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CRISPR Genome Surgery in the Retina in Light of Off-Targeting

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Abstract

Purpose of review—Recent concerns regarding the clinical utilization of clustered regularly interspaced short palindromic repeats (CRISPR) involve uncertainties about the potential detrimental effects that many arise due to unintended genetic changes, as in off-target mutagenesis, during CRISPR genome surgery. This review gives an overview of off-targeting detection methods and CRISPR's place in the clinical setting, specifically in the field of ophthalmology.

Recent findings—As CRISPR utilization in the laboratory setting has increased, as well as the knowledge regarding CRISPR mechanisms including its off-target effects. Although a perfect method for achieving 100% specificity has yet to be determined, the last few years has seen many developments in off-targeting detection and in increasing CRISPR tools efficacy.

Summary—The CRISPR system has high potential to be an invaluable therapeutic tool as it has the ability to modify and repair pathogenic retinal lesions. Although it is not yet a perfect system, with further efforts to improve its specificity and efficacy along with careful screening of off-target

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mutations, CRISPR-mediated genome surgery potential can become maximized and applied to patients.

Keywords

CRISPR; genome surgery; off-targeting; precision medicine; retina

Introduction

Numerous recent studies have pointed towards both potential promises and perils of clustered regularly interspaced short palindromic repeats (CRISPR) genome surgery in the clinical setting[1–10]. Of the potential perils of the CRISPR genome surgery system, one study raised questions about utilizing CRISPR in the clinical setting without further study of off-target effects[1, 11]. While this report raises warranted concern, it should also be considered that the study, though *in vivo*, the CRISPR genome surgery was performed on zygotes, not adult retinae[11]. As such, the off-target effects reported[1] may not necessarily be reflective of therapeutic interventions that may typically occur in postnatal or post-mitotic retinas. Interestingly, the heated debate regarding off-target effects was raised when nine clinical trials utilizing CRISPR to treat various malignancies had already begun (Table 1). Of note, the first patient recruitment for a CRISPR clinical trial occurred in the past year in October 2016 (NCT027933856). This debate and public concern raise the question of whether these clinical trials began too early without proper consideration of safety[1]. Yet, while the debate is a necessary and important consideration, it is also worth noting that all the CRISPR clinical trials that had begun were in phase I or phase II, evaluating safety (Table 1).

It is also important to note that off-targeting effects, unintended mutations that arise from CRISPR-engineering, are not new to the world of genetics, and methods for detecting such changes do exist and are in further development[12–15]. Unintended changes due to DNA engineering in ophthalmology has certainly been encountered before. In 2008, Kleinman et al. in 2008 presented that while choroidal neovascularization (CNV) could be inhibited by small interfering RNAs (siRNAs), the siRNAs may elicit immune effects as the mechanism of action was in suppressing toll-like receptor 3 (TLR3)[16]. This review seeks to address the questions raised by many on the efficacy of CRISPR, safety considerations, and its place in the clinical setting. This review in particular focuses in part on CRISPR-mediated ophthalmic genome surgery.

Brief CRISPR Mechanism

The CRISPR system is a genome engineering tool derived from bacteria and archaea immune systems[17–19] considered to be highly specific[7, 17]. The CRISPR system has been adapted to serve many purposes in genome engineering including DNA modification and repair, transcription modification, and directed evolution[7, 8, 20–23]. Such diverse applications of CRISPR have allowed further study and understanding of CRISPR. As such, much is known about CRISPR and how it can be directed to specific DNA strands[15, 17, 24]. The most commonly used version of CRISPR is comprised of single guide RNA (sgRNA)[19], denoting a chimera of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA), and CRISPR

associated protein Cas9 endonuclease[7, 17, 19]. The sgRNA binds to the Cas9 which activates the endonuclease[24]. In the actual target DNA, another component is necessary for Cas9 to recognize and cleave at the target location: a protospacer adjacent motif (PAM) adjacent to the target site[24–26]. The required PAM sequence for spCas9, the most commonly used Cas9 orthologue derived from *Streptococcus pyogenes*[3], is "NGG," with N denoting any of the four nucleotides. Once Cas9 makes a double strand break (DSB) at the target location, the DSB can be repaired in a variety of ways which determines the application of CRISPR; an example homology-directed repair (HDR) utilizes a template strand[25, 27] which results in DNA repair of a pathogenic allele to wild-type (WT), and non-homologous end joining (NHEJ) without a template strand introduces insertions and deletions (INDELs) which results in gene knockout of pathogenic allele by frameshift caused by the non-specific INDELs[7, 8, 17, 20]. Figure 1 provides a simplified visual schematic.

Along with the development of different CRISPR techniques, studies have also shown that during CRISPR/Cas9 mediated genome engineering, changes to the DNA can also occur at unintended locations, as in off-target sites[15, 28–32]. This type of off-targeting may arise due to a variety of reasons. One such reason may be that though the lack of the presence of a PAM sequence can become a limiting factor in targeting specific sequences in some cases, the opposite case in which the presence of a PAM sequence in unintended locations may also become targeted by Cas9 seems possible. Or perhaps the fact that Cas9 is directed by RNA[33], and inside the eukaryotic cell many non-coding RNAs (ncRNAs) are present playing regulatory roles[34]; it is possible that these ncRNA may direct Cas9 to unintended sites. Thus, many efforts to characterize and predict off-target effects of CRISPR exist[5, 6, 9, 15, 35]. The ultimate goal of these studies is to not only identify off-targeting but determine how CRISPR's specificity can be maximized with minimal off-target effects[5, 9, 15].

