# Advancing CRISPR/Cas-Based Therapies for Genetic Ocular Diseases: A Focus on Glaucoma and Leber's Congenital Amaurosis

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**Abstract**

Glaucoma and Leber's congenital amaurosis (LCA) are serious congenital ocular diseases that result in permanent blindness. Glaucoma is a significant contributor to lifelong blindness worldwide, while LCA is the most severe and earliest type of inherited retinal dystrophy, leading to congenital blindness. Fortunately, recent advancements in genetic editing technology especially CRISPR-cas systems (clustered regularly interspaced short palindromic repeat), have demonstrated significant potential in the treatment of these disorders. Nevertheless, the transportation of the CRISPR-Cas components to specific ocular tissues and cells necessitates the careful utilization of vectors and methods of administration to guarantee safety, effectiveness, and precision. This method has already been successfully employed to deliberately introduce specific mutations in many genes concurrently. This has been extensively tested and has shown promise as a potential treatment for glaucoma and LCA. Several experiments were conducted on mice and nonhuman primates (NHP). Due to ethical concerns regarding human and NHP trials, disparities between the human retina and other organisms, limitations of 2D cultures in interacting beyond a flat surface, and the importance of intricate cell interactions and signals from molecules and the environment, significant advancements have been made in 3D organoid technology derived from hPSCs, tissue-specific adult stem cells, and iPSCs. The study's summary of experiment results indicates that CRISPR-cas9 has enormous promise for curing eye diseases. It can also be used to improve 3D organoids, which will advance the field of genetic technology.

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## I. Introduction

Genome engineering is highly appealing for the treatment of hereditary illnesses caused by genes that have very precise spatial and stoichiometric expressions, such as those present in many inherited, non-syndromic eye diseases [121]. In just one decade, significant advancements have been achieved in the development of gene therapy technologies, resulting in a renewed sense of excitement regarding the potential for treating a wide range of genetic disorders. Notable progress has been made in the field of gene editing with the identification and creation of site-specific nucleases [124]. These nucleases include zinc finger nucleases (ZFNs) [122], transcription activator-like effector nucleases (TALENs) [123], and CRISPR/Cas9. Additional advancements encompass the development of instruments that facilitate the transportation of the cargo to specific cells for genetic editing, both ex vivo and in vivo. In order to fully harness the potential of therapeutic gene editing, it is crucial to carefully evaluate and refine both the gene-editing tool and the delivery system. The corrected gene is maintained under its normal endogenous expression control elements by directly editing the genomic DNA, inducing DNA nicks or breaks, and then repairing them. The CRISPR/Cas technology can prevent ectopic expression and aberrant gene transcription, which can lead to negative outcomes including alterations in immune regulation or the formation of tumors. In addition, the constraints of viral vector capacity, which hinder the broad applicability of existing gene-replacement therapies for numerous disorders, could be overcome by employing a CRISPR-based method [125,126].

Considering the eye's advantageous architectural and immunological features, including as the blood-retinal barrier and ocular immune privilege, it is expected that ocular disorders would be the primary focus of clinical applications for CRISPR/Cas-based therapeutics [127]. Nevertheless, like other developing biotechnologies, the effectiveness of CRISPR/Cas will not just rely on its therapeutic capabilities. Important ethical and regulatory concerns will significantly impact the determination of permitted applications. However, as CRISPR/Cas moves from the laboratory to the clinic, there is an urgent requirement for a well-defined, secure, and standardized clinical pathway for the therapeutic deployment of this technology. This review will present a concise overview of two prevalent and serious congenital Ophthalmological illnesses, followed by a detailed explanation of the CRISPR/Cas9 system. Subsequently, there will be a thorough elucidation of 3D organoids, encompassing their functionality and importance. To recap the preceding material, we will examine the experiments conducted on various organisms and models in order to comprehend the outcomes. Lastly, we will conclude by addressing the ethical considerations surrounding the utilization of CRISPR technology on living beings, specifically about its off-target effects. Additionally, we will examine the ethical implications of conducting studies involving nonhuman primates.

## II. Ophthalmological Congenital Diseases

### A. Glaucoma

Glaucoma is a collection of ocular neuropathies that gradually cause the deterioration of retinal ganglion cells and retinal nerve fiber layers, leading to alterations in the optical nerve head [25]. Glaucoma is linked to the damage that happens to the optic nerve caused by increased pressure inside the eye, known as intraocular pressure (IOP). This damage leads to the death of retinal ganglion cells [26]. There are Multiple types of Glaucoma, and they can be classified into two main categories: primary glaucoma and secondary glaucoma. Both conditions can be classified into two main categories, open-angle, and angle-closure, based on the underlying anatomy and pathology [25,28] Major types of Glaucoma are summarized in Figure 1.

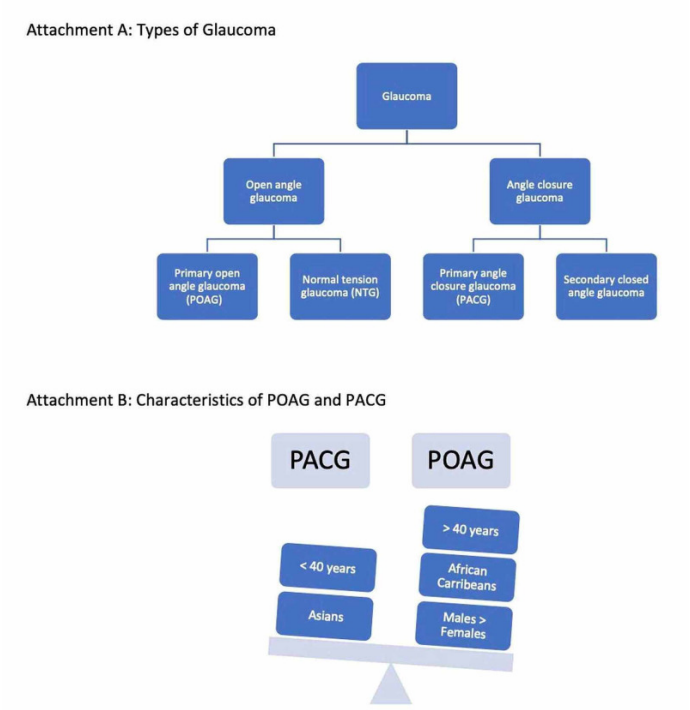



Fig. 1. Types of Glaucoma and characteristics of POAG and PACG. POAG: primary open-angle glaucoma; PACG: primary angle-closure glaucoma [28].

Primary open-angle glaucoma is a major cause of permanent blindness on a global scale as It is estimated that 57.5 million people worldwide are affected by it [25,27]. Primary open-angle glaucoma is usually characterized by a subsiding of the optic disc and distinct visual field abnormalities [27]. Many gene mutations can lead to glaucoma. The myocilin gene (MYOC), which is the first gene known to cause glaucoma, is active in the trabecular meshwork (TM) and ciliary body [132]. The protein produced by this gene may contribute to an increase in intraocular pressure (IOP) by blocking the drainage of aqueous fluid. Mutations in the MYOC gene exhibit autosomal dominant inheritance. Other genes also can cause Glaucoma such as CYP1B and LTBP2 which follow an autosomal recessive pattern; however, the majority of congen-

ital glaucoma cases map to the GLC3A locus on chromosome 2 (2p21)[29].

Several pharmacological treatments have been discovered to reduce the symptoms of glaucoma, for instance, topical medications [130]. A collection of chemicals can be employed to decrease intraocular pressure (IOP) and can be categorized into five primary groups: prostaglandin analogs, beta-blockers, diuretics, cholinergic agonists, and alpha agonists [33]. Another treatment is using laser therapies [31]. The laser's thermal energy is focused on the trabecular meshwork, resulting in localized scarring. This scarring creates openings in nearby structures. The scarring can also be caused by inflammation of cytokines and phagocytosis, which leads to structural modifications that improve the outflow [31,32]. When medication and laser treatment alone are ineffective in lowering intraocular pressure (IOP), surgical therapies can be conducted such as Trabeculectomy, Glaucoma Drainage Implants, and micro-invasive Glaucoma Surgery [30].

