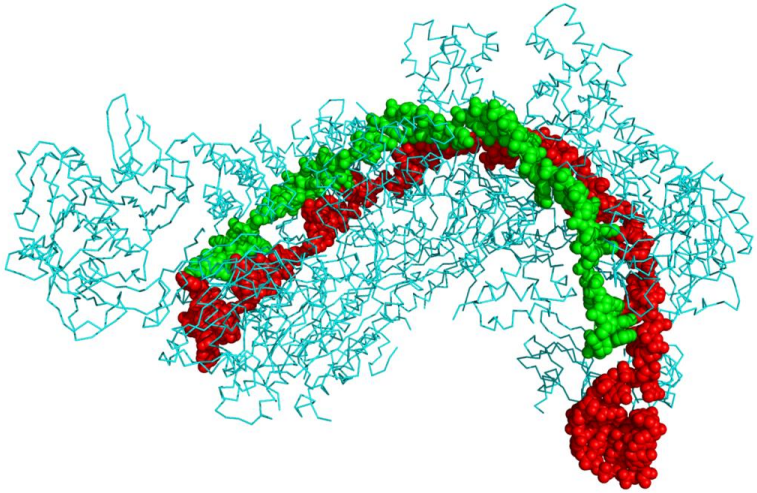

CRISPR-CAS9

A new way of genetic editing

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Table of Content

1.Preface

If you would've told someone in the 1980's that computers would soon take over right about everything; the stock market, our communication systems, even dating and shopping, this person would've looked at in you in disbelief and thought you're delusional and/or crazy. But then, it became reality. We might be at a similar point with genomic editing. Things we thought that might be impossible a few years ago might become reality, thanks to a new technique: Gene editing with CRISPR-Cas. In this paper we want to answer how this interesting technology works, especially the technique using the Cas9, how it is evolving and what could be achieved with it in the future. We'll also talk about the ethics concerning the genomic editing of humans.

2.Introduction

Humans are subject to countless Genetic diseases. They can range from mildly annoying, such as colour-blindness, to deadly, like haemophilia, to causing decades of suffering, like from the Huntington's disease. Also, retroviruses, Herpes or HIV to name some, are diseases that are or get embedded in the genetic code of the affected individual and are currently nearly impossible to completely get rid of. However, CRISPR has the potential to change that.
Genomic editing in eukaryotic cells is possible since the 1980's however the methods used back then depended on the process of homologous recombination and proved to be ineffective and not suited for a widespread application, ending up only being applied on mice and lab-yeast. Later genome editing techniques such as TALENs (Transcription activator-like effector nuclease) were discovered. However, these were very time consuming and expensive. And then was CRISPR discovered.

2.1 History of CRISPR-Cas9

It started in 1993, the researcher Francisco Mojica identified for the first time, what was later known as the CRISPR locus. 12 Years later (2005), during the study of the bacteria Streptococcus thermophilus, the Cas9-Enzyme was discovered by Alexander Bolotin. Luciano Marraffini and Erik Sontheimer discovered in 2008 that the CRISPR system targets and edits DNA-sequences. Moreover, John van der Oost, discovered the crRNA. During 2008 until 2011 all necessary components were discovered for the CRISPR-system to function the year is 2012, the scientist Virginijus Siksnys and his team manage a breakthrough. For the first time ever, they were able to purify Cas9, artificially insert a crRNA sample and were able to confirm its functionality. Also, the ability to reprogram Cas9 to target a site of choosing by changing the sequence of the crRNA were uncovered. In the same year, Charpentier and Jennifer Doudna simplify the usage of CRISPR by creating the single guide RNA. Feng Zhang managed in 2013 to adapt the CRISPR-Cas9 system for genome editing in eukaryotic cells and demonstrated it in human and mouse cells. In 2015, it was finally announced the breakthrough of the year, after being in the runners-up 2012 and 2013. And this year, in the USA the US Food and Drug Administration (FDA) gave their approval to start clinical testing of CRISPR-Cas therapies, so first human trials just begun.

2.2 Before CRISPR-Cas

Before CRISPR two other gene-editing techniques were widely used: Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs). Like CRISPR, these tools can each cut DNA. These tools have some advantages to CRISPR:

- ZFNs have an easier delivery process to the targeted gene of choice.
- TALENs appear to have a higher precision rate than CRISPR and may cause less "off-target mutations" (i.e. unintended consequences) as a result of gene editing.

However, the big advantage of CRISPR is that it is cheap and easy to use. Anyone with a laboratory could theoretically manipulate cells using the CRISPR technique.

