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Gene Editing's Sharp Edge: Understanding Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALEN) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

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Abstract. At present, the main gene editing tools encompass TREN, Zinc Finger Nucleases (ZFN), clustered regularly interspaced short palindromic repeats (CRISPR), and Transcription Activator-Like Effector Nucleases (TALEN). In this study, we introduce an overview of the three gene editing methodologies and discuss their current clinical applications. In addition, we suggest some trends and future applications within the field of gene editing. ZFNs represent one of the pioneering technologies, demonstrating significant efficacy in mitigating a multitude of genetic diseases and finding applications in agriculture. Yet, this technology contains intricate processes and produces substantial costs when implemented. TALENs have already been employed across various domains. In the medical field, they have been successfully applied in the treatment of leukemia in infants. However, TALENs are being replaced by CRISPR due to the superior efficiency of CRISPR. CRISPR, consisting of six components, exhibits considerable promise in the medical realm, particularly in the context of treating diseases such as Alzheimer's disease (AD). In the realm of genetic engineering, it can collaborate with B cells to rectify specific genes within the human genome, which have been tested in experiments. In the future, it can be used in many fields, including agriculture and nucleic acid testing.

Keywords: gene editing; ZFN; TALEN; CRISPR.

1. Introduction

In the 20th century, the elucidation of the Central Dogma of molecular biology represented landmark achievement, with significant contributions from technologies like Reverse transcription and Genes editing. Several biologists harnessed RNA-guided systems from prokaryotes, such as CRISPR to introduce edits in eukaryotic genomes and epigenomes. An illustrative instance of these edits occurs in chromatin remodeling and gene regulation facilitated by long non-coding RNAs (lncRNAs), which has more than 200 nucleotides and can have a great enlightenment to this technology [1]. Gene editing has revolutionized molecular biology and has a wide range of applications. At present, ZFN, TALEN, CRISPR/Cas9 are the most familiar and widely used technologies for Genes editing.

Zinc Finger Nucleases (ZFNs), a remarkable advancement in genetic engineering, are engineered enzymes that can create targeted double-strand breaks in specific DNA sequences, allowing for highly precise genome editing. ZFNs consist of a non-specific FokI endonuclease domain with an array of zinc finger DNA-binding domains [2]. Each zinc finger molecule can recognize a particular 3-base pair segment, and through the arrangement of multiple such zinc fingers with correct spacing, they can target specific genomic sites. When these fingers bind opposite strands with optimal spacing, FokI dimerization occurs, cleaving the backbone.

The induced double-strand break triggers endogenous repair pathways like NHEJ or HDR, enabling targeted mutagenesis or sequence replacement. To edit nuclei, ZFN proteins or constructs must be delivered via methods such as transfection, electroporation, or viral vectors . The zinc finger domains

facilitate sequence-specific binding to both on-target and off-target loci. Subsequent FokI cleavage initiates the repair process, resulting in gene modification frequencies ranging from 1-50%. Possible outcomes include gene knockout through error-prone repair or targeted insertion/replacement using a repair template [3].

Transcription activator-like effector nucleases (TALEN) were among the first user friendly gene editing technologies. A single TALEN comprises two essential components: a TALE domain and a FOKL cleavage domain. The TALE domain identifies and binds to a particular DNA sequence, while the FOKL cleavage domain, as the name suggests, cleaves one strand of the DNA. Since one TALEN can only induce a single-strand break, two TALENs are required to create a double-strand break (DSB), enabling gene editing. Additionally, the FOKL domains must also dimerize to initiate a cleavage [4].

The CRISPR/Cas9 system serves as both a self-protective mechanism in prokaryotes and an adaptive immune system utilized by bacteria to defend against the invasion of exogenous genetic material [5]. It holds a promising outlook for the future and has garnered significant attention for scientists due to its applications across various species.

In this scholarly paper, we delve into the multifaceted applications, encompassing medical applications, of these three gene editing methodologies.

