THE DAWN OF THE CRISPR-FREE GENOME EDITING ERA
—THE DEVELOPMENT OF GENOME EDITING TECHNOLOGY—

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OVERVIEW

- The genome editing technology CRISPR-Cas9, which drew attention in the 2020 Nobel Prize in Chemistry, has been applied in various fields and has a concrete record in improving productivity and others. As such, future social implementation is expected to progress steadily.
- CRISPR-Cas9 has some drawbacks that need to be resolved, such as the possibility of altering the genetic information in unintended locations and the adverse effects to the organism caused by the activated immune functions due to DNA cleavage.
- While there are moves to improve CRISPR-Cas9 currently, research and development are underway into so-called CRISPR-free technologies, such as RNA editing technology and mitochondrial DNA editing technology that edit the genome without cutting DNA. Applications of CRISPR-free technology include RNA therapeutics and the treatment and cure of mitochondrial diseases, and these technological trends warrant attention.

On October 7, 2020, the Royal Swedish Academy of Sciences announced that it would award the Nobel Prize in Chemistry to two scientists involved in the development of genome editing technology. This is a rapid award of the prize, having been just eight years since the paper[1] was published in June 2012, and shows how innovative the **genome editing technology CRISPR-Cas9**[2] is. Since the effectiveness of CRISPR-Cas9 was shown in cultured cells in 2013, it has been used not only in the medical and healthcare fields but also in the areas of agriculture, livestock, and fisheries, and concrete results[3] have been produced.

In this way, CRISPR-Cas9 has attracted attention from all over the world and has a proven track record, but there are some drawbacks to be solved. As will be described later, the immune function can work to adversely affect the organism because CRISPR-Cas9 is a mechanism that "cleaves" DNA, which is extremely important for the organism. Moreover, the technology, albeit with improvement efforts being made, may change utterly unrelated genetic information. Such cases are widely recognized and shared among people involved in life science. Even though some results[4] have been produced by the Center for iPS Cell Research and Application, Kyoto University, unless those issues are to be improved, safety concerns cannot be completely eliminated

[1] "A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity" (PMID: 22745249 PMCID: PMC6286148 DOI: 10.1126/science.1225829) https://pubmed.ncbi.nlm.nih.gov/22745249/

[2] Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated proteins. CRISPR is the name of the place where genes involved in (acquired) immunity are located. It is characterized by a unique arrangement with a palindromic structure, and was discovered by Professor Yoshizumi Ishino of Kyushu University. Cas is the name of a group of proteins. Cas9 is a protein called a nucleolytic enzyme that has the function of cleaving the double helix structure of DNA. See references at the end of the document.

[3] CRISPR-Cas9 is introduced in Mitsui & Co. Global Strategic Studies Institute "Four Technologies to Watch in 2016: Genome Editing" [in Japanese] (author: Tomoyuki Okada) with a focus on case studies.
https://www.mitsui.com/mgssi/ja/report/detail/__icsFiles/afieldfile/2016/10/20/160215mt.pdf

[4] The Center for iPS Cell Research and Application has succeeded in producing iPS cells in which the HLA genome involved in immunorejection has been deleted using CRISPR-Cas9. Also, for Duchenne muscular dystrophy (MDM), iPS cells exon-skipped by CRISPR-Cas9/CRISPR-Cas3 were effectively delivered to cells by virus-like particles developed by the Center, and skeletal muscle stem cells were successfully regenerated. This result was achieved with mice and application to humans is expected in the future. Nippon Shinyaku's MDM therapeutic agent Viltolarsen and Sarepta Therapeutics' Eteplirsen (not approved in Japan) are conventional nucleic acid drugs, not therapeutic agents using genome editing technology.

when CRISPR-Cas9 is fully deployed in the medical field such as in human gene therapy, where it is most desired to be used. Therefore, a technology for overcoming the issues of CRISPR-Cas9 is required. Intensely competitive R&D into genome editing technology is taking place centered in Europe, the United States, and China, and various CRISPR derivative technologies (see Figure 13 below) have been produced. Of these, this report introduces **(1) RNA editing technology** and **(2) mitochondrial DNA editing technology** (Figure 1), which can rewrite genetic information without cleaving DNA like CRISPR-Cas9 and can be applied to medicine.

Figure 1 Genome editing technology introduced in this report

	CRISPR genome editing	Non-CRISPR genome editing	
Editing technology	Genome editing technology CRISPR-Cas9	RNA editing technology	Mitochondrial DNA editing technology
Target	DNA	RNA	Mitochondrial DNA
Editing method	Editing by cutting DNA	Base conversion (replacement) with RNA and mitochondrial DNA intact	
Editing tool (Enzyme used)	Artificially modified nucleolytic enzyme (Nuclease)	Nucleoside metabolic enzyme (deaminase) ・**Adenosine deaminase** ・**Cytidine deaminase**	
Remarks	Has the drawback that unintended genes are edited, but research and development to overcome this is currently progressing	Edits a copy of the genetic information, so eliminates permanent effects on DNA	Good prospects for the treatment of intractable diseases such as mitochondrial disease by mitochondrial DNA editing technology

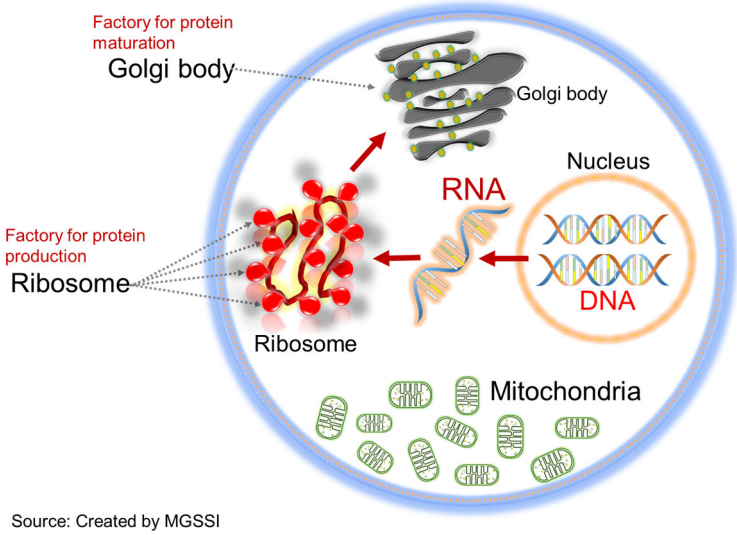
Source: Created by MGSSI

0. PRELIMINARY KNOWLEDGE TO UNDERSTAND GENOME EDITING

0-1. Cells (nucleus, ribosome, mitochondria)

Organisms are composed of an enormous number of cells. An adult human is said to number about 37 trillion cells.[5] While a "cell" is a general term, there are various cells such as nerve cells in the brain, hepatocytes in the liver, and muscle cells. However, the basic contents of the cells are the same, and their structure is as shown in Figure 2.

