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Review

CRISPR-Cas Genome Surgery in Ophthalmology

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Genetic disease affecting vision can significantly impact patient quality of life. Gene therapy seeks to slow the progression of these diseases by treating the underlying etiology at the level of the genome. Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated systems (Cas) represent powerful tools for studying diseases through the creation of model organisms generated by targeted modification and by the correction of disease mutations for therapeutic purposes. CRISPR-Cas systems have been applied successfully to the visual sciences and study of ophthalmic disease – from the modification of zebrafish and mammalian models of eye development and disease, to the correction of pathogenic mutations in patient-derived stem cells. Recent advances in CRISPR-Cas delivery and optimization boast improved functionality that continues to enhance genome-engineering applications in the eye. This review provides a synopsis of the recent implementations of CRISPR-Cas tools in the field of ophthalmology.

Introduction

Precision medicine as a field aims to identify the genetic basis of a disease that is specific to a patient and subsequently treat the individual with a tailored approach. To achieve this, genotype-phenotype correlations in disease processes must be identified. In ophthalmology, the ocular manifestation of disease-causing mutations sometimes is directly observable to the clinician. However, patient-specific treatments are hampered by the considerable heterogeneity of inherited ocular disease. For example, 250 genes to date have been identified or associated in the development of retinal diseases, and numerous more have been implicated in diverse corneal genetic dystrophies.[1–4] Clustered regularly interspaced short palindromic repeats–associated systems (CRISPR-Cas) genome editing may provide relatively easy manipulation of these genetic targets. This review discusses important progress in the use of CRISPR-Cas systems for the study of the human eye and the scope of its application in treating congenital ocular diseases.

A Brief History of Genome Surgery

Stable modification of genetic material within living systems is an essential tool for studying biology

and developing therapeutics capable of ameliorating disease. Beginning with the experiments of Griffith[5] on the transformation of bacteria in 1928, scientists have capitalized on the ability to introduce foreign genetic material into cells to modify their activity. Almost a century later, studies in yeast showed that the induction of double-stranded breaks (DSBs) in DNA could stimulate homologous recombination (HR), a process in which similar or identical DNA sequences are exchanged.[6,7] HR is an intricate process involving endogenous protein machinery that identifies similar or identical DNA sequences, resects nucleotides in the region of the DSB to expose single-stranded nucleotide arms, coordinates strand invasion or strand annealing of complementary base pairs, and resolves intermediate structures to exchange sequences successfully between DNA loci. Additionally, if homologous repeat regions are located near the resected bases within the same DNA molecule, it is possible for the sequence to be repaired without intermolecular exchange using a process called single-strand annealing (SSA) in the case of large homology (>100 base pairs), or microhomology-mediated end joining (MMEJ) for smaller homology.[8] While DSBs can be repaired by HR, an alternative correction pathway called nonhomologous end joining (NHEJ) occurs more frequently. This error-prone mechanism does not rely on any template, but instead simply ligates the ends of the cleaved DNA back together, often resulting in nucleotide insertions and deletions (indels) in the process.

Further studies showed that DSBs can stimulate incorporation of foreign genetic material into mammalian genomes.[8–10] With these early findings, scientists then sought to generate DSBs to induce homologous recombination with foreign DNA; thus, manipulating genes in a targeted fashion. One of the first methods for implementing such breaks was with the use of meganucleases, such as I-SceI, which contained a fixed 23 base pair recognition sequence.[11,12] To generate de novo cut site preferences, scientific interest shifted to engineerable DNA binding proteins, which function as designer endonucleases that cleave and subsequently stimulate HR at genomic positions of interest. The first of these engineered endonucleases was the zinc-finger nuclease, consisting of a modifiable zinc finger domain that targets the protein to a specific, approximately 18 base pair region, which is fused to a FokI endonuclease.[13–15] While efficient, expanding the quick generation of zinc-finger nucleases to other sites has proved difficult.[16,17] Subsequent engineerable nucleases, such as transcription activator-like nucleases (TALENs), can generate DSBs to engineer eukaryotic cells.[18–20] TALENs are an alternative to zinc-finger nucleases, requiring less financial overhead for their generation and quicker turnaround from design to implementation. However, they are not without their downsides, as TALENs are relatively large proteins and often contain repetitive DNA sequences that result in TALEN inactivation due to recombination.[18]

