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PROSPECTS OF GENOME EDITING USING CRISPR/CAS OR HOW TO MASTER GENETIC SCISSORS. NOBEL PRIZE IN CHEMISTRY 2020

S. V. KOMISARENKO, S. I. ROMANIUK

Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kyiv;
e-mail: svk@biochem.kiev.ua

The Nobel Prize in Chemistry 2020 was awarded to two researchers in the field of molecular biology - the French Emmanuelle Charpentier, who now heads the Max Planck Unit for the Science of Pathogens in Berlin, and the American Jennifer Doudna from the University of California, Berkeley - for the 'development of genome editing method'. A press release of the Nobel Committee states that the winners discovered one of the most powerful genetic technology tools - CRISPR/Cas9, or so-called 'genetic scissors'. This method has contributed to many important results of basic research. In particular, plant researchers have managed to create crops resistant to mold, pests and drought. As for medicine, clinical trials of new cancer treatment techniques are underway, and a dream of curing hereditary diseases is about to become a reality. Genetic scissors have brought the life sciences to a new stage of development and greatly contributed to the benefit of mankind.

K e y w o r d s: Nobel Prize in Chemistry, Emmanuelle Charpentier, Jennifer Doudna, genome editing, CRISPR/Cas9.

On October 7, 2020, in Stockholm, as part of the 119-th Nobel Week, the Nobel Committee with the Karolinska Institute of Medicine announced the names of the laureates of the Nobel Prize in Chemistry. Traditionally, on the eve of Nobel Week, the Clarivate Analytics had published a list of the most likely contenders for this award, made by analyzing the number of citations [1].

Three scientists who had made significant contributions to the study of nanocrystals, including the synthesis of nanocrystals with certain properties for a wide range of applications in physical, biological and medical systems, appeared at the top of the ranking. Thus, Taeghwan Hyeon from the National University of Seoul (Korea) invented a new method of producing transition metals` nanocrystals that can be used, for example, as a contrast agent for magnetic resonance imaging. Moungi G. Bawendi from the Massachusetts Institute of Technology (USA) specialized in quantum dots, microscopic semiconductors with special spectroscopic properties. And finally, Christopher B. Murray of the University of Pennsylvania (USA) dealt with improvement of nanocrystals` properties, including enhancement of their conductivity.

The award was also predicted for two American scientists: John F. Hartwig of the University of California, Berkeley, and Stephen L. Buchwald of the Massachusetts Institute of Technology, who discovered a Buchwald-Hartwig amination reaction, which results in the formation of carbon-nitrogen bonds following the reaction of amines binding palladium-catalyzed aryl halides. This method has proven to be very useful for synthesis of many natural alkaloids in laboratory settings.

Makoto Fujita of the University of Tokyo (Japan), a specialist in supramolecular chemistry of complex compounds, was considered the third con-

tender for the Nobel Prize in Chemistry 2020. In particular, he had investigated the self-assembly of three-dimensional structures of organic molecules bonded by metal atoms, which can serve as 'molecular containers' for other substances.

Lately, "classical" chemists have complained that the Nobel Prize in Chemistry has been increasingly awarded for research at the intersection of traditional biological disciplines, for example to scientists working in the fields of biochemistry, molecular biology and immunology. This has happened this year as well. The winners of the 112-th Nobel Prize in Chemistry became two researchers working in the field of molecular biology: Frenchwoman Emmanuelle M. Charpentier of the Max Planck Unit for the Science of Pathogens in Berlin and American Jennifer A. Doudna of the University of California in Berkeley. The Secretary-General of the Swedish Royal Academy of Sciences, Göran K. Hansson, announced the substantiation of the award: the scientists were awarded this prestigious award for 'developing a method of genome editing.' According to the official press release, the winners 'have discovered one of the most powerful tools of genetic technology – the 'genetic scissors' CRISPR/Cas9. Using them, researchers can change the DNA of animals, plants, microorganisms and other living beings quite simply and with extremely high accuracy. This technique has made a revolutionary effect on the life sciences, found its application in new methods of cancer treatment, and is able to bring a dream of treating hereditary diseases to life' [2]. In 2020, the amount of the Nobel Prize is 10 million Swedish kronor, or $ 1.1 million.

Jennifer A. Doudna and Emmanuelle M. Charpentier made a real breakthrough in science in 2012, and certainly deserve the award they have been waiting for the past eight years. By the way, Clarivate Analytics predicted the Nobel Prize in Chemistry for them well back in 2015. However, the Nobel Committee has taken it slow to take such a decision for a long time, probably for years of patent wars and litigation for authorship between different groups of scientists who contributed in the development of the CRISPR-Cas technique. In March 2020, we published the article in the Visnyk NAN Ukraine - Genome Editing or Is CRISPR/Cas9 a Panacea for Many Untreated Diseases or the First Step to Genetic Apocalypse? - about the main aspects of using CRISPR-Cas technique and ethical issues that arose with the advent of the one and caused a hot discussion in the society [3]. This publication predicted a positive decision of the Nobel Committee well six months before the subject meeting (who knows, maybe this contributed to its taking). Therefore, in this article we will only briefly touch the importance of CRISPR-Cas discovery, summarize the achievements of this technique in various fields, and assess the prospects for its development in the future.

So, let's get to know better the Nobel laureates in chemistry in 2020.

Professor Emmanuelle M. Charpentier, 51, now heads the Max Planck Unit for the Science of Pathogens in Berlin.

Emmanuelle M. Charpentier was born on December 11, 1968 in Juvisy-sur-Orge, a suburb of Paris, France. Her father was an officer for the city's greenery and taught his daughter the Latin names of many plants. Her mother was the director of a psychiatric hospital. The girl's parents supported her interest in science, and after graduating the school she entered the Pierre and Marie Curie University of Paris to study biochemistry, microbiology and genetics (now the Faculty of Natural Science at the Sorbonne University). In 1991, E. Charpentier earned a bachelor's degree in biochemistry and announced to her parents the intention to study at the Pasteur Institute's graduate school, which did not surprise them at all. It turns out that at the age of 12 Emmanuelle had already claimed the intention to work there. And so it happened: in 1995, E. Charpentier defended her dissertation on microbiology devoted to the study of molecular mechanisms of antibiotic resistance at the Pasteur Institute.