2.3 CRISPR-Cas in gene therapy

To this point, only a few studies tried to use CRISPR to treat disease. However, there are multiple clinical trials underway, one for example at the University of Pennsylvania using CRISPR for cancer treatment (see under 7, 7.1, 9). It involves removing immune system cells from patients, genetically modifying them in the lab and infusing the modified cells back into the body. These manipulated immune cells are reprogrammed to better recognise and kill cancer cells.
A patient with beta thalassemia, an inherited blood disorder, was treated just recently in Germany.
Another study will try to treat an inherited form of blindness known as Leber congenital amaurosis (see under 7, 7.1, 10/11). What makes this study stand out is that it would be the first time CRISPR would be used to edit genes inside the human body. Usually, cells were removed from patients, the DNA of those cells was edited in a lab and the modified cells get inserted back into patients. In the study a virus would be modified to carry the genetic information the cell needs to create a Cas9 enzyme and its guide RNA. The virus would be directly injected into the patient's eyes and the virus would inject the DNA into the cells, which then would express them and create a Cas9 and its guide RNA.

However, all these studies are mostly aimed at first testing whether this is safe. They're looking for clues to whether it helps the patients. There could be side effects occurring later this year. And it will be many years before any CRISPR treatment could become widely available.

3. Description of technique

When the CRISPR-Cas9 method was discovered for genomic editing it was mainly used for precisely cleaving a specific gene sequence and, if wanted, inserting new genetic material at said cleavage. But scientist started to experiment with the Cas9-Protein and mutating ("hacking") it. New functions of Cas9 were discovered, such as being able activate or suppress the expression of a specific gene.
But to understand these techniques one must first understand how the Cas9 works and what it is.

3.1 CRISPR-Cas from Bacteria

Bacteria need to defend themselves against viruses, more specifically against so called bacteriophages. These viruses are specialised in infecting a specific species of bacteria. If a bacteriophage attacks its prey, it inserts a strand of its own DNA into the bacterium and tricks it to express said DNA, forcing it to produce new phages. Usually the bacterium doesn't survive the attack, often bursting open, as the amount of phages it produced can't fit inside itself. However, if it endures it, the bacterium saves a sample of the virus DNA, 20-30 base pairs long, in its "DNA archive" called the CRISPR locus. This locus is made up of short, palindromic repeats of DNA and the viral DNAs, as spacers in between the repeats. Should a virus attack again, the bacterium makes an RNA copy of the locus, called the pre-crRNA. One palindromic repeat and one spacer (viral DNA sample) then form one crRNA, so there are multiple different crRNA present. For all these processes the bacterium uses different Cas-Enzymes, but the one we are focusing on is Cas9. Each crRNA gets bonded to a tracrRNA and these RNA-complexes are inserted into Cas9s, RNA-guided DNA endonuclease

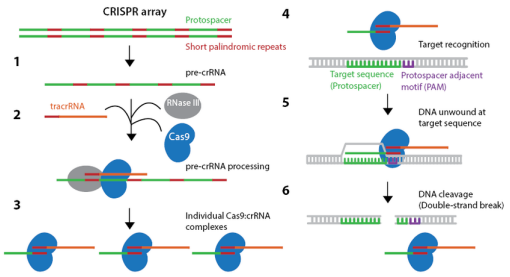

Fig 1: The process of antiviral defence with the CRISPR system in a bacterium

enzymes. The enzyme is activated by the insertion of the RNA-complex and searches the genome of the bacterium for a match of the viral DNA sample(crRNA) it carries. It randomly associates and dissociates with the DNA in the bacteria. It recognizes and binds to a three-nucleotide sequence motif called PAM that is abundant throughout the genome. This PAM usually consists of three bases, any nucleotide (A, C, G or T) followed by two guanines. If attached to a PAM, it unwinds the DNA and compares the crRNA sample to the DNA upstream (in 5'-direction) of the PAM. If it finds an exact match it will make a DSB and disable the viral gene.

3.2 How the CRISPR-Cas9 techniques functions

Scientists made the discovery that Cas9 can be reprogrammed to target a site of their choosing by changing the sequence of the crRNA and inserting it to the Cas9. This process was further simplified when it was discovered that the crRNA and the tracrRNA can be fused together into one molecule with a fuser loop. This RNA-complex was named the sgRNA. This molecule of the custom crRNA and tracrRNA can be inserted to a Cas9 and the Cas9 into any kind of cell. In the cell it will search for a match of the crRNA sample, just as it would do in a bacterium.

