to a retinal cell fate [26–28], we also observed the formation of rudimentary lens-like structures (Fig. 1A2, inset). These lens-like structures were specifically labeled with an anti-αA-crystallin antibody. Regions within the lens-like structure that strongly expressed αA-crystallin in the absence of nuclei could be identified (i.e., no 4′,6-diamidino-2-phenylindole labeling; Fig. 1A2, inset). Taken together, these data show that iPSC-derived lens-like structures can be generated.

Also like the cornea, disorders that affect the lens typically result in opacification and scattering of light as it enters the eye. Genetic defects in lens-specific genes are known to cause cataract formation and loss of vision [29, 30]. Although cataract is readily treatable via surgical intervention in the developed world, in areas where access to ophthalmic care is limited, it is a major cause of blindness. The ability to produce patient-specific lens-like structures in vitro makes it possible to model inherited forms of the disorder in which development of the lens is abnormal.

The trabecular meshwork (TM) is a filter-like structure that lies between the posterior border of the corneal endothelium and the scleral attachment of the iris. The primary function of the TM is drainage of aqueous fluid from the anterior chamber and maintenance of normal intraocular pressure (Fig. 1A3) [31]. Elevation of intraocular pressure is a significant risk factor for glaucoma, which is a leading cause of irreversible blindness worldwide [32]. The genetic causes of glaucoma are complex and undoubtedly have a significant environmental component [33, 34]. However, Mendelian forms of glaucoma have been shown to be caused by mutations in genes such as myocilin (MYOC) [1], optineurin (OPTN) [35], and TANK-binding kinase 1 (TBK1) [33]. The intraocular pressure will be elevated in some subtypes of primary open-angle glaucoma, and although the exact mechanism of this pressure elevation remains unknown, the preponderance of current research supports the hypothesis of an outflow obstruction within the TM [36, 37]. In support of this notion, several studies have shown a decrease in the cell density of the TM in individuals with glaucoma [38]. Using a primary cell coculture paradigm, it was recently shown that iPSCs can be used to generate functional TM cells [38]. iPSC-derived TM cells would be useful to model MYOC-associated glaucoma to investigate cell death and mechanism-specific therapeutics.

Posterior Globe (Retina, Retinal Pigment Epithelium, Choroid-Outer Retinal Unit)

