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Series

CRISPR-Cas Genome editing in medicine

VIB

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Summary

Genome editing is an umbrella term encompassing a series of revolutionary technologies for making changes to the genetic material of living things quickly and precisely. It is possible to do this - in principle - in the hereditary materials (DNA) of plants, microbes, animals and humans. Using these technologies, scientists can very specifically change a single DNA letter, replace a series of DNA letters with another series or switch a well-defined gene on or off.

Genome editing has been propelling research in the life sciences for several years now. This is mainly due to one very successful form of the technology: CRISPR-Cas (pronounced 'Crisper-cas'). In 2015, the journal *Science* declared CRISPR-Cas to be the scientific breakthrough of the year.

The system has two components: a 'scissor' and a 'guide'. Cas is the molecular scissor. It is a protein that cuts DNA into pieces. However, Cas only cuts at a place in the genome where it is directed by a CRISPR molecule. CRISPR consists of an RNA molecule.

Although scientists have only recently discovered the CRISPR-Cas system, it is not really new as bacteria have been using it for a long time to protect themselves against viruses. So rather than being a human invention, CRISPR-Cas was actually devised by nature millions of years ago. With a few modifications, people can now use this system to perform deliberate genome editing.

Just like other genome editing techniques, CRISPR-Cas lets scientists make very precise changes to DNA without having to incorporate 'foreign' genes in the process. CRISPR-Cas differs from these earlier genome editing methods mainly in being cheaper, faster, more efficient, and more versatile.

This mechanism is now being used in countless laboratories around the world. Its use has spread beyond basic research because it has proved to be a very sophisticated tool for gene therapy and crop improvement. Its first applications in medicine and agriculture are therefore well established.

Although this VIB dossier focuses on how CRISPR-Cas technology has already found applications in biomedical research, it also looks at areas where its potential is just emerging.

The dossier is written to be accessible. You don't need to be a specialist in molecular genetics to find something of interest here - just a desire to learn and become better informed. The boxes are written for those who want to learn about the underlying technology in more detail or find out about applications or notable themes.

Facts and figures

DNA is found in the nucleus of each cell. It carries hereditary characteristics and holds the instructions for what the cell is and can do. The whole of the DNA in the cell is called the 'genome'. We call an instruction a 'gene' (see Figure 1 on page 8).

The genome is a sequence of DNA building blocks or DNA 'letters'. The genome of, for example, the intestinal bacterium Escherichia coli *consists of a sequence of about 3 million DNA letters. The human genome has 3.2 billion of these letters.*

*But humans don't hold the record in that field. In the animal kingdom, the axolotl (*Ambystoma mexicanum*), a salamander that lives in parts of Mexico, takes first prize with a genome of 32 billion DNA letters, which is 10 times more than a human. But that pales in comparison to the Japanese canopy plant (*Paris japonica*), which has 150 billion DNA letters. The Japanese canopy plant is a flower that grows in sub-alpine areas of Japan. It is unclear why the canopy plant has so much DNA.*

Genes are first copied to RNA and then translated into proteins. In addition to a structural function in the cell, proteins also have a role in chemical conversions, transport of biomolecules, communication and regulation.

DNA is stable. Nevertheless, the sequence of DNA letters can change permanently. We call this a mutation. Mutations can occur naturally in every gene, at any time and in every cell. Mutations can also be made intentionally by humans - for example by irradiating the cell or by genome editing

The word mutation has a negative connotation. Mutations can change the function of a gene. This can be for the worse: an accumulation of mutations can lead to cancer in humans and mutations are at the basis of hereditary diseases. However, the function of a gene can also be improved by a mutation. The fact that many of us, for example, can digest the nutrients in cow's milk is the result of a mutation that has occurred in one or more of our ancestors. Mutations are therefore not always detrimental. Moreover, many mutations have no effect whatsoever on the function of a gene. We call these 'neutral' mutations.

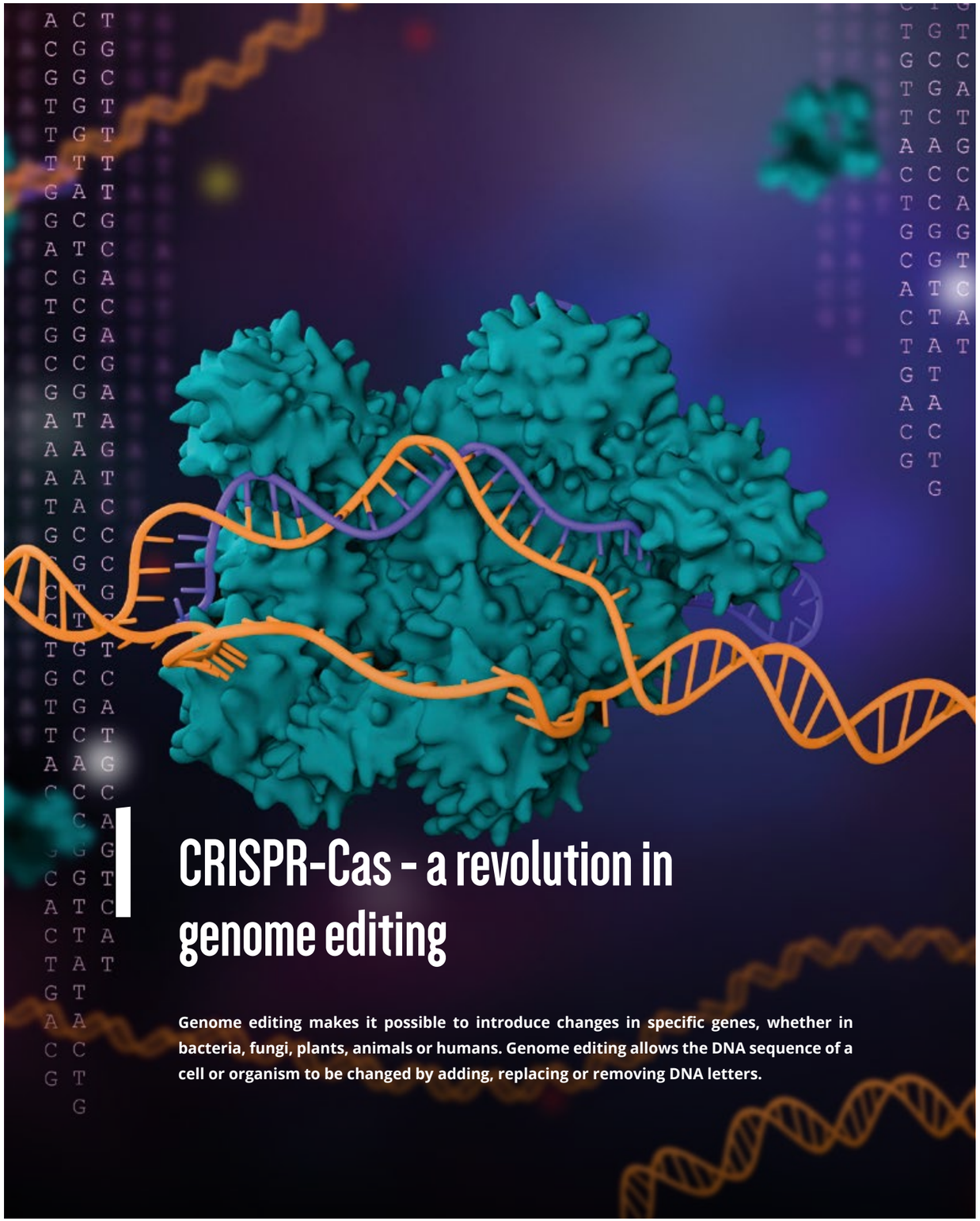
Mutations also create variation within a species. They are essential for living organisms because without mutations there would be no evolution and therefore no biodiversity. There is a delicate balance between DNA stability and evolution.

Genome editing is a method for making one or more mutations in a targeted way and at a predetermined location in the genome.

The fact that genome editing has become widespread in recent years is mainly due to one very successful form of technology: CRISPR-Cas. CRISPR-Cas makes it possible to modify DNA with unprecedented precision and efficiency.

In 2015, the journal Science *called CRISPR-Cas the most important scientific breakthrough of the year. The technology was developed from the CRISPR-Cas system that bacteria use to defend themselves against viruses.*

In CRISPR-Cas, CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats and Cas for Crispr associated protein.



CRISPR-Cas – a revolution in genome editing

Genome editing makes it possible to introduce changes in specific genes, whether in bacteria, fungi, plants, animals or humans. Genome editing allows the DNA sequence of a cell or organism to be changed by adding, replacing or removing DNA letters.

Bacteria protecting themselves against viruses

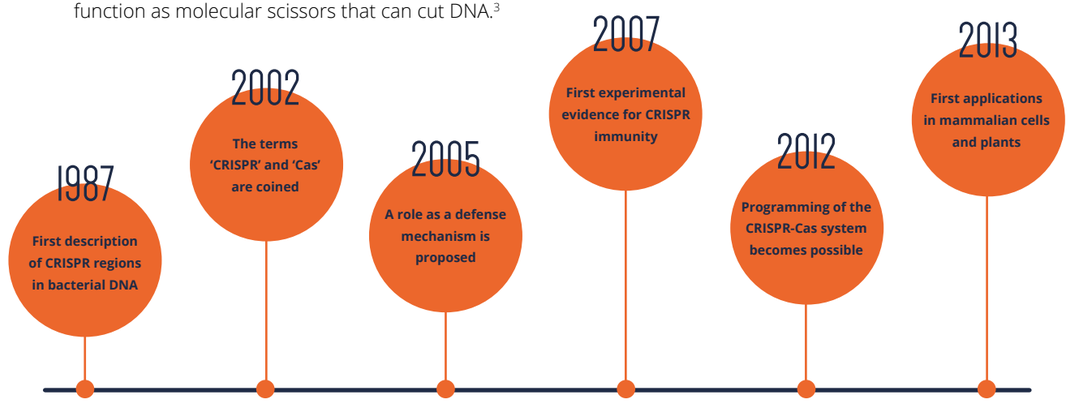
The CRISPR story starts in a bacterium. The initial discovery of CRISPR sequences was announced in 1987 by scientists from Japan who investigated the genome of the bacterium *E.coli*. They identified five identical pieces of DNA that repeated and were separated by non-repetitive DNA sequences of identical size. At that time, these DNA repetitions were seen as a curiosity. After all, they could not be explained.

However, as scientists examined the genomes of more and more bacterial species, they kept seeing these same repeated DNA sequences. These species included the bacteria used to make cheese and yoghurt and the bacteria that naturally occur in our gut. Since then, it has been found that more than half of all bacterial species have CRISPR sequences.[1]

The finding that these regular DNA repetitions always occur together with a common group of genes (CAS genes) only deepened the mystery. In 2002 a team of Dutch microbiologists decided to call the region of DNA with these repeats 'CRISPR', which is an acronym for *'clustered regularly interspaced short palindromic repeats'* and called the associated genes 'CAS' genes, which is short for CRISPR-associated genes.[2] It quickly became clear that the proteins the CAS genes code for function as molecular scissors that can cut DNA.[3]

In 2005 further research showed that the DNA sequences between the repeats are almost identical to the genetic material of viruses - known as bacteriophages - that infect bacteria.[4 5 6] The CRISPR region thus appeared to be a kind of library of viral DNA fragments that the bacterium had built into its own genome. It was then suggested that CRISPR-Cas was a system for protecting bacteria against bacteriophages. The bacterium collects DNA sequences from invading viruses and uses them, in combination with Cas proteins, to cut the DNA of these same viruses and render them harmless (see Figure 2).

In 2007, scientists were for the first time able to demonstrate experimentally that a bacterium - specifically the yoghurt-making bacterium *Streptococcus thermophilus* - uses the CRISPR-Cas system as a defense against viruses.[7] Repeated exposure of the bacteria to a virus caused them to become resistant over time. This resistance was accompanied by the inclusion of fragments of viral genetic code in the CRISPR region of the bacteria's DNA. When the scientists removed the viral sections from this CRISPR region, the resistance disappeared immediately.



Various CRISPR-Cas systems have been identified over the years and, although these systems have different characteristics, the mechanism is always the same: RNA is read off the fragments of DNA in the bacterium's CRISPR library. These pieces of CRISPR RNA then go off in search of viral genes to bind to. Next, the Cas protein, guided by the CRISPR RNA sequence, cuts the viral gene into pieces (see Figure 2). The collection of fragments of virus DNA therefore serves as a kind of memory. This allows the bacteria to quickly recognize and fight off the virus the next time it attacks.[8 9 10]

THE GENOME GOVERNS THE CELL FROM THE NUCLEUS

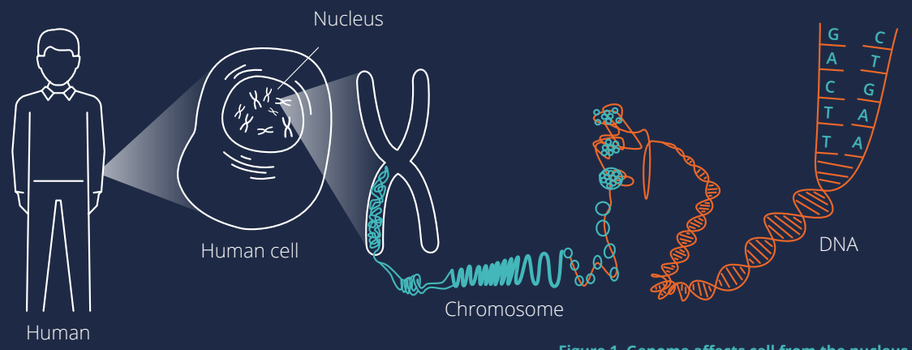


Figure 1. Genome affects cell from the nucleus

The nucleus of each cell contains the 'DNA', the carrier of hereditary information that holds the instructions for what a cell is and what it can do. The whole of the DNA in the cell is called the 'genome'. The human genome has 3.2 billion DNA letters.

