Retinitis pigmentosa - novel treatments and challenges

Clinical Implementation of Gene Supplementation and Genome CRISPR-surgery in RP

Stephen Tsang
CRISPR-mediated Ophthalmic Genome Surgery

**Authors:** Galaxy Y. Cho\(^{1,2,*}\), Yazeed Abdulla\(^3*\), Jesse D. Sengillo\(^{1,4}\), Sally Justus\(^{1,5}\), Kellie A. Schaefer\(^{6,7}\), Stephen H. Tsang\(^{1,2,5}\) and Vinit B. Mahajan\(^{6,7*}\), \(^{§}\)

\(^{1}\)Edward S. Harkness Eye Institute, New York-Presbyterian Hospital, New York, NY, USA

\(^{2}\)Department of Pathology & Cell Biology, Institute of Human Nutrition, College of Physicians and Surgeons, Columbia University, New York, NY, USA.

\(^{3}\)Faculty of Medicine, University of Jordan, Amman, Jordan

\(^{4}\)State University of New York Downstate Medical Center, Brooklyn, NY, USA

\(^{5}\)Jonas Children’s Vision Care, and Bernard & Shirlee Brown Glaucoma Laboratory, Department of Ophthalmology, Columbia University Medical Center, New York, NY, USA

\(^{6}\)Omics Laboratory, University of Iowa, Iowa City, IA, USA.

\(^{7}\)Department of Ophthalmology and Visual Sciences, University of Iowa, Iowa City, IA, USA.

\(*\)These authors contributed equally to this work

\(^{§}\)Corresponding authors
Abstract

Purpose of review: Clustered regularly interspaced palindromic repeats (CRISPR) systems have great potential for clinical applications due to its versatility and programmability. This review highlights the development and use of CRISPR-mediated ophthalmic genome surgery in recent years.

Recent findings: Diverse CRISPR techniques have been developed in order to target the wide array of ophthalmic conditions including inherited and acquired conditions. Gene rescue and disease model rescue in preclinical models (in vitro and in vivo) of ophthalmic conditions point towards the anticipated successes of CRISPR systems as therapeutics. In particular, treatment of Leber congenital amaurosis by CRISPR-mediated genome surgery is expected to be tested in clinical trials in the near future.

Summary: Treatment options for inherited retinal dystrophies are currently limited. CRISPR-mediated genome surgery methods may soon be able to address this unmet need.

Keywords
CRISPR-Cas, induced pluripotent stem cells, genome surgery, retina

Introduction

Inherited retinal degenerations cause irreversible visual impairments rendering many affected persons blind (1, 2). Recent improvements in DNA modification technology utilizing the clustered regularly interspaced short palindromic repeats (CRISPR) system, derived from bacteria and archaea immune systems, is shown to be an effective and precise DNA targeting mechanism and shows great potential for clinical applications to treat inherited conditions (3). In Ophthalmology, applying CRISPR technology to treatment involves modification of pathogenic gene mutations in order to reverse or stop progression of retinal degeneration.
This review gives an overview of the role of CRISPR-Cas systems in treating retinal diseases.

**CRISPR-Cas9**

Genetic modification strategies utilize endonucleases such as meganuclease (MN), zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and CRISPR-Cas9 to introduce double-strand breaks (DSBs) (4). In order to introduce genetic modifications, the induced DSBs are repaired by non-homologous end-joining (NHEJ) in which direct, albeit error-prone, ligation occurs or by homologous recombination (HR) in which a homologous region including the desired mutation to be introduced serves as a template sequence (5, 6). ZFNs and TALENs are guided by protein-DNA binding, which limits broad application due to difficulties in engineering new proteins for each experiment (3). On the other hand, the RNA-mediated CRISPR-Cas9 system offers efficient and versatile gene-modifying allowing for diverse applications including deletions, insertions, knockouts, transcriptional control, epigenetic alterations, and more (3, 7, 8).

