CRISPR applications in ophthalmologic genome surgery

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Purpose of review
The present review seeks to summarize and discuss the application of clustered regularly interspaced short palindromic repeats (CRISPR)-associated systems (Cas) for genome editing, also called genome surgery, in the field of ophthalmology.

Recent findings
Precision medicine is an emerging approach for disease treatment and prevention that takes into account the variability of an individual's genetic sequence. Various groups have used CRISPR-Cas genome editing to make significant progress in mammalian preclinical models of eye disease, the basic science of eye development in zebrafish, the *in vivo* modification of ocular tissue, and the correction of stem cells with therapeutic applications. In addition, investigators have creatively used the targeted mutagenic potential of CRISPR-Cas systems to target pathogenic alleles *in vitro*.

Summary
Over the past year, CRISPR-Cas genome editing has been used to correct pathogenic mutations *in vivo* and in transplantable stem cells. Although off-target mutagenesis remains a concern, improvement in CRISPR-Cas technology and careful screening for undesired mutations will likely lead to clinical eye therapeutics employing CRISPR-Cas systems in the near future.

Keywords
adeno-associated virus, CRISPR-Cas, embryonic stem cells, induced pluripotent stem cells, precision medicine

INTRODUCTION

Inherited genetic disorders of the eye result in severe functional deficits in patients, and to date, many of these disorders have been intractable to precision-based therapies [1,2]. Since the first efforts to sequence the human genome, scientists and physicians have aimed to modify pathogenic genomic loci to alleviate disease burden [3–5]. Recently, with the advent of technologies for direct DNA modification, practicing precision genome surgery in ophthalmology is now closer to becoming a reality. Originally derived from the immune system of bacteria and archaea, clustered regularly interspaced short palindromic repeats (CRISPR)-associated systems (Cas) have become repurposed for DNA manipulation in biomedicine [6]. The present review seeks to highlight the use of CRISPR-Cas systems in ophthalmology, particularly over the past year.

CRISPR-Cas genome engineering

Targeting double-stranded breaks (DSBs) to the desired genomic loci has become a mainstay for the modification of eukaryotic systems [7]. These DSBs can be repaired either by a strategy known as homology-directed repair (HDR), or by nonhomologous end

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KEY POINTS

- CRISPR-Cas genome surgery can make precise modifications in mammalian and nonmammalian preclinical models of eye disease and development.
- Targeting autosomal dominant mutations clinically may be especially effective because the process does not require a corrected version of the gene.
- Delivery of CRISPR-Cas components via AAV vectors is effective in model systems.
- Ex-vivo genome surgery to correct mutations of patient-derived iPSCs and ESCs for future re-implantation is a promising therapeutic.

joining (NHEJ), an error-prone process that often results in insertions and deletions (INDELs) [8]. Over the past decade, meganucleases, zinc-finger nucleases and transcription activator-like effector nucleases have been the dominant tools for the introduction of DSBs in cells [9]. Although these tools can be used to generate precise DSBs, their engineering can be laborious and time intensive because of protein engineering considerations, among other factors [6]. In 2012 the mechanistic actions of the reprogrammable endonucleases from the CRISPR-Cas system of *Streptococcus pyogenes* were first elucidated in detail, and since its first implementation in human cells, the repurposing of this system for genome surgery has skyrocketed [6,10–12].

Because of the ease of re-engineering CRISPR-Cas for diverse targets, this system has become popular for genome surgery in a variety of organisms [13–20]. CRISPR-Cas genome editing from the type II CRISPR system of *S. pyogenes* relies on the RNA-guided endonuclease, Cas9 (SpCas9), and two RNA molecules, a CRISPR RNA (crRNA) and a trans-activating RNA (tracrRNA), which associate and complex with Cas9 to guide cleavage of DNA sequences based on homology between the crRNA and target DNA strand [10,21,22]. A chimeric molecule of the crRNA and tracrRNA was created and subsequently dubbed 'single guide RNA' (sgRNA), which was demonstrated to be as effective as the original system [10]. The sgRNA contains a 20 base pair region that binds to the homologous DNA strand. Cas9 associates with the DNA and generates a blunt cut three base pairs upstream of the protospacer adjacent motif (PAM), a three-nucleotide sequence required for Cas9 association (canonically an NGG sequence) [10,23–25]. Although other endonucleases like the Cas9 derived from *S. pyogenes* have been discovered and optimized for gene editing purposes, SpCas9 has been utilized the most since its introduction [6].

