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REVIEW

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Genome Surgery and Gene Therapy in Retinal Disorders

Lawrence Chan[a,*], Vinit B. Mahajan[b,c], and Stephen H. Tsang[d,e,f]

[a]*Yale School of Medicine, New Haven, CT;* [b]*Byers Eye Institute, Omics Laboratory, Department of Ophthalmology, Stanford University School of Medicine, Palo Alto, CA;* [c]*Palo Alto Veterans Administration, Palo Alto, CA;* [d]*Jonas Children's Vision Care, and Bernard & Shirlee Brown Glaucoma Laboratory, Department of Ophthalmology, Columbia University Medical Center, New York, NY;* [e]*Edward S. Harkness Eye Institute, New York-Presbyterian Hospital, New York, NY;* [f]*Department of Pathology & Cell Biology, Institute of Human Nutrition, College of Physicians and Surgeons, Columbia University, New York, NY*

The emergence of genome surgery techniques like the clustered regularly interspaced short palindromic repeats (CRISPR†) editing technology has given researchers a powerful tool for precisely introducing targeted changes within the genome. New modifications to the CRISPR-Cas system have been made since its recent discovery, such as high-fidelity Cas9 variants to reduce off-target effects and transcriptional activation/silencing with CRISPRa/CRISPRi. The applications of CRISPR-Cas and gene therapy in ophthalmic diseases have been necessary and fruitful, especially given the impact of blinding diseases on society and the large number of monogenic disorders of the eye. This review discusses the impact that CRISPR-Cas has had on furthering our understanding of disease mechanisms and potential therapies for inherited eye diseases. Furthermore, we explore a brief overview of recent and ongoing gene therapy clinical trials in retinal diseases, and conclude with the implications of genome surgery on the outlook of future therapeutic interventions.

INTRODUCTION

Although genome surgery techniques such as transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs) have existed as early as the 1990s, the recent discovery of the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas system has sparked a great amount of excitement in the scientific and medical communities with its precision, affordability, and ease of use in editing genes. The CRISPR-Cas system has been used not only to facilitate the generation of *in vitro* and *in vivo* models for the study of inherited diseases, but also to explore

*To whom all correspondence should be addressed: Lawrence Chan, Yale School of Medicine, New Haven, CT, Email: lawrence.chan@yale.edu.

†Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; TALENs, transcription activator-like effector nucleases; ZFNs, zinc finger nucleases; OCT, optical coherence tomography; ERG, electroretinography; DSB, double-stranded breaks; HR, homologous recombination; NHEJ, non-homologous end joining; crRNA, CRISPR RNA; tracrRNA, trans-activating CRISPR RNA; PAM, protospacer adjacent motif; sgRNA, single guide RNA; dCas9, catalytically dead Cas9; CRISPRi, CRISPR interference; CRISPRa, CRISPR activator; RP, retinitis pigmentosa; adRP, autosomal dominant retinitis pigmentosa; iPSC, induced pluripotent stem cell; AMD, age-related macular degeneration; VEGF, vascular endothelial growth factor; ELISA, enzyme-linked immunosorbent assay; LCA, Leber congenital amaurosis; RPE, retinal pigment epithelium; AAV, adeno-associated virus; RCL, replication competent lentivirus; EIAV, equine infectious anemia virus; CHM, choroideremia; USH1, Usher syndrome type 1.

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potential treatments such as gene therapy and stem cell transplantation for these genetic disorders.

One area of medicine that has been at the forefront of gene therapy development is ophthalmology. The eye has several anatomical and pathophysiological features that lend itself well to gene surgery studies: 1) it is easily accessed by standard instruments and techniques for injection of gene surgery vectors; 2) its disease progression and treatment response can be monitored *in vivo* and longitudinally using non-invasive imaging and function-assessing modalities (*e.g*., optical coherence tomography (OCT), ophthalmoscopy, electroretinography (ERG)) [1]; 3) it is ideal for animal experiments and clinical trials in determining efficacy of treatment because the eyes are one of the few naturally paired organs where the untreated eye can serve as an ideal internal control [2]; 4) the eye is relatively immune-privileged through a reduction in antigen-presenting cells and immunomodulatory factors in the vitreous humor, which allows it to better tolerate the administration of gene surgery vectors [3]; 5) many inherited forms of blindness are caused by different single-gene mutations, and this genetic heterogeneity gives researchers a unique opportunity to target a wide range of genes for correction, even within a single disease [4].