Figure 2 Cell contents



Source: Created by MGSSI

As shown in Figure 2, **DNA** that stores genetic information exists in the **nucleus** of cells. The nucleus, covered and protected by a nuclear envelope, is yet a dynamic world as the genetic information of DNA is frequently referenced by RNA. A **ribosome** is a **protein production factory**: **RNA** copies the genetic information of **DNA** and moves from within the nucleus through the nuclear envelope to the ribosome, where the genetic information brought by **RNA** is used to produce a protein. The various proteins produced by this ribosome fulfill the functions necessary for maintaining vital activity inside and outside the cell.

Mitochondria play the role of an **energy factory** that supplies the energy required for cell activity to cell tissues

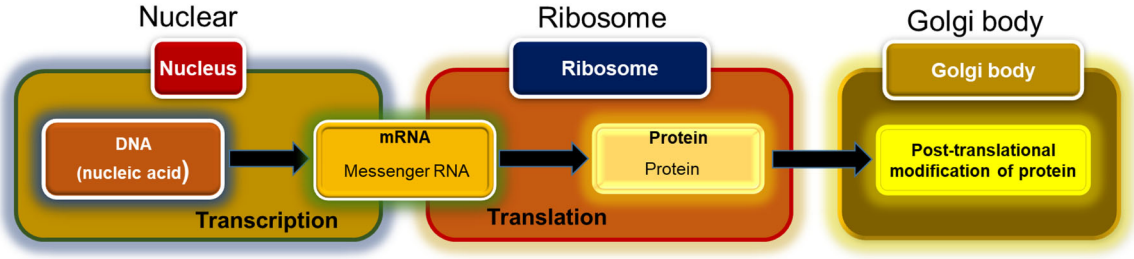
[5] Annals of Human Biology（Volume 40, 2013）"An Estimation of the Number of Cells in the Human Body"
https://www.tandfonline.com/doi/abs/10.3109/03014460.2013.807878

such as the nucleus and ribosomes. A mitochondrion was once an independent bacterium, but it is considered to have come to coexist with cells during the process of evolution and have reached its present form (inhabiting the inside of the cell). For this reason, mitochondria have their own DNA (**mitochondrial DNA**[6] described later), which can be said to be a remnant from the time when they were self-sustaining.

0-2. The Central Dogma and the genome

As the name suggests, the **Central Dogma** is a central concept in biology. It is a theory that the genetic information of an organism is transmitted in the order of DNA (storage) -> RNA (transcription) -> protein (translation). As shown in Figure 3, the genetic information from DNA is transcribed and translated into RNA, and the basic framework of the protein is created by the ribosome. Thereafter, this moves to the Golgi body, where substances (lipids, sugar chains,[7] etc.) are added[8] to modify it and make it react easily to various biological substances. It is then released into the cell.

Figure 3 From Central Dogma to post-translational modification



Source: Created by MGSSI

Genetic information is stored in DNA, and the entire genetic information of a certain species is called the **genome**. There are millions of flora and fauna on the earth, and an equivalent number of genomes exist. Human and animal genomes are composed of a **nuclear genome** and a **mitochondria genome**.[9] The nuclear genome stores genetic information in DNA, and the mitochondria genome stores genetic information in mitochondrial DNA. Usually, the term "genome" refers to the nuclear genome, but mitochondrial DNA, which affects important biological phenomena such as energy production and aging, has also attracted attention in recent years. This report introduces the mitochondrial DNA editing technology published in the UK journal *Nature* in July 2020 in Section 3.

1. GENOME EDITING TECHNOLOGY

DNA, which has important genetic information for cells, exists protected by a nucleus covered with a nuclear envelope. However, in reality, DNA may be broken by external physical influences such as radiation; therefore, cells have the function of repairing damaged DNA. In addition, in order to eliminate or neutralize viruses that threaten one's own survival, cells have protective functions (immunity) such as cleaving the DNA of the organism into pieces and falsifying (replacing) genetic information with meaningless sequences to prevent further proliferation. By utilizing these mechanisms inherent in organisms skillfully, the technology called **genome editing**[10] manipulates genetic information in a form desirable to humans.

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[6] Mitochondrial DNA is circular (16,569 bases) and contains 37 genes. It is more susceptible to mutation than the nuclear genome. Mitochondrial DNA is maternally inherited.

[7] Regarding sugar chains, see "Sugar Chain Technology" [in Japanese] (April 2019), Mitsui & Co. Global Strategic Studies Institute. https://www.mitsui.com/mgssi/ja/report/detail/__icsFiles/afieldfile/2020/01/30/1904t_abe_1.pdf

[8] As shown in Figure 3, this is called post-translational modification of proteins, and biological substances necessary for phosphorylation, methylation, lipid modification, and functioning as proteins such as sugar chain modification are added afterwards (proteins are formed by a series of amino acids that are folded to make a three-dimensional structure).

[9] There is also a chloroplast genome (chloroplast DNA) in the genome of plants that perform photosynthesis.

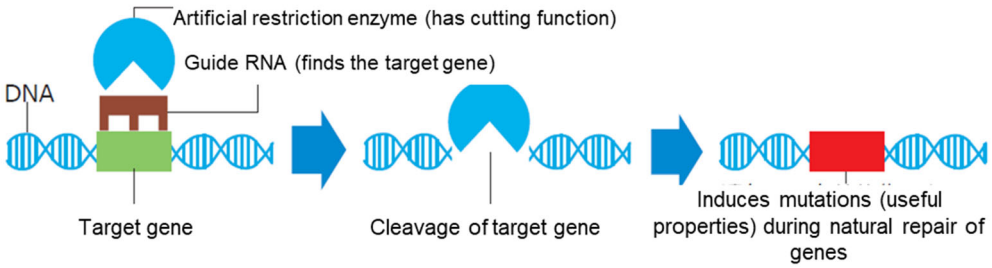
[10] Regarding genome editing technology, refer to "What is Genome Editing?" (in Japanese) (Kodansha Blue Backs, August 2020) by the leading expert in Japan, Professor Takashi Yamamoto of Hiroshima University. https://gendai.ismedia.jp/list/books/bluebacks/9784065194690

1-1. Genome editing technology: CRISPR-Cas9

In 1953, Watson and Crick elucidated the structure of DNA (the double helix), and it became understood how genetic information is retained and how from this information proteins are produced. Since then, research has progressed, and interest has shifted to the decoding of the genomes of organisms, especially the human genome. The completion of the human genome decoding in 2003 raised expectations that the genetic information (genes) decoded would be edited as desired and used for research activities such as investigating the cause of diseases and elucidating life phenomena. Behind this is the clarification of all human genetic information, which led to the acquisition of useful research results. For example, it was identified how a disease develops due to genetic abnormality, and what genes are essential for maintaining vital activity and what their functions are. Initially, it was difficult, costly, and laborious to change the genetic information in the exact form intended, but in 2012, the paper "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity"[11] was published in Science Online, which revealed a technology that can manipulate genetic information by a simple method. This was the **genome editing technology CRISPR-Cas9**.

