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Stem cells for investigation and treatment of inherited retinal disease

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Vision is the most important human sense. It facilitates every major activity of daily living ranging from basic communication, mobility and independence to an appreciation of art and nature. Heritable diseases of the retina, such as age-related macular degeneration and retinitis pigmentosa, are the leading cause of blindness in the developed world, collectively affecting as many as one-third of all people over the age of 75, to some degree. For decades, scientists have dreamed of preventing vision loss or of restoring the vision of patients affected with retinal degeneration through some type of drug, gene or cell-based transplantation approach. In this review, we will discuss the current literature pertaining to retinal transplantation. We will focus on the use of induced pluripotent stem cells for interrogation of disease pathophysiology, analysis of drug and gene therapeutics and as a source of autologous cells for cell replacement.

INTRODUCTION

Vision is the most important human sense. It facilitates every major activity of daily living ranging from basic communication, mobility and independence to an appreciation of art and nature. Heritable diseases of the retina, such as age-related macular degeneration and retinitis pigmentosa, are the leading cause of blindness in the developed world, collectively affecting as many as one-third of all people over the age of 75, to some degree. For decades, scientists have dreamed of restoring the vision of patients affected with retinal degeneration through some type of transplantation of new retinal tissue. Unlike solid organ transplants, which only require the reanastomosis of large vessels, ducts and airways, the transplantation of a whole eye would require the restoration of more than a million axonal connections between the inner retina and the lateral geniculate nucleus of the thalamus, situated several centimeters away. At the present time, the problem of inducing mature mammalian central nervous system (CNS) neurons to successfully and accurately traverse significant distances through inhospitable extracellular environments has not yet been solved. Fortunately, many degenerative retinal diseases spare the inner retina and optic nerve (1,2), reducing the synaptic distance challenge from centimeters to microns. As a result, the majority of the focus in the retinal transplantation field has been on replacement of photoreceptors and retinal pigment epithelial (RPE) cells. Initially, it was thought that intact outer retinal sheets, i.e. photoreceptors with or without attached RPE cells (Fig. 1A), isolated from mature donor retina would be the optimal choice for retinal transplantation. However, the gliosis associated with such outer retinal degenerations (Fig. 1B) significantly limits post-transplant connectivity between host and graft (3). Fortunately, unlike mature photoreceptor cells, retinal stem cells are quite efficient at integrating into and making new connections with the degenerative host retina (3). In addition to the worthy goal of curing blindness, stem cell transplantation in the eye can also be thought of as a model system for investigating cell-based treatments for other degenerative disorders of the CNS. The eye has numerous advantages as a platform for this work. First, all tissues of the eye are surgically accessible, and transplanted cells can be monitored at near-microscopic resolution *in vivo*. Second, the inherent amplification of the visual system means that a relatively small number of rescued or transplanted cells often have a detectable (and clinically meaningful) effect on vision. Finally, genetic disorders of the eye are so numerous that multiple examples exist of conditions that primarily affect photoreceptor cells, ganglion cells, RPE cells, retinal vasculature, choroidal vasculature and eye development. Many of

