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CRISPR challenges in treating retinal disease

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Abstract

Ophthalmic researchers and clinicians arguably have led the way for safe, effective gene therapy, most notably with adeno-associated viral gene supplementation in the treatment of Leber congenital amaurosis type 2 (LCA 2) patients with mutations in the RPE65 gene. These successes notwithstanding, most other genetic retinal disease will be refractory to supplementation. The ideal gene therapy approach would correct gene mutations to restore normal function in the affected cells. Gene editing in which a mutant allele is inactivated or converted to sequence that restores normal function is hypothetically one such approach. Such editing involves site-specific digestion of mutant genomic DNA followed by repair. Previous experimental approaches were hampered by inaccurate and high rates of off-site lesioning and by overall low digestion rates. A new tool, clustered regularly interspaced short palindromic repeats coupled with the nuclease Cas9 (CRISPR/Cas9), may address both shortcomings. Some of the many challenges that must be addressed in moving CRISPR/Cas9 therapies to the ophthalmic clinic are discussed here.

Ethical and Technical Issues

Cultural and social understanding and acceptance of genetic editing varies greatly. Use of CRISPR/Cas9-based gene editing in zygotes has become routine in research for creating transgenic models.^{1–4} However, there is concern about editing genomes in human zygotes and embryos; in some countries it has already been banned (reviewed by Lanphier et al⁵). In adult humans, gene therapy is considered more acceptable. However, gene therapy in adult human eyes poses more difficult technical problems because development has already occurred and cells in the retina are terminally differentiated.⁶ Thus, several different classes of issues, from societal to technical, need to be considered for gene therapy in the clinic to be successful.

Gene therapy in the human eye has progressed faster than in many other tissues for several reasons.^{7,8} Gene therapy in the eye does not require systemic delivery and dilution of therapeutic constructs. Therapeutic agents can be directly delivered by intravitreal or subretinal injection to cells of interest. The structure of the eye retains the gene delivery agent after intravitreal or subretinal injection, resulting in greater transfection efficiencies.

Methods such as magnetofection,⁹ which can be used to concentrate gene delivery agents in target organs after systemic delivery, are not necessary when working with the eye. Very similar gene mutations in animal models and in human disease manifest with similar disease phenotypes, with the result that efficacy has been easy to match and measure between the human condition and animal models.¹⁰ For similar reasons, delivery (often in part by surgery) is similar in humans and animal models.¹¹ Finally, inflammation and immune reactions against vector and against the therapeutic expressing protein are lessened due to partial immune privilege in the eye.¹² Emerging technologies discussed here may permit improved gene editing in the eye, though we need to be aware of the technical hurdles that exist as we move forward with treatment approaches for patients.

Retinal Gene Therapy

The approach to gene therapy is dependent on the underlying genetic defect and the associated disease phenotype. There are ophthalmic diseases that spare the morphological structure, and these are more amenable to in vivo gene therapy, whether the approach is providing exogenous neurotrophic factors such as CNTF,¹³ supplementation with the missing gene product, or gene editing. Diseases that have degeneration of specific cell types in the retina require that those cells be replaced, which poses a different set of problems that are being addressed by research and clinical trials using stem cells for treatment of retinal degenerations (reviewed by Ramsden et al¹⁴). This perspective article will attempt to address the use of CRISPR/Cas9 for treatment in adult retinal disease and provide advice for these issues.

In cases of recessive and null genetic disease, supplementation with functional copies of the missing gene product can restore vision. An excellent example of this is RPE65 insufficiency in Leber congenital amaurosis type 2 (LCA 2) patients with mutations in RPE65. The RPE65 gene provides the retinal isomerase activity in the retinal pigment epithelium (RPE) that is necessary for recycling all-trans-retinal back to 11-cis-retinal. Without this protein, 11-cis-retinal is not regenerated; opsin is left without its chromophore, and photoreceptors cannot detect light. By providing exogenous wildtype RPE65, vision can be restored. This has been demonstrated in human gene therapy trials.^{8,15–18} Although this approach provided initial successes, patients that were treated with adeno-associated viral (AAV) vectors with the RPE65 gene have not maintained vision over long periods.¹⁷ Regardless of ultimate success, supplementation is limited to recessive and null genetic disease.

