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CRISPR/Cas9 technology for targeted genome editing

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CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) are the segments of prokaryotic DNA containing short repeats in its nucleotide sequence. Today we know that this is a bacterial protection system against viral DNA. The molecular components of CRISPR/Cas9 system have been used for a gene editing in eukaryotes since 2013. But as any other method it also has the limitations and drawbacks. Here we are going to review the history of CRISPR biology and to discuss the possibilities that this new technology provides to researchers as well as the prospects for its use in the medical research and treatment.

K e y w o r d s: CRISPR/Cas9, genome targeting, genome editing, personalized therapy, chromosomal translocations, DNA repair.

History

The specific sequences in bacterial genomes, called CRISPR, were discovered in 1987. They consist of repeats, alternating by unique sequences or «spacers» [1] (Fig. 1, *A*). Its function has been unsolved until 2005 when it was found that these «spacers» have a sequence equal to a viral DNA. It was also observed that there are the CRISPR-associated genes and these genes encode nucleases. Afterwards it was supposed that the bacteria use these nucleases to cleave a foreign DNA wherein the spacer sequence is used as an example of which exactly DNA sequence is necessary to be cut [2, 3]. This hypothesis was experimentally proven in 2007 [4]. A detailed mechanism of the complex maturation and directed cleavage of DNA was finally studied in 2011 [5] (Fig. 1, *B*). It was found that the Cas9 enzyme formed a ternary complex with crRNA and tracrRNA encoded in the cluster. This complex unwinds DNA and introduce a double-stranded break in DNA if crRNA binds complementary to one of the DNA chains (Fig. 2, *A*). A year later it was shown that crRNA can be joint to tracrRNA and that this «single guide» RNA (sgRNA) together with the Cas9 enzyme might be used to modify the genome of eukaryotes [6] (Fig. 2, *B*). This idea was realized in 2013 and since then thousands of papers where such technology was applied have been published [7–9].

The approaches to targeted DNA breakage based on the usage of zinc finger nucleases (ZFNs) and TAL effector nucleases (TALENs) were developed much earlier in 2003 and 2009 respectively. Both methods are based on the DNA recognition by the specific proteins designed individually for each par-

ticular sequence [10, 11]. Compared to the these technologies the CRISPR/Cas9 system has proved to be much more simple, available, affordable and three to four times more efficient [12].

The basic principle of CRISPR/Cas9 technology

At the heart of all the methods that use the CRISPR/Cas9 system lies the possibility to introduce a break in DNA in an exactly desired locus. It's enough to deliver the Cas9 gene (or its mRNA) together with sgRNA into the cell, wherein sgRNA at its 5'-end should have 20 nucleotide sequence complimentary
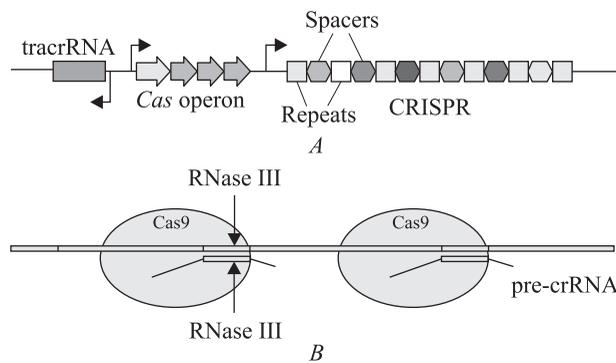
Fig. 1. *A* – The overall structure of the CRISPR loci. *B* – Mechanism of CRISPR/Cas9 complex maturation
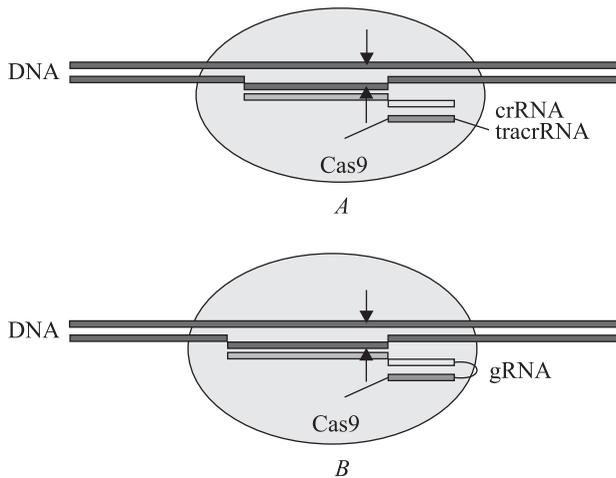
Fig. 2. *A* – Targeted cleavage of DNA by a ternary complex of Cas9 with crRNA and tracrRNA. *B* – Targeted cleavage of DNA by a complex of Cas9 with «single guide» RNA (sgRNA)

