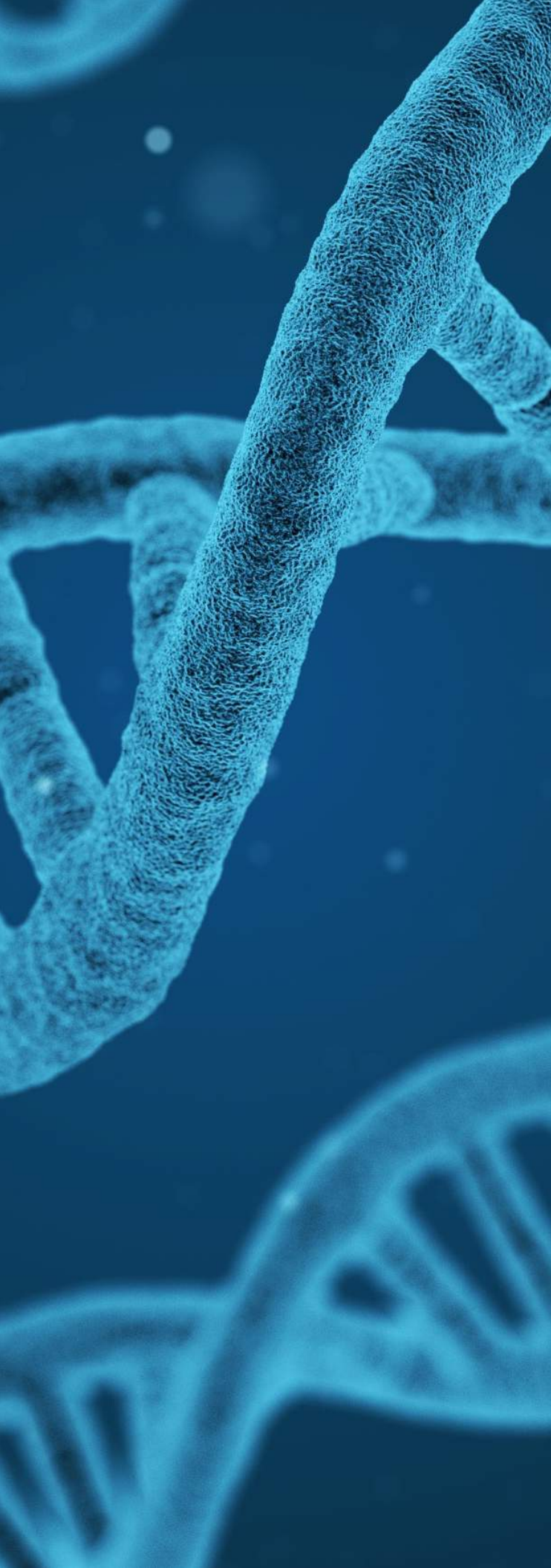

NEXT GENERATION GENOME EDITING TECHNOLOGIES



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ABSTRACT

Gene editing took a new turn with the discovery of CRISPR and its variant CAS9, but along with it came legal battles and a need to find alternate solutions to gene editing. CRISPR not only gave a new image to gene editing but also raised the standards for new findings. Fortunately, tools like Cpf1 and NgAgo were discovered that not only stood up to the standard but proved themselves better. Though, they are in a development stage and proper testing is yet to be done, still they have given a hope to world of both preventative and curative sector.