DNA - a double-stranded molecule in the shape of a helix - is packed in the core in a number of 'chromosomes'. Every cell in our body has 2x23 chromosomes or 2x23 'packets of DNA'. From each of these double packages, one chromosome comes from each of our biological parents. If these chromosomes were to be unrolled and laid end-to-end, they would form a thread about two meters long with a diameter of 2 nm (nanometers), or 2 millionths of a millimeter.
As soon as a cell divides, each daughter cell receives the complete genome - all the DNA packets - from the parent cell. That requires a great deal of copying.

The DNA letter code consists of 4 building blocks represented by the letters A, T, C and G. A letter A on one DNA strand will always be paired with the letter T on the other strand, and vice versa. The same is true for the letters C and G. So when we read one strand, we also know the letter order of the other - complementary - strand.

A sequence of DNA letters encoding an 'instruction' is called a 'gene'. Very often this instruction is the recipe for a 'protein'. In other words, the DNA code, or the gene, is read and translated into a protein via an RNA molecule. Proteins are important in forming the structural parts of the cell, but also perform biochemical tasks. They allow a cell to convert nutrients into energy, a muscle cell to contract, a nerve cell to transmit electrical signals, a salivary gland cell to secrete saliva, etc.

But in reality, only a small part of the human DNA actually codes for proteins. The rest of the DNA is important for the regulation of gene expression, the copying of the DNA, the preservation of the structure of the DNA and the chromosomes, etc.

Every now and then a mistake in the DNA copying occurs. We call such a mistake a 'mutation'. A mutation in a gene can lead to a defective protein. Often, however, a mutation does not lead to a change in the protein. These mutations increase the variation within a species and leave it better able to adapt to changing circumstances and natural selection.

CRISPR-CAS - USED BY BACTERIA TO FEND OFF VIRUSES

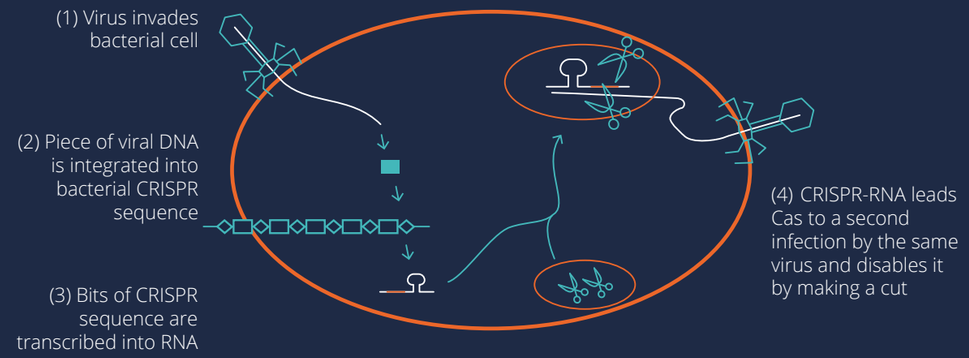


Figure 2. CRISPR-Cas - used by bacteria to fend off viruses

Viruses consist of a protein coat containing genetic material. A virus multiplies by introducing its genetic material into a cell (for example, a bacterium) and using the synthesis mechanisms of that cell to produce new viruses that go on to infect other cells.

Each time a bacterium is attacked by a virus (1) but survives the attack, the bacterium stores a piece of the virus DNA in its own DNA, specifically in the CRISPR library (2). The bacterium translates this library into CRISPR RNA molecules (3) that guide the Cas proteins to new incoming viruses that the bacterium recognizes. Cas then cuts up the viral DNA and, in this way, repels the viral attack (4). (Figure based on reference 11)

Researchers learn from bacteria

Targeted cutting …

The great breakthrough in using CRISPR-Cas as a technology for manipulating the genome of microbe, plant or animal, came in 2012 when a number of researchers - Jennifer Doudna (UC Berkeley in the US), Emmanuelle Charpentier (then at the University of Umeå in Sweden, now at the Max Planck Institute in Germany) and Virginijus Siksnys (University of Vilnius in Lithuania) - independently demonstrated that you can 'reprogram' the CRISPR-Cas complex. By modifying the sequence of the CRISPR RNA molecule, the complex can be made to cut at any desired location in the genome. For this, the sequence of the CRISPR RNA needs to match the DNA sequence where the cut is to be made.[8 9 10]

Shortly thereafter, in 2013, five independent research teams, including Feng Zhang and his colleagues from the Broad Institute (MIT, Boston, USA), showed that the CRISPR-Cas system can also be used to change the DNA in human cells, mice and zebra fish.[11 12 13 14 15] The use of CRISPR-Cas in mammalian cells was a pivotal moment in genome editing. This was quickly followed by countless publications where the system was used in different organisms and for different purposes. Later that year (in August 2013) five research articles were published that discussed the use of CRISPR-Cas in plants.[16 17 18 19 20]

CRISPR-Cas is not the only molecular technology for editing the genome. Several techniques were developed that either use molecular scissors other than Cas9 (Cas12, previously called Cpf1, Cas13 previously called C2c2, etc.) or are based on another mechanism, such as oligonucleotide-directed mutagenesis, TALEN technology, or ZFN technology. However, this goes beyond the scope of this dossier. For an overview, see the VIB dossier 'Van plant tot gewas: het verleden, heden en de toekomst van plantenveredeling' (From plant to crop: the past, present and the future of plant breeding').

… and letting nature tie up the loose ends

Breaks in DNA are harmful. Because of this, living organisms have natural DNA repair mechanisms that detect and repair breaks. And this applies just as well to the breaks caused by Cas. When Cas has made a cut somewhere, one of two natural DNA repair scenarios can occur: 'non-homologous DNA end joining' and 'homology-directed DNA repair'. CRISPR-Cas technology can use both mechanisms.

In 'non-homologous end joining', the cell uses specific proteins to 'glue' the two ends of the DNA break back together. However, this process is error-prone and often leads to random mutations at the site of repair, where one or a few DNA letters often disappear. This can switch off the function of the gene. Often, however, that is exactly the intention of the researcher (see the next section).

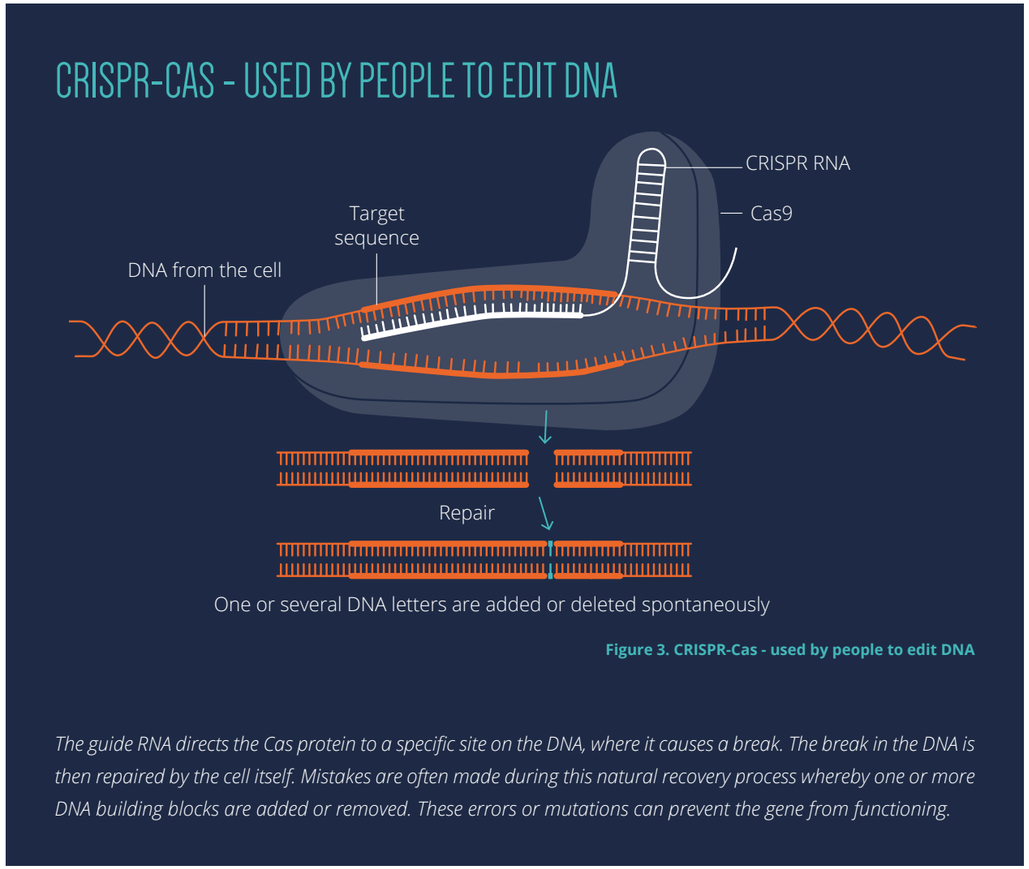


Figure 3. CRISPR-Cas - used by people to edit DNA

The guide RNA directs the Cas protein to a specific site on the DNA, where it causes a break. The break in the DNA is then repaired by the cell itself. Mistakes are often made during this natural recovery process whereby one or more DNA building blocks are added or removed. These errors or mutations can prevent the gene from functioning.

When the cell uses a different DNA sequence as a template for repairing the break, this is called 'homology-directed DNA repair' or 'homologous recombination'. For this, however, the ends of this extra piece of DNA must largely resemble the DNA sequences around the fracture. The break is then repaired by replacing the break region with the help of the DNA piece you provide to act as a template. With this, the DNA around the break is restored to its original state, or a change can be deliberately built in, depending on the template that you provide (see Figure 3).

2 CRISPR-Cas speeds-up research

Various techniques for making changes to DNA have long been available. What makes CRISPR-Cas so revolutionary is that it makes it very cheap and easy to make specific, targeted changes to the genome of plants, animals and humans. Researchers working at universities and companies have therefore started to work with CRISPR-Cas on a massive scale.

The human genome project

All around the world, scientists are trying to decipher molecular mechanisms and life processes in viruses, bacteria, plants, animals and humans. They want to show how everything works and how different genes, proteins and biological processes intertwine. This basic research provides insight into the origins of diseases such as cancer, brain and nerve diseases, cardiovascular diseases, inflammations and infections. Over time, this knowledge leads to new medicines, vaccines, diagnostic tests and treatment methods (see, amongst others, the VIB Fact Series 'Alzheimer' and 'Cancer').

An important element in this fundamental biomedical research is the reading, decoding and mapping of the human genome. This titanic work began with the Human Genome Project and it took more than 10 years to read the first human genome from A to Z. Since then, the genomes of many tens of thousands of people have been mapped. This is becoming faster and cheaper thanks to the introduction of new techniques and reading machines. But simply being able to read a genome and decipher all 25,000 human genes does not mean that you fully understand the human genome. The challenge today is to discover the functions of not only those genes, but also those of the proteins or other biomolecules that they encode. However, it is not only coding sequences that are important. Other genome regions may play a role in the regulation of genes, the structure and stability of chromosomes, the duplication of DNA and so on. These areas are also being intensively investigated.

Investigating the functions of genes

With CRISPR-Cas, scientists can identify the function of a gene or particular DNA sequences much more quickly than before. CRISPR-Cas, after all, lets them switch off the relevant gene and see which characteristics of the cell or organism are affected by this.[21] In addition, multiple genes can be studied at the same time by switching them off simultaneously. Similarly, the sequence of non-coding regions can be changed and the function of those changes on the cell examined. A few examples will show how this works.

The CRISPR-Cas approach has been used several times in cancer cell cultures and in laboratory animals for identifying genes that cancer cells need to survive.[22] [23] [24] [25] Recently, researchers from the British Wellcome Sanger Institute conducted an extensive CRISPR-Cas screening on 339 cancer cell lines from the same number of cancer patients. It involved more than 30 different types of cancer, from colon cancer to breast cancer to pancreatic cancer. They switched off a total of 18,000 genes via CRISPR-Cas (almost every known gene) in each of the 339 cell lines. Then, for each of the switched-off genes, they looked to see whether the cancer cells died or continued to divide. This yielded a list of many hundreds of genes that turn out to be essential for the survival of cancer cells.[26] Each of these genes represent a potential target for the development of new anti-cancer drugs. You can see these genes - and/or the proteins they encode - as switches that stimulate the growth of cancer cells. By developing new drugs that act on these switches, it might be possible to influence the survival of cancer cells. In a similar way, an extensive screening with CRISPR-Cas showed why the tumors of some patients turned out to be resistant to immunotherapy.[27]

Analyses of this kind are also used to detect proteins that are essential for infectious diseases such as AIDS.[28] The virus responsible for AIDS - HIV - recognizes and infects human white blood cells by means of specific receptor proteins that are present on the surface of host cells. Identifying these 'host factors' can lead to new strategies for preventing and treating HIV infections. This is shown by, amongst other things, some recent Belgian research.[29] Similar approaches are used to study other infectious diseases.