In 1996, Emmanuelle M. Charpentier moved to the United States, where she spent the next five years as a researcher with three New York institutions. For a year, she worked for Rockefeller University at the laboratory of microbiologist Elaine Tuomanen, where a mechanism of resistance of *Streptococcus pneumoniae* to vancomycin, which involves mobile genetic elements for changing its genome, was discovered. From 1997 to 1999, E. Charpentier worked as an assistant researcher at NYU Langone Medical Center's Pamela Cowin Laboratory, where she studied skin cells of genetically modified mammals, including mechanisms for regulating hair growth in mice. From 1999 to 2002, Emmanuelle was a researcher at St. Jude Children's Research Hospital in Memphis, Tennessee, and at the Skirball Institute for Biomolecular Medicine in New York.

Later she returned to Europe, where in 2002-2004 she worked as laboratory head and visiting pro-

fessor at the Institute of Microbiology and Genetics of the University of Vienna (Austria). Here, in 2004, she discovered RNA molecules that regulated the synthesis of virulence factor in *Streptococcus pyogenes*. In 2004-2006, she was an associate professor of the Department of Microbiology and Immunobiology, and became a privat-docent of microbiology and earned a qualification certificate from the Center for Molecular Biology in 2006. In 2006-2009, Emmanuelle M. Charpentier worked as a professor at the Max F. Perutz Laboratories, proceeding as a laboratory head at the same time.

That done, E. Charpentier moved to Sweden to a position of professor and head of the Laboratory for Molecular Infection Medicine Sweden (MIMS) at Umeå University. It was the place, where in 2011-2012 she made her major discovery concerning CRISPR/Cas9. Emmanuelle M. Charpentier was a team leader at Umeå University in 2008-2013, and a visiting professor in 2014-2017. In 2013, she moved to Germany, where she worked as a professor and department head with the Helmholtz Center for Infection Research in Braunschweig and the Hanover Medical School until 2015. In 2015, Emmanuelle M. Charpentier accepted the proposition of the German Max Planck Society to become a scientific member of the society and director of the Max Planck Institute for Infection Biology in Berlin. Since 2016, she has been an honorary professor of Humboldt University in Berlin, and since 2018 - the director of the Max Planck Unit for the Science of Pathogens. Emmanuelle M. Charpentier has been a member of the European Molecular Biology Organization (since 2014), the German Academy of Sciences Leopoldina (since 2015), the Berlin-Brandenburg Academy of Sciences, the Austrian Academy of Sciences, the Royal Swedish Academy of Sciences (since 2016), the National Academy of Sciences of the United States, the National Academy of Technologies of France, the French Academy of Sciences (since 2017), and the European Academy of Sciences and Arts (since 2018).

In 2013, Emmanuelle M. Charpentier co-founded CRISPR Therapeutics and ERS Genomics [5].

Jennifer A. Doudna, 56, is now a professor of biochemistry and molecular biology with the University of California, Berkeley.

Jennifer A. Doudna was born on February 19, 1964 in Washington, DC to Martin Kirk Doudna and Dorothy Jane (Williams). Her father earned a

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doctorate in English literature from the University of Michigan in Ann Arbor, and her mother was a housewife, although she had a master's degree in education. When Jennifer turned seven, the family moved to the island of Hawaii because her father, after defending his dissertation, became a professor of American literature at the University of Hawaii at Hilo. Her mother earned a second education degree with the University (a master's degree in Asian history) and taught history at a Community College. While in Hawaii, Jennifer A. Doudna was fascinated by the beauty of the island's nature, its exotic flora and fauna, which fostered her interest in studying the biological mechanisms of life. Her father filled the house with popular science literature and was happy reading Jennifer about science. When she was in sixth grade, he presented her a 1968 book Double Helix by James Watson about the discovery of DNA structure, which inspired her a lot. Jennifer's choice of profession was also prompted by stories of a chemistry teacher, a work on summer holidays at a laboratory of the famous mycologist Don Hemmes at the University of Hawaii, as well as lectures by a scientific fellow of the Honolulu Cancer Center on cancer cell research.

In 1981, Jennifer graduated from high school of Hilo and entered Pomona College at Clare Monto, California. At her sophomore year, Jennifer began to doubt her ability to study science and wanted to quit everything for the sake of learning French, but her teachers disadvised her.

Jennifer A. Doudna had her first research experience at the Professor Sharon Panasenko's laboratory. In 1985, she earned a bachelor's degree in biochemistry and entered the Master's Degree Program of Harvard Medical School, graduating it in

1989 with a dissertation under the direction of Jack W. Szostak on the development of a system that increased the efficiency of self-replicating catalytic RNA.

In 1989-1991, J. Doudna worked at Massachusetts General Hospital and Harvard Medical School in Boston, Massachusetts, and from 1991 to 1994 at the University of Colorado Boulder with Thomas Cech, a Nobel Prize in chemistry winner of 1989 for the discovery of the RNA catalytic properties.

Early in her career, Jennifer A. Doudna studied the structure and biological functions of RNA enzymes, or ribozymes. While working at the University of Colorado, she met Jamie Cate, a four-year graduate student who later became her husband. In 1994, J. Doudna moved to Yale University, where in 2000 she became a professor of molecular biophysics and biochemistry. In 2000–2001, she was a visiting professor of chemistry for Harvard University, and in 2002 she became a professor of biochemistry and molecular biology with the University of California, Berkeley, where she made her major discoveries. Interestingly, Jennifer A. Doudna became the 25-th Nobel laureate from the University of California, Berkeley, and the name of the 24-th, Reinhard Genzel, became known the day before, when the Nobel Prize winners in Physics were announced.

