

CRISPR/Cas9 uses: a review



N. Mimoune^{*#}, O. Benadjel[#], R. Baazizi, D. Kelef and R. Kaidi

Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated system (Cas) is a system that provides immunity to most prokaryotic organisms against viral attacks and other foreign bodies. CRISPR systems consist of a scissor-like protein called Cas9 and a genetic GPS guide "The guide RNA". However, researchers have reoriented and repurposed the primordial immune system to precisely manipulate genomes in most organisms by introducing DNA double-strand breaks at specific genome locations to introduce specific

DNA modifications. More applications of CRISPR have arisen since its discovery, from disabling parasites to correcting mutations and improving crop yields. This review was conceived as a guide to CRISPR technology, from its discovery to the latest breakthroughs. It is hoped that this study will provide a general-based view for this life changing technology, inspiring scientists to go further with CRISPR in the sake of a better life.

Key words: *CRISPR/Cas9; DNA; RNA; genome engineering; bacteria; virus*

Introduction

In the early 20th century, physicians and chemists unlocked the secrets of the atom, changing the world forever. But life remained a profound mystery. Inheritance remained one of life's deepest secrets. Everyone knew that traits, such as the shape of a pea pod, or eye and hair colour, are passed on from generation to generation. But no one knew how such information was stored or transmitted. Scientists were convinced that there had to be a biological molecule at the heart of the process, and that this molecule had

to have certain special qualities. The challenge of solving this mysterious "secret of life" and, furthermore, finding a way to edit it, now known as genetic engineering, was taken up (Doudna and Gersbach, 2015).

With the revolutionary discovery of DNA, scientists tried to manipulate it using different techniques like radiation and chemicals, causing random mutations in the DNA (1960s). In the 1970s, scientists discovered new technologies for sequencing, copying, and manipulating DNA, and they started

Nora MIMOUNE^{*#}, (Corresponding author: nora.mimoune@gmail.com), National High School of Veterinary Medicine, Algiers, Algeria; National Institute of Veterinary Sciences, Laboratory of Biotechnology Related to Animal Reproduction (LBRA), University Saad Dahleb, Blida, Algeria; Oumayma BENADJEL[#], Ratiba BAAZIZI, Djamel KELEF, National Institute of Veterinary Sciences, Laboratory of Biotechnology Related to Animal Reproduction (LBRA), University Saad Dahleb, Blida, Algeria; Rachid KAIDI, Laboratory of Biotechnology Related to Animal Reproduction (LBRA), University Saad Dahleb, Blida, Algeria
*These authors have contributed equally to this work.

using them on many different cells for research and medical reasons (Pray, 2008).

These technologies for manipulating DNA were truly promising and have enabled advances in biology ever since the discovery of the DNA double helix. But until recently, it has been very difficult to rapidly obtain experimental results, as these techniques take a lot of time and effort and sometimes they were inefficient. Furthermore, introducing site-specific modifications in the genomes of cells and organisms remained elusive. Early approaches relied on the principle of the site-specific recognition of DNA. More recently, the site-directed zinc finger nucleases (ZFNs) and TAL effector nucleases (TALENs) were developed using the principles of DNA protein recognition. However, difficulties of protein design, synthesis and validation remained a barrier to widespread adoption of these engineered nucleases for routine use (Doudna and Gersbach, 2015).

However, all this changed with the emergence of a new technology; CRISPR, short for “clusters of regularly interspaced short palindromic repeats”. It was as though someone has pressed fast-forward on the gene-editing field. CRISPR is a simple tool that scientists can wield to snip and edit DNA, thus accelerating the pace of advancements that could lead to treating and preventing diseases (Doudna and Charpentier, 2014).

What is CRISPR?

Definition

The short answer is that the CRISPR technology is a revolutionary new class of molecular tools that scientists can use to make changes in any kind of genetic material. It involves the precise targeting, cutting and pasting of DNA in cells. CRISPR systems are the simplest yet

most powerful methods scientists have ever had to alter any DNA sequence on Earth, including human DNA.

The long answer is that CRISPRs are repeating sequences found in the genetic code of bacteria. They are interspersed with ‘spacers’ - unique stretches of DNA that the bacteria grab from invading viruses, creating a genetic record of their malicious encounters. CRISPR systems consist of a scissor-like protein called Cas9 and a genetic GPS guide “The guide RNA= gRNA”. Such systems inspired by nature and engineered by researchers naturally evolved across the bacterial kingdom as a way to foil attacks by viruses and other foreign bodies. But researchers reoriented and repurposed that primordial immune system to precisely manipulate genomes (Doudna and Gersbach, 2015).

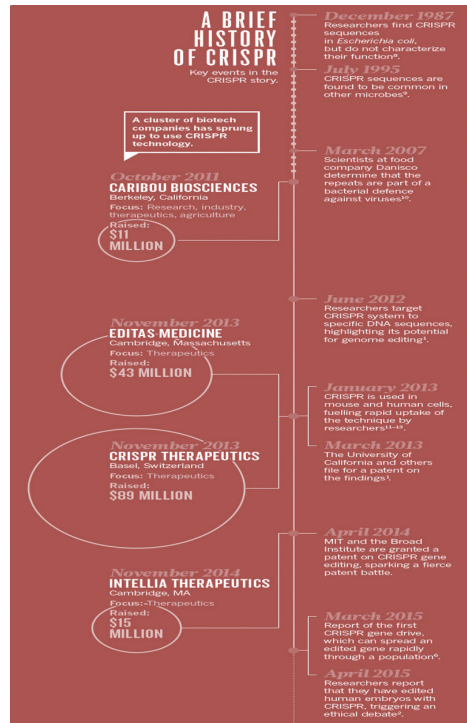


Figure 1. A brief history of CRISPR (Ledford, 2015)

How it was first discovered?

Years ago, sequences of clustered, regularly interspaced short palindromic repeats (CRISPRs) were found disseminated in the genomes of numerous bacteria. In 1987, the first description of a CRISPR array was made by researchers who found repeats of multiple base pairs (bp), interspersed by bp non-repeating spacer sequences in *Escherichia coli*.