to a target DNA fragment. There is also easy way to introduce the multiple breaks at the same time – just to use several different sgRNAs [13].

Application of CRISPR/Cas9 technology

The first example of application of the CRISPR/Cas9 technology is the modeling of tumor-associated chromosomal translocations: CRISPR/Cas9 induces the double-stranded DNA breaks (DSBs) in the precise loci which are known to participate in the oncogenic rearrangements. Typically, DSBs used to be repaired by the mechanism of non-homologous end joining (NHEJ). This error prone mechanism frequently leads to joining the DNA ends belonging to different chromosomes [14]. The cellular models of certain types of cancer might be obtained using this method. Inducing the human specific types of cancer in laboratory animals is also possible. So, the murine model of non-small-cell lung cancer (NSCLC) was developed. Using lentiviral delivery the *Cas9* gene and sgRNA were brought into lung cells in order to induce a cleavage of the murine endogenous *Eml4* and *Alk* loci and to generate the *Eml4-Alk* fused gene found recurrently in NSCLCs. The result was the development of the disease in the treated mice [15]. To date the murine *in vivo* models of human leukemia (namely, AML) have also been developed [16, 17]. These and other similar murine models can be utilized to study the carcinogenesis and to test new treatment approaches.

The next purpose which might be successfully achieved by application of the CRISPR/Cas9 technology is knocking genes out. The DSB formation often leads to the deletion or insertion of several nucleotides in an initial sequence even in case of efficient DSB repair [18]. Thus, introducing a break into a particular gene one may cause its knockout. So the *Cas9*-expressing cell line treated with various sgRNA can be used for searching for the genes responsible for a specific disease. A few years earlier each gene knockout was a complicated time-consuming procedure, but in case of CRISPR/Cas9 it requires only to treat the *Cas9*-expressing cells with a specific sgRNA [19].

In addition, one may insert the desired DNA fragment into the specific broken DNA site. There are

two major mechanisms of DSB repair: homology-directed repair (HDR) and non-homologous end joining (NHEJ). HDR requires a fragment of DNA homologous to the damaged one as a template. Otherwise, in the absence of such a fragment, NHEJ takes place. It is possible to achieve insertion of a desired DNA fragment in both cases. If a DNA fragment with homology arms surrounding a desired gene is available, it will be inserted via HDR. In case of the NHEJ repair it is sufficient to deliver a linear DNA fragment with the ends protected from the action of cellular exonucleases. There is a chance that this fragment will be joined to the ends of the break.

So, the CRISPR/Cas9 technology makes possible the insertion of the desired DNA fragment into the genome. It is widely used in scientific research and offers great opportunities for the treatment of hereditary diseases and viral infections. For example, this technology allows generation of the stable cell lines with the desired inserts in the genome. The CRISPR/Cas9 gene knocking occurs with a much higher efficiency than the random integration of a plasmid into the genome and solely at the particular pre-selected site in almost 100 % of cases. Until recently the generation of a stable cell line usually took at least few months, with the CRISPR/Cas9 technology – up to one month [20].