SpCas9 has been further modified to attenuate its cutting ability to that of a nickase (SpCas9D10A), wherein the enzyme is only able to induce a single-stranded break rather than a DSB [26,27]. SpCas9 has also been modified to completely remove its nuclease activity (termed dCas9 for 'catalytically dead Cas9') to allow for activator and repressor protein fusions [26,27]. Because of the versatile applications of SpCas9 to edit, activate, repress and even epigenetically modify DNA *in vivo*, CRISPR-Cas systems have become essential tools in biotechnology [26,28–32] (Fig. 1a–e). Recently, Komor *et al.* [33] developed dCas9-deaminase and Cas9-nickase-deaminase fusions capable of inducing cytidine to uridine transitions and allowing for directed mutagenesis without introducing a DSB (Fig. 1f).

Off-target binding of Cas enzymes are important considerations, especially when the generation of precise modifications is essential, as it is in clinical situations [34]. Although there are several softwares available to predict off-target binding of RNA-guided endonucleases using sgRNA target sequences and the host genome, a recent experimental study by Tsai *et al.* [35] showed that there is variability in predicted and experimentally verified off-target binding sites. This suggests that examining off-target modifications via CRISPR-Cas in a clinical setting is crucial.

CRISPR-Cas genome editing in mammalian preclinical models of the eye

The ease, reduced cost and rapid speed of generating novel RNA-guided endonucleases have revolutionized the generation of animal models [15,36]. Recently, Wu *et al.* used CRISPR-Cas genome editing to untangle the effects of two potentially pathogenic genetic differences in 'rodless' (*rd1*, *Pde6b*^{rd1}/*Pde6b*^{rd1}) mice, an important preclinical model for retinitis pigmentosa [37▪]. These *rd1* mice exhibit premature rod neuronal death believed to be a result of mutations in the *Pde6b* gene, which regulates intracellular cGMP levels [38,39]. The *rd1* mouse genome contains a nonsense mutation (Y347X) in the *Pde6b* gene and an insertion of a murine leukemia virus (*Xmv-28*) into intron 1 of *Pde6b*. It was unclear if the *Xmv-28* insertion contributed to the *rd1* phenotype, so the group used CRISPR-Cas genome editing, utilizing a single-stranded oligonucleotide as the donor template, to generate mice with a repaired Y347X nonsense mutation in *Pde6b*^{rd1}/*Pde6b*^{rd1} homozygous mice, the background still containing the Xmv-28 insertion. Using fundoscopy, electroretinography, optical coherence tomography and hematoxylin and eosin staining, the group showed that the wild type phenotype