CRISPR-Cas9 is a technology that uses the mechanism (immunity) by which cells protect themselves from things that are harmful to them, and can be summarized by two points. The first is that it uses a substance to cleave the DNA of viruses and the like, and stop their growth to neutralize and eliminate external threats. The second is that it utilizes cells' function to repair DNA that has been cut by the effects of radiation and the like. A substance that cleaves DNA is called a nucleolytic enzyme (expressed as an artificial restriction enzyme in Figure 4). The CRISPR **Cas9**[12] is also a **nucleolytic enzyme**. When Cas9 cleaves the DNA that holds the genetic information, the cell begins repairs to restore the DNA to its original state. During the repair process, the cell uses surrounding biological material to restore the DNA to a double helix. CRISPR-Cas9 tactfully utilizes these two points to modify DNA (edit the genome).

Figure 4 Genome editing mechanism



Source: MGSSI "Four Technologies to Watch in 2016" [in Japanese]

The mechanism is to cleave the target gene (DNA) with Cas9, as shown in Figure 4. When cells detect the abnormality of a DNA cleavage, they immediately try to repair the DNA. At this time, a modification is added to the target gene by the cell using a genetic molecule (molecule that constitutes the gene) prepared in advance for genome editing as DNA repair material (red part on the right in Chart 4).

CRISPR-Cas9 is a technology that is much easier to use than conventional technology, and it is possible to disable abnormal genes or incorporate useful genes at will without labor and cost. As such, it has attracted attention from life science researchers. In the year following the publication of the paper, specific application examples were shown, and their usefulness was confirmed. Thus, genome editing technology became widely known among the media and the general public to this day.

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[11] https://science.sciencemag.org/content/337/6096/816

[12] Cas proteins such as Cas1 to Cas13 including natural/artificial modifications are known. For example, Cas3 chops DNA like a shredder, and Cas13 is artificially modified for use in RNA editing, which will be described later. In this way, they have different roles and characteristics. See references at the end of the document.

1-2. CRISPR-Cas9 application case

CRISPR-Cas9 is already used for livestock, agriculture, fisheries, medicine, etc. In the livestock sector, a research group at the University of California Davis Campus announced in July 2020 at the American Association for Laboratory Animal Science that CRISPR could be used to make 75% of cattle offspring male.[13] Bulls can convert feed to muscle more efficiently than cows. If the number of males can be artificially increased, it may be possible to increase the production of high-value meat while reducing the feed volume consumed. Also, in agriculture, it is widely used for breeding various crops such as rice and wheat, and fruits such as apples. In the fishing industry, Kinki University, which has an established reputation for tuna farming, uses genome editing to turn oversensitive tuna into "insensitive tuna" to improve the cultivation rate.[14] Naturally, CRISPR is also frequently applied in the medical and healthcare sectors, and has recently been used to test for the novel coronavirus (disease name: COVID-19, virus name: SARS-CoV-2) (SHERLOCK in Figure 5. Others in the table are for research use and such). Unlike a PCR test, the presence of the disease is determined based on the unique gene sequence that exists only in the novel coronavirus, which enables extremely accurate diagnosis.

Figure 5 CRISPR-based test method

CRISPR-based test method	Overview	Development companies and other references
SHERLOCK (FDA emergency approval for COVID-19 inspection use)	SHERLOCK uses the nucleolytic enzyme Cas13a, which degrades DNA and RNA, to identify the gene sequence unique to the virus to be tested and make a positive/negative determination.	Massachusetts Institute of Technology McGovern Institute Harvard University Broad Institute Sherlock Biosciences https://sherlock.bio/
SHERLOCK v2	SHERLOCK v2 uses Cas12a and Cas13a to identify the gene sequence unique to the virus to be tested and make a positive/negative determination. It streamlines the SHERLOCK inspection process, shortens the inspection time, and supports various virus inspections.	
SHERLOCK & HUDSON	A test method that combines SHERLOCK with the sample processing method HUDSON (heating unextracted diagnostic samples to obliterate nucleases). HUDSON is a technology that deactivates nucleolytic enzymes, destroys the cell membrane of viruses, and releases the internal DNA and RNA into a solution.	Dengue virus (DENV) Zika virus Yellow fever virus test
DETECTR	DETECTR uses the nucleolytic enzyme Cas12a, which degrades DNA, to identify the gene sequence unique to the virus to be tested and make a positive/negative determination.	University of California, Berkeley Professor Jennifer A. Doudna Mammoth Biosciences https://mammoth.bio/

1-3. Challenges of CRISPR-Cas9

As mentioned at the beginning, CRISPR-Cas9 is a groundbreaking technology, but it has also been revealed that there are challenges to be solved. Since CRISPR intentionally cuts the double helix of DNA, it rarely causes the **off-target** phenomenon, which is characterized by an unexpected immune response caused in cells, and alteration of genes other than the target gene. It is essential that off-targeting does not occur especially in the case of applying genome editing technology to human treatment. In order to overcome such issues, researchers are working to improve CRISPR's shortcomings and seeking editing techniques other than DNA,[15] and one of their targeted areas is RNA. Since the genetic information possessed by RNA is a copy, even if genome editing fails due to the off-target effect and the like, there is no direct effect on the DNA that holds the master copy of genetic information, which may make it a promising post-CRISPR candidate technology. The next section will explain **RNA editing technology**.

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[13] University of California, Davis Campus website.
https://www.ucdavis.edu/news/meet-cosmo-bull-calf-designed-produce-75-male-offspring

[14] Tuna are sensitive to external stimuli and are easily startled by light and other things, which frequently caused them to collide with the cage net at a high speed and die. Kinki University is breeding "easy-to-cultivate" tuna that do not behave in such a way, by tempering the characteristics of the tuna using genome editing technology.
http://www.naro.affrc.go.jp/laboratory/brain/sip/sip1_topix_2-1-06.pdf

[15] See Figure 13 in this report for CRISPR-derived technologies, CRISPR-applied technologies, and CRISPR-free technologies.