CRISPR-associated (Cas) endonucleases identify segments of DNA containing short, repetitive base sequences, which can be targeted for gene modification (7, 9, 10). The most commonly utilized Cas protein is spCas9, a type II CRISPR-Cas system, derived from *Streptococcus pyogenes* (11). The native CRISPR-Cas9 is a four-component system: Cas9, trans-activating CRISPR RNA (tracrRNA), CRISPR RNA, and RNAse-III(12). This four-component system has been simplified by the development of a chimeric molecule which combines the crRNA and tracrRNA into a single guide RNA (sgRNA) allowing further accessibility of CRISPR-Cas9 application to eukaryotes (12, 13). In conjunction to target sequence guiding from the sgRNA, spCas9 function requires a proto-spacer adjacent motif (PAM) adjacent to the target sequence (13-16). The PAM sequence for spCas9 is NGG,
where N stands for any nucleotide but differs between Cas species. the possibility of unintended DNA alterations

**Dominant Mutations**

Treatment methods for dominant mutations are currently limited. Although targeting inherited retinal conditions by gene therapy exists, gene therapies utilize adeno-associated virus (AAV) as a vector to deliver additional wild-type genes by subretinal or intravitreal injections. As such, traditional approaches of gene therapy does not directly target the pathogenic gene and can only be applied to recessive and haploinsufficiency conditions (17). Thus, there is a need for CRISPR application in the treatment of genetic mutations inherited in a dominant manner. Targeting of a dominant-negative allele utilizing CRISPR/Cas9 was demonstrated by Courtney et al. in the *KRT12* gene (18). The C395T mutation in the *KRT12* gene is a dominant-negative mutation which causes Meesmann epithelial corneal dysplasia (MECD). The disease-causing mutation resulted in an alteration to the PAM sequence recognized by Cas9. As a mismatch in sgRNA’s target sequence can ablate spCas9’s cleavage activity (19) Courtney et al. targeted the SNP in a mouse model of MECD by intrastromal injection of sgRNA and SpCas9-GFP plasmids. In the 13 sequences that were analyzed, four resulted in inactivation of the pathogenic allele by early termination (18).

**In vivo Approach**

Delivering the CRISPR system directly into the retina of patients as a therapeutic tool is still in pursuit (7). Efficacy of such a method was examined in adult *Thy1::YFP* transgenic mice via intravitreal injections using dual AAV delivery of SpCas9 and yellow fluorescent protein (YFP) targeted gRNA (20). CRISPR-Cas9 cleavage was repaired by NHEJ in order to decrease YFP expression. Hung et al. used a YFP-sgRNA construct and a noncutting sgRNA
control which showed an 84% reduction in YFP expression in the YFP-sgRNA construct containing cells. AAV2 transduction was the greatest in the retinal ganglion cells (RGCs). Another significance of Hung et al.’s finding was the electroretinography taken five weeks post-transduction; no significant retinal changes were observed suggesting that no toxicity to CRISPR components could be seen (20-26). The potential to utilize in vivo approaches for genome surgery clinically is seen in this genome engineering of adult mouse retina.

Disease Models:

Retinitis Pigmentosa

Diverse CRISPR technologies have been applied to a variety of preclinical models contributing to furthering our understanding of disease models and approach feasible treatment options (7, 17). One disease which has been approached with CRISPR applications in the pre-clinical setting is retinitis pigmentosa (RP). RP is characterized by progressive retinal degeneration that corresponds to a decreasing visual field. It is genetically heterogeneous, as it is known to be caused by mutations in over 70 different genes. Autosomal recessive, dominant, and x-linked inheritance patterns are observed in RP depending on the gene implicated. Current dietary modifications slow the condition but gene-editing strategies may prove to be a more efficacious treatment (17, 27).