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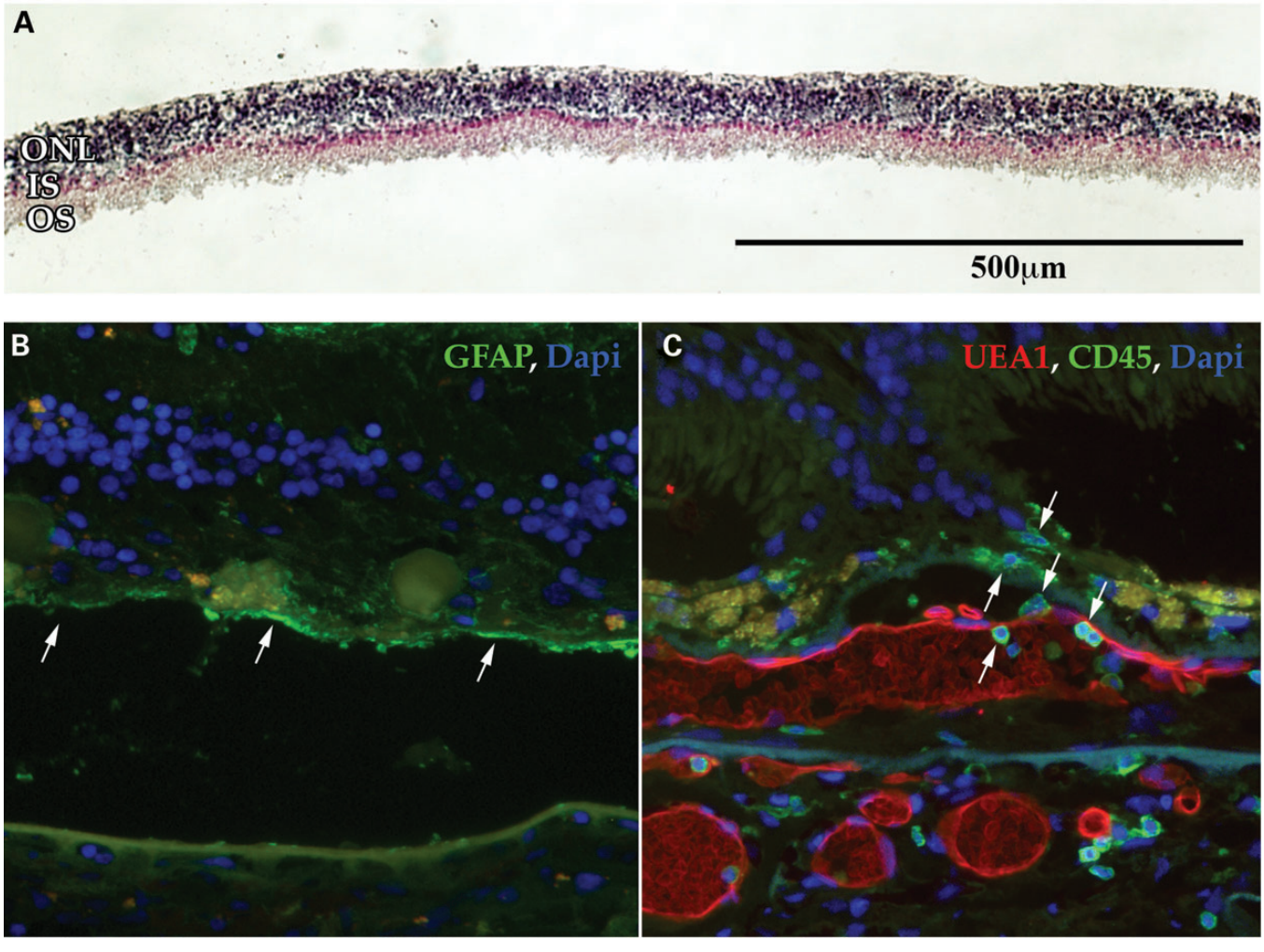

Figure 1. Retinal transplantation. (**A**) H&E staining of a photoreceptor sheet isolated from pig retina. Photoreceptor sheets can readily be isolated via vibratome sectioning and prepared for subretinal transplantation. Resulting sheets are highly organized with proper retinal structure including photoreceptor inner- and outer-segments. (**B** and **C**) Immunhistochemical analysis of human maculas with retinal degeneration (B, Best disease and C, age-related macular degeneration) with antibodies targeted against the gliosis marker GFAP (**B**), the vascular stain UEA1 (**C**) and the lymphocyte antigen CD45 (C). Degeneration of the outer nuclear layer results in glial cell activation, outer retinal scar formation and infiltration of CD45 positive immune cells. ONL, outer nuclear layer; IS, photoreceptor cell inner segments; OS, photoreceptor cell outer segments.

these conditions have pathophysiologic similarities to other CNS disorders that are less surgically and optically accessible.