In 1995, similar CRISPR arrays were found in *Mycobacterium tuberculosis*, *Haloferax mediterranei*, and other bacteria and archaea. Several hypotheses for the function of CRISPRs have been proposed. However, their function as a safeguard and defence system against viruses was not highlighted until 2007, when several research groups reported that the spacer sequences often contained parts of phage-derived DNA or plasmids, and they proposed that CRISPR uses these extra-chromosomal agents to mediate immunity against infection. Other researchers also reported a negative correlation between the number of CRISPR spacers in the genome of bacteria and their sensitivity to phage infection. Several years later, scientists confirmed this hypothesis experimentally by showing that after phage invasion, new spacers were acquired that conferred resistance against the phage (Sorek, 2008).

In 2012, as part of a basic research project on how bacteria fight viral infections, it was invented a new technology for editing the genome using the CRISPR-Cas9 (CRISPR Associated Proteins 9) system (Doudna and Gersbach, 2015) (Figure 1).

How it works? (The CRISPR lexicon)

CRISPR loci are found solely in the genome of archaea and bacteria, they contain the CRISPR arrays including the leader sequence, as the sequence that contains hundreds of nucleotides

and where the transcription of the array CRISPR starts. Researchers found that a CRISPR loci lacking the leader sequence is unable to incorporate new spacers and to execute the CRISPR-Expression and interference (Sinan Al-Attar, 2011).

The associated Cas genes

The CRISPR array: sequences of clustered (cluster means groups), regularly interspaced palindromic (having the property of reading the same forwards as backwards) repeats found in the genome of many bacteria and almost all archaea.

These arrays are composed of direct repeats "short palindromic repeats" that are separated by similarly sized non-repetitive spacers "protospacers" originating from either phages or plasmids and comprise the prokaryotic "immunological memory" (Sorek, 2008).

CRISPR associated proteins (Cas): enzymes having a role in the mediated CRISPR resistance, i.e., nucleases, helicases, integrases and polymerases. These play a role in the addition and suppression of new spacers, processing of the CRISPR-Transcripts and mediation of other CRISPR-defence processes (Sinan Al-Attar, 2011). The Cas9 is an endonuclease found in CRISPR type II and guided by two RNA (Doudna and Gersbach, 2015).

CRISPR RNA (crRNA) is generated from the CRISPR loci. Their function is to guide Cas proteins to silence invading nucleic acids.

The CRISPR array is transcribed to make the precursor-CRISPR-RNA (pre-crRNA). Under the co-processing of transcript-activating crRNA (tracrRNA) and RNAs III, the pre-crRNA matures and becomes crRNA. The dual tracrRNA crRNA with the Cas9 then form a complex to cleave site-specific target DNA (Chylinski et al., 2013). Now, the dual tracrRNA:crRNA is engineered as a single guide RNA (sgRNA).

Protospacer Adjacent Motif (PAM) is a sequence of a few short 2-5 base pairs (bp) found adjacent to one end of the protospacers, linked to the excision of protospacers and their insertion into CRISPR loci (Shah et al., 2013).

Plasmids carrying out a protospacer sequence but no PAM are resistant to CRISPR-Cas9 cleavage (Gasiunas et al., 2012).

Functionality of CRISPR-Cas9

Bacteria have to deal with viruses in their environment. A viral infection is a ticking time bomb, and bacterium have only minutes to diffuse. In their cells, bacteria have CRISPR as an adaptive immune system that allows for the detection of viral DNA; it then stores a record of it and destroys it upon re-exposure (Doudna and Gersbach, 2015).

The CRISPR mediate resistance is a multistage process that functions in three distinct steps that provide DNA-encoded, RNA-mediated and sequence-specific targeting of exogenous nucleic acids (Barrangou, 2015).

Adaptation

When a virus infects a cell and injects its DNA, pieces of this viral DNA are sampled from the invasive by the CRISPR system. Specialized Cas proteins insert it into the "CRISPR loci" and they are acquired as novel "spacers". They serve as a memory bank, enabling bacteria to recognize viruses and fight off future attacks; in a sense, the spacers in CRISPR are an account of the bacteria's battlefield wins. Spacer acquisition is the first step of immunization (Barrangou, 2015). These bits of viral DNA (spacers) serve as a record of infection over time to the viruses they have been exposed to. Moreover, these bits are passed on to the cell's progeny (offspring), resulting in the protection from viruses not only in one generation, as Blake Wiedenheft referred to the CRISPR loci as a genetic

vaccination card (Doudna and Gersbach, 2015).

Expression

After inserting the spacers, the CRISPR array is transcribed and processed to make precursor-CRISPR RNAs (precrRNA) an exact replicate of the viral DNA. The maturation of the precrRNA to the crRNA requires the presence of a trans-activating crRNA (tracrRNA) co-processed by RNase III. The final product is a crRNA with the size of a spacer-repeat unit (Sinan Al-Attar, 2011).

Interference

Through sequence homology, the dual tracrRNA:crRNA guides the Cas9 endonuclease. The recognition of invader nucleic acids then is done by complementarily to the crRNA. The guide RNAs direct the Cas for specific targeting, cleavage and degradation of complementary nucleic acids (Barrangou, 2015). It searches the DNA in the cell, and when matching sites are found, the complex (tracrRNA:crRNA:Cas9) associates with the DNA and allows Cas the cleaver to cut up the viral DNA by making a double-stranded (ds) break into the DNA helix and stopping the virus from replicating (Doudna and Gersbach, 2015).

On a repeat encounter with a virus, a bacterium can produce a stretch of RNA that matches the viral sequence, using the material in its spacer archive (Doudna and Gersbach, 2015).

Finally, there is a built-in safety mechanism, ensuring that Cas9 does not cut just anywhere in a genome. Short DNA sequences known as PAMs "protospacer adjacent motifs" serve as tags and sit adjacent to the target DNA sequence (Sander and Joung, 2014). They are essential for cleavage of the target DNA during the interference stage. If the Cas9 complex does not see a PAM

next to its target DNA sequence, it will not cut. This is a strategy CRISPR uses to distinguish self from non-self sequences, and that is the reason why Cas9 does not attack the CRISPR region in bacteria. Known Cas9 proteins will target only ds DNA sequences followed by a 3' PAM sequence specific to the Cas9 of interest. Cas9 rapidly dissociates from DNA that does not contain the appropriate PAM sequence, whereas it binds for a longer duration at sites containing a PAM sequence, with the dwell time depending on the degree of complementarity between the guide RNA and the adjacent DNA. Once Cas9 has found a target site with the appropriate PAM, it triggers DNA unwinding from the PAM-proximal end to the PAM-distal end of the target site (Chen and Doudna, 2017) (Figure 2).

