Molecules and Cells

Minireview

# RNA in Therapeutics: CRISPR in the Clinic

Dana Carroll*

Department of Biochemistry, University of Utah School of Medicine, Salt Lake City, UT 84112, USA
*Correspondence: dana@biochem.utah.edu
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www.molcells.org

**The advent of the CRISPR-Cas genome editing platform has greatly enhanced the capabilities of researchers in many areas of biology. Its use has also been turned to the development of therapies for genetic diseases and to the enhancement of cell therapies. This review describes some recent advances in these areas.**

**Keywords:** base editing, Cas9, CRISPR, genome editing, prime editing, sickle cell disease

## INTRODUCTION

Today the most familiar use of RNA in medicine is, of course, the mRNA coronavirus disease 2019 (COVID-19) vaccines. Their development required extensive research into RNA biology, RNA chemistry, cellular responses to exogenous RNA, and identifying useful delivery methods. The vaccines have been highly effective, and they promise to have great impact in combatting infectious diseases in the future. In this article, however, we will focus on RNA-involved treatments for genetic diseases, with particular emphasis on genome editing with CRISPR-Cas (abbreviated here as CRISPR).

Humans certainly have known for millennia that traits are passed down in families. With the rediscovery of Mendel's genetic observations (Keynes and Cox, 2008), it became possible to understand and quantitate some such traits, including inherited diseases. With the development of powerful molecular tools in the 1970's, researchers could map, isolate, and ultimately determine the DNA sequence of specific disease alleles.

Still, it has taken several more decades to reach the point where molecular approaches to therapy for genetic diseases has become feasible. Initially, delivery of healthy copies of affected genes were envisioned as cures, and there are some examples in current clinical practice and early-stage trials. The greatest excitement has been generated, however, by tools of genome editing that allow modification of disease alleles at their normal chromosomal locations.

The first clinical trial using genome editing was initiated in 2009 using the first of the editing platforms, zinc-finger nucleases (Tebas et al., 2021; 2014). Now the primary emphasis is on CRISPR-based reagents that include a small RNA to direct sequence changes to the desired location.

## CRISPR GENOME EDITING

The basic components of the CRISPR toolbox are a single protein, Cas9 or a related molecule, and a guide RNA (gRNA) about 100 nucleotides long (Jinek et al., 2012) (Fig. 1). Twenty bases at the 5' end of the gRNA match the intended genomic target; the remainder of the RNA mediates binding to Cas9. The target must also have a specific, very short sequence called a PAM (protospacer adjacent motif) next to the gRNA complement (Fig. 1). Cas9 has no DNA-binding or enzymatic activity on its own, but in complex with a specific gRNA, it binds to a sequence specified by the gRNA, acquires nuclease activity, and makes a double-stranded break at the target.

This cleavage activity has therapeutic potential. Once the break is made, cellular DNA repair processes are activated (Carroll, 2014; Xue and Greene, 2021) (Fig. 2). Nonhomol-

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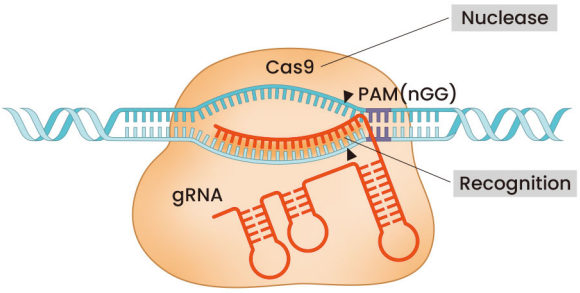


**Fig. 1. Illustration of CRISPR components bound to a DNA target site.** The target DNA is shown as blue lines, the guide RNA as orange lines, and the Cas9 protein as a pale orange shape. Base pairing between target DNA and guide RNA (gRNA) is shown with short, vertical blue and orange lines. The PAM (protospacer adjacent motif) sequence, which is required for target recognition by Cas9, is indicated (purple). PAM is nGG (where n is any base) in the top strand for the commonly used *Streptococcus pyogenes* Cas9. Base pairing between gRNA and DNA is responsible for sequence-specific recognition; Cas9 has the nuclease activity that cuts both strands of the target DNA (black arrowheads).