The outer retinal unit is a laminated structure consisting of the neurosensory retina, the underlying retinal pigment epithelium (RPE), and the choroidal vasculature (Fig. 1A4) [39]. These tissues line the posterior two thirds of the eye and function to detect and process visual information. The neurosensory retina itself can be subdivided into five major layers: (a) the ganglion cell layer (GCL; Fig. 1A4), (b) the inner plexiform/inner synaptic
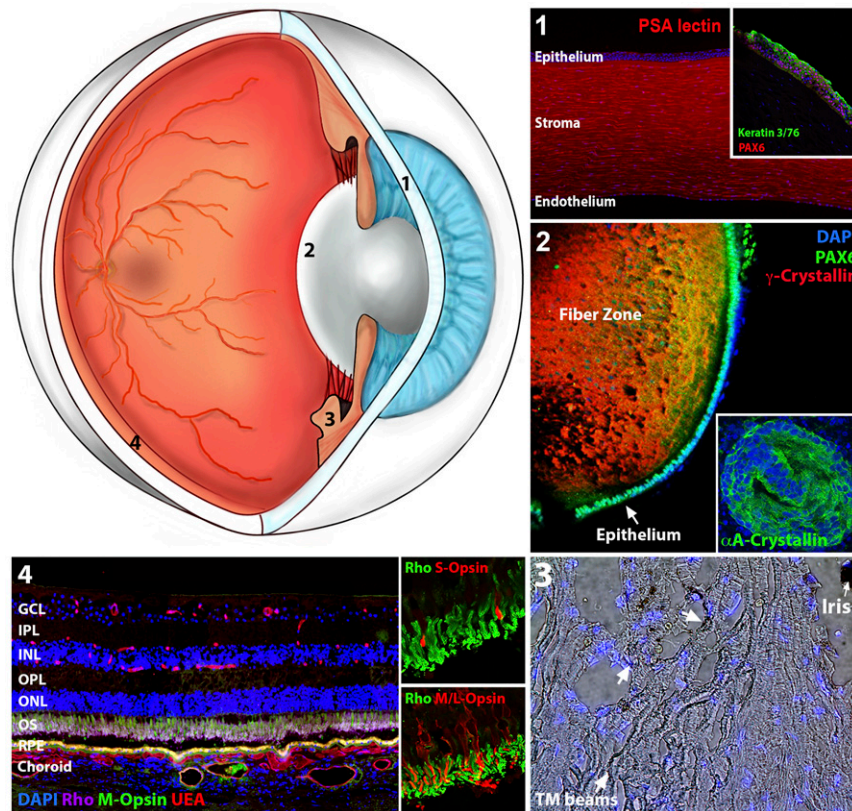
Figure 1. Ocular anatomy. Top left shows a cartoon drawing of the human eye. Numbers in the cartoon correspond to enlarged numbered panels. 1: Human cornea stained with PSA lectin. Inset shows a section of human cornea near the limbus labeled with keratin 3/76 and PAX6. Sections were counterstained using DAPI. 2: Mouse lens labeled with Pax6 that was positive throughout the nuclei of the anterior cuboidal epithelial cells and γ-crystallin, seen throughout the posterior lens fiber cell zone. Inset shows a human induced pluripotent stem cell-derived lens-like structure that was positive for the lens fiber cell-specific crystalline, αA-crystallin. Sections were counterstained using DAPI. 3: Phase contrast of human TM labeled with DAPI. The iris has been identified for the purpose of orientation. TM beams with DAPI stain TM cell nuclei. 4: Montage of the human "outer retinal unit," including the neural retina (GCL, IPL, INL, OPL, ONL, and OS of photoreceptors), the underlying retinal pigmented epithelium (RPE) and the densely vascularized choroid. Cone photoreceptors are labeled with M-Opsin and rod photoreceptors with Rho. The RPE is highly autofluorescent and easily seen underlying the photoreceptor layer. The vessels comprising the choriocapillaris and larger caliber vessels of the choroid were labeled with UEA. Insets show high magnification images of rod photoreceptor outer segments labeled with Rho and S-Opsin (top) and M/L-Opsin (bottom). Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; M-Opsin, green cone opsin; M/L-Opsin, green/red cone opsins; OPL, outer plexiform layer; ONL, outer nuclear layer; OS, outer segments; Rho, rhodopsin; S-Opsin, blue cone opsin; TM, trabecular meshwork; UEA, *Ulex europaeus* agglutinin.

layer (Fig. 1A4), (c) the inner nuclear layer (INL; Fig. 1A4), (d) the outer plexiform/outer synaptic layer (Fig. 1A4), and (e) the outer nuclear layer (ONL; Fig. 1A4) [40]. Each of these layers contains distinct cell types that perform specific functions [41]. The GCL contains retinal ganglion cells, which are the neurons that relay the visual signals obtained from the INL to the lateral geniculate nucleus of the thalamus [42]. The axons of these cells are bundled within fascicles of the optic nerve. In addition to the cell bodies of Müller glia, the INL contains the bipolar cells, amacrine cells, and horizontal cells, which are collectively responsible for first order visual processing and relaying information from the ONL to the GCL [39]. The light sensing photoreceptor spans multiple layers of the retina. The ONL contains the cell bodies of the light sensing cone and rod photoreceptor cells. Photoreceptors consist of an inner segment and an outer segment. The inner segment contains most of a cell's ion channels that contribute to the resting membrane potential [43], the endoplasmic reticulum (ER), the Golgi apparatus, and mitochondria [44]. The highly specialized outer structures (outer segments; Fig. 1A4)

contain the opsin proteins. Opsins are proteins that when bound to the chromophore 11-cis retinal will undergo a conformational change in response to light (Fig. 1A4, insets) [43]. This conformational change initiates a cascade of events that results in hyperpolarization of the photoreceptor cell and an altered release of neurotransmitter [43]. This signal is relayed by the bipolar cells of the INL to the ganglion cells and then to the thalamus.