HOW ARE MUTATIONS MADE IN PRACTICE?

How can CRISPR-Cas be used in practice to make changes in the genome? The desired sequence for the CRISPR RNA can be created quickly and cheaply - for example, by ordering it online from one of the countless DNA and RNA production companies. The CRISPR RNA molecules are then delivered to the laboratory by a courier service. Just like we order shoes, clothes, books or office supplies online.

The supplied CRISPR RNA is inserted into the cells together with Cas proteins, after which a mutation is made at a location in the genome identical to the CRISPR guide RNA. The cells with a mutation are then selected and grown in culture.

There are also 'do-it-yourself' CRISPR-Cas-kits available (http://www.the-odin.com/diy-crispr-kit/) that let people experiment with CRIPSR-Cas in bacteria at school or in the classroom.

The use of the kit outside of a laboratory is permitted in Belgium, provided that certain regulatory obligations of environmental legislation are observed (see also https://www.standaard.be/cnt/dmf20190620_04470564).

Producing disease models

Cell lines and experimental animals

To gain an understanding of diseases, scientists work with 'disease models'. These are cells in culture or laboratory animals in which a human disease is simulated - for example, by means of genetic modifications. The laboratory animals commonly used for this are mice, rats and zebrafish. Disease models are needed in order to study the development and progression of a disease and, in a later phase, to test drugs and other interventions. This knowledge is essential for developing new methods to prevent, diagnose and treat diseases. The great majority of existing medicines were developed with the help of these models.

Many thousands of such disease models have been made worldwide, especially in cell lines but also in mice. The procedure for making these models had remained virtually unchanged over the past twenty years. Thanks to CRISPR-Cas, however, it is now possible to create animal models for studying human diseases much faster and more efficiently. By injecting embryos with the CRISPR-Cas components, precise genetic changes can be made directly in the genome of the experimental animal (see box 'Experimental animals in biomedical research', page 16). This was how in 2013 an American-Chinese research team became the first to succeed in making mice with multiple mutations in one step. It used to take years to make these, but thanks to CRISPR-Cas it could be done in a few weeks.[31]

The technology was also used by the same research team to turn genes associated with a certain type of cancer on and off.[32] Because cancer is often caused by multiple genetic changes, CRISPR-Cas is very useful for simulating these different mutations in an animal. In this way, you can approximate the complexity of a tumor in an animal, which simulates the situation of tumors in a person much more closely than would be possible with cell cultures.

However, the use of genome editing techniques to generate animal models for a human disease is by no means limited to cancer. Using a combination of advanced genome editing tools, researchers were able to develop pigs with Huntington's disease.[33] These pigs display not only the selective brain cell death seen in patients with Huntington's disease, but also a wide range of other symptoms observed in conjunction with this condition. Neurodegenerative diseases, such as Huntington's disease, are particularly difficult for researchers to study because it is not possible to take samples from the damaged organ, which in this case is the brain. In contrast to, for example, liver, kidney, lung or blood diseases. That is why laboratory animals remain very important for studying brain diseases (see box 'Experimental animals in biomedical research').

EXPERIMENTAL ANIMALS IN BIOMEDICAL RESEARCH

Necessity

Biomedical researchers do not always use experimental animals. They often carry out experiments in test tubes or on cell cultures (called in vitro *research). Increasingly, they even make use of computer models (*in silico *research), whether it involves clinical studies, epidemiological or care-oriented research. Despite this, research on laboratory animals (*in vivo *research) is not only important, but even inevitable if we want to understand diseases and develop effective treatments.*

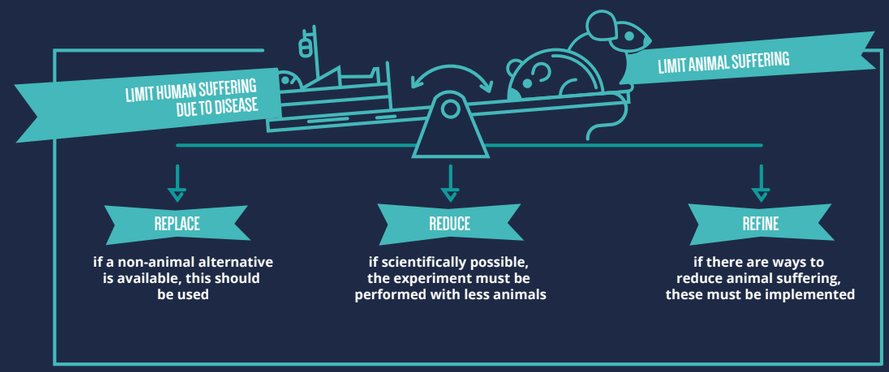
There are important medical and scientific questions that doctors and researchers can only answer through research on living animals in which all complex interactions take place between cells, tissues and organs (a number of examples can be seen on the previous pages).

However, it cannot be emphasized enough that research with laboratory animals is now one of the most heavily regulated research activities. It is often thought that this research is performed on monkeys, cats and dogs, but the most commonly used laboratory animals are, in fact, mice, fruit flies and zebra fish, which are grown specifically for research. The animals are housed under the best conditions. Their welfare is even registered individually (e.g. in mice) and monitored. Obviously, this leads to a significant additional expense for the laboratories. This is one more reason why researchers are as 'economical' as possible with laboratory animals and only use them when there really are no alternatives.

Well-considered

Researchers conduct experiments on animals only after thorough consideration. For every new project, they carefully weigh the use of laboratory animals against its importance for human health. In addition, maximizing animal welfare sits at the top of the list of priorities:

- *Researchers can only work with laboratory animals if they have received education and training in animal welfare and the ethical use of laboratory animals in experiments.*
- *Animal experiments can only be started if they have the approval of the 'animal research ethics committee' of the university concerned. To get this approval, researchers need to outline the case for why they need animals for the research, describe in detail the experiments to be carried out, say how many animals will be used (and why that number is needed) and demonstrate that the experiments have not already been performed.*
- *Researchers are expected to strictly apply the 3R principle: replacement, reduction and refinement of animal experiments. In concrete terms, this means that they must strive to replace animal experiments as far as possible with experiments in test tubes, cell cultures or computer models. Furthermore, they must limit the number of test animals to an absolute minimum and perform the experiments in such a way that animal suffering is reduced as far as possible and so that animal welfare is maximized. The experiential animals used must also be those with the lowest possible level of consciousness: if an experiment gives the same results in fruit flies as in mice or rats, the researchers must invariably use the fruit flies.*



Well-considered animal research therefore has its place in the search for solutions for cancer, cardiovascular diseases, brain diseases and many other disorders. This is a position also held by leading patient organizations and organizations that support research (see also VIB Facts Series Animal Testing).

iPS cells

In addition to animal models, researchers also use cell lines to study diseases. A fairly recent innovation in cell line research is the use of 'iPS' cells - an abbreviation for 'induced pluripotent stem cells'. Researchers make iPS cells by reprogramming body cells (such as skin cells) from an adult human into stem cells. These stem cells can divide in an unlimited way and are able to develop into different cell types. This makes them extremely attractive for use in disease modelling.

To understand this, we need some background in developmental biology. As a fertilized egg develops into an adult individual, different genes are systematically activated and deactivated as cells differentiate. A skin cell differs from a liver cell because it has activated a different set of genes. And different genes are active in a brain cell than in a muscle cell. Once differentiated, human cells usually lose their ability to retrace their steps. In other words, a skin cell will always retain the characteristics of a skin cell, even if you culture it in the laboratory. It will never regain the ability to transform into a nerve cell or a heart muscle cell.

Unless you succeed in reactivating certain genes, such as Oct4, Sox2, cMyc and Klf4. It is then possible to turn a skin cell into a 'pluripotent stem cell' (iPS cell) and, if certain growth factors are added, for it to grow into any desired cell type. By making a patient's body cells pluripotent in this way, the molecular basis of the disease can be examined in a culture dish. So you can, for example, study brain cells from a Parkinson's patient without taking a brain biopsy. A few skin cells can suffice.

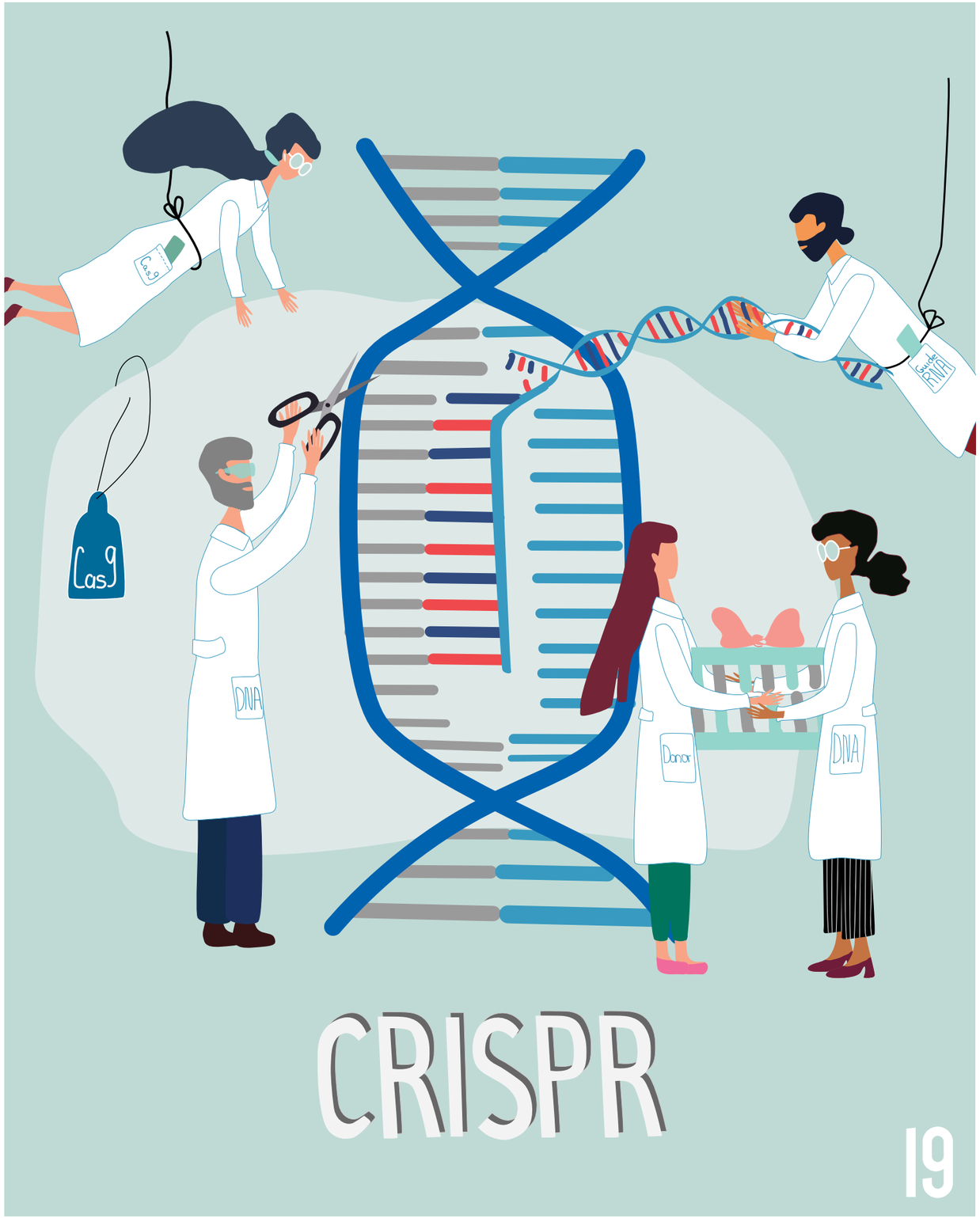
This ground-breaking technology was recently combined with CRISPR-Cas to create tailor-made iPS cells. Researchers recently succeeded in making CRISPR-Cas-altered iPS cell lines to study diseases such as amyotrophic lateral sclerosis (ALS)[34] and Alzheimer's disease[35], GM1-ganlgiosidose[36] (a biochemical storage disorder that can be fatal in children), and hereditary deafness.[37]

