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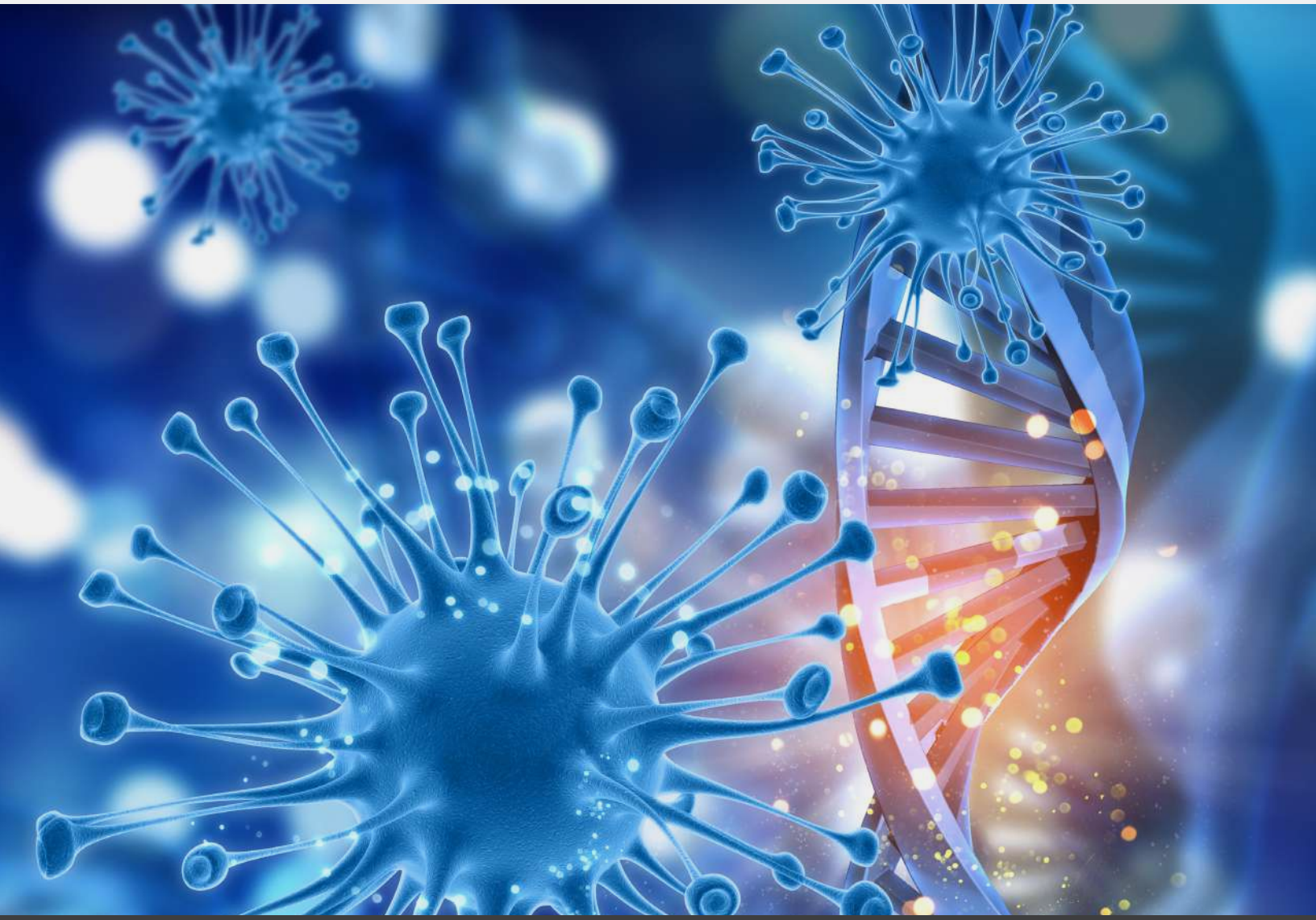
Next Generation Genome Editing Technologies