The societal impact and cost make efforts to cure blindness an urgent priority. Millions worldwide are affected by inherited retinal diseases and have significant struggles with quality of life, employment opportunities, and emotional and psychological well-being [5]. The economic burden of these diseases on patients and society is considerable with estimated global costs to be $3 trillion USD and increasing with the growth of the aging population [6].

Given the aforementioned importance and advantages of studying inherited ophthalmic diseases using genome surgery techniques, research in this area has been progressing at a rapid pace over the past few years. This article will explore the brief history of genome surgery and then focus on the latest applications of the CRISPR-Cas surgery system in the study of retinal disease. We will also look at an overview of gene replacement therapy clinical trials for retinal disease that are ongoing or planned for the near future. Finally, we will conclude with a short discussion on the future directions of genome surgery.

OVERVIEW OF GENOME SURGERY AND GENE THERAPY TECHNIQUES

Ever since landmark experiments on bacterial transformation in the 1920s, scientists have been utilizing transfers and stable modifications to DNA sequences to study the relationship between genotypes and phenotypes [7]. Yeast studies in the 1980s showed that the induction of double-stranded breaks (DSBs) can lead to repair through a process called homologous recombination (HR) [8]. In HR, two identical or similar strands of DNA exchange nucleotide sequences between DNA loci, giving researchers a way to deliver artificial genetic repair template to correct a mutation or add new biological traits. An alternative to repairing DSBs is non-homologous end joining (NHEJ), in which the ends are ligated without requiring a homologous template to guide repair. NHEJ occurs more frequently, but is error-prone and can result in nucleotide insertions and deletions (indels) that result in frameshift mutations or knockout of gene function [9].

Researchers also discovered that they could engineer different endonucleases with affinities for cutting DNA at certain sequences. One of the first engineered endonucleases was the zinc-finger nuclease, which is composed of zinc finger motifs that bind to triplets within the DNA substrate [10]. An alternative to ZFNs is transcription activator-like nucleases (TALENs). The structure of TALENs is similar to that of ZFNs, comprised of an engineerable DNA-binding domain derived from transcription activator-like effectors (proteins secreted by *Xanthomonas* spp. bacteria). These proteins recognize individual base pairs in the DNA through specific tandem repeats [11]. The advantages of TALENs over ZFNs are that they can be custom-engineered with greater ease, affordability, and speed, while maintaining similar levels of efficacy with use.

Most recently, the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas system has emerged as a new tool for genome surgery. The CRISPR repeats were first identified in bacteria and archaea as a method to develop immunity towards invading viruses [12]. The bacterium cleaves the genome of the invading virus and incorporates short viral genetic segments into its own genome, keeping these segments in between short, repetitive sequences called CRISPR. When the same virus tries to re-infect the bacterium, CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) guide the organism's Cas endonuclease to foreign DNA that matches its sequence, thereby degrading the invading viral genome [13]. A protospacer adjacent motif (PAM) sequence in the foreign genome allows the bacterium to distinguish between self and non-self. These motifs are found only in the invading viral DNA and not in the bacterial CRISPR loci; thus, the Cas endonuclease will not cleave the target DNA sequence if the PAM sequence is not present [14]. In 2012, Jinek *et al*. [15] discovered that the Type II CRISPR-Cas system of *Streptococcus pyogenes* could be utilized for genome surgery. They combined the crRNA and tracrRNA guides into a chimeric "single guide" RNA (sgRNA) that could guide the *S. pyogenes* Cas9 endonuclease (SpCas9) to create

DSBs with high specificity [15].

One of the advantages of using an RNA-based system like CRISPR-Cas is the simplicity in engineering custom guide RNAs for targeting within the genome, unlike the protein-DNA interfaces of ZFN and TALEN systems, which are dependent on proteins that are more difficult to construct for a given target [16]. Another feature unique to the CRISPR-Cas system is the ability for multiplexed genome surgery, or the simultaneous editing of multiple loci within the genome using multiple guide RNAs [17].

Despite the relative ease of using the CRISPR-Cas system, several limitations exist as a result of its properties. One example is the requirement that the desired cleavage site be adjacent to the proper PAM sequence. Moreover, studies have revealed that off-targeting effects may occur and create unwanted mutations at off-target sites that are similar to the on-target site sequence. The guide RNA and SpCas9 endonuclease can tolerate a certain degree of base mismatches at different positions in a sequence-dependent manner [18]. This off-targeting characteristic of CRISPR-Cas9 underscores the need to develop algorithms and screening tests to predict and identify unintended mutagenesis processes before any considerations for therapeutic use in a clinical context. One such screening test may be whole genome sequencing, an unbiased approach that can detect a variety of mutations [19].

