CRISPR-Cas, a novel tool for unraveling the mechanisms of eye-related diseases

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Abstract
CRISPR-Cas is a novel tool that may pinpoint the underlying cause of eye diseases. Here, we introduce the different types of CRISPR-Cas systems with an emphasis on type II and present its applications in eye diseases based on previous reports. The CRISPR microbiome is perhaps bigger than the universe. Bacteria contain a self-defense adaptive immune system known as CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated genes) which inactivates invading phages, plasmids, or foreign DNA sequences. CRISPR is an array of repeats separated by spacers along with \textit{cas} genes. In bacteria CRISPR-array and \textit{cas} genes together form a cascade complex to destroy the invading foreign DNA. The two important components, the Cas9 nuclease, and the short guide RNA are introduced for a targeted genetic event. The short guide-RNA is the fingerprint of the target DNA. CRISPR-Cas has been successfully demonstrated in \textit{Rho}, \textit{CRY AA}, \textit{tyr}, \textit{gol}, \textit{ddx19}, and \textit{mitfa} genes in zebrafish, mice, and rabbits. The nuclear homeoprotein \textit{Six3} mutations by CRISPR-Cas showed eye and anterior head defects in zebrafish. Moreover, CRISPR-Cas has been successfully shown in \textit{Crygc} gene repair in the cataract mice model carrying 1bp deletion in exon3 of \textit{Crygc}. The transcription factors like \textit{Rx}, \textit{Six3}, \textit{Pax6}, \textit{Sox2}, and signaling factors involved in eye formation can be further studied by CRISPR-Cas. The first patient ever to receive CRISPR-Cas9 construct for fixing mutations in a centrosomal protein of 290 kDa, \textit{CEP290}, is for the blindness caused by a rare disorder in the retina, Leber’s congenital amaurosis 10 (LCA10). It is injected directly into the eye near photoreceptor cells and is a landmark clinical trial. The present CRISPR-Cas technology opens a new perspective for new treatments of cataract, retinitis pigmentosa, refractive index error, and any other eye diseases.

Keywords: CRISPR-Cas, CRISPR-array, Cas9 endonuclease, crRNA, sgRNA, Protospacer, PAM

Introduction
Bacterial cells have evolved two well-characterized systems to protect themselves against foreign DNA. One is the restriction-modification system and the other is the CRISPR-Cas system. Both systems maintain and promote the survival of the bacterial genome. Restriction endonucleases perhaps provide protospacers for CRISPR-array. CRISPR repeats were first identified in \textit{E. coli} K12 as identical 29-nucleotide repeat sequences separated by 32-nucleotide nonrepetitive sequences [1]. The CRISPR operon is made up of CRISPR array of repeats and spacers along with \textit{cas} genes encoding CRISPR-associated proteins. CRISPR-arrays have been found in both bacteria and archaea. Their prevalence was evident once the microbial genomic sequences were available, particularly \textit{E. coli} strains. The entire system, however, was characterized only after the discovery of a link between CRISPR-array and its role in protection against foreign DNA [2]. There are three main steps of CRISPR-Cas system (Figure 1): 1) Acquisition/Adaptation: foreign DNA fragment is cleaved and become part of a spacer at or near the leader sequence 2) Expression: CRISPR-array containing foreign DNA is processed into mature CRISPR RNAs (crRNAs) 3) Interference: Cleavage of foreign or invader DNA guided by crRNAs [2-4].

Currently, more than 7000 archaea and bacterial genomes have been found to contain one or more kinds of CRISPR-Cas systems [5]. In total, 11 CRISPR-Cas systems have been identified so far and have been classified into Type I, II, III, and their respective subtypes [4-7]. One interesting phenomenon is the co-occurrence of more than one CRISPR-Cas system in some
bacteria. For example, *Tistrella mobilis*, and *Azospirillum* B510 contain III-A, I-C, and II-C [7]. *Streptococcus thermophilus* has four different types of CRISPR-Cas systems: III-B, I-E, II-A, and II-B [6-8].