3.2.1 Cleaving and inserting genetic material

After attaching to a PAM, the crRNA and the unwinded DNA-strand are compared, be trying to align them. If the guide RNA perfectly aligns with the target DNA, the RNA and DNA will form a DNA-RNA helix. This activates Cas9's ability to cut DNA. It makes specific cuts in the DNA at a position three nucleotides upstream from the PAM site. Two active sites on the nuclease domain of Cas9 generate the cuts and cleave both strands of the DNA double helix, resulting in a DSB. Eukaryotic cells can naturally repair the break in one of two ways, either by NHEJ or HDR.

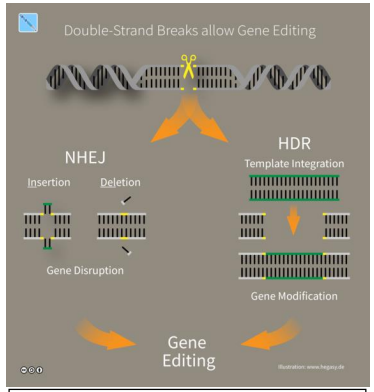

Fig 2: Processes by which a eukaryotic cell repairs a DNA break.

NEHJ is more commonly used and is faster. The cell simply joins the two loose ends together.
The disadvantage is that this process is error prone and can lead to a mutation at the targeted sequence. Often the sequence will be repaired correctly, however, if that's the case, the Cas9 will cleave this sequence again if it comes by it. This process repeats itself until a mutation

occurs and it doesn't match with the RNA guide of the Cas9. The mutations and the type are random but will occur in the exact position that is targeted. If the target sequence is within a gene's coding region, the mutation will likely inactivate that gene. Therefore, scientists will get the cell to use this method if they want to disrupt or inactivate a gene.
The second option, HDR, is less error prone than NHEJ. The cell takes a homologous DNA template, usually form a sister chromatid, and inserts it to the break and repairs it through that. This repairing mechanism can be manipulated if an excess of DNA repair templates is introduced into the cell. If a DSB occurs the cell will use one of the introduced templates, therefore making it possible for scientists to very accurately replace certain sequences in the genome of a eukaryotic cell.

3.2.2 Activating and suppressing Gene sequences

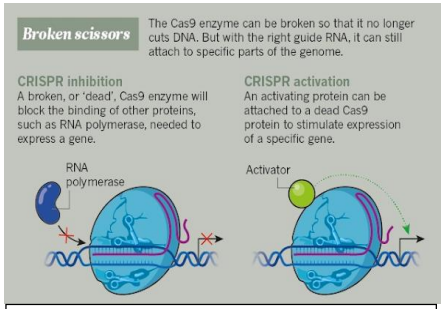

Fig 3: Uses of a mutated Cas9 enzyme

As already mentioned, one of the uses of CRISPR-Cas9 is to disrupt a gene so that the scientist may learn what it does. It reliably makes a cut at the specific sequence it was programmed for; however, the cell's native DNA repair machinery mends the cut and sometimes makes mistakes. This is sometimes wanted, however, if trying to explore what a specific gene does, and therefore turning it on and off, a minor error introduced during repair can completely alter the sequence of the protein it encodes, or halt its production altogether, which can be a problem.
Stanley Qi, a synthetic biologist, found a solution to this. He mutated the Cas9 enzyme. Now, it still bound to the DNA at the site that matched its guide RNA, however it no longer sliced it. Instead, the enzyme stalled there and blocked the access for other proteins, therefore hindering the transcription of that gene from DNA into RNA. The "hacked" Cas9 now allowed for turning off a gene without altering the DNA sequence. Other researchers then further manipulated Cas9, for example by attached a protein to the mutated Cas9-enzyme, one that activates gene expression. he Cas9 would bring the activator to the desired site and release it, therefore activating the gene. With a few other modifications, a reliable tool was made to turn genes on and off at will.

4.Interview:

This interview was held with Prof. Dr. Martin J., Biochemical Institute Zurich, via mails:

Question 1: What ethical questions do come up when working with CRISPR?
There are a lot of questions that are and must be further discussed. For example: Would it be ethically justifiable to artificially alter the human genome? If yes, where do we draw the line of which modification are acceptable or not? Eliminating diseases would most likely be accepted, however what about changing the appearance? In theory one could change hair-, eye- and skin colour and much more with a CRISPR therapy. Would this be acceptable?

Question 2: Do you think that CRISPR can be weaponized?
Yes, just as all great inventions CRISPR will be used for the good and very likely for the bad as well. Dynamite was supposed to revolutionize the mining Industry. It did do so, however the explosives that were made with it killed thousands of people. Hopefully CRISPR will be used only for the good but that most likely won't happen. There will people who'll try to use it to harm others. It could be used in sports as an advanced doping. Or it could become incredibly easy to create genetically modified bioweapons. These are just speculations and with the momentary stand of our technology these speculations can't become reality, but we should make sure that CRISPR stays in safe hands.