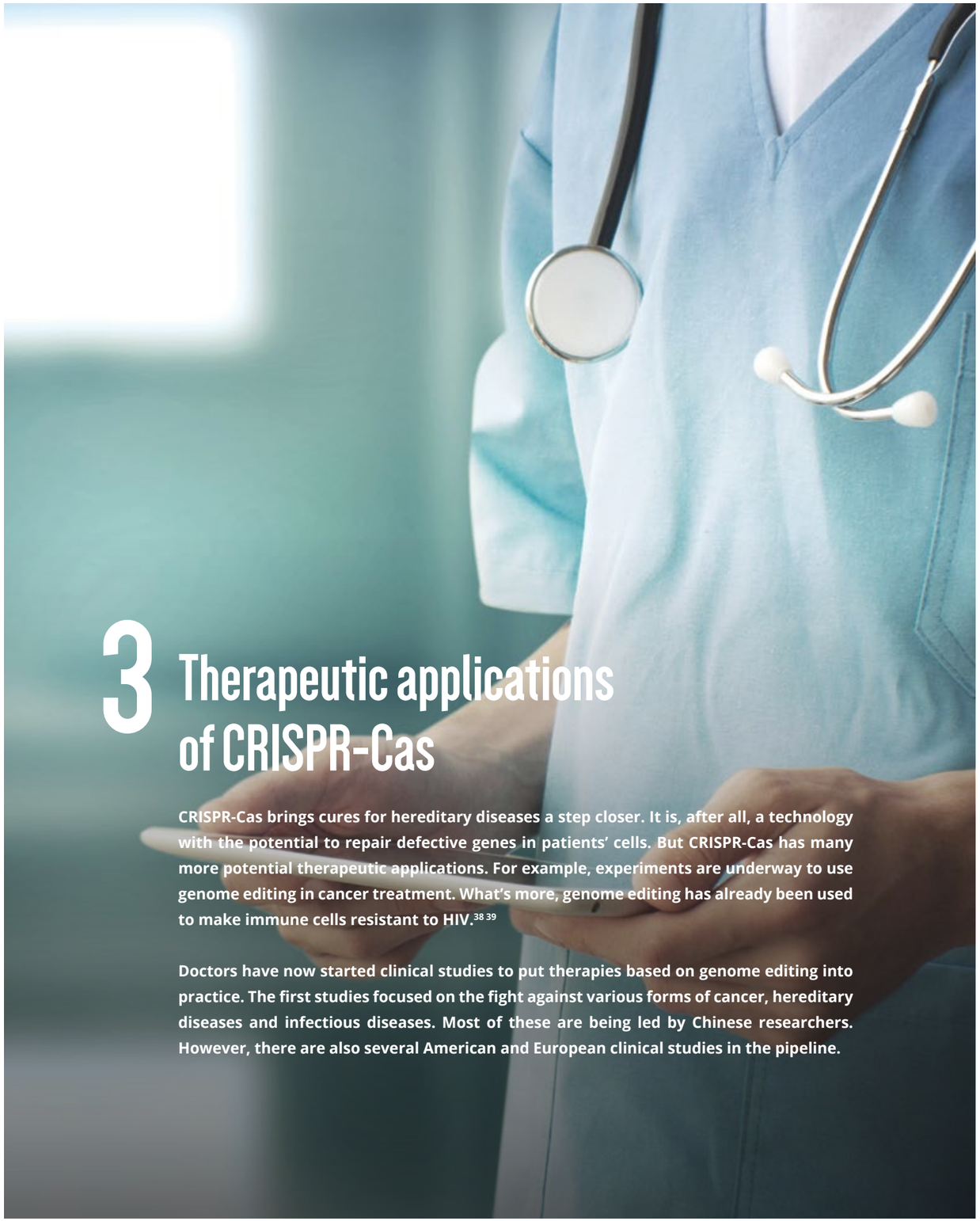
LEGAL WRANGLING

CRISPR-Cas is the subject of a major patent dispute. Various research groups and companies claim that they made an important contribution to the invention CRISPR-Cas and its use as tool for editing genomes. This has created a complex patent landscape, with contradictory arguments about ownership, infringement, and the legality of patents.

Shortly after Jennifer Doudna and Emmanuelle Charpentier showed in 2012 that CRISPR-Cas could be used to edit DNA[10], they applied for a patent on the technology at the American patent office (on 25 May 2013). The patent office, however, granted a patent to a competitor, namely Feng Zhang. He hadn't submitted his application until October 2013 but used a faster procedure. Zhang had published the first use of CRISPR-Cas in eukaryotic cells in 2013.[13] Since then, even more researchers have claimed to have been the inventors, including a Lithuanian team led by Virginijus Siksnys (University of Vilnius, Lithuania) and Luciano Marraffini from Rockefeller University (USA). In addition, hundreds of patents on the use of CRISPR-Cas for specific applications had already been submitted. This has made the patent situation surrounding CRISPR-Cas very complex.

The stakes involved in the patent dispute are high: whoever gets a patent on CRISPR-Cas can determine whether (and, if so, how much) someone has to pay for using the methodology. The way in which previous revolutionary breakthroughs in biotechnology - such as recombinant DNA, RNA interference and PCR - have been dealt with can act as a guide here. These technologies could be freely used by academic and non-commercial research groups, while commercial companies gained access to non-exclusive licenses. This approach facilitated the broad dissemination of these techniques and could therefore also be seen to offer a solution in the CRISPR case.





3 Therapeutic applications of CRISPR-Cas

CRISPR-Cas brings cures for hereditary diseases a step closer. It is, after all, a technology with the potential to repair defective genes in patients' cells. But CRISPR-Cas has many more potential therapeutic applications. For example, experiments are underway to use genome editing in cancer treatment. What's more, genome editing has already been used to make immune cells resistant to HIV.[38] [39]

Doctors have now started clinical studies to put therapies based on genome editing into practice. The first studies focused on the fight against various forms of cancer, hereditary diseases and infectious diseases. Most of these are being led by Chinese researchers. However, there are also several American and European clinical studies in the pipeline.

Hereditary disorders

A spelling mistake in your DNA

The human genome has 3.2 billion base pairs. Minor errors in the DNA code can in certain cases lead to hereditary disorders that are passed from one generation to the next. More than 5,000 of these hereditary diseases are known. One in every 25 children is born with one such condition. However, gene defects can also arise during fetal development or after birth.

Scientists have been looking for years for DNA errors that cause genetic diseases. That search has gained momentum thanks to the human genome project. Detecting DNA errors is one thing, repairing them is quite another matter. It is possible that CRISPR-Cas can generate new momentum.

In theory, genome editing can replace the 'faulty' piece of DNA in a patient with a correctly spelled one. It was quickly demonstrated test tubes that CRISPR-Cas technology can eliminate disease-causing mutations in various tissues. But to take this further by fixing errors in living organisms is a huge step. Nevertheless, recent studies are already raising hope.

In 2014, for example, researchers managed for the first time to cure a genetic liver defect in adult mice with the help of CRISPR-Cas.[40] This involved a mouse model for the human hereditary metabolic disease tyrosinemia, which is caused by a mutation in the FAH gene. Patients with this disease cannot adequately break down the amino acid tyrosine, causing it to accumulate to toxic concentrations. The result is liver and kidney disorders and, often, intellectual disability as well. The researchers succeeded in directly treating the livers of adult mice with a corrective CRISPR-Cas complex. It appeared that the gene was corrected in enough liver cells for the mice to no longer suffer from tyrosinemia.

GENE THERAPY, OLD STYLE - A LONG HISTORY OF TRIAL AND ERROR

As early as 1972 the American doctors Theodore Friedmann and Richard Roblin argued for research into gene therapy as a means to help or even cure people affected by a hereditary disorder. The theory behind gene therapy is simple: the defective gene is replaced by a new, properly-functioning one[41].

However, more than 45 years of research has shown that this simple idea is, in practice, extremely challenging and technically very complex if the aim is to use it safely and efficiently in people.

The first major challenge is introducing the corrective gene into the correct body cells of the patient. There is a problem with selectivity and effectiveness. To correct a defect in the liver, it makes no sense for the gene to end up in brain cells. On the contrary, it is possible that the gene would cause undesirable side effects in the brain. The corrective gene must be selectively incorporated and/or activated in the tissue or cell type that is the real target.

Furthermore, sufficient liver cells have to take up the corrective gene, otherwise there will be too little effect. It is also possible to give cells that have taken up the gene a growth advantage. This way, they will outgrow other cells over time. But this can be a double-edged blade. Nobody wants it to turn into uncontrollable cancer cells.

Significant progress has been made in both areas over the past decades: clinical trials that use inactivated viruses, such as adeno-associated viruses (AAV) and lentiviruses, as 'transport' for the corrective DNA have shown that genes can be introduced into the correct target tissues safely and effectively for various disorders.[42]

The next challenge is the stable incorporation of therapeutic genes into the genome so that they are preserved in dividing cells. A new gene that ends up anywhere in the genome of a cell can have unpredictable effects on gene expression and unintended effects on neighboring cells.

Over the past decades, hundreds of clinical studies have been conducted on patients with the above-mentioned classic form of gene therapy. By no means were all of them successful, although some did reach the finish line.

- *In 2016, for example, the European Medicines Agency (EMA) approved the use of Strimvelis, a form of gene therapy for the treatment of ADA-SCID, a serious immune disorder caused by a defect in the adenosine deaminase gene.*[43]
- *Luxturna was approved in 2018 for the treatment of hereditary retinal dystrophy caused by mutations in the RPE65 gene.*[44] *This rare disease leads to loss of vision and blindness.*
- *In early 2019, EMA issued a favorable decision to Zynteglo, which is a form of gene therapy for treating adults and adolescents who have β -thalassemia, a rare disease that causes severe anemia*[45].
- *In addition, at least 20 forms of gene therapy were recognized by the American and/or the European government as promising. They have a good chance of being approved as a 'medicine' in the future. This concerns treatments against cancer and blood disorders as well as treatments for neurological, liver, muscle and eye diseases.*[42]

Gene therapy, new style

With traditional forms of gene therapy (see the box 'Gene therapy, old style - a long history of trial and error'), corrective genes are usually introduced to 'supplement' the defective gene. Via the new technique of genomic editing with CRISPR-Cas, the defective gene is repaired on-site, without affecting other DNA sequences. Some people see this method of gene repair as a completely new form of gene therapy. Maybe call it 'Gene therapy, new style'.

The first therapeutic experiments based on genome editing focused primarily on blood disorders and immune diseases. Specifically, this concerns *ex vivo* therapies (see the *ex vivo* and *in vivo* therapies box) in which blood-forming stem cells are removed from the patient's body to be processed in the laboratory. It is relatively easy to isolate the stem cells that develop into red and white blood cells from the bone marrow. Gene defects in these cells can then be repaired in the laboratory. The altered cells can undergo various selection and control steps before they are injected back into the patient. A brief overview of the state of affairs of this research, using β-thalassemia as an example, is given below.

EX VIVO *OR* IN VIVO *GENOME CORRECTION*

We make a distinction between the different ways of making corrections in the human genome:

- Ex vivo*: diseased cells are removed from the patient's body, the defective genes are repaired in the laboratory and these corrected cells are injected back into the patient. Since the treatment takes place outside the body, this way of working is called* ex vivo*. The advantage of modifying cells outside the body is that they can be checked before they are returned. This adds an additional level of safety. This is sometimes called cell therapy because it involves trying to cure a disease by administering whole cells. However, it differs from 'conventional' cell therapy in that a genome correction is made, which is not true for all forms of cell therapy.*
- In vivo*, systemic or targeted: on the other hand, an attempt can be made to restore the gene in the patient's body itself by injecting the CRISPR-Cas system (or other systems for performing genomic changes) into the patient. This is referred to as* in vivo *genome editing. If all of the patient's cells are subjected to genome correction, this is called a 'systemic' correction. If the genome correction is aimed at a specific organ, tissue or cell type, then it is called a 'targeted' method.*

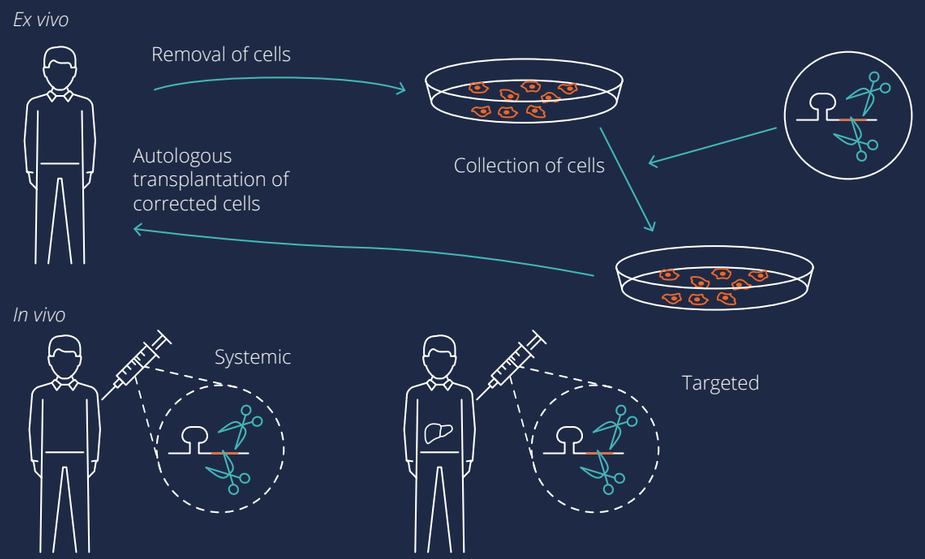


Figure 4. *Ex vivo* or *in vivo* genome correction (Figure modelled to reference 39)

β-thalassemia

β-thalassemia is a hereditary form of anemia. It is one of the most common genetic diseases in the world. Due to a mutation in the β-globin gene, insufficient and/or abnormal hemoglobin is produced. This is the protein that transports oxygen in the blood. People with β-thalassemia need lifelong blood transfusions.

