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CRISPR/CAS9 SYSTEM – A TOOL FOR PRECISE GENOME EDITING

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Abstract

One of the aims of modern medicine is to fight diseases via genome editing. In order to find an even more precise and effective way to modify specific genes, scientists have developed a mechanism mediated by bacterial nucleases called the CRISPR/Cas9 system. This system was first discovered in bacteria where it is used to defend the host cell from foreign DNA, i.e. a type of defense against bacteriophages. After the infection, bacteria can develop "immunity" that can be transmitted to bacterial daughter cells via vertical gene transfer. Since it helps bacteria to recognize and fight new infections of analogous origin, the system was characterized as adaptable bacterial immunity. In this review article we describe the original mechanisms of the bacterial CRISPR/Cas9 system, as well as its applications in higher organisms, including mammals, and discuss its advantages in comparison to other genome-engineering platforms. We also present its possible applications in neuroscientific fields, such as creating brain tumour models for neurooncologic studies, as well as its therapeutic potential in treating diseases with a genetic basis, especially psychiatric ones like schizophrenia. In the end, we discuss the ethical dilemmas associated with the usage of this revolutionary genome editing tool.

KEYWORDS: CRISPR/Cas9, ethics, genome editing, neurooncology, nuclease

INTRODUCTION

One of the aims of today's medicine in fighting diseases is the editing of the genome. In order to find the fastest and most effective way to modify specific genes, scientists have developed a mechanism mediated by bacterial nucleases called the CRISPR/Cas9 system.

It was discovered that certain bacterial strains, as well as most of the Archaea members, have evolved a sophisticated adaptive mechanism which is based on loci in the bacterial genome called CRISPR loci (Clustered Regularly Interspaced Short Palindromic Repeat) and on a variety of Cas genes.[1] Bacteria are simple single-cell organisms at constant risk of other micro-organisms trying to "insert" a part of their genetic code into their own. CRISPR/Cas9 is an adaptive mechanism that allows bacterial strains to fight this menace by gaining resistance to foreign DNAs.[2] Since it helps bacteria to recognize and fight new infections, the system was characterized as adaptable bacterial immunity which is reminiscent of the immune system of more complex organisms.[2] In the present article we will describe the original mechanisms of bacterial CRISPR/Cas9 system, as well as the application of the system in higher organisms, including mammals.

THE ORIGIN OF THE CRISPR MECHANISM

The system was discovered in bacteria where it is used to defend the host cell from viral DNA, *i.e.* a type of defense against phages. The system works in those bacteria that survive viral infection. After the infection, bacteria can develop "immunity" that can be transmitted to bacterial daughter cells through incorporation and storage of the

phage genome in the genome of the first infected bacterium (vertical gene transfer). This allows for molecular memory of previous hostile encounters with foreign genetic material. The result is that all new bacterial daughter cells have a built-in part of the viral genome. They are now "protected". "Protected" how? RNA polymerase synthesizes the mRNA in which CRISPR repeats as well as the viral fragments are transcribed. This mRNA will be cut into small RNA fragments called crRNA (today it is still not clear how). Each CRISPR RNA or crRNA will have CRISPR sequences as well as the sequence of the virus genome. The resulting crRNA ensures that the CRISPR/Cas system has a high degree of specificity and is able to selectively target foreign nucleic acid (eg. in a new viral infection), protecting them from threats that the previous generations have encountered.

There are two major components of the CRISPR locus: *direct repeats* and *Cas genes* (short for CRISPR-associated genes). Direct repeats are 21 to 47 bp long nucleotide repeats that are partially palindromic.[1] These repeats are separated, in other words *regularly interspaced,* by sequences of similar length known as the *spacer sequences*. Spacer sequences may originate from transposons, but mostly they are exogenous DNA fragments adopted from "foreign sources" and thus may have many different origins such as plasmids and viruses.[1] Having that in mind, we may ask ourselves, isn't that what the bacterial cell is trying to prevent? The trick is that the spacer sequences are only fragments of the foreign intruder genomes.[1,2] A bacterial cell that survived the intruder's attack, for example a viral infection, will splice the viral genome into small, and therefore harmless, fragments and incorporate them into its own genome.[2,3] They are not incorporated in a random place, but into a specific locus, the CRISPR locus.[2,3] After this incorporation, they become the aforementioned *spacer sequences*.