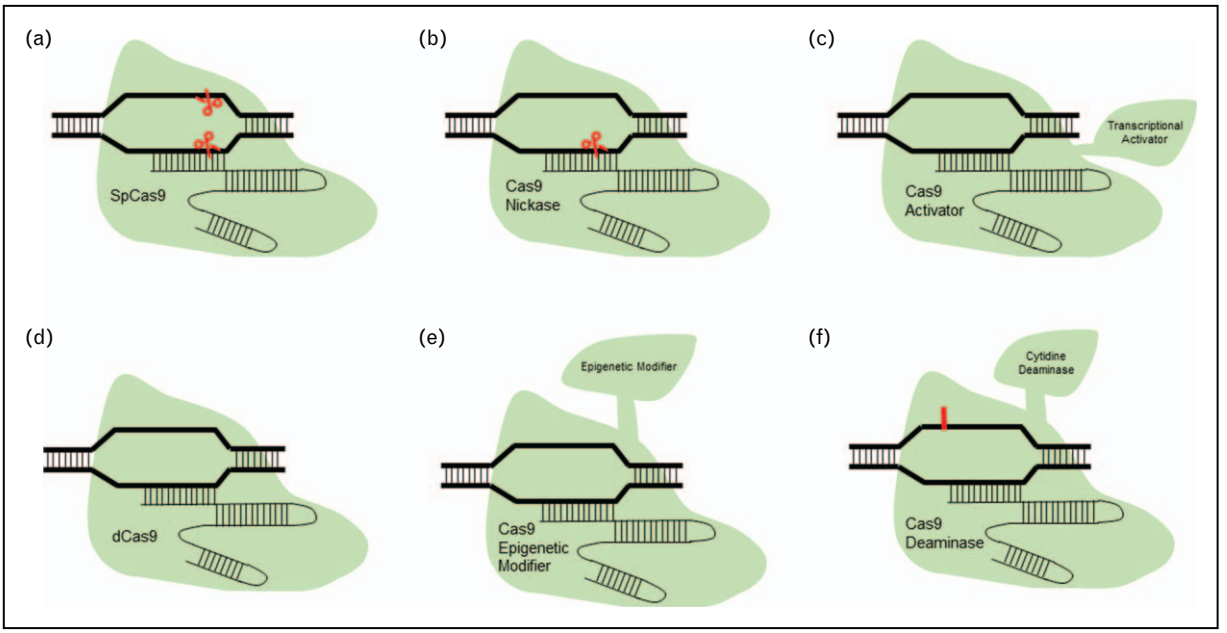

FIGURE 1. Wild type SpCas9 (a) has been modified extensively to attenuate (b, Cas9 Nickase) and remove (c, dCas9) its double-strand DNA cutting activity. It has also been modified via protein fusion to generate targeted transcriptional activators (d), epigenetic modifiers such as methyltransferase and acetyltransferases (e), and domains that allow for precise cytosine to uracil modifications in the case of deaminases (f).

could be restored by repairing the Y347X nonsense mutation alone, suggesting that the *Xmv-28* viral insertion is benign.

CRISPR-Cas genome editing has also been used to generate mosaic tissue models of disease. The *Kcnj13* gene encodes the apically localized, retinal pigment epithelial (RPE)-specific potassium channel subunit Kir7.1. Mutations in this channel are associated with Leber Congenital Amaurosis, and in mice, homozygous null mutants of *Kcnj13* result in postnatal lethality [40▪▪]. Classically, scientists have employed Cre-lox systems to knock out essential genes in adult multicellular organisms and study the resulting phenotype. Briefly, the Cre-lox system enables site-specific recombination at genes of interest that have been modified to contain loxP sites flanking their 5′ and 3′ ends, and it concurrently requires the introduction of a transgenic Cre recombinase. Genes can then be excised via endogenous or exogenous induction of the recombinase [41]. However, generation of Cre-lox systems in mice can take at least one year. To avoid this delay, Zhong *et al.* [40▪▪,42] took advantage of the heterogeneity of the mutations generated by NHEJ. After injection of sgRNA and SpCas9 mRNA into mouse zygotes, the developed animals exhibited tissue mosaicism with respect to the *Kcnj13* locus based on tail genotyping, which is indicative of mosaicism within the retina as well. This model allowed the investigators to study the effects of a loss of function of KCNJ13 at a tissue level, which they concluded correlated with loss of photoreceptors and rhodopsin mislocalization. By employing a model that utilized NHEJ-induced mosaicism instead of conventional knockout via a Cre-loxP system, the generation of CRISPR mice was much more rapid, and diverse genotypes could be examined simultaneously.

Conversely, CRISPR-Cas genome editing can be used to make precise, single base pair changes. To assess the effects of the rs2279744 single nucleotide polymorphism (SNP) implicated in retinal detachment and carcinogenesis in proliferative vitreoretinopathy, Duan *et al.* exploited CRISPR genome editing. They induced the SNP, a T309G mutation in *Mdm2* (murine double minute 2, an implicated inhibitory regulator of p53), into human primary RPE (hPRPE) cells *in vitro* [43▪]. The SNP was imparted using adeno-associated viral (AAV)-delivered SpCas9D10A, which was guided by two sgRNA constructs that flanked the *Mdm2* locus (these guide RNAs were previously tested for activity using WT SpCas9). The group found that although 42.51% of the cells were modified, only 10% of the modified cells were homozygous for the T309G mutation. The group then examined hPRPE responsiveness to apoptotic factors in rabbit vitreous and found that Mdm2 T309G hPRPE cells were less responsive to apoptosis and more proliferative in rabbit vitreous, further implicating the allele in promoting proliferation in vitreous environments. Using CRISPR-Cas

systems to generate human SNPs *in vivo* is a powerful method for screening pathogenicity, a platform that will be used increasingly more with the popularization of personalized medicine paradigms [44,45].