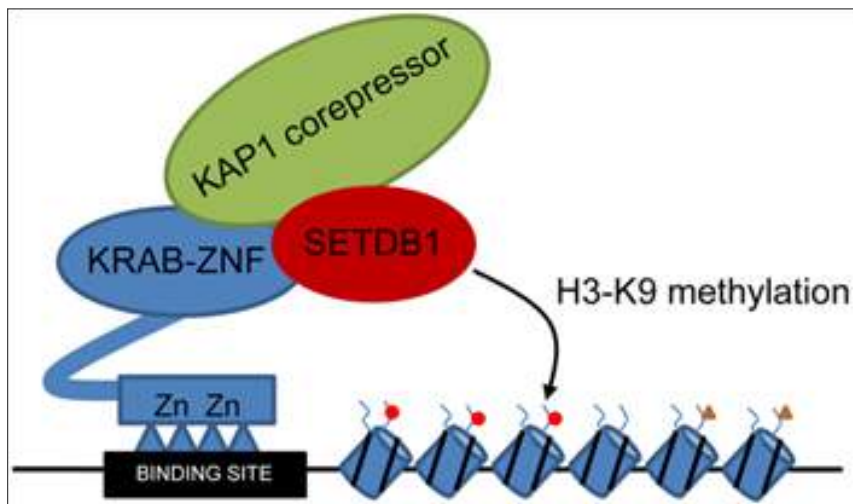


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INTRODUCTION

Renaissance took a lot of pain to take over the dark age of knowledge. Man was unaware of the living beings and varieties of species surrounding him. Nobody in that era would have thought that a day would come when evolution will not seem magical, instead man himself would become capable of instigating evolution. It is said that true powers lie in bringing out a change that we want to see and it is true philosophically as well as genetically. Genetically modified organisms had still not phased out of minds, when hype got created about how these organisms are made. Curiosity lies in the methods devised for genetic modification. Though, there are many methods to do so, but one has made itself stuck in bright minds of researchers.

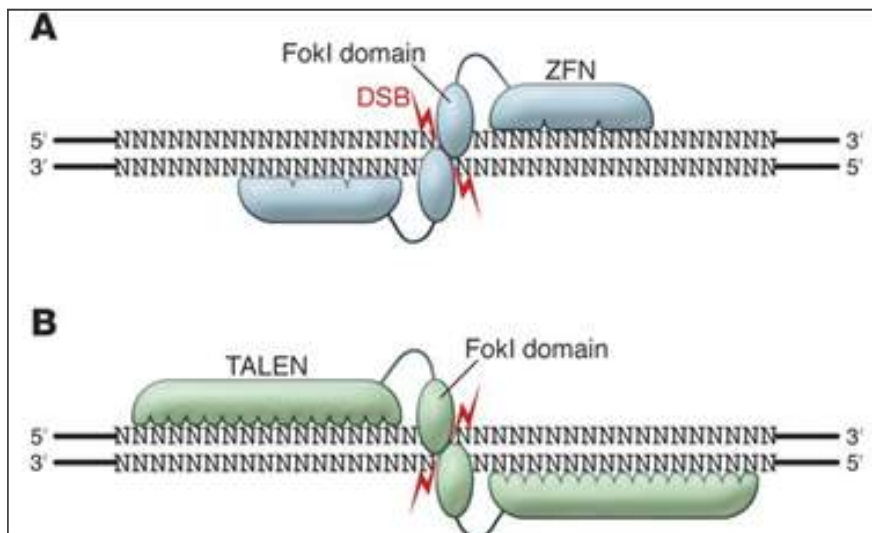


Source: <http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0015082>


Genome editing with engineered nucleases, which can be designed to specifically target a sequence of DNA has been exploited since 1990, when Zinc finger nucleases came into light. Using the property of nucleases along with exploiting endogenous DNA repair apparatus, the DNA of higher forms could be altered with accuracy.

The zinc finger proteins are vast family of proteins that have made themselves useful in numerous ways in biological system. All the Znf found have one thing in common and that is the presence of at least one zinc ion which is required for the coordination of all functional domains present in Znf. The Cys2His2 motif of Znf recognizes the specific nucleotide triplet in the alpha helix and another domain of Znf is that of a nuclease which acts like a sequence specific endonuclease that works to binds and break DNA at specific positions. As said previously, it has two domains, where one of the domains binds to DNA and comprises of eukaryotic transcription factors along with the zinc finger whereas the second domain is comprised of a nuclease FokI enzyme involved in breakdown of DNA (catalytic).

Later, another nuclease TALEN was discovered which began to compliment ZNFs domain and was considered a perfect gene editing tool for years. TALENs are combinations of transcription activator-like (TAL) proteins and a FokI nuclease. TAL proteins are made of repeating motifs which are 33-34 amino acids long with two variable positions that have a perfect recognition for unique nucleotide.