Brief Overview of Current Off-Targeting Detection Methods

In recent years, many methods of genome engineering off-targeting detection have been developed. A brief overview of different methods follows. Figure 2 provides a simplified visual overview. A number of reviews are recommended for a more comprehensive overview[5, 15, 36–38]. Although diverse variations of off-targeting detection methods exist, they can be summarized into the following categories: *in silico* algorithm predictions, *in vitro* selection, and genome-wide assays.

A common method utilized when selecting a sgRNA and checking for off-target mutagenesis is algorithm-based prediction of potential cleavage sites[9, 13, 31, 39]. The algorithms predict off-target cleavage based on sequence similarity to the on-target site. When utilizing *in silico* prediction methods, the predicted sites are checked for INDELs that may have arisen from NHEJ or nucleic acid bulges that occur due to mismatch or gaps in DNA and sgRNA base pairing[31, 32]. Studies of off-targeting using *in silico* prediction have revealed many aspects of off-targeting. Mismatched sites can have high-frequency mutagenesis with five or fewer mismatches[31, 39]. It seems that 10-12 base-pairs proximal to 5′ of the PAM is more determinate of Cas9 specificity than more distal portions[39]. Interestingly, although the PAM sequence is a required component for cleavage, when tested

for spCas9 cleavage of sequences with a 5′-NAG-3′ PAM sequence, cleavage was still achieved at 20% efficiency of that of 5′-NGG-3′ PAM sequence[39]. In consideration of the current knowledge, *in silico* prediction methods have shown that the sequence of the sgRNA is perhaps most indicative of mismatches and off-targeting[9, 13]. Current knowledge has allowed for further development in prediction algorithms including online tools such as CRISPR Design Tool[39], E-CRISP[40] and Cas-OFFinder[13]. But perhaps the biggest consideration of *in silico* prediction methods is that by nature, it is a biased review of off-target sites as the method is based on checking for what is predicted, but not for novel or random potential off-targeting sites that are not yet understood[38].

Another method of detecting off-target mutagenesis is *in vitro* selection combined with high-throughput sequencing; this method was developed in order to more comprehensively characterize spCas9 specificity[30, 41]. This method by Pattanayak et al. utilizes partially randomized concatemeric DNA libraries created by rolling-circle amplification of potential cleavage sites[30]. The libraries are cleaved by CRISPR/Cas9, ligation-tagged, amplified by PCR, then analyzed by high-throughput sequencing computational analysis. Pattanayak et al. reported this method as a comprehensive method; because each selection library includes ~10^{12} target sequence variants, it is theoretically large enough to have tenfold coverage of all sequences with eight or fewer mutations relative to the target sequence. Pattanayak et al. were able to generate over 10^{12} potential off-target sites for the target sequences tested, better defining cleavage characteristics. However, because the randomized oligonucleotide libraries are created to resemble the target sequence, *in vitro* selection may still be a biased method of defining off-target sites[15].

In order to have a true comprehensive understanding of CRISPR/Cas9 actions, especially in off-target sites, an unbiased method is necessary. A number of genome-wide assay methods have thus been developed[15, 36, 38]. In regards to a truly unbiased method, whole-genome sequencing (WGS) could identify even single nucleotide polymorphisms (SNPs) and small INDELs[42]. However, WGS is costly and time-consuming[36]. Another concern with WGS is that although the screening would be accurate for single-cell clones[42], mosaicism is seen in experimental studies of *in vivo* CRISPR/Cas9 editing[11, 43, 44]. It would not be feasible to definitively utilize WGS on every cell in mosaics, but that would be required in order to truly comprehensively identify off-targeting[45]. Thus, even WGS is still an insensitive method of detecting off-target cleavage. Some groups have adapted using whole-exome sequencing (WES) as a more cost-efficient alternative[46] while still detecting changes in the protein-coding regions[47]. However, WES does not detect changes in introns or regulatory regions and is an even more insensitive measure than WGS[36]. Other methods of genome-wide analyses such as integrase-defective lentiviral vector (IDLV) capture, genome-wide unbiased identification of DSBs enabled by sequencing (GUIDE-seq), high-throughput genome-wide translocation sequencing (HTGTS), breaks labelling, enrichment on streptavidin and next-generation sequencing (BLESS), and digested genome sequencing (Digenome-seq) have been developed in recent years to meet the need for better off-target mutagenesis detection[35, 48–51].

Of these, methods that can capture off-target events in living cells is of particular interest as therapeutic applications of genome engineering will need to be applied in living cells.

Gabriel et al. developed IDLV capture in order to show *in vivo* action of endonucleases capture transient DSBs[48]. IDLV capture is a method that detects for DSBs in the genome by essentially tagging DSBs with IDLV that become integrated, or captured, during NHEJ. The sites of integration are amplified by linear amplification-mediated polymerase chain reaction (LAM-PCR) then mapped by high-throughput sequencing. A limitation of IDLV capture however is that IDLVs sometimes randomly integrate into the DNA without endonuclease action. Thus, although sites of high frequency integration can be better identified, and the sites of IDLV capture are not biased by preselection, IDLV capture could show non-specific events. GUIDE-seq, developed by Tsai et al., is a similar method to IDLV capture but integrates double-stranded oligodeoxynucleotide (dsODN) by NHEJ[35]. Another difference is that GUIDE-seq utilizes single-tail adapter/tag (STAT)-PCR, which amplifies only the sequences with integrated dsODN which allows for correction of background and PCR bias. Although GUIDE-seq has high sensitivity with detection of sites with frequencies as low as 0.1% in off-target DSBs in living cells, the detection relies on dsODN insertion, which may not always occur efficiently and is limited by sequencing reads. Another off-targeting detection tool that can be used in living cells is HTGTS, a modified LAM-PCR method by Frock et al.[49]. HTGTS utilizes the detection of translocation events between DSBs created by the endonuclease and off-target DSBs to identify off-target mutagenesis and chromosomal damage[49, 52, 53]. HTGTS does not require any components other than the CRISPR/Cas9 and sgRNA and therefore has the advantage of not introducing other foreign material[49]. However, the rate at which translocations occur is low and are biased towards cleavage sites that are closer in proximity.