### *B. Leber's congenital amaurosis*

Leber's congenital amaurosis (LCA) is a profound and severe type of retinal dystrophy that was named after the ophthalmologist Theodor von Leber [37,38]. LCA usually causes severe visual impairment and blindness from birth [27,34,35,36,37]. This disease comprises approximately 5 percent of all retinal dystrophies and affects around 20 percent of children attending blind schools [34]. Infants with LCA are usually born blind, but it is often unnoticed at a later stage (around 2-3 months) when parents observe that the child does not track objects or respond to light [35]. LCA is unified by severe visual loss, sensory nystagmus, amaurotic pupils, digital-ocular signs, and severely reduced or absent both scotopic and photopic ERG [36,37]. Furthermore, visual function and visual acuity often range from 20/200 to no light perception at all [36]. One of the challenges in diagnosing LCA is the presence of a retinal phenotype that is also found in various complex systemic disorders such as Refsum disease, Senior-Loken syndrome, peroxisomal diseases, Batten disease, Joubert syndrome, and others [36,37].

Multiple genes are associated with LCA and reveal the heterogeneous nature of the disease. LCA-associated proteins show extensive variability in their cellular localization [37,39]. Malfunctions in these proteins have a significant impact on fundamental cellular functions, ranging from gene expression to light signal processing and the metabolism of vitamin A. The AIPL1 gene is observed to be active in photoreceptors as well as in the pineal gland. It plays a crucial role in the process of protein trafficking, and it is needed in the development of the rods and cones[43,44]. There is a complex interaction between this entity and multiple proteins causing it to be expressed in many phenotypes, such as Cone-rod dystrophy; and early macular involvement.

GUCY2D is a gene that is required in the cascade of phototransduction after a light stimulus and is localized to the outer segment membranes of both rods and cones [37,45]. It is induced by the reduction of free Ca2+ radicals and catalyzes the restoration of cGMP levels. Mutations in this gene are the cause of most severe conditions of LCA which are characterized by unmeasured visual fields, poor stable vision, severe cone-rod dystrophy, and a permanent form of blindness present from birth [39].

Another gene is the RPE65 which accounts for 3–16 percent of cases of LCA [39, 40, 41]. It was observed that this protein is highly prevalent in the retinal pigment epithelium (RPE) and plays a crucial role in the metabolism of vitamin A. The lack of RPE65 leads to the buildup of all-trans-retinyl esters because of the inhibition of the conversion from all-trans to 11-cis retinol in the visual cycle [42]. This gene exhibits a wide range of phenotypes, including Rod-cone dystrophy. Early RPE mottling and small white intraretinal spots, with later intraretinal pigmentation, indicate a milder mutation compared to other gene mutations [39].

Additional genes play a role in LCA, as outlined in Table 1. Typically, the inheritance pattern of LCA is autosomal recessive. In some cases, the condition can be passed down through generations in an autosomal dominant manner, as seen with the CRX gene [39].

## III. CRISPR

### *A. What is CRISPR*

Many viruses threaten the survival of bacteria and archaea, so as a defense, prokaryotes developed an adaptive immune system called clustered regulatory interspaced short palindromic repeats (CRISPR). This system is encoded by CRISPR loci and the accompanying CRISPR-associated (cas) genes to provide acquired immunity against bacteriophage infection and plasmid transfer [1,2,3,4].

CRISPR segments are repetitive sequences in DNA separated by short segments called spacer DNA, which are small parts that were cut from the viral DNA of the virus/plasmid and then incorporated into the DNA of the bacteria [6]. CRISPR array allows the bacteria to identify and recognize some types of viruses and then degrade them; therefore, these sequences are known as the defense mechanisms of prokaryotes against foreign DNA [5]. CRISPR/Cas-mediated adaptive immunity occurs over three steps (Fig. 2). Upon transcription of the CRISPR array, the precursor-CRISPR transcripts undergo enzymatic processing through endonucleolytic cleavage to form mature CRISPR RNAs (crRNAs). The crRNA contains the spacer at its 5' end, which is a short segment of RNA that matches with a sequence from a foreign genetic element [1]. The 3' end of the crRNA contains a piece of the CRISPR repeats sequence. Hybridization between the crRNA spacer and a complementary foreign target sequence (proto-spacer) triggers sequence-specific destruction of invading DNA or RNA by nucleases upon a second infection [1].

The nucleases responsible for this process are known as Cas enzymes. These enzymes are RNA-guided DNA endonucleases that have the ability to recognize, analyze, and cut foreign DNA sequences. These enzymes initially recognize and store foreign DNA sequences, then subsequently break them apart during recurrent infections. The process involves attachment to guide RNAs, which direct Cas nucleases to specific target sites for cleavage [7,8].

TABLE I
LCA INVOLVED GENES. ADAPTED FROM [39]

| Gene | Protein function | Physiological defect |
|---|---|---|
| AIPL1 | Protein folding and/or trafficking | Cell cycle progression during photoreceptor maturation are characterized by defective regulation. |
| GUCY2D | Guanylate cyclase (GTPÆcGMP) | The failure to regenerate cGMP and recover the phototransduction cascade |
| RPE65 | Retinyl ester binding | Inability to resynthesize 11-cis retinal |
| SPATA7 | Needed for spermatogenesis | nd |
| LCA5 | Needed for various ciliary proteins | Leads to Rod-cone dystrophy |
| CRX | A transcription factor is crucial for the development of photoreceptors. It plays a key role in encoding the cone-rod homeobox. | Incapacity to develop outer segments of the photoreceptor (PR) and the subsequent inability to produce essential phototransduction proteins. |
| CRB1 | Encodes the protein crumbs homolog 1, which plays a protective role in the outer limiting membrane (OLM) and facilitates the development of photoreceptors. | Results in the development of Nummular intraretinal pigmentation and the maintenance of para-arteriolar RPE, pseudo papilledema, and prepapillary perivascular fibrosis. |
| CEP290 | Encodes for centrosomal protein 290 KD | Hypotonia, ataxia, or intellectual disability |
| RDH12 | Associated with visual cycle with dual specificity for all-trans-retinols and all-cis-retinols | Peripapillary sparing |
| IMPDH1 | Plays a key role in de novo guanine production. | Rod-cone dystrophy |

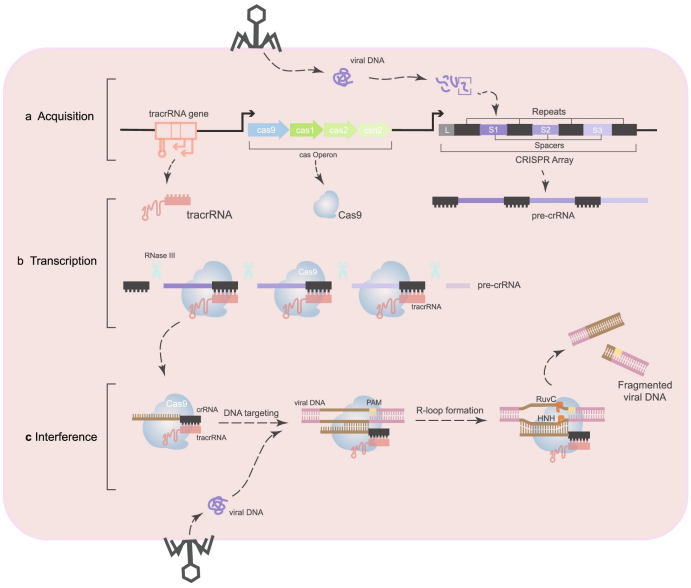



Fig. 2. The mechanism of the type II CRISPR/Cas9 system. During the process of acquisition, following infection by the phage, the DNA sequence of the invading phage is incorporated into the host CRISPR locus as a spacer, which is then separated by repeating sequences. During the transcription stage, pre-crRNA is synthesized and subsequently cleaved to generate mature crRNA. The crRNA consists of a repeating sequence and a spacer sequence that targets the invader. During the interference phase, the Cas protein specifically cuts the foreign nucleic acid at a location that matches the sequence of the crRNA spacer [101].