Jennifer A. Doudna is currently the President and Chairman of the Board of the Innovative Institute of Genomics, funded by the Hong Kong Billionaire Li Ka-Shing Foundation and co-founded by the University of California, Berkeley and the University of California, San Francisco. She is a professor of biomedicine and health, Chair of the Biology Advisory Committee, a member of the Lawrence Berkeley National Laboratory, a researcher with the Howard Hughes Medical Institute, a senior fellow with the Gladstone Institute at San Francisco, and an adjunct professor of Cellular and Molecular Pharmacology at University of California, San Francisco. J. Doudna's laboratory is working on a mechanistic understanding of biological processes involving RNA.

Jennifer A. Doudna is a member of the National Academy of Sciences of the United States (since 2002), the American Academy of Arts and Sciences (since 2003), the National Medical Academy (since 2010), the National Academy of Inventors (since 2014), and a foreign member of the prestigious Royal Society of London (since 2016).

After discovery of CRISPR technology in 2012, J. Doudna co-founded five companies engaged in

Jennifer Anne Doudna

the commercialization of the technique: Caribou Biosciences (2011), Editas Medicinea (2013), Intellia Therapeutics (2014), Mammoth Biosciences (2017), and Scribe Therapeutics (2019) [4].

Both researchers - Emmanuelle M. Charpentier and Jennifer A. Doudna - have an incredible number of scientific awards (almost 40 each). Here are just some companion awards:

- Dr. Paul Jansen Award for Biomedical Research (2014);
- Gabbay Award (2014);
- Breakthrough Prize in Life Sciences (2015);
- Princess of Asturias Award (2015);
- Gruber Genetics Prize (2015);
- Messri Award (2015);
- Canada Gairdner International Award (2016);
- Paul Ehrlich and Ludwig Darmstaedter Prize (2016);
- Tang Prize of the Taiwan Academia Sinica (2016);
- Human Frontier Science Program (HFSP) Award (2016);
- BBVA Frontiers of Knowledge Award (2016);
- L'Oreal-UNESCO for Women in Science Award (2016);
- Warren Alpert Foundation Prize (2016);
- Japan Prize (2017);
- Albany Medical Center Prize (2017);
- Harvey Award (2018);
- Kavli Prize in Nanoscience (2018);
- The American Cancer Society Medal of Honor (2018);
- Wolf Prize in Medicine (2020).

Jennifer A. Doudna and Emmanuelle M. Charpentier were among the world's top 100 thinkers of 2014, the world's 100 most influential people of 2015 according to Time magazine, named Person of the

Year in 2016, and were included in the list of 50 most prominent women in the field of technology of 2018 compiled by Forbes magazine [4, 5].

Then why this same discovery made by this year's Nobel Laureates in Chemistry was recognized a revolutionary and extremely important one by the world? And what is CRISPR/Cas9?

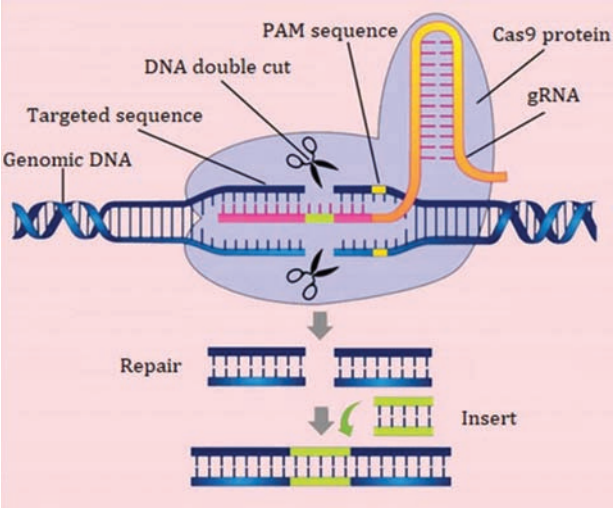
In the 50-70s of the XX century we had already known that bacteria used special restriction enzymes (Latin *restrictio*), which cleave viral DNA and are specific to a certain small DNA sequence. Bacteria protect their own DNA from restriction enzymes by methylating the nucleotide residues of adenine and cytosine. With the discovery of 'crosslinking' enzymes, DNA ligases, it became possible to create artificial structures from DNAs of living organisms. Thus, a new branch of science arose, called genetic engineering, which enabled to create genetically modified organisms (GMOs), recombinant proteins, induced stem cells and more. It turned out later that bacteria protected themselves from viruses not only by restriction enzymes: they had another, more specific mechanism that provided protection following encountering a particular virus. This mechanism implements a system with a rather cumbersome name - CRISPR/Cas9, or crisper.

When virus DNA penetrates a bacterium, a fragment of that DNA is copied and transferred to a special "repository" of information about the virus in its own genome - CRISPR (acronym of the Clustered Regularly Interspaced Short Palindromic Repeats). Here, DNA samples of different viruses (spacers) accumulate between the equal short repeats of bacterial DNA and are used to produce CRISPR RNA - crRNA (also called RNA probes or RNA guides), which specifically recognize the genes of certain viruses and bind them in case of re-infection. Due to cRNA, viral DNA is found by special enzymes - Cas proteins (CRISPR-associated proteins), whose genes are located next to the CRISPR array. These enzymes are endonucleases, like restriction enzymes, meaning they can cut viral DNA to neutralize it, so they are called "genetic scissors".

Let's consider how this system works using the example of CRISPR/Cas9. Following transcription of CRISPR array, a long RNA molecule is formed, to which a small RNA complementary to the repeats (tracrRNA) is attached. Cas9 protein binds and activates tracrRNA, which in turn is bound by enzyme RNAase III, which cleaves long RNA into fragments - cRRNA - and falls off. In fact, as a result of long RNA cleavage, crRNA, tracrRNA complexes and activated Cas9 protein are formed. The spacer part of crRNA recognizes the complementary region of viral DNA, and Cas9 protein locks on two DNA strands and 'cuts' them, causing further degradation of foreign DNA [6]. Successful recognition of the 'target' DNA and its damage requires Cas9 protein to recognize a certain short (3-9 nucleotides) PAM sequence (Protospacer Adjacent Motif), which differs in different bacteria and is located next to the recognized crRNA region of DNA. (see the Fig.). PAM recognition is likely necessary to protect the bacterium's own genes from cleavage by CRISPR/Cas9 system, since CRISPR contains 20% of spacers aimed at its own DNA [7].