Once viral genome fragments became part of a CRISPR locus, they are transcribed together with the whole CRISPR array as a single mRNA molecule.[1] However, the mRNA-processing following transcription will result in the formation of multiple smaller CRISPR-derived transcripts called *crRNA*s.[1,3] For that reason, the long precursor mRNA is also known as the CRISPR precursor RNA or pre-crRNA.[3] crRNAs contain *protospacer regions* named after the fact that they are derived from, and are therefore complementary to, the *spacer sequences* of the CRISPR-array. They are also complementary to the corresponding parts of the specific foreign DNA. Due to this site-specific complementarity they serve as guides to invading foreign DNAs.[1] crRNAs function as a signal that allows an enzyme, a nuclease intended to disable foreign nucleic acid, to recognize its target. There are many Cas9 enzymes, stemming from different bacteria, but the most commonly used is the SPyCas9 enzyme, derived from Streptococcus pyogenes. Every Cas9 enzyme subtype recognizes its specific PAM (protospacer adjacent motif sequence) sequence, a 2-6 nucleotide regulatory sequence found downstream of the DNA target site, which activates the Cas9 endonuclease when recognized.[4]

However, crRNA is not able to direct the nuclease on its own. There is another molecule involved that assists in this process of crRNA and Cas protein collaboration. It is called the trans-activating RNA (tracrRNA) and consists of a short sequence partially complementary to the corresponding crRNA.[1,2] It binds to the crRNA serving as some sort of "bridge" between

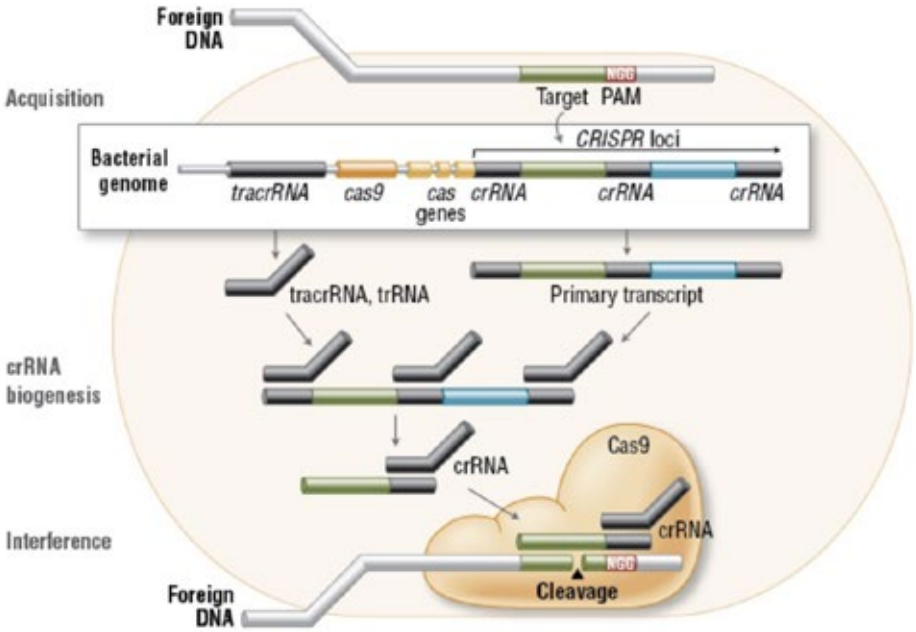

Figure 1. CRISPR/Cas9 mechanism in vivo.
Source: Reis A, Hornblower B, Robb B, Tzertzinis G. CRISPR/Cas9 and Targeted Genome Editing: A New Era in Molecular Biology. NEB expressions. 2014;(1):3-6.