## B. CRISPR Discovery

During the determination of the gene sequence coding an alkaline phosphatase isozyme that converts aminopeptidase in Escherichia coli in 1987, A unique repeat sequence was identified downstream of the gene [9]. This repeat comprised 29-nt (nucleotide) repeats of identical sequences, separated by 32-nt unique sequences, which is a part of 12 repeated repeat loci grouped with the CRISPR-Cas system in E. coli [10]. Additionally, Similar repeated sequences have been found in other strains of E.coli and related enterobacteria, such as Shigella dysenteriae and Salmonella enterica[11]. Likewise, It was discovered that Mycobacterium tuberculosis contains several 36-bp (base paring) Direct Repeats (DRs) separated by distinct spacers ranging from 35 to 41-bp [12].

Then after 6 years, the first Archaeal CRISPR repeat was discovered during investigations on the effect of salinity on the growth of Haloferax mediterranei. This haloarchaeon can only thrive in high salt conditions [9] and after the experiments, researchers discovered that the repetitions of Haloferax mediterranei showed no similarity in sequence with the repeats found in bacteria [9,13]. The sequence of CRISPR segments in Haloferax mediterranei and Escherichia coli in (Fig 3). Finally, Analysis of genomes from bacteria and archaea revealed that approximately 40% of bacteria and 90% of archaea contain CRISPRs [9].

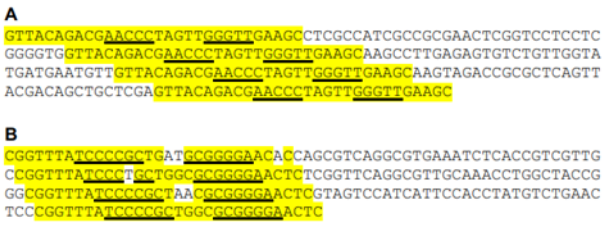



Fig. 3. Sequences of regularly spaced clusters in Haloferax mediterranei (A) [13] and Escherichia coli (B)[10] that were reported, adapted from [120].

Several terminologies were initially proposed for these

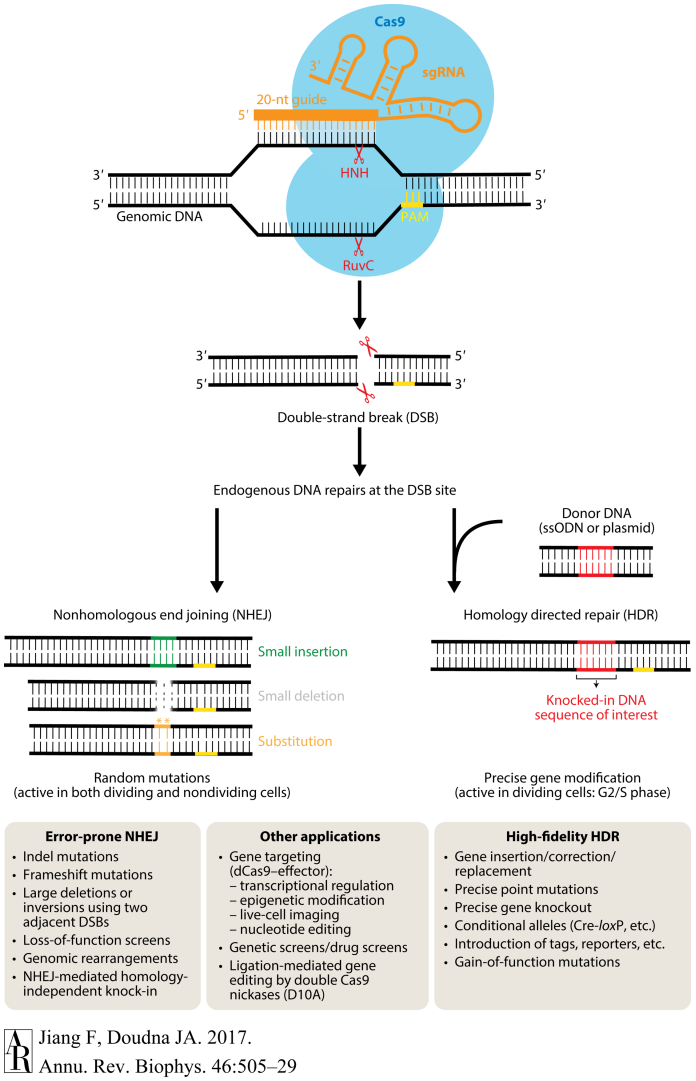
repeats instead of CRISPR. The ones that deserve mentioning include multiple direct repeats (DRs), short regularly spaced repeats (SRSR), and large clusters of tandem repeats (LCTR)[9]. However, Jansen et al was the one who named it CRISPR in 2002. Furthermore, Jansen identified other clusters of genes that are located upstream of the CRISPR loci in species that possess CRISPR but are not present in species lacking any CRISPR components. These genes were defined as CRISPR-associates (cas) genes[15].

The function of the CRISPR system remained unknown until 2005 when a group of researchers found its role in the adaptive immunity of prokaryotes [16]. They observed that spacers found in the array are associated with the Phages DNA and conjugative plasmids. To better understand its function, Barrangou et al. started editing and modifying the spacer sequences in the CRISPR loci in phage-resistant S. thermophiles. When he deleted the spacers, the S. thermophiles were affected by the phage [17].

### *C. Utilizing of CRISPR systems in Genetic editing*

Due to the importance of the CRISPR system in prokaryotes and its potential as a tool, scientists started utilizing the CRISPR systems found in prokaryotes, and they classified them into two systems, Class I (type I, III, and IV) and Class II (type II, V, and VI) [18]. The main difference between these classes is the number of Cas protein sub-units associated with the system. Class I systems contain multi-subunit Cas-protein complexes, whereas class II systems use a single Cas-protein. The initial system to be utilized was the CRISPR/Cas9 system from class II, owing to its relative simplicity, which facilitated significant study on it [19,24].

To utilize the CRISPR systems, single guide RNA (sgRNA) and CRISPR-associated (Cas-9) proteins are the two essential components of the complex needed for genetic modification [18]. The Cas-9 proteins were first extracted from Streptococcus pyogenes; these proteins include multi-domain DNA endonucleases that cleave the DNA that was targeted by sgRNA [20]. The Cas-9 complex consists of two domains, one to recognize the foreign DNA (REC), and the other one is the nuclease region (NUC). The nuclease region consists of RuvC, HNH domains, and Protospacer Adjacent Motif (PAM) interacting domains; they require the presence of a 20-nt sequence and a protospacer in the target DNA, in addition to a conserved PAM sequence next to the target site to be able to identify it [21,18]. After recognition, the nuclease domains start cleaving and cutting DNA leading to double-strand breakage. After the double-strand breakage, the host repair mechanism starts repairing by using two mechanisms non-homologous end joining (NHEJ) and homology-directed repair (HDR). In NHEJ, repair mechanisms join the two DNA fragments without using exogenous homologous DNA and are active in all phases of the cell cycle, leading the process to be error-prone. On the other side, HDR is highly precise and requires the use of a homologous DNA template and it is primarily active at the late S and G2 stages of the cell cycle. In addition, HDR necessitates a high number of donor (exogenous) DNA templates having a desired sequence [18,22,23]. All these Mechanisms are illustrated in ( Fig. 4)

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Annu. Rev. Biophys. 46:505–29

Fig. 4. Summry of CRISPR/Cas system's mechanism in genetic engineering [21].