Scientists from the University of California (USA) were able to correct the gene defect responsible for the disease in human cells.[46] Researchers took stem cells from the body of a β-thalassemia patient and used CRISPR-Cas to restore the β-globin gene. Although the processed cells were not returned to the patient in this study, it was nevertheless an important step towards the treatment of genetic diseases based on genome editing.

A clinical study is now starting in hospitals in Canada, Germany and the United Kingdom in which CRISPR-Cas is used to correct the defective β-globin gene in blood-forming stem cells from patients with β-thalassemia. The stem cells are first isolated from the patient's own bone marrow, then the defective gene is corrected in the lab and the cells put back into the patient. The results of the study are expected by mid-2022.[47]

Sickle cell anemia

Sickle cell anemia, like β-thalassemia, is a hereditary form of anemia but caused by a different mutation in the β-globin gene. What is specific to sickle cell anemia is that, under normal conditions, these patients produce hemoglobin protein molecules in their red blood cells that are capable of binding to and transporting oxygen but which will clump together at low oxygen levels. As a result of this abnormality, the red blood cells become sickle-shaped, hence the name 'sickle cell anemia'. The red blood cells then lose their flexibility and are broken down more quickly. They also have tendency to clump together and get stuck in small blood vessels. This causes blood flow disorders, local oxygen deficiencies and, eventually, the possible death of tissues and organs. Sickle cell anemia affects about 3.2 million people worldwide and led to 176,000 deaths in 2013.

In 2016, American researchers succeeded in using CRIPSR-Cas to correct the faulty β-globin in the blood-forming stem cells of patients with sickle cell anemia. They also introduced these corrected cells into mice and saw that the cells still produced 'healthy' hemoglobin after 16 weeks.[48] Three American hospitals are currently preparing a joint clinical trial to test this technology in 45 patients with sickle cell anemia. These results are also expected mid-2022.[49]

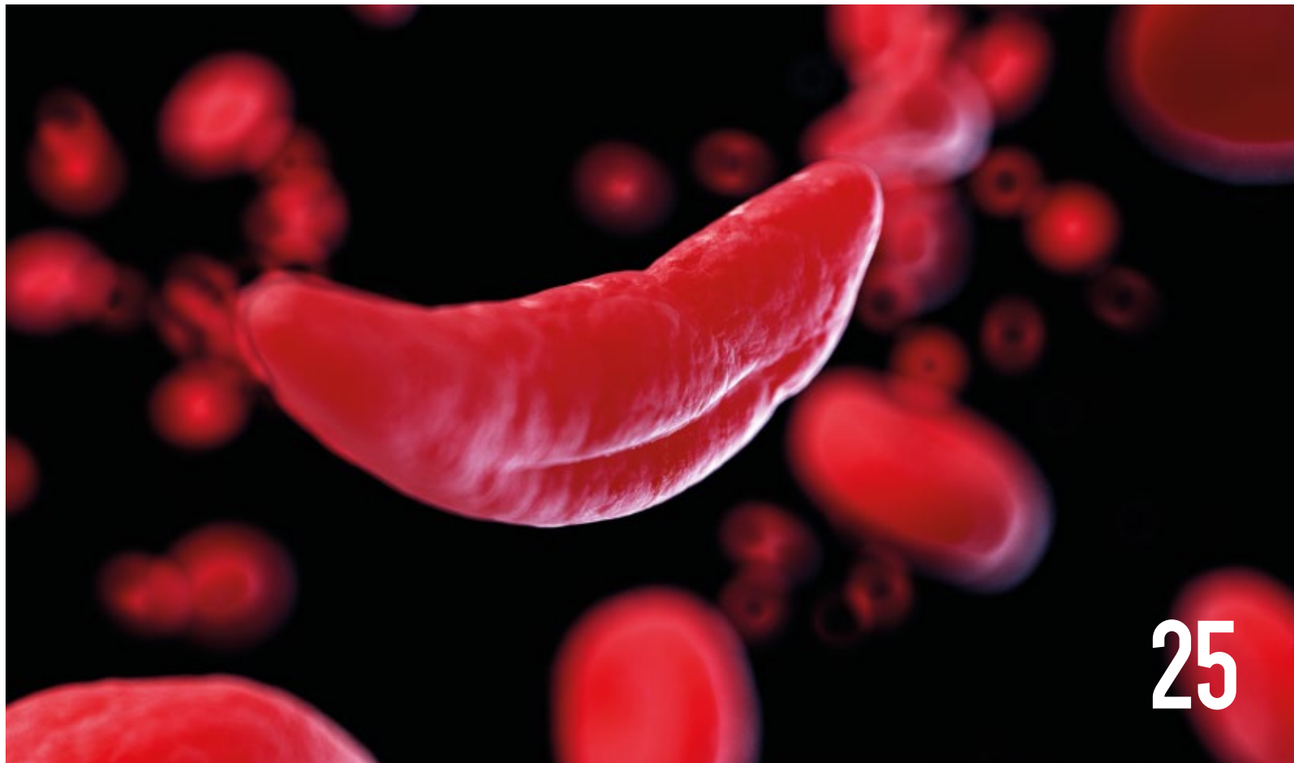
A Japanese-American research group is following a very different strategy. Instead of correcting the mutated β-globin gene, they try to reactivate the fetal γ-globin gene. The rationale behind this approach is as follows: in the red blood cells of children and adults, hemoglobin actually consists of a complex of four proteins (two α-globin chains and two β-globin chains). In fetuses, however, hemoglobin is formed by two α-globin chains and two γ-globin chains. After birth, less and less γ-globin is expressed while production of the β-globin is continually increased. The Japanese-American group has now succeeded in changing the γ-globin gene in blood-forming stem cells so that it is no longer switched off and the γ-globin chains will replace the sickle cell β-globin in children and adults.[50] For the time being this is still an experiment in the test tube. Before starting clinical trials on humans, the process must be further refined and extensively tested on laboratory animals.

Duchenne's disease

Duchenne's disease is a serious hereditary disorder of the muscles caused by mutations in the dystrophin gene. The disease progresses systematically and affects more and more muscle tissue, including the skeletal, heart and respiratory muscles. The average age at which patients with Duchenne's end up in a wheelchair is ten. At a later age the respiratory muscles and the heart muscle are affected so that the patients often cannot breathe without assistance. Because the dystrophin gene is located on the X sex chromosome, the disease primarily affects boys (1 in 5,000). Girls can be carriers of the disease.

At the beginning of 2016, three independent research teams announced that they had restored the dystrophin gene in muscle tissue from mice with muscular dystrophy via *in vivo* gene correction.[51] [52] [53] Instead of first treating stem cells in the laboratory, the CRISPR-Cas complex was administered directly into the muscle tissue of the animals (targeted approach) or via the bloodstream (systemic approach). The researchers succeeded in inducing the expression of a partially functional dystrophin in the muscle tissue of the dystrophic mice. This led to a noticeable improvement in muscle development, but not to a complete recovery.

The patient organization *CureDuchenne* has great confidence in this approach and financed a new start-up company, called Exonics Therapeutics, based on the technology used in mice. They hope to be able to test the CRISPR-Cas correction in people as soon as possible. It is estimated that if 15% of the muscle cells can be corrected, this would be enough to stop disease progression. Although the first tests in humans are still to come, Exonics Therapeutics, in collaboration



with researchers from the University of Texas, achieved a remarkable result in ten dogs with muscular dystrophy in 2018. After a systemic administration of dystrophin-correcting CRISPR-Cas, the researchers saw the expression of functional dystrophin increase by 3% to 90% of the normal level, depending on the muscle type. In heart muscle tissue, a recovery of up to 92% was measured in some animals.[54]

Huntington's disease

Another disorder where systemic administration of genome-correcting CRISPR-Cas could improve symptoms is Huntington's disease. This disease is caused by a mutation in the huntingtin gene (HTT gene). This mutation leads to the production of a faulty, toxic form of the huntingtin protein. This causes brain cells in the striatum to die. This is one of the deeper-lying nerve nuclei in the brain responsible for strengthening, inhibiting and directing motor activity.

The first symptoms of the disease usually occur between the ages of thirty and fifty and include uncontrolled movements, balance problems, mood swings and deterioration of memory and organizational ability. The conventional gene therapy approach, which involves introducing a working copy of a gene, would not work for this disease. This is because the still-present mutated HTT gene will continue to produce toxic protein. It is therefore important to switch off the mutated gene by genome editing or to restore it to its unmutated form. And preferably in as many cells of the striatum as possible.

Using the CRISPR-Cas system, Chinese researchers were able to remove a piece of the mutated gene in a mouse model for Huntington's disease. This reduces the toxic accumulations of the abnormal huntingtin protein.[55] The study also revealed that the brain cells in the striatum not only survive longer but even partially recover when the genetic cause of the toxic proteins was removed. Furthermore, the researchers noticed that the motoric functioning of the CRISPR-Cas injected mice greatly improved when compared to diseased control mice. The same research group published similar results in 2018 in a pig model for Huntington's disease.[56]

The above examples are just some of the large number of disorders that scientists and doctors are hoping to treat with genome editing. You will find a more extensive list in the attached table.

Tabel 1. *Potential of CRISPR-Cas in genome correction therapy*[40]

Condition	*Target gene*	*Model system in which it has already been applied*
Duchenne's disease	Dystrophin	Rats, rhesus monkeys, mice, dogs, muscle stem cells
Mucoviscidosis	CFTR	Intestinal stem cells
Hereditary tyrosinemia	FAH	Mice
Cataract	Crygc	Mice
Lung cancer	KRAS, p53, LKB1	Mice
Cancer resistance	XPO1	T-ALL cells
Severe mental disability	DISC1	iPS cells
Autism	CHD8	iPS cells
Huntington's disease	HTT	Pigs, mice, iPS cells
Microdeletion and micro-replication syndromes	16p11.2 and 15q13.3 copy variants	iPS cells
Epilepsy	SCN1A	iPS cells
Fragile X syndrome	FMR1	iPS cells
ß-Thalassemia/Sickle cell anemia	β-globin	Mice, iPS cells
Urea cycle deficiency	OTC	Mice
Walker-Walburg syndrome	LSPD	Mice
Hepatitis B (HBV)	HBV cccDNA	Mice, cell lines
HIV-1	HIV-1 LTR	Mice, cell lines
Epstein-Barr virus (EBV)	Latent EBV	Cell lines
Human papillomavirus (HPV)	HPV oncogenes E6 and E7	Mice, cell lines

Cancer

CRISPR-Cas also looks promising for the treatment of cancer. Cancer is not one, but a hundred different diseases. Nevertheless, it always involves an uncontrolled growth of abnormal cells. These cells ignore signals and mechanisms that normally keep their growth in check. The cause lies with changes in the DNA that disrupt the regulation of cell growth and cell division. The accumulated DNA changes gradually turn a 'normal' body cell into a cancer cell (see also the VIB Fact Series 'Cancer').

Hereditary disorders and cancer therefore have the common characteristic in that they are caused by DNA mutations. So it's logical that genome correction could be a way to bring cancer cells back under control. However, that is easier said than done.

Tumor growth genes as target

As mentioned earlier, cancer cells have an abnormal cell cycle. This cycle controls the growth and division of a cell, while we know that these things are unrestrained and uncontrolled in cancer cells. Recently, American researchers succeeded in breaking the runaway cell cycle in cancer cells by using CRISPR-Cas to eliminate a gene, called Tudor-SN.[57] Tudor-SN influences the cell cycle by controlling the level of certain microRNA molecules. MicroRNAs are molecules that fine-tune the expression of thousands of genes. By switching off Tudor-SN in tumor cells, their growth - at least in test tubes - was slowed down.