crRNA and the Cas protein. TracrRNAs are also encoded by the CRISPR system. As soon as the crRNA-tracrRNA-Cas complex is made and the foreign DNA is identified, the nuclease creates a double-strand break (DSB) in the foreign genetic material leading to foreign-DNA-silencing.[1]

To sum up the above described mechanisms, naturally occurring CRISPR systems incorporate foreign DNA sequences into CRISPR arrays, which then produce crRNAs bearing "protospacer" regions that are complementary to the foreign DNA sequences. crRNAs hybridize to tracrRNAs and this pair of RNAs can associate with the Cas9 nuclease. crRNA-tracrRNA-Cas9 complexes recognize and cleave foreign DNAs (Figure 1).

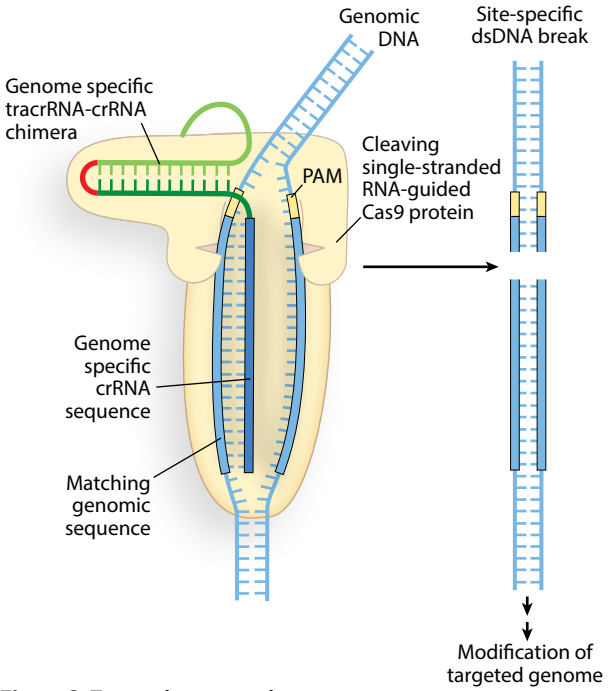

Figure 2. Targeted genome editing. *Image by H. Adam Steinberg, artforscience.com.*

Using CRISPR/Cas9 mechanisms, the bacterial cell creates some sort of a "database" containing information which enables them to recognize threats they have already encountered. Searching for a contemporary analogy in our everyday lives, it could be illustrated by the databases used by antiviral programs on our computers that are continuously being updated and contain information regarding most of currently known malicious software, enabling the antiviral programs to recognize their activity in time to react, before serious damage has been made. Apart from the incredibly precise identification, it also encloses the information for the effector-complex synthesis that disables invading DNA immediately after recognition.

It may seem that bacteria and Archaea kingdom members have won the battle against viruses, but the war continues. Certain bacteriophages can overcome CRISPR interference by different mechanisms, such as indel accumulations throughout their genome; acquiring single mutations around or in the targeted sequence and recombination in case of multiple-variation viral invasion.[3] However, bacteria have provided us with a valuable tool that we already vastly apply in many different areas of gene editing.