One recent effort to address the off-targeting issue has been the creation of modified "high-fidelity" Cas9 variants (SpCas9-HF1) with decreased non-specific DNA binding events [20]. In their characterization of the SpCas9-HF1 variant targeted to non-repetitive sequences, Kleinstiver *et al*. [20] showed that virtually all genome-wide off-target effects were undetectable by GUIDE-seq and next-generation sequencing. Another approach to reduce the incidence of off-targeting has been the attenuation of the Cas9 endonuclease DSB activity to that of a DNA nickase (SpCas9n), which induces single-stranded breaks rather than DSBs [17]. By using two sgRNAs that target separate SpCas9n nickases to induce a DNA break in each strand, the likelihood of off-targeting is reduced since two separate cutting events are needed to induce a single DSB. The Cas9 endonuclease has also been modified to lack any endonuclease activity (dCas9, or catalytically dead Cas9). The complex of dCas9 and guide RNA can reversibly repress target genes (CRISPRi, or CRISPR interference) by silencing transcription initiation and elongation with no detectable off-target effects [21]. In addition to the gene repression activity of CRISPRi, the dCas9 nuclease can also be utilized as an RNA-guided transcriptional activator (CRISPRa, or CRISPR activator) by binding to the promoter regions of endogenous genes [22]. CRISPRa and CRISPRi are different from traditional CRISPR-Cas in that they act to induce/suppress genes at the transcriptome level and do not alter the genome.

GENE THERAPY VS GENOME SURGERY

Gene therapy delivers the gene as a drug and is a less precise yet more conventional alternative to genome surgery techniques like ZFNs, TALENs, and CRISPR. The rationale behind gene therapy is to deliver genetic material that either supplements a wild-type gene copy into patients with homozygous loss-of-function recessive mutations, or silences a gain-of-function dominant mutation. The gene of interest is introduced into the host cell in a viral vector, most commonly AAVs and lentiviruses. AAV, a 25-nm nonenveloped virus containing a linear-stranded DNA genome, is among the most frequently used vectors in ocular gene therapy because of several characteristics: 1) low immunogenicity and pathogenicity; 2) persistence of expression over time in the nucleus; and 3) ability to remain episomal without integration into the host genome [23]. However, one of its limitations is the relatively low carrying capacity of approximately 4.5 kb, which is an issue for delivering larger genes [24]. A strategy to overcome this issue has been to package the large gene into two independent AAV vectors using a trans-splicing approach [25]. Lentivirus, a genus of retrovirus, has the advantages of a larger carrying capacity (~8 kb) and the ability to infect both dividing and nondividing cells such as neurons [26]. The main drawback with lentiviral vectors is safety concerns, including risks of generating replication competent lentiviruses (RCLs) that self-propagate within the host, insertional mutagenesis from genome integration, and germline alterations that transmit the transgene to offspring [27].

Unlike gene surgery, gene therapy does not induce a controlled, stable alteration to the genome. As previously stated, adenoviruses do not integrate their DNA into the host genome, which may necessitate multiple treatments in order to reach efficacious levels of normally functioning protein. Retroviruses can insert genes into the genome but are imprecise with regards to where the gene is integrated. This raises the concerning possibility of insertional mutagenesis, whereby a tumor suppressor gene can be disrupted or an oncogene can be activated, leading to the development of cancer. Another weakness of gene therapy is that the size of the replacement gene is limited by the carrying capacity of the viral vector. Gene surgery can overcome this obstacle by directly correcting defective copies of the gene without the need for gene replacement. Finally, the gene therapy viral vector may elicit a potentially fatal immune response from the host, as was the case in the well-publicized death of Jesse Gelsinger during the infancy of gene therapy trials.