2. Zinc Finger Nucleases (ZFNs)

In recent years, ZFN technology has found numerous real-world applications, particularly in the field of gene therapy for human diseases. Clinical trials have employed ZFNs to modify the CCR5 gene in CD4+ T cells as a treatment for HIV/AIDS. CCR5 serves as a co-receptor that HIV utilizes to infect host cells. Through the disruption of CCR5 using ZFNs, the genetically modified CD4+ T cells become resistant to HIV infection. Patients who underwent the ZFN CCR5-modified transplant exhibited increased CD4+ T cell levels and reduced viral loads, offering promising prospects for HIV therapies.

A Phase 1 clinical trial conducted by Perez et al. and published in the New England Journal of Medicine in 2014 demonstrated both the safety and efficacy of using zinc finger nucleases to genetically modify CD4 T cells as a novel treatment approach for HIV/AIDS. This pioneering study involved 12 HIV-positive individuals who continued taking antiretroviral therapy. Autologous CD4 T cells were collected from each participant and modified ex vivo using ZFNs targeting the CCR5 gene. CCR5 encodes a major co-receptor used by HIV to enter host cells. Disruption of CCR5 aims to mimic the effects of the CCR5-Δ32 mutation that confers natural resistance to HIV infection.

The clinical trial results demonstrated the successful ZFN-mediated modification and sustained presence of CCR5-modified CD4 T cells following a single infusion back into the original participants. At the week 12 post-infusion mark, the proportion of CCR5-modified cells ranged from 5.4% to 27.7% of the total circulating CD4 T cells within the cohort. Importantly, one of six participants who were evaluated demonstrated undetectable HIV RNA levels after the infusion of CCR5-modified cells, suggesting a promising level of efficacy. Moreover, no serious adverse effects were observed, underscoring the safety of this approach. Collectively, this Phase 1 trial provided critical proof-of-concept for the utilization of ZFN-based gene editing in autologous cells as an innovative therapeutic strategy for the treatment of HIV/AIDS. However, larger-scale studies are imperative to comprehensively evaluate its efficacy. Nonetheless, these results represent an exciting and pioneering application of advanced gene editing technology within the clinical context. [6]

Another groundbreaking clinical application of ZFNs is in the treatment of Hunter syndrome, a genetic lysosomal storage disease. In 2016, Sangamo Therapeutics employed ZFNs to introduce a functional copy of the missing enzyme iduronate-2-sulfatase directly into liver cells in vivo, resulting in substantial reduction of the toxic accumulation of glycosaminoglycans. This achievement marked

the inaugural instance of in vivo genome editing in humans and established a significant precedent for the potential treatment of other monogenic diseases.

Hunter syndrome is a rare X-linked genetic disorder caused by a deficiency in the lysosomal enzyme iduronate-2-sulfatase (IDS). In a study published by Santiago-Fernandez et al. Molecular Therapy in 2016, the application of zinc finger nucleases (ZFNs) for rectifying the genetic defect in Hunter syndrome was demonstrated. The researchers collected fibroblasts affected by Hunter syndrome patients and reprogrammed these cells into induced pluripotent stem cells (iPSCs). Subsequently, ZFNs were utilized to disrupt the mutated IDS gene in the iPSCs, leading to ZFN-mediated cleavage and error-prone DNA repair by non-homologous end joining, ultimately resulting in gene knockout.

This IDS gene disruption approach aims to mitigate the accumulation of glycosaminoglycans, which underlies the pathological symptoms of Hunter syndrome. Excitingly, the gene-edited iPSCs exhibited a restoration of IDS enzyme activity to levels comparable to those of normal control cells. Moreover, the differentiation of IDS knockout iPSCs into neural stem cells, hepatocyte-like cells, and cardiomyocyte-like cells demonstrated phenotypic correction. The differentiated cells exhibited a normal lysosomal appearance and reduced glycosaminoglycan storage when compared to unmodified iPSCs derived from individuals with Hunter syndrome.

Overall, this study offers crucial proof-of-concept that ZFN-mediated gene disruption has the potential to rectify the fundamental enzyme deficiency observed in Hunter syndrome iPSCs. The capacity to ameliorate the disease phenotype across multiple clinically relevant cell types holds substantial promise for future therapeutic interventions. Further studies are still requisite to translate these findings into practical treatments for patients. Nonetheless, the results demonstrate innovative application of advanced ZFN-based gene editing tools in addressing genetic lysosomal storage disorders such as Hunter syndrome.