Source: <https://www.jci.org/articles/view/72992/figure/2>




Science keeps on looking for more efficient tools than we have at a certain point of time and so CRISPR was introduced to mankind. CRISPR/Cas9 nucleases have several advantages over ZNFs and TALEN, such as target design simplicity, efficiency and multiplexed mutations.



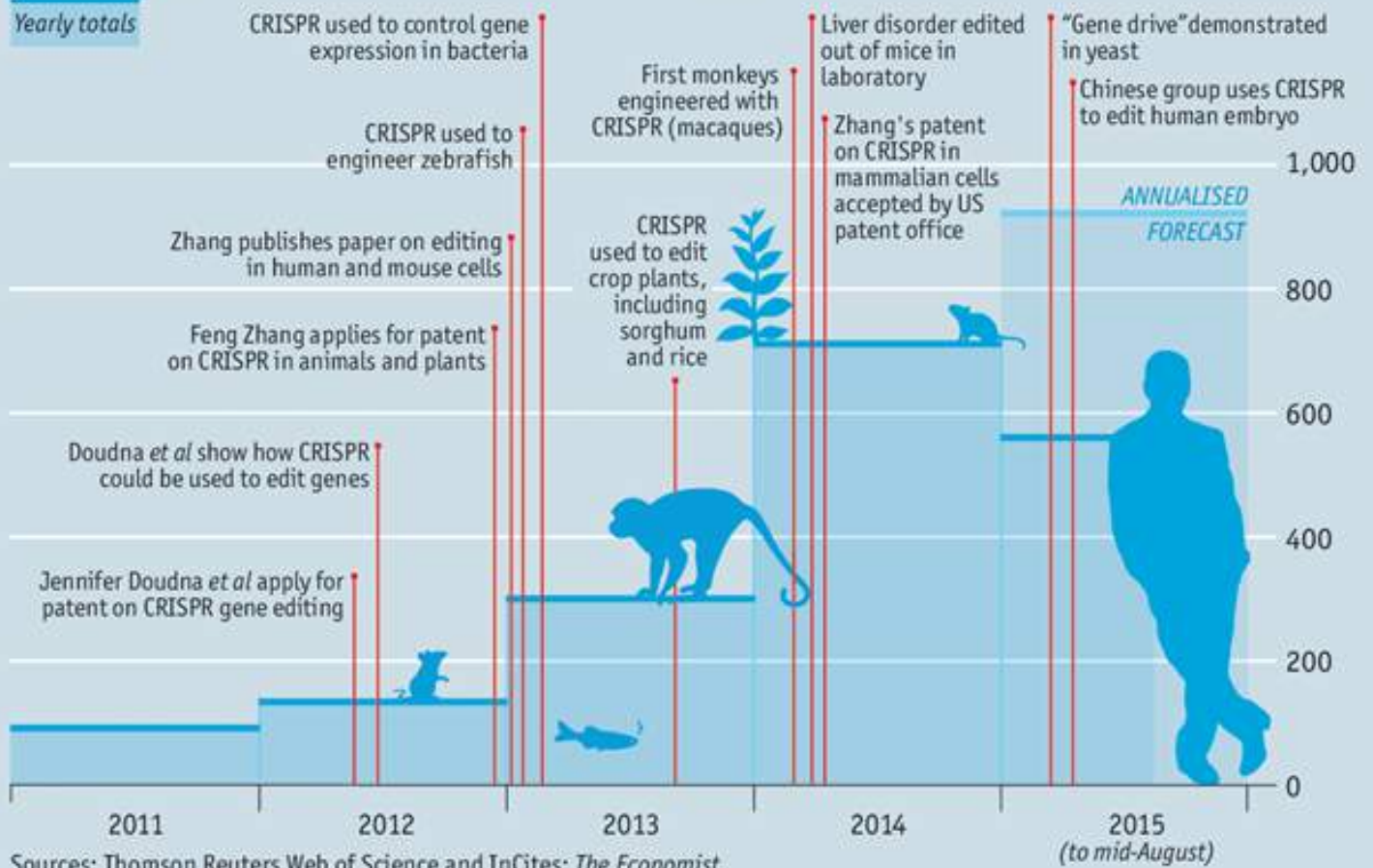
HISTORY OF CRISPR

Clustered regularly interspaced short palindromic repeats; are part of prokaryotic genome and as their name suggests they are short repetitive base sequences, which have an important role in bacterial defense mechanism. They are not only vital to prokaryotic defense mechanism but are also considered as well-established pillars of modern day gene editing. These repeats have repeated themselves in history many times even when they were not fully understood:

1. In 1987, Yoshizumi Ishino of Osaka University accidentally cloned a part of CRISPR together with target gene of his interest, *lap*.
 2. In 1993, while studying *Mycobacterium tuberculosis*, researchers in Netherlands published two articles about a cluster of interrupted direct repeats. They exploited this discovery to design a typing method known as Spoligotyping, which is used till today.
 3. At the same time repeats showed themselves in archaeal species, *Haloferax* and *Haloarcula*. The function was then studied by Francisco Mojica, at University of Alicante, Spain; who also showed that transcription of these repeats was also possible.
 4. Finally, in 2007, Barrangou and colleagues demonstrated that *S. thermophilus* can acquire resistance against bacteriophage by inserting a genome fragment of infectious virus into its CRISPR locus.
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Stepping up

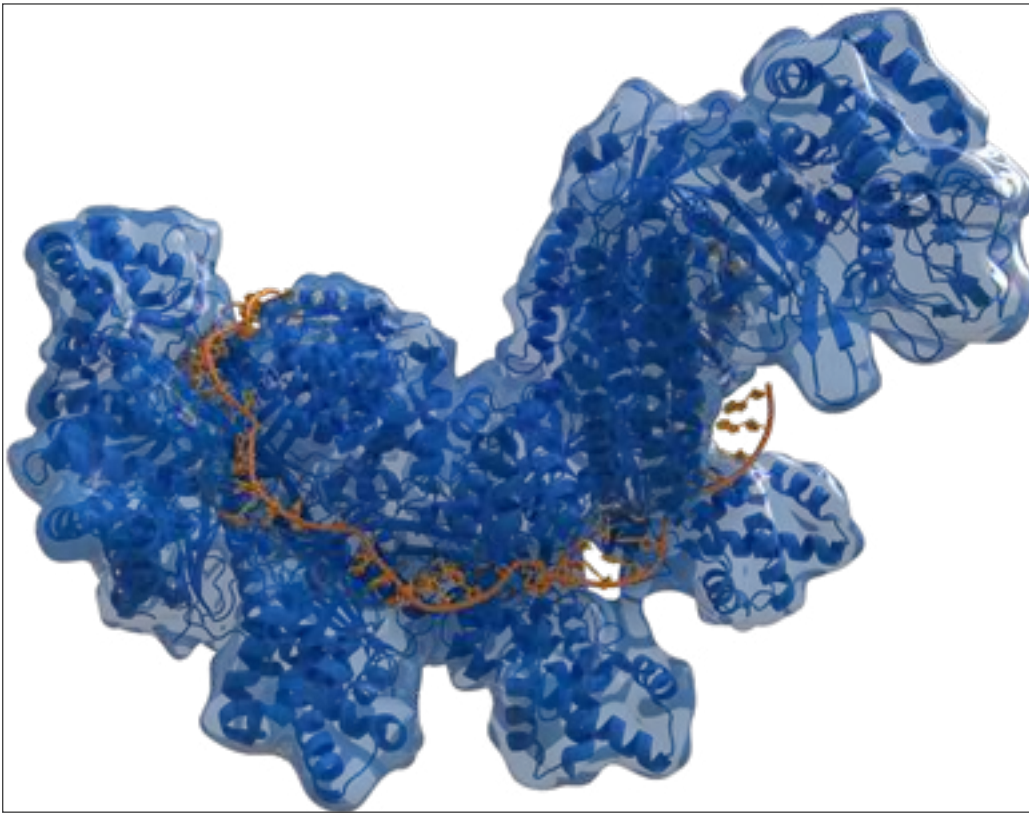
Number of CRISPR papers published and some research highlights