Scientists have aimed to understand the activity of cas9 and how they could harness its function as a genetic engineering technology to offer opportunities to do things that have not been previously possible. They found that it can be programmed to recognize particular DNA sequences and make a break at that site, and this activity is now used in genome engineering. That allowed scientists to perform very precise changes in the DNA at the site where the break was introduced (Doudna and Gersbach, 2015).

The reason why the CRISPR system can be used for genome engineering is that cells have the ability to detect and repair broken DNA (Eastman and Barry, 1992), so when a plant or an animal cell detects a ds break in its DNA, it fixes it either by pasting together the ends of the broken DNA or it can repair the break by integrating a new piece of DNA at the site of the cut. Therefore, CRISPR is the way to introduce ds breaks into DNA at precise sites and by that we can trigger cells to repair those breaks either by disruption or incorporation of new genetic information (Doudna and Charpentier, 2014).

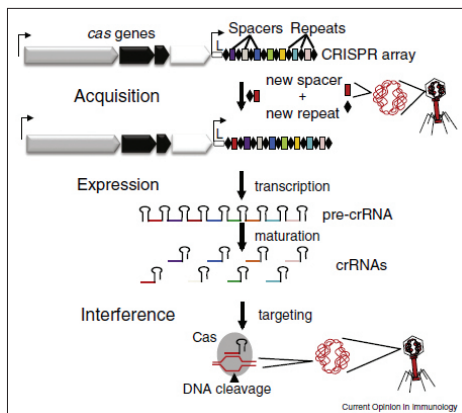


Figure 2. CRISPR–Cas immune systems
CRISPR-encoded immunization and interference
(Barrangou, 2015)

Genome engineering using CRISPR

CRISPR/Cas technology has emerged as the most popular tool for the precise alteration of the genomes of diverse species and different organism models. CRISPR/Cas9 system has taken the world of genome editing by storm in recent years, making it one of the hottest technology breakthroughs. Its popularity as a tool for altering genomes is due to the ability of Cas9 protein to cause double-stranded (ds) breaks in DNA after binding with short guide RNA molecules, which can be produced with dramatically less effort and expense than required for production of transcription activators, like effector nucleases (TALEN) and zinc-finger nucleases (ZFN). This system has been exploited in many species from prokaryotes to higher animals, including human cells, and the literature has shown increasing sophistication and ease of CRISPR/Cas9 and a wider range of species for which it is applicable. This technology is poised to solve several complex molecular biology issues currently faced in life science research. The invention of single guide RNA (sgRNA) by fusing crRNA and tracrRNA was an important breakthrough in this

field because it simplified the task of programming Cas9 to create breaks at specific DNA sites *in vitro*. Following this advance, this technology has been adopted for genome engineering in cells and different model systems

The first step towards gene alteration is precise generation of single- or double-stranded breaks (SSB or DSB) in the genome. Cas9/sgRNA complexes can generate accurate breaks in the genomes of bacteria, yeast, plants and animals. CRISPR-Cas9 is the latest inclusion in the genome editing toolbox that already contains ZFNs and TALENs (Figure 3).

The CRISPR/Cas9 system is currently the most desirable tool for genome engineering for several reasons. Cas9 is programmed by readily engineered sgRNAs. The ease of CRISPR/Cas9 use has opened new prospects for studying functional genomics of diverse organisms in a more precise manner with less effort when compared to other techniques like TALENs and ZFN. The variety of applications is growing at a rapid pace, including different types of knock-outs at single-gene or genome-wide scale, knockings, activating and inhibitory Cas9 versions, visualization and even biochemical tools (Ceasar et al., 2016).

Generating a knockout using CRISPR

Knockout: Permanently disrupts gene function in a particular cell type or organism without a specific preferred mutation.

Cas nucleases enable efficient and precise genetic modifications by inducing targeted DNA double-strand breaks (DSBs) that stimulate the cellular DNA repair mechanisms, including error-prone non-homologous end joining (NHEJ) and homology-directed repair (HDR). The Cas9:sgRNA complex binds to the target DNA to cleave the target DNA from 3' to 5'. Then the Cas9 undergoes a conformational change that positions the nuclease domains (RuvC and HNH) to cleave opposite strands of the target DNA that results in a ds break. This break in the DNA incites repair pathways, the efficient but error prone NHEJ or the less efficient but high-fidelity HDR. The NHEJ is the most active repair mechanism that causes small nucleotide insertions/deletions at ds break sites. Diverse arrays of mutations appear that causing amino acid insertion/deletion/frameshift mutation that leads to premature stop codons of the target genes. The ideal result is a loss-of-function mutation within the targeted gene resulting in the knockout of the phenotype (Chen et al., 2015).

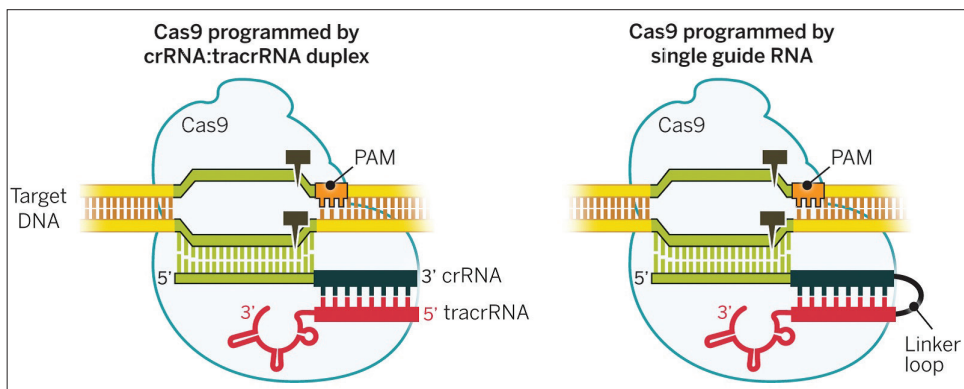


Figure 3. Evolution and structure of *S. pyogenes* Cas 9 (Doudna and Charpentier, 2014)

Enhancing specificity with Nickases

Cas 9 generates ds breaks through the combined activity of the two nuclease domains RuvC and HNH. Cas nickase is another advantage of CRISPR that can be converted to a nickase that creates single-stranded (ss) breaks by retaining only one nuclease domain and generating a DNA nick rather than a ds break. Either of the two endonuclease domains of Cas9, HNH and RuvC can be mutated to form nickases. That transforms the Cas9 complex into a strand-specific nicking endonuclease (Gasiunas et al., 2012).