USE OF STEM CELLS FOR RETINAL REGENERATION

Stem cells are an attractive option for retinal cell replacement, because they are pluripotent (i.e. they have the potential to become any cell type in the body) and they have an unlimited capacity for self-renewal (i.e. they can divide an unlimited number of times with each daughter cell being identical to its parent). Initial subretinal transplant studies employed tissue-specific stem cells, known as retinal progenitors, that can be isolated from the developing retina during the peak of rod photoreceptor cell histogenesis [postnatal Day 0 in mice, (4)]. For example, several groups have demonstrated that subretinal transplantation of green fluorescent protein-positive retinal progenitor cells into retinal degenerative recipients results in migration of the transplanted cells into the outer nuclear layer, differentiation into immunohistochemically identifiable rod photoreceptor cells and functional rescue of blindness in the form of improved pupillary light responses (5). Extension of these initial studies revealed that post-mitotic photoreceptor precursor cells, which are isolated a bit later than retinal progenitors [postnatal Day 4–5 in mice, (4,6)], are optimal for retinal transplantation in that they have the greatest capacity for cellular integration and restoration of retinal structure (6,7). Unfortunately, in human embryonic development, both retinal progenitor and photoreceptor precursor cells are generated relatively late [i.e. between 14 and 24 weeks of gestation, (8–11)]. The serious ethical implications of this fact, coupled with the practical reality of the relatively small number of photoreceptor precursor cells that can be obtained from a pair of fetal donor eyes make it quite unlikely that this cell type will be used to any meaningful extent in the treatment of human retinal disease.

At present, the two most promising options for human retinal transplantation are: (i) embryonic stem cells (ESCs), which can

be isolated from developing embryos ~4–5 days after fertilization (12) prior to implantation and (ii) induced pluripotent stem cells (iPSCs), which can be generated from adult cells like dermal fibroblasts via viral transduction of a variety of transcription factors including Oct4, Sox2, Klf4 and c-Myc. Both cell types can be expanded to yield sufficient cell numbers for clinical applications and both have the capacity to generate tissues of the three primary germ layers. To date, differentiation protocols capable of generating photoreceptor precursor cells from both ESCs and iPSCs have been developed (13–23). Likewise, cellular integration and restoration of retinal structure and function have been demonstrated in mouse models of retinal degeneration following subretinal transplantation of retinal precursor cells derived from both ESCs and iPSCs (13,15,16,24).

One potential advantage of iPSC-derived retinal precursor cells is the opportunity to derive them from the patients for whom the transplants are destined; thereby reducing the risk of post-transplant attack by the host immune system. The eye is generally considered an immune privileged organ because the inflammatory responses of a healthy eye differ markedly from those in other tissues. For example, the anterior chamber of the eye exhibits a striking tolerance of antigens injected into the front of the eye [i.e. the anterior chamber-associated immune deviation (25,26)]. Allogenic transplantation of the avascular cornea is a commonly performed and highly successful procedure that requires no human leukocyte antigen matching of donor and recipient, and nothing more than topical immunomodulation. Although the retina does not enjoy the same degree of immune privilege as the anterior chamber, in the healthy state it is nonetheless 'immune-advantaged' compared with other tissues in the body. The blood–retinal barrier (a component of the blood–brain barrier) consists of a non-fenestrated retinal vasculature ensheathed by pericyte and astrocyte processes on the inner aspect and by tight junctions between adjacent RPE cells on the outer aspect. In the healthy state, this blood–retinal barrier would likely afford some protection from the systemic immune system to cells transplanted within or beneath the retina. However, such protection is much less likely to occur in patients with advanced retinal degenerative disease for at least two reasons. First, the maintenance of the blood–retinal barrier requires an intact monolayer of RPE cells with intact tight junctions. In most degenerative diseases of the retina, the RPE is significantly injured. For example, studies by our group using human donor eyes have shown loss of RPE in a wide range of retinal diseases including age-related macular degeneration (Fig. 1C), *ABCA4*-associated photoreceptor degeneration and Best disease (1,27). Second, there is evidence for immune surveillance in the subretinal space, and the retina becomes much more pro-inflammatory in acquired or inherited photoreceptor degenerations (1,28,29). There is a particularly extensive body of literature demonstrating a major role for inflammation in the pathogenesis of age-related macular degeneration (30,31). There is also some evidence that genetic or pharmacological modulation of the immune response may be beneficial in some types of photoreceptor disease (32).