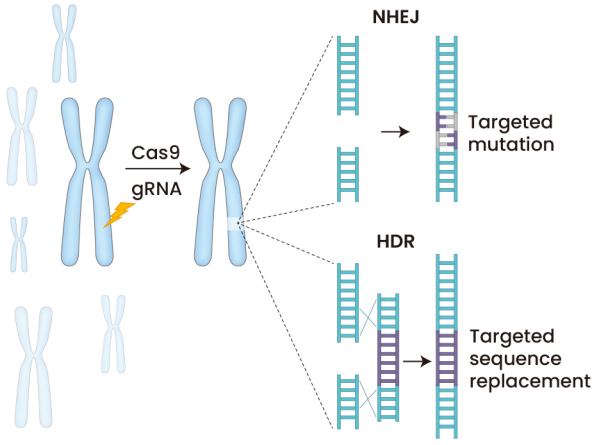


**Fig. 2. Illustration of the cellular repair activities and outcomes following a unique, targeted double-strand break induced by CRISPR.** Nonhomologous end joining (NHEJ) often leads to insertions and/or deletions (indels) at the target. Homology-dependent repair (HDR) uses a template to copy sequences into the break site; the template can be one provided by the researcher. gRNA, guide RNA. Adapted from the article of Carroll and Charo (2015) (Genome Biol. *16*, 242) under the terms of the Creative Commons Attribution 4.0 International (CC BY 4.0) license.

ogous end joining (NHEJ) directly rejoins the DNA ends, but sometimes incorporates insertions or deletions (indels) at the junction. Such sequence alterations can inactivate a gene that is disease-causing. Homology-directed repair (HDR) relies on a closely related template, often the sister chromatid, to restore chromosomal integrity. When an appropriate template

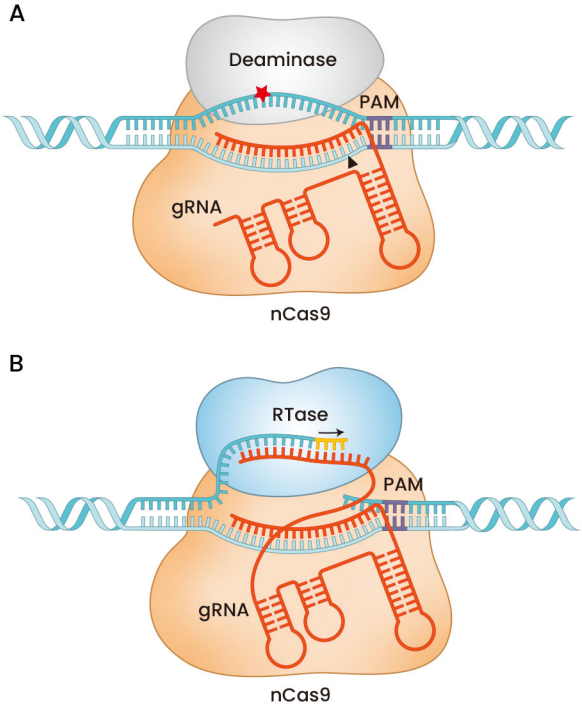


**Fig. 3. Additional CRISPR tools.** (A) Generalized picture of the cytosine and adenine base editors. A version of Cas9 that cuts only the RNA-bound strand (nCas9) is fused to a relevant deaminase that modifies one or a few bases on the displaced DNA strand (red star) at some distance from the PAM (protospacer adjacent motif). The cytosine base editor is also linked to two copies of UNGi, an inhibitor of the cellular uracil N-glycosylase enzyme that would normally remove uracil from DNA (not shown). The nicking activity of Cas9 directs the cellular mismatch repair system to use the modified DNA strand preferentially as the template for repair. (B) Schematic illustration of the prime editor. Cas9 is modified so it only cuts the displaced strand at the target, and it is fused to a viral reverse transcriptase (RTase). The guide RNA (gRNA) is extended at its 3' end (orange line) with a sequence that has homology to the displaced DNA strand and carries a desired sequence modification to the right (in this picture) of the break in that strand. The RNA-DNA hybrid (orange-blue) is treated as a primer-template complex by RTase, which extends the 3' end of the DNA across the modification in the RNA. Additional steps lead to the incorporation of this modification into the target. As in the base editors, a second gRNA is provided to create a nick nearby in the bottom strand of the DNA to favor the modified strand as the ultimate template for repair (not shown).

is supplied by the researcher, a disease allele can be replaced with a non-disease-causing sequence. Unfortunately, in most human cell types, HDR is less efficient than NHEJ.