The RPE (Fig. 1A4) is a monolayer of pigmented cuboidal cells, the apical surface of which forms long microvilli that are interdigitated with the photoreceptor cell outer segments [45]. The RPE plays a vital role in the turnover of these outer segments, the exchange of metabolic nutrients, the removal of waste [45], and the recycling of 11-cis retinal in a process known as the visual cycle [46]. The choroidal vasculature lies below the RPE and consists of deep, large-caliber choroidal vessels and a more superficial fenestrated choriocapillaris immediately adjacent to the RPE. This vascular system supplies the RPE and outer retina with oxygen and nutrients and removes the carbon dioxide and waste generated by the neurosensory retina and RPE [47].
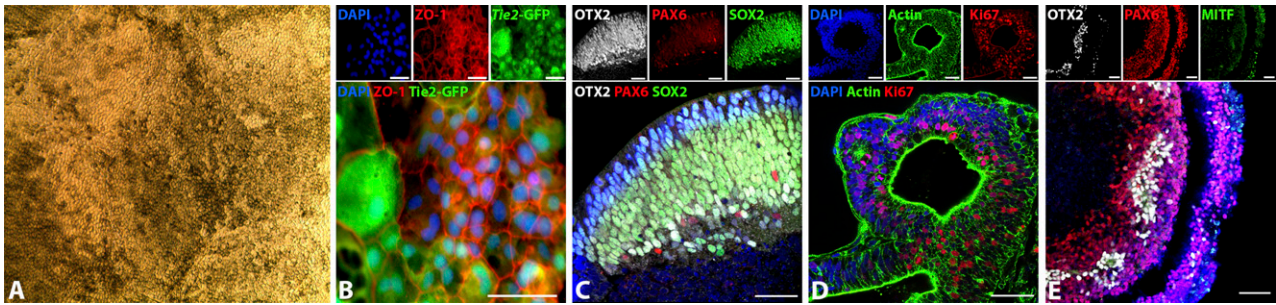
Figure 2. Using induced pluripotent stem cells (iPSCs) to model cells of the posterior pole. **(A):** A sheet of pigmented, hexagonal retinal pigmented epithelium (RPE) cells derived from the skin of an 89-year-old man with age-related macular degeneration. The patient was homozygous for the high-risk complement factor H haplotype and homozygous for the low-risk 10q haplotype. **(B):** iPSC-derived mouse choroidal endothelial cells expressing green fluorescent protein, driven by the Tie2 endothelial cell-specific promoter, and ZO-1. **(C):** Three-dimensional (3D) iPSC-derived eyecup structure at 30 days after differentiation from a patient with *USH2A*-associated retinitis pigmentosa expressing SOX2 and the early retina-specific transcription factors, OTX2 and PAX6. **(D):** A 30-day 3D eyecup from the same patient in **(C)** stained with phalloidin to visualize filamentous actin organization and labeled with anti-Ki67 to demonstrate proliferating cells within the neural epithelial cell layer. **(E):** A 63-day 3D eyecup differentiated from an unaffected, control iPSC line displaying the development of retinal lamination. Note the development of the outer layer of presumptive RPE cells expressing the RPE-specific transcription factor, MITF, and an underlying layer of OTX2- and PAX6-positive photoreceptor precursor cells.