### *D. Delivering to the eye*

In selecting an appropriate vector for delivering the CRISPR system, several factors must be taken into account. These include the vector's capacity, immunogenicity, minimal cytotoxicity, and longevity [131]. AAV (Adeno-Associated Virus ) is a collection of non-harmful viruses that cannot reproduce and carry a genome made up of a single strand of DNA measuring 4.7 kilo-nucleotides (4.7knt)[46] . The wide range of 13 AAV serotypes and other variations obtained through isolation, rational design, or controlled evolution has significantly broadened the range of tissues and organs that AAV may infect [47.48]. AAV vectors are now the preferred option for in vivo gene transfer experiments because they do not cause disease, do not provoke a strong immune response, and can maintain long-term expression of the transferred genes. Over 40 gene therapy trial protocols have examined the safety and efficiency of gene transfer for treating eye disorders [47].

Another type is Adenoviral vectors. Adenovirus is a type of virus that does not have an outer envelope and contains

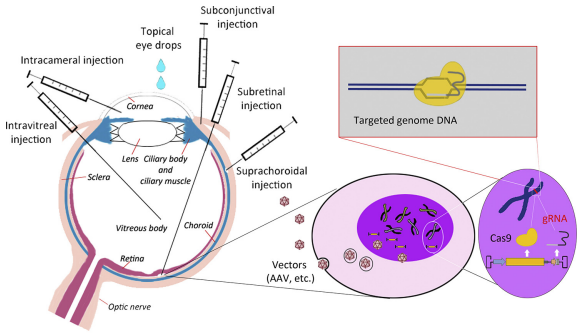


Fig. 5. Typical methods of administering genes to the eye. The front part of the eyeball consists of the cornea, ciliary body, and lens, whereas the back part of the eyeball consists of the vitreous body, choroid, retina, and optic nerve [106].

a 36-kilobase pair linear, double-stranded DNA genome. It does not become part of the genome, which means there is very little chance of causing changes in the genetic material by insertion. Adenoviral vectors were the first method of delivering genes to the eyes of mice, and they were able to successfully express reporter genes in both retinal pigment epithelium (RPE) and photoreceptor cells [49, 50]. Nevertheless, previous iterations of adenoviral vectors provoked robust immunological responses, such as the cytotoxic T lymphocyte (CTL) reaction, resulting in the elimination of the transduced cells [51].

Lentiviruses (LV) are retroviruses with an enclosed structure that consists of two copies of a single-stranded RNA genome with a positive-sense orientation. Lentiviral vectors can efficiently and durably introduce genetic material into both actively dividing and non-dividing cells while eliciting minimal or no immune response in living organisms. They can transport 8 to 10 kilobases (kb) of genetic material, enabling the transfer of a wide range of therapeutic genes or the simultaneous delivery of numerous genes [52]. Lentiviral vectors can integrate into the genome, allowing them to facilitate long-lasting gene expression in the cells they infect. Nevertheless, due to the inability to correctly estimate the integration location and copy quantity, the therapeutic applicability of these methods has been restricted by the possibility of insertional mutagenesis [53].

Since ocular tissues cannot receive therapeutic levels of biologics due to the blood-ocular barrier, the only method to deliver CRISPR systems to the eye is by direct injection. Main ocular routes are summarized in Figure 5 and their features are compared in Table 2.

As mentioned before, the ciliary body and trabecular meshwork play crucial roles in the production and drainage of aqueous humor, as well as in the regulation of intraocular pressure (IOP) and the development of glaucoma [106]. Therefore, Intracameral injection is usually used as it delivers genes to the corneal endothelium, ciliary body, and trabecular meshwork [102,103] leading it to be used in many trials of glaucoma gene therapy [104,105]. Furthermore, Intravitreal is a multi-target injection as its main target is the posterior segment of the eye. Particularly, its main focus is on transferring genes to the inner layer of the retina, with a particular emphasis on targeting retinal ganglion cells [107]. Additionally, it can also target tissues in the front part of the eye, such as the ciliary body [108], corneal endothelium, and trabecular meshwork [109]. This is probably due to the spread of the vector from the vitreous at the back of the eye (vitreous) to the fluid in the front part of the eye (aqueous humor). Moreover, it can transport genes to the outer retina. Intravitreal injection is preferred over other posterior injections because of its lower invasiveness and the potential for the vector to disperse to wider parts of the retina. Nevertheless, the majority of viral vectors are unable to access the outer retina after being injected into the vitreous cavity. Recently, certain newly created AAV variants have demonstrated the potential to enter several layers of the retina and reach to cells in the outer retina [110,111]. It is important to highlight that the humoral immune reaction against the AAV capsids might happen after intravitreal injection but can be prevented by using subretinal injection [112,113,114].

Another widely recognized method to transfer genes to the outer retina is subretinal injection. The subretinal injection method is a widely recognized way to deliver genes to the outer retina. After being administered subretinally, vectors are transported to the subretinal space, which is situated in the space between photoreceptors and the RPE cells [106]. Consequently, AAV vectors can effectively transduce photoreceptors and RPE cells when specific AAV serotypes or variations are employed. The technique of subretinal injection has been widely used in clinical trials for ocular gene therapy, including the FDA-approved Luxturna for the treatment of LCA2 [115]. Furthermore, In contrast to intravitreal, subretinal injection is focused on the injected retinal area, which minimizes post-injection immune reactions in addition to needing fewer doses to achieve the expected effect [115]. The subretinal delivery of CRISPR components demonstrated effective gene editing in retinal structures, particularly in photoreceptors. Photoreceptors are the most prevalent cell types in the retina and are commonly affected in inherited retinal dystrophies [116,117]. This breakthrough has significant implications for treating diseases like Leber's congenital amaurosis. Nevertheless, vitrectomy is necessary for subretinal injection in patients, a procedure that is linked to a limited number of complications. A study was conducted where AAV vectors were injected with another route which is suprachoroidal injection, they tried it on mice, pigs, and nonhuman primates. The results showed that this method of injection produced equivalent transduction and therapeutic effects compared to the traditional method of subretinal injection [118] Due to its minimally invasive nature and ability to be performed on an outpatient basis, the suprachoroidal injection has the potential to be extensively utilized for delivering vectors to the outer retina in the future.

## IV. 3D ORGANOIDS

Tissue and organ biology are very challenging to study in humans due to their limited accessibility and ethical concerns [64]. Furthermore, some organs show many differences from those of other organisms and even with our close relatives, like primates. Moreover, attempting to utilize stem cells or HeLa

TABLE II
COMPARISON OF ADMINISTRATION ROUTES FOR OCULAR VECTOR DELIVERY, ADAPTED FROM [106].

| Delivery route | Invasiveness | Cornea | Ciliary body | Trabecular meshwork | Inner retina | Outer retina |
|---|---|---|---|---|---|---|
| Suprachoroidal injection | + | - | + | - | + | +++ |
| Intravitreal injection | ++ | + | +++ | + | +++ | ++ |
| Subretinal injection | +++ | - | - | - | ++ | ++++ |

cells to replicate the exact conditions of organ interactions would likewise yield insufficient accuracy needed to ensure the validity of the treatment. Cells are found in tissues that experience intricate interactions between other cells, signals from molecules outside the cell, and signals from the surrounding environment [63]. Cellular phenotypes and functions, particularly in normal conditions, are primarily influenced by these microenvironment interactions. Thus, cells in 2D cultures are only able to interact in a horizontal plane, resulting in uniform exposure to stimuli or medications. As a result, this model fails to replicate the complex interactions found in a natural microenvironment.

Due to the previous reasons, advances in stem cell culture have made it possible to derive in vitro 3D tissues called 3D organoids [64]. Organoids are three-dimensional cell cultures that are derived from pluripotent stem cells [65]. These cultures accurately replicate the structure, function, and cellular complexity of human organs. In vitro, tiny organ models are particularly suitable for investigating intricate multicellular organ structures, including the brain, retina, kidney, lungs, and, in our case, eyes. Scientists can generate a wide variety of organoids from human pluripotent stem cells (hPSCs), tissue-specific adult stem cells, and induced pluripotent stem cells (iPSCs) obtained from patients. By putting these cells under specific protocols with specific growth factors and proteins, they start differentiation into an organoid. These organoids are highly suitable for drug screening and tailored cancer treatments [133]. Continuous advancements in organoid culture methods provide researchers with an opportunity to concentrate on innovative and specific therapies [66, 67]. while mitigating the potential hazards associated with human experimentation. The protocols for deriving various organoids differ based on the initial state of the cell population. Based on the initial conditions, a group of cells may go through all or only a portion of the steps related to self-organization.