Type II CRISPR-Cas system is the most widely used system for gene editing, gene repair, gene regulation, and gene visualization experiments. It can be rapidly used for single or multiple gene function analysis and it has been successfully used for making deletions of up to 1 Mb [9]. Our review is an attempt to discuss this phenomenal bacterial defense system in the understanding and treatment of eye disorders.

We have discussed the known types of CRISPR systems with emphasis on type II. We are also presenting the experimental data employing CRISPR-Cas in the study of different eye genes and discuss the future of this technology.

**Review**

**Type I CRISPR-Cas System**

There are three known types of CRISPR-Cas systems: Type I, II, and III. Each is made up of a CRISPR array and cas genes associated with the array (Table 1). Type I is classified into six subtypes from I-A to I-F. Type I-E has been studied in *E. coli*.

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![Type II CRISPR-Cas System of *Streptococcus pyogenes*.](image)

Figure 1. Type II CRISPR-Cas System of *Streptococcus pyogenes*. shows three important steps: acquisition, expression, and interference by the CRISPR-Cas system of Streptococcus pyogenes. The Cas operon, CRISPR array, and tracrRNA encoding bacterial genomic regions are shown. The tracrRNA is transcribed from the minus strand and CRISPR-array from the plus strand. After transcription, the processing of pre-crRNA involves the base pairing between a 25 nucleotide sequence of tracrRNA and the complementary repeat region of the pre-crRNA transcript which triggers the cleavage by the housekeeping ribonuclease RNAse III. Cas9 is required specifically in the production of mature crRNAs. Once mature crRNAs are formed, they direct Cas9 for cleavage of target DNA. (Fig1 is adapted from ref 6 and 17).

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**Table 1. Major CRISPR-Cas systems, their signature genes, and PAM sequence.**

| System | Subtypes | Signature genes | Acquisition | Expression | Interference | PAM |
|--------|----------|-----------------|------------|-----------|-------------|-----|
| Type I | I-A to I-F | Cas1, Cas2, Cas3, Cas6 | Cas1, Cas2 | Cas6 | Cas3 | 5’-CTT-3’ (E. coli) |
| Type II | A, B, and C | Cas1, Cas2, Csm2, Cas9 | Cas1, Cas2 | RNase III, tracrRNA | Cas9 | 5’-NGG-3’ (S. pyogenes) 5’-NNAGAAw-3’ (S. thermophilus) 5’-NGGNG-3’ (S. thermophilus) |
| Type III | A and B | Cas1, Cas2, Csm or Cmr complex, Cas6, Cas10 | Cas1, Cas2 | Cas6, Csm or Cmr complex | Not clear | No PAM |

Adapted from Staals et al., 2013 [3], Makarova et al., 2011 [4], and Carte et al., 2014 [6], Semenova et al., 2011 [10], Wiedenheft et al., 2011 [11], Mojica et al., 2009 [13], Jinek et al., 2012 [14], Brouns et al., 2008 [62], Jore et al., 2011 [63], Westra et al., 2012 [64], Garneau et al., 2010 [65], Saprauskas et al., 2011 [66].
Type I-E locus consists of 8 genes: cas3, cse1, cse2, cas7, cas5, cas6e, cas1, and cas2 [3]. These cas genes are located upstream of the CRISPR array. CRISPR arrays are typically made up of 29 nucleotide palindromic repeats that are separated from each other by 32-33 nucleotide spacer sequences [3]. Some of these repeats make secondary structures which are important for CRISPR-RNA (crRNA) maturation. Cas6e is a signature gene, a metal-independent endoribonuclease; it cleaves the repeat sequence at the conserved position, 8-nucleotides upstream of the repeatspacer boundary, and helps in the formation of mature crRNAs. Once the mature crRNA is made, Cas6e guides the cascade complex to complementary target DNA [3,4]. Another signature gene Cas3 is a nuclease/helicase and is required for the target degradation along with Cas6e [3] (Table 1). In Type I-E system PAM (protospacer adjacent motif) sequence CTT is the key for recognition [3]. PAM sequence is present in the foreign DNA (protospacer). PAM and a minimum of seven crRNA-complementing nucleotides in the protospacer (the so-called ‘seed sequence’) allow for CRISPR interference to take place [3,10,11].