Other genome-wide methods cannot be completed in living cells, but offer other advantages. Crosetto et al. developed BLESS which detects telomere ends and endonuclease-induced DSBs for visualization of a genome-wide DSB landscape[50]. BLESS is able to capture transient DSBs by *in situ* labeling of DSBs by ligation of biotinylated linkers. Although BLESS is able to detect DSBs after CRISPR/Cas9 delivery *in vivo* and is a high-resolution method, BLESS requires cell fixation and lysis for analysis. Another approach developed by Kim et al. specifically for human cells, Digenome-seq, is an *in vitro* approach[51]. Digenome-seq is performed by isolating then digesting the genome *in vitro* using Cas9 ribonucleoprotein (RNP), preassembled Cas9 protein and sgRNA. The digested genome is analyzed by WGS. *In vitro* delivery allows the DSB events to be isolated from other *in vivo* factors such as cell conditions and chromatin accessibility. The preassembled Cas9 RNP also allows for maximization of cleavage events identifying off-target sites with frequency of 0.1% or lower. Other than it being an *in vitro* approach, Digenome-seq is also limited by WGS background information, as well as potential inefficient sequencing.

Some Characteristics of Off-Targeting

As described above, no current method of off-target detection is completely sensitive or specific, and more developments are needed. Still, despite limitations of off-targeting detection methods, the development of these methods has given a better understanding of Cas9-induced DSBs and off-targeting events. A finding of greatest concern is that some off-target mutagenesis occurs at frequencies equivalent to or higher than the target site[31, 35] which further emphasizes the need to better define off-targeting. Current understanding of

how the CRISPR/Cas9 system determines DSB locations has led to studies focusing on sgRNA's effects on off-targeting[5, 9]. O'Geen et al. report based on a review of *in silico* predictions, *in vitro* screenings, and genome-wide detections that what determines CRISPR/Cas9 specificity most is the sgRNA and suggest that it is not Cas9 that should be categorized as specific or nonspecific but rather the sgRNA[9]. Though efforts toward better defining sgRNA sequence for higher specificity exist, further study is needed[54, 55]. In regards to the targeted genome, some studies have shown a correlation between off-targeting and chromatin accessibility and non-methylated regions[56, 57]. Another characteristic that has been observed by genome-wide assays is up to six mismatches in the PAM and non-canonical PAM sequences seem to be tolerated by Cas9[15]. It has also been shown that Cas9 will cleave DNA sequences in the presence of DNA or RNA bulges created by mismatched target DNA and sgRNA[32].

CRISPR as a Therapeutic and Safety Considerations

In light of current understanding of off-target mutagenesis, safety considerations for CRISPR/Cas9 as a therapeutic tool is critical. Wu et al.'s study "CRISPR Repair Reveals Causative Mutation in a Preclinical Model of Retinits Pigmentosa" is a relevant and important preclinical model of CRISPR utilization for therapeutic purposes[11]. Wu et al.'s success in disease model rescue following CRISPR-mediated correction of a pathogenic point mutation is an example of CRISPR's potency as a clinical tool. In recent years, CRISPR has been applied to many different species and to a variety of diseases[5, 58] and the successful applications of CRISPR in disease model rescue has built much anticipation for the benefits of CRISPR[7, 17]. Furthering the anticipation is Ma et al.'s recent report in August 2017 of CRISPR/Cas9-mediated correction of *MYPC3* in human preimplantation embryos[59]. Yet, some respond that correction as described by Ma et al. to be unlikely, but rather what likely occurred is deletion of the gene of interest[60], which raises uncertainty and consideration of safety. In evaluating CRISPR as a therapeutic tool, this review focuses on the retina as Wu et al.'s experiment is a retinal disease study, as the vast volume of recent CRISPR-mediated genome engineering does not allow for a comprehensive overview.

The retina is of particular importance as one of the most feared illnesses in America is blindness[61]. This is unsurprising as blindness or low vision can cause impairment in mobility and activities of daily living, and has been linked to depression and anxiety[61]. Retinal pigment epithelium (RPE) disorders, which affect more than 10 million Americans[62], lead to retinal degenerations causing irreversible blindness, as the RPE has little regenerative potential[63]. At this time, retinal degenerations with genetic cause have no curative treatments available. It is hoped that these previously unapproachable genetic retinal dystrophies may be treated by gene and cell-based therapies[63]. An example of such an approach currently undergoing clinical trials is gene therapy. Gene therapy uses viral vectors to insert therapeutic (or wild-type) genes[64]; clinical trials of this therapeutic model have had varying degrees of success[63, 65–72]. The variability of success has been theorized to be attributable in part to the timing of gene therapy; beyond a certain degree of disease progression there may be a "point of no return" due to cell death[65]. Another great limitation of gene therapy is its inability to treat dominant mutations; only recessive conditions and haplo-insufficiency can be approached through gene therapy as it is a gene addition

method[63]. Thus, a different treatment approach is needed. Figure 3 demonstrates this need. The therapeutic method that is expected to have the most potential for success in treating inherited disorders at this time is the CRISPR system[8, 63, 73].