CRISPR-Cas systems represent a novel and efficient method of gene editing. Before the advent of this technique, conditions inherited in an autosomal dominant or dominant negative fashion theoretically were less approachable. Previous gene-based therapies that translated to clinical trials for inherited ocular disease largely treated autosomal recessive conditions. In this context, a cell harboring biallelic pathogenic mutations in a disease-associated gene could be supplemented with the wildtype, functional version of the gene.[21,22] However, the same strategy could not be applied to dominant conditions, as the pathogenic gene needed to be repaired or down-regulated instead. CRISPR-Cas has the potential to correct dominantly inherited mutations; thus, expanding the repertoire of conditions that scientists and physicians alike can approach.

CRISPR-Cas Systems in Genome Editing

The field of engineerable nucleases was transformed following the discovery of a bacterial immune system mechanism occurring in *Streptococcus pyogenes*. CRISPR and Cas proteins provide an important mechanism of immunity against viral invasion in bacteria and archaea. These systems "remember" previous viral infections, store a DNA copy of the viral sequence, and then use that sequence to cleave future invading viruses.[23,24] While these systems were first noticed in bacterial genomes in 1987, it was not until 2012 when Jinek et al.[25] and others[26–28] discovered the mechanisms of a Type II CRISPR-Cas system of *Streptomyces pyogenes*, allowing for future breakthroughs in genome editing. In their work, Jinek et al.[25] found that *S. pyogenes* Cas9 (SpCas9) protein was capable of cleaving double strand DNA when two RNA molecules were present, a CRISPR RNA (crRNA) and a trans-activating RNA (tracrRNA). crRNA guides the complex to a region of DNA specified by a 20 base pair sequence, while trans-activating RNA (tracrRNA) associates

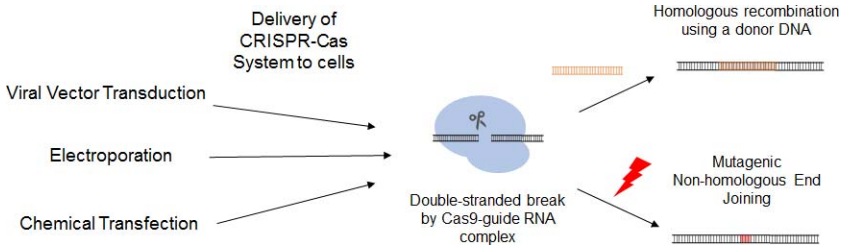

Figure 1. Delivery of CRISPR-Cas systems to live cells to generate DSBs. DSBs can be repaired via multiple pathways, including homologous recombination or nonhomologous end joining.

with crRNA and SpCas9 and allows the latter to function as a nuclease. Another requirement is an approximately 3 base pair region (encoded by a NGG trinucleotide) adjacent to the 20 base pair, crRNA-specified region, termed the protospacer adjacent motif (PAM). By fusing the tracrRNA and the crRNA, termed single guide RNA (sgRNA), Jinek et al.[25] could guide the SpCas9 enzyme to cut DNA with high specificity, thus simplifying the system for future use.[29] Not long after these developments, the SpCas9 system was rapidly repurposed to provide gene repair in human cells as well as numerous other organisms (Fig. 1).[30–36]

After elucidating the mechanism of SpCas9, numerous modifications were made to this enzyme, such as the removal of endonuclease activity (dCas9), attenuating the endonuclease activity to that of a nickase (SpCas9D10A).[29,37–39] Moreover, dCas9 has been used to repress genes directly (termed CRISPR interference or CRISPRi) or modified to act as a gene activator (CRISPRa), DNA epigenetic modulator, or DNA cytidine deaminase, further modifying the effects of this programmable, DNA-binding, RNA-protein complex.[40–42] Initial studies with wild-type SpCas9 showed that the potential for off-target cleavage was dependent on the sgRNA used and that the 12 base pairs of the target sequence closest to the PAM were the most important for determining specificity.[29,43,44] Further refinement of SpCas9 by the generation of a high-fidelity mutant (SpCas9-HF) to increase accuracy also was accomplished by generating mutants with decreased nonpecific DNA interactions, leading to undetectable off-target activity.[45] While these results are promising for the increased specificity of newer SpCas9 mutants, testing off-target activity for each sgRNA used in therapeutics may be advisable to avoid unintended mutagenesis.[44]