Economist.com


Source: <https://alfinnextlevel.wordpress.com/2015/08/25/enhancement-of-human-intelligence/>

PROBLEMS IN HAND: CASE OF CAS9: CRISPR ASSOCIATED SYSTEM



Structure of CrRNA-guided *E. coli* Cascade complex (CAS, blue) bound to single-stranded DNA (orange)


Source: Thomas Splettstoesser (www.scistyle.com)



Making precise and correct changes in target genome has been a dream for researchers. Luckily, we live in an era where dreams can become reality. A similar boon was given to the domain of gene editing when a tool based on bacterial nuclease known as CRISPR -associated protein-9 (CAS9) came into spotlight. Isolated from *Streptococcus pyogenes*, CAS9 became most efficient tool after TALENs. It is a known fact that CRISPR and CAS are essential in providing adaptive immunity to bacterial and archaeal species, by enabling them to eliminate the invading genetic material. There are three types of CRISPR mechanisms and type II is studied most:

- The attacking viral DNA is cut into small fragments and integrated into CRISPR locus between small repeats.
- These loci are then transcribed and are processed to give CRISPR RNA or CrRNA.
- CrRNA then guides endonucleases which cleave the target foreign DNA based on complementary sequence.

Disadvantages:

- **Offsite effects:** There were cases when changes were made to similar but not identical sites. This led to formation of offsite mutation that had potential to cause collateral damage.
 - **Mosaicism:** Organism with a mutant allele in only some of its cell can also result in a problematic production, as nucleases may not have cut DNA at one of the cell stages of embryonic development.
 - **Multiple alleles:** Production of organism with multiple mutation sites is also possible. Therefore, extra step of breeding might be required to separate organism with single mutation.
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LITIGATIONS SURROUNDING CAS9:

The ease with which CAS9 has shown its productivity, it is not hard to visualize it as highly utilized technology in academic and commercial laboratories across the world. In the landscape of CRISPR patent, there are two major role players, who could secure rights to CRISPR system. The first group is led by Jennifer Doudna, a professor of chemistry and Molecular and cellular biology at University of California, Berkeley, USA. In collaboration with Dr. Emmanuelle Charpentier's group, Jennifer Doudna in 2012 published the first characterization of function CAS9 within the CRISPR system that reflected its ability to induce site specific double stranded breaks in target DNA.

The second major party is that of Dr. Feng Zhang's group from Broad Institute of Harvard and MIT USA. He published a paper reporting guide sequences that could simultaneously cut genome in eukaryotic cells, making CRISPR widely applicable. Zhang was awarded the first patent on the basic CRISPR technology - US8697359; CRISPR-Cas systems and methods for altering expression of gene products'.

Doudna/ Charpentier had filed their patent in March 2013 and Zhang had filed in October 2015, so then why did Zhang's patent get approved? The answer lays in the difference between cell types i.e. prokaryotic v/s eukaryotic cells. PTAB has recently decided that both parties can maintain patents based on this differentiation. But University of California and its co-owners disagree with the decision as they believe that using CRISPR-CAS9 system for eukaryotic cells is not different from using it from other cell types. But the major question that develops itself in whole scenario is that whether a technique like CRISPR which being used worldwide should be patented, should one organization get all the rights of using it or should it be accessible to other parties as well?






ALTERNATIVE SOLUTIONS:


With the ongoing strife for rights to CRISPR acquisition, other researchers who have keen interest in gene editing tools or who need developing a much better tool than CAS9 which could overcome the problems related to it have laid their eyes on two new emerging tools:

1. Cpf1

The history of gene editing became interesting when a new nuclease came into picture. Zhang's lab recently published a paper describing two genes from Cpf1 family that have cleaving ability in mammalian cells.

1. It is a class II nuclease (CRISPR from *Prevotella* and *Francisella*) and is classified as type V CRISPR system.
2. It has RuvC-like endonuclease which is similar to CAS9's but lacks in HNH domain.
3. It requires only one RNA whereas CAS9 required two (trRNA and crRNA).
4. It cleaves DNA in a staggered manner and gives 5' overhang 18-20 bp away from PAM sites. CAS9 produces blunt ended double strands (3nt upstream of PAM sites).
5. The Cpf1 crRNA has a much simpler structure (short stem loop in direct repeat).