In addition, other groups have used CRISPR-Cas systems to knockout genes in parallel with short-interfering RNA (siRNA), which are short (~20–30 nucleotides), noncoding RNA sequences that can be designed to repress the expression of genes of interest [46,47]. Priglinger *et al.* [48] used CRISPR-Cas genome editing to study key processes involved in the epithelial-to-mesenchymal transition of RPE. This transition is a crucial step in proliferative vitreoretinopathy, and understanding its mechanism is essential for developing therapeutics [49]. The investigators found that CRISPR-Cas-induced knockout of β1,6-*N*-acetylglucosaminyltransferase V (*MGAT5*) in human RPE cells, along with siRNA knockdown in a separate experiment, decreased the binding of galectin-3 (Gal-3) to glycans on RPE cells *in vitro*. Gal-3 is an important modulator of RPE growth *in vivo*, and this binding modulation provided by MGAT5 makes it an interesting target for therapy [48]. This study shows the use of CRISPR-Cas genome surgery as a parallel tool to classic techniques such as siRNA knockdown.

Advances in ocular genetics using CRISPR-Cas genome editing in zebrafish

In addition to mouse and human tissue, zebrafish represent an attractive model for human disease because of their rapid lifecycle ease of manipulation, and highly orthologous correlation with human genes [50]. CRISPR-Cas genome editing has previously been applied to zebrafish [20]. Yin *et al.* [51] used retina tissue-specific delivery (injection and electroporation) of sgRNA targeted to the zebrafish retinal regeneration gene *Ascl1a* in a strain background constitutively expressing SpCas9. Targeting *Ascl1a* for cleavage resulted in a reduction of retinal proliferation compared with controls, as expected [52]. This study shows that spatial and temporal delivery and expression of CRISPR components are a powerful way to regulate gene activity in zebrafish, especially when targeting small cell populations in specific tissues such as the eye.

Zebrafish are a powerful model for studying development. Taylor *et al.* [53] induced mutations in the gene *NeuroD*, a potential player in retinal regeneration in zebrafish, via CRISPR-Cas genome editing. The group targeted *NeuroD* using sgRNA and SpCas9 to induce deleterious mutations in zebrafish embryos, and they also suppressed the gene using morpholinos oligonucleotides to provide a point of comparison. Their data suggested that *NeuroD* is a regulator of Notch signalling, confirming its important role in photoreceptor regeneration [53]. By using both classical genetic techniques such as morpholino knockdown and CRISPR-Cas genome editing, the findings of this study were strengthened by the rigorous experimental design.

Targeting dominant-negative alleles using CRISPR-Cas *in vivo*

Mismatch in the 20 base pair target sequences of the sgRNA can have severe effects on SpCas9 cleavage, especially if the mismatch occurs in the 12 base pair sequence closest to the PAM (seed sequence) [54]. In addition, alterations in the PAM sequence from canonically acceptable sequences can ablate cutting ability. Courtney *et al.* [55] used this characteristic of sgRNA–SpCas9 complexes to specifically target the C395T mutation in the *KRT12* gene, a dominant-negative allele for Meesmann epithelial corneal dysplasia (MECD) in heterozygous patients. This particular SNP results in a novel PAM sequence which the investigators targeted with a sgRNA. Their goal was to either induce inactivating mutations (nonsense or frameshift mutations) via NHEJ or correct the allele using the wild type sequence.

After validation of sgRNA activity *in vitro*, the investigators used intrastromal injection of sgRNA and SpCas9-GFP plasmids to target the SNP in a humanized heterozygous mouse model of MECD. The *Krt12* locus was then amplified from the treated mice, and of the 13 sequences that were analyzed, five contained NHEJ-derived mutations, four of which resulted in early termination of the pathogenic allele. This study illuminates the potential of CRISPR-Cas genome editing to not only insert beneficial alleles, but also to inactivate deleterious ones.