The rd1 mouse, a preclinical model of RP, harbors a mutation in each Pde6b allele leading to rod cell death (28-30). In this model, there is a nonsense mutation (Y347X, C to A) in exon 7 and a murine leukemia virus insertion (Xmv-28) in intron 1 of Pde6b (28, 31). After nearly a century long debate concerning the origin of this phenotype, a CRISPR-mediated homology-directed recombination (HDR) of the nonsense mutation (Y347X) by Wu et al. resulted in disease model rescue, demonstrating Y347X to be pathogenic (28).
In another preclinical model of RP, the transgenic S334ter-3 rat holds the mutation \( \text{Rho}^{S334} \), which yields a truncated \( \text{RHO}^{S334} \) protein lacking the signal sequence for RHO trafficking resulting in rapid degeneration of photoreceptors (32-34). The PAM sequence in \( \text{RHO}^{S334} \) (5'-TGG-3') differs from the PAM sequence in \( \text{RHO}^{WT} \) (5'-TGC-3') by one nucleotide. Bakondi et al. were able to disrupt \( \text{Rho}^{S334} \) without disrupting the \( \text{Rho}^{WT} \) by utilizing the difference in PAM sequence (34). Subretinal injection of CRISPR/Cas9 and a sgRNA yielded photoreceptor rescue in transfected regions with retinal synapse preservation. Bakondi et al. reported a 53% increase in visual acuity measured by optokinetic response and a nine-fold increase in photoreceptor nuclei density (34).

**Retinitis Pigmentosa: Stem Cell Modification**

Another approach to treating retinal degeneration is stem cell transplantation. Recent clinical trials of subretinal transplantation of retinal pigment epithelium (RPE) derived from human embryonic stem cells (hESCs) have shown potential efficacy and safety (35). Due to the ethical controversy surrounding the use of hESCs and potential immune rejection, the use of patient-derived induced pluripotent stem cells (iPSCs) is a topic of interest (36). Although iPSCs contain the same genetic mutation that caused the degeneration in the patient and the disease model would persist, CRISPR-mediated correction of patient-derived iPSCs could theoretically yield a useful cell-based treatment method that doesn’t require immunosuppression (36, 37). In 2016, Bassuk et al. were the first to report CRISPR correction of a pathogenic mutation causing photoreceptor degeneration in patient-derived iPSCs. The iPSCs were derived from patients with X-linked RP caused by a retinitis pigmentosa GTPase regulator (RPGR) mutation (c.3070G > T, pGlu1024X). The iPSCs were induced from fibroblasts collected through a skin-punch biopsy. The iPSCs were transfected
with spCas9, sgRNA g58, and a WT RPGR anti-sense single-stranded oligonucleotide donor template. Correction was seen in 13% of the cells establishing proof of concept of utilizing iPSCs for subretinal transplantation in the future (36).

**Age-related Macular Degeneration**

Age-related Macular Degeneration (AMD) is associated with over-expression of vascular endothelial growth factor A (VEGFA). Although AMD is not caused by monogenic mutations, Kim et al. sought to harness the many potentials of the CRISPR systems by utilizing $\text{Vegfa}$ gene-specific Cas9 ribonucleoproteins (RNPs), preassembled Cas9 protein complexed with *in vitro* transcribed gRNA, to inactivate expression of $\text{Vegfa}$. AMD was modeled by laser-induced choroidal neovascularization (CNV) in adult mice. Sub-retinal injection of Cas9 RNPs led to a reduction in VEGFA expression followed by a reduction of CNV. This preclinical model is of particular significance because of the expansion in potential treatment methods utilizing CRISPR to non-genetic diseases and to the adult eye (38).

**Leber Congenital Amaurosis**

Leber congenital amaurosis (LCA) is a genetically heterogeneous retinal dystrophy which can manifest within the first months of life (39, 40). One of the mutations associated with LCA is in the $\text{KCNJ13}$ gene which encodes an RPE-specific potassium channel subunit Kir7.1

In the mouse model, traditionally scientists would have needed to employ Cre-lox systems to study this disease model as a homozygous null mutation of $\text{Kcnj13}$ is lethal (41). Although a useful method, generation of Cre-lox systems can be time-consuming. In seeking a faster method for disease model generation, Zhong et al. utilized CRISPR/Cas9 with NHEJ to
introduce mutations in the *Kcnj13* locus to generate a LCA disease model. Mouse zygotes were injected with sgRNA and spCas9 mRNA. Tail genotyping of the developed mice exhibited tissue mosaicism in the *Kcnj13* locus as expected from heterogeneous mutations that arise from NHEJ (41, 42). Study of the generated disease model showed that loss of function of KCNJ13 corresponded with loss of photoreceptors and rhodopsin mislocalization. As demonstrated by Zhong et al., CRISPR is useful not only in correction of disease, but study of disease as well (41).