2. RNA EDITING TECHNOLOGY

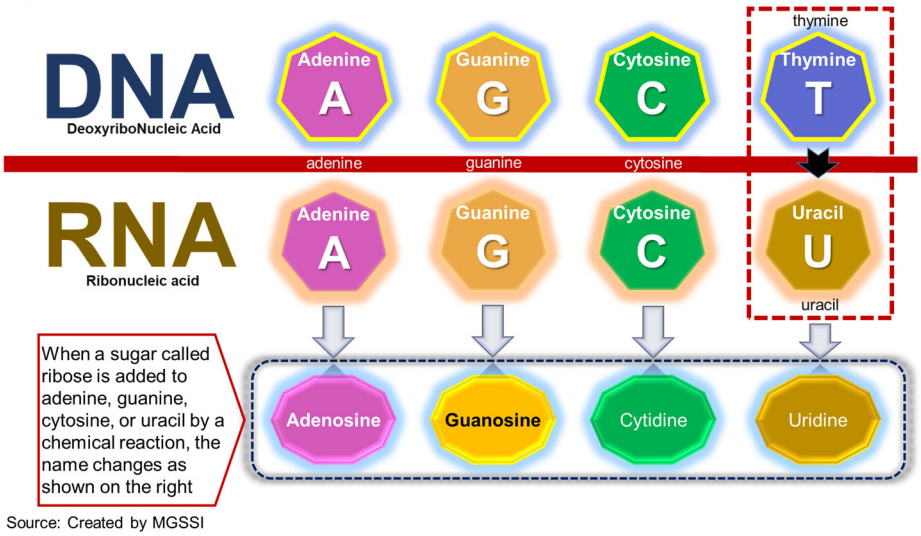
RNA[16] plays an important role: it stores a copy of the genetic information held by DNA and uses this genetic information to pass on the information necessary for the ribosome to produce proteins. RNA editing technology is a technology for editing the genetic information on the RNA copied from DNA. RNA editing technology does not directly edit the DNA that is the master copy of genetic information in the way that CRISPR-Cas9 does; therefore, there is no risk of causing undesired mutations in the DNA. In addition, since cells have the function of rapidly degrading RNA that has finished its role, the fact that edited artificial RNA does not remain in the body is another reason why RNA is attracting attention from researchers.

2-1. RNA editing technology: Pinpoint editing without cutting

It is known that flora and fauna frequently change the genetic information of RNA in the natural world. For example, RNA editing may be applied to a part of the genetic information copied from the mitochondrial DNA of a plant to RNA, and there are research results indicating that the protein does not function sufficiently without this RNA editing. Apart from making proteins straight from the genetic information of DNA, there is a mechanism to edit genetic information at the RNA level and make proteins, which is thought to exist in order to respond to factors such as changes in the natural environment and biological tissues. That mechanism for adjusting functions as necessary is considered **RNA editing**. In this RNA editing, biological control relies on a protein called an **RNA editing enzyme**. RNA editing enzymes have the **property of replacing** only specific genetic information on RNA without cleaving like the nucleolytic enzyme Cas9. The replacement of genetic information by this RNA editing enzyme specifically changes a substance called a **base**.

Genetic information is composed of four bases, as shown in Figure 6. DNA is a combination of **adenine (A)**, **guanine (G)**, **cytosine (C)**, and **thymine (T)**, and RNA is a combination of adenine (A), guanine (G), cytosine (C), and **uracil (U)** (when genetic information is copied from DNA to RNA, **thymine (T) is converted to uracil (U)**[17]). These bases are easily affected by biological substances in cells, and when a sugar called ribose attaches to the base, its name changes as shown in the lower part of Figure 6. As shown in Figure 7, the binding partners of the four bases are strictly determined as well. This is called a **base pair**.

Figure 6 DNA and RNA bases

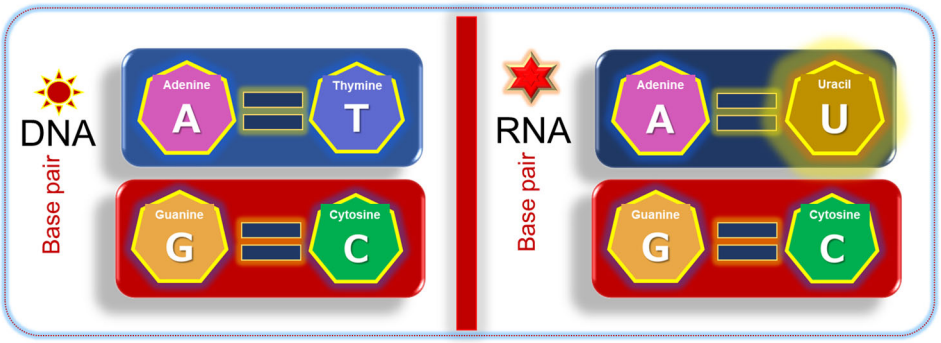


Source: Created by MGSSI

[16] In this report, RNA refers to messenger RNA (mRNA), which carries a copy of the genetic information.

[17] DNA bases may change to other substances due to chemical changes in the organism. In particular, cytosine has the property of being easily converted to uracil (it is known to change frequently), and the DNA repair mechanism works to return uracil to cytosine each time. If uracil is used instead of thymine from among the DNA bases, when cytosine is converted to uracil, the DNA repair mechanism cannot determine whether the substance was uracil from the beginning or cytosine that was changed into uracil, and it becomes impossible to repair. It is thought that thymine, which can be synthesized based on uracil, was adopted as the base of DNA in the process of evolution to solve this problem.

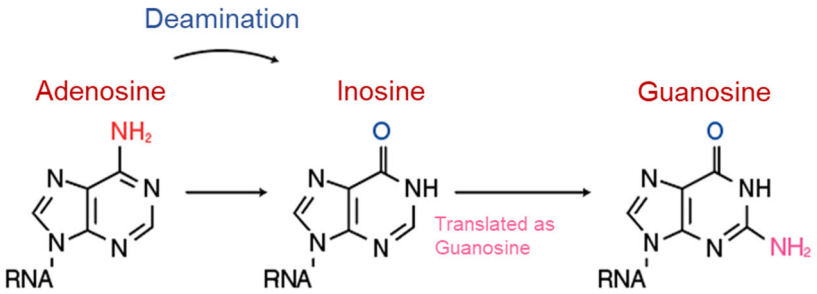
Figure 7 Base combinations (base pairs)



Source: Created by MGSSI

For example, **adenosine deaminase** is an RNA editing enzyme that **converts adenosine (A) in RNA into inosine (I)**. Inosine (I) is a substance that does not appear in the "DNA and RNA bases" of Figure 6, but **adenosine deaminase** changes adenosine (A) to inosine (I) by a chemical reaction (Figure 8). When RNA undergoing this chemical change is transported to the ribosome and the genetic information is read, inosine (I) is translated as guanosine (G) (the chemical structure of inosine is so similar to guanine that it is misidentified when translating genetic information). For this reason, a protein produced is different from the one based on the genetic information of the original DNA.