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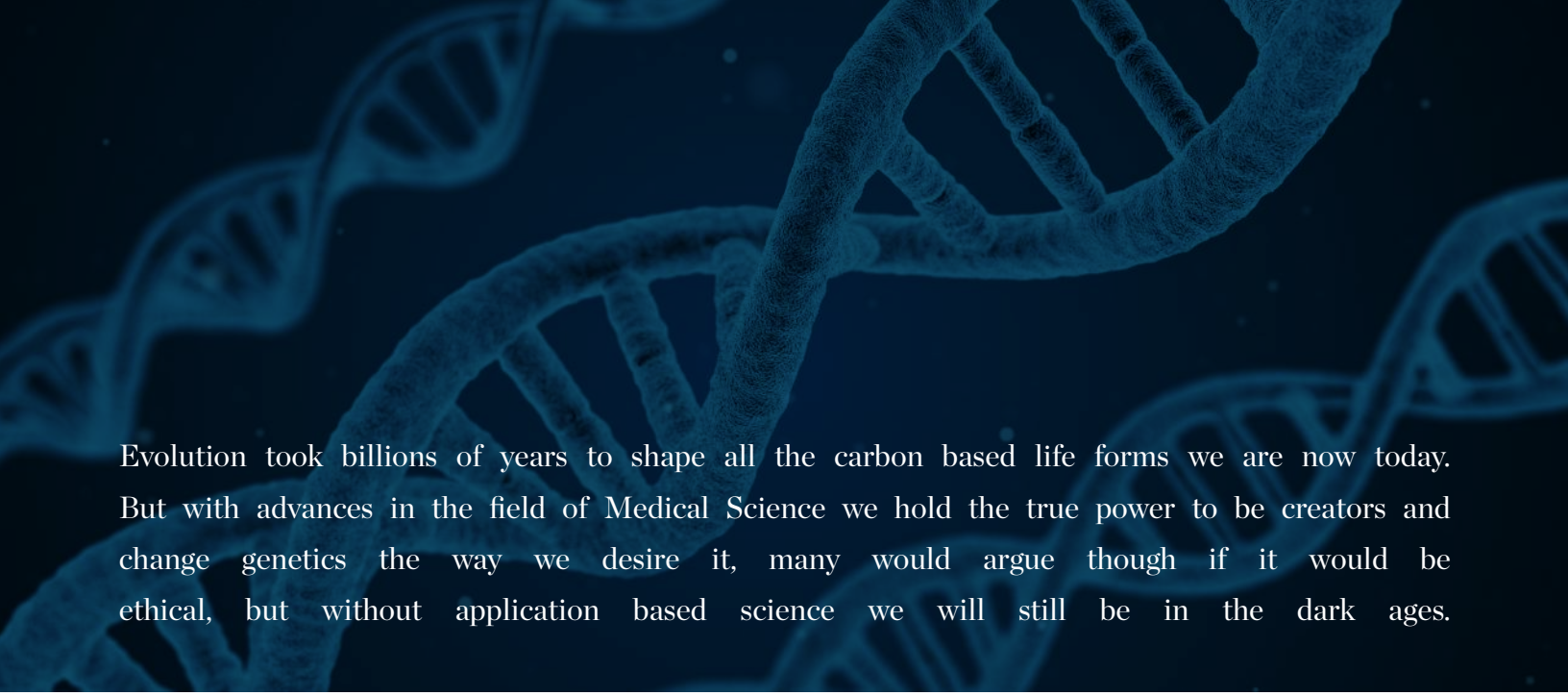


Evolution took billions of years to shape all the carbon based life forms we are now today. But with advances in the field of Medical Science we hold the true power to be creators and change genetics the way we desire it, many would argue though if it would be ethical, but without application based science we will still be in the dark ages.

