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Crispr-Based Gene Editing Technology and its Application in Microbial Engineering

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ABSTRACT

Gene editing technology involves the modification of a specific target gene to obtain a new function or phenotype Recent advances in clustered regularly interspaced short palindromic repeats (CRISPR)-Cas-mediated technologies have provided an efficient tool for genetic engineering of cells and organisms. Here, we review the three emerging gene editing tools (ZFNs, TALENs, and CRISPR-Cas) and briefly introduce the principle, classification, and mechanisms of the CRISPR-Cas systems. Strategies for gene editing based on endogenous and exogenous CRISPR-Cas systems, as well as the novel base editor (BE), prime editor (PE), and CRISPR-associated transposase (CAST) technologies, are described in detail. In addition, we summarize recent developments in the application of CRISPR-based gene editing tools for industrial microorganism and probiotics modifications. In this review, the potential challenges and future perspectives of CRISPR-based gene editing tools are discussed.

KEYWORDS: CRISPR-Cas system, Gene editing ,Industrial microorganisms ,Phenotype,Probiotics

I. INTRODUCTION

We have progressed from the "reading" stage of genetic information to the post-genomic era, where "rewriting" and even "new design" of the genome are becoming realities, thanks to the quick development of biotechnology and the life sciences. With constant research and development, gene editing technologies based on clustered regularly interspaced short palindromic repeats (CRISPR) have gained popularity. Their ease of use and effectiveness have significantly boosted the advancement of numerous life science disciplines. When Watson and Crick suggested the double helix structure of the DNA molecule in 1953, the history of gene editing technologies began [1]. By this time, scientists had come to understand that DNA plays a crucial role in biological traits and phenotypes. This important discovery also sparked the idea of artificial DNA modification. From this foundation, gene editing technology has evolved steadily. Early gene editing methods depended on organisms' spontaneous homologous recombination (HR) mechanisms, which produced changes by exchanging and rearranging target sites and supplying homologous donors [2]. However, HR only occurs in eukaryotes at a frequency of about 10−6 to 10−9 [3]. The stimulation of host damage repair pathways through DNA double-strand breaks (DSBs) was then demonstrated to enhance the frequency of HR occurrences [4–5]. A number of nuclease-based gene editing tools have consequently surfaced. In summary, programmable nucleases induce double-strand breaks (DSBs) at particular genomic locations, which are followed by the introduction of mutations via subsequent damage repair mechanisms, including as homology-directed repair (HDR), microhomology-mediated end joining (MMEJ), and non-homologous end joining (NHEJ) [6]. Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR-based technologies are the most popular and well-known of these nuclease-based gene editing methods (Fig. 1). Because they are both designed nucleases, ZFNs and TALENs are extremely similar. Fused Fok I endonuclease is in charge of DNA cleavage, whereas zinc finger protein (ZFP) or transcription activator-like effectors (TALEs) are in charge of target recognition. Although the development of ZFNs represented a significant breakthrough in gene editing technology, it has some drawbacks, including high cost, intricate design, high off-target

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rate, and challenges in doing multi-target editing [7–9]. ZFNs served as the foundation for the enhanced technology known as TALENs. The cleavage efficiency of TALENs and ZFNs were found to be similar for the identical target locations; however, TALENs exhibit decreased intracellular toxicity [8, 10]. In addition to being simpler to build than ZFNs, TALENs can potentially be built for any target sequence [7]. But because each base in the target sequence needs its own TALE recognition module, TALENs come with a significant manufacturing burden. The effectiveness of gene knockout can be increased by ZFNs and TALENs, however both require modified proteins for particular DNA recognition, necessitating the creation of new proteins for various target sites. Their use in gene editing is hampered by their difficult operation and high technical threshold [11]. The subsequent development of the CRISPR-Cas system, which uses short RNAs to identify target sites, fully made up for the drawbacks of these several previous gene editing methods.

HUMAN GENE THERAPY (HGT)

One kind of gene alteration that aims to treat aberrant cells is called gene therapy. To prevent or treat disease, it entails deactivating or replacing the gene that causes it. The creation of gene therapy has faced numerous obstacles and setbacks, yet it has consistently been regarded as one of the greatest medical advances as it offers the potential to treat numerous illnesses that were previously believed to be incurable. An 18-year-old child who enrolled in a gene therapy trial died in 1999 as a result of an immunological reaction to the treatment, following the first successful gene therapy trial in 1990. Gene therapy's advancement was consequently slowed down and subject to additional safety and precautionary procedures. However, it returned to the therapeutic arena in 2003 after China approved gendicine, the first gene therapy for head and neck cancer. In 2011, the European Commission authorized Glybera for lipoprotein lipase deficiency, and China, Russia, and Neovasculgen for peripheral artery disease. Unfortunately, due to its excessive cost of €1M for a single dose, Glybera was forced to depart the medical field in 2017. A number of developments in vector engineering, gene editing, and gene delivery methods enabled gene therapy to evolve at such a quick rate that, by 2025, the Food and Drug Administration (FDA) estimates that between 10 and 20 cell and gene therapies will be approved annually.

Approximately 2600 gene therapy trials are currently underway to treat a variety of illnesses, including congenital abnormalities like hemophilia, cystic fibrosis, cancer, cardiovascular diseases, uncommon diseases, and neurodegenerative diseases. Monogenic illnesses are the second most common condition treated by gene therapy, after cancer. More than 65% of the more than 2597 gene therapy clinical studies that were carried out up until November 2017 were for cancer. However, just 22 gene therapy products for the treatment of various diseases were licensed by August 2019 for unknown reasons. Immunotherapy, particularly the well-known one that uses CAR-T cells that have been created, has shown promise in the treatment of cancer.Zolgensma, Zynteglo, and Abecma are some of the most recent gene therapy drugs that regulatory agencies have approved. The FDA approved Zolgensma for spinal muscular atrophy in May 2019. To restore the normal function of motor neurons, zolgensma, which has a healthy copy of the human SMN gene, is administered to affected children under the age of two. In May 2019, Zynteglo, which has a healthy copy of the gene that produces hemoglobin, was approved by the European Union to treat beta thalassemia in people aged 12 and upto18. In March 2021, the FDA authorized Abecma, the first gene therapy based on CAR-T cells, to treat multiple myeloma in adults.