Type III CRISPR-Cas System

Type III CRISPR-Cas systems are present in 80% of archaea and 40% of bacteria [12]. It has been identified in bacterial pathogens such as Staphylococcus and Mycobacteria and a clinical Staphylococcus epidermidis isolate. The signature genes of type III are Cas6 and Cas10 (Table 1). The other hallmark of type III is RAMPs; multiple genes that encode “repeat-associated mysterious proteins or RAMPs” [12]. RAMPS are a superfamily of Cas proteins part of Csm or Cmr complex, possessing one or more RNA recognition motifs and a characteristic glycine-rich loop [4,12]. Cas6, Cas10, and RAMPS help in processing and maturation of crRNAs and play roles in the defense phase of CRISPR interference [12]. PAM sequence is not required for targeting in type-III systems [12,13]. There is a beautiful example of how bacteria prevent auto-immunity while protecting their genome from foreign invasion. The 5’-tag on the crRNAs of Staphylococcus epidermidis is essential for targeting and most likely constitutes a seed sequence [12]. The flanking repeat sequence upstream of 5’ region licenses the foreign DNA for interference, how; the perfect complementarity between these two regions prevents targeting, the mismatches result in targeting foreign nucleic acid [4,12]. That is how S. epidermidis distinguishes self from non-self [12].

Type II CRISPR-Cas System

Type II is the most widely used system for genome editing. It is the rarest of all CRISPR-Cas systems missing in archaea and present in 3% of the bacterial genomes [4-7]. Type II has three subtypes based upon their characteristic operon organization: II-A, II-B, and II-C. Type IIA contains an additional gene, csn2, which is involved in spacer integration, for example in S. pyogenes SF370 [58]. Csn2 protein exists in both long and short forms [7,8]. Type IIB has cas4 instead of csn2 and it strictly maintains the spacer selection with the right PAM sequence, for example, Synnechocystis sp. 6803 [7,8]. Type IIC has been identified in Neisseria meningitides. It is different from the other type II forms because crRNAs in this type are transcribed from promoters that are embedded within each CRISPR repeat, so crRNA 5’ ends are transcribed [7] and crRNA 3’ end formation requires processing by RNAase III and tracrRNA [60].

Type-II system minimally contains CRISPR-array and 3-4 cas genes [7]. The ubiquitous casI and cas2 genes are required for spacer acquisition and the signature cas9 gene (Table 1) has multiple roles. The cas9 gene is the main endonuclease which recognizes and cleaves target DNA [7,10-17]. Cas9 is a large protein with two distinct domains: MsrA/HHN and RuvC/RNaseH [17]. The HNH domain cleaves DNA strand that is complementary to the guide RNA sequence (target strand) and RuvC/RNase H cleaves DNA strand that is opposite to the complementary strand (non-target strand). Both HNH and RNase H of Cas9 act as a molecular anchor to permit the pairing of tracrRNA with pre-crRNA [6,14] and finally cleave the foreign DNA.