In the laboratory setting, CRISPR/Cas9 has proven to be a useful genome engineering tool in preclinical models, especially in ophthalmology (see Table 2, Table 3). The advantage of CRISPR is that it has the potential to directly correct the genetic defect in induced pluripotent stem cells (iPSCs)[74], which allows corrections for both dominant and recessive genetic mutations. These corrected iPSCs could be transplanted into the patient's eye as a cell-based therapy potentially curing blindness[63, 75]. In 2015, Bassuk et al. were the first to report correction of a retinal dystrophy causative point mutation in patient-derived iPSCs using CRISPR/Cas9[74]. Since then, a number of groups have reported successful correction of pathogenic mutations in patient-derived iPSCs (see Table 3).

Of the numerous CRISPR system applications, Leber congenital amaurosis (LCA) is of particular interest to the current discussion of CRISPR as a therapeutic in the retina, as Editas and Allergan announced in March 2017 plans to develop CRISPR-mediated treatment of LCA10 and begin testing the treatment in 2018. LCA is also important to the discussion of CRISPR correction in general as affected individuals show symptoms within a few months from birth, and the disease-causative mutations are genetically heterogeneous[76, 77]. Of these, LCA10 is caused by the most frequently detected amongst the affected individuals: an intronic mutation in *CEP290* (c.2991+1655A>G). *CEP290* is challenging to target with CRISPR due to its size exceeding adeno-associated virus (AAV) delivery capacity[78, 79]. A dual AAV approach of pAAV-SpCas9 and pAAV-sgRNA has been shown to circumvent the AAV carrying capacity limitation[78]. Another approach which utilized a smaller S. aureus-derived Cas9 with two guide RNAs has also been shown to be effective in circumventing the AAV carrying capacity limitation[79]. These successful corrections are demonstrative of the accelerated developments for potential application that the field has seen in recent years.

Further, expectations for advances in CRISPR applications in ophthalmology is greater than in other fields as the human retina is one of the simplest areas to evaluate experimental gene and cell therapies because of its relative immune privilege by the blood-retina barrier and easy accessibility for monitoring without invasive techniques[80]. That is to say, one of the safest platforms for evaluating CRISPR's safety in clinical trials may be the eye.

Even still, as discussed above, CRISPR has yet to be developed into a perfect system and there are many considerations especially for clinicians to navigate, including lack of accurate and specific off-target mutagenesis detection tools[15]. When safety is not properly assessed, even in a more ideal platform such as the eye, adverse events can occur. Related to the use of CRISPR is the use of stem cells as a therapeutic as mentioned above. Unfortunately, public perception of stem cells, driven in part by deceptive advertising, has led patients to seek stem-cell therapy under unregulated conditions leading to deleterious effects[81]. In such a case, a patient with an inherited retinal dystrophy who was expected to maintain useful vision for many years sought unregulated stem cell treatment and returned with sudden central vision loss in the treated eye[82]. Additionally, recent article "Immunity to CRISPR Cas9 and Cas12a therapeutics" highlights the potential of the CRISPR system to

mount a host immune response, via innate, cellular, and humoral immunity[2]. Further, it is noted that CRISPR correction that introduces new protein products, while therapeutic for the treated disease, may introduce immunologically foreign proteins to the host[2]. Thus, in applying CRISPR to patient-derived iPSCs for autologous transplantation, and all other applications of CRISPR as a therapeutic, all of the benefits and risks must be considered. Without careful consideration of treatments and all of their effects, clinicians may cause more harm than good. For such reasons, efforts to improve CRISPR efficacy, reduce off-targeting, and better understand CRISPR mechanism and its effects are in progress.

Safer CRISPR?

A brief overview of experimental methods of increasing CRISPR/Cas9 specificity is given. For a more complete mechanism of individual methods, studies which report the use of these methods are recommended[4, 29, 83–93]. As discussed above, the specificity of Cas9 is dependent on the sgRNA sequence. Thus, some methods to improve Cas9 specificity utilize the sgRNA sequence[54]. One such example is truncated gRNA (tru-gRNA) which is 2-3 nucleotides shorter than traditional sgRNAs[83, 94]. Tru-gRNA has been shown to reduce off-target mutagenesis in comparison to traditional sgRNA by two to fivefold, but does not reduce off-target mutagenesis to undetectable levels[35]. Another method uses the opposite approach and elongates the sgRNA by two additional guanine nucleotides to the 5′ end[29]. Although the extended gRNA has been shown to reduce off-targeting, it has also been shown to reduce actual on-target site activity as well. Thus, though these two methods of sgRNA manipulation seem to have some success, these methods alone do not seem to be enough for the specificity desired.

Additionally, some groups have modified the Cas9 protein itself in order to improve CRISPR/Cas9 specificity. One method utilizes paired Cas9 nickases (Cas9n) which create single-strand breaks instead of DSBs[84, 85]. This is achieved by deactivating one of the two nuclease domains of the Cas9. Two separate offset nicks, or single-strand breaks, are made on each strand of DNA, guided by two different sgRNAs. This method has been shown to reduce off-target mutagenesis by 50 to 1500-fold in human cells. It is not yet clear whether the usage of a second sgRNA may cause off-target mutagenesis at other locations. Cas9n has also been observed to have an increased frequency of point mutations, which are more difficult to detect than INDELs[15, 87]. Another variation of the Cas9 protein is dimeric RNA-guided FokI-dCas9nuclease (RFN) which has been created to reduce the off-target effects of Cas9n's activity as a monomer[86–88]. The variation is created by fusing catalytically inactive Cas9, or dead Cas9 (dCas9) to dimerization-dependent FokI nuclease domain[95], meaning two co-localized RFN are necessary for cleavage. This dimerization dependent activity also allows extended double-length target sites to be recognized for cleavage. RFN in combination with tru-gRNA, also known as tru-RFNs, has been shown to especially reduce off-target monomer activity[86]. The requirements of the dimerization however also limit the target range of the endonuclease.