Bakondi *et al.* have applied a similar approach to ablate the autosomal dominant Rho^{S334} allele in a retinitis pigmentosa rat model, a mutant allele that results in misprocessing of the RHO protein and apoptotic death of photoreceptors. The Rho^{S334} allele contains a novel PAM sequence which the investigators targeted with a sgRNA that was first tested *in vitro* for targeting efficacy. sgRNA and SpCas9 plasmids were introduced via subretinal injection and electroporated to stimulate uptake into photoreceptors. Two rats in the study showed an INDEL frequency of 33 and 36% as a result of NHEJ in the Rho^{S334} locus. In the CRISPR-Cas ablated Rho^{S334} rats, the authors reported a 53% increase in visual acuity as assessed by the optokinetic behavioral response and a 35% increase in visual acuity using untreated contralateral eyes as controls within individuals. Finally, the investigators reported a

nine-fold increase in nuclei density in photoreceptors at P33 [56▪▪]. These beneficial effects, both functionally and histologically, of pathogenic allele ablation further support the use of CRISPR-Cas for therapies that require the removal of dominant-negative alleles (Fig. 2a and b).

In-vivo delivery of CRISPR-Cas systems

Direct delivery of CRISPR-Cas components to retinal tissue for genome surgery in patients is an important goal that is still being studied and pursued. To this end, Hung *et al.* examined the efficacy of dual AAV delivery of SpCas9 and validated yellow fluorescent protein (YFP)-targeted guide RNA components were introduced into *Thy1*::YFP adult transgenic mice via intravitreal injections [57▪]. These mice have a *YFP* gene inserted into the *Thy1* locus, and CRISPR-Cas induced cleavage and the error-prone NHEJ repair would result in a decrease in functional YFP expression. Post-delivery, the group found an 84% reduction in YFP expression in cells that contained YFP-sgRNA constructs as compared to a noncutting sgRNA control. Using whole retinal mounts, the group could determine the efficacy of knockout across retinal layers, with AAV2 transduction being the greatest in retinal ganglion cells (RGCs). Without selection for sgRNA-containing cells, there was a 50% reduction in YFP expression in RGCs. Using electroretinography, no significant retinal changes could be observed 5 weeks posttransduction, indicating a lack of toxicity to CRISPR components. Recently, delivery of CRISPR-Cas components *in vivo* and modification of endogenous loci has been successful with varying degrees of efficacy [58–63]. Similarly, AAV delivery of therapeutic DNA to eye tissue has been increasingly promising due to tropism preferences of AAV and the immune status of the eye [64–66]. This study shows the potential of *in vivo* genome engineering in adult animal retina, a technique that will likely become more translationally relevant in the near future.

Modification of stem cells using CRISPR-Cas genome editing

Patient-specific induced pluripotent stem cells (iPSCs) may be an ideal source of autologous cells to ameliorate many diseases [67–71]. Bassuk *et al.* showed that CRISPR-Cas genome editing could be used to correct a novel mutation in the retinitis pigmentosa GTPase regulator (RPGR) locus (c.3070G > T, pGlu1024X) which results in a relatively severe X-linked RP phenotype [72▪▪]. Using skin-punch biopsy, cells were collected then transformed into iPSCs, which have the potential to be differentiated into retinal cells. Because of the high GC content and repetition in the locus, 21 sgRNA constructs were tested *in vitro*, and one was chosen based on specificity and efficacy (sgRNA g58).