The adaptability of protocols varies due to their complexity and the variability inherent in experimental procedures. While many protocols can be adapted using standard equipment in a typical tissue culture room, alterations and variations may be necessary to accommodate specific experimental requirements. During the process of generating organoids using human pluripotent stem cells (hPSCs), the pluripotent cells are first cultured alongside a population of feeder cells. These feeder cells play a crucial role by providing the necessary growth factors that help maintain the pluripotency of the stem cells. (Fig.6) summarizes the steps of creating a 3D organoid.

These organoids will be valuable to ophthalmological congenital diseases as the growth and maturation of the retina are controlled by a sequence of interconnected signaling networks that facilitate the metamorphosis of the front part

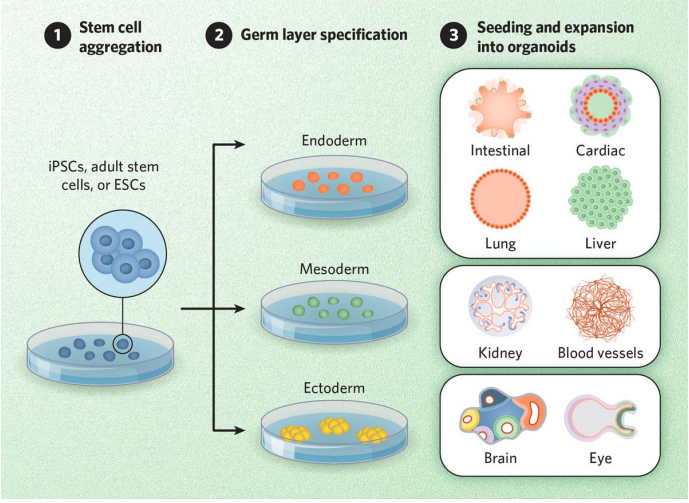


Fig. 6. Stem cells are cultivated by scientists using various substrates to replicate the process of development and intercellular interactions. Stem cell aggregates are commonly harvested and induced to generate cell types that are present in one of the three germ layers. Throughout typical development, the germ layers play a crucial role in generating distinct tissue types. To mimic this natural process, scientists have been able to cultivate organoids in the lab that closely resemble the structural and functional characteristics of various tissues [68].

of the growing brain [69]. Research conducted on model organisms continues to clarify the intricate sequence of events. Nevertheless, the human retina exhibits numerous disparities compared to those of other organisms, and the study of human eye development currently receives advantages from stem cell-derived organoids. Sasai's groundbreaking work involved creating a self-organizing 3D optic cup and stratified neuroepithelia from mouse pluripotent stem cells (mPSCs). This achievement has opened up new possibilities for developing retinal models that closely mimic the natural development process in vivo [70]. Eiraku et al. utilized a modified version of the serum-free floating culture of embryoid bodies (SFEB)-like aggregation method to develop embryonic bodies (EBs) produced from mouse pluripotent stem cells (mPSCs). The EBs were suspended in a low-growth factor environment and supplemented with Matrigel, which served as an extracellular matrix (ECM). This resulted in the spontaneous development of Rax+ retinal progenitor cells (RPCs) in the optic vesicles. These RPCs then fold inward to create optic cup-like structures with a pattern that extends from the proximal to the distal region, thereby designating the retinal pigment epithelium (RPE) and neural retina (NR) [71]. Presently, the use of 3D organoids in conjunction with CRISPR technologies enables scientists to generate precise disease models, facilitating the testing of medicines on these organoids.

Huang et al. established hPSC models of glaucoma and used

CRISPR/Cas9 to investigate a severe glaucoma mutation in the Optineurin (OPTN) gene, along with matched isogenic controls [72]. Using these human pluripotent stem cells (hPSCs), retinal ganglion cells (RGCs) were generated to study the neurodegenerative characteristics linked to glaucoma. At first, they examined the phenotypic changes, such as the remodeling of neurites, and the modifications in the autophagy process caused by this mutation. They found that the retinal organoids derived from these human pluripotent stem cells exhibited self-organization that accurately replicated the temporal and spatial characteristics of human retinal development. Extended cultivation of OPTN(E50K) retinal ganglion cells (RGCs) resulted in a decrease in the expression of important transcription factors. Furthermore, OPTN(E50K) retinal ganglion cells (RGCs) exhibited neurite retraction and heightened functional excitability when compared to genetically identical control cells. This implies that excitotoxicity may contribute to the neurodegeneration of RGCs. The findings of this study will enable the investigation of specific cellular processes that result in the death of retinal ganglion cells (RGCs) in glaucoma. Additionally, it will facilitate the development of new methods for treating glaucoma, such as screening potential drugs and exploring cell replacement therapies.

Furthermore, Leung et al. created retinal organoids by utilizing induced pluripotent stem cells (iPSCs) taken from renal epithelial cells collected from four children with AIPL1 mutations [73]. The photoreceptors generated from induced pluripotent stem cells (iPSCs) showed the characteristic molecular features of LCA4, such as the absence of AIPL1 and rod cyclic guanosine monophosphate (cGMP) phosphodiesterase (PDE6), when compared to control or CRISPR-corrected organoids. The LCA4 retinal organoids displayed minimal restoration of full-length AIPL1. Nevertheless, this was inadequate to completely reinstate PDE6 in photoreceptors and diminish cGMP. LCA retinal organoids serve as an important tool for studying and testing innovative treatments in a laboratory setting. Other expermeint was made by Kruczek and his team [74] . They made an in vitro model of CRX-LCA in retinal organoids using induced pluripotent stem cells (iPSCs) from a patient who had the CRX-I138fs48 mutation. The organoids exhibited impaired maturation of photoreceptors, as observed through histology and gene profiling, characterized by reduced expression of visual opsins. Then, they used gene augmentation to treat the mutation.

Advancements in stem cell culture enabled the creation of in vitro 3D tissues called organoids, produced from pluripotent stem cells. Organoids mimic the architecture, functionality, and cellular function of human organs, rendering them invaluable for investigating complex multicellular formations like the retina. Researchers have created retinal organoids using human pluripotent stem cells, which imitate the natural development process. This allows for the investigation of disease causes and the evaluation of prospective treatments. Research has shown that retinal organoids are useful in studying disorders like glaucoma and Leber congenital amaurosis (LCA). They help us understand the causes of these diseases and make it easier to design new treatments. In addition, the integration of organoid models with CRISPR technology facilitates the development of accurate illness models, enabling the exploration of distinct cellular mechanisms and the evaluation of new pharmaceuticals.

## V. Trials

### A. Mice

As we said before, chemical chaperones and cyclodiode lasers can be used to reduce the IOP stress that happens due to protein misfolding causing endoplasmic reticulum (ER) stress in the trabecular meshwork (TM) [134]. However, the sustained generation of mutated myocilin necessitates the prolonged use of these techniques to alleviate the symptoms associated with glaucoma.

Several studies have been conducted to gain a deeper understanding of the disorders and ultimately discover a therapy. An example of these experiments is Jain and his team. Jain et al. conducted an experiment where they developed a mouse model that replicates the POAG phenotype (Tg-MYOCY437H) [54]. After that, they used the CRISPR-Cas9 system in both lab experiments and studies done on mice to mess up the MYOC gene in those animals. In the experiment, they targeted the first exon, which should be equally effective for numerous different MYOC mutations. They were able to effectively lower IOP in mutant MYOC ocular hypertensive mice, disrupt the gain of function of mutant myocilin, reduce the misfolded protein load in TM cells, and prevent ER stress in vitro. Using the same CRISPR system, the researchers were also able to stop the progression of glaucoma in young Tg-MYOCY437H mice, even before any symptoms like high intraocular pressure were seen. Furthermore, genome editing was employed to address glaucoma in older Tg-MYOCY437H mice with elevated intraocular pressure (IOP) for 9 months before treatment. Furthermore, It is anticipated that there will be a rise in viable and functional TM cells following the disruption of the mutant transgene. While the researchers were able to preserve RGC function in younger mice, the reduction of IOP in older mice didn't allow significant RGC recovery due to the high sensitivity to pressure variation and irreversible RGC loss. This experiment is summarized in (Fig.7).