THE PRINCIPLE OF USING CRISPR/CAS9 IN GENE EDITING

A paper published in 2012 by Jennifer Doudna and Emanuelle Charpentier is considered to be of paramount importance in starting the so-called CRISPR revolution. It highlighted the potential of this bacterial acquired immune system in genome editing.[2] The CRISPR/Cas9 mechanism, based on the previously described processes, can be divided into 3 phases: adaptation, expression and interference. The adaptation phase is characterized by integration of spacer elements into CRISPR loci. They are then transcribed into precursor crRNAs and spliced into smaller fragments known as crRNAs, during the expression phase. Production of a double-strand break in the exogenous DNA is mediated by the tracrRNA, by binding to the crRNA with a sequence partially complementary to it on one end, and a 'docking site' for the Cas9 enzyme on the other end.[4,5]

The idea of using CRISPR/Cas9 in gene editing is simple. By producing a crRNA nucleotide sequence complementary to our region of interest, along with a matching tracrRNA, it is possible to create tailor-made double-strand breaks via Cas9 endonuclease activity. The crRNA and tracrRNA are introduced either separately, to form a duplex, or engineered into a single-guide RNA (sgRNA or gRNA) molecule where two RNAs are bound by a linker loop. Due to its origin from two gene loci, it is also referred to as chimeric RNA.[6] The most widely used CRISPR-Cas system utilizes a fusion between a crRNA and part of the tracrRNA sequence. These single gRNAs form complexes with Cas9 and mediate cleavage of target DNA sites that are complementary to the 20 nucleotides 5′ (upstream) of the gRNA and that lie next to a sequence called PAM (protospacer adjacent motif sequence). Such RNA designed according to our wishes specifically mediates the cutting of the target locus thus forming double stranded breaks. Such breaks will activate the mechanisms of homologous recombination repair. It should be emphasized that homologous recombination is the naturally occurring cellular repair mechanism of double strand breaks. This type of genetic recombination is initiated by a fracture of both strands of the DNA molecule and involves physical changes in the DNA duplex material and substitution of homologous DNA sequences. It is so precise that a single base pair will not be lost or added. In that way these intrinsic cellular mechanisms are used as a tool for precise gene modification, i.e. corrections (Figure 2).

An important paper was published in the journal Cell in which the CRISPR/Cas9 system was applied to target monkey genomes.[7] By coinjection of Cas9 mRNA and sgRNAs into one-cell-stage embryos, precise gene targeting in cynomolgus monkeys was achieved. Two target genes, *Ppar-g* and *Rag1*, were also disrupted using this system.

Cell type or organism	Cas9 form	Cell type	Reference numbers
Human cells	Cas9 nuclease	HEK293FT, HEK293T, HEK293, K562, iPSC, HUES9, HUES1, BJ-RiPS, HeLa, Jurkat, U2OS	9,13–16,47, 49–51,54,59, 84,85
	Cas9 nickase	HEK293FT, HEK293T	13,14,47,49
	dCas9 (gene regulation)	HEK293FT, HEK293T	70–72,74,82
	dCas9 (imaging)	HEK293T, UMUC3, HeLa	81
Mouse or mouse cells	Cas9 nuclease	Embryos	14,24–26
	Cas9 nickase	Embryos	47
	dCas9 (gene regulation)	NIH3T3	74
Rat	Cas9 nuclease	Embryos	26,36
Rabbit	Cas9 nuclease	Embryos	27
Frog	Cas9 nuclease	Embryos	28
Zebrafish	Cas9 nuclease	Embryos	17,33,37,60,85
Fruit fly	Cas9 nuclease	Embryos	29,30,61
Silkworm	Cas9 nuclease	Embryos	31
Roundworm	Cas9 nuclease	Adult gonads	32,62–67
Rice	Cas9 nuclease	Protoplasts, callus cells	21,23
Wheat	Cas9 nuclease	Protoplasts	21
Sorghum	Cas9 nuclease	Embryos	23
Tobacco	Cas9 nuclease	Protoplasts, leaf tissue	19,20,23
Thale cress	Cas9 nuclease	Protoplasts, seedlings	19,23
Yeast	Cas9 nuclease	*Saccharomyces cerevisiae*	18
Bacteria	Cas9 nuclease	*Streptococcus pneumoniae, E. coli*	8
	dCas9 (gene regulation)	*E. coli*	69,70

HEK, human embryonic kidney; iPSCs, induced pluripotent stem cells; UMUC3, human bladder cancer.