Activation or repression of target genes using CRISPR

Repress (knockdown): reduce expression of particular gene(s) without permanently modifying the genome. Activate (CRISPRa): increase expression of an endogenous gene(s) without permanently modifying the genome.

RuvC and HNH domains can be rendered inactive by point mutations, resulting in a nuclease dead Cas9 (dCas9 lacking endonuclease activity) molecule that cannot cleave target DNA. The dCas9 molecule retains the activity to bind to target DNA based on the gRNA targeting sequence. This system is called CRISPR interference (CRISPRi). Early experiments demonstrated that targeting dCas9 to transcription start sites was sufficient to repress transcription by blocking initiation. dCas can also be tagged with transcriptional repressors or activators (Gilbert et al., 2013), and targeting these dCas9 fusion proteins to the promoter region (the section of DNA that controls the initiation of RNA transcription) results in robust transcriptional repression or activation (it can upregulate endogenous expression in human cells (Cheng et al., 2013), of downstream target genes (towards the 3' end). The simplest dCas9-based activators and repressors, consist of dCas9 fused directly to a single transcrip-

tional activator or repressor. Importantly, unlike the genome modifications induced by Cas9 or Cas9 nickase, dCas9-mediated gene activation or repression is reversible, since it does not permanently modify the genomic DNA (Qi et al., 2013).

CRISPR base editing without double-strand breaks

Base editing is a different approach to genome editing that enables the direct, programmable, targeted point mutations without inducing ds breaks by the conversion of a C:G base pair to a T:A base pair.

CRISPR base editors fuse Cas9 nickase or dCas9 to a cytidine deaminase. Base editors can convert cytidine to uridine within a small single-stranded DNA bubble at a guide RNA-specified locus near the PAM site. Base excision repair (BER) is the cell's primary response to G:U mismatches, creating a C->T change (or G->A on the opposite strand) conversion in mammalian and plant cells. In addition, new base editors have been engineered to convert adenosine to inosine, which is treated like guanosine by the cell, creating an efficient and permanent A->G (or T->C) change on the opposite strand (Rees et al., 2017).

Epigenetic modification using CRISPR

Epigenetics can have several meanings. To Conrad Waddington, it was the study of epigenesis or how genotypes give rise to heritable phenotypes changes without altering the DNA sequence (Waddington, 1957).

Cas enzymes can be fused to epigenetic modifiers to create programmable epigenome engineering tools that could be used to precisely control cell phenotype or the relationship between the epigenome and transcriptional control. Like CRISPR activators and repressors, these tools alter gene expression without inducing a ds break. However, they are much more specific for particular chro-

matin and DNA modifications, allowing researchers to isolate the effects of a single epigenetic mark. Another potential advantage of CRISPR epigenetic tools is their persistence and inheritance. CRISPR activators and repressors are thought to be reversible once the effector is inactivated/removed from the system. In contrast, epigenetic modifiers may be more frequently inherited by daughter cells (Hilton et al., 2015).

Visualize genomic loci using fluorophores

The fluorescence labelling of endogenous genomic DNA by CRISPR using a dCas9 has greatly simplified the study of the spatial organization of the genome in live cells, with numerous advantages over other techniques, including the simplicity of programming to target a wide array of deferent genomic sequences, even detecting multiple genomic loci. This method offers a unique detecting of the chromatin dynamics in living cells (Ma et al., 2015).

Multiplex genome engineering with CRISPR

CRISPR cas9 allows for simultaneous targeting of multiple genomic loci. This multiplexing feature uses multiple guide RNA sequences that can be encoded into a single CRISPR array to enable simultaneous editing of several sites within the mammalian genome, demonstrating easy programmability and wide applicability of the RNA-guided nuclease technology.

The ability to carry out multiplex genome editing in mammalian cells enables powerful applications across basic science, biotechnology, and medicine (Cong et al., 2013).

RNA targeting

In some bacteria, type VI CRISPR systems recognize single-stranded RNA

(ssRNA) rather than dsDNA. RNA-guided RNA-targeting type VI CRISPR is capable of highly efficient and specific RNA knockdown and degradation in mammalian cells. Type VI CRISPR-Cas systems contain the programmable single-effector RNA-guided ribonuclease Cas13. Similar to Cas9, Cas13 can be converted to and RNA-binding protein through mutation of its catalytic domain (Cox et al., 2017).

Gene Drive

Gene drives are a particularly powerful application of CRISPR technology. They are genetic elements that insert themselves into target sites lacking that element, converting heterozygous alleles to homozygous alleles within an organism by allowing CRISPR on one chromosome to copy itself to its partner in every generation. In organisms that support sexual reproduction, gene drives enable non-Mendelian inheritance of alleles that can spread throughout a population. Usually, a genetic change in one organism takes a long time to spread through a population. That is because a mutation carried on one of a pair of chromosomes is inherited by only half the offspring. But a gene drive allows a mutation made so that nearly all offspring will inherit the change. This spread can be rapid for species with short reproductive generation times and it can quickly sweep an edited gene through a population. This means that it will speed through a population exponentially faster than normal. A mutation engineered into a mosquito could spread through a large population within a season if that mutation reduced the number of offspring a mosquito produced, then the population could be wiped out, along with any malaria parasites it is carrying. The work is at an early stage, but such a technique could be used to wipe out disease-carrying mosquitoes or ticks, eliminate invasive