Another variation or approach is engineered variants of SpCas9. SpCas9 high-fidelity variant 1 (SpCas9-HF1)[4] and enhanced specificity SpCas9 version 1.1 (eSpCas9 1.1)[89] have alanine substitutions at the predicted Cas9 protein residue that makes contact with the target DNA.

This method disrupts the non-specific DNA contact points and diminishes off-target events. SpCas9-HF1 and eSpCas9 1.1 both show target site cleavage activity at rates comparable to SpCas9. More recently, another interesting variation of Cas9 has been reported: an expanded PAM SpCas9 variant (xCas9), engineered by phage-assisted continuous evolution (PACE)[96]. xCas9 is unique in that it recognizes a broader range of PAM sequences including NG, GAA, and GAT. The wider PAM recognition widens the scope of genetic sequences accessible to CRISPR. Current experimental data shows that though PAM compatibility is broadened, xCas9 actually yields lower rates of off-target activity with increased specificity. Still, while the engineered variants show great specificity with great reduction in off-target events, some off-targeting is observed.

Other methods of improving Cas9 specificity limits the duration of Cas9 activity to reduce the likelihood of off-target events. These methods include utilization of ribonucleoproteins (RNPs) which have been shown to be degraded within 24 hours versus several days of traditional Cas9 delivery[90]. Inducible mechanisms also exist including split Cas9 which is induced to dimerize in the presence of rapamycin for activation of enzymatic activity[91]. Another inducible mechanism represses the enzymatic activity of Cas9 by intein insertion[92]. This particular insertion is cleaved in the presence of 4-hydroxytamoxifen (4-HT), inducing Cas9 activity. Even a photoactivatable Cas9 (paCas9) exists[93]. PaCas9 is composed of split Cas9 fragments that are fused to dimerization domains called Magnets which dimerize upon blue light irradiation, activating endonuclease activity. Optogenetic control allows an inducible system that is also reversible. A very recent advancement in limiting Cas9 activity duration is the use of anti-CRISPR protein, AcrIIA4, or anti-CRISPR DNA mimic[97]. AcrIIA4 mimics DNA and binds to Cas9-sgRNA complexes, interfering with PAM recognition. AcrIIA4 could be administered after a specified time in order to stop Cas9 activity. Overall, reducing the duration of Cas9 activity does reduce off-target events, but still does not yield perfect specificity.

Another CRISPR approach that is in development as an alternative to HDR-dependent repair is the use of base editing[98–101]. Base editing systems are unique as they substitute nucleotides, unlike endonuclease cleavage systems which make DSBs. A third-generation base editor (BE3), which can permanently covert C:G base pairs to T:A base pairs, was introduced in the past year[99]. The BE3 mechanism is similar to a traditional CRISPR system in that a gRNA targets Cas9 to a specific locus. The difference lies in Cas9 engineering; BE3 utilizes inactive Cas9 tethered to a cytidine deaminase enzyme[99, 100, 102, 103]. Upon binding of the engineered Cas9 complex to DNA, a small window of DNA is exposed allowing for any cytidine to be deaminated to uracil. Through a series of steps, the mutated U:G is converted to a T:A[104]. Base editing has been applied to induction of STOP codons (iSTOP), which works by converting codons CAA, CAG, CGA, and TGG into STOP codons, TAA, TAG, and TGA[105]. iSTOP is advantageous as it could potentially treat dominant negative disorders without the need for HDR or NHEJ. Another application of base editing is CRISPR-X, which pairs dCas9 with activation-induced cytidine deaminase (AID), resulting in a diverse library of point mutations[106]. While base editing methods are promising, there are still drawbacks including: conversion of base pairs to T:A at undesired locations, limitation to the C:G to T:A conversion, and varied efficiency depending on the target. Recently, fourth-generation base editing (BE4) has been unveiled which increases editing

efficiency and specificity[104]. BE4 is a fusion of BE3 and Gam, a bacteriophage Mu protein which binds ends of double-stranded DNA. This fusion seems to reduce INDEL formation leading to greater efficiency and specificity, but is still confined to some of the limitations of BE3, specifically the inability to make edits beyond the C:G to T:A conversion.

Conclusions

In recent years, immense research has been completed to improve genome engineering techniques and better understand the mechanisms and effects of genome engineering. Despite great successes and progress, limitations to genome engineering and our understanding of it exist. These limitations bring safety concerns to the usage of CRISPR-mediated genome surgery in the clinical setting. Yet, the CRISPR system has the potential to cure illnesses previously unapproachable. Further efforts to improve CRISPR/Cas9 specificity and efficacy and better define and understand off-target mutagenesis should be made in order to maximize the potentials of the CRISPR system and apply to clinical therapeutics. There is still great anticipation and expectation for CRISPR to be utilized as a therapeutic tool, given that it is with warranted judicious care.

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Summary Statement

This review gives an overview of off-targeting detection methods and CRISPR's place in the clinical setting, specifically in the field of ophthalmology.