Thus, while allogenic cells may be tolerated in an otherwise healthy retina, cells transplanted into a retina damaged by disease are likely to be at much higher risk of immunological attack. As a result, we believe that transplantation of iPSC-derived autologous cells currently represent the best approach for restoring vision in the advanced stages of retinal degenerative diseases. Although the use of patient-specific iPSCs will reduce the likelihood of immunological injury to a transplant, for patients affected with a genetic retinal disease, the newly transplanted cells would be at risk of succumbing to the same mutations unless these mutations were corrected in some fashion before transplantation. This would be especially true for disorders with a very early onset of disease. As discussed more fully below, the expression of a genetic disease in patient-derived retinal cells does have one useful attribute: it can be used to investigate the pathophysiology of rare diseases and develop effective treatments for them.

iPSCs FOR DEVELOPMENT OF GENE AND DRUG THERAPY

There are now numerous examples from animal models (33–49), and a few from human clinical trials (50,51), of successfully mitigating an inherited eye disease with a gene-replacement or gene silencing strategy. A sobering aspect of this otherwise exciting progress is the extremely slow pace of moving these therapies from a 'proof of concept' stage in animals to a fully approved treatment that is available to anyone who needs it. For example, convincing efficacy of RPE65 gene replacement was demonstrated in dogs in 2001 (52), and 13 years later, fewer than 100 patients have been treated world-wide and the phase 3 clinical trial is still underway. This raises the possibility that some diseases might be so rare in the population that it is not economically possible to bring a treatment through the regulatory gauntlet and into clinical availability. An additional concern is that some genes will have a very narrow range of therapeutic 'dose' such that overexpression could be as harmful as underexpression (34). If the ideal therapeutic expression level of a therapeutic gene is different in different patients (because of differences in genotype or genetic background), it is hard to imagine how such a treatment could be commercially viable. One possible solution for disorders that are so rare that they are below the commercial viability threshold is to use cells or tissues created from a patient's own cells to test the molecular efficacy of a viral-mediated gene therapy—including the optimal level of expression—and then deliver that therapy to one eye in a compassionate use manner. Patients treated in this way can be followed at intervals using conventional clinical measures such as visual acuity, Goldmann perimetry, ganzfeld electroretinography and optical coherence tomography. Whenever treated eyes fare worse than the untreated ones, the compassionate use treatment can be stopped until the reason for the poorer outcome can be identified and an improved treatment can be devised. Whenever treated eyes do noticeably better than untreated eyes over time, the treatment can continue to be offered to new patients and the second eyes of the initial patients can also be treated. In this manner, a series of increasingly predictable, reusable parts (vectors, promoters and surgical approaches) can be assembled and used to devise a compassionate use therapy for retinal disorders, regardless of their rarity. For diseases such as Batten disease and mucolipidosis type 4 that affect other parts of the CNS in addition to the retina, the treatment can be tried first in the very accessible and assessable retina, and if it shows a therapeutic benefit there, the treatment can be tried in other parts of the

CNS affected by the disease. Retinal cells derived from iPSCs are sufficiently faithful representatives of their *in vivo* counterparts (53) that they also present an excellent avenue for identifying and evaluating pharmacological agents capable of mitigating degenerative retinal diseases. High-throughput drug screens of differentiated iPSC-derived cells have been suggested as a means for drug discovery and personalized medicine (54). Screening extra-ocular neuronal cells derived from iPSCs from patients with rare diseases has shown excellent promise (55,56). Combining the power of differentiated ocular cells with high-throughput screens will likely prove effective for discovering new pharmacological treatments for delaying the progression of retinal degenerative diseases.

CRISPR-BASED GENOME EDITING

The use of genome editing tools, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system, is particularly attractive for treating genetic diseases caused by genes with very specific spatial and stoichiometric expression. That is, by editing the mutations in the patients' genomic DNA through double strand break (DSB) induction and subsequent homology-directed repair (HDR), the corrected gene will remain under the normal endogenous expression control elements (i.e. promoters, enhancers and repressors); thereby avoiding expression in inappropriate cell types and incorrect levels of expression that can occur with conventional viral-mediated gene replacement.