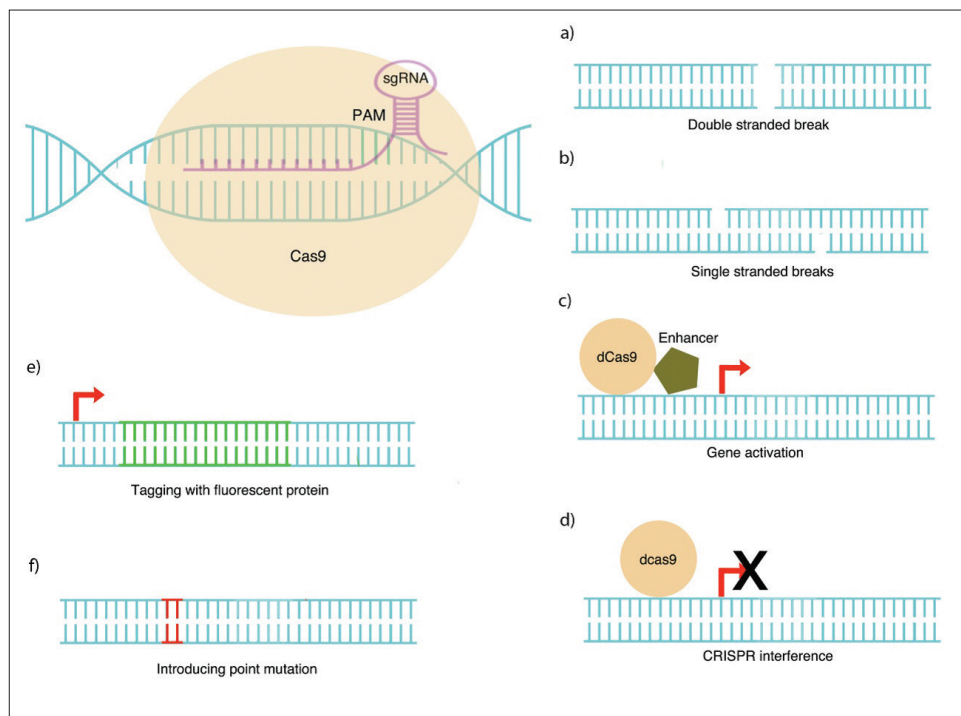


Figure 4. Overview of possible experimental designs of CRISPR/Cas9 (Ceasar et al., 2016)

plants or eradicate herbicide resistance (Komor et al., 2016) (Figure 4).

CRISPR applications

CRISPR is incredibly powerful. It has already brought a revolution to the day-to-day life in most laboratories, according to molecular biologist Jason Sheltzer. Programmable DNA cleavage using CRISPR–Cas9 enables efficient, site-specific genome engineering in single cells and whole organisms. It has been used in various ways, to alleviate genetic disorders in animals and is likely to be employed soon in the clinic to treat human diseases (Barrangou and Doudna, 2016). Of course, humans are not the only species with a genome. CRISPR has applications in animals and plants, from disabling parasites to improving the crop yields. Here, we take a look at

the recent advances that demonstrate CRISPR's capabilities.

Disease fighter

Cancer

Figure 5 shows the cancer therapeutics arising from the CRISPR system as cited by multiple authors.

HIV

HIV still infects more than 35 million people worldwide according to the Global Health Observatory data (updated November 2017). Not only does the virus infect the very immune cells in the body that attack viruses, but it is also a notorious mutator. Currently, highly active antiretroviral therapy (HAART) is able to suppress HIV infection below detectable levels in HIV patients. However, HAART is limited in its high

Cancer type	Modification	Contribution to therapy
Breast cancer	Knock-out of miR-644a	Inhibition of tumor growth, metastasis, and drug resistance
Breast cancer	Knock-out (KO) BC200 lncRNA by CRISPR system	BC200 may serve as a prognostic marker and possible target for attenuating deregulated cell proliferation in estrogen-dependent breast cancer
Endometrial cancer	Knock-out of <i>MUC1</i> at cells by CRISPR system	Concomitant decrease of MUC1 and EGFR can be prognostic markers in human endometrial tumors
Lung adenocarcinoma and endometrial carcinoma	Deletion of super-enhancers 3' to <i>MYC</i> in cells by using CRISPR system	Super-enhancers stimulate cancer driver genes in diverse types of cancer
Endometrial cancer	<i>ERα</i> -null endometrial cancer cells	Inverse relationship between the tumor suppressor PR and the oncogene Myc in endometrial cancer
Prostate cancer	<i>NANOG</i> and <i>NANOGP8</i> knockout DU145 prostate cancer cell lines	Attenuation of malignant potential of prostate cancer

Figure 5. Cancer therapeutics arising from the CRISPR system [Stella et al., 2018]

cost, patient compliance, side effects from long-term therapy, and emergence of drug resistance. Most of all, it does not cure HIV infection. Therefore, there is a continuous need to develop more effective therapeutics and cure strategies for HIV infection. Researchers now are using CRISPR to snip the virus from the cell it was infecting, shutting down the virus's ability to replicate. The first use of the CRISPR technique to eradicate the HIV virus was in 2017, demonstrating a way to eliminate HIV from infected cells, resulted in reduced viral RNA expression and successful proviral excision. One of techniques used was inhibiting the

gene coding sequences which resulted in a strong inhibition of HIV-1 by Cas9/gRNA. This inhibition was obtained due to the deletions introduced into the viral DNA due to Cas9 cleavage in the cytoplasm and in the nucleus (Yin et al., 2018).

Cystic Fibrosis

Cystic fibrosis is a progressive, genetic disease originating from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) in the gastrointestinal and pulmonary tract. Mutations in the CFTR gene disable the CFTR protein, an ion channel

regulating epithelial fluid transport. Loss-of-function alleles lead to an accumulation of a thick and sticky mucus in the gastrointestinal and pulmonary tract, causing a number of symptoms such as difficulties in breathing and recurrent infections. In the lungs, the mucus clogs the airways and traps germs, like bacteria, leading to infections, inflammation, respiratory failure, and other complications (Savić and Schwank, 2015). It was recently shown that the CFTR gene can be corrected using CRISPR-Cas9. The obtained results were possible by targeting the CFTR gene in cultured intestinal stem cells isolated from a cystic fibrosis patient (Schwank et al., 2013).