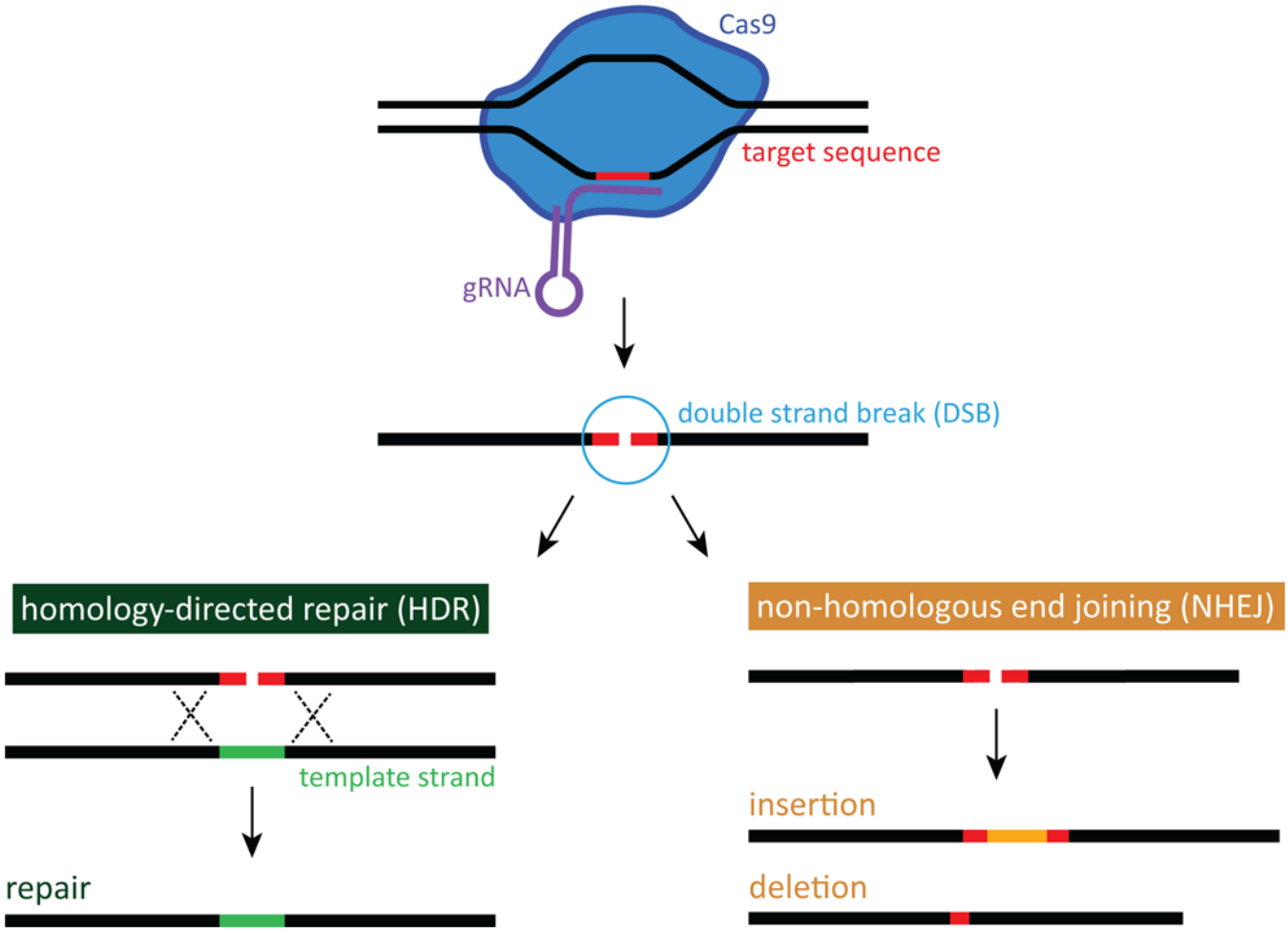

Figure 1.

CRISPR-mediated genome surgery simplified schematic. Homology-directed repair (HDR) and non-homologous end joining (NHEJ). Cas9 endonuclease creates a double strand break (DSB) at the target location. DSB repair by HDR (left) utilizes a wild-type template strand resulting in DNA repair. DSB repair by NHEJ (right) introduces insertions and deletions (INDELs).

