

CRISPR/Cas9 mediated genome editing: Applications and clinical studies



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Introduction

Eukaryotic species' genomes are made up of billions of DNA bases. The ability to modify these DNA bases at specific predetermined positions is extremely valuable not only in molecular biology, but also in medicine and biotechnology. Genome editing has long been a priority in molecular biology. The late 1970s discovery of restriction enzymes that usually defend bacteria against phages was a defining moment that fueled the era of recombinant DNA science. It was later discovered that the introduction of a double-strand break (DSB) at a target site increases the frequency of selective gene incorporation by many orders of magnitude. While artificially conceived meganucleases, followed by Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), enhanced genome editing performance, re-design or re-engineering of a new category of proteins was needed to target different sites in the genome. The challenge in cloning and protein engineering ZFNs and TALENs hampered their widespread adoption by the scientific community. CRISPR has transformed the industry in this regard because it is as powerful as, if not more so than, existing editing tools (1). Furthermore, it is much simpler and more adaptable to use. A brief timeline of key events in the discovery and subsequent repurposing of CRISPR/Cas9 system for gene editing is mentioned in table 1.

What is CRISPR/cas9

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)–CRISPR-associated (Cas) (CRISPR–Cas) systems originate from Prokaryotes, where they serve primarily as a defensive mechanism against mobile genetic elements like phages and plasmids. It is now being utilized as potent a gene editing tool that allows highly specific and rapid modification of DNA. The CRISPR-Cas system, which has been modified for use in lab settings relies on three main components: a guide RNA (gRNA) a cas nuclease, and the target DNA. The various components are depicted in figure 1.

Table 1: A brief history of CRISPR/Cas9 gene editing system

Year	Discovery	Referenc
1987	The CRISPR sequence first identified, (not known as CRISPR)	(2)
2000	More clustered repeats of DNA identified in other bacteria and archaea, termed Short Regularly Spaced Repeats (SRSR)	(3)
2002	Term CRISPR-Cas9 published for first time	(4)
2005	Jennifer Doudna and Jillian Banfield started investigating CRISPR	(5)
2005	French scientists suggested CRISPR spacer sequences can provide cell immunity against phage infection and degrade DNA	(6)
2007	Experiments demonstrate for the first time the role of CRISPR together with Cas9 genes in protecting bacteria against viruses using the lactic acid bacterium <i>Streptococcus thermophilus</i>	(7)
2008	Created the first artificial CRISPR arrays—programming CRISPR to target four essential genes in lambda phage	(8)
2008	Proved that the target of Cas9 is DNA, not RNA. Recognized that CRISPR was essentially a programmable restriction enzyme.	(9)
2010	Showed that Cas9's nuclease activity cut DNA at precise positions encoded by the specific sequence of the crRNAs.	(10)
2011	Discovery of tracrRNA (trans-activating CRISPR RNA).	(11)
2011	Reconstitution of CRISPR in a Distant Organism.	(12)
2011-12	Reprogramming Cas9 with custom-designed spacers in the CRISPR array to cut a target site of their choosing in vitro. In addition, Charpentier and Doudna showed that the two RNAs could function in vitro when fused into a single-guide RNA (sgRNA) (Lander 2016).	(13,14)
2011-13	Genome editing transferred to mammalian cells.	(15)
2018	First CRISPR-Cas9 clinical trial launched.	(16)
2020	Nobel Prize in Chemistry awarded 'for the development of a method for genome editing'. To Emmanuelle Charpentier and Jennifer Doudna.	(17)