Variations on the basic CRISPR system take advantage of gRNA-based sequence recognition to bring alternative activities to the target. Base editors have a cytosine or adenine deaminase linked to a Cas9 variant that cuts only the RNA-bound strand (Anzalone et al., 2020; Gaudelli et al., 2017;

Komor et al., 2016) (Fig. 3A). Cytosine base editors (CBEs) convert cytosine to uracil at specific sites within the RNA-bound target sequence, which leads to replacement of a C:G base pair with T:A after repair and/or replication. Similarly, adenine base editors (ABEs) convert adenosine to inosine, ultimately replacing an A:T pair with G:C. These conversions are possible on either DNA strand, so all disease-causing single-base pair transition mutations can potentially be reversed.

A recent addition to the CRISPR armamentarium is prime editing (Anzalone et al., 2019; 2020) (Fig. 3B). In this case, a Cas9 variant engineered to cut only the displaced DNA strand is fused to a reverse transcriptase. The 3' end of the gRNA is extended with a sequence that will serve as a template to introduce the desired sequence change. Although the range over which modifications can be introduced is rather short, this approach, while homology-dependent, does not produce a double-strand break or rely on HDR factors.

## CLINICAL APPLICATIONS

Genome editing with CRISPR reagents for clinical therapies requires a number of things to be true: (1) The disease must be the result of a mutation in a single gene. (2) The mutation must be confidently identified as the cause of the disease. (3) The mutation must be capable of being changed productively with the available tools. (4) High specificity must be achievable—i.e., the gRNA must direct Cas9 only to the desired target, since cleavage or modification at other genomic sequences can lead to undesirable off-target changes that could be deleterious. (5) The efficiency of the desired modification at the target must be high enough to be clinically effective; the required level will differ among targets and situations. (6) It must be possible to deliver the reagents to the affected tissues and cells with adequate efficiency.

Despite these challenges, a number of promising CRISPR therapeutics are on the horizon (Table 1). An example that illustrates several approaches is sickle cell disease (SCD). The characteristic sickle shape of patients' red blood cells was described in 1910 (Herrick, 1910); a corresponding alteration in hemoglobin was reported in 1949 (Pauling et al., 1949); and the single responsible amino acid substitution in the β-globin chain was identified in 1957 (Ingram, 1957). The human β-globin gene was isolated in 1978 (Lawn et al., 1978; Wilson et al., 1978), which led to the discovery that essentially all SCD sufferers share the same single-base change in DNA that results in a glutamic acid being replaced by valine in the protein. This, in turn, leads to polymerization of hemoglobin and the alteration of red blood cell shape and flexibility.

As soon as the gene was isolated, attempts at gene therapy using a normal β-globin gene were begun. Those failed, but recent approaches have shown promise (Kanter et al., 2022). Gene editing using CRISPR is also underway and has advantages over gene addition: (1) Induced changes are targeted to the natural locus, under the control of normal regulatory sequences. (2) There is no danger of adverse effects due to integrating a transgene at random locations in the genome. (3) Both recessive and dominant mutations can potentially be reversed.

At least two early-stage trials for SCD have been initiated using Cas9 protein, a specific gRNA to induce a break very close to the sickle mutation and a DNA molecule carrying the non-mutant sequence to act as a template for repair by HDR (Lattanzi et al., 2021; Magis et al., 2022). We await results that will show whether this approach can be efficient enough to be clinically effective.

The most advanced CRISPR therapy for SCD takes an alternative tack. Rather than restoring the β-globin sequence, which depends on HDR, this approach relies on the more efficient NHEJ process to reactivate fetal γ-globin gene expression (Liu et al., 2018). The specific genomic target is a lineage-specific enhancer in the gene for *BCL11A*, the protein that is responsible for turning off β-globin expression after birth. It has been known for decades, based on rare human mutants, that the presence of fetal hemoglobin largely reverses the effects of SCD. Because this approach relies on inducing expression of fetal hemoglobin, it is applicable to β-thalassemia as well, and very exciting data have been reported for both types of patients (Frangoul et al., 2021).