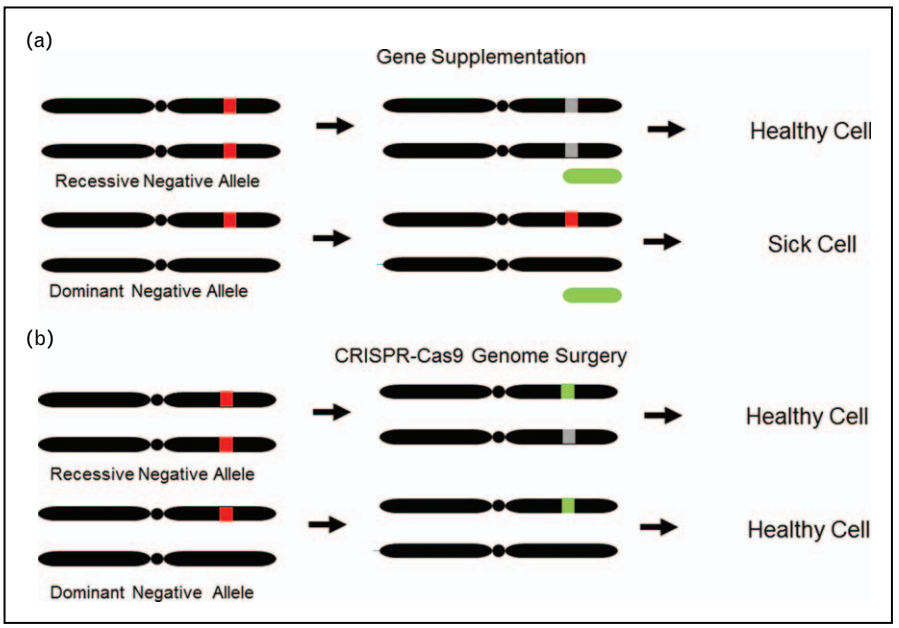

FIGURE 2. (a) Gene supplementation approaches using traditional gene delivery is useful for adding additional copies of wild type alleles in the case of recessive negative allele genotypes, but ineffective at removing pathogenic dominant negative alleles, where the mutant allele causes damaging effects. (b) CRISPR-Cas9 genome surgery has the benefit of precisely removing pathogenic alleles, regardless if they are dominant or recessive, resulting in permanently modified healthy cells.

Patient-derived iPSCs were modified via transfection of SpCas9, sgRNA g58, and a WT RPGR anti-sense, single-stranded oligonucleotide donor, resulting in 13% of the cells containing the corrected allele. Although not completed in this study, correctly differentiated iPSCs could theoretically be autologously transplanted back into patients.

Human embryonic stem cells (hESCs) also serve as a potential source of cells for allotransplantation, in addition to representing important tools for studying human development. Sluch *et al.* [73,74] sought to ease the isolation of hESC-derived RGCs by using CRISPR-Cas genome editing to link mCherry, a red fluorescent protein that enables genome engineering to be visualized (among other purposes), to the BRN3B gene (an important marker for RGCs) using a P2A self-cleaving peptide linker in h7 hESCs. The group first targeted the stop codon of BRN3B by electroporation of sgRNA with spCas9 plasmids and an mCherry gene containing a 5′ P2A linker with homology arms to the cut locus. The cell line was then differentiated to RGCs and then sorted by flow cytometry using the mCherry reporter. The mCherry-enriched cells were then analyzed for markers of RGCs via real-time quantitative PCR, used to quantitatively measure DNA amplification over time, and fluorescence staining. Ultrastructural and electrophysiological properties, assayed by transmission electron microscopy and whole-cell current clamp, respectively, further confirmed RGCs phenotype. The investigators also examined the frequency of off-target mutation at predicted sites and were unable to find off-target mutagenesis. In summary, tagging of a marker of RGC lineage using CRISPR-Cas editing allowed for rapid isolation of RGCs derived from hESCs.

CONCLUSION

CRISPR-Cas-derived tools will continue to revolutionize modern biology. In ophthalmology, these tools allow for exquisite manipulation of the genomic content in ocular cells. Because of the power of precision medicine, it is likely that the pace at which both mammalian and nonmammalian models for eye disease are used to test other therapeutics in patient-specific backgrounds will increase greatly, finally allowing for treatment plans that are tailored to each patient and genetically informed. As patient safety is at the utmost importance, vigorous screening for off-targets using experimental methods may be strongly encouraged before implantation of CRISPR-Cas edited cells to avoid off-target mutations resulting in malignant or ineffective transplants. Assuming these safeguards are in place, CRISPR-Cas technologies have great potential to benefit countless patients with inherited retinal dystrophies.

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Conflicts of interest

J.E.D. is an author of a patent application filed on a CRISPR/Cas9 application for genome engineering in eukaryotes (PCT/US2014/045691).

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This study shows the use of CRISPR-Cas genome surgery to a validate potentially pathogenic SNP, process that will become increasingly important in verifying unique disease causing mutations in the age of personalized precision medicine.

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