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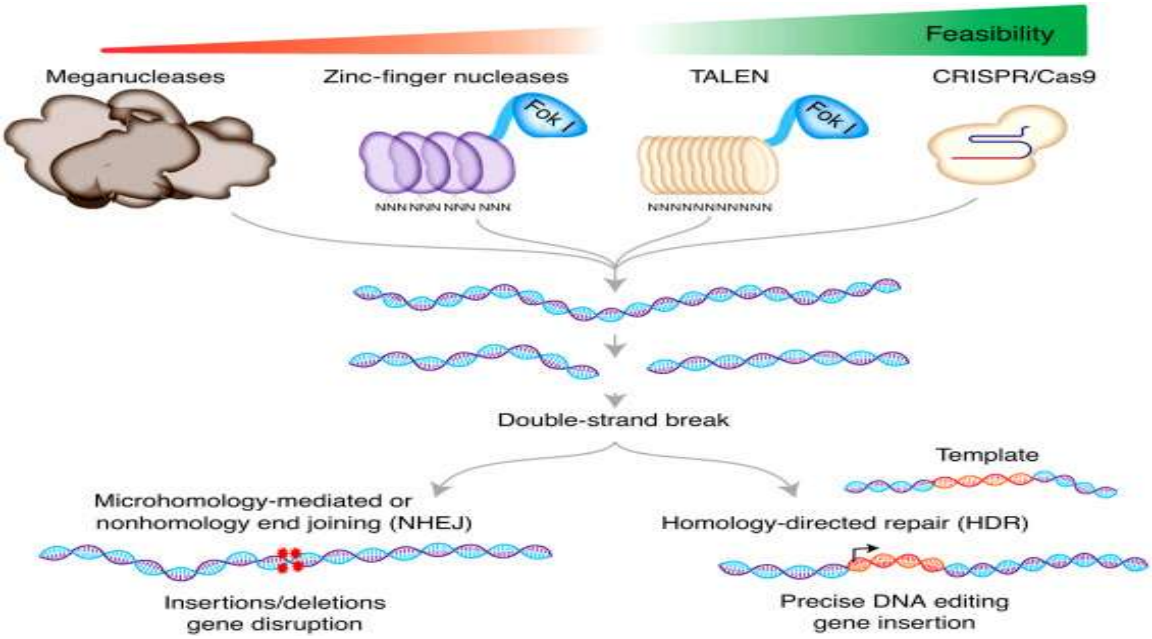

Figure 1. Comparison of gene editing methods based on ZFNs, TALENs, and CRISPR-Cas9 technologies.

Through the intricate protein engineering involved in ZFNs and TAL-ENs, the CRISPR-Cas system allows for the targeting of various sequences by merely altering the guide RNA. Furthermore, compared to protein-based recognition, base-pairing-based target site recognition hasa better specificity [12]. Eukaryotes, prokaryotes, and archaea are the three domains of life that have recently made extensive use of CRISPR-based gene editing technologies [13–15]. Furthermore, a number of derivative technologies constructed using the CRISPR-Cas system have also shown strong gene manipulation capabilities. These include tools for gene transcriptional regulation, CRISPR interference (CRISPRi), and CRISPR activation (CRISPRa) [16–18], as well as new gene editing tools that do not depend on DSBs, base editors (BEs) [19–23], prime editors (PEs) [24–27], or CRISPR-associated transposases (CASTs) [28]. The use of gene editing technology to modify the genetic makeup of microbes is crucial for making use of microbial resources. Probiotics and the genetic modification of industrial chassis bacteria are two significant research topics among them. The creation of industrial chassis microorganisms can yield superior cell factories that can support the realization of sustainable economic growth and environmental preservation. Furthermore, probiotics have been utilized extensively in food and medicine for a long time, and gene editing will enhance their probiotic qualities, which has promising industrialization potential. The specific mechanics of various CRISPR-Cas systems and their uses in higher species, like plants and animals, have been the main focus of previous studies of CRISPR-Cas technology [6, 29–31].

The present study aims to provide an overview of both established and new CRISPR-based gene editing technologies and their uses in modifying microorganisms, such as probiotics and industrial platelets. The classification and mechanism of the CRISPR-Cas system are explained in this article, along with a variety of gene editing methods that are based on it. We also provide an overview of how CRISPR-based gene editing techniques have been applied to probiotics and industrial microbes. Additionally, the limitations of the available CRISPR-based gene editing techniques and their future prospects are examined.

DEFINITION AND PRINCIPLE OF THE CRISPR-CAS SYSTEM

Prokaryotes have an acquired immune system called the CRISPR-Cas system that defends them against invading genetic material. About 50% of bacteria and 90% of archaea species that have been sequenced have it [32, 33]. CRISPR arrays and a set of cas genes that encode Cas proteins are the two genetic components that make up the CRISPR-Cas system. An AT-rich leader sequence, which serves as a promoter and recognition site for new spacer sequence integration, is the first

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sequence in the CRISPR array [30]. Following the leader sequence, distinct spacer sequences and identical repeat sequences are alternately placed. The repeat sequences of homogeneous CRISPR arrays exhibit great conservation, but those of distinct CRISPR array types exhibit variations in both sequence and structure [32]. Different cas genes that encode specific Cas proteins with endonuclease domains, RNA and DNA binding domains, and transcriptional regulatory domains border the CRISPR array [34–36].

Three stages are often included in the immunological process of the CRISPR-Cas system: interruption, processing, and adaptability (Figure 2). The process of adaptation, often referred to as spacer acquisition, involves capturing additional spacers from invasive nucleic acids and integrating them into the CRISPR array as new spacers that sequentially follow the leader sequence. A stable complex including two Cas1 dimers and one Cas2 dimer is often necessary for this stage [37], and certain CRISPR-Cas systems also require the involvement of other auxiliary proteins, like Cas4 and reverse transcriptase [38].CRISPR RNA (crRNA) maturation is the second stage. Driven by promoter regions embedded in the leader sequence, the entire CRISPR array is first transcribed into a lengthy precursor crRNA (pre-crRNA). Some housekeeping non-Cas nucleases, like RNase III, RNase E, and PNPase, as well as Cas nucleases, like Cas6, Cas5d, and Cas12, are among the ribonucleases implicated in pre-crRNA maturation [30, 39–41]. After maturing, the crRNA forms a ribonucleoprotein complex (RNP) via binding to effector proteins or multi-subunit effector complexes. In the last phase of target interference, the RNP uses complementary base pairing between the target nucleic acid and the crRNA to identify and break the invasive DNA or RNA [30, 34].