Figure 8 Chemical reaction of adenosine deaminase



Source: Medical and Biological Laboratories website
https://ruo.mbl.co.jp/bio/product/epigenetics/article/RNA-modification.html

Cytidine deaminase, which converts cytidine (C) to uridine (U), is an RNA-editing enzyme other than adenosine deaminase. Converting just one base using such an RNA-editing enzyme is called **single base editing**. Enzymes that can be used for this single-base editing include **APOBEC,**[18] which belongs to the cytidine deaminase family, and **ADAR,**[19] which belongs to the adenosine deaminase family. Since this single base editing is effective not only for RNA but also for DNA editing, such enzymes are collectively called **base editors (BE)**. Typical bass editors include the **adenine base editor** and the **cytosine base editor** (Figure 9).

Currently, base editor research and development is progressing at a tremendous pace, and more than 50 methods including derivative types have been published.[20] As a unique base editor, **REPAIR** combines an RNA-editing enzyme with **Cas13**, which is a Cas protein. Other than that, there is **RESURE**, which enables the conversion of cytidine (C) to uridine (U). **RESTORE** and **LEAPER**[21] do not require RNA editing enzymes by

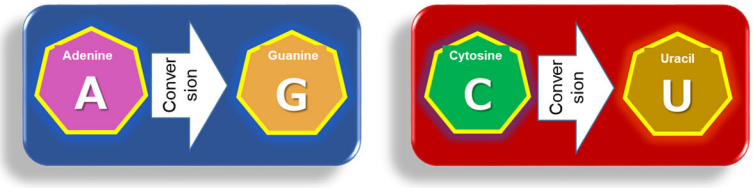
[18] Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like (APOBEC) is called a deamination enzyme. It removes amino (NH^2) from cytidine and converts it to uridine. Eleven types of APOBEC have been confirmed in humans.

[19] Three types of Adenosine Deaminase Acting on RNA (ADAR) have been confirmed in humans, and it is understood that ADAR1 and ADAR2 have editorial activity. Also, about three million adenine (A) → inosine (I) conversion sites have been identified in the human body. (Picardi, E. et al., Sci Rep 2015, 5, 14941.)

[20] CRISPR-Cas9 DNA Base-Editing and Prime-Editing (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7503568/)

[21] The current genome editing technology CRISPR and RNA editing technology have achieved gene editing by utilizing the function of enzymes. RESTORE and LEAPER, which do not require enzymes, are **CRISPR-free editing technologies** that minimize the risk of the off-target effect.

Figure 9 Typical base editors

Single base editing		
Base Editor Architecture		
Name	**Adenine Base Editor** Adenine Base Editor (ABE)	**Cytosine Base Editor** Cytosine Base Editor (CBE) Target-AID
Enzyme	Adenosine deaminase	Cytidine deaminase
Conversion		

Source: Created by MGSSI

giving the function of single-base editing to the guide RNA itself—a guide to the target gene not mentioned in this report (Figure 10). Then, from the research and development on these base editors, there appeared an **almighty editing technology called prime editor (PE)** that can convert all base patterns. The prime editor is said to revolutionize the world of genome editing, and there are research papers[22] stating that, in principle, it can correct 89% of human pathogenic genetic variations.

Figure 10 Main RNA editing technologies

Name	Structure	Conversion	Remarks
ADAR1	Adenosine Deaminase ADAR1 + guide RNA	**A to I conversion** (Adenosine → Inosine)	First RNA single base editing technology
REPAIR	Adenosine Deaminase ADAR2 + dPspCas13	A to I conversion (Adenosine → Inosine)	Successful conversion in human cells
RESURE	Cytosine Deaminase + dRamCas13	C to U conversion (Cytosine → Uracil) A to I conversion (Adenosine → Inosine)	Expansion of application of single base editing
RESTORE	Modified oligoRNA (+ utilizing ADAR2 existing in cells)	A to I conversion (Adenosine → Inosine)	First CRISPR-free RNA single base editing technology
LEAPER	Antisense RNA (+ utilizing ADAR2 existing in cells)	A to I conversion (Adenosine → Inosine)	CRISPR-free Various cell introduction methods

ADAR is present in RNA editing enzyme (Adenosine deaminase acting on RNA) cells

REPAIR : RNA editing for programmable A to I replacement
RESURE : RNA editing for specific C to U exchange
RESTORE : recruiting endogenous ADAR to specific transcripts for oligonucleotide-mediated RNA editing
LEAPER : leveraging editing endogenous ADAR for programmable editing of RNA

Source: Created by MGSSI

The RNA editing technology explained so far is the basic technology for RNA therapeutics[23] described later, and it is thought that it will assist in curing difficult-to-treat diseases radically by editing the genetic information held by RNA and sending it to the ribosome to produce a protein that can be used as a therapeutic agent.

2-2. Application of RNA editing technology: RNA therapeutics

Applications of RNA editing technology include the research field of RNA therapeutics, in which the genetic information of RNA is edited to produce the desired protein. One example is when it is confirmed that a kidney

[22] See "Search-and-replace genome editing without double-strand breaks or donor DNA" published in Nature. The paper reports that human cells were used to correct the genetic mutation of Tay–Sachs disease and sickle cell disease.
https://www.nature.com/articles/s41586-019-1711-4

[23] For a discussion of the differences between RNA therapeutics in general and nucleic acid therapeutics, see "Global Trends in Development of mRNA Therapeutics" [in Japanese] (Pharmaceuticals and Medical Device Regulatory Science).
http://nats.kenkyuukai.jp/images/sys/information/20190717095649-6ABC2FA50410294C82EBEF7D74463510333BCF1FB717B3F864612BCB0CA9F6B2.pdf

disease develops due to the absence or non-functioning of a particular protein. The disease could possibly be cured if RNA is made to carry the genetic information necessary to produce the protein and then sent to kidney cells (ribosomes) to produce the necessary protein.[24] Because RNA therapeutics using RNA capable of retaining genetic information can (in theory) produce any protein, their development is proceeding for a vaccine for individual cancer therapy, virus mutation measures, and a therapeutic agent for the novel coronavirus. More than 20 types of RNA therapeutics have already entered clinical trials[25] overseas.

RNA therapeutics can be expected to have a high therapeutic effect, but the problem is that when the RNA with genetic information comes out of the cell where it is supposed to be and moves in the body with blood or other biological fluid, nucleolytic enzymes detect and capture the abnormality, and decompose it. Countermeasures against this immune function require a drug delivery system (DDS) that protects RNA therapeutics and delivers them to target tissues. Such a system is under development. However, as reported in "Lectins and Exosomes" [in Japanese] (May 2020),[26] the development of DDS using knowledge of synthetic biology and other latest technologies is steadily progressing, and RNA therapeutics may soon be used for actual treatment.

3. MITOCHONDRIAL DNA EDITING TECHNOLOGY

A paper on mitochondrial DNA editing technology, "A bacterial cytidine deaminase toxin enables **CRISPR-free mitochondrial base editing**," was published in *Nature* on July 8, 2020. According to this paper, **DddA** produced by a bacterium called Burkholderia cepacia is used when editing mitochondrial DNA. The original role of DddA is to neutralize bacteria that have invaded a cell. Cas9 attaches to the bacterial DNA and physically cuts it into pieces, while DddA binds to the bacterial DNA that has invaded the cell and converts all cytosine (C) in the DNA to uracil (U) to neutralize the bacteria. In other words, this method stops the growth of bacteria by overwriting the genetic information to make it meaningless.