One of the major advantages of ZFNs lies in their exceptional target specificity, a feature facilitated by the modular zinc finger DNA-binding domains [7]. Through the engineering of novel combinations of zinc fingers, ZFNs can be tailored to cleave virtually any DNA sequence, endowing them with remarkable flexibility for genome editing. However, it is important to note that the design of zinc fingers to bind to new targets can be challenging and often requires extensive screening of large libraries. This process can elevate both the cost and effort required to generate functional ZFNs that specifically target desired genes. Another significant consideration is the potential for off-target binding and cleavage, especially in therapeutic applications that demand the utmost precision. Additionally, ZFNs exhibit variable activity levels across different cell types, contingent upon the intracellular delivery method and the protein's functionality.

ZFN technology is currently encountering significant competition from newer CRISPR nucleases, such as Cas9, which offer simpler and more cost-effective options for genome editing [8]. Cas9 has gained popularity in recent years owing to its ease of use and versatility. In contrast to ZFNs, Cas9 is simpler to design and can target multiple sites simultaneously, enabling more efficient gene editing. However, the ultra-high specificity of ZFNs may still hold value for applications necessitating minimal off-target effects, such as gene and cell therapies. Ongoing advancements in rational design, protein engineering, and delivery methods are expected to further improve the targeting range and functionality of ZFNs across various biotechnology sectors. Despite the persisting challenges and the competitive landscape, ZFNs have firmly established themselves as fundamental tools for targeted genome editing, contributing significantly to genome understanding and biomedicine advancements. Despite the challenges and competition, ZFNs have demonstrated tremendous utility in the realms of biological research and biomedicine.

Beyond human gene therapy, ZFNs have facilitated a broad spectrum of genome editing applications within the realm of basic biology, agriculture, and industry. ZFNs can generate gene knockouts in model organisms like zebrafish, providing valuable insights into developmental and disease pathways [9]. In the agriculture sector, ZFNs find utility in EXZACT precision technology, which is used to introduce or modify genes in crops, bestowing upon them valuable traits such as pest resistance [10].

Synthetic biology companies like Sangamo Therapeutics employ ZFNs to engineer yeast and other microbial organisms to facilitate industrial-scale production of chemicals, pharmaceuticals, and biofuels [11].

3. TALEN

Numerous successful applications of Transcription Activator-Like Effector Nucleases (TALENs) in gene editing have been documented. For example, Li et al. achieved an enhancement in herbicide resistance in rice (Oryza sativa) through the utilization of TALENs. The TALEN construct and a DNA template containing the desired mutations were introduced to the rice genome. This targeted the Acetolactate synthase gene (OsALS), a pivotal regulator of resistance to specific herbicides. The outcome was the modification of up to 6.3% of the target genes. Subsequently, the next generation of rice exhibited increased resistance to the herbicide. [12]

TALENs have also found application in the clinical domain. In 2015, they were used to ameliorate the condition of an 11-month girl in the advanced stages of leukemia. In this specific intervention, scientists used modified T cells obtained from a donor, which were engineered to selectively target leukemia cells. Subsequently, TALENs were employed to delete the receptor gene within T cells to prevent the T cells from recognising the body cells as foreign and attacking them. Additionally, the infant received an antibody known as Campath, which was administered to deplete the baby's immune cells, preventing them from attacking the donor T cells. Notably, Campath targets the immune cell marker CD52, and TALENs were also used to eliminate the CD52 marker from the donor T cells. This dual approach served to safeguard the donor T cells from being attacked as well as preserving other essential immune cells. [13]

After the baby received a bone marrow transplant, her conditions improved, and the T cells did not attack the patient [13]. Within a month, the baby's health had sufficiently improved for her to return home. Subsequent assessments indicated that the infant had achieved full donor chimerism in the bone marrow and tested negative for minimal residual disease (MRD) markers. The baby remained in good health 18 months after the therapy. In another case, a similar therapy was administered to a different patient in the same year. As of the 12-month mark following the therapy, this infant remained in robust health, with no detectable MRD markers [13].