Huntington disease

This is a fatal genetic neurodegenerative disorder that causes nerves in the brain to deteriorate over time. The condition results from a faulty gene that becomes larger than normal and produces a larger-than-normal form of a protein called huntingtin. The elongated protein is cut into smaller, toxic fragments that bind together and accumulate in neurons, disrupting the normal functions of these cells. This process particularly affects regions of the brain that help coordinate movement and control thinking and emotions. The dysfunction and eventual death of neurons in these areas of the brain underlie the signs and symptoms of Huntington disease. Although suppressing the expression of mutant HTT (mHTT) has been explored as a therapeutic strategy to treat Huntington's disease (Shin et al., 2016). Considerable efforts have gone into developing allele-specific suppression of mHTT expression. Researchers reported that they have reversed the disease by permanent suppression of endogenous mHTT expression in lab mice engineered to have a human mutant huntingtin gene in place of a mouse huntingtin gene. CRISPR/

Cas9-mediated inactivation was effectively used to snip out part of the mutant huntingtin gene that produces the toxic bits, resulting in the evacuation of HTT aggregates and attenuating early neuropathology. The reduction of mHTT expression in neuron cells in adult mice did not affect viability, but alleviated motor deficits. This study suggested that CRISPR/Cas9-mediated gene editing could be used to efficiently and permanently eliminate the gene expansion-mediated neuronal toxicity in the adult brain and the obtained results showed the potential of CRISPR to help fight this condition (Yang et al., 2017).

CRISPR and COVID-19

Coronaviruses are a large family of viruses which may cause diseases in animals or humans. In humans, multiple coronaviruses are known to cause respiratory infections ranging from the common cold to more severe diseases such as Middle East Respiratory Syndrome (MERS) and Severe Acute Respiratory Syndrome (SARS). For the third time in as many decades, a zoonotic coronavirus has crossed species to infect human populations. This new virus and disease were unknown before the outbreak began in Wuhan, China in persons exposed to a seafood or wet market, in December 2019. At 9 a.m. on 7 January 2020, the virus was identified as a novel coronavirus and officially named by the WHO as 2019-nCoV, the novel 2019 coronavirus. COVID-19 is now a pandemic affecting many countries globally (WHO, 2020a).

The information provided by the Chinese public health, clinical and scientific communities facilitated the recognition of the disease. Like outbreaks caused by two other pathogenic human respiratory coronaviruses (SARS) and (MERS), 2019-nCoV causes respiratory disease that is often severe. As of 24 January 2020, there were more than 800 reported cases, with a mortality rate of

3%. Globally, as of 4:03pm CEST on 30 June 2020, 10,185,374 cases of COVID-19 were confirmed, including 503,862 deaths reported to WHO (WHO, 2020b).

After sequencing the viral genome, the information provided showed that it is 75 to 80% identical to the SARS and related to numerous bat coronaviruses. It can be spread in the same cells that attract the SARS and MERS viruses, though notably, 2019-nCoV grows better in primary human airway epithelial cells, unlike SARS or MERS (Perlman, 2020).

The structure of COVID-19

Coronaviruses are enveloped, non-segmented, positive-sense single-stranded RNA virus genomes. It is the largest known viral RNA genome with a size ranging from 26 to 32 kilobases. The virion has a nucleocapsid composed of genomic RNA and phosphorylated nucleocapsid (N) protein, which is covered with phospholipid bilayers and ornamented by two different types of spike proteins: the spike glycoprotein trimmer (S) that can be found in all CoVs, and the hemagglutinin-esterase (HE) present in some CoVs. The membrane (M) protein (a type III transmembrane glycoprotein) and the envelope (E) protein are located among the S proteins in the virus envelope. CoVs were given their name based

on the characteristic crown-like appearance. The structure of the CoV virion is shown in Figure 6 (Li et al., 2020).

Authorizing the use of the CRISPR diagnostic tool during the pandemic

The recent outbreak of the novel coronavirus COVID-19 can be diagnosed using qPCR, but the exhausted stock of reagents and equipment has slowed disease detection in some areas. To reduce the shortage and help increasing the testing, the U.S. Food and Drug Administration granted its first-ever use authorization for the gene-editing technology CRISPR on 7 May 2020. The kit was granted approval under 'emergency use' provisions, and should help to ease testing backlogs in the country. Average testing has reached nearly 250,000 tests per day, causing shortages in some places. This Greenlighted corona diagnostic test, developed by Sherlock Biosciences (Cambridge, Massachusetts, USA) is the first authorized use of CRISPR technology for an infectious disease test. (Guglielmi, 2020).

The Sherlock CRISPR SARS-CoV-2 Kit is a CRISPR-based SHERLOCK (Specific High sensitivity Enzymatic Reporter unLOCKing) diagnostic test. It works by programming the CRISPR machinery to detect a snippet of the SARS-CoV-2 genetic material (DNA or RNA) in samples as is used for qRT-PCR assays (in upper respiratory specimens, such as nasal swabs, and bronchoalveolar lavage specimens, such as from fluid in the lungs), from individuals suspected of COVID-19. If viral genetic material is found, a CRISPR enzyme generates a fluorescent glow. The test can return results in about an hour using a dipstick (Guglielmi, 2020).

The SHERLOCK COVID-19 detection protocol works in three steps, starting from nucleic acid extraction as used for qRT-PCR tests:

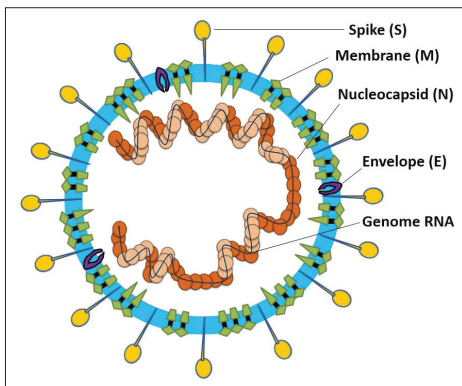


Figure 6. Coronavirus particle (Li, 2020)

- Step 1: (25 min incubation) isothermal amplification of the extracted nucleic acid sample using a commercially available recombinase polymerase amplification (RPA) kit.
- Step 2: (30 min incubation) detection of pre-amplified viral RNA sequence using Cas13.
- Step 3: (2 min incubation) visual read out of the detection result by eye using a commercially-available paper dipstick (Zhang et al., 2020).