Benefits:

- Due to staggered cleavage, directional gene transfer is possible
 - Sticky end mediated gene transfer can be used to target nondividing cells, which are difficult to modify through HDR.
 - AT- rich regions that did not have enough 3'-NGG PAM sites can also be treated as targets.
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- The synthesis of crRNA is economically cost effective because when one uses Cpf1 guide RNAs they have to work upon only 42 nucleotides. Whereas in CAS9 cost of 100 nucleotides has to be bared as it is a hybrid of (crRNA/trRNA).
- It is easier to deliver them as their size is small. Low capacity vectors can be used like adeno-associated viral vectors.
- 'Second chance mechanism' can be carried out as PAM sites are not destroyed during cleavage. This is because they are located 18-20 bp away. So a second HDR edit can be made.

2. NgAgo: The experimental child

Gene editing has never been so interesting like it is being right now. While CRISPR is making scientist gaga over its abilities, there has been a new development to its genre. NgAgo, a DNA cleaving argonaute from *Natronobacterium gregoryi*, plays an essential role in eukaryotes during RNA interference. It binds guideRNA to cleave foreign DNA. They are also present in prokaryotes and help them protect against foreign DNA. The orthologs like NgAgo which are thermophilic in nature can cut plasmid and genomic DNA in mammalian cell lines. It can also mediate HDR, when a template is supplied.

Cpf1	CAS9	NgAgo
Endonuclease Lacks HNH domain	Has HNH domain	Argonaute protein
Requires only one guide RNA	Requires trRNA as well as crRNA	Requires guide DNA
Produces sticky end	Produces blunt end	Base removal technique
PAM sites are not destroyed	PAM sites destroyed	PAM sites are not destroyed
Easier to deliver, low capacity vectors can be used	Low capacity vectors cannot be used	Low capacity vectors cannot be used

Smaller in size i.e. 40nt	100nt hybrid of trRNA and crRNA	24bp small
Synthesis is cheaper	Costly	Cheaper. Can be ordered as oligonucleotide
AT rich regions can be targeted	AT rich regions cannot be targeted as they lack PAM sites	Has lower potential for off-target effects

Benefits:


- Does not require PAM sequence, so choosing a target is more flexible than in CAS9.
- Uses DNA guides instead of RNA guides, which are short 24bp DNA.
- Editing efficiency is not affected as transcription isn't there, so changes in gRNA secondary structure is not possible.
- Small capacity viral vectors can be used.
- Follows the concept of base removal i.e. randomly removing 1-20 nucleotides from cleavage site given by gDNA. Therefore, foreign DNA cannot recover its original sequence.

CONCLUSION

It does not matter who wins the legal war or which tool is better as long as health and heredity is in hands of gene editing. Though it may seem like a work of science fiction that DNA could be edited and modified according to our needs but we are indeed living in the era of technical magic. Extinct animals which most of the modern generations does not know about or have seen in history books could be brought back to life by working with their recovered genes. Unintended results may take place, but what is work without a little pain.




Source: <http://news.nationalgeographic.com/2015/12/151203-gene-editing-terrific-terrifying-science/>



Apart from bringing extinct animals back to life, gene editing tools could be used for curing diseases like Alzheimer's. Then too targeting the correct gene still remains a challenge because of deleterious effects, collateral damage and ethical concerns as even airplanes once were considered a myth. Nevertheless, gene therapy is already in use to treat Sickle cell anemia, blood thalassemia etc.


Heredity is reserved as it will be possible to stop the diseases from being inherited. In vitro fertilization is already in use for avoiding such inheritance, but recently George Church, a geneticist at Harvard Medical School showed that CRISPR edited sixty pig embryonic genes simultaneously. Though doing the same with human genes is still not advisable as most of our genes are in dormant stage and are still to be characterized. Deletion of such genes may prove fatal to human kind.