A New Gene Editing Tool

The “holy grail” of gene editing is an approach in which, regardless of phenotype or genotype, genomic mutations can be corrected in affected cell types. Clustered regularly interspaced short palindromic repeats coupled with the nuclease Cas9 (CRISPR/Cas9) theoretically can be used as the basis for this approach. The CRISPR/Cas9 system in bacteria was described in 2000,^{19,20} although it has been known since 1987 that clustered repeats are present in the genomes of bacteria.^{21,22,23} In bacteria, the CRISPR/Cas system serves to provide adaptive immunity for combatting viral pathogens. The clustered repeats

are separated by spacers derived from bacteriophage DNA and provide a template for generating an RNA sequence that is homologous to bacteriophage DNA. The bacteria use the RNA in conjunction with endonucleases (such as Cas9) to specifically cut and inactivate invading bacteriophage DNA (reviewed by Mali et al²⁴).

In 2012, the CRISPR/Cas9 system was adapted by Doudna and Charpentier for use in molecular biology for gene editing.²⁵ They described the synthesis of guide RNA (gRNA) molecules that contained the tracer RNA sequence for recruitment of Cas9 endonuclease and RNA sequence specific to desired cut sites. Since 2012, there has been a tremendous amount of work dedicated to understanding and improving this tool for use in the laboratory, for development of gene editing tools, and to improve agriculturally important plants and livestock. This system has been predominantly studied in cell culture models and is being used for the generation of transgenic organisms including mice, rats, plants, and even fungi (mushrooms).

As a molecular tool, the CRISPR/Cas9 system provides one of the functions necessary for gene editing. It allows us to cut DNA at a precise location dependent on the gRNA sequence.²⁴ Once the DNA has been cut, other mechanisms endogenous to the cells must come into play to complete the gene editing. There are 2 major DNA repair systems that can be employed by the cell to repair DNA that has been cut.

Nonhomologous End Joining

As described by Moore and Haber,²⁶ nonhomologous end joining (NHEJ) repairs double-stranded breaks by ligating 2 ends of double-stranded DNA together. This is an error-prone process, as the ends of the DNA tend to be shortened or extended in the cell before the ligation can occur, resulting in deletions and insertions of sequence. If the double-stranded break is in a gene, the mutations created by NHEJ are disruptive because of loss of DNA sequence and frameshifts. This is a useful mechanism when trying to create knockout mutations to disrupt the function of a dominant-negative allele. Repair of double-stranded breaks in quiescent cells such as photoreceptors occurs primarily through NHEJ mechanisms.²⁷ However, as a gene editing treatment it is not ideal, as it is unpredictable how much sequence will be lost and the new allele that is created potentially has novel functions that could be detrimental.

Homology-Directed Repair and Homologous Recombination

Homology-directed repair (HDR) and homologous recombination (HR) are endogenous mechanisms present that repair double-stranded DNA breaks with high fidelity in cells.²⁸ They require the presence of exogenously supplied DNA sequence that is homologous to the region where the break occurred, and that HDR/HR machinery is present in the cells. The homologous DNA is used as a template to repair the broken DNA strand. For the purpose of gene editing, HDR/HR allows us to replace sequence at the cut site with our desired sequence. Therefore, for gene editing, DNA can be cut at or near the site of a mutation using CRISPR/Cas9, and then HDR/HR can replace the mutant sequence with wildtype sequence derived from the provided donor template.

Disruption of Dominant-Negative Alleles

New technologies such as CRISPR/CAS9 are allowing for treatment of dominant-negative alleles by providing a mechanism to digest at specific gene sequences. This has not been tried in human patients yet; however, in trials with rats, disruption of the dominant-negative allele of rhodopsin (S334Ter) by inducing such “knockout” mutations specifically in the mutant allele have restored normal function to photoreceptors.²⁹ The NHEJ repair function after cutting results in the disruption of the mutant allele, such that the misfolded rhodopsin protein does not accumulate in photoreceptors and lead to death of the cells.

Repair to Functional Sequence

CRISPR/CAS9 may be useful in an approach to therapeutically modify the sequence of mutant alleles. Homologous repair mechanisms in the cell can use provided donor template to change the sequence where the genome has been cut. This methodology can be used for dominant or recessive alleles. Evidence from earlier approaches in mouse and cell models of retinal degeneration^{30–32} suggest that strategies invoking HDR/HR may eventually be clinically relevant if repair rates can be significantly enhanced.

Technical Challenges to Gene Editing

Gene editing approaches must overcome technical challenges associated with delivering molecular tools into cells where they can be used. These challenges are compounded in fully developed tissues where the cells are terminally differentiated.

Delivery In Vivo

Various gene delivery methodologies exist for delivery to the eye/retina/RPE. The cheapest and easiest to produce are plasmids. Plasmids are small circular DNA molecules that contain the minimum bacterial replication sequences and selectable markers. They are easy to propagate and purify in large quantities in the laboratory setting. There are many plasmids that have been designed to make subcloning DNA sequences of interest easy and efficient. The cost of making and propagating plasmids is very low, making them attractive for customized gene editing approaches. It is even possible to remove the bacterial replication and selection sequences from the plasmids to make what are called “mini-circles.” This is advantageous for gene editing approaches because only the DNA of interest is delivered into the target cells, which helps to preserve highly active transcription of the therapeutic gene.