As mentioned in the section on RNA editing, uracil (U) is originally a substance that does not exist in DNA, and when DddA changes cytosine (C) in all the genetic information to uracil (U), the DNA loses its function. On top of that

, cytosine (C) is a substance that easily changes to uracil (U), and the cell repair mechanism constantly restores cytosine (C) that has changed to uracil (U). DddA destroys the genetic information of DNA while inhibiting the normal functioning of this repair mechanism (incidentally, both Cas9 and DddA play a role in the immunity that defends cells from external harmful bacteria, and genome editing takes advantage of the immune system of cells).

The paper describes the approach as follows: If DddA is used as it is, mitochondrial DNA will be neutralized; therefore, DddA is divided into two and inactivated, and only when these two DddA halves are combined, mitochondrial DNA is changed from cytosine (C) to uracil (U). When the cell senses that a change has occurred in the DNA, it starts repairs by replacing uracil (U), which is an abnormal substance for DNA, with thymine (T). Here it should be recalled that DNA has a double-stranded structure and that it is strictly determined which base the four bases bind to. Cytosine (C) pairs with guanine (G), and adenine (A) pairs with thymine (T). If one DNA base is cytosine (C), the opposite base is determined to be guanine (G). Studies have shown that when DddA converts cytosine (C) to uracil (U), DNA repair results in the conversion to a pair of adenine (A) and thymine (T).

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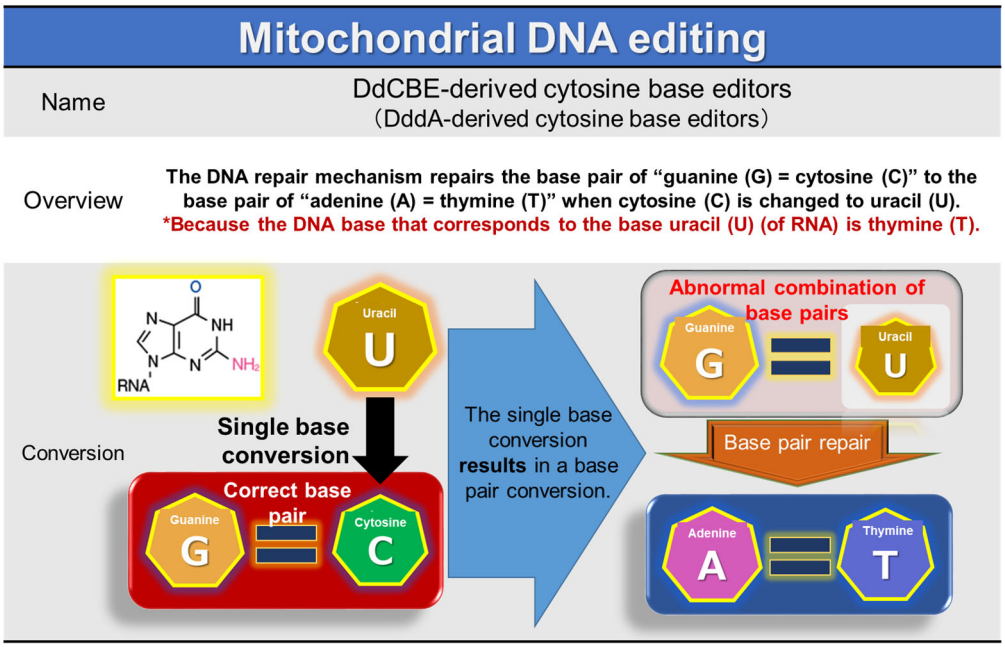
[24] At present, it is possible to artificially synthesize DNA and RNA, and there are companies that manufacture and sell synthetic DNA/RNA by receiving orders from research institutes and others. The technology to artificially write the necessary protein information in the synthesized RNA or edit a part of it and send it to the necessary organs and tissues to produce the protein is becoming feasible.

[25] The US company Moderna started vaccine design after the genome decoding of the novel coronavirus was completed. It takes about 62 days to manufacture an investigational drug for clinical trials. Phase 1 clinical trials of the vaccine mRNA-1273 began on February 24, 2020. According to the company's website, Phase 3 testing has been underway since July 27, 2020 (For more information, search for the identifier: NCT04470427 at ClinicalTrials.gov. Or see the Moderna website https://www.modernatx.com/cove-study).

[26] https://www.mitsui.com/mgssi/ja/report/detail/__icsFiles/afieldfile/2020/05/21/2005t_abe.pdf

This editing technology was named a **DddA-derived cytosine-based editing tool**[27] **(DdCBE)** (Figure 11) by the research team that published the paper. Expansion of its application to others than mitochondrial DNA is being investigated, but since the paper has only just been published, it is necessary to continue research and accumulate reliable results in the future. However, there are great expectations in the groundbreaking technology, particularly in solving the biological mysteries of mitochondria involved in various life phenomena such as energy production and aging. It is also anticipated to open up a new path to the treatment and cure of **mitochondrial diseases** caused by mitochondrial abnormalities.

Figure 11 Base pair conversion in mitochondrial DNA editing



Mitochondrial diseases

Mitochondria are one of the organelles present in almost all cells other than erythrocytes, and produce the energy source (**adenosine triphosphate: ATP**[28]) required for cell activity. Mitochondria are abundant in cells. There are hundreds to thousands of mitochondria in cells particularly active in metabolism, such as the brain, liver, and muscle cells, and it is said that about 10% of adult body weight comprises mitochondria. When mitochondrial abnormality occurs, a group of diseases called **mitochondrial disease**[29] develops (Figure 12). Mitochondrial disease has the characteristic of developing in any organ or tissue, regardless of age. It is an intractable disease that is associated with neurological disorders such as **Alzheimer's disease**[30] and **Parkinson's disease**.[31] The disease is said to occur in one out of every 5,000 babies born, and when it does, the symptoms are diverse and severe, and mortality is high. The cause of this mitochondrial disease is nuclear DNA abnormality in three-quarters of childhood cases and mitochondrial DNA abnormality in the remaining quarter. When it develops in adulthood, this ratio is reversed and mitochondria DNA abnormalities account for three-quarters of cases.