Type II is different from type I and type III because it uses a specialized RNA molecule known as trans-activating crRNA (tracrRNA) and RNAse III instead of Cas-endoribonuclease (in type I and III, mainly Cas6). The tracrRNA sequence is located either between cas9 and casI or upstream of the cas9 gene [6,17,18]. The tracrRNA orthologs are characterized by the presence of an antirepeat sequence homologous to cognate CRISPR repeats [7,8]. The first processing event involves base-pairing of tracrRNA with pre-crRNA repeats in the presence of Cas9 to form RNA duplexes that are cleaved by the endogenous RNase III. Cas9 is an anchor for the base-pairing of tracrRNA with pre-crRNAs [17,18]. The intermediate crRNA species or crRNA1X [6] undergoes a further maturation event resulting in mature individual smaller units called crRNAs that remain duplexed with tracrRNA in a complex with the Cas9 protein [11]. Cas9 is only required during the second cleavage step from crRNA1X to mature crRNAs (Figure 1) [6]. The tracrRNA also undergoes cleavage in parallel with crRNAs [6]. In S. pyogenes, the final crRNA molecule is made up of a single spacer containing ~1/3 of guide sequence and a partial CRISPR repeat of 19-22 nucleotides [12] (Figure 1). The way pre-crRNA is processed from long precursor to final mature CRISPR-RNA varies among different species.

A PAM sequence is present near the protospacer region. PAM sequences in CRISPR-Cas of S. thermophilus are 5’-NNA-GAAW-3’ (W for A/T) and 5’-NGGNG-3’ while in S. pyogenes is 5’-NGG-3’ (Table 1) [8]. PAM sequence helps in recognizing the foreign protospacer region. The PAM-interacting domain of Cas9 guides Cas1-Cas2 complex in selecting spacers [61]. CRISPR protein complex cleaves the protospacer DNA and is then incorporated into the leader end of CRISPR array. During this spacer acquisition stage, a new repeat is also added by an unknown mechanism. Deletions of one or more old spacers have been also detected [8].

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CRISPR-Cas system containing cells are primed to efficiently combat a foreign element that contains a matching protospacer and PAM. Cleavage occurs within the protospacer specifically 3 nucleotides upstream of the PAM sequence in most of the cases of S. thermophilus DGCC7710 and S. pyogenes [8]. In the interference stage, it is assumed that the crRNA and its associated Cas proteins are guided to the matching foreign dsDNA. The specific domains of Cas9 recognize this hybrid structure and cleave the target dsDNA.

CRISPR-spacers mainly appear from the phage genome, mobile genetic elements, and mysterious elements that comprise the 'dark matter' of CRISPR [61]. It was found that one-third of the spacer sequences had no obvious extrachromosomal origin [19,20]. Three CRISPR1 spacers and one CRISPR3 spacer showed 100% identity to S. thermophilus chromosomal sequences, notably in DtpT (a proton symporter), RexA (an ATP-dependent exonuclease), and Ster_0775 (a phage-associated DNA primase) and an intergenic region (between Ster_0810 and Ster_0811 genes) [8,14]. Thus, it is proposed that CRISPR-Cas systems may play a role in microbial genome regulation by controlling mRNA transcript levels [8,15,19,20]. The dark matter of the CRISPR array, the unknown spacers may also comprise the missing phage and mobile genetic elements sequences [61].

**Applications of CRISPR Technology in Ophthalmology**

The discovery of Cas9 endonuclease has resulted in a faster and less expensive way of genome editing or genomic surgery [20-23], but one must be careful for mismatches or off-targets. Cas9 endonuclease has two highly conserved domains: RuvC and HNH as mentioned before. The RuvC domain targets the non-complementary strand and the HNH domain cleaves the complementary target DNA strand [17,57]. Upon complementarity of guide RNA and target DNA, a heteroduplex is formed and then Cas9 cleaves the target DNA [23,57]. Sequences fully complementary to the guide RNA but lacking a nearby PAM are ignored by Cas9 [17,23,57]. In the absence of recognition of PAM sequence the residency time of Cas9 is very short [17,57]. The proximal 10-12 nucleotide near 3' end of the spacer are the seed region, any mismatches in the seed region would prevent Cas9 from executing the cleavage of target, and any homologies in the seed region would cause promiscuity of Cas 9 [57]. Once PAM is bound it results in the unwinding of target DNA and efficient DNA cleavage [17,57]. The Cas9 along with dual guide RNA and tracrRNA is a great external artificial system for mutating genes in a variety of organisms from bacteria to humans. To make mutations, Cas9 nuclease and short guide RNA (containing both crRNA and tracrRNA) are introduced into the host of choice (Figures 2a and 2b).