Plasmids can be delivered into cells by a variety of methods that vary greatly in cost and efficiency. The easiest method is to simply deliver the plasmids into the vitreous or subretinal space using intravitreal or subretinal injection coupled with ultrasonic microbubble disruption or electroporation to create small pores in the plasma membrane of the target cells.^{33–37} This method has the advantage of potentially delivering large number of plasmids (and other DNA or RNA constructs) into each transfected cell, as we have used successfully for various ocular targets.^{33,38,39} For HDR, more copies of donor template increase the chances that donor template will be available at the cut site for repair. Disadvantages are that the method of making pores in the cell membrane can lead to death of a significant number of the target cells.

Plasmids can be packaged into lipid small unilamellar liposome vesicles (SUVs) that are able to fuse with the target cell membrane. There is little cost or difficulty to packaging plasmids in SUVs, making it attractive for customized medicine. The SUVs are delivered to the target tissue by intravitreal or subretinal injection. The plasmid contents of the SUVs are released into the cytoplasm of the target cells. This method also delivers large numbers of copies of the plasmid into each cell, which is advantageous for HDR. The downside of SUV technology is that the chemicals used to make the lipid vesicles are toxic to cells. Recent work is leading to less toxic vesicles for in vivo delivery. There are many commercially available kits for making vesicles for cell culture applications; however, there are currently few options for in vivo delivery.

Plasmids can also be packaged into nanoparticles for delivery. Nanoparticles are solid particles 30–100 nm in size that can be made of many different materials and can have many different deliverables embedded. The nanoparticles need to be able to disrupt the plasma membrane of target cells and pass into the cytoplasm where they release their plasmid cargo. Nanoparticle technology has been developing at a rapid pace in the past decade. Many of the issues being addressed with current research in nanoparticle technology deal with keeping the nanoparticles stable in the blood stream until they reach their intended targets. Because of the unique opportunities we have in the eye for direct delivery and holding the nanoparticles at the site of the target tissue by intravitreal or subretinal injection, issues of stability in the blood stream are not a concern.

As an alternative to plasmids, AAV and lentiviral delivery systems use viral infection mechanisms to delivery synthetic nonviral DNA into cells. The serotype of the viral coat determines which cell surface antigens must be present on the target cells; therefore, it is possible to more specifically target the cells of interest for delivery. Viral titer can be increased to improve transduction efficiency; however, this is limited by the number of cell surface antigens to which the virus binds on the target cells. Generally, this methodology does not deliver large numbers of copied DNA to each cell, so it may be problematic if HDR is the goal. If using viral vectors for delivery, all components must be encoded in the vector DNA. This may require co-transfection with multiple viral vectors because of limits on vector size for viral packaging.

Components to Deliver for CRISPR/CAS9-Mediated Gene Editing

To make use of the CRISPR/Cas9 system in vivo, 2 or 3 components must be delivered to the target cells. If NHEJ is the goal, 2 components must be delivered: the Cas9 gene and 1 or more gRNA(s). If using plasmids, both components could be cloned into the same plasmid or co-transfected in 2 plasmids.

For HDR/HR, 3 components must be delivered: Cas9, gRNA(s), and donor template. The donor template must be delivered in relatively large quantities so that it is available at the cut site. If using plasmids, the components can be subcloned into 1 to 3 plasmids. Donor template design should include silent mutations that prevent recognition of the donor template sequence by the gRNA to prevent cutting of the supplied template and/or cutting of the repaired genomic DNA. For the HR mechanism, the double-stranded DNA donor template must have a large region of homology at the cut site, at least several hundred bases

upstream and downstream of the cut site. The donor template could be subcloned into one of the plasmids used for the Cas9 gene or the gRNA(s), or provided on its own plasmid. For viral-mediated delivery, the size of the donor template necessitates that it be cloned into its own vector. For HDR, donor template is provided as a small single-strand DNA oligonucleotide of at least 100 bases. If using electroporation, ultrasonic microbubble disruption, SUVs, or nanoparticles for delivery, the short repair template oligonucleotide can be formulated with the plasmid for co-transfection.

Delivery to the Nucleus In Vivo

For gene editing using plasmids in vivo in adult tissues where cells are quiescent, plasmids must be actively transported into the nucleus.⁴⁰ Genes encoded in plasmids are not expressed without targeting the plasmids into the nucleus, and donor template must be present at the cut site in the nucleus. Viral vector methodologies include mechanisms of delivery to the nucleus. For HDR, the small size of the single-stranded repair oligo allows it to pass through the nuclear pores.