CLASSIFICATION AND INTERFERENCE MECHANISM OF THE CRISPR-CAS SYSTEM

There are now six forms of the CRISPR-Cas system, which are separated into two classes (class 1 and class 2) based on the architectural makeup of Cas proteins and effector complexes (types I to VI). There are 33 subtypes and many variations for each kind, each of which has its own hallmark cas gene [42]. Types I, III, and IV of the class 1 CRISPR-Cas systems employ a complex of several Cas protein subunits to provide immunological defense. Types II, V, and VI of the class 2 systems defend against intruders by using a single Cas protein effector (Figure 3). With the exception of type III, practically all known CRISPR-Cas systems require the identification of a brief sequence in the 5′ flanking region.

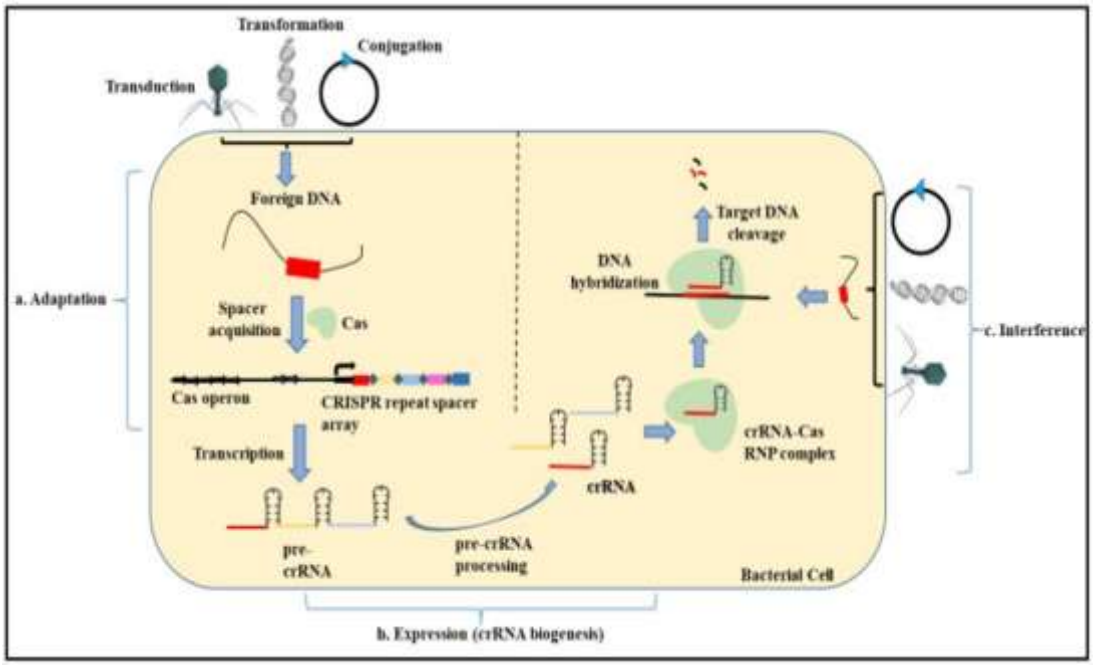

Figure 2. The CRISPR-Cas immunization against invasive genetic elements is divided into three stages: interference, processing, and adaptability.

International Journal of Pharmaceutical Research and Applications
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To create an immunological memory, brief segments of foreign nucleic acids are incorporated into the CRISPR array during the adaption step. A similar ribonuclease then converts the pre-crRNA produced by transcription of the CRISPR array into mature crRNA. During the last phase of target interference, mature crRNAs use complementary base pairing between the target nucleic acid and the crRNA to direct the CRISPR-Cas effector complex to cleave foreign nucleic acids.

To mediate interference and adaptation, the protospacer (also known as the protospacer adjacent motif, or PAM) [30]. In class 1 CRISPR-Cas systems, type I systems recognize and cleave target DNA via a multi-subunit RNP known as CRISPR-associated complex for antiviral defense (Cascade) [34]. The distinctive protein Cas3 is usually present in type I systems together with one Cas5 subunit, one Cas6 subunit, and many Cas7 subunits.

Cas6 family proteins process nearly all type I pre-crRNAs, with the exception of the I-C subtype system, which employs Cas5d . When the type I system functions, Cascade first looks for the PAM sequence on the target DNA using Cse1 (Cas8) . Following the identification of the particular PAM sequence, Cascade causes the target DNA strands to separate and pairs the target DNA with the crRNA to create an R-loop for Cascade immobilization . Following this, Cascade changes its shape and brings Cas3 on board via the complex's Cse1 subunit. Cas3.

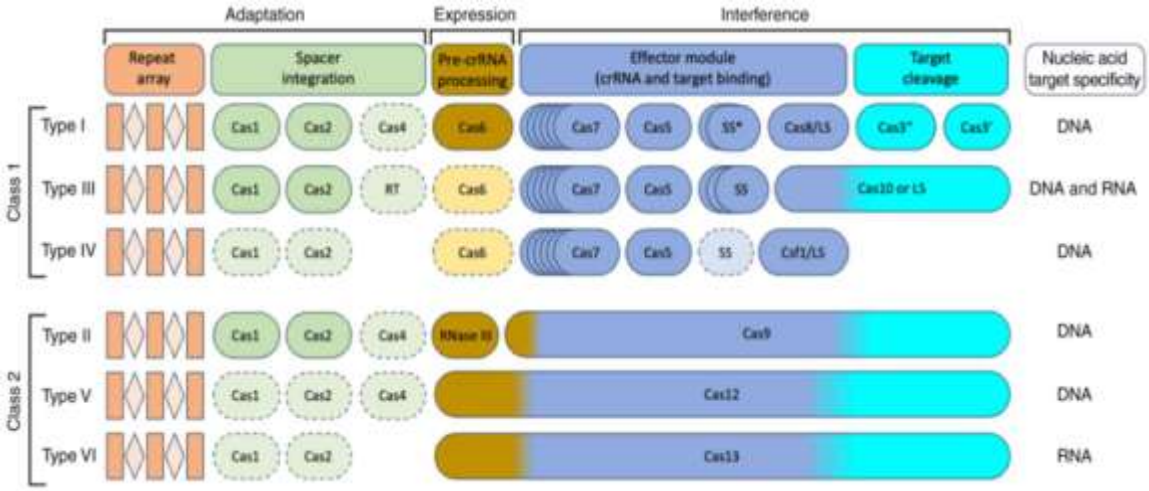

Figure 3. Schematic diagram of the classification and architecture of different types of CRISPR-Cas systems.