Emmanuelle M. Charpentier and Jennifer A. Doudna received the Nobel Prize for discovering the technology of genome editing with the help of CRISPR/Cas9. Perhaps, it is no longer possible to discover something alone in modern science. The history of the discovery that revolutionized genetic engineering lasted long 25 years, is contributed by many scientists.

In 1987, a group of Japanese researchers led by Yoshizumi Ishino, together with the iap gene they studied, accidentally cloned a large fragment of *Escherichia coli* genome that did not encode any of the proteins [8]. This was really strange, since bacteria usually do not have extra sequences. The site consisted of repeating DNA sequences and spacers - variable DNA fragments between them [9].



Mechanism of action of CRISPR/Cas9 system, which allows using it for genomic DNA editing (adapted from www.labiotech.eu)

In 1993, scientists from the Netherlands under the leadership of Jan D.A. van Embden studied the variety of interrupted direct repeats among different strains of the TB causative agent (*Mycobacterium tuberculosis*) and developed a new method of strain differentiation, which is still in use [10].

Later, the Spaniard Francisco Juan Martínez Mójica found similar sites in other bacteria and archaea species and proposed to combine them into a family called SRSRs (Short Regularly Spaced Repeats) [11] in 2000, which was two years later renamed CRISPR.

In the same 2002, a group of scientists from the Netherlands led by Ruud Jansen discovered Cas protein genes, which were located next to the CRISPR site [12].

And in 2005, three research groups independently found sequences identical to the DNA sequences of bacteriophage viruses and plasmids often happening between CRISPR repeats [13-15].

These results and assumptions about CRISPR antivirus protection property appeared to be not of much interest at the time.

Everything changed following publishing a scientific article in the Science journal in 2007 [16]. Its authors were scientists led by Philippe Horvath from Danisco (USA), a manufacturer of famous Danone yogurts (acquired by DuPont for $ 6.3 billion in 2011). The point is that produced fermented milk products are at risk of infecting lactic acid bacteria with viruses-bacteriophages, which may result in huge losses. Therefore, Danisco decided to select lactic acid bacteria clones resistant to the virus for production purposes. And since the company used CRISPR to classify its commercial strains, the researchers immediately noticed new spacers appeared in the CRISPR regions of the selected clones that were identical to those of the viral genome. The researchers then artificially inserted a spacer with the DNA sequence of the virus into the CRISPR site of the bacterium, which immediately made it resistant to the virus.

In 2007-2008, the important details of CRISPR mechanism were discovered. The group led by John van der Oost, for example, showed that CRISPR protection was implementing through small RNAs [17]. Luciano A. Maraffinni and Erik J. Sontheimer from Northwestern University at Evanston (USA) proved that the CRISPR system was aimed at DNA cleavage [18]. They were the first to suggest the possibility of using CRISPR for editing genome in heterologous systems and even applied for a patent, but eventually abandoned it due to lack of experimental confirmation [19].

It later became known that CRISPR protective function required interaction with many Cas proteins, some of which bound to small RNAs, some recognized the virus's DNA, and others cut it. However, Emmanuelle M. Charpentier, who then worked at the Umeå University (Sweden), discovered a variant of the CRISPR system, which needed only one very large Cas protein - Cas9 - for full protection. In parallel, Jennifer A. Doudna of the University of California, Berkeley, was studying the functioning of the CRISPR system from a structural point of view.

E. Charpentier and J. Doudna met in 2011 at a conference in Puerto Rico. They met in a cafe and decided to cooperate at a walk around the city. J. Doudna, paying much interest to Cas9 protein, tried to reproduce the work of CRISPR/Cas9 system in vitro, but without any particular success. In the same year, E. Charpentier discovered tracrRNA, which plays a crucial role in CRISPR-mediated protection against viruses [6]. After adding tracrRNA to a test tube, J. Dudney finally managed to cleave DNA using the CRISPR/Cas9 system. In 2012, Martin Jinek from Jennifer Doudna`s team combined tracrRNA and crRNA into a single sgRNA (single-guide RNA) molecule to create a vector for cloning this RNA. It turned out that such synthetic sgRNA correctly worked in a cell in complex with Cas9 protein. In the meantime, scientific teams of Emmanuelle M. Charpentier and Jennifer Doudna published an article in Science [20], in which described a method of using CRISPR/Cas9 system for cutting DNA ‘targets’ selected in a cell and demonstrated a possibility of such an approach in *in vitro* experiments. Almost simultaneously, a similar article was published by a group of Lithuanian biochemist Virginijus Siksnys from the Institute of Biotechnology at Vilnius University [21]. Interestingly, V. Siksnys obtained the result earlier, but its publication was pending for six months due to an unjustified rejection of the article by Cell magazine.

In early 2013, teams leaded by George Church [22] and his former graduate student Feng Zhang [23] of the Broad Institute of MIT and Harvard University of Cambridge, Massachusetts, demonstrated that artificial crRNA and Cas9 protein were fully functional in cells of higher organisms. Almost simultaneously, the effectiveness of this approach was

confirmed by scientists from South Korea in experiments with human cell culture [24]. Immediately after that, everyone forgot about the problems of bacteria in their difficult struggle with viruses. Scientific and social magazines seemed to "explode" with publications on only one topic: CRISPR/Cas9 as a fantastic tool for editing genomes of higher organisms.

The discovery of CRISPR/Cas9 caused a real turmoil, as this technique proved to be much better than previous methods of genetic engineering. Previously, to create a GMO, it was necessary to carry out a very painstaking and time-consuming work, rejecting a significant number of unsuccessful options. CRISPR/Cas9 offered an easy way to introduce a gene insert into a specific DNA sequence and enabled working in cells of all organisms from bacteria to mammals. The CRISPR/Cas9 system can be used not only *in vitro* as a restriction enzyme, but also directly in a living cell. It makes possible simultaneous editing an unlimited number of genes in a genome, as well as to introduce components of the system not only in individual cells, but also in different tissues or whole organisms.

