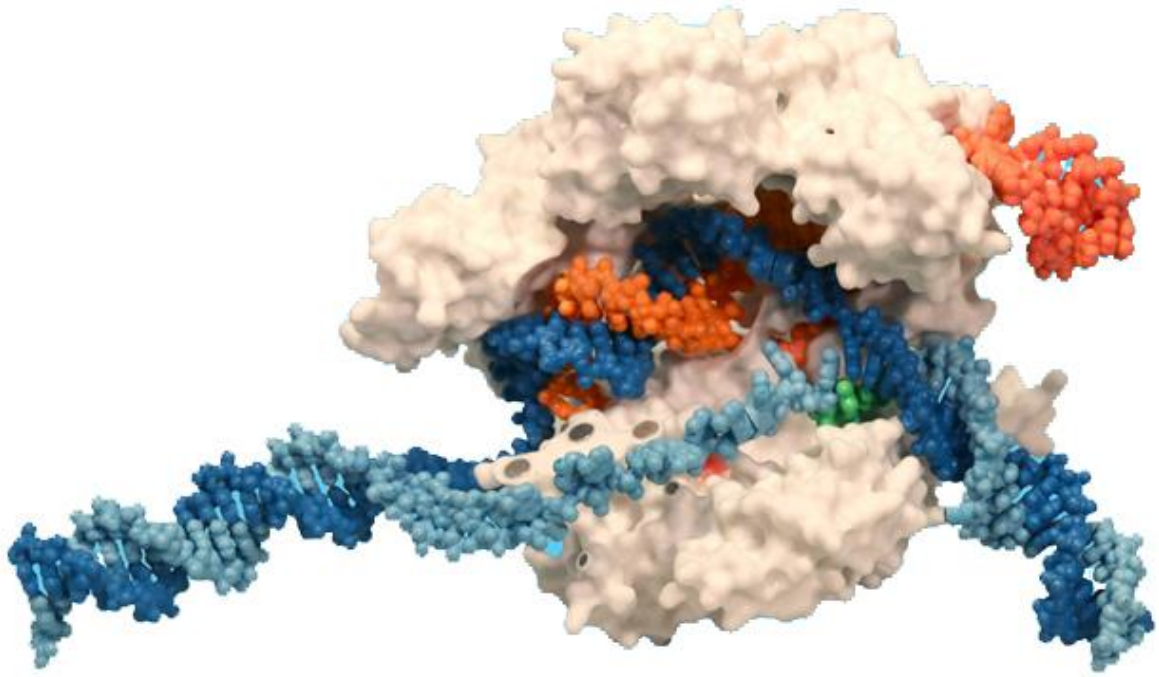
Clustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats and the **C**RISPR **as**sociated protein **9**



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Gymnasium Kirschgarten
Biology Class 2019

Preface

As we were evaluating possible topics for this term paper, we soon remembered the Biovalley-College-Day at the University of Basel, where many interesting topics in all different sectors of Biology were presented. One topic CRISPR/Cas, presented by Dr. Jean Charles Paterna from the University of Zürich, was particularly fascinating. We wanted to learn more about this technique of gene alternation and therefore decided to dedicate this term paper in Biology to this topic.

CRISPR/Cas opens an enormous variety of possibilities, such as changing genetic traits and treating various illnesses. It is interesting because its research is still in his infancy and because there is still a lot of controversy about ethical aspects and implications of genome editing.

In the following paper we wanted to answer the following questions:

- What is CRISPR/Cas?
- How does CRISPR/Cas work?
- What are the areas in which CRISPR/Cas is used?
- What are the chances and risks of CRISPR/Cas?
- What is the potential of CRISPR/Cas in the future?

Introduction

CRISPR/Cas is a new technique, which could have a dramatic impact in many areas. We are still in early stages of research, but scientists expect to largely improve the efficiency of agriculture and hope to treat diseases, which are still untreatable, e.g. cancer or genetic diseases. In the future we could even edit the genomes of babies improving their resistance against diseases, choosing how they look or making them more intelligent or muscular. It is therefore understandable, that designer babies are a very controversial topic inside the field of genome editing.
Recently a Chinese scientist, He Jiankui, used the CRISPR/Cas method to genetically modify human babies for the first time. He altered the gene CCR5, which codes for a specific protein used by the HI-Virus to enter host cells, thus claiming to have made the twin sisters Lulu and Nana resistant against the HI-Virus. (Wikipedia, 2019)