Another proof-of-concept experiment was conducted by Wu et al., who selectively disrupted Aqp1 in adult mice using the ShH10 serotype of AAV to deliver the S. aureus-derived CRISPR-Cas9 system to the eye [55]. A unilateral intravitreal injection of the combined vector was administered to wild-type C57BL/6J mice. After 3 weeks, the ciliary body was isolated for further analysis, which confirmed successful genomic DNA editing within exon 1 of Aqp1. The researchers found a 22% reduction in IOP levels. Following successful IOP reduction in normal eyes, the method was tested in two experimental mouse models of glaucoma. The ocular hypertension caused by corticosteroids results in a slight increase in intraocular pressure (IOP), similar to what is observed in humans. However, this does not cause a significant loss of ganglion cells when using a depot model. Thirty-six interventions in this model led to a significant decrease of approximately 20% in intraocular pressure (IOP) and levels of AQP1 protein in the ciliary body.

Leber's congenital amaurosis captured the attention of scientists for testing CRISPR technologies on it, as there has

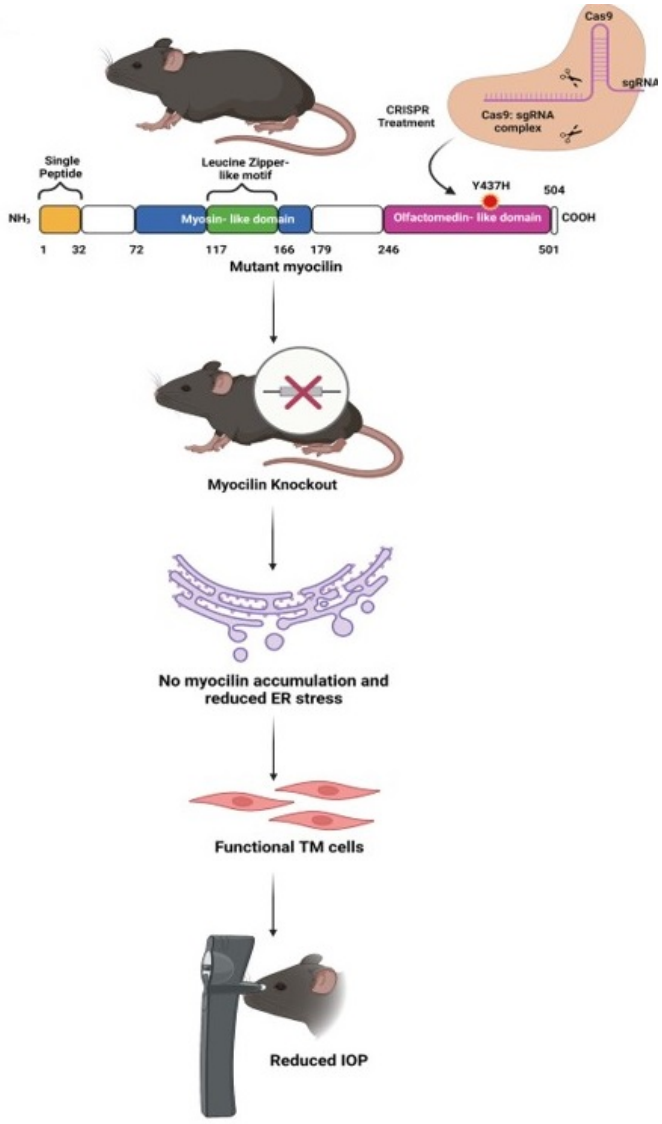


Fig. 7. The mutant MYOC gene was successfully knocked down in vivo using CRISPR/Cas9-based gene editing, effectively preventing its accumulation in the ER. The observed outcome led to the normal functioning of TM cells, which played a significant role in reducing intraocular pressure (IOP) [56].

been no approved treatment for LCA until today. Jo et al. targeted mutations found in the Rpe65 sequence in rd12 mice, a model of human LCA [60]. They deployed CRISPR-Cas9-mediated HDR that was delivered with dual AAV-mediated vectors with a Rpe65 donor sequence to correct the mutations found in the gene and enhance retinal function. In addition, they developed the $TS4^{rd12}$ sgRNA that precisely corresponds to the sequence of the Rpe65 exon 3 mutation site in rd12 mice. The researchers discovered that the treatment resulted in the restoration of RPE65 expression in RPE cells six weeks after the injection, as well as elevated RPE65 gene expression in RPE cells seven months after the injection. In addition, the a- and b-waves of electroretinograms in the mice that received the injection were restored to values of 21.2 ± 4.1% and 39.8 ± 3.2%, respectively, compared to their counterparts in wild-type mice, when exposed to bright stimuli after 7 months of dark adaptation.

Another experiment was conducted by Maeder et al., where they developed a potential genome-editing approach called EDIT-101. Its goal is to eliminate the abnormal splice donor caused by the IVS26 mutation in the CEP290 gene and reinstate normal CEP290 expression [61]. They employed a pair of Staphylococcus aureus Cas9 guide RNAs that were highly active and identical to the human CEP290 target sequence. They delivered the system by subretinal injection into humanized CEP290 mice. On day 3 following injection, Cas9 mRNA was easily visible, and the expression of both Cas9 mRNA and gRNA increased considerably by week 2 and remained stable thereafter. By week 6, SaCas9 and gRNA expression had peaked by week 6. Also, mice showed rapid and sustained CEP290 gene editing. These results support further development of EDIT-101 for LCA and additional CRISPR-based medicines for other inherited retinal disorders. Targeting the same gene, Ruan et al. experimented to correct the IVS26 splice mutation in CEP290 [62]. In this study, the researchers employed dual recombinant adeno-associated virus (rAAV) vectors to cause the removal of a particular intronic segment of the Cep290 gene in the photoreceptor cells of mice. Furthermore, they developed a “hit and go” approach to control the expression of SpCas9 in the transfected cells to reduce the impact of a host immune response to the exogenous enzyme.

### *B. Non-human Primates*

Before implementing CRISPR technologies in humans, it is crucial to bridge the anatomical and psychological disparities between mice and humans. Non-human primates (NHP) are the most suitable model for this study due to their possession of a crucial feature of human vision [57]. Specifically, they have a central visual pathway that originates from the macula, located in the center of the retina. This area is primarily composed of cone photoreceptor cells. Therefore, conducting experiments on non-human primates (NHP) can provide valuable insights into the potential effects that may arise during human clinical trials.

Barraza R et al. injected lentiviral vectors into the anterior chambers of the eyes of five Cynomolgus monkeys to assess the potential for glaucoma gene therapy [58]. The monkeys were observed for in vivo transgene expression and clinical parameters. Notably, there was a significant presence of GFP fluorescence in the trabecular meshwork (TM), which remained observable for a duration of up to 15 months, as confirmed by goniophotographic monitoring. Out of all the eyes that were injected, only the three with the lowest dose did not show any detectable TM fluorescence in vivo. However, when the enucleated eyes were examined at 2, 7, or 15 months post-injection, five out of the eight vector-injected eyes still exhibited significant GFP expression. Cells that underwent transduction were also observed in the iris and ciliary body. The findings indicate that the nonhuman primate aqueous humor outflow pathway can be effectively transduced using a lentiviral vector. These results suggest that this system could be used for gene therapy in humans with glaucoma.