Of these three technologies, the CRISPR/Cas system is particularly attractive for therapeutic purposes. Unlike ZFNs and TALENs, which use protein-based DNA targeting motifs, the guide RNAs used for CRISPR-based genome editing can be generated relatively easily. The guide RNAs used in this system require the presence of an NGG sequence, termed a protospacer adjacent motif, downstream of a 17–20-nucleotide DNA target, termed the protospacer (57–61) (Fig. 2). Complementary guide oligonucleotides can be synthesized, annealed and subsequently ligated into a bicistronic vector expressing scaffold RNA and a modified Cas9 nuclease optimized for efficient targeting of human cells. Combined with the use of commercially available gene blocks for the efficient generation of HDR constructs, CRISPR-based genome editing is a relatively straightforward technology for correction of genetic mutations in patient-specific cells *in vitro*. The utility of this approach was recently demonstrated by Mali *et al.* who employed humanized CRISPR/Cas reagents to target the endogenous AAVS1 locus

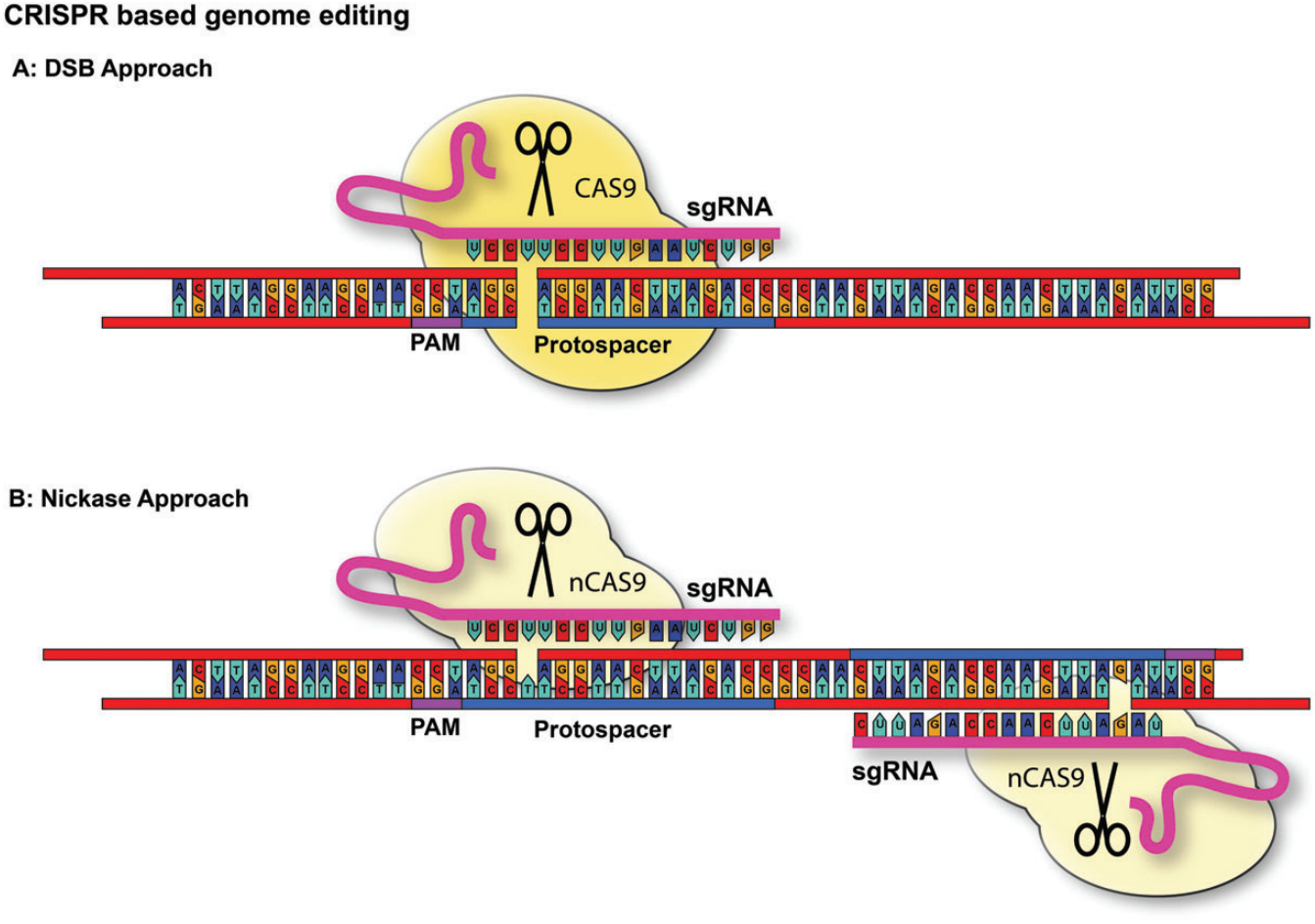

Figure 2. Schematic diagram depicting the functionality of the CRISPR/Cas9 system. (**A**) Use of wildtype humanized Cas9 and CRISPR RNA for induction of double strand breaks. (**B**) Use of modified Cas9 (nCas9) and CRISPR RNA for induction of single-strand nicks. PAM, protospacer adjacent motif; sgRNA, single-guide RNA.