Several groups, including our own, have developed differentiation protocols capable of producing retinal ganglion cells, photoreceptor cells, and retinal pigment epithelial cells from iPSCs [13, 28, 48–59]. Diseases in which these different cell types are involved range from very common disorders, such as AMD [60, 61] and glaucoma [62], to much rarer conditions, such as retinitis pigmentosa [63], Leber congenital amaurosis (LCA) [64], Stargardt disease [65], and Best disease [66]. Although there have been many different variations on this theme, there are two major differences in the way these cell types have been generated. The first uses an adherent two-dimensional (2D) culture system in which exogenous factors known to drive forebrain and eye field development are used [28, 48, 50–54, 67–69]. The second uses a floating three-dimensional (3D) culture system that couples the cells' intrinsic ability to spontaneously differentiate and self-organize with the experimenters' ability to positively identify and enrich for the desired tissue types [13, 55, 57, 58, 70]. Although each of these different approaches have advantages and shortcomings, to date they have proved useful for the production of the desired retinal cell types and, in turn, for the investigation of disease pathophysiology. Figure 2 illustrates the use of a 2D system for the generation of RPE (Fig. 2A) and choroidal endothelial cells (Fig. 2B) and a 3D system that faithfully recapitulates retinal development (Fig. 2C–2E). We have found the latter method to be highly efficient and quite useful for studying diseases that affect photoreceptor cells.

APPROACHES TO DISEASE MODELING

Inherited retinal dystrophies are collectively the most common and well-studied of all the inherited eye diseases. They are also genetically heterogeneous, and, as a result, many individual disease-causing genes are involved in only very small fractions of the total disease burden. To date, more than 190 different genes and thousands of different mutations have been shown to cause a retinal degenerative phenotype [71]. For many of these genes, even the normal function of the gene in the retina has yet to be determined, much less the pathophysiologic mechanisms of the associated retinal disease. The following sections summarize the published examples of the use of iPSCs to study three major

categories of disease: glaucoma, AMD, and Mendelian retinal degenerations.

USING iPSCs TO MODEL GLAUCOMA

The blindness associated with glaucoma is caused by the death of retinal ganglion cells (RGCs). The intrinsic regenerative capacity of the central nervous system is extremely limited, and, unlike the outer retina, replacement of lost RGCs would require newly transplanted RGC axons to traverse large distances through the relatively inhospitable environment of the mature optic nerve. Thus, most treatment approaches for glaucoma have focused on prevention. The most well-studied risk factor for glaucoma is elevated intraocular pressure (IOP); thus, most glaucoma treatments developed to date, whether pharmaceutical or surgical, have targeted the TM in an attempt to lower the IOP and slow the disease progression. However, some people experience a disease known as low-tension glaucoma, in which progressive retinal ganglion cell death occurs in the absence of an elevated IOP. For these patients, traditional TM-based therapies are largely ineffective. Mutations in two genes, *OPTN* and *TBK1*, have been associated with familial low-tension glaucoma and directly linked to retinal ganglion cell death and permanent loss of vision [33]. In a recent study, patient-specific, iPSC-derived retinal ganglion cells were generated from dermal fibroblasts of normal nonglaucomatous individuals and from a patient with *TBK1*-associated low-tension glaucoma. Using these cells, we were able to demonstrate that duplication of the *TBK1* gene increased activation of the autophagy pathway via upregulation of the lipidated form of LC3 (LC3-II) [72]. Although physiologic levels of autophagy are essential for normal cell function, excessive levels have been shown to induce cell death [73]. These findings suggest that one could use patient-derived *TBK1*-deficient retinal ganglion cells in a high-throughput screen to identify the small molecules capable of regulating autophagy and preventing ganglion cell death.