**Table 1**. Clinical trials for disease gene editing with CRISPR

| Disease | CRISPR target(s) | Drug name | Origin |
|---|---|---|---|
| Sickle cell disease, β-thalassemia | *BCL11A* | CTX001 | CRISPR Therapeutics |
| Sickle cell disease | β-globin | CRISPR-SCD101 | Mark Walters |
| | | GPH-101 | Graphite Bio |
| β-thalassemia | β-globin | ET-01 | Edigene |
| | | BRL-101 | China |
| Leber congenital amaurosis type 10 | *CEP290* | EDIT-101 | Editas |
| TTA polyneuropathy, cardiomyopathy | Transthyretin (TTR) | NTLA-2001 | Intellia |
| Hereditary angioedema | Kallikrein (KLKB1) | NTLA-2002 | Intellia |
| AIDS | HIV-1 genome | EBT-101 | Excision Bio |
| Cervical neoplasia | HPV E6/E7 | CRISPR/Cas9-HPV16 E6E7T1 | China |
| Herpes virus keratosis | HSV genome | BC111 | China |
| HIV-AIDS | *CCR-5* | | China |

Data retrieved in October 2022, from the ClinicalTrials.gov website (https://clinicaltrials.gov/), curated by the U.S. National Library of Medicine.

Whether there will be adverse long-term consequences remains to be seen.

Because only a single base pair is altered in SCD, you might think that base editors would be good therapeutic candidates. As noted above, however, it is possible only to make base transitions (purine for purine, pyrimidine for pyrimidine) with current base editors, and the sickle mutation is a transversion (A:T to T:A). In a clever alternative approach (Newby et al., 2021), rather than converting T back to A, the A base editor is used to convert A to G (T to C, in the coding strand), and the resulting sequence encodes alanine. This variant has been found, albeit quite rarely, in humans and appears to have little if any effect on hemoglobin function.

SCD has many advantages as an initial clinical target: (1) It is due to mutation in a single gene. (2) All patients carry the same mutation. (3) That mutation has been shown to be responsible for the disease. (4) Delivery can be carried out *ex vivo*. This last feature owes a lot to the developments in bone marrow transplantation. Current protocols for SCD involve isolating hematopoietic stem and precursor cells (HSPCs) from a patient, treating them in the laboratory with the CRISPR reagents, characterizing them thoroughly, returning them to the same patient, and monitoring engraftment. Although simple to describe, this process has its complexities. Both the treatment of the patient and the manipulation and characterization of the cells are time consuming and expensive and add to the ultimate cost of the therapy (Wilson and Carroll, 2019).

Several other disease targets are being addressed with CRISPR in early-stage clinical trials (Table 1). Inherited eye diseases are promising candidates because the reagents can be delivered by direct injection, and immunological reactions are blunted in the eye. Leber congenital amaurosis type 10 is caused by a mutation that creates a novel splice site in the intron of the *CEP290* gene. The approach taken by Editas Medicine is to deliver coding sequences for Cas9 protein and two gRNAs that direct cleavage on either side of the mutation (Maeder et al., 2019). Deletion or inversion of the segment between the cuts removes the cryptic splice site and restores normal splicing. Use of a smaller version of Cas9 makes it possible to deliver all three coding sequences in an adeno-associated virus (AAV) vector that is injected sub-retinally.

Diseases with a primary defect in the liver are also good candidates for genome editing because injected materials are efficiently taken up from the circulation by hepatocytes. Transthyretin amyloidosis is caused by aggregation of the transthyretin (TTR) protein and accumulation in neural and heart tissue. Intellia has devised a strategy to reduce TTR production and aggregation by introducing knockout mutations in the gene with Cas9 cleavage (Gillmore et al., 2021). This works for both the acquired form and the inherited autosomal dominant form of the disease. In this case, the mRNA for Cas9 and the gRNA, both synthesized *in vitro*, are delivered via lipid nanoparticles (LNPs, similar to the COVID-19 vaccines) that are readily taken up in the liver, which is the site of TTR synthesis.

CRISPR-mediated gene disruption is also being used to enhance the effectiveness of cancer immunotherapy with CAR (chimeric antigen receptor) T cells (Stadtmauer et al., 2020). Patient T cells are recovered and cultured in the laboratory and endowed with a specific, synthetic anti-tumor T cell receptor. They are further treated with CRISPR to inactivate two of their own genes: the T cell receptor and the gene for PD-1. The latter is a cell surface protein that is involved in terminating T cell activation; its knockout prevents what is called T cell exhaustion. Because the cells are manipulated in culture, the CRISPR reagents can be delivered by electroporation *ex vivo*, as is done with HSPCs for SCD and β-thalassemia. Multiple clinical trials with similar approaches are ongoing.