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**Figure 2. CRISPR-Cas design for eye gene of interest.**

Adapted from the Addgene website (https://www.addgene.org/CRISPR/guide/). The short guide RNA and Cas9 endonuclease can be on the same or two different plasmids. Cas9 applications: endonuclease, nickase, by inactivating Cas9 catalytic domain- Cas9 can be used as a fusion protein containing DNA binding domain of Cas9 + transcriptional activator or repressor, cytidine or thymidine deaminase, the fusion of histone modifiers for epigenetic regulation, the fusion of fluorescent proteins for imaging of genomic loci.
Cas9 endonuclease makes a double-stranded break and can tolerate certain mismatches in the complementary sequence and results in off-target events [22-25]. Off-targets are highly undesirable, especially for therapeutic applications. The enzyme Cas9 nickase only makes nicks in single strands and nicks can be repaired with high fidelity. Mutated Cas9 HNH (H840A) and RuvC (D10A) have been engineered as a Cas9 nickase (dCas9); they result in a single-stranded DNA break [24,25], and use the same base-excision repair pathway, but with little or no damage to the genome or off-targets. So, dCas9 is used with two guide RNAs separated by no more than 10 bps to make nicks on the opposite strands.

Experiments have demonstrated that dCas9 can be used for gene activation or repression studies [26,27]. The dCas9 fused with activation domain VP64 along with multiple sgRNAs showed enhanced expression of ASC1, NANG, HBG1 and HBG2, MyoD1, VEGFA, TERT, IL1B, and IL1R1 promoters in HEK293T cells [26]. Similarly, it has been used for targeted repression by fusing dCas9 with transcriptional repression domain (the Krüppel-associated box (KRAB) domain) [27].

CRISPR-Cas mediated dsDNA breaks can be repaired by either nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) pathways. These pathways exist in all types of cells and organisms [28]. HDR pathway can use another homologous strand which is nearby, for example, in 5-phase or it can be delivered exogenously in the form of oligonucleotide fragment or a plasmid containing homologous regions [29]. During repair by NHEJ pathway, there is an efficient introduction of insertion/deletion mutations (indels) of various lengths [29]. But in HDR, we can insert the desired point mutation or sequence at dsDNA break. Indels are possible even with HDR. The Mutation rate by any of these methods is typically greater than 1% and in some cases over 50% [29].