Other researchers used CRISPR-Cas technology to smuggle a gene into cancer cells that converts

the drug Ganciclovir into a toxic product that causes the cancer cells to die. The treatment also seemed to work well in mouse models with cancer.[58]

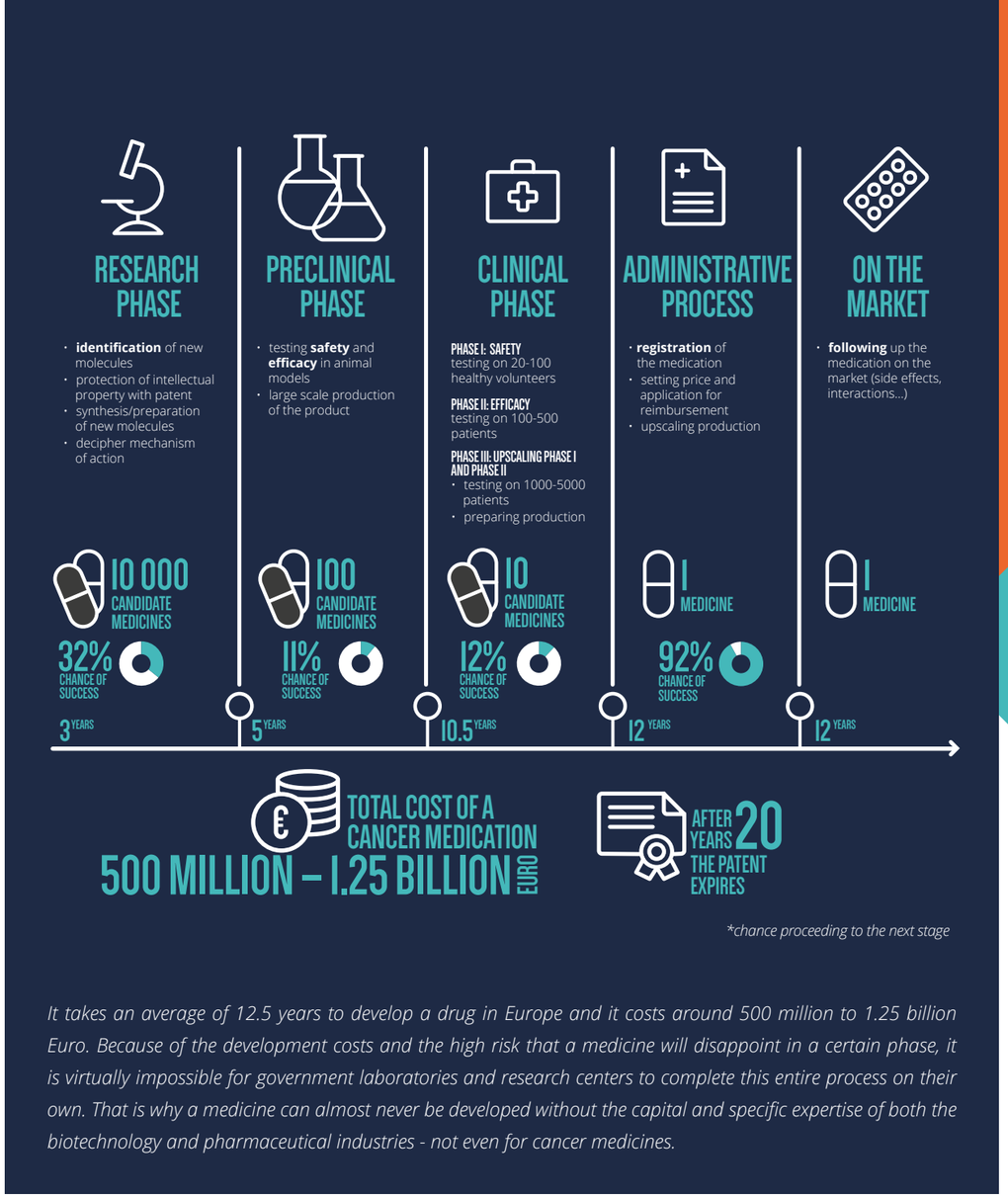
South Korean researchers, on the other hand, were able to eliminate mutated KRAS - an important oncogene - so that the growth of tumors was strongly inhibited.[59]

Despite the advances in therapeutic approaches based on directly modifying the DNA of cancer cells, we need to be cautious and not overestimate their therapeutic potential. Cancer cells are, after all, masters in circumventing treatments (see VIB Fact Series 'Cancer'). They will also develop resistance to these CRISPR-Cas-based treatments. Cancer experts are already convinced of this.[60] Moreover, a lot of extra work and research is needed to put these potential treatments into clinical practice. Because what works in cell cultures, in a mouse or in a rat, is not yet ready for use in humans. Before a new medicine reaches the patient, it needs to go through a time-consuming, complex, intensive and costly process. (See 'The development of a medicine').

THE DEVELOPMENT OF A MEDICINE

The procedure by which a molecule or experimental treatment becomes a recognized medicine is long and complex. Before it comes onto the market, every candidate drug is extensively tested for safety and effectiveness - does the medicine do what we expect and does it do so in a safe way? This evaluation first takes place in the laboratory and on experimental animals (preclinical phase), and then in clinical studies, which we generally subdivide into three to four phases:

- *In a phase I clinical study, the safety and effect of the drug is analyzed on a small number of volunteers. Checks are made to see whether serious side effects occur.*
- *If everything turns out to be safe, a phase II clinical trial begins in which a small group of patients is tested to see if the drug has an effect in treating the disease (e.g. slower tumor growth, less chance of metastases, longer survival or improved quality of life).*
- *If phase II yields promising results, a phase III clinical trial is performed in which the safety and efficacy of the drug is further investigated in a large group of patients. Only when these results are positive will a candidate drug be admitted to the market by the competent government authorities.*
- *In phase IV, when the medicine is on the market, a larger group of patients is monitored to detect any undetected side effects over the long term.*



It takes an average of 12.5 years to develop a drug in Europe and it costs around 500 million to 1.25 billion Euro. Because of the development costs and the high risk that a medicine will disappoint in a certain phase, it is virtually impossible for government laboratories and research centers to complete this entire process on their own. That is why a medicine can almost never be developed without the capital and specific expertise of both the biotechnology and pharmaceutical industries - not even for cancer medicines.

CRISPR-Cas in anti-cancer immunotherapy

Immunotherapy is a new addition to innovative cancer treatments. There are different forms of immunotherapy, but what they all have in common is the aim of strengthening and stimulating the patient's own immune system to get rid of cancerous cells (see the VIB Fact Series 'Cancer').

CAR T-cells

A specific form of immunotherapy, recently approved by the drug authorities in both Europe and the US, is based on 'CAR T-cell' technology. 'CAR' stands for 'chimeric antigen receptor' while T-cells are a class of white blood cells that are also involved in clearing-up cancer cells. The therapy consists of taking the T-cells from the patient and putting a new gene into them in the laboratory so they can better recognize the cancer cells. The implanted gene codes for an artificially constructed receptor protein (hence 'chimeric antigen receptor') that recognizes specific proteins found on the surface of cancer cells. Once the genetically modified T-cells are reintroduced into the patient, they will, directed by their newly-designed receptor, bind to cancer cells and initiate a mechanism to kill them. Moreover, these T-cells will multiply in the body of the patient so that they also have an anti-cancer effect over the long term.[61]

Hundreds of additional clinical studies are currently being conducted with CAR T-cells around the world. The first approved therapies primarily dealt with specific forms of blood cancers (leukemia) and lymphomas. New studies, however, focus also on solid tumors.

A major limitation for this form of therapy is the need to use only autologous T-cells. That is, each patient must be treated with their own T-cells to avoid the risk of rejection. The need to use the patient's own cells makes the process time-consuming, complex and extremely expensive.[62]

How can CRISPR-Cas make a difference to CAR T immunotherapy?

Until now, CAR genes have been introduced into T cells through viral vector systems - the 'old-style' gene therapy, so to speak. CRISPR-Cas clearly offers possible alternatives for incorporating CAR genes.[60]

But even more, CRISPR-Cas is seen as a way to produce 'universal' CAR T-cells that do not cause rejection in patients. This would mean that CAR T-cells could be 'taken from a hospital pharmacy rack' instead of having to be individually tailored to each patient (see also Figure 5 'Possible role of CRISPR-Cas in producing autologous and universal CAR T-cells' on page 31).

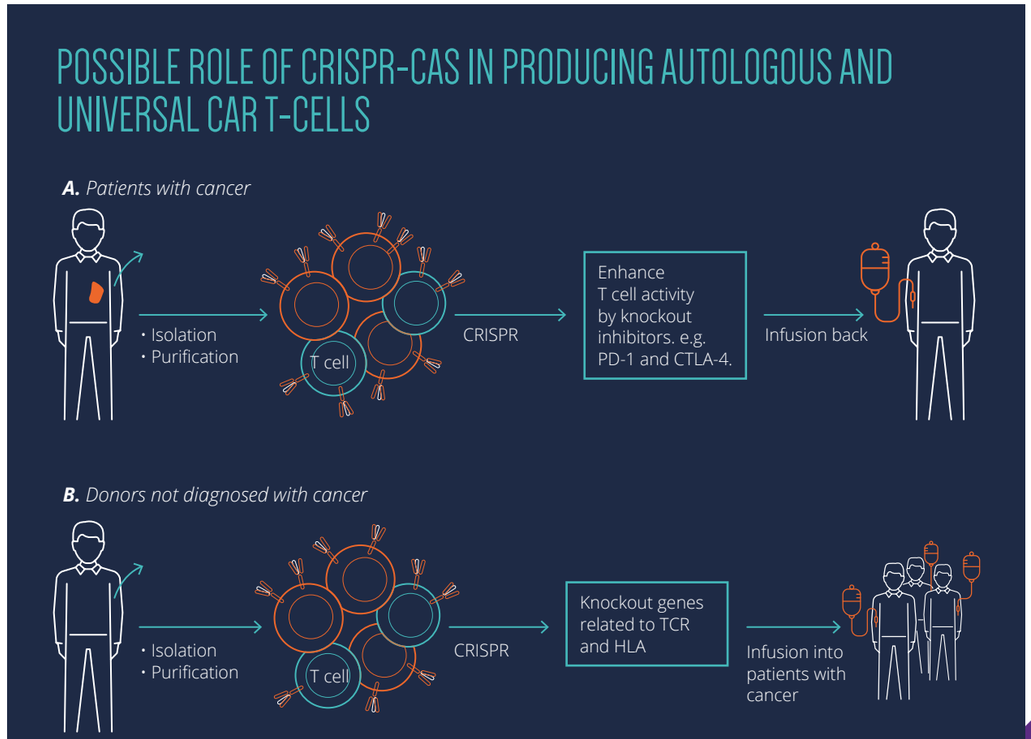


Figure 5. Possible role of CRISPR-Cas in producing autologous and universal CAR T-cells (Figure based on reference 60)

A. T-cells are taken from the patient with cancer by drawing blood. They are then separated out and purified. A chimeric antigen receptor (CAR) is introduced using CRISPR-Cas. This receptor increases the anti-cancer activity of the T-cells. The CAR T-cells are then injected into the same patient. This is strongly reminiscent of existing CAR-T therapy, but the insertion of a CAR-gene would be easier with CRISPR-Cas.

B. T-cells are isolated and purified from a blood sample from a cancer-free donor. A chimeric antigen receptor (CAR) is introduced using CRISPR-Cas. CRISPR-Cas is also used to switch off 'rejection genes'. This includes genes encoding the T-cell receptor (TCR) and the human leukocyte antigen system (HLA). The universal CAR T-cells can then be injected into multiple cancer patients.

T-cells can be made so that they no longer evoke rejection responses by switching off genes that are responsible for those rejection responses. Such a procedure certainly looks possible as two girls with leukemia were recently treated in a London hospital with CAR T-cells from a donor (see 'Designer cells stop cancer in a one-year-old baby'). The girls themselves did not produce enough healthy T-cells for these to be used for CAR T-therapy.[63] That is why it was necessary to switch to a donor. The technology used for this was TALEN, which is a form of genome editing related to CRISPR-Cas.

PD1 immunotherapy against cancer

It is not just CAR T-cells that hold great promise in anti-cancer immunotherapy. Similar immunotherapies are also under development (see VIB Fact Series 'Cancer'). CRISPR-Cas genome editing can contribute to these too. For example, Chinese researchers have used CRISPR-Cas to disable the PD1 protein in T-cells. PD1 is a receptor protein that inhibits the reactivity of T-cells against cancer cells. T-cells without this protein react more aggressively to cancerous cells. Chinese doctors are now testing this form of immunotherapy in clinical studies in patients with lung, bladder, prostate and kidney cancer. The first results are expected sometime in 2020.

Infectious diseases

A third area of medicine in which genome editing could play an important role is that of infectious diseases, for example to stop HIV and other viral diseases or to help address the acute lack of new antibiotics.

HIV and AIDS

HIV, the virus that causes AIDS, infects and kills immune cells, specifically the CD4+ T-cells. But HIV is a stealthy rather than an acute T-cell killer. This is because the virus inserts its own genetic material into the genome of its host. It can hide there for years in the form of a 'provirus', only to become active again at unexpected times and order itself to be translated into new virus particles. These will, in turn, threaten other immune cells. Antiviral HIV therapy, which is already particularly successful, is directed against the replication of virus particles in the host cell but fails to address the 'dormant' proviruses.

Does CRISPR-Cas genome editing offer a solution that could permanently stop HIV and AIDS, which is still a major worldwide medical problem? The answer could be yes, and in different ways too.

Recently a team of Chinese and American researchers managed to use CRISPR-Cas to disable HIV proviruses in mice.[65] The team was the first to show that HIV can be completely removed from the bodies of mammals. The technique needed only one treatment to wipe out all traces of the HIV infection. The next phase is to repeat the study in apes, which is a more appropriate animal model for HIV infections and AIDS than mice.

CRISPR-Cas could even be used to immediately cut up incoming viruses so that they cannot replicate themselves or insert themselves as proviruses in the host genome. This was successfully tested on cell cultures, although the viruses managed to build up resistance after a while.[66 67]

A third strategy is to deny HIV access to the CD4+ T-cells in the first place. The viruses, after all, use receptors present on the outside of T-cells to infect them. The most important receptors and co-receptors are CD4, CCR5 and CXCR4.