CRISPR-Cas IN RETINITIS PIGMENTOSA

In the study of retinal diseases, animal models have played important roles in the understanding of disease mechanisms, particularly in genetic disorders. One such disease is retinitis pigmentosa (RP), the most common genetic cause of progressive blindness, affecting 1 in 4000 worldwide [28]. It is a genetically heterogeneous disorder associated with mutations in at least 79 genes, and inherited through multiple modes of transmission (i.e., autosomal dominant, autosomal recessive, X-linked) [28]. Characterized by the progressive degeneration of rod and cone photoreceptor cells, RP initially presents as night blindness and peripheral vision loss, eventually resulting in loss of central vision later in life [29]. One of the most studied animal models of RP is the *Pde6b*^{rd1}/*Pde6b*^{rd1} ("rodless") mouse, whose mutation was discovered in 1924 by Keeler [30]. This mouse model has two homozygous mutations, an intronic insertion of a leukemia virus (*Xmv*-28) and an exonic nonsense point mutation (Y347X) in the *Pde6b* locus [31]. The *Pde6b* gene encodes for a protein complex that plays an important role in mediating the phototransduction cascade. The question of which mutation on the *Pde6b* gene is the causative variant in RP has been a longstanding and controversial one [32,33]. Using a single-stranded donor template, Wu *et al*. [31] performed CRISPR-mediated repair of the Y347X point mutation in the *rd1* mice. The corrected mice were found to have both structural (H&E staining, fundoscopy, optical coherence tomography) and functional (electroretinography) restoration to wild-type levels, suggesting that the Y347X mutation is pathogenic and the remaining *Xmv*-28 insertion is clinically irrelevant to the RP disease phenotype in these mice.

In addition to being used to elucidate causative mutations in RP pathogenesis, CRISPR-Cas has also been used in the study of therapeutic strategies for autosomal dominant retinitis pigmentosa (adRP). Bakondi *et al*. [34] successfully applied CRISPR-Cas9 gene surgery to ablate the dominant mutation (*Rho*^{S334}) on the rhodopsin gene of a rat model for severe adRP. The gain-of-function S334ter mutation results in an early termination, leading to truncation of the RHO peptide, prevention of normal photoreceptor deactivation following light exposure, and interference with proper trafficking to photoreceptor outer segments [35]. The team took advantage of a base pair difference between the wild-type and mutant *Rho* alleles (5′-TGC-3′ and 5′-TGG-3′, respectively) that presented a PAM sequence unique to the *Rho*^{S334} locus, allowing them to target with the Cas9 nuclease. Removal of the S334 mutation was found to be effective in preventing the accumulation of mutated proteins in photoreceptor cell bodies and restoring the trafficking of wild-type rhodopsin to the outer segments. Immunohistological studies revealed a decrease in retinal degeneration, and optokinetic testing of visual acuity showed improved visual acuity [34]. Thus, this was a proof-of-concept demonstration that CRISPR-Cas9 can be used to silence a dominant gain-of-function mutation *in vivo* and prevent retinal degeneration.

While Bakondi *et al*. [34] specifically targeted the 334ter mutation for ablation, Latella *et al*. [36] developed a strategy for editing any mutation on the human *RHO* gene. Using the P23H humanized transgenic adRP mouse model containing a human minigene, they demonstrated feasibility of using the CRISPR-Cas9 system to ablate the human *RHO* gene *in vivo*. The researchers combined two sgRNAs into a single effector plasmid designed to target opposite DNA strands, and the generation of small indels within the exon by NHEJ repair leads to frameshift mutations knocking out *RHO* transcription. Retinas transfected with the sgRNAs and Cas9 were found to have a significant decrease in RHO protein, supporting the feasibility of using CRISPR-Cas to knock-out gain-of-function mutations on human *RHO* via NHEJ frameshift mutations [36].

Another approach to potentially treating inherited diseases is by autologous transplantation of a patient's own stem cells that have been corrected for the underlying mutation [37]. This method involves generating induced pluripotent stem cells (iPSCs) from fibroblasts obtained from the patient [38], correcting the pathogenic mutation through gene surgery (*e.g*., CRISPR-Cas) while leaving the genetic background unaltered, and then transplanting the graft as a tissue replacement therapy with minimal risk of rejection and thus no need for immunosuppression [39]. In addition to precluding the possibility of immune-mediated rejection, iPSC-based treatments also circumvent the controversial use of human embryonic stem cells. Bassuk *et al*. [37] explored the genetic repair of iPSCs using CRISPR-Cas9 to correct a novel *RPGR* point mutation found to cause X-linked retinitis pigmentosa (XLRP). The *RPGR* gene encodes for the retinitis pigmentosa GTPase regulator protein, which plays a role in protein transport within the connecting cilium of photoreceptors [40]. Dermal fibroblasts were first cultured from a skin-punch biopsy obtained from a patient with a novel *RPGR* mutation (c.3070G > T, pGlu1024X within the ORF15 exon). Next, the cells were transformed into iPSCs and confirmed to be expressing four markers confirming pluripotency (Sox2, Oct-4, TRA-1-60, SSEA4) and capable of differentiation into all three germ layers, including retinal cells [37]. After 21 gRNAs were screened for specificity and efficacy of targeting the mutation site, g58 was determined to be the most ideal gRNA. The patient-specific iPSCs were transfected with the g58/Cas9 expression plasmid and an *RPGR* single-stranded oligodeoxyribonucleotide (ssODN) as the

donor homology template during homology-directed repair. Deep sequencing revealed that 13 percent of transfected cells were corrected for the mutation [37]. This study showed that patient-specific iPSCs containing a pathogenic RP mutation are amenable to correction by CRISPR-Cas9, laying the groundwork for further study of transplantation of corrected cells into the host.