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[27] DddA-derived cytosine base editor (https://www.nature.com/articles/s41586-020-2477-4)

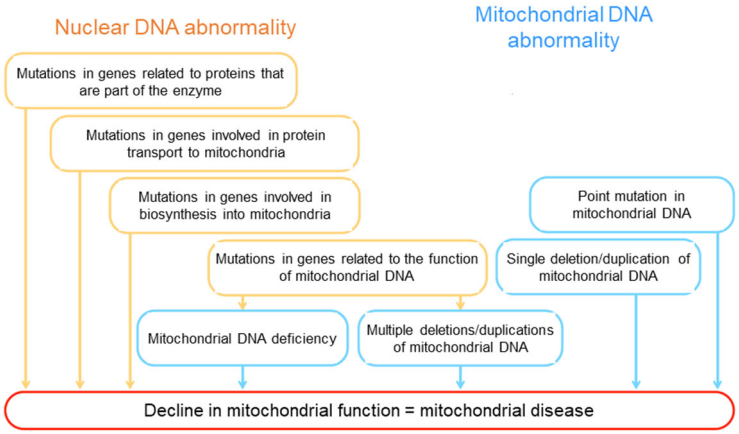
[28] Adenosine triphosphate is a biological substance that is an energy source with three phosphate groups attached to an adenosine molecule (adenine with a ribose sugar attached as in the explanation of RNA editing).

[29] Regarding mitochondrial disease, refer to the website of **KOINOBORI Associate Inc.**, which provides support for establishing effective treatment methods for mitochondrial disease: http://koinobori-mito.jp/. The site provides a list of major mitochondrial diseases and information on clinical trials in Japan and overseas.

[30] "Enhancing mitochondrial proteostasis reduces amyloid-β proteostasis" *Nature* (December 2017) https://www.nature.com/articles/nature25143

[31] Research on the relationship between Parkinson's disease and mitochondria is progressing, and it has been found that mutations in the molecules Parkin and Pink that process mitochondria that have declined in function cause Parkinson's disease. *Nature* (July 17, 2019) https://www.nature.com/articles/d41586-019-02094-6

Figure 12 Causes of mitochondrial disease



Source: National Center of Neurology and Psychiatry Hospital Genetic Counseling Unit "Mitochondrial Disease Handbook" [in Japanese]
https://www.nanbyou.or.jp/wp-content/uploads/upload_files/mt_handbook.pdf

Since the 21st century, advances in analysis technology for biological substances such as DNA and RNA have significantly improved the accuracy and speed of analysis of mitochondrial DNA. Thanks to these technological advances, a new causative gene for **Leigh's encephalopathy**, which often occurs in infants, has been discovered. The main symptoms of Leigh's encephalopathy are developmental retardation, muscle weakness and hypotonia, respiratory disorders, intellectual regression, etc., which are said to occur in one in 50,000 people. It is a particularly intractable disease with a poor prognosis. Analysis of mitochondrial DNA revealed that the cause of Leigh's encephalopathy was a gene called **PTCD3**. PTCD3 is an essential gene for mitochondria to make the enzyme necessary for making ATP. It was found that an abnormality in this gene prevented ATP from being made correctly and caused the disease. On July 6, 2020, Juntendo University also announced that it had discovered **NDUFA8**, the causative gene of mitochondrial disease that causes developmental retardation, microcephaly, epilepsy, and others. It has been identified that an abnormality in NDUFA8 inhibits mitochondrial energy production due to the inability to produce normal proteins. If the causative gene is identified in this way, it is also conceivable to use an above-mentioned RNA therapeutic to make RNA carry information on the NDUFA8 protein and promote the production of the NDUFA8 protein in the ribosome. In the case of seeking a radical cure, one possible measure is to normalize the genes with mitochondrial DNA editing technology so that the PTCD3 and NDUFA8 genes of mitochondrial DNA work normally. Even though this mitochondrial DNA editing technology—which can convert bases without cutting DNA—has just emerged, it is anticipated for the technology to be matured and applied as soon as possible to the treatment of patients suffering from intractable diseases.

4. BEYOND CRISPR

CRISPR appeared in 2012, and that year also saw remarkable achievements in the fields of artificial intelligence (AI) and quantum IT, other than life science. A team led by Professor Hinton of the University of Toronto won the ILSVRC 2012,[32] a competition in the recognition rate of objects, with a machine learning (deep learning) method that surpassed conventional methods, which had a significant impact on the IT industry. This triggered a third AI boom, which raised the general awareness of machine learning and made great strides in social implementation of AI. A research team at Harvard University has used an annealing-type quantum computer from D-Wave Systems to calculate and obtain a solution to the protein folding problem[33] (Miyazawa and

[32] ImageNet Large Scale Visual Recognition Challenge 2012.
http://image-net.org/challenges/LSVRC/2012/index#timetable

[33] Amino acids are linked together and folded to form a complex shape, but the mechanism by which proteins are folded has not been elucidated and is called the "protein folding problem."

Jernigan model[34]).[35] Although perhaps controversial, this is undoubtedly the first example demonstrating the industrial utility value of quantum computers' ability. This was the advent of CRISPR-Cas9, which has opened up a new era in life science. The year 2012 will be remembered for a long time as a year in which innovative technologies such as AI (deep learning), quantum computer, and genome editing appeared one after another and had a tremendous impact not only on academia but also on society.

However, no perfect technology exists in the world. AI and quantum computing are still in the development phase, and there have been shocking events surrounding CRISPR, such as the birth of babies by **Human Genome Editing**, which poses serious problems concerning research ethics, moral norms, and the like. As already mentioned, CRISPR-Cas9 cuts and edits DNA, which is the master copy of genetic information. Consequently, the potential for unintended effects on the body cannot be denied. In addition, although not a technical problem, intellectual property and other rights are complexly intertwined, and caution is necessary in use. Furthermore, there is the problem that cost is high when it is used for commercial purposes. Under these circumstances, there are moves to improve CRISPR-Cas9 and vigorous research and development into so-called CRISPR-free editing technologies, such as RNA editing technology and mitochondrial DNA editing technology that edit the genome without cutting DNA. Among these, interesting new technologies (Figure 13) are appearing one after another, such as **epigenome editing** that controls target genes without severing or base conversion, and the use of **DNA as a storage device** by applying genome editing technology.[36] In this way, the technological trends related to genome editing technology deserve attention.