In addition to SpCas9, many groups are actively searching for CRISPR-Cas systems with different specificities and improved qualities, such as smaller Cas gene sizes that are more suitable to the carrying capacity of various gene delivery techniques.[46–48] In addition to SpCas9, the CRISPR-Cpf1 system identified in *Acidaminococcus* and *Lachnospiraceae* bacteria was used in human cells effectively and offers different PAM site specificity.[47,49] Recently, Kim et al.[48] identified a small, 2.9 kilobase pair (kb) orthologue of SpCas9 in *Campylobacter jejuni* (CjCas9), whose reduced size compared to a 4.1 kb SpCas9 makes CjCas9 more attractive for gene delivery purposes. This enzyme will be discussed in more detail in later sections, as it relates directly to applications in ophthalmology.[48] As this technology is further developed, it is likely that future CRISPR-Cas systems will continue to improve in efficiency and targeting specificity.

CRISPR-Cas Genome Surgery in Animal Models of the Eye

Zebrafish Models of Human Eye Development

Zebrafish represent a powerful model for studying vertebrate eye development. With ease of access to eye tissue for imaging and drug/gene delivery, short lifecycle, and genetic similarities to higher vertebrates and mammals, zebrafish have many advantages to other model organisms. [50] Several groups have used zebrafish models to explore the applications of CRISPR-Cas gene editing in the study of important pathways and regulators in the development of ocular tissue. An example of such work was by Collery et al.,[51] who used CRISPR-Cas gene editing to examine the role of membrane frizzled related protein (MFRP) in the development of nanopthalmic-hyperopia. The MFRP protein, when mutated in humans, can cause extreme hyperopia, but developing an animal model previously was difficult, as *Mfrp* mutant mice do not faithfully recapitulate this phenotype.[52,53] By injecting

mRNA for Cas9 and sgRNAs targeting two exons of *Mfrp* into the embryos of zebrafish, the group was able to generate heterozygote missense mutants for this gene that then were crossed to generate homozygotes.[51] These $Mfrp^{-/-}$ mutants have nanophthalmos, hyperopia, and increased retinal folding, which data suggest is from a reduction in scleral proportions, not retinal cell proliferation. Additionally, the $Mfrp^{-/-}$ mutants show a reduced optokinetic response.[51] This work demonstrates the power of CRISPR-Cas genome editing in zebrafish and the important role this organism has in studying diseases of the human eye.

One of the most popular methods for modifying gene expression in zebrafish is the use of a morpholino oligonucleotide, which are short oligonucleotides containing morpholine bases that can inhibit gene translation or splicing.[54,55] For example, Serifi et al.[56] combined CRISPR and morpholino techniques and found that the *Setb* gene in zebrafish, a paralog of the human *SET/I2PP2A* gene, has a role in the development of the retina.[56] Another example of using SpCas9-generated knockouts and morpholinos together was shown by Taylor et al.,[57] who targeted a notch-signaling pathway protein, *NeuroD,* to show its important role in photoceptor regeneration.

Yin et al.[58] modulated SpCas9 expression to specific tissue types to specify the cleavage of specific genes by SpCas9 to certain tissues after being injected with sgRNA.[58] In addition to specific tissue promoters, they also expressed SpCas9 under the *Actb2* promotor, a broadly expressed gene, and then electroporated two sgRNAs that targeted *Ascl1a,* an important gene involved in zebrafish retina regeneration and Müller glia de-differentiation.[58,59] After 48-hours of photodamage, the zebrafish containing the sgRNA targeting *Asc1a* had an inner nuclear layer with 5-fold less proliferating cells. This proof-of-concept study illustrated the use of combining broadly expressed promoters with specific sgRNA delivery to target gene regulation in desired cell types.[8]