CRISPR-Cas IN AGE-RELATED MACULAR DEGENERATION

Age-related macular degeneration (AMD) is the leading cause of blindness in the developed world, and its prevalence will only increase in the United States as the aging population rapidly grows [41]. The neovascular form of AMD, also known as wet AMD, is characterized by central vision loss due to abnormal choroidal vessel proliferation behind the macula, which is the region of the retina responsible for high-resolution central vision [42]. Since the neovascularization in wet AMD is caused by an excess production of vascular endothelial growth factor (VEGF), the current standard treatment is intravitreal injections of anti-VEGF agents such as bevacizumab, aflibercept, and ranibizumab [43]. However, some of the limitations and risks associated with these frequently used (approximately once monthly) treatments include high cost, endophthalmitis, retinal detachment, and vitreous hemorrhage [44]. This provides a potential advantage in studying the use of *in vivo* gene surgery to treat wet AMD, as the changes produced would be persistent even after a single injection.

In one study exploring new gene surgery options in the treatment of AMD, Kim *et al*. [45] demonstrated efficient targeting of the *Vegfa* or *Hif1a* gene in mice using a small Cas9 ortholog (CjCas9) derived from *Campylobacter jejuni*. HIF-1α (hypoxia-inducible factor) is a protein that activates VEGF transcription in response to hypoxic conditions [46], making it a therapeutic target for inactivation in the treatment of AMD. The small size of the CjCas9 ortholog makes it a theoretically better alternative to the larger traditional *Streptococcus pyogenes*-derived Cas9 (SpCas9) because the CjCas9 gene and sgRNA sequence can be packaged together within a single adeno-associated virus (AAV) vector for efficient delivery into cells, while SpCas9 requires its own AAV vector or splitting into multiple parts and co-delivery of two AAV vectors, both of which decrease its activity and efficiency [47,48]. After determining the PAM sequences recognized by CjCas9 and optimizing the sgRNA length, the group packaged the CjCas9 gene with sgRNA specific to either the *Vegfa or Hif1a* gene into AAV9 vectors and administered them into the eye through intravitreal injections. Targeted deep sequencing of the retinal pigment epithelium (RPE) cells, the primary site of dysfunction in AMD, at 6 weeks post injection confirmed the presence of CjCas9-induced indels at frequencies of 22±3 percent and 31±2 percent for *Vegfa* and *Hif1a* target sites, respectively. Enzyme-linked immunosorbent assays (ELISA) also revealed the expected decrease of VEGFA protein levels in RPE and retinal cells as compared to controls. After using a laser to induce choroidal neovascularization as a model of AMD, the group showed that mice transfected with either AAV-CjCas9:*Vegfa* or AAV-CjCas9:*Hif1a* had decreased surface areas of choroidal neovascularization without any decline in visual function testing [48]. This study demonstrates efficiency of the small CjCas9 endonuclease as well as a new potential gene surgery approach in treating wet AMD.

CRISPR-Cas IN LEBER CONGENITAL AMAUROSIS

CRISPR-Cas has recently been used as a timesaving alternative to the Cre-loxP recombination system in the study of mosaic tissue models of disease. The Cre-loxP system is a method of inducible recombination and conditional gene-knockout that requires at least one year to implement in mice. This system is particularly useful in the study of homozygous lethal alleles, as it allows for researchers to limit the homozygous deletion of the allele to certain cell/tissue types and to study the null phenotype of specific cells and tissues in living animals. One such homozygous lethal allele is the *Kcnj13* mutation, which has been found to be associated with an early-onset form of blindness called Leber congenital amaurosis (LCA) [49]. LCA is a group of autosomal recessively inherited rod-cone dystrophies characterized by severe and early visual loss from birth [50]. The *KCNJ13* gene encodes for the inwardly rectifying potassium channel subunit Kir7.1, which is expressed at the apical surface of RPE cells [51]. The mechanisms by which *KCNJ13* mutations cause LCA have not been explored, and because murine and human KCNJ13 proteins have a high degree of similarity, the *Kcnj13* mutant mice may yield useful insight into LCA pathogenesis [49].