Correction and Modeling of Cataract-Inducing Mutations

A cataract is a clouding of the lens and for a patient with cataract; vision is like seeing through a frosty or fogged-up window. A cataract is mainly an age-related disease but congenital cataracts are a leading cause of blindness in newborns. Congenital cataracts are mainly caused by autosomal dominant or recessive mutations in the αA-crystallin gene (CRYAA) and αB-crystallin (CRYAB) gene. Congenital cataract has been modeled in rabbits by knocking out αA-crystallin gene. The rabbit zygotes were co-injected with Cas9 mRNA and sgRNA against αA-crystallin gene. These rabbits showed phenotypes of congenital cataracts, microphthalmia, obscurity, early atrophy of the lens, and failed differentiation of lens fibers and these rabbits showed a lack of αA-crystallin protein expression [29]. This new CRISPR-Cas created cataract rabbit model is not only useful for studying the role of crystallin in human cataract but can also help in conducting drug screening for cataract prevention in clinical practice. Recently, the CRISPR-Cas system has been used to reverse the cataract phenotype of mice carrying a dominant mutation in the Crygc gene [30]. These mice carry a 1bp deletion in exon 3 of the Crygc gene which results in a shifted reading frame and introduction of a stop codon at the 76th amino acid of exon 3. The consequence of this deletion is the production of a truncated, dominant-negative γC-crystallin and ultimately, the formation of cataracts [30,31]. To repair this mutation, the mammalian codon-optimized Cas9 mRNA and single-guide RNA (sgRNA) with or without single-stranded oligonucleotide donor were injected into B6D2F1 mouse zygotes carrying one allele of mutant Crygc. When Cas9 mRNA and sgRNA were injected without oligo-donor, out of 135 transferred blastocysts, a total of 22 live pups were born and 4 were corrected of cataracts with HDR-mediated repair by internal wild allele and 2 by NHEJ-mediated indels. By injecting oligo-donor1, 9 out of 29 live pups were free of cataracts and 5 pups showed correction by HDR-directed repair. To further investigate the efficiency of HDR-repair by external oligo-donor, another oligo-donor carrying two synonymous mutations were used. In this case, 9 out of 27 were free of cataracts and 4 were cured via HDR; three of them carried modified Crygc genes with a DNA sequence that was the same as the exogenous oligo-donor, and remaining one had a DNA sequence identical to that of the WT allele [30]. In this experiment mutation repair by using NHEJ or HDR did not show a big difference. The exogenous oligonucleotide can increase the efficiency of homology-directed repair by acting as a template for repairing Cas9-mediated double-strand breaks and replacement of mutant alleles and is especially useful when endogenous wild type allele cannot be used as the template [30,31]. Out of 12 of 22 mice, 2 showed off-target mutations at one of the potential off-target sites. In this study, however, the frequency of correction or repair was low and this may be improved by better optimized Cas9 endonuclease, or Cas9 nickase, better sgRNA or improved injection methods.

A similar CRISPR-Cas-mediated correction has been done in the Fah5981SB mouse model of human hereditary tyrosinemia (HTI). The Fah5981SB mouse is homozygous for a G to A point mutation of the last nucleotide of exon 8 in the fumarylacetoacetase (FAH) gene results in truncated, unstable FAH protein, which mimics human HTI. FAH deficiency causes accumulation of toxic metabolites, such as fumarylacetoacetate, in hepatocytes, resulting in severe liver damage. The plasmid pX330 coexpressing Cas9 and one sgRNA along with a 199 nucleotide, single-stranded DNA donor were given to adult Fah mut/mut mice by hydrodynamic tail vein injections [32]. This resulted in wild-type FAH protein in ~1/250 liver cells [32]. This was sufficient to rescue the body weight loss phenotype, as improvement of liver histology and reduction of serum markers for liver health like aspartate aminotransferase, alanine aminotransferase, and bilirubin [32]. The above type of experiment was also performed in human induced pluripotent stem cells (iPSCs) using a combination of zinc finger nucleases (ZFNs) and piggyBac9 transposon. The piggyBac9 transposon is used for precise and scarless modification of the genome.
Some interesting experiments are done by ectopic expression of Pax6. Elucidation of Eye Formation Pathways involves vacuole formation in the lens, incomplete denucleation, and eye lens with the central cloudy region [34]. In this manner, the human cataract phenotype was just recapitulated in zebrafish embryos. Mutations in the PITX3 and FOXE3 genes, which are expressed in the lens epithelium, cause anterior segment defects, including cataracts, have been also modeled in zebrafish [35-37]. The availability of zebrafish genome sequence information and mutants from Sanger Institute makes modeling multiple cataract-inducing mutations in zebrafish possible. Experiments similar to those on Crygc can likewise be performed on a wide variety of heretofore, in the context of eye diseases, unexamined crystalline genes, connexins, and transcription factors such as FoxC1, Pax6, Six3, PITX3, and HSF4.