The type III system is composed similarly to the type I system, however Cas10 is its characteristic protein. The more intricate type III system has the ability to break down both DNA and RNA . The type III system targets RNA substrates selectively during the interference phase. Furthermore, base pairing between crRNA and developing RNA transcripts can trigger a variety of type III system functions. First, single-stranded DNA is cleaved non-specifically by activating the histidine-aspartate (HD) nuclease domain of Cas10 . In parallel, Cas10's palm domain is activated as a cyclic oligonucleotide (cOA) synthetase to produce cOAs from cytoplasmic ATP. The CRISPR-associated Rossman fold (CARF) domain-containing ribonucleases (Csm6/Csx1 families) are subsequently activated by these second messengers. Sensing cOAs triggers the allosteric CARF structural domain of Csm6/Csx1 to activate its higher eukaryotic and prokaryotic nucleotide-binding (HEPN) domain, which can degrade RNA without specificity. As a result, host and exogenous transcripts are often depleted, which stops the spread of invaders in a way akin to abortion infection (Abi) and stops host growth. Unlike type I and type III systems, which are frequently found on plasmids, type IV systems encode Cas5 (Csf3), Cas7 (Csf2), and the distinctive protein Csf1. They also lack the Cas1 gene, Cas2 gene, and nuclease gene (Cas3 or Cas10) for interference. Nevertheless, there is currently a dearth of functional research on the type IV system. Its

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evident target interference with DNA nuclease activity has not been seen in the few published investigations, which have solely detailed its crRNA maturation and PAM preference. In order to protect against external invaders, the class 2 CRISPR-Cas systems—type II, V, and VI—most notably employ a single Cas protein. Cas9, Cas12, and Cas13 are the corresponding hallmark proteins of type II, V, and VI CRISPR-Cas systems.

GENE EDITING BASED ON THE CRISPR-CAS SYSTEM

Utilizing biological resources, releasing biological mechanisms, and studying gene function all depend on the capacity to manipulate and control genetic information. Basically, programmable nucleases constitute the foundation of today's well-established gene editing methods. Among these, CRISPR-based gene editing methods are the most advanced and widely used. As was previously indicated, the primary difficulty with ZFNs and TALENs for gene editing is that different target-binding proteins need to be developed and synthesized for different editing sites. Making these takes a lot of time, effort, and labor [11]. By simply changing the guide RNA sequence, however, the RNA-guide nuclease utilized in the CRISPR-Cas system can be customized for different target spots. It is a simple and efficient process with enhanced specificity based on complementing base pairing [12]. CRISPR-based gene editing tools have quickly and completely replaced ZFNs and TALENs as the next generation of gene editing technology due to these many advantages. After much iteration and research, two generations of CRISPR-based editing tools have been produced. First-generation techniques rely on DNA damage repair pathways and create DSBs at specific sites to enable gene editing.What's Because they use Cas proteins with decreased nuclease activity, which avoids DSBs, innovative second-generation techniques fuse other functional proteins to Cas proteins, enabling precise gene editing.

CRISPR-based gene editing dependent on DSBs

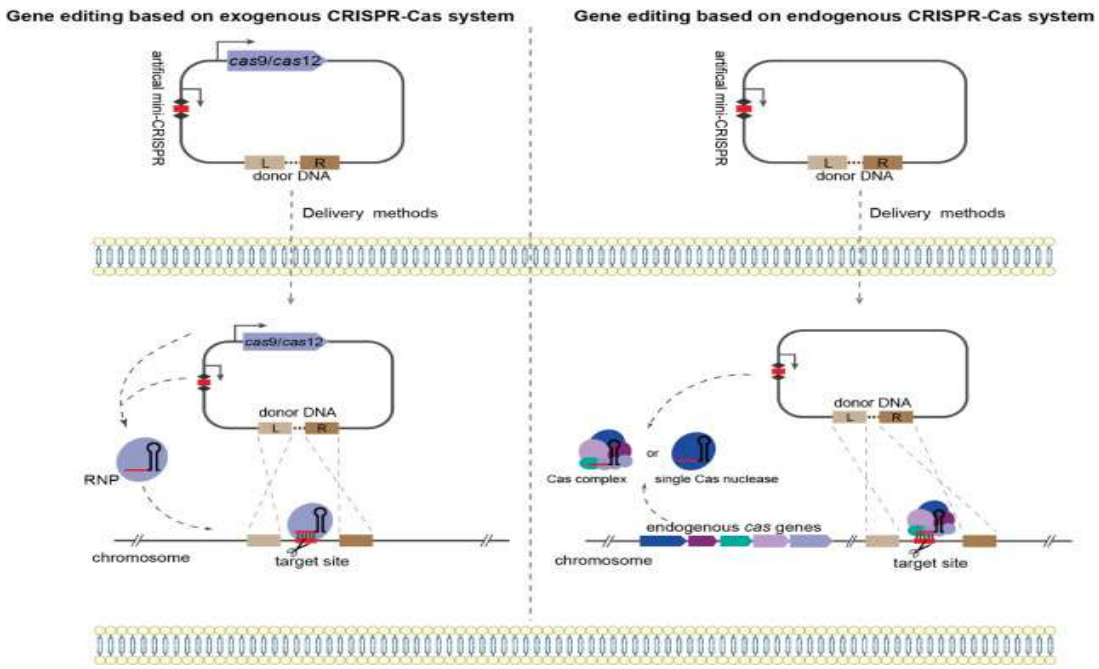

Figure 4. CRISPR-based gene editing dependent on DSBs

In gene editing methods, both external and endogenous CRISPR-Cas systems are employed.

This results in the desired mutation by homologous recombination between the donor DNA and the host genome.

