Cutting and Splicing For CRISPR Vision

How this particular type of gene editing works and a discussion of its potential uses in the future.

Mark B. Abelson, MD, CM, FRCSC, FARVO, Connie Slocum, PhD, and David A. Hollander, MD, MBA
Andover, Mass.

Despite modest success to date, gene therapy remains the elusive magic bullet in the post-human-genome era. Ophthalmologists, who often bridge the divide between medicine and surgery, know well the benefit of surgical versus pharmaceutical treatments; gene therapy can be thought of as a kind of surgery on a molecular scale, a scalpel-less cure for rare genetic conditions. Sight-threatening disorders such as Leber congenital amaurosis or retinitis pigmentosa have been targeted as the test cases for genetic approaches and serve as the basis for treatments on a broader scale in the future.[1] While the culpable genes have been identified, and early trials have met with some success, development of a reliable means to correct genetic defects *in situ* has been much more difficult.

Recently, an entirely new approach to genetic modification therapies has caused considerable excitement: Clustered Regularly Interspaced Palindromic Repeats.[2] The CRISPR system of gene editing, found originally in prokaryotic organisms, is already a prominent method for introducing genes into eukaryotic cells and has even made its way to market in genetically engineered mushrooms.[3] The simplicity and versatility of CRISPR provide a path forward for introducing repaired or therapeutic genetic material into affected tissues in humans in the future. This month we explore the phenomenon that is CRISPR: what it is, what it can do, and why it's such an important new therapeutic technology.

Gene Therapy So Far

The earliest efforts to introduce rescue genes in humans focused on immune system defects such as severe combined immunodeficiency syndrome, where copies of the repaired gene were introduced into marrow cells using retroviral-mediated gene transfer.[4] The first successes reported in 2000 were followed by some cases of leukemias associated with the treatment, but progress in this area continues.[5] What about ocular gene therapy? LCA has been the primary test case, and several trials have been conducted using adeno-associated viral vectors to deliver functional genes for retinal pigment epithelium-specific 65 kDa protein.[6,7] In general, these studies have been able to demonstrate some degree of successful expression of introduced genes, and most trials report modest improvements in visual function. But progress has been slow, and the variability in viral vector design, patient genotype and disease progression have all hampered significant progress.

While viral-based expression of RPE-65 kDa is robust, one disadvantage of this therapy is its localization to peripheral regions of the retina; there is also some concern about immune responses to these viral proteins. But the most significant limitation to these treatments is that they generally fail to halt progression of the disease. It's thought that timing of rescue gene delivery may be important, as most patients in LCA trials to date have been adults. In addition, delivered genes must "battle" the resident mutation that is the ultimate cause of the disorder. What if, instead of delivering a rescue gene, it was possible to deliver a gene repair kit?

The CRISPR/Cas9 system is ideally suited to the task of repairing genetic defects *in situ*.[2,8]

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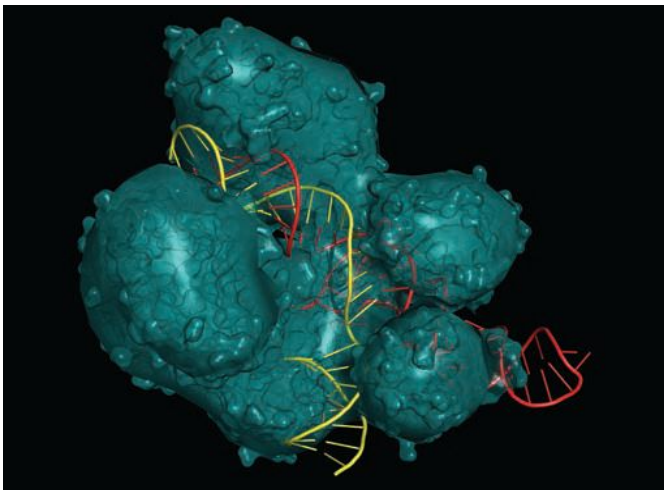
The concept is relatively simple: The CRISPR complex includes an RNA template to identify the region of the genome to target for splicing, as well as an endonuclease Cas9, the molecular scissors that cut and splice the targeted gene where needed. By targeting a defective gene with the code identified within the appropriately designed RNA template, cutting and splicing in any region of the entire genome become possible.



A rendering of a CRISPR/Cas9 complex with associated RNA (yellow) and DNA (red) strands. The CRISPR complex brings together three elements: an RNA template; a DNA target; and the Cas9 endonuclease (pictured here as part of the green structure) to execute sequence-specific DNA editing.

Genomic Editing

The CRISPR system was originally identified in bacteria as an antiviral defense mechanism that provides adaptive immunity for a host bacterium against extrachromosomal genetic material. Nucleases and nucleic acid polymerases are often called housekeeping enzymes, because they function in part to routinely repair genetic material that suffers spontaneous strand breaks and nicks. In bacteria, the CRISPR/Cas complex co-opts this housekeeping function to prune foreign (viral) DNAs from the bacterial genome. The foreign viral DNA sequences attempt to hide within the bacterial genome, but are given away by the characteristic presence of clustered, regularly interspaced palindromic repeats.[10] CRISPR sequences "remember" previous encounters with foreign DNA, and thus initiate a defense against future viral encounters. The CRISPR sequences are transcribed into an RNA that serves as a guide to direct endonucleases (such as Cas9) to the foreign DNA, which is cut and removed from the bacterial genome.