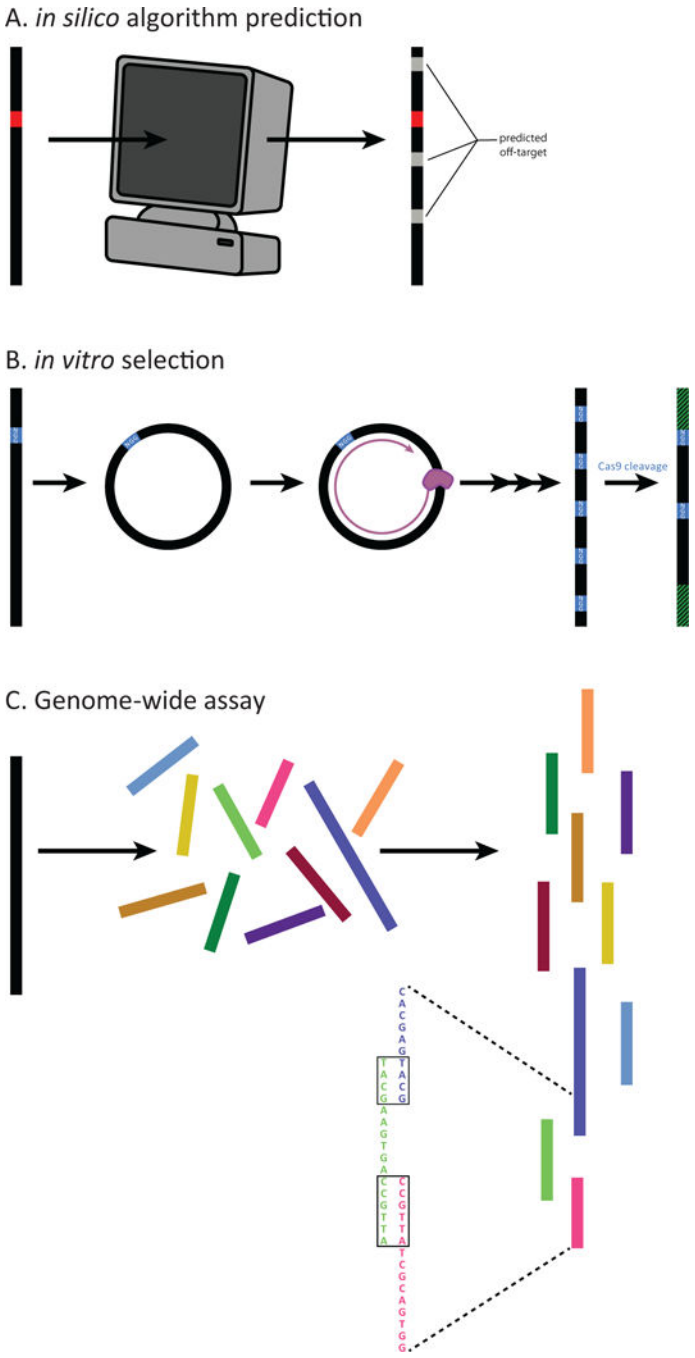

Figure 2.

Off-target detection methods simplified overview. (A) *in silico* algorithm prediction. Computer algorithms are utilized to predict off-target cleavage sites based on sequence similarities. The predicted sites are checked for off-target cleavage that may have occurred but does not allow detection of novel or random off-target sites. (B) *in vitro* selection. Partially randomized concatemeric DNA libraries are created via rolling-circle amplification of potential cleavage sites, which are checked for off-target cleavage. While a large library is created, the sequences are selected for by sequence similarities and may miss some off-

target sites (C) Genome-wide assay. High-throughput sequencing of the entire genome is performed, essentially being an unbiased method as the entire genome is checked for off-target cleavage, although mosaicism may be missed.

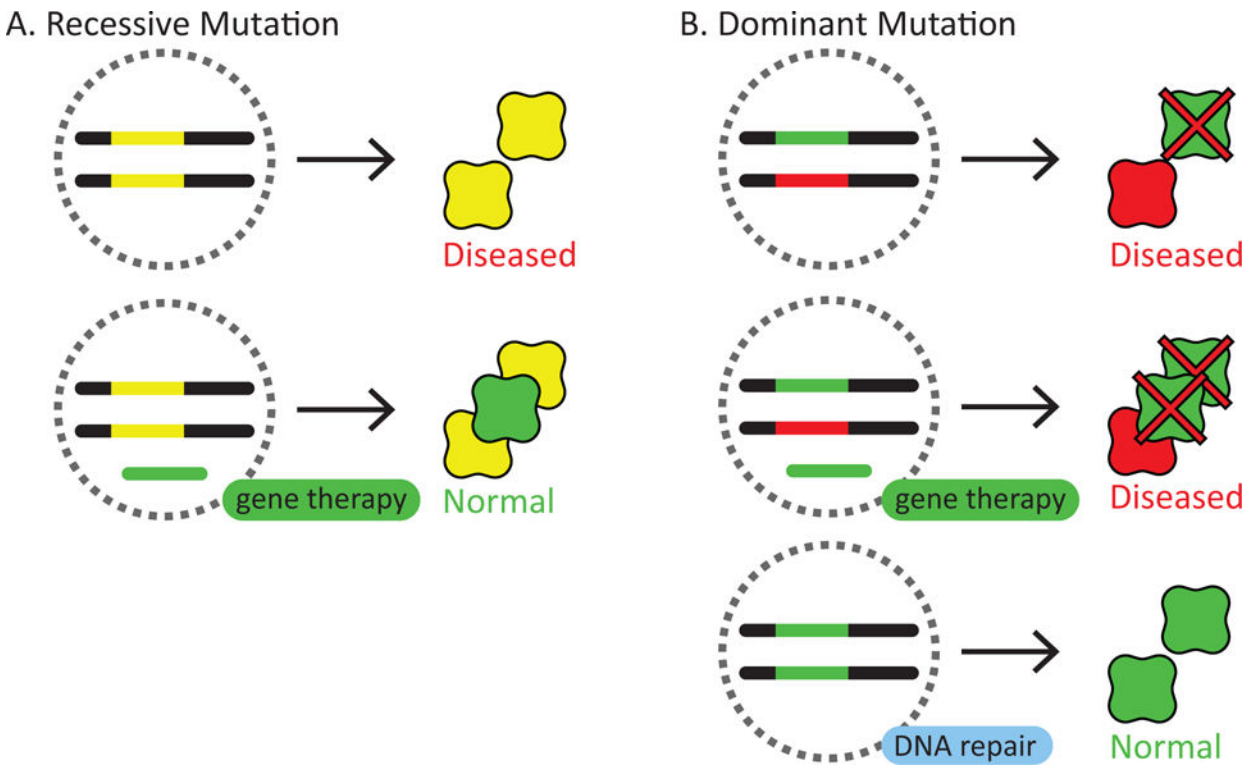

Figure 3.

Therapeutic options for recessive and dominant conditions. (A) Diseases resulting from recessive mutations can be corrected by the addition of a wild-type gene, as in gene therapy. (B) Diseases resulting from dominant mutations cannot be corrected by the addition of a wild-type gene. Correction of the pathogenic dominant mutation by DNA repair, e.g. CRISPR-mediated genome surgery, is needed.