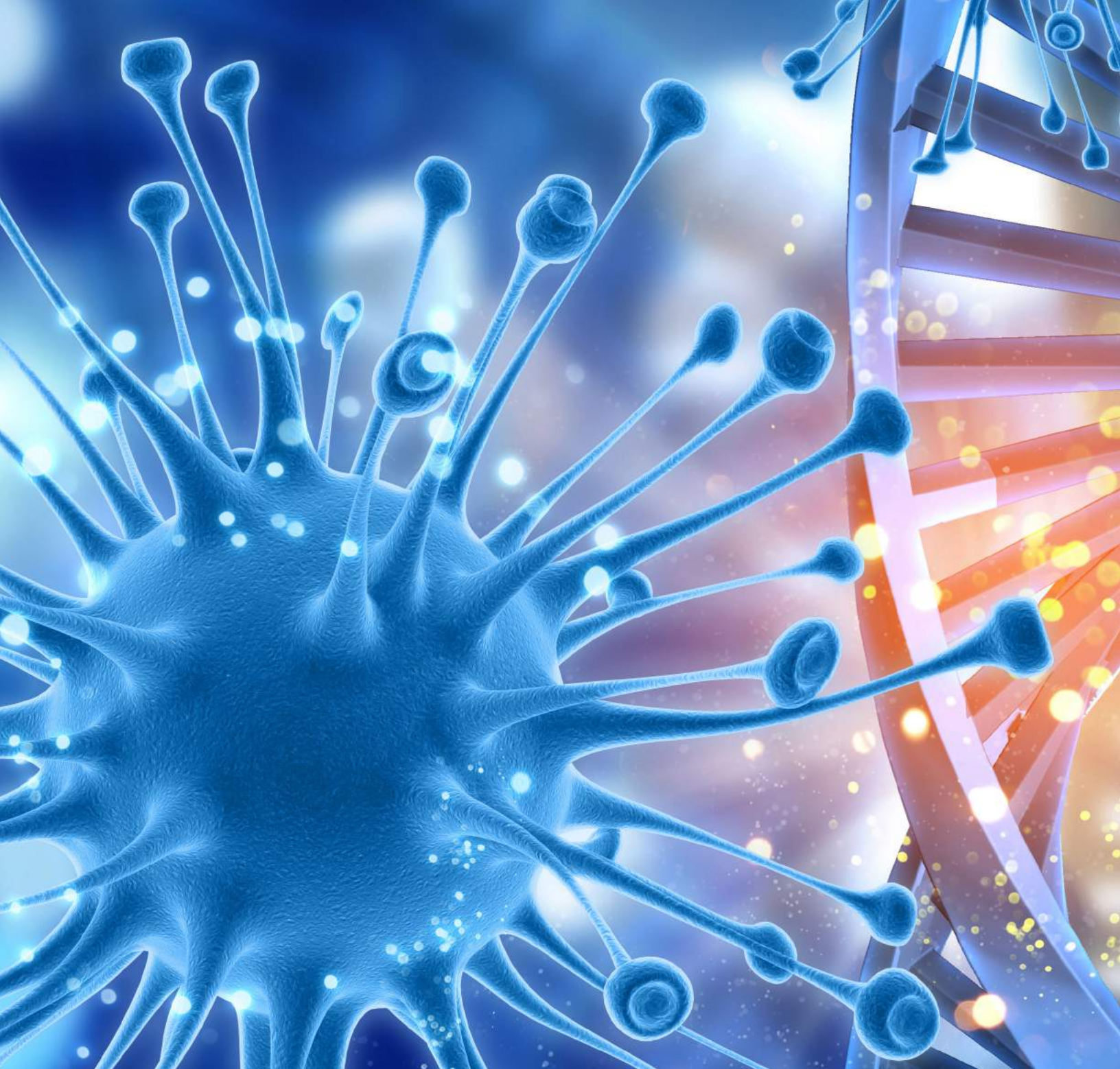
Whatever may be the benefits of gene editing or it may or may not seem like magic, the question still lingering in minds of young scholars and researchers is 'Are we on a verge of genetically engineered evolution?'





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