In another experiment, Teresa et al. injected four Cynomolgus monkeys with AdenoGFP into the anterior chamber [59]. The results showed that the expression of the transgene in monkeys that received lower doses was observed non-invasively, with the transgene remaining active for 3–4 weeks and exhibiting minimal to no indications of clinical inflammation. On the other side, monkeys receiving the high viral dose exhibited corneal cloudiness, corneal epithelial edema, and a significant presence of protein in the anterior chamber (AC), which hindered the ability to conduct a thorough clinical or photographic assessment of fluorescence. These results showed that the transfer of genes to the TM and cornea can be observed without causing harm in non-human primates. This enables us to study how gene transfer relates to physiological factors. Due to the phenomenon of ocular immune privilege, the repeated administration of adenoviral vectors expressing specific genes into the anterior chamber could potentially be a therapeutic approach for glaucoma.

After Maeder et al. conducted experiments on mice, they continued their research on NHP, particularly cynomolgus monkeys [61]. They developed a surrogate pair of gRNAs (cynoCEP290 gRNAs 21–51) and delivered it in AAV5 NHP vectors using subretinal injection. The transport of genetic material to photoreceptor cells in the retina was found to be highly effective using in situ hybridization. This technique demonstrated a precise binding of the vector genomes, mostly in the outer nuclear layer, with some extra signal seen in the retinal pigment epithelium. The non-human primate (NHP) vector successfully edited the NHP CEP290 gene at levels that reached the desired therapeutic threshold, proving the effectiveness of CRISPR/Cas9 in editing somatic primate cells in live organisms. The results support continuing to work on EDIT-101 for LCA10 and looking into other CRISPR-based treatments for retinal diseases that are passed down through families.

### C. 3D organoids

Considering the minor differences between the human retina and those of other non-human primates (NHPs), it is imperative to conduct trials on humans to fully comprehend the effects of any experimental intervention, scientists started to embrace the 3D organoids approach to conduct their experiments on them. Chirco et al. created a 3D human retinal model system from iPSC lines that were derived from patients with Leber congenital amaurosis (LCA) who had dominant disease-causing mutations in the CRX gene [75]. The hiPSC lines were subsequently differentiated to produce retinal organoids with LCA7 mutations: $CRX^{T155ins4/+}$ *or* $CRX^{K88Q/+}$. A proof-of-concept study was conducted, utilizing allele-specific CRISPR/Cas9-based gene editing to disable the mutant CRX gene. The $CRX^{K88Q/+}$ organoids exhibited noticeable variations in the photoreceptor phenotype compared to the $CRX^{T155ins4/}$ organoids. Specifically, the *K88Q* mutation led to a more pronounced reduction in mRNA and/or protein levels for several impacted indicators, including *CRX, RCVRN, AIPL1, SAG, and ARR3.* Additionally, the *K88Q* mutation resulted in the downregulation of two important photoreceptor markers, NRL and NR2E3, which were not affected by the T155ins4 mutation at D180. On the other hand, the M-opsin/OPN1MW and L-opsin/OPN1LW genes were more significantly suppressed in the $CRX^{T155ins4/+}$ organoids compared to the $CRX^{K88Q/+}$ organoids. Finally, a partial improvement of photoreceptor characteristics in our organoids. This study presents preliminary evidence for a successful treatment strategy for LCA7, which has the potential to be applied to a wider range of dominant genetic disorders. In the experiment, an allele-specific CRISPR/Cas9 system was employed to disable the mutant CRX gene. As a result, a significant improvement was observed in the photoreceptor abnormalities in the organoids. This study presents preliminary evidence for a successful treatment strategy for LCA7, which has the potential to be applied more widely to other genetic illnesses with dominant inheritance.

Additional trials were done to verify the efficacy of utilizing CRISPR as a treatment for LCA, as there are no alternative treatments available. Afanasyeva et al. generated a patient-specific cellular model to study LCA5-associated retinal disease [76]. The CRISPR-Cas9 technique was employed to rectify a homozygous nonsense mutation in the LCA5 gene (c.835C¿T; p.Q279*) in induced pluripotent stem cells (iPSCs) obtained from the patient. Whole-genome sequencing was used to verify that there was no off-target editing in the gene-corrected (isogenic) control iPSCs. We subjected the patient, gene-corrected, and unrelated control induced pluripotent stem cells (iPSCs) to a process of differentiation, resulting in the formation of three-dimensional structures resembling retinas, commonly referred to as retinal organoids. The researchers noted that opsin and rhodopsin were found to be incorrectly located in the outer nuclear layer in organoids developed from patients, but not in those that had been genetically repaired or in unrelated control organoids. In addition, they also verified the recovery of lebercilin expression and localization along the ciliary axoneme within the gene-corrected organoids. In this study, we demonstrate the capability of integrating accurate single-nucleotide gene editing with the iPSC-derived retinal organoid system to create a cellular model of early-onset retinal illness.

### D. Clinical trials

Following EDIT-101 successful trials on mice and NHP [61], Editas Medicine is currently conducting the first RNP in vivo clinical trial in the eye (NCT03872479) to treat LCA10 patients with intronic mutations in CEP290 that result in splicing defects [128]. The drug, EDIT-101, utilizes AAV5 as a vector to encapsulate two gRNAs and Cas9. The expression of the transgene for Cas9 is limited to PRs using the hGRK1 promoter [129]. Participants of different age groups were administered a single subretinal injection with varying concentrations of virus. In the interim report, no significant adverse events or dose-limiting toxicities were observed across all the doses. During this phase, fourteen participants underwent treatment with EDIT-101 and experienced no serious adverse events related to the treatment or injection procedure. The treatment was well tolerated by the participants. In addition,

no DLTs were defined [128]. There were positive results observed in the efficacy data for each endpoint. Specifically, 4 out of 14 participants showed improvement in BCVA (best corrected visual acuity), 5 out of 14 in FST (full-field stimulus threshold), and 4 out of 14 in VFN. In the study, a total of 8 out of 14 participants experienced an improvement in their quality of life. Among these individuals, six also showed improvement in either their BCVA, FST, or VFN.

## VI. Bioethics

### A. CRISPR off-target Effects

The field of life sciences has seen a significant transformation due to the advent of genome editing (GE) technologies. These techniques, include zinc finger nucleases, transcription activator-like effector nucleases, and CRISPR. CRISPR/Cas9 is a highly adaptable method for enabling precise modifications to be made to the DNA of all organisms [77,78]. It is extensively employed in the examination of genetic elements, the production of genetically modified creatures, and the preclinical investigation of genetic illnesses. Nevertheless, there are many ethical considerations about it. The significant occurrence of off-target activity (50%)—mutations caused by RGEN (RNA-guided endonuclease) at locations other than the intended target site—is a serious problem, particularly for therapeutic and clinical purposes [77].

Of the things that cause off-target effects is the sgRNA.The sgRNA consists of two parts: the seed sequence and the nonseed sequence. Preliminary research has indicated that the 10-12 base pairs at the PAM (3' end of the guide RNA), known as the "seed sequence," play a crucial role in determining the specificity of Cas9. This seed sequence is generally more significant than the remaining sequences of the guide RNA [79, 80]. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) of DNA bound to catalytically dead Cas9 (dCas9) in murine embryonic stem cells reveals that only a small portion, specifically one to five base pairs, of the immunoprecipitated DNA aligns with the guide region. This finding suggests that the one to five base pairs of the guide region that are closest to the PAM sequence are the true "seed region" [81]. The seed sequence exerts an influence on the specificity of Cas9-sgRNA binding through various potential methods. The arrangement of the seed region dictates the occurrence rate of a "seed + NGG" in the genome and regulates the concentration of the Cas9-sgRNA complex (either through Cas9 binding or sgRNA abundance and specificity) [81, 82]. Conversely, the presence of U-rich seeds is expected to lead to a reduction in sgRNA levels and an increase in specificity. This is because the occurrence of many U's in the sequence can cause the cessation of sgRNA transcription [81,82].