With precise investigations of plant, animal and human genome facilitates astounding chances of acquired information application in biotechnology and medicine. Though there are various arms of biotechnology which are popular these days, the red hot share is genome editing with engineered nucleases. Research is going on around the globe to exploit these engineered nucleases in health sector as well as in industries. Though the effort for synthesizing these nucleases started as early as 1990, Zinc finger proteins (ZNFs) were the first genome editing nucleases to hit the spotlight. Later on, another mega nuclease TALEN joined ZNFs domain and was standard in the gene editing field for years. However, it was CRISPR/Cas9 nucleases, which brought the latest exciting development in genome editing technology with many advantages over ZNFs and TALEN such as target design simplicity, efficiency and multiplexed mutations.

CRISPR v2.0: The scion of gene editing

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 gene editing when a tool based on bacterial CRISPR –associated protein-9 nuclease from *Streptococcus pyogenes* came into spotlight. They comprise of a Cas endonuclease that is coordinated to sever an objective grouping by a guide RNA (gRNA). Clustered regularly interspaced short palindromic repeats (CRISPR); is part of prokaryotic gene and are short repetitive base sequences, which have an important role in bacterial defense mechanism. They are not only vital to prokaryotic defense mechanism but also is a base of modern day gene editing.

ThedominanceofCRISPR/Cas,whencomparedtoothergeneeditingtoolsisduetothefollowingreasons:

- Target design simplicity
- Better efficiency
- Ability to perform multiplexed mutations
- Highly economical than other engineered nucleases

It is difficult to accurately estimate the value of the emerging market for CRISPR RNA-guided nucleases in the various sector, but documents from the initial public offering (IPO) of Horizon Discovery Group, plc., which has in-licensed Cas9 IP, indicate a market size of $46 billion . The recent private equity financings of Cas9-based genome engineering companies include: Caribou Biosciences (undisclosed venture estimated at $2.9 million from Novartis), CRISPR Therapeutics ($25 million), Recombinetics, Inc. ($5 million), Intellia Therapeutics ($15 million), and Editas Medicine ($43 million). In total, companies with an interest in using Cas9 for applications related to gene therapy have raised over $600 million in venture capital and public markets since the beginning of 2013.

Business enthusiasm for Cas9 IP has not gotten away from the interest of big pharmaceuticals. Intellia Therapeutics, a leading gene-editing company focused on the development of curative medicines using CRISPR/Cas9 technology, has closed a Series B investment round with $70 million in additional financing led by Orbi Med HealthCare Fund Management and earlier backers Atlas Venture and Novartis AG. Similarly Pfizer partner Cellectis SA will be using Cas9-based technologies to make T-cells with Chimeric Antigen Receptor T-cell (CAR-T).

Shortcomings

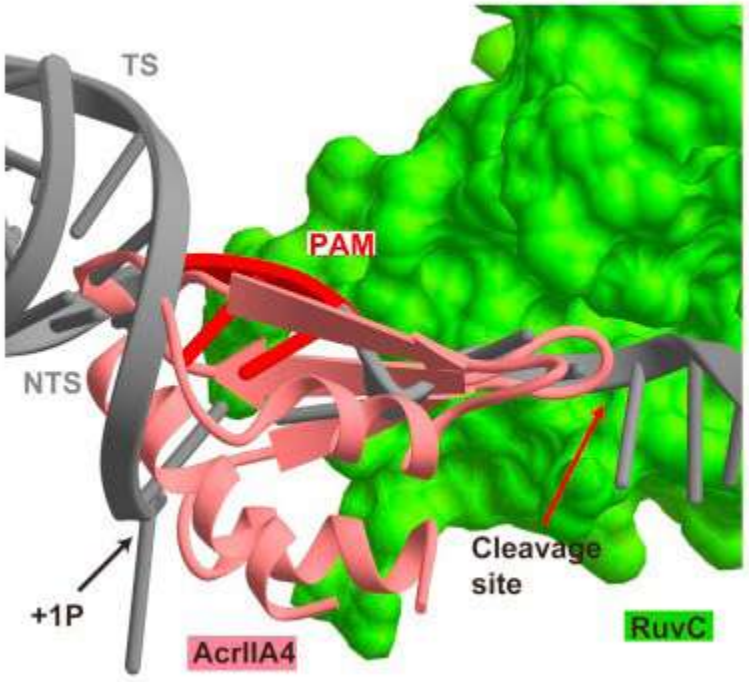
As curious CRISPR/Cas could get, so can its disadvantages:

- **Offsite effects:** Sometimes mutations are introduced at similar but not identical target sites that could lead to collateral damage.