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There are two primary methods for employing CRISPR systems to generate DSBs at specific loci in the genome: either the host is given a complete set of exogenous CRISPR-Cas systems, or the CRISPR-Cas system expressed by certain prokaryotes is used directly for gene editing (Fig. 4). When using exogenous CRISPR-Cas systems for gene editing, class 2 CRISPR-Cas systems are typically selected. Because only one Cas protein is needed to completely recognize and cleave target sites, class 2 systems are simple enough to allow for customisation and exploitation. As the first class 2 system to be identified, Type II CRISPR-Cas9 was also the first to be used for gene editing. Recent years have seen the use of the CRISPR-Cas9 system to modify the genomes of numerous organisms [13, 14].

CRISPR-BASED GENE EDITING INDEPENDENT OF SBs

DNA DSBs are induced at target sites by early gene editing methods based on ZFNs, TALENs, and CRISPR-Cas systems. This triggers host DNA damage repair mechanisms, such as MMEJ, NHEJ, and HDR. The host might suffer greatly from random insertions and deletions that are easily caused by MMEJ and NHEJ, two of these repair mechanisms. Despite HDR's ability to induce precise mutations of interest based on the donor DNA we supply, the effectiveness of homologous recombinant repair in cells is very modest (about 0.1% to 5% for eukaryotes). Additionally, DSBs brought on by possible CRISPR system off-target effects could be extremely dangerous. The host is therefore at risk when DSBs are introduced. The CRISPR-Cas system has been improved to produce BE and PE technologies in order to solve this issue. Further broadening the range of applications for gene editing techniques that do not depend on DSBs is the development of CASTs technology (Figure 5).

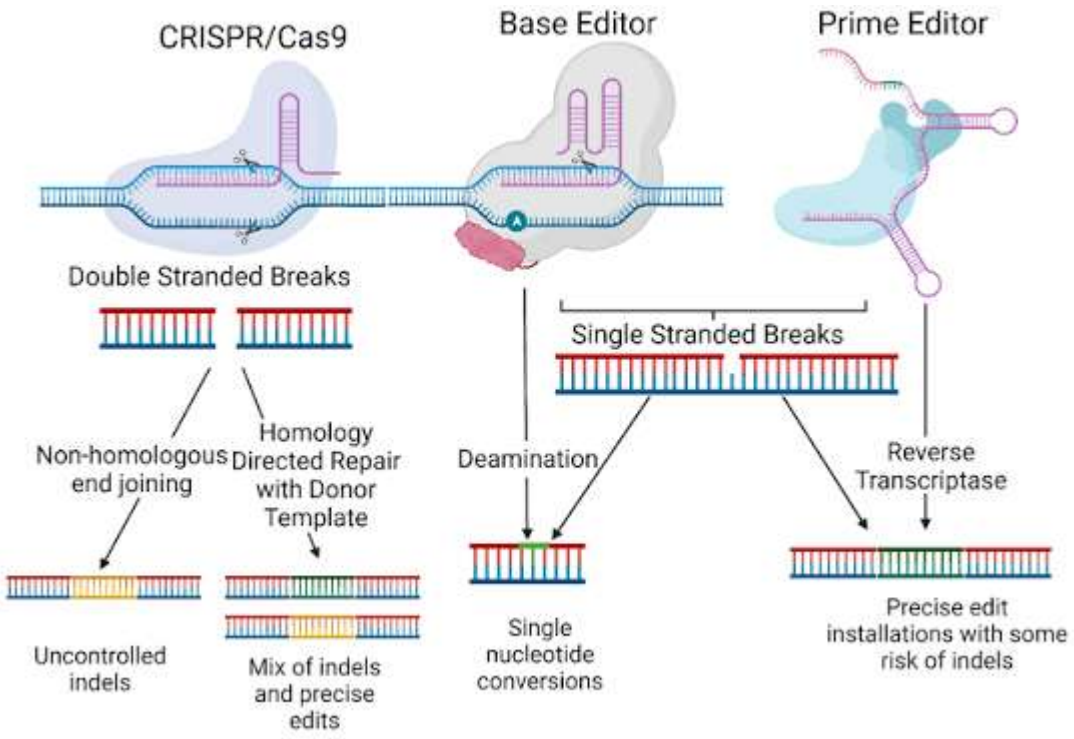

Figure 5. CRISPR-associated transposases, base editors (BEs), and prime editors (PEs) are CRISPR-based gene editing instruments that do not cause DSBs. Deaminase is coupled with either dCas9 or nCas9 for BEs.

International Journal of Pharmaceutical Research and Applications
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Deaminase deamidates the unwinding single-stranded DNA after the sgRNA directs Cas9 to attach to the target, which ultimately leads to base conversion. The sgRNA was modified to become a more complicated pegRNA for PEs, and nCas9 is coupled with reverse transcriptase. After nCas9's operations, the 3 ′ end of the pegRNA can link with the broken single-stranded DNA, allowing reverse transcriptase to deliver the intended changes into the target location. First, TnsA and TnsB collect and process transposon donor DNA for CRISPR-related transposase (CAST)-mediated gene integration. Following processing, an integrated complex is formed by TnsA, TnsB, and the substrate. With the help of crRNA, the CRISPR elements of CAST systems attach to a particular location and enlist the help of the TniQ, TnsC, and TnsA/B complexes to integrate the donor DNA fragment downstream of the target site.