Moreover, until recently, the gene therapy implied the insertion of the desired gene into the random place in the genome. In its turn, the CRISPR/Cas9 system allows changes in a certain place, where one may not only insert the gene but also remove or replace the certain DNA fragments. Several *in vivo* studies in mice have shown the possibility of gene therapy by means of the CRISPR/Cas9 application. A deletion of 1 bp in the *CRYGC* gene is known to cause the cataract in mice. After injection of the Cas9 mRNA, sgRNA and the DNA fragment containing a wild-type allele into the mouse embryos with this mutation the mousekins were born healthy [21]. In a recent work the researchers have successfully applied the CRISPR/Cas9 system in order to reduce the level of cholesterol in the blood of mice by turning off the *PCSK9* gene in the liver cells. They used adenovirus particles to deliver the *Cas9* gene and sgRNA into hepatocytes. As a result more than 50 % of hepatocytes were «edited»

and it resulted in a decrease in the cholesterol level to a value that is typical for the *PCSK9* knockout mice [22]. Striking results were achieved with CRISPR/Cas9 in the prevention of Duchene's muscular dystrophy (DMD) in mice by correcting the *dmd* dystrophin gene. A copy of the normal *dmd* gene was integrated in the zygotes via the CRISPR/Cas9 system. The born animals were mosaic in the *dmd* gene but nonetheless even a partial gene editing has prevented the disease development [23].

The ability to deliver the CRISPR/Cas9 system elements into the brain cells has been also recently demonstrated. Because of the packaging size limitation of the adeno-associated viral (AAV) vectors, the dual-vector system was designed composed of two separate vectors that package the SpCas9 (AAV-SpCas9) and sgRNA (AAV-SpGuide) expression cassettes. However, the co-transfection efficiency was shown to be rather high [19].

Taken together, these *in vivo* studies on animals prove the excellent prospects for the genome editing via application of the CRISPR/Cas9 technology.

On the other hand, many studies *ex vivo* show the ability to edit DNA in human cells. The particular mutations in the *CCR5* gene which occur in some people are known to make it resistant to HIV. A recent study has established that the CRISPR/Cas9-based editing of *CCR5* in the induced pluripotent stem cells (iPSCs) may lead to the HIV-1 resistance of descendant lymphocytes [12].

The possibility to treat the latent virus infection has also been recently demonstrated: the patient-derived cells from a Burkitt's lymphoma with latent Epstein–Barr virus infection showed a proliferation arrest and a concomitant decrease in viral load after targeting the viral genome by the CRISPR/Cas9 system elements [24]. Finally, the healthy gene copy was introduced in cells of the patients with cystic fibrosis via CRISPR/Cas9. As a result the cells demonstrated a healthy phenotype [25].

Taken together with the fact demonstrating that the «*in vitro* corrected» organs might be successfully transplanted back into the body in mice [26], the above experiments give us a hope for the successful application of this experience in the treatment of pa-

tients. This considerably strengthens the position of gene therapy among the advanced treatments of the genetic and viral diseases in humans.

An especial theme is the editing of the human embryo genome. Such possibility opens up broad prospects for the prenatal correction of the «broken» genes. The researchers from Sun Yat-sen University, China, have already tested this potential. They used triplonuclear zygotes (for ethical reasons) and replaced the *HBB* gene using the CRISPR/Cas9 technique. As a result it was shown that: 1) the effectiveness of the replacement of a normal gene copy by means of HDR in these cells was relatively low; 2) the edited embryos were mosaic; 3) the mutations in the genome located in not-planned loci was identified (so-called «off-target» mutations); 4) another highly homologous gene (*HBD*) could serve as a donor template for HDR. However, despite the difficulties this study shows the fundamental possibility of the genome editing in human embryos [27].

Drawbacks of CRISPR/Cas9 technology

The common problem of all genome editing methods is so called «off-target» effects – when a nuclease cleaves DNA not in the place where it was intended. It may impair the function of any incidental gene or may lead to integration of the desired gene in the wrong place and genome surrounding. In case of human embryos such a risk is absolutely unacceptable. In the abovementioned work with the human embryos several off-target mutations have been found and documented, but later the authors noticed that three out of four of them were polymorphisms already presented in the germline and were not associated with the CRISPR/Cas9 action. The special studies show that the level of off-target mutations in case of the CRISPR/Cas9 application is in fact extremely low [28]. Today, several methods for measuring the off-target editing have been developed. The first method is based on the fluorescent *in situ* hybridization (FISH): one fluorescent probe anneals to the insertion fragment and another fluorescent probe detects the insertion site. Normally these two probes have to be colocalized otherwise it is the erroneous insertion [29]. Other method (GUIDE-seq) is based

on the addition of short DNA fragments which would be integrated at the breaks. These fragments of DNA are then used as anchors when surrounding DNA is amplified by PCR and sequenced. So one we can see all sites of insertion of these fragments and count off-target mutations [30]. These evaluation methods have shown that not all of the off-target sites might be predicted by special computer software and that the different sgRNA provides a different number of these effects.