Scientific history

The research of CRISPR-sequences and the CRISPR/Cas system in bacteria began in the 1980. Especially in the 2000s the connection between the CRISPR-sequences and DNA as well as Cas9-genes became clearer. Also, the importance of CRISPR/Cas in the immune system of bacteria was discovered. In 2011 the two researchers Emmanuelle Charpentier, Jennifer Doudna and their research group discovered how the CRISPR/Cas-system of these bacteria could cut DNA at specific sights. For this work they were awarded with the "Breakthrough Prize in Life Sciences". The neuroscientist Feng Zhang was able to apply the technique not only in bacteria but in all cells. (Wikipedia, 2019)

Applications of CRISPR/Cas

The CRISPR/Cas method is applied in a variety of different areas. Its applications are still experimental at the moment but as the method progresses more direct and specific uses will most likely be developed.
It is used in cancer research, where cancer cells are produced on purpose by mutating specific genes in order to study the biology and progression of cancer. Scientists are even trying to modify and therefore improve our immune cells to enable them to attack cancer cells more efficiently. (Alford, 2017) Other research groups are working on treating the HI-Virus with the help of CRISPR/Cas, they were able to eliminate HIV-1 DNA from the genome of T-cells and by adding mutations to the viral genome, making it unable to reproduce. (Sciencealert, 2016) Researchers are trying to develop alternatives to antibiotics by "equipping" bacteriophages with CRISPR/Cas-systems. This would not only be very specific for different types of bacteria but it would also be nearly impossible for the bacteria to build a resistance against the CRISPR/Cas-system itself, as it would have to destroy molecules of its own immune system. But there is still the possibility of the bacteria developing a resistance against the bacteriophage. To this point the only other way to inject the CRISPR/Cas-system is by shocking the bacteria, which would not be a suitable method to use inside the human body. (Bent, 2015)
With CRISPR/Cas we could eventually eliminate malaria by introducing genes into malaria flies, which make them resistant to the parasite and are even transferred to their offspring in the majority of cases. (Ledford & Callaway, 2015)
The CRISPR/Cas technology could also be used to protect plants, produce food and some scientist are even planning to revive extinct animal species. There is a long list of applications, the potential of CRISPR/Cas is enormous.

Alternative treatments

Before CRISPR/Cas rolled over the genetical research there were already other methods in place to get the same job done. Both of the examples given followed the same principle of fusing a non-specific nuclease, a protein that can cut DNA, to a sequence specific DNA binding domain of a protein.
Zinc Finger Nucleases, a method by which restriction enzymes, similar to nucleases, are fused to Zinc fingers, proteins with integrated zinc atoms that can bind to DNA, are one example. The Zinc fingers can be engineered to identify specific locations in the range of nucleotide triplets. However, there is the chance that off-target cleavages occur. (Gene Therapy Net, 2011)
Then there are also the TALENs. **T**ranscription **a**ctivator-**l**ike **e**ffector **n**uclease. TAL effectors are proteins which can bind to a promotor and are able to recognize certain DNA sequences. If a TAL effector is now fused to a nuclease a TALEN is formed. As they recognize DNA series, they can also be engineered to target specific DNA sequences, however off-target cleavages are still a problem. (Wikipedia, 2019) (Wikipedia, 2019)
Both of those alternatives are beaten by the simplicity of CRISPR/Cas. Once it was modified to work not only in prokaryotic cells, it offered more advantages over the other methods. Not only is it simpler, it is also more efficient and offers the advantage of introducing other genes into the genome while cleaving in the same process. (Yeadon, 2014)

Description of engineering technique

Before explaining how the CRISPR/Cas method works it is important to understand what CRISPR/Cas means. CRISPR stands for **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats. Meaning a repeating sequence of base pairing, that comprise different viral genetic material, so-called spacers, within them. Those sequences are naturally found in prokaryotes due to the defensive use of CRISPR against viruses. The exact function of those genes will later be explained. Cas on the other hand is the abbreviation of **C**RISPR-**as**sociated protein. The correct scientific name would actually be CRISPR/Cas9 as it then is clear, that the CRISPR-associated protein 9 (Cas9) is meant. The ability to interact with RNA is the most important feature of the Cas9 protein. To this day Cas9 is the most used endonuclease for the CRIPSR/Cas job.