Table 1. Published examples of organisms and cell types modified by Cas9.
Source: Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol. 2014;32(4):347-355. doi:10.1038/nbt.2842.

Another ethically controversial paper was published in Protein Cell in 2015, describing the use of CRISPR/Cas9 technology in human pre-implantation embryos where it effectively cleaved the endogenous β-globin gene (*HBB*).[8]

There are two mechanisms of recombination DNA repair: non-homologous end joining (NHEJ) and homology-directed repair (HDR). Non-homologous end joining is an error-prone mechanism of DNA repair that relies on microhomology of single-stranded overhangs, which may or may not be compatible, to determine the precision of repair. Therefore it can result in insertions or deletions (commonly known as indels). Contrary to this, homology-directed repair uses a template, either a sister chromatid, or an exogenous complementary DNA to repair double-strand breaks. It is useful in gene editing because it enables the insertion of entire genes. The cell's DNA is cut via CRISPR/Cas9 at a precise location, and if a donor DNA molecule is simultaneously introduced, carrying sequences homologous to upstream and downstream regions of the cut ('right and left homology arms'), it serves as a template for homology-directed repair.[9] HDR mostly occurs during the G2 and S phases of the cell cycle, thereby being relatively ineffective in non-mitotic cells. There are a couple of new approaches which try to overcome mitotic inactivity of target cells.[10]

Although highly specific, it is possible for the Cas9 enzyme to exert mutagenic effects away from the designated target. There are reports on the off-target activity of Cas9, which needs to be disabled for obvious reasons. Various strategies are being developed to disable off-target activity, paving the way for safe clinical application of this method in the future. One is to produce nickases, by inactivating one of Cas9's two domains: HNH or RuvC. Consequently, the enzyme is unable to produce double-strand breaks, and instead produces only single-strand breaks. Another approach is to add two gRNAs, in both sense and antisense directions; in this manner highly specific double-strand breaks will be produced. Off-target single-strand breaks can occur, but are much easier to repair as opposed to double-strand breaks.

Since the first paper on CRISPR/Cas9 appeared in 2012. it has been shown in a great number of publications that the system could be programmed to cut various DNA sites in a variety of cells and organisms (Table 1). It has successfully been used in cultured transformed human cancer cell lines and human pluripotent stem cells. It has also been used in bacteria, zebrafish, yeast, tobacco, rice, wheat, mice, rats, rabbits, frogs, fruit flies, etc.[11]

EX VIVO VS. *IN VIVO* DELIVERY OF CRISPR/CAS9 SYSTEM

It is possible to deliver the CRISPR/Cas9 system to cells both *ex vivo* and *in vivo*. *Ex vivo* delivery is used for creating animal models of disease in order to study gene functions.

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Table 2. Methodical comparison of the ZFN, TALEN and CRISPR/Cas9 genome-engineering platforms.
Source: Eid A, Mahfouz MM. Genome editing: the road of CRISPR/Cas9 from bench to clinic. Exp Mol Med. 2016;48(10):e265. doi:10.1038/emm.2016.111.

	ZFN	*TALEN*	*CRISPR/Cas9*
Construction	Protein engineering for every single target	Protein engineering for every single target	20-Nucleotide sequence of sgRNA
Targeting	Protein–DNA interaction, less predictable	Protein–DNA interaction, less predictable	DNA–RNA interactions, highly predictable
Delivery	Two ZFNs around the target sequence are required	Two TALENs around the target sequence are required	sgRNA complementary to the target sequence with Cas9 protein
Multiplexing	Challenging	Challenging	Highly feasible
Feasibility of library construction and transformation for genome-wide screens	Technically challenging	Technically challenging	Highly feasible
Affordability	Resource intensive and time consuming	Affordable but time consuming	Highly affordable

Abbreviations: CRISPR/Cas9, clustered regularly interspaced palindromic repeats/CRISPR-associated-9; sgRNA, single-guide RNA; TALEN, transcription activator-like effector nuclease; ZFN, zinc-finger nuclease.