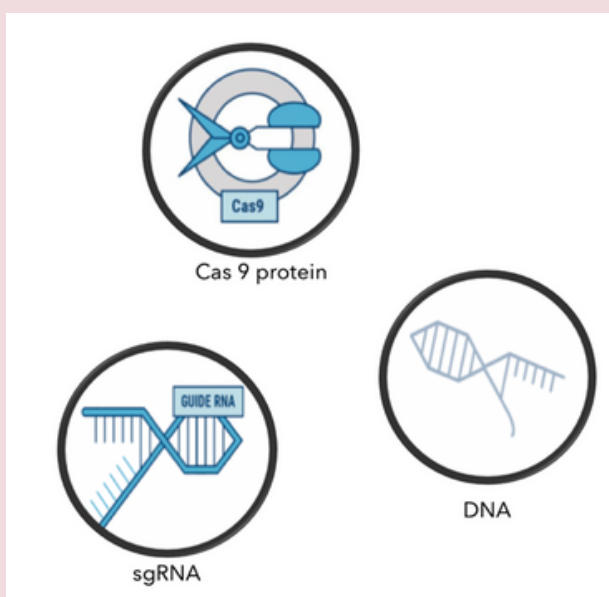


Figure 1 Main components of the CRISPR/cas9 system (19)

1. The guide RNA is a specific RNA sequence that recognizes the target DNA region of interest and directs the Cas nuclease there for editing. The gRNA is made up of two parts: CRISPR RNA (crRNA), a 17-20 nucleotide sequence complementary to the target DNA, and a tracrRNA, which serves as a binding scaffold for the Cas nuclease. The crRNA and tracrRNA can be combined to form a single guide RNA (sgRNA).
2. The CRISPR associated protein (Cas) is a bacterial RNA-guided endonuclease. It is directed to the specific DNA locus by a guide RNA (gRNA), where it makes a double-strand break. There are several versions of Cas nucleases isolated from different bacteria. The most commonly used one is the Cas9 nuclease from *Streptococcus pyogenes* (18).
3. The DNA sequence contains the sequence which we want to replace or the site where we want to insert our gene of interest. Once this DNA sequence interacts with the sgRNA-Cas9 complex, Cas9's HNH domain cleaves the DNA strand complementary to sgRNA and Cas9's RuvC-like domain cleaves the other DNA strand. It is necessary for this DNA sequence to show complementarity to the sgRNA and the presence of a PAM motif, for target recognition. This schematic has been diagrammatically depicted in figure 2.

Applications of CRISPR/Cas9 gene editing

Some of the many applications of this technology are as follows:

1. Generation of cellular/animal models:

The pathological analysis of different disorders relies heavily on genetically engineered animal models that mimic human diseases. Cas9-mediated genome editing has sped up the generation of transgenic models and broadened biological science beyond conventional, genetically tractable animal model organisms. CRISPR editing can be used to conveniently and rapidly model genetic disorders, research anatomy, disease development, and develop novel drugs for diseases by repeating the common mutations present in disease state populations. To prepare cell models and animal models (injection in zygote), direct transient transfection of cells with plasmids containing the CRISPR/cas9 system can be used. However, gene targeting has limited applications in some organisms due to time-consuming procedures and the lack of available embryonic stem cells (21).

2. Treatment of infectious diseases:

Given that the CRISPR-Cas system was designed to be an antiviral adaptive immune system in bacteria, it has the potential to be used to cure infectious diseases by eradicating pathogen genomes from infected individuals. According to recent research, the CRISPR-Cas9 mechanism will eliminate the HIV-1 genome and avoid new HIV infections. A sgRNA expression vector targeting the long terminal repeats (LTR, necessary for gene expression) of HIV-1 efficiently cleaves and mutates LTR target sites and suppresses LTR-driven viral gene expression when transfected into HIV-1 provirus-integrated human cells. Furthermore, this system has been shown to delete viral genes from host cell chromosomes (22).

3. Correction of genetic disorders:

This is a very exciting application of the CRISPR/cas9 technology for curing genetic diseases. The discovery and development of CRISPR/Cas9 has re-opened the door for gene therapy and changed the way scientists can approach a genetic aberration—by fixing a non-functional gene rather than replacing it entirely, or by disrupting an aberrant pathogenic gene.

This system has shown potential in efficiently correcting mutations in various animal models, and there are ongoing human clinical trials for curing genetic disorders like Beta thalassemia, Leber Congenital Amaurosis 10 (LCA 10) (22). A description of how CRISPR/Cas9 is used to treat LCA 10 is depicted in figure 3. Various genetic disorders, for which therapies based on CRISPR/Cas9 are currently in clinical trials, have been summarized in table 2.