USING iPSCs TO MODEL AMD

AMD is one of the leading causes of blindness in people older than age 50 in the developed world [74]. The genetic component to this

complex disease is significant and has been estimated to explain 45%–71% of the variation in disease severity [75]. To date, 19 genetic risk loci have been identified with genome-wide significance [76]. Drug therapy using agents that inhibit vascular endothelial growth factor has been quite effective for the neovascular form of the disease [77–79]. However, no similarly efficacious treatment has been developed for the non-neovascular form, which accounts for as much as 90% of the disorder. The high prevalence and mechanistic complexity of this disease might limit the use of conventional gene therapy for the prevention of this condition. However, strategies designed to arrest vision loss or even restore vision by replacing lost RPE cells are under intense investigation [27, 53, 54, 80–84]. If one could better understand how different genetic risk factors contribute to the disease pathogenesis, one could conceivably develop preventative small molecule therapies that could have a major impact on the disease. For example, in a recent study by Yang et al., iPSC-derived RPE cells were generated from patients with both high- and low-risk 10q haplotypes, and these were exposed to A2E for prolonged periods of time [85]. The investigators identified a reduction in antioxidant defense mediated by superoxide dismutase 2 in cells generated from patients with the high-risk haplotype [85]. These findings suggest that one or more of the genetic variants within the 10q locus (a nonconservative polymorphism in *ARMS2* [Ala69Ser], a complex 144-base pair [bp] deletion and a 54-bp insertion in the 3′ UTR of *ARMS2*, and a promoter polymorphism in the *HTRA1*) induce RPE cell death via oxidative stress. These findings suggest that drugs specifically targeting oxidative stress pathways might be useful for the treatment of AMD and that cells derived from patients with high-risk haplotypes could be used to demonstrate such benefit.

The variants within the 10q locus are in strong linkage disequilibrium. That is, only a few percentage of 10q alleles that harbor *ARMS2* Ala69Ser lack the *HTRA1* promoter variant and vice versa. Thus, it has been difficult to determine the specific contribution made by each variant to the pathogenesis of AMD. To unravel this mystery, one could generate iPSCs from AMD patients homozygous for the low-risk 10q haplotype and then use clustered regularly interspaced short palindromic repeats (CRISPR)-mediated genome editing to create patient-specific cell lines with each of the *ARMS2* and *HTRA1* variants in isolation. After differentiation into the appropriate AMD-specific cell types (e.g., iPSC-RPE generated from an AMD patient with the low-risk 10q haplotype [Fig. 2A]; and iPSC-choroidal endothelial cells generated using a *Tie2*-green fluorescent protein endothelial cell reporter line [Fig. 2B]), one could independently analyze the effect of *ARMS2* and *HTRA1* mutations on cellular function.

USING iPSCs TO MODEL MENDELIAN DISEASES OF THE OUTER RETINA

Diseases such as retinitis pigmentosa (RP), LCA, Usher syndrome, and Best disease are all the product of single gene defects. These disorders are inherited in dominant, recessive, and x-linked fashions and vary significantly in the age of onset, severity, and pathophysiologic mechanism. With the growing ability to replace and repair defective genes through gene augmentation and CRISPR-based genome editing, it is increasingly important to be able to accurately identify and understand how individual patients' genetic variants cause their disease. However, as noted, it is often difficult to be certain whether newly identified genetic mutations

are truly pathogenic, especially in situations in which the disease-causing gene is associated with a very small fraction of a rare disease, and when the mutation is a rare or novel missense variant.

PHOTORECEPTOR CELL DISORDERS

During the past 5 years, significant progress has been made in developing patient-specific iPSC model systems to investigate the pathogenesis of Mendelian retinal degenerative disorders. In 2011, we published one of the early examples of the use of patient-specific iPSC-derived photoreceptor precursor cells, generated from dermal fibroblasts, to demonstrate how mutations in a newly identified *RP* gene cause disease [26]. In that study, next-generation and Sanger sequencing were used to identify a novel homozygous Alu insertion in exon 9 of the gene encoding male germ cell-associated kinase (*MAK*) [26]. Using iPSC-derived retinal cells, we were able to demonstrate that this novel mutation induced loss of the *MAK* transcript, presumably via nonsense-mediated decay [26]. We also discovered that the retina, unlike any of the other tissues that were investigated, has a novel splice variant that contains an extra 75 bp in frame, which is now known as exon 12 [26]. Expression of this retinal-specific exon was found to be dependent on expression of the exon 9-containing splice form. Not surprisingly, loss of the retina-specific transcript resulted in the inability to produce the full-length retinal-specific MAK protein [26].