With their successful genome manipulation outcomes, gene editing techniques based on exogenous class 2 systems, such as CRISPR-Cas9/Cas12a, have unquestionably garnered a lot of attention. Cells that do not encode their own CRISPR systems, however, might benefit more from this tactic. Moreover, there are still some serious drawbacks to gene editing instruments based on exogenous CRISPR systems. First, the activity of widely utilized Cas nucleases may be impacted by the complex intracellular environment and growth conditions of extremophiles. In addition to Because heterologous Cas nucleases are intrinsically proteotoxic, their huge size may make it impossible to introduce them into some species. Repurposing endogenous CRISPR-Cas systems is also a preferable approach for gene editing in prokaryotes, considering the broad distribution of CRISPR-Cas systems in these organisms (found in around 50% of bacteria and 90% of archaea) [32, 33]. Since all of the components of the endogenous CRISPR-Cas system are found in the cell, the toxicity associated with heterologous nucleases is avoided, unlike when exogenous class 2 systems are introduced. Additionally, endogenous processing proteins are the only proteins required for crRNA maturation; no extra external proteins are needed. The ability to precisely edit target sites requires only an artificial CRISPR array and a homologous DNA template for damage repair, which is especially useful for simultaneous multi-gene editing and metabolic engineering regulation. At this time, many bacteria and archaea, including extremophiles, use various forms of endogenous CRISPR-Cas system-mediated gene editing, which are quite well established in prokaryotes. For instance, genome editing of the thermophilic archaeon Sulfolobusislandicus has been successfully accomplished using the endogenous I-A and III-B systems. Interestingly, the foreign Cas9 system can only perform extremely ineffective gene editing on the Zymomonasmobilis strain, which is valuable for producing ethanol. By describing and repurposing its endogenous I-F type CRISPR-Cas system, recent research has made efficient gene editing possible. Furthermore, when it came to genome editing, the halophilic archaea Haloarculahispanica's type I-B system and the lactic acid-producing bacterium Pediococcusacidilactici's type II-A system both showed great editing efficiency. Our most recent research has demonstrated that a variety of endogenous CRISPR-Cas systems, such as types IIIA/B, I-B, and I-C, encoded by Thermusthermophilus, a model strain used to study thermophiles, may be utilized to edit their genomes with excellent editing efficiency. Several distinct CRISPR-Cas systems are frequently encoded at the same time in prokaryotes, particularly in archaea [32, 33]. It is also possible to overcome the restrictions of a single system for PAM sequences by identifying and using their many endogenous CRISPR-Cas systems. As sequencing and bioinformatics tools advance quickly, it will be possible to quickly define endogenous CRISPR-Cas systems from particular genomes. This will also make it easier to create endogenous CRISPR-Cas systems, which are the next generation of prokaryotic genome editing technology.

BASEEDITORS (BEs)

The team led by David R. Liu first presented BEs, a revolutionary gene editing approach that enables single-base copying without homologous templates or DNA DSBs, in 2016 [19]. Based on the CRISPR-Cas9 system, this method uses Cas9 nickase (nCas9) or dead Cas9 (dCas9) that lack intact nuclease function. HNH and RuvC, two nuclease domains found in Cas9, are in charge of cleaving the target and non-target strands, respectively. Since both of dCas9's nuclease domains are altered, its ability to cleave DNA is eliminated. It still retains the capacity to connect to target DNA, though. In contrast, nCas9 can only cleave one DNA strand since it only has one nuclease domain mutation.In addition to employing dCas9 or nCas9, the combination of

International Journal of Pharmaceutical Research and Applications
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deaminase and dCas9/nCas9 is the basis of BEs. When cytosine and adenine deaminase were introduced produced adenine base editors (ABEs) and cytosine base editors (CBEs), which allowed C-G to be converted to T-C and A-T to G-C, respectively [19]. The host then fixes the U-G mismatch to U-A, ultimately creating a T-A base pair, after cytosine deamidates cytosine (C) in exposed ssDNA to uracil (U) for CBEs. Furthermore, the insertion of a uracil glycosylase inhibitor (UGI) inhibited the excision of uracil, hence increasing the editing efficiency of CBEs [28]. The enzyme adenine deaminase transforms adenine (A) into inosine (I) for ABEs, and cells mistake inosine for guanine (G).

PRIME EDITORS (PEs)

PEs were presented by David R. Liu's team in 2019 to get around the drawbacks of BEs. This method eliminates the need for DSBs and external template DNA and permits a variety of base substitutions as well as accurate DNA insertions and deletions. A primary editing guide RNA (pegRNA) is used to direct the fusion protein of Cas9 nickase (H840A) and reverse transcriptase (RT) to accomplish gene editing at the desired location. Primarily, the pegRNA modifies the single guide RNA's (sgRNA) 3′ end. In order to initiate reverse transcription, the pegRNA is made up of three main components: the sgRNA portion at the 5 ′ end, a hairpin structure at the 3 ′ end of the sgRNA, and a primer binding site (PBS) for hybridization with the 3 ′ end of the nicked DNA strand. At the 3′ end of the pegRNA, the PAM-containing target DNA strand is cut by nCas9 under the guidance of the pegRNA. The broken strand is then base-paired to the PBS sequence. Using the RTT sequence as a template for RT, a reverse transcription reaction Following the cleavage of the 5 ′ DNA flap and the repair of the genome, the 3 ′ DNA flap created by reverse transcription pushes the 5 ′ DNA flap on the target DNA strand, ultimately introducing the desired mutation into the target spot.

CRISPR-ASSOCIATED TRANSPOSASE (CASTs)

Furthermore, the CAST system has been developed as a gene editing method independent of DSBs, in addition to BEs and PEs. In the process of investigating the diversity and evolution of the CRISPR-Cas system, it was recently found that the Tn7 transposon diverted certain prokaryotic CRISPR elements, leading to the co-evolution of the CAST system. When used as a programmable gene integration method, CAST systems can mediate DNA transposition under the direction of crRNA. Usually, CRISPR nucleases are not active in CAST systems. For instance, it is anticipated that some type V systems would not exhibit nuclease activity, and some type I systems will not have the crucial Cas3 helicase-nuclease domain. This CRISPR-Cas system serves as a genomic targeting module for CAST systems because it is nuclease inactive. Improved knowledge of the Tn7 transposon's structure and function will aid in the development of this novel instrument. Donor DNA placed between left end/right end (LE/RE) sequences and the tnsA, tnsB, tnsC, tnsD, and tnsE genes are commonly found in tn7-like transposons. TnsB usually attaches to TnsA, which processes the 3′ and 5′ ends of the substrate, respectively, after recognizing the transposon end sequence. After processing, TnsA, TnsB, and the donor create an integrated complex (other systems do not have TnsA). Target selection is accomplished by a sequence-specific DNA-binding protein called TnsD/E. TnsA/B and TnsD/E complex communication is facilitated by the protein TnsC, which is hydrolyzed by ATP. TnsC communicates with TnsD/E to Incorporate donor sequences into conserved Tn7 sites and organize transposase recruitment.