Table 1

CRISPR Clinical Trials at time of debate

NCT ID	Phase	Intervention	Conditions Targeted	Study Sponsor
NCT03057912	I	TALEN- or CRISPR/ Cas9- mediated disruption of HPV E6/E7 DNA	Human Papillomavirus-Related Malignant Neoplasm	First Affiliated Hospital, Sun Yat-Sen University
NCT0381715	II	Autologous CRISPR/ Cas9-engineered PD-1 knockout-T cells	Esophageal Cancer	Hangzhou Cancer Hospital
NCT03044743	I/II	Autologous CRISPR/ Cas9-engineered PD-1 knockout-T cells	Stage IV Gastric Carcinoma; Stage IV Nasopharyngeal Carcinoma; T-Cell Lymphoma Stage IV; Stage IV Adult Hodgkin Lymphoma; Stage IV Diffuse Large B-Cell Lymphoma	Yang Yang, The Affiliated Nanjing Drum Tower Hospital of Nanjing University Medical School
NCT02793856	I	Autologous CRISPR/ Cas9-engineered PD-1 knockout-T cells	Metastatic Non-small Cell Lung Cancer	Sichuan University
NCT02867332	I	Autologous CRISPR/ Cas9-engineered PD-1 knockout-T cells	Metastatic Renal Cell Carcinoma	Peking University
NCT02867345	I	Autologous CRISPR/ Cas9-engineered PD-1 knockout-T cells	Hormone Refractory Prostate Cancer	Peking University
NCT02863913	I	Autologous CRISPR/ Cas9-engineered PD-1 knockout-T cells	Invasive Bladder Cancer Stage IV	Peking University
NCT03164135	–	CRISPR/Cas9 CCR5 gene modification of CD34+ hematopoietic stem cells	HIV-1-infection Hematological Malignancies	Affiliated Hospital to Academy of Military Medical Sciences
NCT03166878	I/II	Autologous CRISPR/ Cas9-engineered universal CD19-specific CAR-T cells	B Cell Leukemia; B Cell Lymphoma	Chinese PLA General Hospital

Table 2

CRISPR/Cas9 Applications for the Retina in Non-human Preclinical Models

Gene of Interest	Associated Human Disease	CRISPR method	Purpose	Model Used or Generated	Reference
Reep6	arRP	CRISPR/Cas9 *Reep6*$^{L135P/L135P}$ knock-in	Disease model generation	*Reep6* knock-in mouse model	107
Reep6	arRP	CRISPR/Cas9 *Reep6* knock-out	Disease model generation	*Reep6* knock-out mouse model	108
Mertk	arRP	CRISPR/Cas9 mediated HITI	New application and Gene rescue	RCS rat	109
Pde6b	arRP	CRISPR/Cas9 HDR	Discern causative mutation in *rd1* mouse model and gene rescue	*rd1* mouse	11
Nrl	–	CRISPR/Cas9 knock-down	Improve rod survival in multiple mouse models of retinal degeneration	*Rho*$^{-/-}$ mouse, *RHO-P347S* transgenic mouse, *Rd10* mouse	110, 111
Rho	adRP	CRISPR/Cas9 ablation of pathogenic mutation	Disease model rescue	Transgenic S334ter rat	112
RHO	adRP	CRISPR/Cas9-induced knock-down of P23H-mutant *RHO*	Feasibility of knock-down *in vivo*	P23H *RHO* transgenic mouse	113
Kcnj13	LCA	CRISPR/Cas9-induced mosaicism of *Kcnj13* function	Disease model generation	*Kcnj13*-related LCA mouse model	44
Mfrp	Multiple including: nanophthalmia, hyperopia, RPE atrophy	CRISPR/Cas9-induced mutagenesis	Disease model generation	*Mfrp* zebrafish	114
Vegfa	Multiple including: CNV, AMD	Cas9 RNP-mediated gene inactivation	Feasibility of gene editing therapy for a non-genetic CNV disease model	laser-induced CNV mouse	115
Pax6	–	CRISPR/Cas9 knock-out	Expand understanding of eye development	*Pax6* knock-out mouse model	43

abbreviations: arRP, autosomal recessive retinitis pigmentosa; adRP, autosomal dominant retinitis pigmentosa; LCA, Leber congenital amaurosis; RPE, retinal pigment epithelium; CNV, choroidal neovascularization; AMD, age-related macular degeneration; HITI, homology-independent targeted integration; HDR, homology-directed recombination; RNP, ribonucleoprotein

Table 3

CRISPR/Cas9 Applications for the Retina in Human Cell Line Preclinical Models

Gene of Interest	Associated Human Disease	CRISPR method	Purpose	Cell Type Used	Reference
MAK	arRP	CRISPR/Cas9 HDR	Gene rescue	Patient-derived iPSCs	108
RPGR	XLRP	CRISPR/Cas9 HDR	Gene rescue	Patient-derived iPSCs	71
CEP290	LCA	Targeted genomic deletion with self-limiting CRISPR/Cas9	Gene rescue	HEK293FT cells with IVS26 mutation in *CEP290*	75
CEP 290	LCA	CRISPR/Cas9 NHEJ of splice site mutation IVS26	Feasibility of protein expression restoration via splice site correction	Patient-derived iPSCs	108
PROM1	STGD4	CRISPR/Cas9 *PROM1* knock-out	Expand understanding of disease mechanism	ARPE-19 cells (human RPE cell line)	109
VEGF-A	Multiple including: CNV, AMD	CRISPR/Cas9-mediated INDEL formation	Feasibility of gene editing therapy in human RPE cells	ARPE-19 cells (human RPE cell line)	110

abbreviations: arRP, autosomal recessive retinitis pigmentosa; XLRP, X-linked retinitis pigmentosa; LCA, Leber congenital amaurosis; STGD4, Stargardt-like macular dystrophy; CNV, choroidal neovascularization; AMD, age-related macular degeneration; HDR, homology-directed recombination; NHEJ, Non-homologous end joining; INDEL, insertions or deletions; iPSCS, induced pluripotent stem cells; RPE, retinal pigment epithelium