Elucidation of Eye Formation Pathways

Some interesting experiments are done by ectopic expression of an eye on antennae or legs. The mouse transcription factor Pax6 (paired box 6) can induce the formation of the functional ommatidial eyes in Drosophila antennae or legs [38-40]. This shows that Pax6 may be a switch for eye development. The expression of mouse Six3 in the ear placode of the medaka fish led to the development of a lens [41]. Similarly, the injection of Six3 mRNA into medaka embryos resulted in ectopic Pax6 expression and the formation of ectopic retinal primordia [41]. Human mutations in Six3 lead to holoprosencephaly; in some cases, the phenotype is milder and manifests a microphthalmia and iris coloboma [41-45]. In humans, Pax6 mutations mainly cause aniridia, isolated cataracts, and Peters anomaly. The significance of Pax6 has been further demonstrated through the use of injected TALENS (Transcription activator-like effector nucleases) against Pax6 which resulted in deformed eyes in Xenopus laevis embryos [45]. CRISPR-Cas induced mutations in the Six3 coding regions as well as in its promoter region showed anterior head and eye defects [46]. The experiments on Pax6 and Six3 suggest that they play critical roles in early eye development. Six3 is also activated by Pax6 and Prox1 [43]. Prox1 is another transcription factor important for the differentiation of lens fiber cells and a-cristallin expression [44]. RX and SOX2 are retinal and anterior neural fold homeobox genes, critical for eye development [44]. RX-deficient mouse embryos lack eye anlagen and Pax6 expression in the eye field [44]. Therefore, Rx, Six3, Pax6, and Sox2 are involved in early eye development.

Defects in signaling molecules such as sonic hedgehog (SHH) also appear to play important roles in eye development [42]. This can be seen in SHH knock-out mice and humans with SHH mutations which both show holoprosencephaly with ocular manifestations that range from microphthalmia to cyclopia [42]. The exact contributions of SHH and other signaling molecules such as BMP4 and BMP7 to eye development are not known. These molecules may regulate for early eye development transcription factors like RX, Six3, Pax6, or other specific eye region transcription factors. Gene editing tools such as CRISPR-Cas can be used to elucidate these pathways. By mutating genes using Cas9 endonuclease or nickase and comparison of phenotypes, additional important genes can be identified. CRISPR-Cas can also be used for visualization of endogenous genomic loci, repetitive, and non-repetitive genomic sequences [47]. Endonuclease deficient Cas9 fused with GFP can be directed by specifically designed sgRNAs for dynamic imaging. This system helped in robust imaging of repetitive elements in telomeres and coding genes in living cells [47]. We can similarly utilize this technique for imaging human eye coding genes. A new technique with inactive Cas9 fused to FokI nuclease has been shown to enhance target specificity and less off-target effects [48]. All these new types of Cas9 fusion proteins will provide more flexibility and understanding of the eye development pathways.

Use of Gene Editing in the Study of Other Eye Disorders

Retinitis pigmentosa (RP) is caused by the progressive degeneration of the retina and retinal pigmented epithelium. It is characterized by the progressive peripheral and night vision loss, which depending on the phenotypic subtype, results in a fast or slow loss of central vision [49]. About 25% of autosomal dominant RP cases are connected to over 100 point mutations in the rhodopsin gene [49]. Identification of different mutations in the rhodopsin gene suggest that misfolding and misfolding of rhodopsin may be the cause of severe changes in vision loss though the mechanism behind these mutations is not understood. The Pro23H mutation is commonly associated with autosomal dominant RP in North America and a stable human embryonic retinoblast cell line containing Pro23H rhodopsin has been created [50]. Zinc finger nucleases (ZFNs) against human rhodopsin gene in human embryonic retinoblasts showed a 12-fold increase in homologous recombination and an absolute frequency of ZFN-directed homologous recombination as high as 17% in human rhodopsin gene [50]. But CRISPR-Cas mediated ablate-and-replace gene therapy showed promising results by rescuing the RhoP23H mutation defect. By injecting codon-optimized Cas9, double gRNA to make a 300 bp deletion containing RhoP23H mutation, and hRHO cDNA to replace the wild allele, driven by mouse RHO promoter. The RhoP23H mice showed remarkable recovery from autosomal dominant RP. Histological and electroretinographical analysis showed 17%
to 36% more outer nuclear layer thickness at three months after AAV injection; the a and b waves were preserved more significantly than just using gene replacement monotherapy [51]. The same strategy also showed promising results in RhoD190N mutation. This is a promising future therapy for human RP. Just like glial cells, Muller glial cells (MG) are adult retinal stem cells that have been implicated in retinal regeneration [52]. Muller glial cells are an injury-induced stem cell model in zebrafish. Zebrafish have a robust capacity for retinal repair and can regenerate whole retinas, somal layers, and even discrete retinal sub-neurons with help of highly regenerative MG cells. In humans, MG cells cannot regenerate, but cultured human MG cells can produce neurons capable of restoring visual function when transplanted into retinal degeneration models, hence they have the potential to act as stem cells and hold the strong therapeutic capacity for retinal regeneration. Using multiplexed CRISPR-Cas9 targeting of genes, 614 sites in 312 regeneration genes, and 14 sites in 8 RP-linked genes were screened by Automated Reporter Quantification assay. This led to the isolation of a novel mutation in rhodopsin (rhod503), which encodes the G-protein coupled receptor necessary for phototransduction in rod cells [52].