Table 2. List of registered clinical trials of CRISPR/Cas for treatment of various diseases
 HPV- Human Papilloma Virus; LNP- Lipid nanoparticle; AAV- Adeno-associated virus; HBB- Hemoglobin Subunit Beta; iHSC- induced hematopoietic stem cells; CAR: Chimeric antigen receptor

Disease condition	Ex vivo/ in vivo	Delivery system/ modification	Gene targeted	Route	Cargo	If in clinical trial-stage	ClinicalTrials.gov identifier	Sponsor
Refractory herpetic viral keratitis	In vivo	Lentivirus	Herpes Simplex virus type 1	Corneal injection	CRISPR-Cas9 mRNA	Phase 1/2	NCT04560790	Shanghai BDgene Co., Ltd.
Human papillomavirus related malignant neoplasm	In vivo	Gel	HPV16 and HPV18 E6/E7	Topical application	CRISPR-Cas9 plasmid	Phase 1	NCT03057912	First Affiliated Hospital, Sun Yat-Sen University
Hereditary transthyretin amyloidosis	In vivo	LNP	Transthyretin	IV administration	CRISPR/Cas9 system	Phase 1	NCT04601051	Intellia Therapeutics
Leber congenital Amaurosis 10 (LCA 10)	In vivo	AAV	Centrosomal protein 290	Subretinal injection	CRISPR/Cas9 system	Phase 2	NCT03872479	Allergan
β-thalassemia	Ex vivo	Not specified	HBB gene	IV injection	HBB gene correction in patient specific iHSCs	Early Phase 1	NCT03728322	Allife Medical Science and Technology Co., Ltd
β-thalassemia, Sickle Cell Disease	Ex vivo	Ribonucleo protein electroporation	Enhancer of the BCL11A gene	Single infusion through a central venous catheter.	Autologous CD34+ HSPCs modified at the enhancer of the BCL11A gene	Phase 2/3	NCT03655678, NCT03745287	Vertex Pharmaceuticals Incorporated
AIDS and hematological malignancies	Ex vivo	Gene knockout	CCR5 gene modification	Cell transplant	CCR5 Gene Modified CD34+ Hematopoietic Stem/Progenitor Cells	N/A	NCT03164135	Affiliated Hospital to Academy of Military Medical Sciences
Metastatic Gastrointestinal Cancers	Ex vivo	Gene knockout	CISH	Cell infusion	Tumor-Infiltrating Lymphocytes (TIL), Cyclophosphamide, Fludarabine, Aldesleukin	Phase 1/2	NCT04426669	Intima Bioscience, Inc.
CD19+ Leukemia or Lymphoma	Ex vivo	lentiviral vector and electroporated (in cell), infused by IV injection	HPK1	IV infusion	XYF19 CAR-T cell, Cyclophosphamide, Fludarabine	Phase 1	NCT04037566	Xijing Hospital
Sickle Cell Disease	Ex vivo	RNP	Not specified	IV infusion	sickle allele modified CD34+ HSPCs	Phase 1/2	NCT04774536	Mark Walters, MD
Sickle Cell Disease	Ex vivo	Gene editing	HbS to HbA by SNM	IV infusion	GPH101:CD34 + Hematopoietic Stem Cells (HbS to HbA)	Phase 1/3	NCT04819841	Graphite Bio, Inc.
T or B Cell Malignancies	Ex vivo	Gene editing	CAR-T Therapy	IV infusion	CTX130: Anti-CD70	Phase 1	NCT04502446	CRISPR Therapeutics AG