Using a similar strategy, we recently used iPSC-derived photoreceptor precursor cells to confirm the pathogenicity of a novel intronic *USH2A* variant in a patient with nonsyndromic RP [27]. In that study, patient-specific, keratinocyte-derived iPSCs were differentiated into laminated eyecup-like structures containing RPE and photoreceptor precursor cells that expressed the mature photoreceptor cell markers recoverin and rhodopsin [27]. Analysis of the patient's *USH2A* transcripts revealed that the novel IVS40 mutation induced exonification of a portion of the intron, which in turn created a translation frameshift and a premature stop codon [27]. This mutation, in conjunction with the mutation on the patient's opposite allele (Arg4192His), was found to cause elevated levels of ER stress-related proteins in the patient's photoreceptor precursor cells, suggestive of protein misfolding, a common pathophysiologic mechanism in other forms of RP. In a similar study, Jin et al. demonstrated that patient-specific photoreceptor precursor cells derived from fibroblasts, generated from patients with autosomal dominant rhodopsin-associated RP, underwent premature ER-stress-induced cell death [86]. The idea that the *USH2A* variants identified in our patient act via a postdevelopmental process is supported by the finding that after transplantation into immunodeficient retinal degenerative mice, the patient's photoreceptor precursor cells developed into morphologically normal photoreceptors with discernable inner and outer segments.

In addition to using iPSCs to determine the pathogenicity of novel mutations, disease-specific phenotypes are proving invaluable for evaluating novel drug and gene-based therapeutics. For example, in a recent study, we demonstrated that patient-specific fibroblasts and, in turn, iPSC-derived retinal progenitor cells could be used to test the efficacy of a lentiviral-based gene augmentation approach for the treatment of *CEP290*-associated LCA [87]. The *CEP290* gene encodes a 290 kDa centrosomal protein. Mutations in this gene have been shown to inhibit primary cilia formation and elongation [87, 88]. Using cells obtained from patients

with *CEP290*-assciated LCA, we have demonstrated the ability to restore wild-type CEP290 protein and, in turn, rescue the disease-associated ciliogenesis defects. Importantly, by using fibroblasts obtained from patients with various combinations of *CEP290* alleles, we were also able to demonstrate that CEP290 is stoichiometrically sensitive (i.e., overexpression of *CEP290* is toxic) [87]. Lustremant et al. performed a genome-wide transcriptome analysis of LCA-human iPSC derived from patient fibroblasts to uncover the molecular mechanism and potential treatment targets [89]. Another study used a patient-specific iPSC model of rhodopsin-associated RP derived from fibroblasts to screening therapeutic reagents as a method to reduce the ER stress of photoreceptor cells [90].

RPE DISORDERS

The vision loss associated with RPE gene defects typically results from disruption of the visual cycle and/or secondary loss of photoreceptor cells. One of the most notable RPE-specific diseases is *RPE65*-associated LCA, an autosomal recessive disorder characterized by significantly reduced visual acuity, nystagmus, and night blindness that is first noticeable shortly after birth. *RPE65* encodes a protein that catalyzes the isomerization of all-trans-retinyl to the light-sensitive chromophore 11-cis-retinal [91]. Loss of *RPE65* results in an inability to reactivate rhodopsin and thus an inability of the rod photoreceptor cells to detect light [91]. As this disease progresses, both RPE and photoreceptor cells are lost, and irreversible blindness ensues. The recent success of gene augmentation for the treatment of this disorder [92–95] underscores the need for rapid and accurate identification of a patient's disease-causing genetic variants. In a recent study, we were able to show how iPSC-derived RPE generated from a 2-year-old girl of Haitian ancestry could be used to investigate the pathogenicity of a novel mutation in intron 3 of *RPE65* (IVS3-11) [96]. Specifically, we were able to demonstrate that the novel IVS3-11 mutation interfered with normal splicing, inducing a frame shift and insertion of a premature stop codon in the patient's *RPE65* transcript.