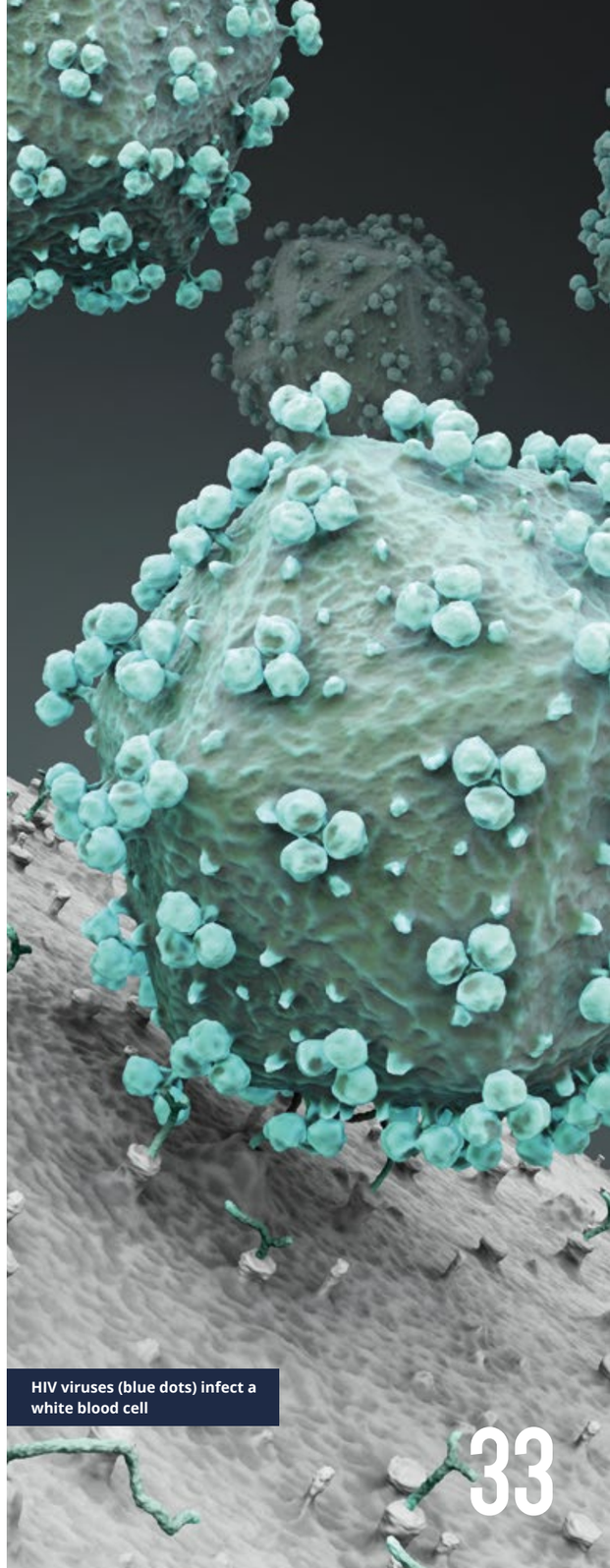
Disabling CD4 in human T-cells to prevent HIV uptake would be a bad idea, however, as the CD4 protein is critical to the proper functioning of the immune system. CCR5 and CXCR4, on the other hand, would be good candidates. After all, there are people who do not naturally produce CCR5 and who are immune to HIV as a result. In recent years, mainly Chinese researchers have demonstrated in cell culture that disabling both CCR5 and CXCR4 led to long-term resistance to HIV infection.[68 69]

The first clinical studies are also underway: in the Beijing military hospital (China), HIV patients are being treated with their own blood stem cells in which the CCR5 gene has been switched off.[70] In addition, twins were recently born in China whose CCR5 genes had been intentionally altered to prevent the children from being infected by their father, who was HIV positive. But that is another story, which we will return to in detail in the next section.

Herpes virus

The herpes virus remains latent in the body for life because the virus builds its genome into that of the host. Herpes viruses cause, amongst other things, chicken pox, shingles and cold sores on the lips. So far, there is no method to completely eliminate the virus from its host. The latent virus does not multiply and remains unnoticed by the immune system. Until it occasionally flares up.

Current treatments suppress the symptoms but cannot remove the infection. In 2016, Dutch researchers were able to control the multiplication of the virus in mammalian cells using CRISPR-Cas.[71] In additional experiments, the herpes virus was even completely removed from the genome of the cells.[72]



HIV viruses (blue dots) infect a white blood cell

Human papillomavirus (HPV)

The human papillomavirus (HPV) causes, among other things, cervical cancer. Infection normally occurs through sexual contact. Once someone is infected, it is difficult or impossible to remove the virus. That is why it is best for women to have a regular cervicovaginal smear to screen for uterine or vaginal cancer. Vaccines against certain HPV strains have also been in existence for more than 10 years. In Flanders, these vaccines are offered (free) to all girls in the first year of secondary education, and, starting with the 2019-2020 school year, also to the boys of the same age.

In 2014, a Chinese research group demonstrated that they could induce cell suicide (apoptosis) in cervical cancer cells infected with HPV by means of a CRISPR-Cas induced attack on the HPV genome.[73] Their method also proved effective in inhibiting tumor growth in animal models.[74]

A clinical study was recently started at the University of Guangdong, in China, based on similar CRISPR-Cas genome editing. In this non-invasive treatment, 60 women who are positive for the HPV16 and/or HPV18 strains receive a vaginal gel containing CRISPR-Cas against these viruses.[75] No clinical trial results are yet known and the primary purpose of this study is to determine whether the treatment is safe.

A potentially life-saving antibiotic

CRISPR can also serve as a new type of antibiotic.[76] Although CRISPR-Cas is naturally used by bacteria to protect themselves against invading viruses, it can also be reprogrammed to cut bacterial DNA. Bacteria themselves do not have an advanced DNA repair system like ours. This makes them very susceptible to DNA damage and, in most cases, a CRISPR-Cas attack on bacterial DNA will lead to the destruction of the bacterium. This fact has opened the doors for numerous research projects that investigate whether CRISPR-Cas can be used as a very specific antibiotic.[77 78 79 80]

Equally, CRISPR-Cas can eliminate the genes responsible for antibiotic resistance in bacterial strains. It would make bacteria sensitive to existing antibiotics again. This could be a solution against the current increase in multi-resistance in many bacterial strains.[81]

But CRISPR-Cas can also be used to change the composition of the human gut flora for the benefit of our health or to prevent obesity, amongst other things.[82 83]

These are just a few options from the larger range of opportunities that genome editing has created in the field of infectious bacterial diseases.

STOP MALARIA!

Can CRISPR-Cas be used to fight malaria? It would be welcome, because the fight against malaria has been going on for more than 100 years with varying degrees of success and the disease still kills half a million people, mainly children. Malaria causes illness in 200 million people every year.

This infectious disease is caused by single-celled parasites of the genus Plasmodium. *The parasites are transmitted to humans and animals by mosquitoes of the genus* Anopheles.

CRISPR-Cas as a 'gene driver'

Several research groups are trying to use CRISPR-Cas as a 'gene drive' system to reduce the number of malaria mosquitoes in high-risk malaria areas. Gene drive is a powerful genetic technique for spreading desirable DNA changes in populations of wild animals, insects or plants. The aim is for the desired DNA change to be present in more and more individuals as the generations follow each other. Although the concept was described more than 80 years ago, it is only now that the tools are available to apply the principle in reality.

Scientists from Imperial College London in the UK are experimenting with three genes of the Anopheles gambiae *mosquito that, if mutated, lead to infertility in female mosquitoes provided that they have two mutated copies of the gene (the mosquitoes have two copies of each gene - one from each parent).[84] The fertility of male mosquitoes is not affected by the mutation status of the three genes.*

Natural selection bypassed

Such mutations would have little chance of survival under normal natural selection in the environment because female mosquitoes with two mutations cannot have offspring. As a result, it is mainly non-mutated copies that are passed on to the offspring. However, by incorporating a complex CRISPR-Cas DNA fragment into the genome of male mosquitoes, the British researchers managed to bypass natural selection and ensure that mutated versions of fertility genes are given the upper hand (see figure 6 on the next page).

The secret of their gene drive technique is hidden in the contents of the built-in CRISPR-Cas DNA fragment. This contains:

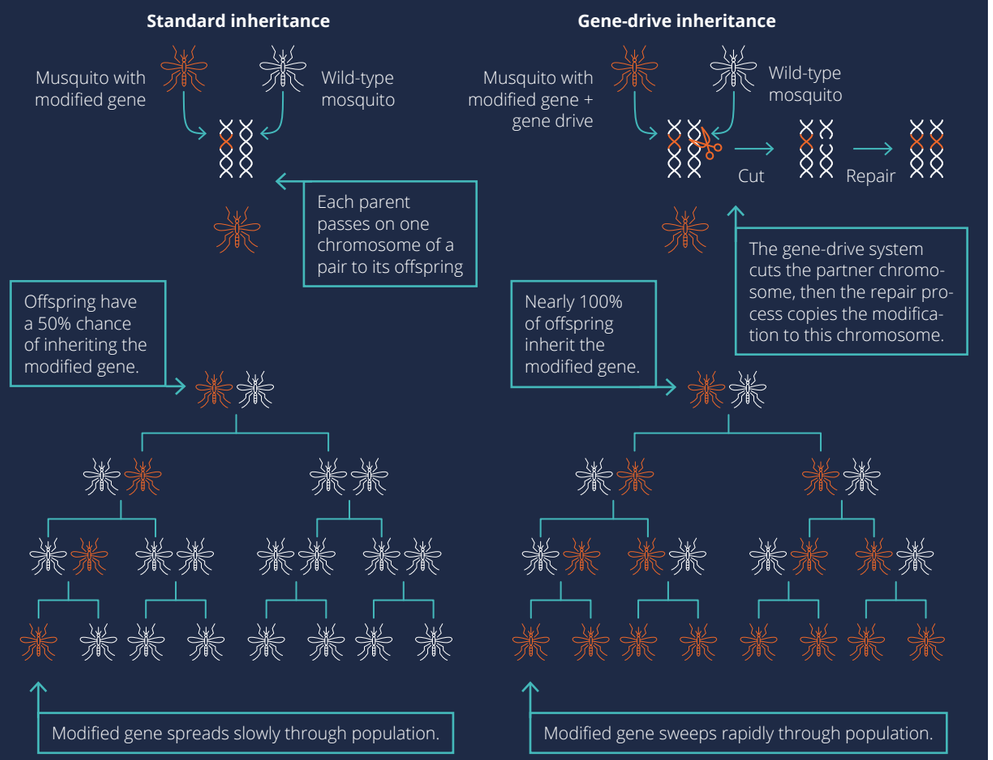
- *A CRISPR guide sequence that consists of a normal piece of the fertility gene*
- *The DNA code for a Cas protein*
- *Homologous, but mutated, DNA sequences of the fertility gene*

For mosquitoes with a CRISPR-Cas fragment, the CRISPR guide sequence will search for and bind to the normal fertility gene, after which the Cas protein will cut the DNA strand. Via homologous recombination (see page 8), the healthy copy of the fertility gene will be replaced by the mutated fertility gene. The result is that both chromosomes now carry a mutated fertility gene.

Each descendant of a double-mutated male mosquito will carry a fertility mutation on one of the two chromosomes in addition to a healthy gene from their mother (see Figure 6). But the molecular genome editing mechanism will repeat itself in them too and they will end up with two mutated fertility genes. Female offspring will no longer be able to have offspring themselves, but the male mosquitoes will.

Despite the evolutionary disadvantage, the mutation will spread rapidly in this way and eventually eradicate the mosquito population due to a lack of fertile female mosquitoes. This will also reduce the transfer of the malaria parasite. The figure below shows how gene drive inheritance spreads a desired mutation much faster through a population, even if it is a mutation that eventually eradicates the population completely.[85]

The British researchers also carried out a proof-of-concept experiment: they brought together 600 mosquitoes under controlled conditions, half of which had a non-mutated genome and the other half carried the CRISPR-Cas-gene-drive fragment. After four generations, 75% of the mosquitoes carried the mutations for infertility. This is in line with the theoretical expectations of how the mutations would spread. They recently introduced refinements to their technique and demonstrated in a controlled field trial that a mosquito population really can be eradicated.[86]



Principals of gene drive used to propagate genetic modifications rapidly through generations of a population. Figure modelled to reference 85

Beyond malaria

Other diseases spread by insects or other disease vectors could be tackled in a similar way. This might include dengue fever, yellow fever, the West Nile virus, sleeping sickness, Lyme disease or Zika virus. Gene drives can, in theory, not only prevent the spread of a disease, they could also undo pesticide and herbicide resistance in insects and weeds or control harmful invasive species.

The risks of destroying ecosystems

But not everyone is enthusiastic about this technology. In the past, the malaria parasite and the mosquito have repeatedly succeeded in building up resistance to the means and technologies people have developed to combat them. Even now we run the risk that the parasite and the mosquito will find a way out.[87] *This argument is countered by the proponents by saying that three fertility genes could be switched off together, which would minimize the risk that resistance would develop.*

Other researchers are very concerned because eliminating an entire natural population can have unpredictable consequences for the ecosystem. It is possible that other pests will emerge, or that the entire ecosystem will collapse. Moreover, it is not inconceivable that the guide CRISPR RNA will mutate over time so that it targets another part of the genome or other organisms. This mutation could then run unchecked through populations, with totally unpredictable results. They therefore demand that the government takes measures to subject gene drive experiments, whether in laboratories or in the wild, to strict safety rules.[87 88]

CRISPR-CAS IN AGRICULTURE

Genome editing technology is not only used in healthcare. CRISPR-Cas has also become a welcome new tool in plant research, crop breeding and agriculture. By switching off genes in plants, researchers gain insight into their growth and disease mechanisms, into which genes contribute to the yield of agricultural crops in normal and extreme conditions (e.g. drought) or what role they play in pest infestations and diseases.