Zhong *et al*. [49] used CRISPR-Cas9 to cleave the *Kcnj13* start codon in zygotes and generate mice with homozygous null *Kcnj13* mosaicism. Genotyping of the tail DNA confirmed mosaicism, and electroretinogram (ERG) functional testing and retinal immunohistochemical analyses revealed a range of KCNJ13 mosaic expression throughout the retina. Using these mosaic mice, the investigators observed that areas of the retina with lack of KCNJ13 function in RPE cells were associated with rhodopsin mislocalization and loss of photoreceptors, while areas containing wild-type RPE cells with detectable KCNJ13 expression had preserved

photoreceptors. Furthermore, photoreceptors in small regions of KCNJ13 loss were able to be rescued by adjacent areas with KCNJ13 expression, suggesting that KCNJ13 expression in RPE cells can rescue adjacent photoreceptor cells through an indirect mechanism. CRISPR-Cas9 enabled the researchers to quickly and affordably create a mosaic model for LCA in which mutant and wild-type cells are juxtaposed, allowing for the study of the role of KCNJ13 in pathogenesis and overcoming the issue of homozygous lethality.

GENE THERAPY CLINICAL TRIALS FOR RETINAL DISORDERS

Currently there are multiple ongoing and planned clinical trials for the use of gene therapy in the treatment of various retinal diseases. While the safety and efficacy profiles of CRISPR-Cas gene surgery have not yet been fully validated for use in human clinical trials within the United States, multiple groups in China have begun recruiting patients in trials testing the use of CRISPR-engineered immune cells in the treatment of lymphoma, non-small cell lung, esophageal, and renal cell cancers. In October 2016, a team at Sichuan University in China became the first to take autologous T cells whose PD1 gene, which is used by cancer cells to prevent immune cells from attacking them, was knocked out using CRISPR and then inject them into a patient with metastatic non-small cell lung cancer (ClinicalTrials.gov Identifier: NCT02793856). This trial is assessing the safety profile of using these engineered T cells and is estimated to be completed in April 2018. Another group at Sun Yat-Sen University in China is planning to run the first trial of CRISPR being used to edit cells inside the body. The goal of the trial is to determine the safety and dosing regimen of using TALENs and CRISPR-Cas9 to disrupt the genes responsible for causing HPV-related cervical cancer (ClinicalTrials.gov Identifier: NCT03057912).

These initial trials are creating a rush among the scientific community to discover the first gene surgery cure for use in patients. Even in the United States, where scientists have been taking a cautious approach to fully understanding the efficiency and off-target effects of CRISPR-Cas9 using preclinical models, there has been a push from biopharmaceutical companies to begin clinical trials. In March 2017, Editas and Allergan, two leading biopharmaceutical companies, announced plans for a 2018 trial in which CRISPR-Cas9 will be used to correct the mutated *CEP290* gene that causes Leber Congenital Amaurosis type 10 (LCA10). Although LCA10 is a rare disorder, treating this monogenic ophthalmological disease is Editas's lead program for the reasons mentioned earlier regarding the attractive properties of the eye for gene surgery. Furthermore, the large size of the *CEP290* gene lends itself well to a CRISPR-mediated correction approach as opposed to a gene-supplementation strategy limited by the carrying capacity of viral vectors.

Other than these gene surgery trials that are already underway or planned for the near future, there are currently many active gene therapy clinical trials utilizing different viral vectors and delivery methods as therapies for inherited retinal disease.

Leber Congenital Amaurosis

One of the earliest breakthroughs in gene therapy has been the results of clinical trials studying *RPE65*-associated LCA, or LCA2. In 2001, a University of Pennsylvania team demonstrated restoration of visual function in a naturally occurring *RPE65*$^{-/-}$ canine model using a recombinant AAV carrying wild-type *RPE65* (AAV-RPE65) [52]. RPE65 is a membrane-associated protein involved in 11-*cis* retinoid metabolism for the visual cycle in the retinal pigment epithelium [53]. Since the success of that trial, multiple studies have shown safety and modest efficacy with the administration of AAV-RPE65 in humans [50,54-57]. However, follow-up studies revealed a more nuanced therapeutic response: the restored RPE65 enzymatic cycle had markedly slowed kinetics and although the patients were found to have sustained improved visual acuity, their photoreceptors showed unabated degeneration, reflecting a need for additional approaches to slow retinal degeneration [58]. At present, Spark Therapeutics (Philadelphia, PA) is sponsoring a Phase III randomized controlled gene therapy trial testing bilateral subretinal administration of AAV2-hRPE65v2 (ClinicalTrials.gov Identifier: NCT00999609) [59]. This trial's primary outcome is mobility testing, and the estimated study completion date is July 2029.