In a bacterium the process starts with the copying of gene sequences of invading viruses and a following insertion of those sequences (spacers) into their own genome. If the same viruses attack the bacteria it starts the CRISPR/Cas defense by transcribing CRISPR-sequences with the virus-specific spacer into RNA. The so-called crRNA or guide RNA. After the transcription there is a molecule that contains both virus RNA as well as bacteria RNA. The bacterial RNA will later get important.

Now so-called tracrRNA (trans-activating RNA) binds itself to the bacterial RNA (Figure1) and together they can fit into the active center of Cas9. Only together with the tracrRNA the crRNA can be set up in the right place to form a Cas9 complex. Scientist found a way to join crRNA and tracrRNA into one molecule, therefore simplifying the method by a lot.

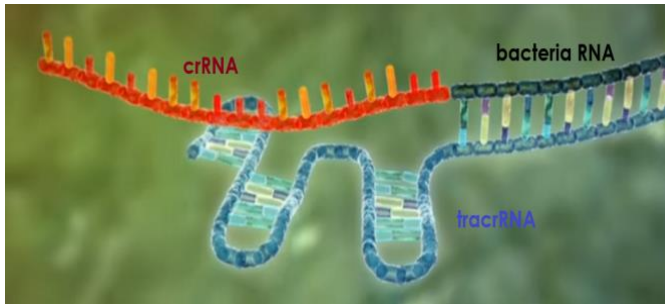

Figure 1 (MaxPlanckSociety, 2016)

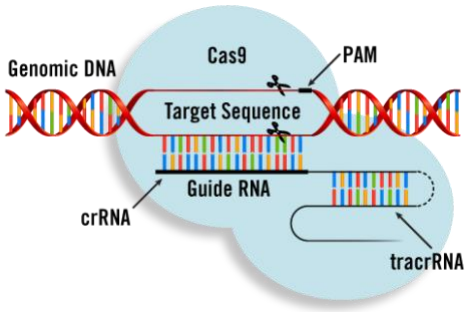

Figure 2 (Levien, 2017)

If now a double-stranded (ds) DNA of the virus enters the Cas9 complex, the Cas9 can bind to the viral dsDNA if there is a specific base sequence called PAM. Once it is bonded, the crRNA unwinds the DNA and the Cas9 cuts the dsDNA. With the cleavage the function of CRISPR/Cas9 is completed. The DNA can repair itself but as there is no strand to give information about which bases are needed the fixing process often fails and therefore deactivates the gene or changes it.

As there has been and still is a lot of research going on, the Cas9 complex can further be modified and has been. E.g. Cas9 had first been modified to work not only in prokaryotic cells but also eukaryotic. The complex can still be further modified, so it does not only cut but also inserts specific bases or even changes them without cutting. The vast number of possibilities on how to modify the complex is still to be investigated.

Discussion

Past progress

When CRISPR/Cas first was discovered in bacteria cells it quickly became clear what potential it had and still has. But back then the first problem to solve was that it became possible to use CRISPR/Cas not only in prokaryotic cells but also in eukaryotic cells. The research developed into modifying the Cas9 protein. As CRISPR/Cas became more popular the variety of modifications expanded just as quickly. Some scientist deactivated the active centre in the Cas9 protein to then fuse other proteins with Cas9 that then change just one base specifically without cutting the dsDNA. In this example researchers used an adenosine deaminase to specifically change A-T to G-C. The CRISPR/Cas was used as a guide to the correct DNA sequence. The variety of different CRISPR/Cas forms and uses increased over the past years. From a defence mechanism in bacteria/archaea cells to a tool that allows us to change bases, shut down certain genes, cut them out or even insert new ones. (Li, et al., 2018)

Future research steps

As CRISPR/Cas is widely usable, more and more areas and applications will be studied. In general, the future research of CRISPR/Cas will mostly focus on making the technique more efficient. A lot of work is still needed to make it more specific and to minimize its off-target effects. (Paterna, 2019) At the moment the risk of failure and unintended effects of the method are very small, but still not at an acceptable level to apply this method to humans outside of heavily regulated research contexts.