Of course, there other tools for genome editing are also available, namely: meganucleases, TALEN genetically engineered proteins (based on effector's domain, similar to TALE transcription activator), and nucleases with ZFN 'zinc fingers' (containing a domain of the eukaryotic transcription factor). However, application of these systems for genome editing requires the creation of a separate artificial protein for each new target DNA. In contrast, the CRISPR system employs the universal Cas9 protein, and only sgRNA needs to be altered. It is much simpler and cheaper, because any RNA can be easily synthesized. Another advantage of the CRISPR/Cas9 system is the ability to apply it to RNA, which provides more flexibility to influencing biochemical processes in a cell.

Realizing the undeniable benefits of CRISPR/Cas9 technique, scientists around the world have rushed using it for editing genomes of viruses, bacteria, plants and animals. Almost limitless prospects for creation of GMOs to control diseases, improve the breeds of farm animals and plant varieties, create models for studying genetic diseases of humans and animals, test new therapies and even bring to life new pet species, have emerged.

CRISPR/Cas9 technique can improve the efficiency of agriculture and help feed the growing population of the planet, simultaneously reducing the negative impact of mankind on the environment. Over the past four years, the US Department of Agriculture has authorized the cultivation of six CRISPR-modified organisms, including *Agaricus bisporus*, which are deprived of the ability to darken with mechanical damage and lose their marketability; *Camelina sativa* - an oil crop that contains more omega-3 fatty acids than average plant, as well as a drought-resistant soybean variety. Researchers plan to rear chickens whose meat does not cause allergies in humans, restore the population of honey bees suffering from diseases and parasites around the world, and use CRISPR to control the sex of farm animals. However, because CRISPR genome modification may alter non-target genes, it has been suggested to apply carefully, as it may affect animal health, meat and milk contents. Therefore, consumers have been so far demanding caution in the application of new techniques for productive animals [26].

The possibility of modifying genomes of exotic and little-studied animals caused a 'wave' of mass creation of new model organisms. In March 2019, the first genetically modified reptile, *Anolis sagrei*, was brought to life using CRISPR [27]. CRISPR technique has become a valuable tool for fundamental research that allows researchers to 'turn off' certain genes and establish their biological function in a body.

The gene can be inactivated without damaging it by CRISPR interference (CRISPRi). When using this method, the mutant dCas9 protein, in which both nuclease centers do not function, binds to the target DNA and prevents the advancement of RNA polymerase, which leads to the cessation of transcription [28].

Once a transcription factor domain is 'bound' to dCas9 protein, which increases or decreases gene activity, it becomes possible to directly influence gene functioning and the functioning of the whole organism. Gene expression activity can also be regulated by influencing epigenomic processes (e.g., DNA methylation or histone acetylation). For this purpose, artificial proteins may be used, which consist of the dCas9ia catalytic domain of corresponding enzyme (DNA methyltransferase or histone acetyltransferase) [29]. The CRISPR/Cas9 system may also target long noncoding RNAs (lncRNAs) or enhancer RNAs (eRNAs), which regulate gene expression and epigenetic processes [30]. This is very important for studying the functions of regulating RNAs and establishing their role in the pathogenesis

of diseases, because in more than 90% of diseases associated with a single nucleotide substitution, this substitution occurs in non-coding DNA regions [31]. And by attaching a fluorescent GFP protein to dCas9 protein, it makes possible to mark a certain area in a chromosome of a living cell and observe it under a microscope during the cell cycle or visually determine the telomere length [32].

In 2019, a CRISPR-Chip biosensor was created based on ultrasensitive graphene and CRISPR/Cas9 system, which allows to detect certain DNA sequences without its amplification with a sensitivity of 1.7 fM (1.7×10^{-15} Mol) for 15 min. [33]. Such biosensors involving different types of Cas enzyme may be aimed at detecting specific sequences in single- or double-stranded DNA and RNA of infectious agents. Such a system, for example, is able to determine the amount of coronavirus RNA, its type (SARS-CoV or SARS-CoV-2) and even to differentiate individual substitutions in RNA [34]. Therefore, as we can see, the CRISPR/Cas9 system properties are not limited to only cutting certain DNA sequences. This system is a universal mechanism for delivery of any molecule to any 'point' of the genome, which opens up fantastic opportunities for medicine and basic science.

In January 2014, Chinese scientists modified the macaque genome using the CRISPR/Cas9 system [35]. After their success with the monkeys, everyone realized that human is the next in the line. In fact, it is very tempting to heal a patient from a severe inherited disease, such as hemophilia, by correcting just one DNA nucleotide site. However, it is still unknown how the human body will react to interference with the genome. Therefore, the ability to edit human genes immediately raised a number of ethical issues, especially critical in the case of editing human embryo genes. Jennifer A. Doudna and other leading scientists have repeatedly stated the dangers of reckless using the CRISPR/Cas9 technique and called for a moratorium on clinical experiments with human genes until the outcome is understood and proper rules introduced [36]. After all, the simplicity of the technique and the availability of reagents for its implementation in the United States have allowed biohackers to intervene with genome of living organisms at home, even without special skills and equipment. However, the WHO Expert Advisory Committee at its meeting in August 2019 put off the issue of a moratorium, although created a global registry to track all types of research on human gene editing and offered a regulation on managing such techniques [37].

Despite calls for a ban on human genetic modification experiments, an article by Chinese geneticists led by Junjiu Huang of Sun Yat-sen University at Guangzhou was published in Protein&Cell magazine on April 14, 2015, describing an experiment involving CRISPR/Cas9 for editing a genome of human embryo [38]. The effort was aimed on correcting a mutation in one of hemoglobin genes, which resulted in a blood disease - beta-thalassemia. This was the first attempt in history to genetically modify a human. As a result, of the 86 fertilized ovicells, the mutation was corrected in only 4 embryos (approximately 5%). Moreover, a significant number of new mutations in other genes have emerged in all embryonic cells as a result of non-specific interaction of crRNA with other similar DNA sequences, as well as due to errors in repair enzymes. Such results have raised objective fears that CRISPR/Cas9 will never be used in human experiments at all.