Another LCA causative mutation is an intronic mutation in the *CEP290* gene (c.2991 + 1655A to G) known to cause LCA10; it is the most frequently detected mutation causing LCA in patients. Targeting *CEP290* proves a challenge due to its large size exceeding the capacity of adeno-associated virus (AAVs) delivery (43, 44). Ruan et al. reported successful deletion of *CEP290* in 293FT cells utilizing a dual AAV delivery approach of pAAV-SpCas9 plasmid and pAAV-sgRNA plasmid, circumventing the carrying capacity limitation (43). Another approach to circumvent the carrying capacity limitation was reported by Maeder et al. utilizing the smaller *S. aureus*-derived CRISPR/Cas9 which allowed a single AAV vector delivery of two guide RNAs to perform a dual-cut excision of the *CEP290* gene in LCA10 patient-derived primary fibroblasts (44).

Whether CRISPR will truly be a viable treatment option for ophthalmic conditions has not yet been tested in clinical trials. This may soon change. A press release in March 2017 from Editas and Allergan announced plans for a research and development alliance to develop CRISPR-utilized treatment of LCA10. Editas’s pipeline for 2018 includes plans to test treatment of LCA10 via NHEJ small deletion via AAV local injection delivery of the treatment product.
Potential Limitations to using the CRISPR system in patients

Although the CRISPR system has shown promising preliminary results for a variety of ophthalmic conditions, limitations still exist. One such limitation is the possibility of unintended DNA alterations. Because CRISPR modifies DNA (as opposed to incorporating extra copies of the wild-type gene like AAV does), off-targeting mutations, the unintended editing of other regions in the genome, could lead to the formation of secondary phenotypes.

A variety of methods to test for off-target mutations have been developed (45-47). The most common method for determining potential off-targeting of a sgRNA is to use algorithms to computationally detect homologous regions in the genome that are most likely to be mis-targeted. Although most studies subsequently sequence the predicted off-target sites to test for mutation, it is currently not clear if Cas9 has the potential to target other, non-homologous sequences.

Additionally, some studies have begun to perform whole exome sequencing (WES) in CRISPR-treated cells and organisms (48, 49). While these studies are the most accurate and comprehensive to-date, they still only detect approximately 1% of the genome, and recent findings reveal that non-coding regions play crucial roles in the cell (50).

In the past decade, the role of the CRISPR-system for disease gene editing has been realized and developed into a potential therapeutic tool. The final, and perhaps most important, step before translating CRISPR to clinical use is to develop a complete, non-biased method for detecting potential off-targeting mutations at the whole organism level.
Conclusion

The reprogrammable ability of CRISPR allows much versatility in approaching disease treatment. In combination with the eye’s easy accessibility, relative immune-privileged status, CRISPR-Cas therapeutic strategies show great promise in the field of ophthalmology. CRISPR technologies have furthered our understanding of ophthalmic diseases in significant ways. Given the successes of CRISPR techniques in preclinical models and plans for clinical trials, therapeutic uses of CRISPR in ophthalmology may become available to patients in the near future.
Acknowledgements

VBM is supported by NIH grants [R01EY026682, R01EY024665, R01EY025225, R01EY024698 and R21AG050437]. VBM is supported by The Doris Duke Charitable Foundation Grant #2013103, and Research to Prevent Blindness (RPB), New York, NY. GV is supported by NIH grant T32GM007337. The Barbara & Donald Jonas Laboratory of Regenerative Medicine and Bernard & Shirlee Brown Glaucoma Laboratory are supported by the National Institute of Health [5P30EY019007, R01EY018213, R01EY024698, R21AG050437], National Cancer Institute Core [5P30CA013696], the Research to Prevent Blindness (RPB) Physician-Scientist Award, unrestricted funds from RPB, New York, NY, USA. S.H.T. is a member of the RD-CURE Consortium and is supported by the Tistou and Charlotte Kerstan Foundation, the Schneeweiss Stem Cell Fund, New York State [C029572], the Foundation Fighting Blindness New York Regional Research Center Grant [C-NY05-0705-0312].
References