When introduced into a zygote, CRISPR/Cas9 is able to rapidly produce multiple mutations at once, an achievement formerly accomplished only by cross-breeding of numerous single-mutant generations. A downside of this method is its relatively low efficiency, and potential for mosaicism occurrence. Multiple strategies are being developed in order to ameliorate these pitfalls.[7,12] Another example of *ex vivo* delivery is a currently ongoing trial on people with lung cancer, in which peripheral blood lymphocytes are taken from patients, and CRISPR/Cas9 is used to knock out a gene called Programmed cell death protein 1 (PDCD 1). Afterwards, selected lymphocytes are infused back into patients. This is the first clinical trial where CRISPR/Cas9 is used.[13] *Ex vivo* manipulation of human zygotes is currently an area of great ethical dilemmas.

In vivo delivery is a promising therapeutical strategy for many Mendelian, as well as non-Mendelian diseases. There are viral and non-viral delivery systems available. Viral delivery systems are used due to their high efficiency. On the basis of integration into host chromatin they are divided into either integrating (retroviruses, lentiviruses) or non-integrating, persisting viruses (adenoviruses, adeno-associated viruses (AAV)). Non-viral delivery methods use nanoparticles: cationic nanocarriers, liposomes and polymeric materials. They are a safer option, easier to produce, but it is more difficult for synthetic particles to overcome the cell's membranous barriers.[14]

CRISPR/CAS9 VS. OTHER GENE EDITING TECHNIQUES

Before the invention of CRISPR/Cas9, two older programmable nuclease methods were used for genome engineering: zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). The ZFN system consists of two components: zinc finger proteins, responsible for DNA sequence recognition, and FokI DNA cleavage domains, with restriction endonuclease activity. Although highly specific, their engineering is more difficult than CRISPR/Cas9 construction and requires substantial technical knowledge.[14] TALENs consist of two components as well: the same FokI nuclease as ZFNs, and a DNA binding domain known as transcription activator-like effector (TALE) domain. The TALE domain consists of 33-35 amino acid repeat domains, each recognizing a single base pair. Although TALENs are easier to construct than ZFNs, they are significantly larger, thereby impeding their delivery to target cells. Their prokaryotic origin can also induce an immune response.[14,15]

Due to its RNA-DNA interaction, as opposed to protein-DNA interactions occurring in ZFNs and TALENs, CRISPR/Cas9 has a more predictable effect and is easier to use for targeting of multiple loci, by introducing multiple gRNAs. This is more difficult to achieve with the two aforementioned techniques. CRISPR/Cas9 is also more affordable than ZFNs and a less time consuming technique than TALEN (Table 2).[16]

APPLICATIONS OF CRISPR/CAS9 IN DISEASES OF THE CENTRAL NERVOUS SYSTEM

CRISPR/Cas9 can be applied for therapeutic purposes in several ways: it can correct causal mutations in monogenic diseases, it can alter the genomes of pathogens (e.g., HIV) or it can induce a protective role of the gene in the host tissues. CRISPR/Cas9 has also shown potential use in cancer gene therapy by specifically targeting and inactivating oncogenes or activating tumour suppressor genes. The senescent, non-dividing nature of most neurons presents a challenge in gene editing via HDR, and the aforementioned strategies are being used to bypass this obstacle. The blood-brain barrier is another problem in delivery of the system.