Stargardt Disease

Stargardt disease, the most common form of juvenile onset macular degeneration, is an autosomal recessive genetic disorder caused by mutations in the ATP-binding cassette, subfamily A, member 4 (*ABCA4*) gene [60]. It is characterized by central vision loss due to progressive accumulation of cytotoxic lipofuscin within the RPE [60]. The pharmaceutical company Sanofi (Paris, France) is currently conducting a Phase I/IIa study in which SAR422459, an Equine Infectious Anemia Virus (EIAV) based lentivector containing a normal copy of the gene is administered by subretinal injection into patients with Stargardt disease [61]. The study is estimated to enroll 46 patients with a completion date of November 2018.

Choroideremia

Choroideremia (CHM) is an X-linked retinal dystrophy affecting roughly 1 in 50,000 males. It is

caused by loss-of-function mutations in the *CHM* gene, which encodes for Rab escort protein 1 (REP1), leading to choroid atrophy with pallor of the fundus and progressive loss of vision that starts with peripheral and night blindness [62]. Ongoing Phase I/II clinical trials are studying the safety and efficacy of the rAAV2.REP1 vector as a gene replacement therapy. A trial sponsored by Spark Therapeutics and in collaboration with Children's Hospital of Philadelphia, University of Pennsylvania, and Massachusetts Eye and Ear Infirmary is seeking to compare the outcomes between a single low dose and single high dose range of AAV2-hCHM (ClinicalTrials.gov Identifier: NCT02341807). In the initial findings of a University of Oxford trial, MacLaren *et al*. [63] reported a mean gain in best corrected visual acuity of 3.8 letters despite two patients undergoing retinal detachment, suggesting that the rod and cone function improvements from the AAV.REP1 administration outweigh any negative effects of retinal detachment. A follow-up study after 3.5 years revealed sustained improvements in the treated eyes of the two patients who had advanced disease, while their untreated control eyes had progressive degeneration [64].

Usher Syndrome

The most common cause of deaf-blindness in humans, Usher syndrome type 1 (USH1) is characterized by profound congenital deafness, vestibular dysfunction, and retinitis pigmentosa and is inherited in an autosomal recessive fashion [65]. One of the mutated genes responsible for USH1 is myosin VIIA, which encodes for a protein involved in organelle transport within the RPE [66,67]. UshStat®, a recombinant EIAV-based lentivector expressing functional human myosin VIIA, was found to protect the *shaker1* mouse model of Usher type 1B from light-induced photoreceptor degeneration [68]. Following successful safety studies in primate models, Sanofi initiated Phase I/IIa trials of UshStat® (SAR421869) unilateral subretinal administration in patients with Usher syndrome type 1B (ClinicalTrials.gov Identifier: NCT01505062). The clinical trial began in March 2012 and is estimated to be completed in April 2019.

Neovascular Age-related Macular Degeneration

As previously mentioned, neovascular (or wet) AMD, a leading cause of visual impairment in the United States, is caused by overproduction of vascular endothelial growth factor (VEGF) resulting in choroidal vessel proliferation behind the macula. The current standard of care involves frequent anti-VEGF injections, and although AMD is not a monogenic disorder like the other diseases mentioned, efforts are ongoing to discover a gene therapy approach that would reduce the number of intravitreal injections needed. One Phase IIa study (ClinicalTrials.gov Identifier: NCT01494805) at Lions Eye Institute (Perth, Western Australia) investigated the safety, immunologic, and other secondary endpoints (*e.g*., best-corrected visual acuity, foveal thickness) of subretinal rAAV.sFLT-1 gene therapy in patients with active wet AMD [69]. Soluble fms-like tyrosine kinase-1 (sFLT-1) is a protein that binds to and inactivates VEGF; serum levels of sFLT-1 have been found to be decreased in patients with wet AMD [69]. The results of the study showed no serious adverse events or side effects, yet no conclusions could be drawn about the efficacy of rAAV.sFLT-1 as the study was underpowered [69]. Similarly, a Phase I trial sponsored by Regenxbio Inc. (Rockville, MD) is testing an AAV8 vector containing a gene encoding for a monoclonal antibody fragment against VEGF (RXG-314) with an estimated study completion date of February 2020 (ClinicalTrials.gov Identifier: NCT03066258). In the beginning of 2017, Oxford BioMedica (Oxford, UK) published its results on the safety and expression profile of a lentiviral EIAV vector expressing angiostatin and endostatin (RetinoStat®), which was injected subretinally into wet AMD to suppress neovascularization [70]. A lentiviral approach was preferred over an adenoviral vector because of the former's stable long-term transgene expression, which is advantageous in a chronic disease like AMD. The team found that each of the doses were well-tolerated with no vector-related adverse events. Long-term follow-up (2.5 years in eight subjects and > 4 years in two subjects) revealed sustained expression levels of endostatin and angiostatin [70]. This follow-up study will examine the incidence of adverse events in these patients over a period of 15 years and is expected to be completed in November 2027 (ClinicalTrials.gov Identifier: NCT01678872).