APPLICATIONS

1. CRISPR-BASED GENE EDITING INENGINEERING MICROBIOLOGY

Numerous industries, including food, health, manufacturing, and agriculture, have long used microorganisms. Modern industrial biotechnology revolves around them, and their significance for human life is becoming more and more clear. Scholars are gradually moving from only isolating strains from nature to artificially creating superior strains utilizing gene editing techniques as a result of the discovery and use of contemporary biological procedures. To increase the quality and production of fermentation industrial products, superior microbial strains are essential.

2. INDUSTRIAL MICROORGANISMS

For bioindustrial production, high-quality chassis microbial cells are crucial. Targeted genetic enhancement of strains is made possible by gene editing technologies. Moreover, the creation of the CRISPR-Cas system has made it possible to modify and enhance industrial chassis cells using a

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relatively easy and effective gene editing technology. We can create a cell factory that is nearly optimal by artificially creating the metabolic network of microorganisms, fine-tuning the metabolic flow, and improving the appropriateness of components and chassis. This might aid in producing the desired number and quality of products. It is possible to artificially modify the microorganisms used in this production process to make substances that have not yet been synthesized or are challenging to manufacture through the use of genetic engineering techniques. Finding and refining chassis microorganisms can also lead to the development of novel, more effective industrial processes that result in purer and less expensive goods. The most widely utilized industrial chassis microorganisms as cell factories at the moment are Saccharomyces cerevisiae, Escherichia coli, Corynebacteriumglutamicum, and Bacillus subtilis. E. coli is one of the most commonly employed of these in industrial fermentation due to its high environmental tolerance, short growth cycle, and ease of cultivation. Jiang et al. (2013) confirmed that the CRISPR/Cas9 system could specifically target the cleavage of the E. coli genome and cause mutations in the host by repairing the λ-Red proteins. With a mutation rate of 65%, this demonstrated that the system could effectively integrate recombinant systems for gene editing in E. coli. Gene editing techniques based on CRISPR-Cas9 have since been extensively employed to enhance E. coli output. A CRISPR-Cas9-based technique for metabolic engineering and iterative editing of E. coli was created by Li et al. With about 100% editing efficiency, we have been able to introduce many kinds of genomic alterations using this technique. Through this platform, they also built a library of more than 100 mutations and optimized the pathways linked to E. coli's production of β-carotene. A model strain of Gram-positive Bacillus, B. subtilis is non-pathogenic, has a high capacity for extracellular protein production, and does not exhibit any discernible codon preference. It is a very promising strain for the development of chemical goods, enzyme preparations, and nutraceuticals. It is a GRAS (generally recognized as safe) grade food safety strain. The CRISPR-Cas system has also been used to produce gene editing and regulatory tools for subtilis B. Altenbuchner et al. and Wu et al. employed the CRISPR-Cas9 and CRISPR-Cpf1 systems, respectively, to accomplish multi-point mutation and long fragment gene deletion of B. subtilis. Zou et al. recently used an all-in-one plasmid-based CRISPR-Cas9 system to efficiently iteratively edit B. subtilis genes with over 100% efficiency for various mutation types. The creation of these effective CRISPR-based B. subtilis gene editing instruments has significantly aided in the industrialization of B. subtilis and encouraged the modification of its chassis cells. In B. subtilis, Fehler et al. similarly employed the CRISPR-dCas9 system to reduce the transcript levels of genes linked to flagella, which led to a notable two to three-fold increase in α-amylase production.

3. PROBIOTICS

Probiotics are live microorganisms that are good for the host's health if taken in enough amounts. Probiotics have been employed for many years in the food and clinical industries, and their efficacy and safety have progressively come to light. The majority of recent research has been on how they affect the intestinal microbiome in relation to their particular active components and health-promoting functions. Recent research has demonstrated that probiotics can support intestinal health in a number of ways, such as by enhancing the intestinal microflora's structure, preventing the growth of infections, and immunological response. The two primary groups frequently utilized for probiotic production and food addition are, in particular, strains from Lactobacillus and Bifidobacterium. Additionally, probiotics from additional genera, including Bacillus coagulans, Streptococcus thermophilus, and Lactococcuslactis, are available. In the colon, these strains can typically ferment food fiber into short-chain fatty acids (like acetate and butyric acid) that give the host energy. Additionally, they may be crucial for vitamin production. As genome sequencing technology has become more widely used and microbial genome editing tools based on the CRISPR-Cas system have been developed, researchers have gradually moved their focus from isolating probiotics from the natural environment to altering probiotics to create customized ones. To enhance the yield of target metabolites, we can optimize and reconstruct metabolic pathways and add target genes with positive effects via probiotic gene editing. However, the well-liked CRISPR-based gene editing technology has only been established in a few number of probiotics since the majority still lack a robust genetic transformation mechanism. Although there are fewer reports of significant parts of foreign functional genes being knocked in and persistently expressed, and even fewer animal experiments, the majority are still

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being studied in areas like gene knockout and regulation. For instance, the first time the exogenous CRISPR-Cas9 system was employed as a screening tool to aid in ssDNA recombination, a codon saturation mutation was achieved in Lactobacillus reuteri. Later on, Lactobacillus casei also successfully produced Cas9 nickase-based gene editing tools, which use nCas9 to create ssDNA breaks on chromosomes to initiate the HDR process and accomplish precise alteration.

More recent research has demonstrated that a range of Lactobacillus species, including Lactobacillus acidophilus, Lactobacillus gasseri, and others, may effectively modify their genes using the CRISPR-Cas9 system. Lactobacillus paracasei as well. The findings of Tian et al. modified Lactobacillus paracasei strain that combines an adaptive evolutionary approach with CRISPR-Cas9 gene editing technology to produce high-purity L-lactic acid efficiently. All things considered, these adaptable exogenous Cas9-based systems make it easier to change the genome of probiotics and offer a useful method of gene editing in animals without an endogenous CRISPR-Cas system.