Thus, TALENs have demonstrated a potential to engineer cells used in leukemia treatment. A notable advantage of this therapeutic approach lies in the comprehensive bone marrow transplant, which significantly diminishes the likelihood of residual cells triggering relapsing, therefore effectively keeping the disease under control. Additionally, this treatment does not eliminate all B cells, as some treatments do. Consequently, it obviates the requirement for additional treatments aimed at B cell replacement [13].

However, it's essential to note that this clinical trial involved only two patients, and in both cases, the T cells were obtained from the same donor. Therefore, there remains an inherent uncertainty regarding potential off-target cleavages by TALENs in other patients, which results in genotoxicity. In addition, treatments as such require a bone marrow transplant, which carries the risk of graft-versus-host disease (GVHD) in patients. Indeed, both patients mentioned above experienced mild skin GVHD, although after treatment with steroids, neither displayed no signs of GVHD in the next months [13]. Nevertheless, this therapy can still be especially useful in situations where the patient is desperate for treatment, when the benefits of the treatment outweigh the risks.

In recent years, the TALENs used in this therapy have been supplanted by CRISPR. In a subsequent phase 1 clinical trial, the same therapy was delivered to 6 children with leukemia, but CRISPR was employed to edit the T cells, instead of using TALENs. Although several of these patients suffered complications such as neurotoxicity and skin GVHD, the overall outcome of the therapies was successful, therefore validating CRISPR as a potential therapeutic tool [14]. Therefore, although

TALENs can be effective in therapy, they tend to be replaced by newer, more modern technologies such as CRISPR. This is because TALENs carry some disadvantages over them.

One of the disadvantages lies in the less efficient process of engineering TALENs: given that two TALENs are required to induce a DSB, the engineering of two TALENs becomes mandatory for accomplishing gene editing. Moreover, many repeat units must be engineered, which imposes a technical challenge. [15]

However, the advantages of TALENs should not be neglected. One of their primary advantages lies in their remarkable specificity: each TALEN targets a sequence of 16-24 base pairs, and since two TALENs are required to induce a double-stranded break, this confers a combined specificity spanning 32-64 base pairs [15]. As a result, off-target cleavages are infrequent - unlike CRISPR - making TALENs a preferred choice in some cases. Another advantage is their degeneracy. Certain repeat units within the TALE structure can recognize more than one nucleotide. Thus, TALENs can still achieve gene editing despite mutations within the target DNA. Additionally, TALENs have the unique ability to target mitochondrial DNA (mtDNA), a task beyond the reach of CRISPR/Cas9. Consequently, TALENs have an enormous advantage over CRISPR to treat mitochondrial diseases caused by mutations in mtDNA [16]. Overall, TALENs should still be considered as a gene editing tool under some circumstances.

4. CRISPR/Cas9

The CRISPR-Cas9 system, a formidable genome-editing tool, traces its origins to the realm of genetics. Researchers have demonstrated the reciprocal interactions among P53.1, P53.2, P53.3, P53.4, and P53.5, enabling precise gene inactivation. This system plays a pivotal role in DNA repair, with p53 swiftly defending against genetic damage [17]. In the field of biology, the adaptable immune systems of many bacteria and archaea primarily hinge on CRISPR and CRISPR-associated proteins. These systems utilize RNA-directed nucleases to cleave foreign genes, thereby countering viral and phage invasions.Over the past decade, numerous researchers devoted themselves to unraveling the biology of CRISPR and advancing CRISPR technology. Based on the characteristics of the endonuclease, the CRISPR/Cas system is classified into six main types, with type II being CRISPR/Cas9 [18].