CRISPR-Cas Editing in Mammalian Models of Human Eye Development

Genome editing in mouse embryos became quicker and more cost-efficient with the advent of CRISPR-Cas technology.[33] In ophthalmology, these animal models represent important first steps for examining genetic conditions as well as assessing therapeutics for such genetic diseases. Sometimes, animal models used to study diseases contain mutations or other genetic elements of unknown consequence that affect the mechanistic insight gained from such models. CRISPR-Cas genome editing has deconvoluted long-standing controversies surrounding these interactions by adjusting pre-existing models of human disease. One example of this is provided by Wu et al.[60] and Keeler[61] who used SpCas9 to correct a nonsense mutation (Y347X) in the *Pde6b* gene in the rodless rd1 $Pde6b^{rd1}$/ $Pde6b^{rd1}$ mouse, a model system for retinitis pigmentosa (RP). These mice also contain an *Xmv*-28 viral intronic insertion in *Pde6b,* and it was unclear whether the *Xmv*-28 insertion or the Y347X mutation was the major contributor to the RP phenotype. After correction of the Y347X mutation using a single stranded oligonucleotide donor DNA template, the group showed that retinal structure and function could be rescued, suggesting that the *Xmv*-28 insertion does not drive the degeneration in this model.[60]

In vitro experiments using mammalian cells also provide insight into the development of genetic disease in humans. Lv et al.[62] recently showed that the *RP9* gene, a pre-mRNA splicing factor thought to be implicated in autosomal dominant RP, has a key role in proliferation and migration of photoceptor cells in vitro. The group used SpCas9 to knockout *Rp9* and knock in a patient-specific *RP9* mutant (c.A410T, P.H137L) in 661 W mouse retinal photoreceptor cell line. This resulted in decreased proliferation and cell migration.[62] They also found that the edited cell lines had altered pre-mRNA splicing profiles, and specifically, splicing of intron 3 and intron 4 of the retinal *Fscn2* gene, which is an RP-associated gene, was affected, further providing a basis for the role of mutant *RP9* alleles in retinal degeneration.[62]

CRISPR-Cas systems were applied to mammalian models of the eye, such as in a study of a particular Leber's congenital amaurosis (LCA) model.[63,64] These investigators examined the *Kcnj13* gene, a Kir7.1 retinal pigment epithelium potassium channel whose mutants have been implicated in human LCA, although homozygous mutants of this gene are inviable in mice. They took advantage of CRISPR-Cas genome editing to generate mutant *Kcnj13* genes by targeting SpCas9 to cleave the locus in mouse zygotes, without supplying a homologous donor template, leading to NHEJ-derived mutations and a mosaic mutant pattern, with 80% mutant cells in the

RPE. They found that RPE cells with mutant *Kcnj13* are viable but subsequently degenerate, and that wild-type cells in close proximity help rescue degeneration. These investigators chose CRISPR-Cas editing instead of a Cre-loxP system (a system that allows for inducible recombination and knock-out of specific genes) to generate knockout at the tissue level. This was advantageous, as Cre-loxP systems require more time to engineer the mouse strain, while CRISPR-Cas systems offer less steps to generate knockouts and still afford a high degree of control.[63]

CRISPR-Cas Therapeutic Tool Development in Mammalian Models

In addition to generating more ways to study ocular disease, CRISPR-Cas genome editing in animal models has been useful in developing and testing therapeutic techniques that may render sight-saving effects in patients. Targeting pathogenic variants that are inherited in a dominant fashion using CRISPR-Cas gene targeting is an illustration of such therapeutics. Courtney et al.[65] used a similar technique to inactivate the C395T pathogenic mutant of *KRT12* involved in the development of Meesmann epithelial corneal dysplasia (MECD).[65] This mutant generates a de novo PAM sequence that could be targeted specifically with sgRNA constructs for cleavage, which is accomplished by intrastromal injection of plasmids encoding SpCas9/GFP and a sgRNA targeting *Krt12* (C395T).[65] The group found that 38.5% of the clones analyzed contained NHEJ-derived mutations of the pathogenic allele, suggesting this approach in humans could be viable for knocking down autosomal dominant pathogenic alleles that can be targeted specifically with CRISPR-Cas systems.[65]