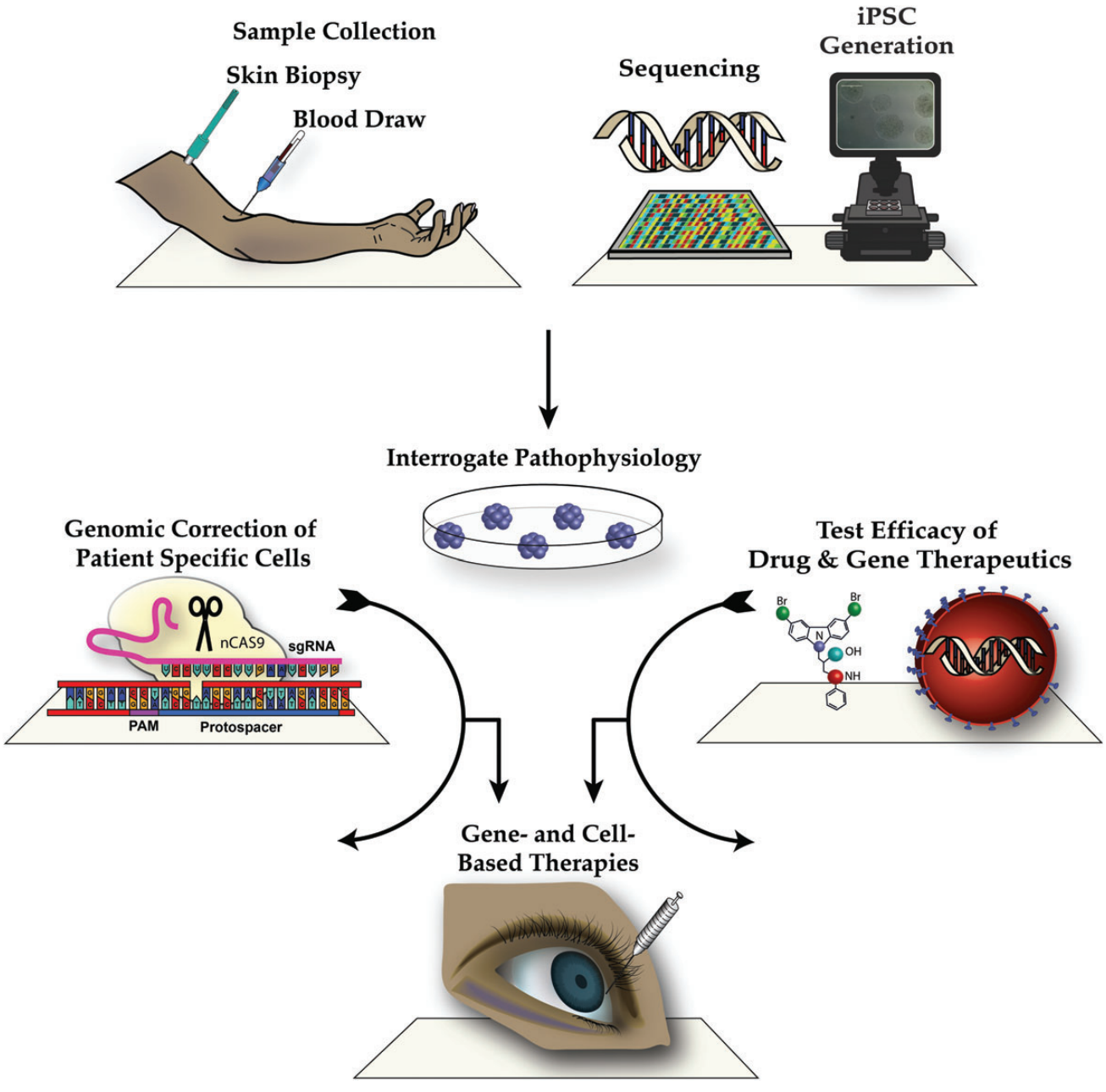

Figure 3. Schematic diagram summarizing the proposed pipeline from patient identification to gene discovery and treatment of inherited retinal degenerative disease.

in human iPSCs (58). They successfully achieved HDR of fibroblast-derived iPSCs generated from participants in the personal genome project (58).

CRISPR/Cas targeting can tolerate significant mis-pairing of guides and genomic DNA, and as a result, induction of DSBs in undesired locations is of some concern (57,62–64). However, strategies to significantly decrease the risk of off-target DSBs are being developed. For example, Ran *et al*. recently demonstrated that by mutating a single amino acid in the catalytic domain of the Cas9 nuclease, they could generate a 'nicking' enzyme that only cleaves a single strand of DNA. The majority of single-strand breaks are repaired by endogenous mechanisms in the base excision repair (BER) pathway (65). Thus, by employing paired 'nickases' that recognize sequences separated by 4 to 20 base pairs (Fig. 2B), Ran *et al*. were able to achieve efficient (>40%) modification of three distinct genetic loci with a 200–1500-fold increase in specificity (66). These findings were extended *in vivo* by co-injecting guide RNAs targeting the *Mecp2* locus with mutant Cas9 mRNA into single-cell mouse zygotes. These experiments yielded >80% modification at the intended locus (66). Collectively, these experiments demonstrate the potential utility of employing nickases to increase the specificity and safety of the CRISPR/Cas genome editing technology for both *in vitro* and *in vivo* studies.

FUTURE