The various methods to improve the accuracy and efficiency of insertion were proposed. For example, in order to obtain a higher specificity a «double nicking» method might be used [31]. It consists in special modification of Cas9 resulting in an ability of the enzyme to introduce break in only one DNA strand («nick»). Thus, for the formation of a double-stranded break two such nickases should work vis-a-vis. Furthermore, each of the enzymes has to bind its own sgRNA which should recognize its own (mutually complementary) DNA sequence. Thus, the probability of double-stranded breakage in the off-target sites reduces dramatically.

In order to increase the efficiency of the insertion in the genome the HDR mechanism should prevail. To achieve this goal the NHEJ-inhibiting agents might be used. In particular DNA ligase IV which plays an important role in NHEJ might be turned off by using the Scr7 agent. This may increase the efficacy of DNA integration up to 19 times [32].

The selection of sgRNA sequences should be performed in such a way to reduce the probability of the off-target effects. The special algorithms have been developed for this purpose (CRISPR Design Tool: http://tools.genome-engineering.org). If there is need for especial accuracy it is necessary to check a number of the off-target sites by applying the GUIDE-seq technique.

Finally, the integration of new genes requires a special attention when selecting the integration site. Such target should be a locus where the inserted DNA fragment would be «protected» against the epigenetic effects, would work in a predictable manner and would not cause negative effects on a cell, for example, induce carcinogenesis. Such loci are called

«genomic safe harbors» (GSHs). They should be located far from other genes especially protooncogenes and at the same time should be outside the transcription units and the ultra-conserved DNA regions. The sites suitable as GSHs might be identified by the bioinformatics methods but also require experimental confirmation [33].

Conclusion

So far we can say with confidence that the CRISPR/Cas9-based technologies have revolutionized the biomedical science and are spreading at a remarkable speed by virtue of their simplicity and efficiency. The CRISPR/Cas9 application dramatically facilitates the manipulation with DNA in terms of providing the targeted genome editing, foremost deletions or gene replacement. Despite some drawbacks this technology has a wide perspective in the fields of basic research, biotechnology, translational and personalized medicine.

Acknowledgments

Competing interests

The authors declare no competing interests.

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CRISPR/Cas9 технологія
для цільового редагування геному

Н. А. Ломов, В. В. Борунова, М. А. Рубцов

CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) – послідовності в геномі прокариот, які складаються з коротких повторів, що перемежовуються унікальними послідовностями. Це система бактеріальної захисту від вірусної ДНК. Молекулярні компоненти даної системи з 2013 року використовуються як інструмент редагування еукаріотічесого геному, хоча дана технологія і має деякі обмеження і недоліки. У даному огляді ми торкнемося історію застосування системи CRISPR / Cas9 і обговоримо можливості, які дана технологія надає для дослідження і лікування різних захворювань.

Ключевые слова: CRISPR/Cas9, тагретінг генома, редагування геному, персоналізована терапія, хромосомні транслокації, репарація ДНК.

CRISPR/Cas9 технология
для целевого редактирования генома

Н. А. Ломов, В. В. Борунова, М. А. Рубцов

CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) – последовательности в геноме прокариот, которые состоят из коротких повторов, перемежающихся уникальными последовательностями. Это система бактериальной защиты от вирусной ДНК. Молекулярные компоненты данной системы с 2013 года используются как инструмент редактирования эукариотического генома, хотя данная технология и имеет некоторые ограничения и недостатки. В данном обзоре мы затронем историю применения системы CRISPR/Cas9 и обсудим возможности, которые данная технология предоставляет для исследования и лечения различных заболеваний.

Ключевые слова: CRISPR/Cas9, таргетинг генома, редактирование генома, персонализированая терапия, хромосоные транслокации, репарация ДНК.