Targeting a de novo PAM sequence of pathogenic autosomal dominant allele without providing a repair template for repair by homologous recombination can lead to ablation of the allele, a strategy that was used in models for retinitis pigmentosa. Specifically, Bakondi et al.[66] targeted a de novo PAM in the Rho^{S334} allele of an RP rat model that results in *RHO* protein processing errors and degeneration of photoreceptors. This group introduced sgRNA and SpCas9 plasmids into rats through subretinal injection, resulting in a 33% and 36% mutation frequency of the Rho^{S334} allele in two separate animals.[66] These percentages were sufficient to result in a 9-fold increase in photoreceptor nuclei density, a visual acuity increase of 53% by optokinetic assessment, and a 35% increase compared to untreated contralateral eyes.[66] These functional improvements in sight in such model organisms buoy the promise for beneficial targeting of autosomal dominant pathogenic alleles in humans. Similar to the work of Bakondi et al.,[66] Latella et al.[67] used CRISPR-Cas genome engineering to target the *RHO* gene in a transgenic mouse model for RP encoding the patient-derived human P23H mutant *RHO* minigene. Latella et al.[67] showed robust editing knockout capabilities by the use of two sgRNA (designed to cut 5′ and 3′ regions in exon 1 of the *RHO* gene) encoded on a single plasmid delivered with SpCas9 via subretinal injection and electroporation. Their data suggested a significant reduction in expression of the human *RHO* gene, further supporting the use of CRISPR-Cas systems to target autosomal dominant disease alleles.[67]

While genomic editing using CRISPR-Cas tools is effective and easy, delivery of these tools to cells in vivo presents similar challenges as previous gene-based therapy techniques. Viral vectors, such as adeno-associated viral (AAV) vectors, serve as important tools for delivering CRISPR RNA, Cas protein, and even donor DNA components often required for precise genome engineering to cells in specific tissues in live animals, as well as in isolated cells.[68,69] Hung et al.[70] examined the efficacy of AAV2 delivery of SpCas9 and sgRNA encoding DNA targeting an endogenous Yellow Fluorescent Protein introduced in the *Thy1* locus. By measuring the inactivation of YFP expression by NHEJ-induced mutations, the group found that, after intravitreal injection, there was an 84% reduction of YFP expression in cells that received the sgRNA targeting *YFP*.[70] They also found a 50% reduction in *YFP* expression in retinal ganglion cells (RGCs). Five weeks after injection, no significant changes in retinal structure or health by electroretinography were detected, indicating few off-targeting effects and limited impact of the CRISPR-Cas components targeting neutral genes on cellular health in the eye.[70] AAVs serve as a promising vector for gene therapy in human eyes, and studies such as the one by Hung et al.[70] and others[71–73] are important stepping stones for improving CRISPR-Cas delivery using viral vectors.

As mentioned previously, other CRISPR-Cas systems could provide improved qualities for genome engineering currently lacking in SpCas9. The smaller Cas9 orthologue CjCas9 has great gene delivery potential due to its 2.9 kb gene size for vectors, such as AAV vectors whose maximal carrying capacity is

4.7 kb.[48,74,75] After teasing out the required guide RNAs and PAM sequences, Kim et al. showed efficient programmable DNA cutting in vitro and in vivo using CjCas9.[48] Moreover, this group packaged CjCas9 as well as guide RNA targeting *Vegfa* or *Hif1a* genes into an AAV9 vector, then delivered this to the retina in a mouse model for choroidal neovascularization (CNV).[48] The group found a reduction of CNV by 24 ± 4% with the *Vegfa* guide RNA and 20 ± 4% with the *Hif1a* guide RNA after 1 week.[48] The group also found that the AAV vector with the *Vegfa* guide RNA cause a reduction in opsin-positive area (a marker for cone cell functionality) of the retina, compared to the AAV vector with the Hif1a guide RNA which did not. This indicated that Hif1a could be an attractive and safer target.[48] This small Cas9 protein and its efficient editing in retinal cells show promise for more widespread use of this system in gene editing in the future.

While *VEGFA* has been a common target for inhibitors used to decrease neovascularization in the eye, another potential target is a receptor of VEGFA, *VEGFR2*.[76] Huang et al.[76] used SpCas9 to edit exon 3 of *VEGF2* in primary human retinal microvascular endothelial cells and found that mutating VEGF2 significantly decreased VEGF stimulated proliferation in these cells, and did so to a greater extent than aflibercept and ranibizumab (two common anti-VEGF proteins used to inhibit the growth of damaging abnormal blood vessel formation in patients). They also found that the mutant *VEGF2* cell population was less responsive to VEGF when forming tubes (a marker of angiogenesis) compared to wild-type cells, again with the *VEGFR2* mutated cells being inhibited more than the aflibercept and ranibizumab treated cells.[76] This study exemplifies the use of CRISPR-Cas gene editing to directly modify a receptor for vascular growth factors and could stimulate novel therapeutic targets going forward.