- **Mosaicism:** Organism with a mutant allele in only some of its cell can also be a problematic production, as nucleases may not have cut DNA at one cell stage of embryonic development.
- **Multiple alleles:** Production of organism with multiple mutation sites is also possible. Therefore, extra step of breeding might be required to segregate organism with single mutation.

Technology Updates: CRISPR/Cas

Recently, two anti-CRISPR proteins (AcrIIA2 and AcrIIA4 from Listeria monocytogenes prophages) were identified which interact with SpyCas9 in sgRNA dependent manner. AcrIIA4 hinders SpyCas9 movement by basically mimicking the PAM to involve the PAM-interacting site in the PAM area, in this manner blocking recognition of double stranded DNA substrates by SpyCas9, further the AcrIIA4 affects the SpyCas9 endonuclease activity by shielding RuvC active site, thus acts as a "switch" off tools for SpyCas9 mediated unwanted genome edits within cells and tissue.



Cpf1: Rise of the second son

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

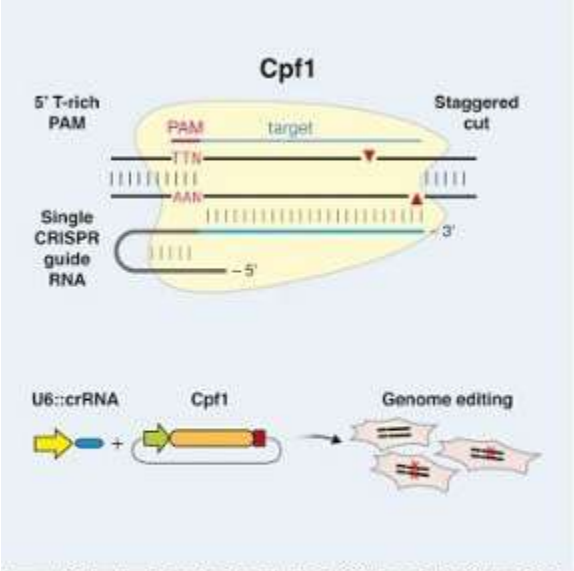


Image Attribution: *Zetsche, Bernd, et al. "Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system." Cell (2015). PubMed PMID: 26422227.*

Benefits:

- Due to staggered cleavage, directional gene transfer is possible
- Sticky end mediated gene transfer can be used to target non dividing cells, which are difficult to modify through HDR.

- AT- rich regions can also be targeted that lacked 3'-NGG PAM sites.
- The synthesis of crRNA is cheaper when one uses Cpf1 as guide RNAs are only 42nt long. Whereas in CAS9 100nt hybrid (crRNA/trRNA) is used.
- 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.

Technology Updates: Cpf1

- Agriculture mammoth Monsanto has licensed CRISPR/Cpf1 genome altering technology from Broad Institute. Under the terms of assention, Monsanto increases overall selective rights to agricultural uses of the CRISPR/Cpf1 framework. In addition, to this Editas pharmaceutical has authorized the same for human therapeutic applications.
- Center for genome engineering, within the institute for basic research (IBS) in South Korea has effectively altered two genes that add to the fat content of soybean oil utilizing the new CRISPR-Cpf1 innovation.
- German Merck KGaA has also developed a new genome editing tool called "proxy CRISPR' that allows access to microenvironments of the genome by modification of chromatin. It prevents time consuming re-engineering of natural bacterial CRISPR systems blocked at several genomic levels and allowing working into human cells. Thus allowing widening the range of druggable genomic targets.