Much of the work that has been done with CRISPR/CAS9 in cell culture and in generation of transgenic animals has not needed to address issues with plasmid delivery to the nucleus because cells in culture and in developing embryos are actively dividing. With each division the nuclear membrane is disrupted, allowing the delivered components entry into the nucleus. For gene editing in the immediate future, we are dealing with fully developed and terminally differentiated cells that are not actively dividing. Therefore, we must incorporate into our strategy the means to translocate our components into the nucleus.

When using an expression construct for Cas9, both the plasmid and the Cas9 protein that is produced must be localized to the nucleus. For Cas9, much work has already been done to humanize the Cas9 sequence to give better expression in mammalian cells. A nuclear localization tag has also been previously designed and added to the humanized *S. pyogenes* Cas9, which allows the Cas9 protein to be imported into the nucleus. However, the plasmid construct must be imported into the nucleus for expression.

As described by Young et al in 2003,⁴¹ the inclusion of a 72 bp sequence from the SV40 enhancer is sufficient to allow import of plasmids into the nucleus of nondividing cells and allow gene expression from those plasmids. They termed this sequence a DNA nuclear targeting sequence (SV40 DTS). Other DTS sequences have since been described and rely on the binding of transcription factors to the plasmid in the cytoplasm and cotransport into the nucleus using the nuclear localization signal on the transcription factor. Plasmid designs for in vivo gene editing should include a DTS sequence such as the SV40 DTS on both the Cas9 and gRNA(s) expression constructs and on the HR template plasmid.

Efficiencies

One of the greatest challenges to gene editing in vivo is efficiency. This is a compounded problem because the overall efficiency is dependent on the efficiency of transfection, the number of components that are transfected into the cell, the rate of cutting of the CRISPR/Cas9 system, and the amount of HDR/HR that occurs. Studies using CRISPR/Cas9 on dividing cells in culture have demonstrated rates of NHEJ repair at 1–10% and HDR/HR

repair rates at appreciably less, unless specific enhancement strategies are attempted that may not translate clinically.^{42–46} In vivo in retina, our target cells are quiescent, therefore HDR/HR enzymes are expressed at a much lower level than in dividing cells where HDR/HR enzymes are normally upregulated during mitosis.^{27,32,47} Further research needs to be done to suppress NHEJ^{46,48–51} or increase expression of endogenous HDR/HR if we hope to use gene editing to restore functional alleles in vivo for treatment.⁴⁹ It is notable that one approach to circumventing the challenges inherent in treating quiescent cells is the use of CRISPR/Cas9 to edit retinitis pigmentosa genes in induced pluripotent stem cells (iPSCs) generated from patient fibroblasts, with the eventual goal being to transplant the “repaired” cells back into patients’ retinas.⁵²

Off-Target Cleavage

The CRISPR/Cas9 system is not perfect when recognizing the target sequence and is able to cut with several bases of mismatch.⁵³ Several software programs have been developed for predicting off-target cleavage sites.^{54–60} In addition, a new version of Cas9 has been engineered with higher fidelity.⁶¹ Guide RNA sequences should be tested in tissue culture for off-target effects before being used for in vivo gene editing.^{62–64}

Summary

The development of the CRISPR/Cas9 site-specific DNA cutting mechanism has great potential for gene editing and gene therapy. News reports have propagated this excitement and made it seem like a simple system ready to be employed in patients. We must understand that gene editing relies on high levels of delivery and good construct design⁶⁵ and must be applied in the context of the patient’s disease. CRISPR/Cas9 certainly makes targeting specific mutations more accurate, and new developments in CRISPR/Cas9 are improving this accuracy. CRISPR/Cas9 is a powerful new tool to our gene editing toolbox, but our excitement over this potential must be tempered by the understanding that substantial improvements are needed before application, even in experimental clinical trials.

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Figure 1.

Example of CRISPR/Cas9 with donor template-directed homologous recombination. The diagram represents a 2-plasmid system with Cas9 with a nuclear localization signal (NLS) in 1 plasmid and a second plasmid with the gRNA construct and donor template DNA. In this example, the plasmids are delivered into the cytoplasm of the target cell with electroporation. The DTS on each plasmid promotes nuclear import of the plasmids. Cas9 is expressed, and CAS9 protein with NLS is produced in the cytoplasm and then imported into the nucleus. The gRNA is generated and, together with the CAS9 protein, cuts the target in the genomic DNA. Homologous recombination between the donor template sequence and the cut genomic DNA replaces the genomic sequence resulting in gene editing.