4.1. In Medical Fields

It has shown promise in the treatment of Alzheimer's disease (AD), a condition characterized by anomic aphasia, disorientation, mood swings, and behavioral disorders. CRISPR-Cas9 can be a linchpin part of setting up the model of the animal and cell-based, shedding light on the clinical AD phenotypes, deciphering the intricate pathobiology, and facilitating the study of specific sequences.[19] In an experiment, CRISPR-Cas9 can select the PSEN1M146L allele as an aim and have a valid way to oppose some phenotype related to AD which is supported by researchers. Following this line of thinking, it has the potential to evolve into a novel approach for treating AD patients with dominant mutations [20]. Dealing with breast cancer, CRISPR/Cas9 encompasses two major genres and six subdivisions. Due to their function, they can not only check the target, but also cut the single protein. During this process, Class 2 Cas effectors, specifically Type II (Cas9), Type V (Cas12), and Type VI (Cas13), play a significant role. It is very effective in testing nucleic acid, relayed on CRISPR RNA (crRNA) or single-guide RNA (sgRNA). The pathognomonic nucleic acid can interact with the Cas effector proteins, leading to either further fragmentation or union. Cas9, dCas9, Cas12a, Cas13a, and Cas14 are some of the most frequently used Cas effector, all of which contribute significantly to molecular diagnosis through the adorn of crRNA sequence [21] (Figure 1).

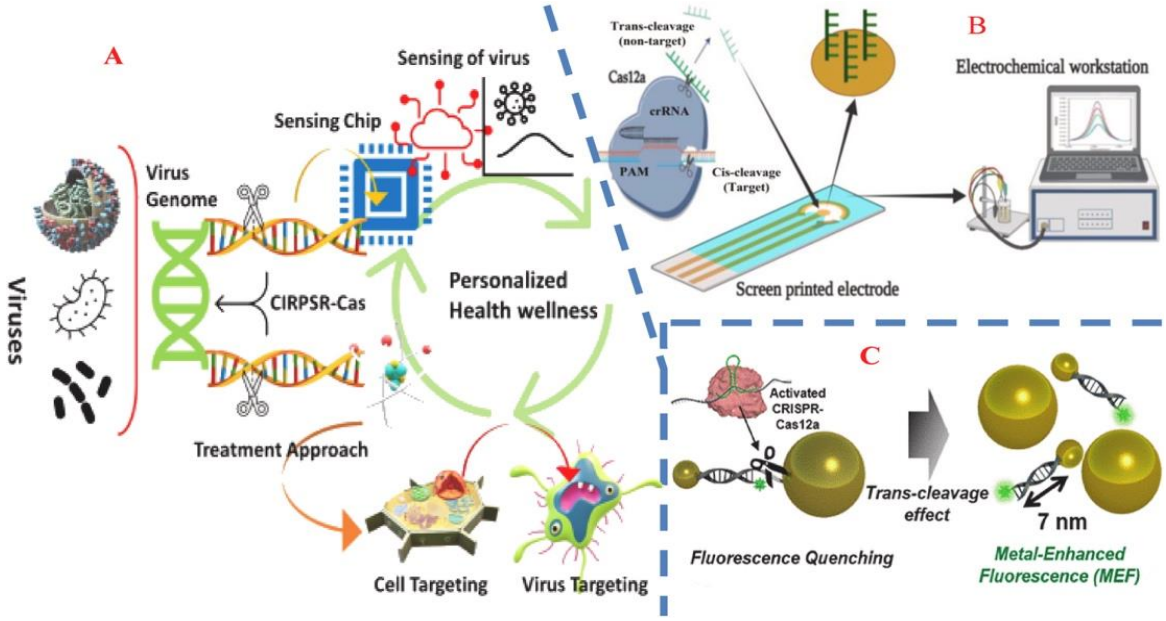

Figure 1. Detection methods based on CRISPR/Cas for breast cancer biomarkers [22]

4.2. In Genetic Engineering

The editing techniques based on CRISPR have explicit applications in B cells and can be extended to humans. Compared to traditional methods, CRISPR enables detailed research at the molecular and mechanistic levels. In pursuit of this objective, researchers have developed new methods involving electroporation to introduce CRISPR-Cas9 ribonucleoproteins (RNPs) into human peripheral blood or tonsil B cells independently. During these experiments, researchers conducted genetic editing and extensively studied the behavior of B cells. One approach involved culturing cells to create a supportive environment that maintains B cells in an undifferentiated state. Another approach allowed for the analysis of B cell differentiation induced by soluble factors. RNPs have the capability to simultaneously edit single or multiple genes. Finally, a single-stranded DNA oligonucleotide (ssODN) is introduced for homology-directed repair (HDR), establishing a novel method [23] One of the most prevalent gene deletions in patients with Duchenne muscular dystrophy (DMD) is Δ50;h51. Gene editing of the DMD exon 51 using CRISPR can be beneficial for approximately 13% of DMD patients. The mutation in exon 51, which results in an out-of-frame configuration in humans, can be corrected through single-cut gene editing with CRISPR. In this strategy, a single sgRNA guides the cleavage of DNA double strands (DSB) at the 5′-AG-3′ splice acceptor and the 5′-TGA-3′ premature termination codon. The insertion and deletion events occurring at the termination site of non-homologous dystrophin protein can either insert one nucleotide (3n+1) or remove two nucleotides (3n−2), thereby restoring exon 51 [24] (Figure 2).