Since our audience is mostly interested in the central nervous system (CNS), we decided to briefly mention the

use of CRISPR/Cas9 in two CNS diseases which can be potentially cured using this technique. Schizophrenia is a genetically related psychiatric disorder characterized by abnormal social behaviour. Among other things, it is associated with an abundance of regulatory non-coding RNAs. Since psychiatric disorders are difficult to study via animal models of disease, this area of research has potential in editing these ncRNAs via CRISPR/Cas9 in humans, once it becomes cleared for clinical application.[17]

Neurooncology is another area which could benefit greatly from the arrival of the CRISPR/Cas9 technique. As was previously mentioned, cancer models are difficult to construct due to the multitude of concomitant mutations. CRISPR/Cas9 offers the ability of multiplexing mutations, and thereby presents endless possibilities in brain tumour modelling. Zuckermann et al. have demonstrated this by producing models of medulloblastoma and glioblastoma by deleting a single gene (*Ptch1*) or multiple genes (*Trp53, Pten, Nf1*). This method provides an opportunity to study brain tumours, allowing more specific investigations *in vitro* and greater potential in translation to the clinic.[18]

ETHICAL CONSIDERATIONS

CRISPR/Cas9 is a revolutionary genome editing tool, with practically unlimited potential. Its biggest limits will not be determined by its methodological aspects, but rather by the ethical threats it poses. A moratorium for research involving the human germline and its clinical application was held in Washington in 2015, named the International Summit on Human Gene Editing. Shortly after, 2 articles were published describing the application of CRISPR/Cas9 on human tripronuclear embryos, sparking great controversy.[19] At the meeting it was concluded that basic and clinical research will continue with the appropriate legal and ethical guidelines. They emphasized the distinction between the clinical use of CRISPR/Cas9 methodology in somatic cells, where the effects of a correction are limited to one person, opposed to the use of technique in the germ cells, where repair of the genome would be inherited by future generations. Modification and repair of stem cells would have extensive and unintended consequences on human evolution from both a genetic perspective (interaction of genes and the environment) and a cultural perspective (social Darwinism). Therefore the modification of gametocytes and embryos for the purpose of obtaining human hereditary changes was declared ineligible. There is still an ongoing debate about whether the findings from this type of research outweigh the moral and ethical dilemmas. Nevertheless, CRISPR/Cas9 meticulousness has made it one of the most desirable tools in scientific experiments requiring genetic modifications, as well as in ones in quest of its clinical applications.

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SUSTAV CRISPR/CAS9 – ALAT ZA PRECIZNO UREĐIVANJE GENOMA

Sažetak

Jedna od težnji današnje medicine jest mogućnost preinake genoma kao oruđa za borbu protiv bolesti. U želji da pronađu još precizniji i učinkovitiji alat za modifikaciju gena, znanstvenici su razvili mehanizam za uređivanje genoma posredovan bakterijskim nukleazama, nazvan sustavom CRISPR/Cas9. Sustav je prvotno otkriven u bakterija koje ga koriste u obrani stanice domaćina od strane DNA, primjerice prilikom borbe protiv bakteriofaga. Po infekciji, bakterijska stanica razvija „imunitet" koji može prenijeti na stanice kćeri. Na taj je način bakteriji omogućeno pravovremeno prepoznavanje te obrana od novih infekcija analognog podrijetla, zbog čega se taj sustav smatra mehanizmom adaptabilne bakterijske imunosti. U ovom preglednom radu opisan je izvorni princip djelovanja CRISPR/Cas9 sustava u bakterija, ali i njegova primjena u viših organizama, uključujući sisavce. Uz to donosimo i prikaz njegovih prednosti u usporedbi s drugim platformama za modifikaciju genoma. Također, opisujemo i mogućnosti njegove primjene u neuroznanostvenim područjima, kao što je kreiranje modela tumora za neuro-onkološke studije, kao i njegov potencijal u liječenju poremećaja i bolesti s genetskom predispozicijom, primjerice psihijatrijskih entiteta poput shizofrenije. Naposljetku, razmatramo neke od etičkih dilema povezanih s upotrebom tog revolucionarnog alata za uređivanje genoma.

KLJUČNE RIJEČI: CRISPR/Cas9, etika, uređivanje genoma, neuroonkologija, nukleaza

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