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 important role in eukaryotes during RNA interference. It binds RNA guides to cleave foreign DNA. They are also present in prokaryotes and help them protect against foreign DNA. The orthologs similar to NgAgo which are thermophilic in nature can cleave plasmid and genomic DNA in mammalian cell lines. It can also mediate HDR, when a template is supplied.

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 effected 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.

The discovery of NgAgo has fascinated many researchers, but due to uncertainty over reproducibility of the experiments the scientific community is divided. The results were based on a single scientific paper by Han Chunyu, a biologist at Hebei Univerty of Science and technology in Shijiazhuang published on Nature Biotechnology. Though CRISPER-Cas9 increased the effectiveness of scientists laboratory tool kits, many scientists wanted NgAgo to be the next phase in genome editing, but due to lack of reproducibility it may not happen soon.

Technology Updates: NgAgo

One of the leading enzyme manufacturers in January 2017 has signed a deal with Chinese University to work on CRISPR/Cas alternatives. It is not clear, however recent news indicates that Novozymes might be planning to use NgAgo for gene editing.

CRISPR/Cas vs. Emerging Gene Editing Tools

Features	CRISPR/Cas	CRISPR/Cpf1	NgAgo
HNH Domain	HNH endonuclease domain present	Lack HNH endonuclease domain	Lack HNH endonuclease domain
Structure	Requires trRNA as well as crRNA (CRISPR based)	Requires only one guide RNA (CRISPR based)	Requires guide DNA (non-CRISPR based)
Nucleotide Removal	Produces blunt end	Produces sticky end	Random nucleotide removal
Effect on PAM Sites	PAM sites destroyed	PAM sites are not destroyed	PAM sites are not destroyed
Delivery	Low capacity vectors cannot be used	Easier to deliver, low capacity vectors can be used	Low capacity vectors cannot be used
Nucleotide Size	100nt hybrid of trRNA and crRNA	Smaller in size i.e. 40nt	Smaller in size i.e 24bp
Synthesis Cost	Costly	Cheaper	Cheaper
AT/GC-rich Target Efficiency	AT-rich regions cannot be targeted, Less efficient for GC-rich regions also	AT-rich regions can be targeted	More efficient with GC-rich region

Though the Cpf1 and NgAgo are quite nascent genome engineering molecules with few patent filings in the recent years, there is no doubt that with various advantages over Cas, it may give strong fight to all the existing genome editing nucleases.

While the key assignees active in patent filing of Cpf1 are Pioneer Hi-Bred INT, Harvard University, University of California and Broad institute, for NgAgo, major assignees are Agenovir Corp, Arc Bio LLC, University Nantong and DSM IP Assets BV.

Future Prospects:

The drawbacks of CRISPR/Cas have brought CRISPR/Cpf1 & NgAgo in the limelight. In their paper portraying Cpf1, Zetsche et al. recommended that Cpf1 may enhance the recurrence of homology-coordinated repair (HDR) over non-homologous endjoining (NHEJ). Cas9-mediated NHEJ usually destroys the PAM site due its close proximity to the cleavage site, which eliminates possibility of future alteration of DNA. Conversely, since Cpf1 performs its function without being in close proximity of PAM, NHEJ may hold the PAM site. Therefore, with Cpf1, subsequent round of DNA targeting can be performed easily.

Talking about NgAgo, a non-CRISPR based endonuclease, it offers higher sensitivity to off-targets when compared to Cas9.

In light of what we know now about Cas9, NgAgo and Cpf1, the later might be the best alternative for HDR; however this plausibility has not been tentatively affirmed. Scientists have additionally not yet described the potential “sticky end” transgene inclusion interceded by Cpf1-created overhangs. Investigating the proficiency of these two procedures will enable us to better comprehend where Cpf1 fits into the genome altering scene.

In the near future, it seems that the main aim of the biologist would be to make engineered nucleases which are cheap, target specific, reusable and can be manipulated at will. In addition, from IP perspective, it is also important that authorities in research as well as in IP should discourage broader patent claims around engineered nucleases, which was not effectively implemented during CRISPR/Cas.