A novel approach to delivering CRISPR-Cas genome editing tools to eye cells is by directly delivering Cas9/sgRNA ribonucleoproteins instead of DNA vectors, which require transcription and translation before expression of desired proteins.[77,78] In a CNV mouse model of age-related macular degeneration, Kim et al.[77] administered ribonucleoproteins of SpCas9 and sgRNA targeting *Vegfa* by subretinal injection and found a 25% ± 3% mutation frequency in RPE cells 3 days after injection. They also discovered by Western blot analysis that SpCas9 was degraded by this time, as predicted due to rapid degradation of introduced proteins without stable expression.[77] CNV mouse models were provided subretinal injections with these complexes, resulting in a significant decrease in neovascularization. In the same study, 42 potential off-target cut sites by the *Vegfa* SpCas9-sgRNA complex were identified, but the researchers were unable to identify evidence of such cleavage in deep-sequencing of ribonucleoprotein-treated animals.[77] No cone dysfunction was observed 7 days after injection, which was surprising given that researchers have noticed cone damage after *Vegfa* deletion in other studies. The lack of cone dysfunction may be due to the unique delivery method of gene editing components used in this study.[77,79]

In addition to designing more efficient and effective strategies to deliver CRISPR-Cas components to cells, scientists also have focused on controlling expression of the components post-delivery. For example, to study the use of SpCas9 and sgRNAs targeting the human splice mutation IVS26 in the *CEP290* gene involved in LCA, Ruan et al.[80] incorporated an additional sgRNA into the AAV transduced construct: a sgRNA that targeted the CRISPR construct itself. As prolonged expression of CRISPR-Cas components can be undesirable due to off-targeted activity and potential toxicity, this self-limiting system was able to successfully reduce expression of the IVS26 splice mutation as well as destroy the CRISPR-Cas components shortly after introduction, limiting potential deleterious effects.[80] This approach may be used in the future to reduce prolonged expression of CRISPR-Cas systems in the eye post-modification, which could serve to diminish off-target effects of the Cas endonuclease.

Targeting specific mutations using CRISPR-Cas systems as well as generation of patient mutations in models allow for the rapid study of human diseases of the eye in a precise manner. The easily reprogrammable nature of the Cas9 endonuclease allows for mutation-specific targeting to occur. Table 1 summarizes specific mutations targeted or generated in eye cells mentioned in this review.

CRISPR-Cas Genome Surgery in Human Eye Tissue

Since SpCas9 was first applied in human cells, there has been a steep rise in the use of CRISPR-Cas systems to edit human cells in vitro.[29–31] A example of this use in ophthalmology was the recent use of SpCas9 to knockout the *PAX6* gene in human corneal

Table 1. Summary of Specific Gene Mutations Created or Targeted Using CRISPR-Cas Systems in This Review

Disease Studied	Gene/Mutation	Organism/Cell Line	CRISPR-Cas System	Delivery Mechanism
RP	*RP9* (c.A410T, P.H137L)	Mouse retinal cell line 661W	SpCas9	Chemical transfection
Meesmann epithelial corneal dysplasia	*KRT12* (C395T)	Corneal epithelium in mice	SpCas9	Intrastromal injection
RP	RHO^{S334}	Retinal cells in mice	SpCas9	Subretinal Injection
Leber's congenital amaurosis	*CEP290* splice mutation IVS26	Retinal cells in mice	SpCas9	Subretinal injection of adeno-associated virus serotype 5 vector
Proliferative vitreoretinopathy	*MDM2* rs2279744	Human primary retinal pigment epithelial	SpCas9D10A nickase	Adeno-associated virus vector in vitro
RP	*RPGR* c. 3070G>T, pGlu1024X	Induced pluripotent stem cells	SpCas9	Chemical transfection
RP	$Pde6b^{rd1}$ Y347X	rd1 mice	SpCas9	Zygote injection

epithelial cells (CEC) in vitro by Kitazawa et al.[81] This group found that retrovirally delivered constructs encoding SpCas9 and two sgRNAs targeting *PAX6* were able to generate knockouts of the gene, which caused decreased expression of corneal-specific genes and an increase in epidermis-related genes compared to controls as analyzed by microarray and qPCR.[81] This suggested that *PAX6* is important in the development of nonkeratinized epithelial qualities of CEC cells and further validates the use of CRISPR-Cas systems for editing the human genome in vitro for ocular applications.[81] The importance of *Pax6* in eye development was suggested further by Yasue et al.,[82] who used SpCas9 to make *Pax6* mutant mouse embryos with a varying degree of mosaicism at the *Pax6* locus. They found that the degree of eye malformation depended on the type of mutation generated, with the most severe malformations correlated with null *Pax6* mutants.[82]