Question 3: Will CRISPR be able to cure cancer?
***Yes,** I believe so. There were some studies that showed promising results, but we are not there yet. Therapies still must be fully developed and undergo many safety checks and it's all very time consuming. It is better we take our time and make it right.*

Question 4: Would a CRISPR therapy be affordable to the average citizen?
Yes, I do believe it will be affordable. A good indicator to this is how easily one can use CRISPR-Cas9 without highly specialized equipment. In theory can be done in any laboratory. But unfortunately, we are still far from a useable therapy and the price could change a lot.

Question 5: Would you participate in CRISPR therapy as a patient if you could? And for what reason?
It would depend on the situation. If conventional therapies would have failed me and I'd have to turn to experimental drugs/therapies, yes, I would. However, as this would

still be experimental and would have potentially unknown long- and short-term side effects. I wouldn't just do it for a non-serious disease, one I could live with. In the past we encountered things that seemed great and revolutionary at first, but they turned out to be dangerous and a health hazard. E.g. asbestos. From 1900-1950 it was used in nearly every house in some shape or form. It was cheap and great at doing its job. But then, after nearly 50 years of usage and high exposure, it was identified as a highly cancerogenic. So, we shouldn't rush the development of CRISPR-Cas techniques and make sure it is done right.

Pictures of laboratories in the UZH (sent to us by Dr. Matrin J.):

5. Discussion

5.1 The Achievements and the Future of gene editing

In the field of gene editing CRISPR is a huge breakthrough. One nice comparison was that whereas the older techniques were could be compared to a paper map, CRISPR-Cas is a GPS. It is a method with great potential because it is so inexpensive and easy to use. It is used to develop therapies to fight cancer or gene-based diseases like Huntington's disease. I believe, in a best-case scenario, any genetic diseases could vanish simply by receiving an injection of a CRISPR-Cas based drug. Wait a few minutes or hours depending on how severe the disease is, and the person is healthy again. Other areas where this gene editing could be used is in the food industry. I could imagine a world in which genetically modified food is normal and a necessity to be able to provide something to eat for an earth with double the population as the current. But these were only a few of the possible application of gene editing and as with most thing concerning CRISPR-Cas, we are still far away from it.

5.2 Ethical Aspects

New technologies can be beneficial and are always encouraged. For example, new drugs and medical technologies can be developed to ensure patients get the best we can provide. But new technologies often bring ethical questions with them.

For example, one of the ethical questions CRISPR-Cas comes along with is the one of genetically engineered or modified humans. CRISPR-Cas could be used to grant people immunity to diseases with a modified immune system, maybe even slow down, stop or reverse aging. In nature there are animals that can do so, so maybe we could insert some of their genes into us? However, next to these health-related modifications, it would be possible to make cosmetic changes. The appearance of a human could be changed (not of an adult but an embryo could be manipulated). People could create their "perfect baby". It should be smart, strong, tall, a certain hair colour with this eye colour and a different skin tone, this list could go on and on and on. There are so many possibilities to manipulate, that an unmanipulated baby could become rare. This is where the hard questions come up. Is it unfair towards the baby, as it can't decide for itself? Or is it only fair, as this baby would have a chance at having a better live? Also, sooner or later, the modified genes would enter the human gene pool and alter it. Is that justifiable?

6. Summary

CRISPR is a, by the time of Mai. 2019, 7 years old gene editing technology that can manipulate targeted genes with a protein called Cas9 and a sgRNA. This originates as a natural defence system of bacteria against viral attacks. The enzyme that was responsible for disabling the viral DNA in the bacteria, Cas9, showed great capabilities to be a cure to a lot of diseases that were believed to be incurable. It is very easy and cheap to use and precise. It searches the genome of the cell it's in for a match of the guide RNA it carries and it if a match is found a double strand break is induced. Eukaryotic cells can repair this break in one of two ways, one simply joining the ends together and the other by inserting a DNA template. The advantage of the end joining is that is fast, while the other is less error prone and allow for manipulating gene sequences.

Until recently CRISPR-Cas was never used directly in humans, because it was unknown what risks it would bring when cleaving certain sequences. But, just recently, human trials have started.

However, there are still ethical questions to be answered and precautions to take as CRISPR is still in development.

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7.2 Figures

Fig 1:

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Fig 2:

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Fig 3:

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