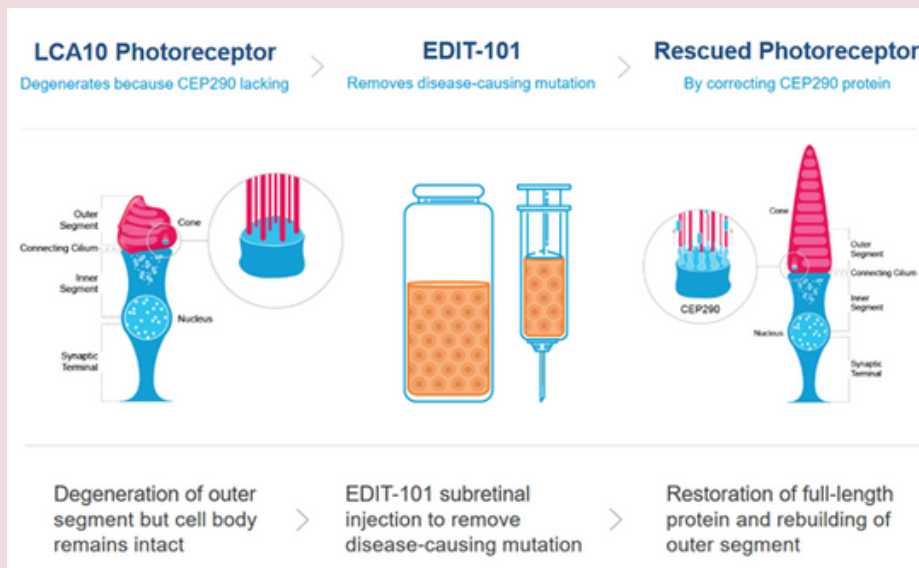


Figure 3: Successful editing by EDIT-101 restores functionality (23)

Challenges/Risks of using CRISPR/cas9 as a gene editing system

1. Off-target mutations:

The main concern of the CRISPR/Cas9 system is the problem of off-target effects, which cause genomic toxicity, carcinogenesis, genome instability, functional gene disruptions, and epigenetic alterations. Given that genomic changes caused by the CRISPR/Cas9 system are permanent, these effects should be carefully identified. The rate of off-target effects is affected by the composition and structure of the sgRNA, so shorter and unique sgRNA, which has a decreased mismatch tolerance, should show lesser off-target effects. Such a target site should be selected such that it shows no homology anywhere else in the genome. Shortening the sgRNA by 2-3 nucleotides, in the protospacer portion reduces the tolerance of the created mismatch and hence reduces the off-target effects.

2. Rate of INDEL formation

Another concern of the CRISPR/Cas9 system is the unwanted insertions and deletions (INDELS) (<20 bp) that rarely occur, but if the INDELS are too long (up to 600 bp or 1.5 kb) it can lead to pathological defects. Moreover, the size of the Cas9 protein is a key disadvantage, which is larger than a TALEN monomer and much larger than a ZFN monomer and causes Cas9 delivery by viral vectors to be challenging (24).

3. Delivery

The efficiency of delivery depends on the target cell type and the delivery method chosen. Due to the large size of the CRISPR complex, efficient and specific delivery into the cell is a major challenge. Currently, a number of viral, non-viral and physical methods are being employed for targeted cell delivery. Further development of suitable carriers will require long-term studies (25).

Conclusion

Innovation in the development of tools and technologies is indispensable for scientific progress. The CRISPR/Cas9 system is a versatile gene-manipulating tool consisting of a guide RNA sequence (sgRNA) and a DNA splicing protein complex (e.g. Cas9). The development of this

has allowed inducing DSB at selected sites determined by the modification of the guide RNA as required. Despite the availability of gene-editing tools such as ZNFs and TALENs, CRISPR/Cas9 has facilitated reaching this goal at a much faster pace, primarily owing to the ease of use and its high versatility. This technology is now available to quickly bring rapid and precise alterations to the genome. However, the road ahead is not free of obstacles. Additional research is required to better understand CRISPR/characteristics, Cas9's including its specificity, off-target effects, and delivery strategies. For example, the results of current genome-wide deep sequencing will be useful for choosing appropriate target sites and creating highly specific gRNA.

List of Abbreviations:

DNA- Deoxyribonucleic acid; DSB- Double stranded break; ZFN- Zinc Finger Nucleases; TALENs- transcription activator-like effector nucleases; CRISPR- Clustered Regularly Interspaced Short Palindromic Repeats; Cas- CRISPR associated; RNA- Ribonucleic acid; gRNA- guide RNA; sgRNA- single guide RNA; PAM- Protospacer adjacent motif; LTR- Long Terminal Repeats; LCA 10- Leber Congenital Amaurosis 10; bp- base pairs

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