Another method used for knockdown of gene expression that came before the advent of CRISPR-Cas systems for genome editing is the use of small interfering RNA (siRNA), which target mRNA for degradation by endogenous processes.[83] CRISPR-Cas genome editing and siRNA tools can be used in combination similar to the use of CRISPR-Cas tools and morpholinos as described previously in zebrafish. An example of this was the SpCas9-derived knockout and siRNA knockdown of the β1,6 N-acetylglucosaminyltransferase V (MGAT) gene in RPE cells by Priglinger et al. in their study of the epithelial-to-mesenchymal transition (EMT).[84] This transition is involved in proliferative vitreoretinopathy, a phenomenon that produces complications during retinal detachment. Changes in the glycome are representative of the EMT shift. This group found that downregulation of MGAT expression in RPE cells in vitro resulted in reduced galectin-3 protein binding to surface glycans, confirming the connection of MGAT5 to EMT and identifying galectin-3 as a potential future therapeutic target.[84]

Studying pathogenic human single nucleotide polymorphisms (SNPs) in vitro can lead to understanding of the mechanisms and interactions of specific gene mutations and potentially can lead to future patient-specific therapeutics. Rapid screening of SNPs for pathogenicity also aids in the understanding of high-coverage genetic testing, such as whole exome sequencing in patients.[85] In the eye, work by Duan et al.[86] shows the promise of such a screening technique. In human primary retinal pigment epithelial (hPRPE) cells, Duan et al.[86] used an AAV vector to deliver SpCas9D10A nickase along with two sgRNA constructs and a single-stranded

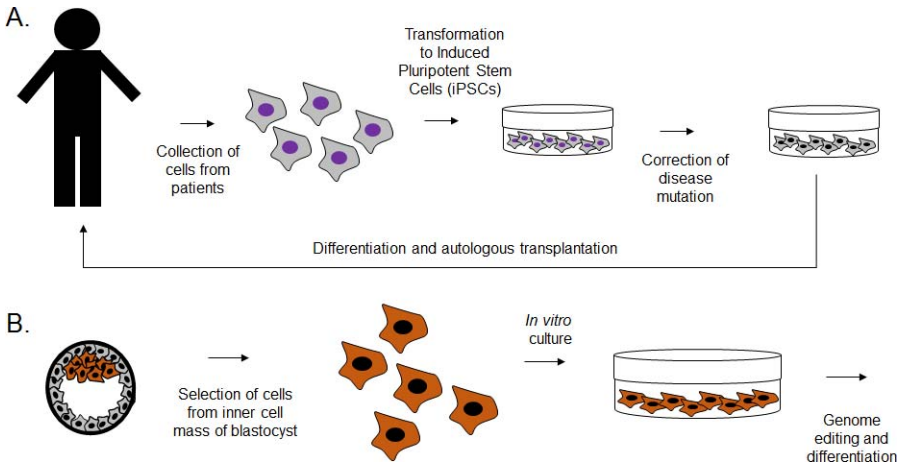

Figure 2. (A) Process of selection of somatic cells from patients, reprogramming to iPSCs, correcting by genome editing, and then implanting through autologous transplantation. (B) Generation of embryonic stem cells (ESCs) by selection of inner cell mass from a blastocyst, culture, and then reprogramming/genome editing.[90,93]

oligonucleotide donor DNA template, which was electroporated into cells to revert the human rs2279744 SNP to the *Mdm2* (murine double minute 2) gene, a likely regulator of the tumor suppressor protein, p53.[86] This SNP had been associated with retinal detachment and vitreoretinopathy previously in patients and, as such, this group wanted to assess its effect in vitro.[86] They found that this SNP made hPRPE more likely to proliferate and less likely to induce apoptosis when rabbit vitreous was applied to the cells, validating the SNP's pathogenic potential.[86]