Ethical discussion

As the potential applications of CRISPR/Cas are extremely diverse, so are the opinions on where and how far it should be used.
One could claim, that the editing of genomes is simply unnatural. Even though it could start with single point mutations it could also reach a point, where mankind loses its humanity. It could lead to a generation of "Superhumans", who could be made more intelligent, muscular, and efficient, which could create an even larger social gap as the treatment would probably favour already rich societies. Also we have not yet seen the impact of genome editing on future generations of genetically modified humans.
With CRISPR/Cas we could potentially delay or stop ageing, which could be seen as a positive or negative aspect. On one hand we could prevent many diseases, which come with ageing, but it would also have a tremendous impact on our society, which we cannot foresee.
As for now CRISPR/Cas is the simplest, cheapest, and most efficient genome editing tool. It could and is to some extent already used in agriculture.
Not only the productivity of plants could be improved, but also their resistance against pests.
Most importantly CRISPR/Cas could be an effective treatment for many diseases, which to this day cannot be cured or provide an alternative to other limited treatments.
In the end the difficulty remains in drawing the line on how far genome editing should be allowed. To tightly and the technique might be further developed and used by underground organizations and to loose and our society could change drastically.

Interview with Dr. Jean-Charles Paterna of the University of Zurich

- What is your field of research and in how far is CRISPR/Cas important?

The VVF is a central technology platform located at the University of Zurich and as such, it provides viral vectors and associated services to the research community on a worldwide basis. The delivery of genetic information by viral vectors is a rather efficient means and therefore, research aiming at expressing CRISPR/Cas within particular cells relies to a certain extend on viral vectors.

- How did you have to alter the CRISPR/Cas method to fit your needs in research?

The VVF does not modify the methodology. Rather, as a service unit, we produce viral vectors based on existing DNA sequences. However, the VVF sometimes is asked to modify existing DNA sequences to fit specific needs.

- Do you think the method could be improved, if yes how? Or would we have to find new more effective methods?

CRISPR/Cas methodology will certainly be improved (there is heavy ongoing research activity towards this goal), mainly to increase specificity and efficiency and to minimize/abrogate off-target effects. It is not excluded that new methods - that will be more effective - will be discovered or developed.

- Does CRISPR/Cas research help to advance in the decryption of DNA?

CRISPR/Cas certainly helps to identify the function of particular DNA sequences by modifying them in a targeted and rational manner. Therefore, the answer to this question is "Yes".

- Where do you see risks and consequences of the method?

Off-target modifications (alteration of DNA sequences at non-targeted sites) that may cause negative effects and prolonged expression of CRISPR/Cas in eukaryontic cells that may not be well tolerated. The consequences of this is to generated highly site-specific CRISPR/Cas systems and to express those system only transiently.

- Could you comment on the following statement?
  "As the technology improves it will become more unethical not to use genetical engineering."

If CRISPR/Cas proofs efficient in ameliorating or preventing the course of a disease that is caused by alteration(s) in the DNA sequence (for which no other and more suitable therapies exist), then it may be not ethical to grant access to this methodology.

- What could be achieved using CRISPR/Cas, either good or bad?

Pros:
Genetic modifications to
a) ameliorate or prevent a disease caused by alterations in the DNA sequence
b) study the function of DNA sequences

Cons:
Genetic modifications to
a) alter the genomic DNA for non-live threatening phenotypes (either by deleting or adding new properties)

- Where would you draw the line at using CRISPR/Cas with all its potential?

This is currently not possible, simply because the methodology has not yet unrevealed its potential. As stated above, alterations of DNA sequences that are merely based on achieving improved or new phenotypes may not be ethically acceptable.

- How do you think CRISPR/Cas will affect our society?

CRISPR/Cas may add a new tool for the treatment of life-threatening diseases (such as cancer).

Summary

Clustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats and the **C**RISPR **as**sociated protein 9, a complex of DNA and proteins stemming from the immune system of bacteria, is an important mean for gene alteration. By locating a gene sequence and cutting it at the specific sight CRISPR/Cas is a very precise and efficient method, its potential is enormous.
Its applications are very diverse. It is predominantly used for research, such as cancer research, where it is used to purposely mutate cells for studying purposes. Researchers are developing alternatives to antibiotics, searching possibilities to treat the HI-Virus, eliminate Malaria or even trying to revive extinct animals.
CRISPR/Cas opens possibilities to heal before untreatable diseases. To this day the technology has not yet been perfected to all extent. There is still a small chance of failure, such as off-target alterations, which is why it is not used in humans. But once improved the the vast possibilities range from simple single point changes to designer babies.

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Figures

FIGURE 1 5
FIGURE 2 5