CRISPR-Cas is also seen as a tool for precision breeding: the technology makes it possible to make DNA changes in plants and crops in a very controlled manner so that undesirable properties can be weakened and desired properties enhanced. This makes genome editing an advanced form of mutation breeding, as this has been used for decades. In addition, CRISPR-Cas breeding differs in a number of fundamental ways from 'conventional' genetic modification technology.

Various crops have already been refined thanks to genome editing: from mildew-resistant wheat, grapes and tomatoes to grapefruit resistant to citrus cancer, disease-resistant bananas, soya and rapeseed with a healthier fatty acid composition and maize that is more resistant to drought. Most of these crops are still at the research stage, although it is expected that the first products will come onto the market at some stage.

An overview of the use of CRISPR-Cas technology in agriculture can be found in the VIB Fact Series 'Precision plant breeding via CRISPR-Cas'.

4 Ethical and social discussion

The emergence of CRISPR-Cas and similar techniques for editing human genomes also exacerbates some old ethical and social discussions. The technology can be used, for example, to cure hereditary diseases in an embryo where the corrected genes are passed on to subsequent generations. This form of genetic modification, which influences the human germ line, has been approached with great caution.

The technology could in principle also be used for non-therapeutic purposes. What if people use the technology to fully design their child according to their preferences? With the desired appearance, intelligence level and character? Although there is no clear relationship between most of those traits, talents and competencies with specific genetic variations, the risk remains that some parents still want to have genetic characteristics built in that give their children an advantage.

Genome editing in embryos

From moratorium …

At a meeting in December 2015, an international group of scientists and ethicists discussed the ethical implications of genetically adapting human embryos. Participants represented various bodies, including the Academies of Sciences and Medicine of the US, the Royal Society of the UK and the Chinese Academy of Sciences. At the end of the meeting, the participants called for a moratorium on modifying the DNA of human embryos intended for implantation (and thus creating a pregnancy) because of the 'unforeseen effects on future generations'.[89] They did, however, state that there are good reasons for allowing genome editing of embryos for basic research. The scientists did not want a total ban on research into genome editing because the technology could have many useful applications.

The scientific community therefore issued a voluntary moratorium on any genome editing in human embryos that aims to create genetically modified children. Other researchers supported this call for a moratorium in various publications.[90] [91]

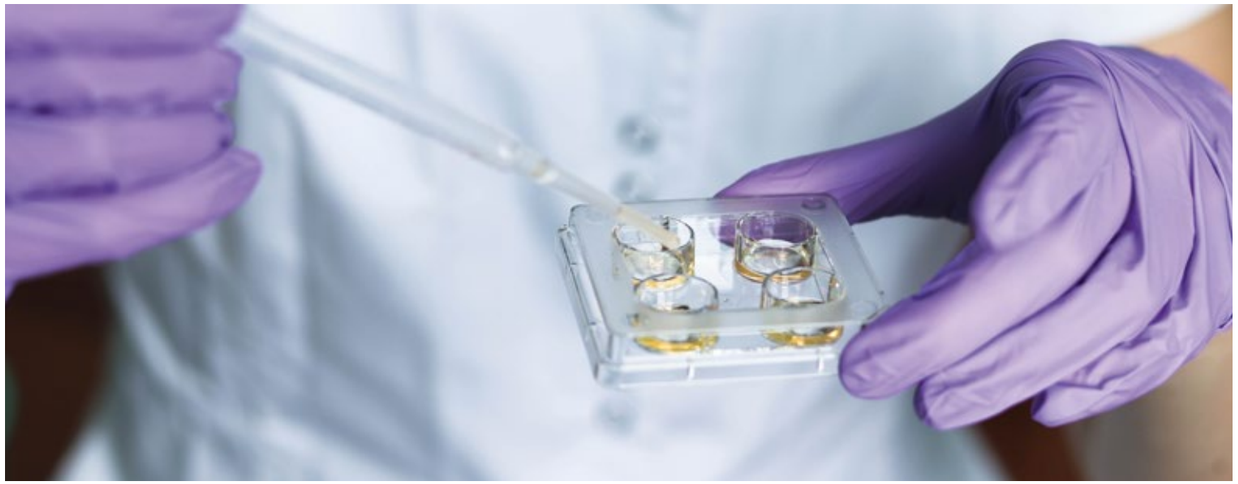
… over careful experiments …

The American National Academies of Science, Engineering, and Medicine made a similar recommendation. Their report, published in 2017, states that genome editing of human embryos is currently only desirable for research purposes.[92] They did, however, consider it likely that in the future the technique would be accepted for modifying embryos before implantation, provide that this is for treating an incurable disease and that no viable alternatives are available.

By then several research groups had already demonstrated that editing the genomes of human embryos was technically possible. In 2015, Chinese scientists succeeded for the first time.[93] They used human embryos that were not viable for those experiments. Their research, incidentally, was not a complete success as only slightly more than half of the embryos tested contained the desired change in the DNA and, moreover, this change was not present in all cells. The publication received a lot of media attention and started a worldwide debate.

In 2016 another research team from China was able to use the technology to make human embryos resistant to HIV[94], and in the same year Kathy Niakan from the Francis Crick Institute in London received permission from the British government to use CRISPR-Cas in research on very early embryonic development. Specifically, British scientists wanted to eliminate various genes that they suspected played a role in that development. With this research, the researchers hoped to better understand why some pregnancies end early in miscarriage.[95] The Karolinska Institute also received permission from the Swedish government to learn more about the developmental biology of human embryos through genome editing in embryos.[96]

Even more recently, an American, South Korean and Chinese consortium of researchers used CRISPR-Cas to rectify a disease-causing mutation in the MYBPC3 gene in human embryos.[97] Mutations in this gene lead to hypertrophic heart muscle disease in patients. In contrast to earlier studies, genome editing was particularly successful this time because it succeeded in all cells of the treated embryos.



… to a bolt out of the blue: the Chinese CRISPR-Cas babies

On November 26, 2018, the media announced that the very first genome-edited babies had been born in China, twin girls called Lulu and Nana. The genome editing procedure with CRISPR-Cas was performed by researcher He Jiankui, as part of an *in vitro* fertilization procedure. After the sperm was introduced into the egg, He Jiankui added components that made a small targeted change to the CCR5 gene of the embryo. Before the embryo was replaced in the womb, the researchers removed one cell from it to check whether the desired change had actually been made. That check would also have been carried out after the babies were born. Moreover, a check was made to see whether there had been any other changes to the genetic material.[98] To ensure the chance of a successful pregnancy, he placed several genome-edited embryos into the prospective mother's womb.

He Jiankui defended his approach two days later during a congress in Hong Kong ('The Second International Summit on Human Genome Editing'). According to the researcher, the procedure was justified because the father of the babies was a HIV carrier. By making a variation in the CCR5 gene, the children would be genetically protected against every possible HIV infection. At the same time, He Jiankui posted a video message on YouTube in which he explained his intervention (www.youtube.com/watch?v=th0vnOmFltc&app=desktop). In addition, the Chinese researcher confessed that a third pregnancy with a CRISPR baby was underway.

Global condemnation

Instead of applause, He Jiankui garnered worldwide denunciation. Even before the congress ended on November 29, one day after his presentation, the organizers made the following statement: "We were informed of the unexpected and very disturbing news that the genome of human embryos had been edited and then implanted. This resulted in a pregnancy and the birth of twins. Even if the genome changes were verified, the procedure was irresponsible and not in accordance with international standards." [99]

All the other responses were also outright negative. Fellow scientists, policy makers, academic institutions and doctors' organizations called He Jiankui's research misleading, irresponsible and in violation of Chinese law and international rules. Or in simple terms, this is about unethical experimentation on people using technology that was not yet ready.[100]

Many critics argued that an ethical distinction should be made between genome editing for 'serious' life-threatening hereditary diseases for which there is no treatment and for genome editing that has the purpose of tackling less serious matters, or that even has the aim of human enhancement. According to them, the CCR5 modification cannot be seen as the treatment or prevention of a (serious) hereditary disease, and certainly not in embryos.

In addition, there are researchers who suggest that there are few situations in which genome editing of embryos would have a medical advantage over the currently prevailing technique of pre-implantation genetic diagnosis (PGD) for preventing hereditary diseases from being passed on.[101] PGD involves testing embryos for the presence of a disease following *in vitro* fertilization. Only embryos without the mutation are replaced in the womb. Compared to PGD, genome editing appears to be technically difficult, expensive and uncertain and offers few advantages.

Vanished from the face of the earth

He Jiankui did not avoid the consequences: the Chinese authorities forced him to halt his research with immediate effect. A few months later he was fired by his employer, the Southern University of Science and Technology in Shenzhen. References to his research were removed from official Chinese websites, discussions about the researcher were censored on Chinese social media and a judicial investigation was started against him.[100] China also promised to amend its regulations on genome editing in humans.[102]

WHY THE BIRTH OF LULU AND NANA WOULD BE HIGHLY UNLIKELY IN BELGIUM AND THE REST OF WESTERN EUROPE

Before a treatment can be applied to humans, the law requires many stages to be completed. There must first be extensive pre-clinical research, then a number of phases of clinical research, and then a market authorization is required before a treatment can be applied in large-scale clinical practice. In addition, it is legally forbidden in many countries to make genetic changes to humans that are passed on to children ('germ line gene therapy').

In Belgium, the Law on Research on Embryos In Vitro *(Wet betreffende het onderzoek op embryo's* in vitro*) regulates the use of embryos for research purposes. Research on embryos is only permitted under very strict conditions. The law does not regulate the application of therapies to embryos in clinical practice. This falls under the general legislation that controls the marketing of medicines and other therapies.*

Clinical research related to genome editing in humans must always be approved by a medical ethics committee. It seems extremely unlikely that an ethics committee in Belgium would approve genome editing for a pregnancy, either at present or in the near future.

In the meantime, He Jiankui seems to have vanished from the face of the earth. The Chinese silence around him is so remarkable that some are starting to doubt the authenticity of his claims. Could it be a scientific hoax, they wonder?[102] So far there has been no scientific confirmation that the DNA of Lulu and Nana had actually been successfully edited. Nor has He Jiankui's research been published in a peer-reviewed scientific journal.

The call for international regulation

International regulation of genome editing in embryos is being advocated for from various quarters, although researchers themselves are not sure what exactly should be done. Some repeat the call for a moratorium with a strict prohibition on genomic editing of the human germline during a certain period.[103] Others, including leaders of the American National Academy of Sciences, the National Academy of Medicine and the British Royal Society, emphasize the need for a broad social consensus before deciding anything. They consider this global consensus necessary given the wide-ranging implications of hereditary genome editing.[104] They have already set up a working group of experts, consisting of researchers and ethicists, to draw up specific standards and criteria for this type of research. Dozens of scientific institutions around the world have pledged their cooperation.

The World Health Organization (WHO) has also rolled up its sleeves. The WHO wants to establish a worldwide register of all studies in which the human genome - including that of embryos - is edited. That way it can keep an overview of who is doing what. It also wants to compel researchers to be transparent about their work by using this register. The WHO asks organizations that sponsor research and publishers that publish research results to ensure that studies they support or publish are registered.[105] The chief editors of the journals *Nature*, *Science* and *Cell* have already responded positively to this proposal.

In the coming months, the WHO will work on an international framework to regulate the use of genome editing internationally, whether or not it is for clinical purposes.

The future will show where we are going with genome editing.

5 Conclusion

Genetics has developed at a breathtaking pace. In less than one human lifetime we have gone from the discovery of the double helix structure of DNA (1953) by James Watson, Francis Crick and Rosalind Franklin to genetic manipulation with restriction enzymes and PCR in the 1980s, large-scale genome analysis since 2000, and now the development of genome editing.

Future fundamental research using CRISPR-Cas will concentrate on, amongst other things, the development of new methods for the efficient and safe introduction of Cas proteins and their guide CRISPR RNAs into the cells and tissues of complex organisms. However, these rapid advances in technology are already allowing us to make unprecedentedly accurate changes in the DNA of almost all living things. This helps researchers to gain in-depth insights into all sorts of human diseases.
In addition, many new applications are beginning to emerge in both agriculture and healthcare. We have never been as close to successfully implementing gene therapy as we are today thanks to the new 'genome editing toolkit' we now have. But the new technology can also offer unprecedented opportunities for treating cancer and infectious diseases.

As with many other new technologies, genome editing raises social and ethical questions. These questions include the possibility of genetically improving humans, animals and plants, or passing on edited genomes to subsequent generations.

The relevant regulations are a long way from being clear and therefore remain a challenge for policy makers and regulatory bodies, both nationally and internationally. Technologies and their products evolve rapidly and must be continuously monitored and regulated, that much is clear. The negative effects they might have on health must be kept to a minimum. However, regulation should not paralyze innovation or block investments in and development of useful new therapies.

That is why a dialogue with the end user of these new technologies - which in this case would be the patient, the potential future patient, the citizen, in short, you and me - is also important. Two-way communication means that scientists and doctors listen to the concerns and arguments of those end users. Not only the what, how and why should be discussed, but above all we have to get together to discuss and think about which direction we wish to take.

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