In addition to the diagnosing tool, CRISPR can be used as a therapeutic to fight COVID-19. By activating and inactivating the different genes in our cells we can better understand how the coronavirus infects cells, this way can lead to discovering a drug to cure the disease or even to destroy the virus (Li et al., 2020).

Mutation corrector

Blindness

One of the most common causes of childhood blindness is a condition called Leber congenital amaurosis (LCA), which affects about 2 to 3 per 100,000 newborns. LCA is a group of inherited retinal diseases causing blindness or severe vision loss in early childhood. The condition is caused by mutations that lead to the degeneration and/or dysfunction of photoreceptors, the cells in the retina that make vision possible. Photoreceptors capture light, converting it to electrical signals which are sent to the back of the brain to create the images we see. Mutations in one of more than two dozen genes can cause LCA (Perrault et al., 2004). A study explored the potential of CRISPR/Cas9-mediated gene editing to correct the mutation, and found that the use of the CRISPR system could efficiently correct the mutation by deletion of the mutant genes. These

researchers used the CRISPR-Cas strategy as a treatment and they noticed a remarkable reduction in the number of mutant cells. These results showed the therapeutic potential of CRISPR-Cas strategies in treating patients with LCA (Ruan et al., 2017).

Duchenne Muscular dystrophy

Duchenne muscular dystrophy (DMD) is a debilitating condition that develops because of a mutation in of the longest genes in the body that codes for the dystrophin protein. Because of the mutation in the dystrophin gene, the body does not make a functional form of the protein dystrophin, which is essential for muscle fibre health and for the integrity of muscle cell membranes of striated muscles. Over time, the lack of this protein causes progressive muscle degeneration and weakness. There is no effective treatment for this disease. Numerous approaches to rescue dystrophin expression in DMD have been attempted. However, these approaches cannot correct *DMD* mutations or permanently restore dystrophin expression (Pichavant et al., 2011).

In April 2017, a team of researchers used CRISPR to find ways to fight Duchenne muscular dystrophy. They had used a variation of the CRISPR tool, called CRISPR-Cpf1 (CRISPR from *Prevotella* and *Francisella*) to correct the mutation that causes Duchenne muscular dystrophy. They fixed the gene in human cells growing in lab dishes and in mice carrying the defective gene by deletion of some of the Exon in that mutated gene. They showed that Cpf1 provides a robust and efficient RNA-guided genome editing system that can be used to permanently correct *DMD* mutations by different strategies, thereby restoring dystrophin expression and preventing progression of the disease. These findings showed the efficiency of Cpf1-mediated correction

of genetic mutations in human cells and an animal disease model and represent a significant step toward therapeutic translation of gene editing for correction of DMD (Zhang et al., 2017).

Fanconi Anaemia

Fanconi Anaemia (FA) is a rare potentially life threatening autosomal recessive disorder characterized by progressive pancytopenia, multiple congenital anomalies with multiple type of cancer risk. It is classified into the chromosomal instability syndromes (Ataxia telangiectasia, Bloom syndrome and Werner syndrome). Chromosomal instability syndromes are groups of disorders due to the defects in DNA repair, increased risk of cancer, and other phenotypic changes. FA is also classified into another group of syndromes, inherited bone marrow failure syndromes by its haematological abnormalities (Ahammad et al., 2017). The main malformations concern the extremities, rachis, skin, kidneys, urinary tract and ear, nose and throat. Advances in molecular biology have made it possible to identify 15 genes involved, whose mutation is responsible for haematological damage and increased risk of neoplasms (Hammoud et al., 2020).

Cas9-mediated genome editing has been used to correct the mutations in this disease where some researchers employed fibroblasts derived from a patient with Fanconi anaemia as a model to test the ability of the CRISPR/Cas9 nuclease system to mediate gene correction. They showed that the Cas9 nuclease and nickase each resulted in gene correction, but the nickase, because of its ability to preferentially mediate homology-directed repair, resulted in a higher frequency of corrected genes (Osborn et al., 2015).

These studies collectively demonstrate significant progress toward devel-

oping treatments for genetic diseases and even potential cures (Komor et al., 2016).

Life improvement

Agricultural applications

Just as CRISPR can be used to modify the genomes of humans and animals, in 2013, its application in plants was successfully achieved. This breakthrough has opened up many new opportunities for researchers, including the opportunity to gain a better understanding of plant biological systems (Barrangou and Doudna, 2016).

The use of CRISPR/Cas systems covers various applications, from biotic stress tolerance to abiotic stress tolerance, and also includes the achievements of improved yield performance, biofortification and enhancement of plant quality by reducing disease in some crops, while making others more robust. The most important group of target applications relates to yield traits followed by the achievement of biotic or abiotic stress tolerance. Biotic stress tolerance includes induced tolerance to viral, fungal and bacterial diseases. As for abiotic stress tolerance, the two main objectives are to achieve herbicide and natural environmental stress tolerances. Environmental stress includes cold, salt, drought and nitrogen stress. All of these trait improvements are related to economic and agronomic challenges faced by farmers as pathogens, and environmental conditions are important threats that need to be dealt with in agriculture. Furthermore, plant breeders are continually trying to increase yield performances. The most studied crop is rice (*Oryza sativa*) followed by other major crops: maize (*Zea mays*), tomato (*S. lycopersicum*), potato (*Solanumtuberosum*), barley (*Hordeumvulgare*) and wheat (*Triticumaestivum*).

Humans have been improving the yield and disease resistance of crops and to ameliorate the quality and quantity of

nutrition for hundreds of years through traditional agricultural methods based on lucky hits. But now scientists are using CRISPR to perform the genetic modification of food plants, which is known in the world of science as GMOs (genetically modified organisms), as organisms created in the laboratory using genetic engineering techniques. These techniques consist of removing one or more genes from the DNA of another organism, such as a bacterium, virus, animal, or plant, and “recombining” them into the DNA of the plant they want to alter. By adding these new genes, genetic engineers hope the plant will express the traits associated with the genes. For example, genetic engineers have transferred genes from a bacterium known as BT into the DNA of corn. BT genes express a protein that kills insects, and transferring the genes allows the corn to produce its own pesticide (Ricroch et al., 2017).