Stem cells are likely to make important contributions to the cure of human blindness on both sides of the commercial viability threshold. However, their most important role is likely to be in the development of therapies for conditions that are so rare (e.g. one person per million) that the involvement of for-profit entities is simply not possible. For such diseases, we envision stem cells being used to: (i) identify disease mechanisms; (ii) demonstrate the pathogenicity of unusual mutations in individual patients; (iii) test the efficacy of gene- and drug-based therapies and (iv) replace lost outer retinal elements in patients with advanced stages of degenerative disease. The enabling infrastructure for this approach would be small, dedicated cGMP laboratories based in academic institutions where viral gene therapy vectors and patient-specific, genetically corrected, iPSC-derived retinal precursor cells can be made. Figure 3 summarizes the steps in this process. Skin biopsies and blood draws will be offered to every new patient suspected to have an inherited retinal disease. The blood sample will be used to look for the patient's disease-causing mutation(s) using a combination of conventional allele-specific screening, single gene automated DNA sequencing, and exome sequencing depending on the specific clinical findings. The skin biopsy will be used to create a patient-specific cell line. These cells will be used to help demonstrate the pathogenicity of unusual mutations that are identified in the blood samples. They will also be used to test the efficacy of gene- and drug-based therapies for the patient's disease, or to develop transplantable retinal cells if the patient has sufficiently advanced disease to warrant them.

Conflict of Interest statement. None declared.

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