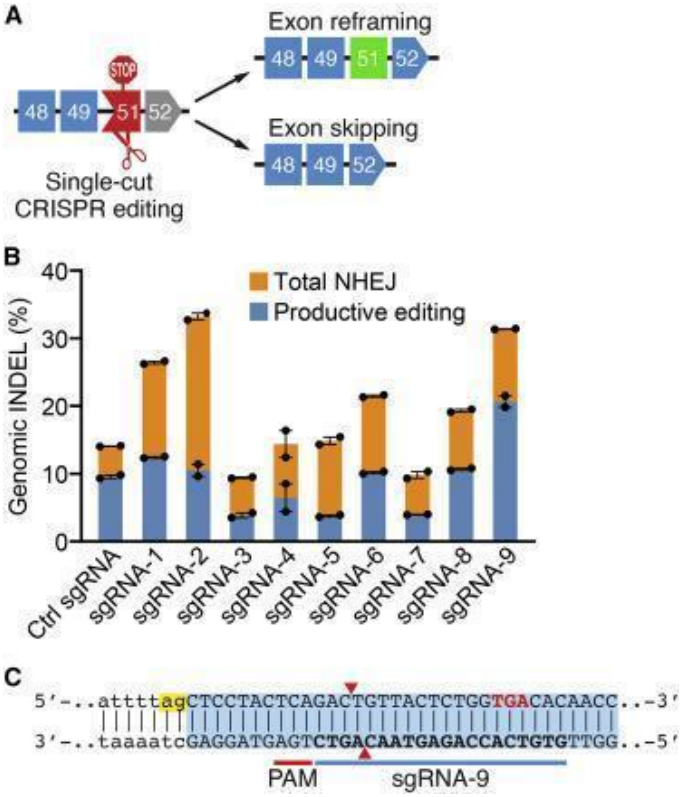

Figure 2. Splice acceptor: A humanized knockin mouse model and correction by CRISPR/Cas9 of Duchenne muscular dystrophy [24]

4.3. Advantage and Disadvantage of CRISPR

In many detection platforms, various technologies are integrated with the CRISPR systems. These integrations allow even the weakest signals to be detected and amplified, thereby improving efficiency. However, CRISPR still has several limitations. For instance, biosensors based on CRISPR-Cas have strict restrictions concerning the size of the target objects.

4.4. Future Study

Gene editing in plants has opened new avenues for plant researchers to study genes and improve specific trails of plants. Tools for gene editing have already been applied to precision breeding and the improvement of crop characteristics, with CRISPR/cas9 being particularly noteworthy [25]. In the construction of microbial cell factories, such as Saccharomyces cerevisiae and Corynebacterium glutamicum, these tools have played a role in metabolic engineering. Genome manipulation and expression have been achieved through CRISPR/Cas9 technology [26]. Some functional elements like Cas12 and Cas13 exhibit multifunctional capabilities, including the dependence of nuclease, high resolution of gene editing in muti-biological system, gene regulation conducted by CRISPR DNA/RNA base-editing and testing nucleic acid effective [27] (Figure 3).