Because there is a specific RNA template, for genomic engineering purposes the CRISPR/Cas complex can be designed to target virtually any sequence in the genome, and introduce a controlled break in the DNA.[9] These breaks are then mended by cellular DNA repair mechanisms, resulting in the incorporation of deletions or new sequences into the genome.

Although initially described in *E. coli* in 1987, the tremendous potential of CRISPR wasn't understood until 2012 when it was shown that the CRISPR complexes could be used to direct any DNA cleavage in any cell type.[11] So while the principle of this directed gene deletion has been known for decades, it wasn't until Jennifer Doudna, PhD, Emmanuelle Charpentier, PhD, and their colleagues at UC Berkeley showed that the technique could be used for direct editing of any genome that its true value was appreciated: Any DNA sequence in the genome could be edited, deleted or replaced.[12]

The ability of CRISPR/Cas technology to alter gene sequences with high efficiency and accuracy has transformed biomedical research.[13] It's been rapidly adopted in laboratories around the world as a primary genomic editing tool because it's cost-effective, easy to use and operates with high fidelity.[2,8] Multiple studies in mammalian and murine cell lines have demonstrated that CRISPR-driven editing can correct disease-causing mutations and lead to reversion of disease phenotypes.[9] CRISPR is also being used to generate transgenic mice as a means of improving our understanding of disease mechanisms and potential therapeutic targets *in vivo*.

CRISPR in the Clinic

As with all gene therapies, a major technical hurdle involves delivering the therapeutic treatments to the appropriate tissues. CRISPR can partner with all existing technologies, but is particularly well suited for use in combination with stem cell or allogenic transplant-based therapies. Stem cells or other cells from the affected individuals can be genetically repaired with CRISPR and delivered to affected tissues, such as the retina. This approach is being applied to blood disorders, and is in use in treatments being developed for conditions such as β-thalassemia and sickle cell disease.[14]

Viral-based delivery systems have been adapted to carry the entire CRISPR functionality, and can deliver the gene editing apparatus with high efficiency to both dividing and quiescent cell types.[15] Viral packaging is all about the size of the message, and despite their complex function CRISPR genes can be efficiently integrated into AAV2 vectors. This advance comes at a time when improvements in viral delivery to the retina offer the prospect of much

greater infection efficiencies, insuring that expression of the genetic rescue reaches the entirety of the target tissue.[16]

With such promising technologies, it's not surprising that biopharmaceutical entrepreneurs are leading the race to exploit the technology. Editas, a Boston-based biotech company focused on application of CRISPR technology, appears to be in the lead for the first Phase I trial, which is slated for 2017. Ophthalmology is a core program for Editas, with five inherited retinal diseases being investigated, including LCA.

One difficulty with inherited retinal disease is the genotypic heterogeneity of patients, meaning that treatment may involve multiple genomic targets. Data presented earlier this year at the annual meeting of the Association for Research in Vision and Ophthalmology demonstrated that genetic editing of three distinct classes of variants can be successfully accomplished using mutation-specific variations of genetic editing. *(Burnight EM, et al. IOVS 2016;57:ARVO E-abstract 1157)* Pairing of AAV delivery with these CRISPR technologies is particularly well-suited to the favorable anatomical and immunological profiles of the eye, compared to other tissues in the body, so the future for application of CRISPR to genetic therapies in the eye seems bright.

The Way Forward

The ease with which the CRISPR methodology can be applied might lead to ethical concerns. In 2015, a team of Chinese researchers reported their efforts to genetically modify human embryos using CRISPR/Cas9 technology, and although the study did not yield a successfully engineered human, it set off a firestorm of controversy worldwide on the use of human embryos in research.[17] Many have voiced a concern that the simplicity of editing the human genome will result in the production of "designer babies."[13] Others claim that these efforts are doomed because they are attempting to leap beyond our existing technological capabilities.[17] In either case, as the CRISPR technology advances, it's likely that the ethical debate will continue regarding which applications of CRISPR/Cas are universally acceptable.[10]

Current limitations of CRISPR revolve around a few imperfections in the technology. For one, our cells have several different DNA repair mechanisms, and while the repair is predictable, it's not entirely controllable. Developing a better understanding of the mechanisms involved in the repair of breaks induced by CRISPR/Cas9-induced DNA remains a crucial step towards maximizing this technology.[9] Off-target editing of the genome is also a major concern with the CRISPR-based gene editing technology. Although CRISPR is highly specific, it has the potential to cleave other areas in the genome that have DNA sequences similar to the site of interest. Further optimizations and improvements are continually being developed, and will ultimately increase the specificity and power of the CRISPR/Cas system.

The future of CRISPR will also be impacted by an ongoing patent battle over who owns the rights to this cutting-edge technology. The feud began back in 2012, when Drs. Doudna and Charpentier submitted a patent application for the technology. In 2013, Feng Zhang, PhD, from the Broad Institute of Harvard and MIT, submitted a similar patent application, but requested a fast-track process, and thus received the official patent in 2014. Since then, the two parties have been locked in a legal battle over patent rights to this tremendously promising technology. Perhaps the best indication of its promise is the fact that despite this ongoing litigation CRISPR development continues, with the many scientists conducting the research presumably planning on figuring out who gets the royalties for the technology at some point in the future. REVIEW

Dr. Abelson is a clinical professor of ophthalmology at Harvard Medical School. Dr. Slocum is a medical writer at the ophthalmic research and consulting firm Ora in Andover. Dr. Hollander is chief medical officer at Ora, and assistant clinical professor of ophthalmology at the Jules Stein Eye Institute at the University of California, Los Angeles. Comments for Dr. Abelson can be sent to MarkAbelsonMD@gmail.com.

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