Editing human embryos

The speed at which CRISPR-based studies can go from hypothesis to result is astounding. Experiments that used to take months now take weeks. And since these experiments have been performed on different species, some have also been applied to early human embryos. In 2017, a report issued by the National Academies of Sciences and Medicine recommended that trials on human embryos can be performed, but under certain conditions, and altering the cells in embryos, eggs and sperm was ethically permissible provided that it was done to correct a disease or a disability, not to enhance a person’s physical appearance or abilities, but to prevent genetic diseases from being passed to future generations (Saey, 2017).

More than 10,000 inherited disorders have been identified, affecting millions of people worldwide. Often there are no cures for these diseases. Correcting

disease-causing genetic defects in human zygotes was previously unthinkable because the efficiency would be too low to be of any practical value. CRISPR/Cas9 offers, for the first time, a tangible potential to allow for the correction of genetic defects (Tang et al., 2017). Among these diseases are autosomal dominant mutations, where inheritance of a single copy of a defective gene can result in clinical symptoms. Genes in which dominant mutations manifest as late-onset adult disorders include *MYBPC3*, in which a mutation causes hypertrophic cardiomyopathy (HCM). Because of their delayed manifestation, these mutations escape natural selection and are often transmitted to the next generation. HCM is a myocardial disease characterized by left ventricular hypertrophy, myofibrillar disarray and myocardial stiffness; it has an estimated prevalence of 1:500 in adults and manifests clinically with heart failure. HCM is the most common cause of sudden death in otherwise healthy young athletes. HCM, while not a uniformly fatal condition, has a tremendous impact on the lives of individuals, including physiological (heart failure and arrhythmias), psychological (limited activity and fear of sudden death), and genealogical concerns. *MYBPC3* mutations account for approximately 40% of all genetic defects causing HCM, and are also responsible for a large fraction of other inherited cardiomyopathies, including dilated cardiomyopathy and left ventricular non-compaction. *MYBPC3* encodes the thick filament-associated cardiac myosin-binding protein C (cMyBP-C), a signalling node in cardiac myocytes that contributes to the maintenance of sarcomeric structure and regulation of both contraction and relaxation. Recent developments in precise genome-editing techniques and their successful applications in animal models have provided an option for correcting human germline mutations.

In particular, CRISPR–Cas9 where it was used in different division phases in a zygote carrying the previous MYBPC3 mutation. The injection of CRISPR–Cas9 during S-phase resulted in mosaic embryos consisting of non-targeted mutant, targeted NHEJ-repaired and targeted HDR-repaired blastomeres (Figure 7). These trials confirmed the efficiency, accuracy and safety of the CRISPR–Cas9 mediated-gene correction, suggesting that it has the potential to be used for the correction of heritable mutations in human embryos. However, much remains to be considered before clinical applications, including the reproducibility of the technique with other heterozygous mutations (Ma et al., 2017).

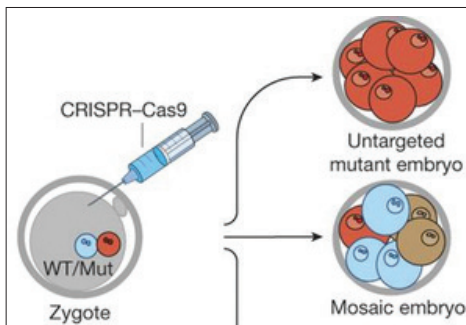


Figure 7. Gene correction in S-phase-injected human embryos (Ma et al., 2017)

Conclusions

CRISPR, this scientific breakthrough, has the ability to eliminate diseases, solve world hunger, and provide unlimited clean energy. It has potentially given us direct access to the source code of life and at the same time has given a great amount of hope to billions of people. The technology's promising ability to deliver is a major reason why so many investors are spending millions of dollars on it. In fact, the sector CRISPR belongs to is experiencing something of a 'gold

rush' due to massive investments, not to mention that about 80% of rare diseases are caused by faulty genes, as stated by the Rare Diseases Organization. These figures alone show what a huge impact the technology would have in our lives if used appropriately and for the right reasons.

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Uporabe CRISPR/Cas9: pregledni članak

Nora MIMOUNE, High School of Veterinary Medicine, Algiers, Algeria; National Institute of Veterinary Sciences, Laboratory of Biotechnology Related to Animal Reproduction (LBRA), University Saad Dahleb, Blida, Algeria; Oumayma BENADJEL, Ratiba BAAZIZI, Djamel KELEF, National Institute of Veterinary Sciences, Laboratory of Biotechnology Related to Animal Reproduction (LBRA), University Saad Dahleb, Blida, Algeria; Rachid KAIDI, Laboratory of Biotechnology Related to Animal Reproduction (LBRA), University Saad Dahleb, Blida, Algeria

Grupirane pravilno razmaknute kratke ponavljajuće palindromske sekvence (CRISPR)/CRISPR-povezani sustav (Cas) je sustav koji osigurava imunost većine prokariotskih organizama na napade virusa i drugih stranih tijela. CRISPR sustavi se sastoje od bjelančevine nalik škarama naziva Cas9 i genetskog GPS vodiča „vodičke RNK“. Međutim, istraživači su preorijentali i prenamijenili iskonski imunostni sustav za preciznu manipulaciju genomom u većini organizama uvodeći dvolančane lomove na DNK na određenim lokacijama genoma radi uvođenja specifičnih izmjena DNK.

Od njegova otkrića CRISPR ima sve više primjena; od onemogućavanja parazita do ispravljanja mutacija i poboljšavanja prinosa u poljoprivredi. Ovo istraživanje provedeno je da bi poslužilo kao vodič za CRISPR tehnologiju, od povijesti njegova otkrića do najnovijih saznanja. Nadamo se da će ova studija osigurati općeniti pregled ove tehnologije koja mijenja živote, a i inspirirati znanstvenike da nastave istraživanja s CRISPR-om, za bolji život.

Ključne riječi: *CRISPR/Cas 9, DNK, RNK, genetski inženjering, virus*