Various ideas have been proposed to improve the CRISPR/Cas9 accuracy. For example, Cas9-nikase, a modified Cas9 protein with only one nuclease center allowing only single-stranded DNA cuts, was isolated. Using two such nicases with different sgRNAs, it is possible to significantly improve the accuracy of DNA cleavage in a desired location [39]. In August 2017, encouraging research results were published under the leadership of Shoukhrat Mitalipov, a researcher known for his successful experiments on cloning primates and humans. The research team managed to increase to 72.4% the yield of embryos with a corrected mutation of MYBPC3 gene, which caused hypertrophic cardiomyopathy - an inherited heart disease, without the emergence of other mutations [40].

In November 2018, Chinese researcher He Jiankui from the Southern University of Science and Technology at Shenzhen unexpectedly announced that he had created the world's first genetically engineered children, Lulu and Nanu twin girls, who were unable to contract the human immunodeficiency virus (HIV) due to modification of CCR5 gene. In 2019, another modified child was born within the framework of this project. These statements provoked a scandal and a police investigation in China followed by indignation of the world scientific community [41]. He Jiankui was arrested and sentenced to three years in prison by a closed court session and fined 3 million yuan ($ 430,000) for 'illegal medi-

cal practice'. His colleagues Zhang Renli and Qin Jinzhou were sentenced to 24- and 18-month probation, respectively. All three pleaded guilty and were permanently barred from research related to reproductive medicine. By the way, He Jiankui confessed that the re-editing attempt was not fully successful, as the editing system entailed a mutation, yet not the one he expected. What is happening now with the modified children is unknown, since Chinese authorities are hiding them.

In 2019, two more scientists announced their intention to create babies with edited genes: Denis Rebrikov from the Pirogov Russian National Research University in Moscow and Gianpiero D. Palermo from New York. It is not known how close these plans are to completion, but such statements warn us that in the near future the other people will also attempt to amend the human genome that can be inherited by future generations [37, 43]. Scientists set for creating children with edited genes may dream of improving the world, ridding humanity of dangerous inherited diseases. Perhaps, the mutations introduced, eliminating one danger, can inflict others to the body due to the multifunctionality of most genes, which in the case of gene mutations may lead to changes in the whole complex of features, in fact not always desirable and useful.

Therefore, scientists have focused on other promising areas of CRISPR/Cas9 application, such as editing the genome of bacteria or yeast for synthesizing completely new substances, creating animal models of human diseases, combating antibiotic resistance of microorganisms, eliminating insect pests and transmissible infections, solving the problem of lacking donor organs for transplantation, protection against dangerous viruses, treatment of oncological diseases, etc.

It has been experimentally confirmed that the CRISPR/Cas9 can be successfully used to demolish genes of enzymes responsible for resistance of bacteria to antibiotics [44]. Even bacteriophages have been obtained that selectively neutralize antibiotic-resistant bacteria [45]. Biotechnology company Oxitec (UK) uses CRISPR/Cas9 to create genetically modified insects, which after mating with their wild relatives, kill some of their offspring. In October 2019, in Indaiatuba (Brazil), researchers successfully conducted tests on modified mosquitoes intended to control dengue fever and other diseases, as well as modified diamond moths, responsible for damaging various types of cabbage. [46].

In August 2017, a team of scientists led by George Church of Harvard University released stunning results of research on cloning genetically modified pigs using CRISPR/Cas9, in which PERV viruses were completely inactivated. Inactivation of these viruses opens the possibility of using pig organs with artificially created transplant antigens identical to a particular human for transplantation [47]. Researchers from the University of Alabama (USA) used gene editing and cloning to create pigs without specific carbohydrates on the surface of their organs. Baboons who received heart and kidney transplants from these pigs lived for more than a year. In July 2019, 30% of transgenic 'humanized' mice that had been previously infected with HIV were completely removed from T-cells using combination of CRISPR/Cas9 technique and long-acting antiretroviral therapy. After testing this technique on monkeys in 2020, the first tests involving humans are planned [49].

The CRISPR/Cas9 system is also a valuable tool for creating viruses with certain properties, such as attenuated viruses for vaccines or oncolytic viruses [50]. This system makes possible improvement of immunotherapeutic approaches to cancer treatment, including creating genetically modified T-cells with chimeric antigen receptors (CAR-T), which contain scFv antibody against a specific cancer cell antigen. Thus, in 2017, the FDA approved the Kymriah (Tisagenlecleucel) manufactured by Novartis (Switzerland), which successfully treated B-cell lymphoma using T-cells with chimeric receptors against the CD19 antigen [51]. More than 300 different CAR-T therapy options are currently being studied and tested. Along with this, CRISPR/Cas9 technique opens up real possibilities for eliminating cancer-causing mutations [52].

The simplest option for using CRISPR/Cas9 in practical medicine for treatment of hereditary diseases caused by a single mutation of a particular gene is the therapy of blood diseases, for which cell techniques are already well suited. Late last year, a group of scientists from the University of California, Berkeley, reported the successful use of CRISPR/Cas9 for correction of a genetic mutation of stem cells in patients with sickle cell anemia [53]. Not surprisingly, biotechnology companies are investing more and more in developing CRISPR/Cas9 technique and implementing ideas for its medical application.

Scientists who have made the greatest contribution to the invention of CRISPR/Cas9 technique

have become co-founders of the first seven companies developing the application of this method in medical practice. Emmanuelle M. Charpentier is a co-founder of two companies: CRISPR Therapeutics and ERS Genomics, while Jennifer A. Doudna is a co-founder of five companies: Caribou Biosciences (develops therapeutic modified cells and gut bacteria), Editas Medicine (medicines and modified cells for therapy), Intellia Therapeutics (approaches to cancer therapy, genetic and autoimmune diseases), Mammoth Biosciences (diagnostic test systems involving new Cas12-14 proteins), and Scribe Therapeutics (approaches to the therapy of neurodegenerative diseases, including using CasX protein).

Jennifer A. Doudna had nothing to do but to break off relations with Editas Medicine, since her partner Feng Zhang unexpectedly registered a patent for using CRISPR/Cas9 technique in eukaryotic cells (including humans) for himself and his institute. Tens of millions of dollars were spent in litigation over the patent priority lasted for several years, and the court has eventually taken a decision in favor of the Doudna-Charpentier team. Pharmaceutical companies have already invested more than $ 300 million in the above-mentioned startups. If they get encouraging results, investments will increase, and administration of developed medicines for practical treatment of patients promises billions in profits.