Figure 13 Genome editing technology

Category	Technology	Name	Notes and remarks
CRISPR technology	DNA editing	CRISPR-Cas9	Genome editing technology using acquired immune function that appeared in 2012
		CRISPR-Cas12a	CRISPR using RNA-dependent endonuclease (Cpf1) that works the same as Cas9
		CRISPR-Cas14a	Overwhelmingly lower molecular weight than CRISPR-Cas9; suitable for delivery to microtissues
		CRISPR-Cas3	The first CRISPR (type 1) using multiple Cas; can also be used for human iPS cell editing
	RNA editing	CRISPR-Cas13a	Cas13a is a Cas protein artificially modified by RNA editing CRISPR
CRISPR improvement technology	PAM-free	SpRY	Realizes editing of all gene regions without the need for PAM, which is the starting point of genome editing
	CRISPR transposon	CAST, CRISPR-Cas12k, CRISPR-Cascade	Highly efficient genetic sequence insertion combining transposon (translocated DNA) and CRISPR
	Local tethering	Cas9-TREX2, 2C-HR-CRISPR	Modification and improvement of homology directed repair (HDR) and microhomology-mediated end-joining (MMEJ)
	Genome self-repair	IHR	Knock-in that does not require foreign donor DNA; genome self-repair by homologous interchromosomal recombination
	Promotion of genetic sequence mutation	EvolvR	Promotes gene mutation (enCas9-Po1I5M has 7.77 million times the mutation rate of natural strains)
CRISPR application technology	DNA virus test	DETECTOR	Uses Cas12a to scan and identify virus-specific gene sequences to determine the presence or absence of disease
	RNA virus test	SHERLOCK	Uses Cas13 to scan and identify virus-specific gene sequences to determine the presence or absence of disease
	DNA & RNA virus test	SHERLOCK v2	Virus testing using Cas12a and Cas13
	DNA storage	GESTALT, scGESTALT	Accumulates mutations made by Cas9 in cells and reads information by sequencing
		TRACE	Uses Cas1 and Cas2 and records the effects on bacteria (heavy metals, sugar metabolism, small molecules, etc.)
		CAMERA	Uses single base substitution (BE) to record on a plasmid etc. in response to a stimulation
		DOMINO	Records information using Target-AID for real-time confirmation without disrupting cells
	RNA storage	stgRNA	Randomly records information by combining self-targeting RNA (stgRNA) and Cas9
CRISPR-derived technology	Single base edit	ABE (Adenine base editor)	Adenosine deaminase (converts adenosine (A) → inosine (I))
		CBE (Cytosine base editor)	Cytosine deaminase (converts cytosine (C) → uracil (U))
		Target-AID	Converts activation-induced cytidine deaminase (AID) cytidine → uridine
		BE3 (Base editor3)	Uses APOBEC1 to convert uracil (U) → cytosine (C)
	Prime editing	PE (Prime Editor)	Innovative almighty editing technology that uses Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) · dSpCas9-MMLV-RT + pegRNA (prime editing guide RNA) · nSpCas9-MMLV-RT + perRNA
	Epigenome editing	SunTag, SAM	Gene expression control by combining dCas9 such as DNA methylase and histone modifying enzyme
		Adenosine deaminase ADAR1	First RNA single base edit (October 2012)
		REPAIR	Adenosine deaminase ADAR2 + CRISPR-dPspCas13
		RESURE	Cytosine deaminase + CRISPR-dRamCas13
CRISPR-free technology	RNA editing	RESTORE	Modified oligoRNA (RNA editing is realized by guide RNA alone)
		LEAPER	Antisense RNA (RNA editing is realized by guide RNA alone)
	In vivo editing	AAV－HDR	A technology that enables genome editing of non-proliferating cells using adeno-associated virus (AAV). Possibility of genome editing therapy for human genetic diseases
RISPR-free technology & RNA-free technology	Mitochondrial DNA editing	DdCBE (Ddda-derived cytosine-based editing)	Mitochondrial DNA single base editing using Ddda toxin produced by Burkholderia cepacia, a glucose non-fermentative gram-negative rod

Source: Created by MGSSI

[34] The Miyazawa and Jernigan model is a computational biology model that shows how proteins are shaped by the combination of amino acids, taking into account the unique interactions between all 20 amino acids.

[35] *Nature* (August 13, 2012) https://www.nature.com/articles/srep00571

[36] See Emerging Technologies (Page 137) or the Mitsui & Co. Global Strategic Studies Institute "Technology Foresight Book 2020."

Reference: Main Cas Proteins

Cas protein	Function
Cas 1	When a virus invades a cell, Cas1 forms a complex with Cas2, cleaves the DNA of the virus, inserts the DNA of the cell into the CRISPR site, and records it (**immunological memory**). Immunological memory is separated by repetitive sequences. Recognizes the **PAM (Proto-spacer Adjacent Motif)** sequence in genome editing.
Cas 2	Forms a complex with Cas1 and attaches to the virus, cleaving DNA/RNA. Recognizes PAM.
Cas 3	Cas3 is used in the **genome editing technology CRISPR-Cas3 developed by Osaka University**, and is an editing technology with almost no off-target. Unlike Cas1/Cas2, Cas3 cleaves DNA/RNA into pieces.
Cas 4	The viral DNA fragment excised by the Cas1/Cas2 complex is molded into the CRISPR site and inserted in the correct direction. Cas4 has the function of unraveling double-stranded DNA and also breaking down single-stranded DNA.
Cas 5	Cas5 forms **Cascade (CRISPR-associated complex for antiviral defense)** by binding to **crRNA (CRISPR RNA)** biosynthesized from the CRISPR sequence by Cas6 as part of the multi-subunit with Cas1 & Cas2 / Cas6 / Cas7 / Cas8, etc.
Cas 6	Cas6 is a **CRISPR-derived endoribonuclease** that cleaves precursor-crRNA transcribed from the CRISPR site that retains immunological memory. Cas6 is a **pre-crRNA biosynthetic enzyme**.
Cas 7	Cas7 is a unit that makes up Cascade. Cas7 retains the double-stranded structure of the DNA unraveled by Cas4, and maintains the **crRNA:DNA bent structure**, which bends every 6 bases and winds around the center of the molecule in a spiral form.
Cas 8	Cas8 is a unit that makes up Cascade and recognizes PAM.
Cas 9	Cas9 forms a **guide RNA (gRNA) and Ribonucleoprotein (RNP) complex**. The target site is recognized by collating the DNA sequence with the gRNA, and the DNA is cleaved within 3 bases from the PAM (NGG) sequence.
Cas 10	Cas10 is a complex containing DNA polymerase and nucleotide cyclase. Research is progressing on **CRISPR-Cas10** for the genetic manipulation of phages, the most abundant entity in the biosphere. Pathogenic phage and other **prokaryotic genome editing technology**.
Cas 11	Constitutes an **RNP complex** (recognizing a target sequence). The main subunits are Cas5 and Cas7, and Cas11 is a small subunit.
Cas 12a	Cas protein developed for genome editing, also known as Cpf1. Cas9 cleaves double-stranded DNA vertically, while Cas12a creates a cut surface 5'-end. One of the platforms for editing the human genome along with Cas9 and Cas12b.
Cas 12b	One of the human genome editing platforms. Since it is smaller than Cas9 and Cas12a, it may be a tool for delivery to human cells by a viral vector using adeno-associated virus etc.
Cas 13	**RNA-edited Cas protein** (Cas13a, C2c2). Subtypes of Cas13 include Cas13a, Cas13b, Cas13c, and Cas13d. Technologies using Cas13b include **A-to-I base substitution methods REPAIR and SHERLOCK**.

Source: Created by MGSSI