Modification of Human Stem Cells for the Treatment of Eye Disease

While gene-based therapeutics may slow the progression of disease, stem cells offer the potential of reversing inherited ocular conditions. The differentiation and proliferative potential of these cells theoretically can lead to replacement of diseased tissue in patients and allow scientists to study human disease in vitro with increased precision, potentially tailored to the individual patients from which the cells were derived.[87–89] Deriving stem cells from patients offers specific advantages for testing targeted therapeutics and autologous transplantation with reduced risk of rejection.[88] Induced pluripotent stem cells (iPSCs) are stem cells that can be generated from patient tissue by reprogramming processes, and they have greatly enhanced the study of human disease.[90–92] Figure 2A shows an example workflow by which these cells are generated and modified. Recently, Bassuk et al.[93] demonstrated the potential of patient-specific disease mutation correction using CRISPR-Cas genome editing to correct the c.3070G>T, pGlu1024X mutation in the GTPase regulator (*RPGR*) locus in patient-derived iPSCs acquired from individuals with X-linked RP. In their study, they tested 21 sgRNA constructs to identify the ones with the highest targeting efficiency, since the targeted mutation is located in a repetitive region of the genome that has a high GC content.[93] After identification of a suitable sgRNA, they used SpCas9 to correct the mutation using a single-stranded oligonucleotide donor template encoding the wild type sequence, resulting in a 13% correction efficiency.[93] This study demonstrates that a high specificity of Cas targeting is possible even in genetic loci that previously were considered inaccessible due to their highly repetitive nature, and future work may allow for corrected iPSCs to be differentiated into photoreceptors, perhaps even enabling autologous transplantation one day.

While iPSCs offer great potential in terms of immunomatched cells for autologous transplantation, human embryonic stem cells (hESCs) offer extensive differentiation capabilities and allow for the study of development in very early stages of life.[95,96] Figure 2B shows a schematic by which these cells are generated and modified. After in vitro differentiation of hESCs, verification of differentiated cell-type represents an important step before further study can occur. To assist in the process of identification of RGCs derived from hESCs, Sluch et al.[97] used SpCas9 and a sgRNA targeting the *BRN3B* gene to introduce an mCherry fluorescent protein gene with a P2A self-cleaving peptide in frame with the *BRN3B* gene. This gene is

RGC-specific, and by adding an mCherry under the same transcriptional control, the group allowed for faster determination of properly differentiated hESCs via flow cytometry and selection for mCherry-expressing cells.[97,98] After flow cytometry, they confirmed these cells were of RGC identity using whole-cell current clamp and transmission electron microscopy.[97] Both iPSCs and hESCs will continue to make an impact on the study and treatment of human disease, and CRISPR-Cas tools enable precise manipulation of these stem cells for ophthalmic applications, scaffolding a pipeline for correcting deleterious mutations in a patient's own cells.

Conclusion

While CRISPR-Cas systems allow relative ease and precise manipulation of the genome, improvements in the development of more efficient delivery methods and more precise CRISPR-Cas components could bring targeted genome editing closer to the ophthalmology clinic. Moreover, novel techniques, such as delivery of preformed CRISPR-Cas complexes, could serve as an alternative to methods that require transcription and translation of Cas components and allow for a more transient presence of CRISPR-Cas elements.[77,78] As mentioned previously, measuring off-targeted mutations from each reprogrammed endonuclease is crucial for ensuring the purity of the experimental model and avoiding malignancy. Prediction software for sgRNA binding is crucial for sgRNA design, but does not always capture off targeting in vivo or in vitro (which can show different cutting profiles).[44,45,77,78]

With current technology, the carrying capacity of delivery modules can be limited, so some of the most appealing opportunities for CRISPR-Cas therapies in ophthalmology lie in the targeting of autosomal dominant pathogenic alleles as well as the knockdown of specific wild-type genes that produce pathogenic phenotypes in specific locations, such as *Vegfa* in causing neovascularization of the choroid.[66,77] Both types of therapies do not require a donor DNA sequence for homologous recombination — reducing the size requirements of the delivery vector — but instead rely on error-prone NHEJ repair of the cut site. Further development of our ability to differentiate CRISPR-Cas modified iPSCs into retinal, corneal and other cell types, as well as methods for autologous transplantation, could make sight-saving advances in numerous genetic diseases of the eye possible.[99–101] Overall, the future is bright for the use of CRISPR-Cas systems in ophthalmology, and it is likely that the important experiments discussed in this review represent a small sampling of what is to come.

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