For a long time, the FDA barred federal-funded clinical trials of the CRISPR/Cas9 system. However, in early 2019, the first CRISPR/Cas9 clinical trial on humans outside China, where such trials had been conducted in the past two years, was finally officially approved. This year, pharmaceutical companies CRISPR Therapeutics and Vertex commenced trials of CTX001 therapy for treatment of beta-thalassemia and sulfur-like anemia caused by a single gene mutation. This therapy involves modification of patient's stem cells by introducing a single genetic change that will lead to an increase of hemoglobin levels in fetal red blood cells [55].

In the United States, more than 20 clinical trials of CRISPR-based therapeutic medicines are currently being conducted for a number of diseases, including orphan monogenic hematological and ocular ones, and polygenic oncological diseases [56]. Among the inherited diseases caused by the point mutation, the attention of scientists is attracted mainly by such diseases as congenital Leber Hereditary Optic Neuropathy (retina lesion), Usher syndrome (deafness with gradual loss of vision), Duchenne muscular dystrophy, cystic fibrosis (cystic fibrosis affecting the respiratory and digestive systems), transthyretin deficiency (peripheral nervous system damage), alpha-1 amyloidosis, and type I primary hyperoxaluria (kidney damage). However, the high cost of CRISPR-based medicines could be an obstacle to their widespread use, since neither government budgets nor insurance companies are prepared for such costs. Moreover, CRISPR/Cas9 has a number of shortcomings, the main of which consists in the large number of editing errors, the inefficiency of off-cell DNA editing, and the large size of Cas9 enzyme, which complicates its delivery into the cell, thus causing strong immunogenic properties.

At the same time, there is no doubt that CRISPR/Cas9 technique is revolutionary and very promising one. With the discovery of CRISPR/Cas9, many opportunities have emerged to address the pressing problems of humanity that science has not yet been able to deal with. However, to realize all these possibilities in full, it is necessary to make the technique safe: to exclude all side effects, as well as to improve the systems of CRISPR/Cas9 components' delivery to cells. Having this done, the idea of applying CRISPR/Cas9 to human embryos for excluding serious hereditary diseases will probably no longer seem so questionable.

By the way, Jennifer A. Doudna believes that editing the human embryo genome should not be completely banned. This technology should be thoroughly researched and improved, and its clinical application should be limited to cases of serious genetic diseases, when there no other treatment options exist. Perhaps, such cases are rare, so it is much easier to get rid of genetic defects not by genome editing, but by mere screening and selecting embryos following *in vitro* fertilization. According to J. Doudna, a more promising direction in the development of CRISPR/Cas9 technique is using it for regulation of protein expression and control of cell function. There are also many other possibilities for using CRISPR/Cas9 in the near future. J. Doudna's laboratory plans to study the functioning of the CRISPR system in various microorganisms, as well as a possibility of genome editing in natural microbial communities and manipulation of certain microbes. One of its companies, Mammoth Biosciences, which develops CRISPR/Cas9-based diagnostic tests, plans to conduct the trial of test for diagnostics of COVID-19 in November 2020 [57].

We are now experiencing a surge of enthusiasm for CRISPR/Cas9 technology. Over the past eight

years, about 17,000 scientific articles on the subject have been published, millions of dollars have been spent for the research, and a number of startup companies is growing steadily. Not surprisingly, Science, the authoritative scientific journal, has published a review of achievements in this field called The CRISPR Craze [58]. By 2025, more than $ 5.3 billion is expected to spend on the development of CRISPR/Cas9 technique and genome editing in the world [59].

The main merit of Jennifer Doudna and Emmanuelle M. Charpentier is that the discovery of CRISPR/Cas9 genome editing technique marked the beginning of a new era in the history of genetic engineering and stimulated its further development. After all, progress is not standing still, as scientists are trying to improve CRISPR/Cas9, combine it with other methods, and are constantly looking for new techniques of genome editing.

One way to improve the technique has become finding other Cas proteins that may be more effective. Thus, ScCas9 [60], CasX and CasY [61], Cas12a (Cpf1) [62], Cas13 (a, b, c, d) [63–65], and Cas14 (a, b, c) proteins were discovered [66], which have unique properties and capable of more efficient and safer CRISPR-based genome editing. These efforts have led to the development of three new methods of genome editing, which employ such tools as transposases/recombinases, base editors and prime editors [67].

Transposases are enzymes able to bind single-stranded DNA and integrate it into genomic DNA. Recombinases are enzymes involved in the processes of homologous recombination - the exchange of genetic material between two DNA molecules that have homologous nucleotide sequences and certain specific sites. The discovery of CRISPR-associated transposases in some organisms, as well as the fusion of transposases and recombinases with Cas proteins and their artificial evolution, have made it possible to use these enzymes for editing genomes. For example, a team led by Feng Zhang has recently discovered a new gene editing technique that allows to insert genes into DNA without cleavage using CRISPR-associated transposase from cyanobacteria *Scytonema hofmanni* (ShCAST), consisting of Tn7-like transposase subunits and Cas12k enzyme [68].

Researchers at Harvard University, led by David R. Liu, have proposed a new method of genome editing - base editing, which presumes changing the nucleotide sequence of living cells by chemical conversion of one base to another without making cuts of double-stranded DNA, which may result in editing errors due to improper repair. The method is based on a combination of CRISPR system and fused proteins consisting of Cas9-nicase (Cas9, which cleaves only one DNA strand) and an enzyme that modifies a specific nucleotide base. The cytidine deaminase is used, which provides direct conversion of cytosine to uracil in the composition of the corresponding nucleosides in 15-75% of cell DNA, thereby substituting cytosine for thymine (or guanine for adenine) [69], as well as so-called adenine base editors (ABE) - enzymes resulted from artificial evolution based on adenosine deaminase tRNA responsible for reverse conversion of thymine to cytosine (or adenine to guanine) in 50% of cell DNA [70].