In addition to *RPE65*-LCA, several groups have demonstrated the utility of iPSC-RPE cells for pathophysiologic investigation of other degenerative disorders. A recent report from Meyer et al. showed that iPSC-derived RPE cells generated from a patient with mutations in *OAT1*, a gene known to cause the retinal degenerative disorder gyrate atrophy, were found to have low OAT activity, which could be enhanced by the addition of vitamin B_6 [97]. When the same experiment was performed using an *OAT1* line that the investigators had previously corrected using a bacterial artificial chromosome-mediated homologous recombination [98], they found that vitamin B_6 supplementation had little to no effect on OAT activity [97]. A study by Li et al. demonstrated that iPSC-derived RPE cells generated from a patient with *MFRP*-associated RP have distinct abnormalities in their actin cytoskeleton, cellular pigmentation, and transepithelial resistance [99]. Delivery of wild-type *MFRP* via adeno-associated virus 8 successfully rescued this cellular phenotype [99]. Another group generated patient-specific iPSC-derived RPE from fibroblasts cells to study an *RP2* stop mutation, which leads to a loss of endogenous protein levels [100]. Using a translational read-through inducing drug, the protein was restored to 20% of endogenous levels and the cellular phenotype was reversed [100]. A study by Singh et al. showed that dominant mutations in *BEST1,* a gene responsible for Best macular dystrophy, caused abnormal fluid flux and an increased

accumulation of autofluorescent material after long-term feeding of photoreceptor outer segments (POSs) [66]. In addition, degradation of rhodopsin, a normal RPE function, was delayed compared with cells derived from normal individuals [66]. These investigators also noted that after POS feeding, intracellular calcium homeostasis was disrupted and oxidative stress increased, indicated by a decrease in the enzymes GPX1, SOD2, Trf, and TrfR [66]. Collectively these findings implicate impaired POS handling in Best disease.

CONCLUSION

As ophthalmology enters the era of patient-centered precision medicine, the ability to generate disease-specific cell types that would be otherwise inaccessible will become increasingly important. Not only will they greatly accelerate our ability to evaluate novel drug and gene-based therapies, they will also, in some cases, allow the restoration of vision by replacing cells that have already been lost to disease. One can begin to envision a multimodal, reusable-parts strategy, which could be used regardless of the stage or rarity of the patient's disease. For those who present early in the course of their disease, the primary goal will be to arrest the disease and prevent vision loss. The most promising method to date has been gene augmentation, but this requires accurately identifying the patient's disease-causing genetic variants and ensuring that overexpression of the gene will not be harmful. For individuals with significant vision loss at the time of presentation, a combined gene augmentation and cell replacement approach could be useful. For patients who have already lost most of their vision due to photoreceptor cell death, conventional gene augmentation will not be helpful. In such cases, very advanced restorative strategies such as polymer-supported grafts with multiple cell types are likely to be needed. As discussed throughout the present review, patient-specific iPSCs will play important roles in each step of this strategy. iPSCs will be used to investigate the pathogenesis of newly identified genetic variants, test the efficacy of novel therapies, and when combined with CRISPR-based genome editing, provide an autologous cell source for vision-restoring transplantation.

AUTHOR CONTRIBUTIONS

J.C.G. and E.R.B.: conception and design, manuscript writing; L.A.W. and R.F.M.: conception and design, manuscript writing, collection and/or assembly of data; A.E.S.: collection and/or assembly of data; E.M.S.: conception and design, manuscript writing, collection and/or assembly of data, final approval of manuscript; B.A.T.: conception and design, manuscript writing, final approval of manuscript.

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