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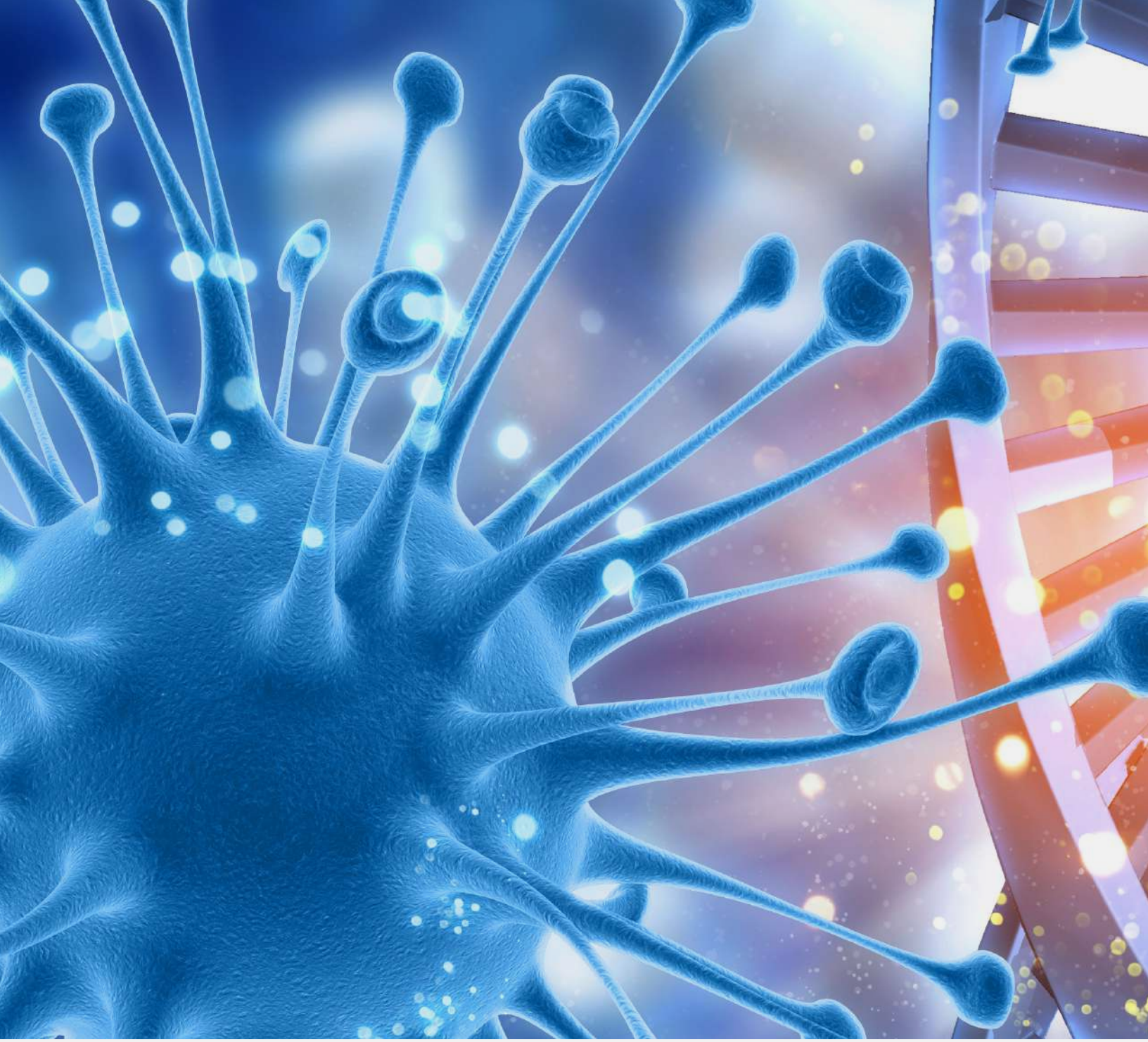

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