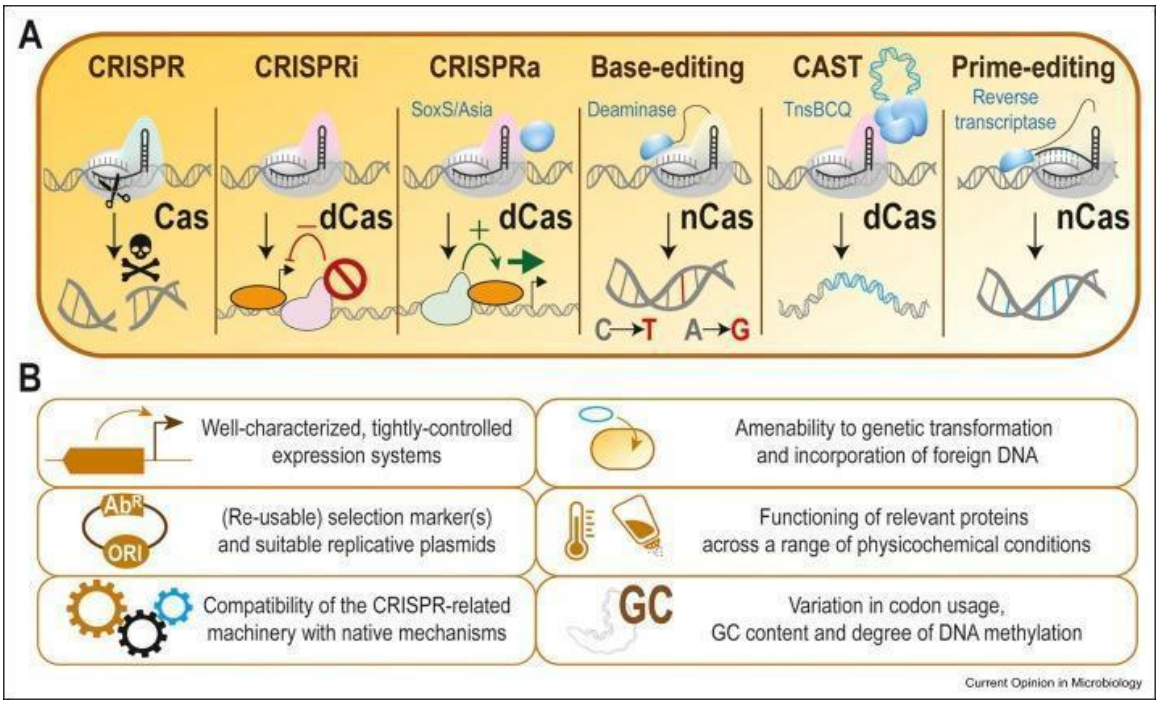

Figure 3. Engineering non-model bacteria using emergent CRISPR–Cas-based technologies [27]

In some species, CRISPR-Cas can play a part in virulence factor independently or in virulence positively or negatively modulating [27] (Figure 3).

5. Conclusion

In conclusion, the field of genome editing has witnessed remarkable advancements over the years, driven by the relentless pursuit of precision and efficiency in altering genetic material. Zinc finger nucleases (ZFNs) initially paved the way for targeted genome editing by harnessing the power of double strand breaks and endogenous repair pathways. However, the limitations in their specificity spurred the development of transcription activator-like effector nucleases (TALENs), offering a highly specific alternative for genetic manipulation. Nonetheless, the rise of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) has revolutionized the landscape of gene editing, overshadowing the prior methods due to its unparalleled efficiency and cost-effectiveness. CRISPR/Cas9 has rightfully claimed its place as the preeminent gene editing technology, promising even greater precision in DNA targeting as it continues to evolve. Looking ahead, the future of genome editing appears promising and transformative. The continued maturation of CRISPR/Cas9 technology holds immense potential to address critical concerns in diverse fields. In the realm of agriculture, it may enable precise modifications to crops, alleviating concerns about food safety, sustainability, and security. Moreover, in the domain of medicine, CRISPR/Cas9 offers a powerful tool for developing more effective treatments for genetic diseases, where traditional therapies often fall short. The prospect of editing genetic factors at their root provides hope for patients and families affected by inherited disorders. The journey of genome editing has evolved from the pioneering days of ZFNs to the precise and versatile capabilities of CRISPR/Cas9. As we move forward, we must continue to explore and harness the potential of this groundbreaking technology while addressing ethical and safety considerations. Ultimately, the future of genome editing holds the promise of a brighter and healthier world, where the potential for precise genetic modifications paves the way for innovative solutions to some of humanity's most pressing challenges.

Authors Contribution

All the authors contributed equally, and their names were listed in alphabetical order.

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