The activity of sgRNA is also influenced by the sequence of the PAM [77]. The preliminary findings suggest that the canonical sequence for the PAM is NGG, where N represents any of the nucleotides A, T, C, or G. Recent findings indicate that the type II CRISPR system can utilize NRG (where R is G or A) as a PAM sequence. However, its binding efficiency is only one-fifth of that observed with NGG. Multiple studies have indicated that the NRG sequence is the most common noncanonical PAM (Protospacer Adjacent Motif) for CRISPR/Cas9-mediated DNA cleavage in the human EMX gene [83, 84]. The occurrence rate of each nucleotide in the PAM sequence varies. The first nucleotide is the least conserved, with G present in about 50% of binding sites. In contrast, the second position has G present in over 90% of the binding sites, indicating that NRG is not the ideal PAM for building CRISPR/Cas9 sequences. Consequently, the precise impact of the NRG PAM sequence on the process of DNA cleavage by Cas9 remains mostly uncertain.

Furthermore, the delivery of purified Cas9 protein and sgRNA directly into cells has been shown to decrease off-target effects compared to the delivery of plasmid sequences encoding Cas9 and sgRNA. This is because Cas9-sgRNA ribonucleoprotein complexes quickly cleave chromosomal DNA after delivery and are rapidly degraded in cells [85,86]. The occurrence of off-target effects may vary depending on the specific cell type and the functionality of the double-stranded breaks repair pathways in that particular cell type [87]. For instance, off-target effects of nucleases can happen in human cell lines that have been transformed and have disrupted DNA repair pathways. On the other hand, when healthy human pluripotent stem cell clones with intact DNA repair capabilities are sequenced, only a small number of off-target mutations caused by the nucleases have been identified [88,89].

### B. Using NHP in trials

Animal models that are not human are used to study human diseases, which helps us understand the genetic basis of these diseases and develop new treatments for humans. Although mice are frequently used as model organisms, their use is restricted [90]. While larger animals have the potential to offer more precise and effective disease models, the task of developing such models has been difficult until recently. Genome editors, such as Clustered Randomised Interspersed Palindromic Repeat (CRISPR), address these obstacles and make it possible to routinely modify the genomes of larger animals including non-human primates (NHPs). Due to the genetic similarities found between humans and NHP, Non-human primates (NHPs) serve as an excellent experimental model for testing before conducting trials on people. However, there a type of NHP which is called apes, they share common ancestors with humans as Darwin and Huxley proposed more than a century ago [95]. As they are very similar to ours, there are strict ethical regulations on using them for research and experiments. Lately, there has been a change in preference toward utilizing transgenic non-human primate (NHP) models for illness research. In 2001, the first transgenic non-human primate (NHP) was created [92]. Then, in 2008, a rhesus macaque with characteristic symptoms of Huntington's disease became the first transgenic monkey model for a human disease. This development raised hopes for the future development of new treatments [93,94]. The objective of creating novel therapeutic ways using these techniques is commendable, but it also brings up significant ethical concerns regarding the potential expansion of research involving transgenic apes to explore the function of HLS sequences. We contend that it

TABLE III
SUMMARY OF CRISPR-BASED EXPERIMENTS ON EYE DISEASES

| Disease | invloved Gene | Animal Model | Main Findings |
|---|---|---|---|
| Glaucoma | MYOC | Tg-MYOCY437H mice | Lowers IOP disrupts mutant myocilin function, reduces misfolded proteins, prevents ER stress |
| Glaucoma | Aqp1 | C57BL/6J mice | Reduces IOP by 22%, effective in corticosteroid-induced glaucoma |
| LCA2 | Rpe65 | rd12 mice | Restores RPE65 expression, improves electroretinogram responses |
| LCA10 | CEP290 | Humanized CEP290 mice | Restores CEP290 expression, rapid and sustained gene editing |
| LCA10 | CEP290 | Mice | Corrects CEP290 splice mutation using dual rAAV vectors |
| Glaucoma | Not specified | Cynomolgus monkeys | Lentiviral vector transduces aqueous humor outflow pathway |
| Glaucoma | Not specified | Cynomolgus monkeys | Adenoviral vectors have the potential for therapeutic approach |
| LCA10 | CEP290 | Cynomolgus monkeys | AAV5 NHP vector edits CEP290 gene in retina |
| LCA7 | CRX | T155ins4/+ and K88Q/+ 3D model | Edited mutant CRX, improved photoreceptor abnormalities |
| LCA5 | LCA5 | $c.835C>$T ;(p.Q279*) 3D model | Corrected nonsense mutation, restored protein localization |
| LCA10 | CEP290 | Clinical trials on humans | Treatment well tolerated, positive results in vision improvement |

is necessary to differentiate between the utilization of apes and all other non-human primates (referred to as 'monkeys' henceforth) for such scientific investigations [95].

The feasibility of carrying out transgenic research on non-human primates varies by country. Currently, there are no international regulations specifically addressing animal welfare in this context. However, the OIE World Assembly of National Delegates recently endorsed a fifth strategic plan to implement global objectives for animal health and welfare [95]. At present, certain nations such as the United States and China do not have any explicit restrictions on transgenic research with non-human primates (NHP). Therefore, such a study could potentially be authorized in these countries [95-96]. Great apes are prohibited from being used for study in the United Kingdom, New Zealand, the Netherlands, Austria, and Sweden [97-98]. Additionally, Austria and Sweden have extended this restriction to include lesser apes [99]. The European Union is currently contemplating revisions to its animal welfare legislation that would align with the standards set by the United Kingdom [100]. Monkeys are utilized for research purposes in the United Kingdom; nevertheless, strict rules mandate the implementation of additional supervision. This includes the necessity of obtaining a particular license for the researcher, project, and institution involved.

Other concerns are that the animals utilized for scientific research are often subjected to procedures that hinder their well-being and reduce their overall quality of life. They will experience pain as a component of the trials [90]. Furthermore, the confinement, limitation of mobility and physical exertion, and restricted dietary options all have a significant effect on the overall welfare. This section specifically addresses factors involved in the development of NHP model organisms that impact the well-being and overall quality of life of the animals. In essence, it covers concerns linked to the welfare of these animals. An issue arises when model organisms are created, as they may acquire abilities that elevate their moral standing above their non-genetically modified counterparts. Model organisms can be generated by injecting human stem cells into animal embryos, resulting in the formation of a non-human/human chimera. chimeras are creatures that possess human elements, such as tissues, organs, DNA, or stem cells, despite being non-human animals. While not all model organisms exhibit chimerism, some do. An ethical concern arises with the moral permissibility of intermingling human and non-human creatures. Another issue to consider is that incorporating genetically human organs, neurons, or stem cells into chimeric animals could potentially increase their moral standing, such as by increasing their self-awareness. However, these animals would not have the corresponding moral safeguards in place [91]. This criticism of the construction of chimeric model creatures, however, overstates the degree to which most chimeric model organisms are "humanized". The presence of human neurons in mouse brains allows for basic functionality, but due to the significant structural and physical disparities between mouse and human brains, it is highly improbable for the chimeric mouse to possess cognitive or emotional capacities similar to those of humans [91].

## VII. CONCLUSION

The CRISPR/Cas system is poised to have significant impacts in the field of clinical medicine specifically in the area of ophthalmology. Due to the eye's susceptibility to gene editing and the abundance of inherited eye illnesses that can be modified by gene manipulation

Although there are technological obstacles to overcome, such as the off-target effect, the significant advancements in the 3D organoid and the results presented demonstrate the efficacy of CRISPR-Cas9 in treating not only glaucoma and Leber congenital amaurosis (LCA) but along with other congenital disorders. Researchers were able to use CRISPR to lower intraocular pressure (IOP) in mice by targeting mutant genes, fixing protein misfolding in the trabecular meshwork, and lowering stress in the endoplasmic reticulum. Non-human primate studies further supported CRISPR's potential for glaucoma therapy. In LCA, CRISPR corrected genetic mutations, restoring retinal function and improving photoreceptor abnormalities. Furthermore, the ability to generate Patient-specific retinal organoids derived from induced pluripotent stem cells (iPSCs) validated CRISPR's gene editing precision and therapeutic potential to be a trusted treatment for LCA. These

findings confirm CRISPR's potential as a targeted therapy for ocular diseases and emphasize the importance of advanced models in disease modeling and treatment validation.

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