CRISPR-Cas was used for mutations in tyrosinase (tyr), golden (gol), mitfa, and ddx19 genes [53] in zebrafish. The tyr gene mutation showed mottled pigmentation, patchy loss of pigmentation in the retinal pigment epithelium, golden gene showed hypopigmentation in RPE, mitfa involved in neural crest development did not show detectable phenotype and ddx19 showed brain necrosis, small eyes, and curved body axis. Biallelic mutations by using TALENS in the tyrosinase (tyr) gene also showed ocularcutaneous albinism in Xenopus tropicalis [54].

Conclusion and Perspectives
CRISPR-Cas has been successfully utilized in the study of pigmentation genes like tyrosinase (tyr) and golden (gol). It has been used for mutations in ddx-19, mitfa, Pax6, gata4, and gata5 [55]. ZFNs have shown successful homology-directed repair of Pro23His mutation in the rhodopsin gene. CRISPR-Cas was applied to repair 1 bp mutation of crygc gene in the mouse cataract model. Many genes, transcription factors, and signaling molecules are common to the brain and eye development. For example, Walker-Warburg syndrome (WWS) and muscle eye brain disorder (MEB) caused by glycosyltransferases which result in defective glycosylation. They both show common brain and eye abnormalities along with muscular dystrophy [56]. A new marker fukutin related protein (FKRP) with novel mutations has been discovered in two new patients of MEB and WWS [56]. We can visualize these common pathways or targets by exploiting inactive Cas9 fused with fluorescence reporter GFP. The new form of inactive Cas9 fused with Fok1 endonucleases may enhance gene specificity and less off-target effects. CRISPR-Cas can be used to rapidly assess single or multiple gene functions. Large inversions/deletions from several 100 bps to 1Mb (981kb) have been achieved in coding regions as well as in microRNA genes of zebrafish [55]. More efficient CRISPR-Cas systems with better gene targeting ability will soon offer new treatments for eye diseases. For example, CRISPR-from Streptococcus thermophilus, Neisseria meningitidis and Treponema denticola have been characterized. Hopefully, new systems will allow better recognition, fewer PAM sequence restrictions, and reduced off-target activity. In the future, more types of cas9 endonucleases, nickases, and Fok1 nucleases will revolutionize mutant gene repair in a variety of eye disorders.

List of Abbreviations
Protopspacer: Short fragment of foreign nucleic acid
PAM: Protospacer adjacent motif
CRISPR-array: DNA sequence of repeats and spacers
Cas9: CRISPR-associated protein 9
crRNA: CRISPR RNA
sgRNA: short guide RNA
tracrRNA: trans-activating crRNA
Indels: Insertions/deletions
NHEJ: Nonhomologus end joining
HDR: Homology-directed repair

Competing interests
The author declares that she has no competing interests.

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