DISCUSSION

The rate at which research results have been generated due to the accessibility and ease of genome manipulation with CRISPR-Cas-based tools has been unprecedented. In particular, ophthalmology has been at the forefront of advances in the genome surgery field because of the amenable properties of the eye. Despite the excitement about the potential of this still nascent technology, much work remains to be done in improving our understanding of the minutiae of the CRISPR-Cas system before therapeutic uses in humans can be considered. Specifically, safeguards must be implemented to prevent off-target mutations that can cause malignancies or germline alterations. Other priorities include control of the endogenous repair pathway to undergo NHEJ or HDR, and algorithms to accurately predict sgRNA binding.

The recent publication "Unexpected mutations

after CRISPR–Cas9 editing in vivo" [19] has raised controversial concerns that the CRISPR-Cas9 editing technology may introduce hundreds of single nucleotide mutations and large insertions/deletions that were previously never characterized in CRISPR studies. As described earlier, multiple CRISPR clinical trials have begun in China and one is slated to begin next year in the US, prompting public apprehension as to whether these trials were started prematurely without full considerations of safety. These findings underscore the importance of performing well-powered and controlled follow-up studies using a variety of CRISPR protocols and high-coverage whole genome sequencing with the primary outcome of looking for single nucleotide differences throughout the entire genome, which includes noncoding regions. These studies will fully characterize the off-targeting profile of CRISPR and will be crucial to our understanding of how to safely bring CRISPR-based therapies to patients.

As this review has summarized, CRISPR-Cas has great utility in both elucidating mechanisms behind pathogenesis and potentially treating previously incurable genetic disorders. The toolbox for CRISPR has been expanded further with the recent discovery of using the catalytically dead dCas9 to enable transcriptional activation and silencing with CRISPRa and CRISPRi. These technologies have dramatically reduced off-targeting effects and can help with the study of dose-dependent effects of target genes through controlled incomplete activation and repression of genes without the complete silencing effects of normal CRISPR-Cas9.

In July 2017, Shin *et al*. [71] found that the recently discovered "anti-CRISPR" proteins that viruses use to evade the bacterial immune system can decrease off-target effects by a factor of four when administered hours after delivering CRISPR-Cas9. These results give researchers yet another tool for controlling the undesirable off-targeting effects of CRISPR.

Another promising use for CRISPR is the genetic modification of iPSCs derived from patients with genetic disorders [37]. These corrected induced stem cells are capable of differentiation into a variety of cell types for autologous transplantation with minimal risk of graft rejection. With the recent surge in the amount of diagnostic and genetic information that can be obtained from patients, there are exciting possibilities of developing patient-specific cell-based therapies using CRISPR-modified iPSCs in the age of personalized medicine.

Most gene clinical trials in ophthalmology are Phase I/II gene therapy trials and thus are primarily focused on safety, which means more time is needed to monitor for adverse effects before proceeding to trials that assess efficacy. Currently, the most progress has been made in the Leber congenital amaurosis clinical trials, but the long-term follow-up studies have shown modest improvements and the need to address the issue of how to halt progression of photoreceptor degeneration. In gene surgery, the first CRISPR trials abroad are focused on immunotherapy approaches to treating cancer, as the recently developed antibody "checkpoint inhibitors" have shown incredible promise in the treatment of certain cancers. Although these trials are not in ophthalmology, they will yield invaluable data that answer questions about safety, effective dosing, number of cells that need to be corrected for therapeutic benefit, frequency of treatments, and persistence of effects. All of these trials will certainly inform clinical applications in all fields, including the planned retinal LCA10 trials in the US. The excitement for rapidly advancing CRISPR-Cas genome surgery technologies to treat previously incurable diseases will have to be tempered with the prudence and due diligence of carefully understanding the risks. Nevertheless, CRISPR holds great potential in translating successes in the laboratory into new treatments at the patient's bedside.

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