Some progress has been made in developing a CRISPR treatment for Duchenne muscular dystrophy (DMD), and good results have been reported in a dog model using both intramuscular injection and systemic delivery of paired AAV vectors—one encoding Cas9, the other carrying 3 copies of a specific gRNA (Amoasii et al., 2018). Cleavage leads to a variety of NHEJ products, some of which are effective in restoring expression of a nearly full-length dystrophin protein in the case of one specific causative mutation. The situation is complex, however, because there are many different individual DMD mutations scattered throughout the gene, and many muscle groups are affected, so delivery presents a daunting challenge.

In other cases where *ex vivo* culture is not an option and the affected cells and organs are not readily accessible, some success has been achieved with AAV vectors and with LNPs, but getting these specifically to the affected sites remains a very challenging problem. In the case of AAV, researchers are working to find or to evolve variants that will use tissue-specific cell-surface proteins as receptors (Li and Samulski, 2020; Wang et al., 2020). An additional complication with base editors and prime editors is that the Cas9-fusion proteins are so large that they far exceed the packaging capacity of AAV and must be expressed from two vectors as "halves" that will assemble in co-infected cells (Levy et al., 2020). Other viral vectors and various nanoparticle compositions are also being developed.

## ADDITIONAL ISSUES

No CRISPR-based therapy is currently on the market, but predictions are that they will be very expensive, in the range of one to two million dollars or more per treatment. The cost reflects the investment in perfecting and testing the technology for each case, the treatments necessary for the patients, the production costs (particularly high for viral vectors), as well as the expectation of profits for the companies and investors. In principle, genome modifications should require only a single treatment for life-long benefit, and a high one-time cost could replace many years of continuing drug treatments.

Do high costs mean genome editing therapies will be available only to wealthy countries and individuals? Unfortunately, this will certainly be the case in the foreseeable future. Insurance companies may be willing to pay in the cases of very rare conditions, since the total cost will be limited. This is currently the case for the gene addition therapy for spinal muscular atrophy but is not likely for a more common condition like SCD. In the long run, significant advances will have to be made in delivery modalities and production to make the

therapies more broadly and more equitably accessible (Wilson and Carroll, 2019).

I want to mention briefly two other issues without going into great detail. The first is off-target effects—i.e., the observation that modifications can occur elsewhere in the genome in addition to the intended target. This concern was raised soon after the initial demonstrations of CRISPR as an effective genome editing tool. Since then, we have developed methods to identify sites that are at risk with any particular gRNA and to reduce the frequency of off-target alterations with changes in procedures and the reagents themselves. Furthermore, for any specific therapy, the range of unintended modifications we need to worry about may be limited. For example, disruption of globin genes would be unlikely to have consequences for editing that is confined to the liver. Some adverse effects may be reversible, and careful characterization of editing outcomes in preclinical research and early trials will minimize the chances of serious outcomes. I have written about this in more detail elsewhere and have concluded that it should be possible, with careful research, to make CRISPR treatments safe at the genomic level in somatic therapies (Carroll, 2019).

Second, small RNAs have also been used in other clinical applications. These include delivering anti-sense and small interfering RNAs. A very successful example is Nusinersen, a highly modified RNA that is used to treat spinal muscular atrophy with dramatic outcomes (De Vivo et al., 2019). In some situations, this drug is being replaced with gene addition therapy (Dabbous et al., 2019). The latter has the advantage that a single treatment can provide extended—perhaps lifetime—relief, while the RNA therapy must be repeated several times annually.

I have intentionally avoided discussing possible uses of CRISPR for heritable human genome editing (HHGE). This is a complex topic that requires serious consideration of multiple aspects, including extensive public discussion of the needs, ethics, and advisability of moving forward. Several recent international reports (National Academies of Sciences, Engineering, and Medicine, 2017; National Academy of Medicine et al., 2020; World Health Organization, 2021a; 2021b) and multiple opinion pieces have delved into these issues and are worth reading. Suffice it to say here that the technology cannot currently ensure the levels of safety and efficacy required for clinical applications. All proposed uses of HHGE should be deferred at least until this standard can be achieved.

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## AUTHOR CONTRIBUTIONS

D.C. wrote the manuscript.

## CONFLICT OF INTEREST

D.C. receives license royalties from Sangamo Therapeutics for use of zinc finger nucleases in genome editing.

## ORCID

Dana Carroll https://orcid.org/0000-0002-3241-3331

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