A similar approach can be used for RNA editing. In 2019, the method developed in 2012 by German scientists Thorsten Stafforst and Marius F. Schneider from the University of Tübingen [71] excited great interest in scientific circles (more than 400 publications), which was then ignored being overshadowed by a sensational discovery of CRISPR/Cas9. This method allows to modify proteins by editing RNA, which solves the problem of risks associated with permanent changes in human DNA and a likelihood of burdening edit errors. Changes in mRNA are made by adenosine deaminases acting on RNA, which form a complex with adRNAs (ADAR guide RNAs) and are capable of changing adenosine to inosine in the formed double-stranded RNA, which is distinguished as guinosine during protein synthesis. An important advantage of using human ADAR enzymes over Cas9 protein of bacterial origin is the absence of adverse reactions from the immune system. Other RNA editing agents are now known: APOBEC cytidine desaminases and certain grape enzymes convert cytosine to uracil in nucleosides, and some tumor enzymes can convert guanine to adenine, etc. Despite the limited methods of changing the RNA sequence and problems with the delivery of RNA to cells, several startups have already embarked upon using RNA editing systems for development of therapies for cancer, muscular dystrophy and other diseases (not just genetic), as well as for correction of various pathological conditions such as acute pain or high cholesterol [72].

The aforementioned group of scientists led by David R. Liu developed a new universal method of genome editing - a prime editing, which also did not require cuts of double-stranded DNA, but allowed

for any transformations, including insertion and deletion of DNA fragments with high accuracy (10% error rate) and greater efficiency (20-50%) compared to the classic CRISPR/Cas9 (3-20%). The method is based on a combination of CRISPR, a fused protein consisting of Cas9-nicase and a reverse transcriptase enzyme capable of building DNA on RNA basis. What is more, pegRNA is used instead of gRNA, which not only directs the fused protein to the desired site in the genome, but also contains 'primer' - a sequence required for self-completion of DNA chain enzyme (in CRISPR/Cas9 system, DNA is completed with repair enzymes, often making mistakes). The possibility of prime editing was demonstrated on the example of sickle cell anemia, Tay-Sachs disease (early childhood amaurotic idiocy) and prion infection, when researchers introduced mutations into the human cell genome that caused these diseases, and corrected them having this done. The analysis of the database of harmful human mutations that cause various diseases by scientists showed that 89% of these mutations can be corrected with the help of prime editing [73]. This method has become of great interest not only in scientific circles; its discoverer David R. Liu became a co-founder of several companies - Editas Medicine, Beam Therapeutics and Prime Medicine, which are engaged in the development of therapeutic substances based on the prime editing method.

It is quite possible that the microorganisms that opened CRISPR/Cas9 for us still hide many little-studied and completely unknown molecular mechanisms that we can use for our own purposes, in particular for genome editing. For example, scientists have recently clarified the biological function of retrons - natural bacterial DNA elements encoding reverse transcriptase, which synthesizes many copies of a single-stranded DNA followed by formation of DNA-RNA-enzime complexes, discovered well in the 1980s. It turned out that retrons, like CRISPR, contribute to antiviral protection of bacteria and activate a protein that damages the cell membrane of infected bacteria in case of inactivation of other defense systems by bacteriophages and causes its death before phages have time to reproduce and spread [74]. Since retrons are able to convert any desired RNA sequence into DNA, even in yeast and mammalian cells, scientists of Stanford University led by Hunter Fraser, have combined retrons and CRISPR/Cas9 to develop a new method of genome editing called CRISPEY (Cas9 Retron precISe Parallel Editing via homology). This method has demonstrated high efficiency (92%), throughput and accuracy (4.6% of errors) and allowed to obtain tens of thousands of mutant yeast clones, each of which differed by only one base in the nucleic gene sequence [75].

It is likely that this year's Nobel Prize in Chemistry is not the last one in the field of genome editing, since the pace of its development is simply amazing. Despite the fact that the current editing systems have certain shortcomings and limitations, and the methods of their delivery to cells need improvement, it has become clear that genome editing technologies will achieve greater efficiency and safety in the near future, and long-awaited intervention in the human genome can evolve in a usual practice. David R. Liu's statement makes us believe this: "If CRISPR resembles scissors and the base editors look like pencils, then the prime editors are a word processor suitable for precise search and replacement. All genome editing tools will play their roles... This is just the beginning, not the end" [76].

ПЕРСПЕКТИВИ РЕДАГУВАННЯ ГЕНОМУ ЗА ДОПОМОГОЮ CRISPR/CAS, АБО ЯК ОПАНУВАТИ «ГЕНЕТИЧНІ НОЖИЦІ». НОБЕЛІВСЬКА ПРЕМІЯ З ХІМІЇ 2020 РОКУ

С. В. Комісаренко, С. І. Романюк

Інститут біохімії ім. О. В. Палладіна
НАН України, Київ;
e-mail: svk@biochem.kiev.ua

Нобелівську премію з хімії у 2020 р. присуджено двом дослідницям у галузі молекулярної біології – француженці Емманюель Шарпантьє (Emmanuelle Charpentier), яка нині очолює Відділення наук про патогени при Товаристві Макса Планка в Берліні, та американці Дженніфер Дудні (Jennifer Doudna) з Каліфорнійського університету в Берклі – за «розвиток методу редагування геному». У пресрелізі Нобелівського комітету зазначено, що лауреатки відкрили один з найпотужніших інструментів генної технології – CRISPR/Cas9 – або так звані «генетичні ножиці». Цей метод сприяв отриманню у фундаментальних дослідженнях багатьох важливих результатів. Зокрема, дослідники рослин змогли створити культури, стійкі до цвілі, шкідників та посухи.

У медицині тривають клінічні випробування нових методів лікування раку, а мрія про те, щоб вилікувати спадкові захворювання, ось-ось стане реальністю. «Генетичні ножиці» вивели науки про життя на новий етап розвитку і дають людству величезну користь.

Ключові слова: Нобелівська премія з хімії, Еммануель Шарпантьє, Дженніфер Дудні, редагування геному, CRISPR/Cas9.

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