In addition, it has been shown that over 60% of the 1,262 pub- licly accessible Lactobacillus genomes can be recognized by CRISPR ar- rays, and about 40% have intact CRISPR-Cas systems. This suggests that CRISPR-Cas systems are found in Lactobacillus in a comparatively broad range. S. thermophilus, Lactobacillus sakei, Pediococcusacidilac-tici, and other probiotics' encoded endogenous CRISPR-Cas systems are being progressively characterized as more genome sequences are determined. There have already been instances of harnessing the endogenous CRISPR-Cas system to manipulate the genomes of different probiotic bacteria. The endogenous type I-E CRISPR-Cas system has been utilized for effective gene editing in Lactobacillus crispatus, where plasmid transformation and gene editing have proven challenging in the past. Pediococcusacidilactici's type II-A CRISPR-Cas system was discovered by Liu et al., who then turned it into a powerful gene editing tool. P. acidilactici's growth performance and lactate production were similarly enhanced by this. Bifidobacterium is a crucial intestinal commensal and the second most often employed genus in commercial probiotic products, after Lactobacillus. 954 public Bifidobacterium genomes were examined by researchers, who found that 57% of these strains have CRISPR-Cas systems. They also further defined the crRNA and PAM sequences. For a variety of uses, this offered a molecular foundation for the creation of novel genome editing instruments. The genome engineering of Bifidobacteriumlactis has been demonstrated to be possible using both exogenous CBE and the endogenous type I-G system of Bifidobacterium.

II. DISCUSSION

The field of gene therapy may see substantial changes as a result of CRISPR-Cas9 gene editing. By circumventing many of the drawbacks of traditional gene therapy techniques, the field has undergone a revolution thanks to the prospective application of this cutting-edge gene editing technology. The key benefits of CRISPR are its simplicity, effectiveness, adaptability, and ease of usage. It has aided scientists in precisely editing the gene of interest that causes the disease in the right places. One But CRISPR technology also has drawbacks and difficulties of its own. The inability to directly administer CRISPR/Cas9 because to its instability and vulnerability to endonuclease digestion is one of the primary challenges facing CRISPR technology. The development of an effective technique for delivering gDNA to cells has been the subject of much research in order to prevent off-target effects and ensure that the maximum number of necessary cells receive it. When it comes to delivering the therapeutic gene, AAV, the most widely used viral vector in gene therapy, is highly effective when compared to non-viral vectors. Consequently, the delivery of Cas9 and gRNA via AAV vectors continues to be a crucial delivery method in CRISPR gene therapy too. A number of problems with viral vectors, such as insertional mutagenesis, smaller insertional size, off-target effects, immunogenicity, cytotoxicity, and costly large-scale production, could be avoided if scientists could optimize non-viral systems like plasmids, nanoparticles, electroporation, microinjection, and liposomes for the effective delivery of CRISPR/Cas9 in cells.

In addition to liposomes, additional nanocarriers such polymers, peptides, inorganic nanoparticles, and LNPs are also being extensively studied in clinical settings for the delivery of CRISPR-Cas9 in vivo. When compared to viral vectors, the effectiveness of polymer and lipid-based approaches for CRIPR-Cas 9 delivery remains questionable, despite their usage in clinical trials for a variety of illnesses. Researchers recently became interested in selective organ targeting

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(SORT), a unique customizable LNP platform, because of its potential uses in delivering CRISPR-Cas9 payloads to particular tissues such the brain, kidney, and heart.Patients with transthyretin amyloidosis have been treated with LNP-based CRISPR-Cas9 in 2020 as part of Intellia Therapeutics' ongoing clinical trial, NTLA-2001. The initial clinical data from the trial, which Intellia Therapeutics presented in June 2021, demonstrates a good safety profile and encouraging outcomes in the management of disease. To increase the number of CRISPR-based gene therapies with 100% safety assurance in the medical industry, more advancements in the field of non-viral vectors are required, as the safety and effectiveness of gene editing tools hinge more on their mode of delivery in cells.

In China, the first human clinical trial using CRISPRCas9 to treat metastatic non-small cell lung cancer was carried out in 2016. In that ex vivo cell-based gene therapy, the PD1 gene was knocked out using CRISPR to aid cells in reactivating the immune response. In 2018, the FDA approved the first Phase 1 clinical trials utilizing CRISPR-based gene editing in the United States. It enabled researchers to modify healthy T cells obtained from patients with various malignancies, such as melanoma, multiple myeloma, and synovial sarcoma, and then employ the altered T cells for immunotherapy.The majority of the CRISPR-based trials that have since been made available are still for cancer immunotherapy. In addition to cancer, the FDA authorized CRISPR-mediated human trials in 2018 for conditions like β-thalassemia and sickle-cell anemia. The FDA authorized EDIT101, also known as AGN-151587, in 2018 as the first in vivo CRISPR gene therapy. In order to treat blindness, EDIT-101 is intended to fix a point mutation in CEP290. EDIT-101 and NTLA-2001 are two ongoing in vivo CRISPR-based treatments that have shown encouraging early outcomes in terms of efficacy and safety.

Remarkably, two novel approaches to the technology are suggested by recent developments in CRISPR research to improve gene editing accuracy without producing DSBs. Prime and base editing are what they are. Point mutations may be effectively introduced into the genome through base editing, while prime editing is more flexible as it can insert, replace, and remove any desired sequence segment in the designated region of the genome. Using an appropriate distribution strategy, these two innovative techniques potentially lead to important developments in the field of therapy. In the rapidly evolving area of medical science, where nothing seems to stay the same for very long, CRISPR-edited human embryos are not a miracle. Even though it is still quite contentious, He Jiankui may have already verified that CRISPR has the ability to change the course of human history. In October 2018, he declared the birth of the world's first gene-edited twins, reportedly resistant to HIV. Even if there is not any solid proof to back up his claim of gene-edited offspring, the announcement has generated a lot of interest among researchers.

III. CONCLUSIONS

The advancement of science surely depends on the creation of new technologies and research instruments. One of the most fruitful scientific endeavors of the past ten years has been the utilization of CRISPR-Cas systems that are derived from bacteria and archaea. Thanks to CRISPR-based biotechnology, scientists now have access to an incredibly powerful toolkit. Nowadays, many kinds of Cas nucleases and their modified forms are employed in both industrial production and a variety of scientific fields. With the emergence of several ground-breaking new technologies, like BEs, Pes, and CASTs, the development of gene editing tools based on the CRISPR-Cas system has also proceeded extremely quickly. This has increased their range of application even more.

The most well-known CRISPR-Cas9 and CRISPR-Cpf1 systems are utilized extensively in gene editing due to their usage of single-protein RNP complexes for target cleavage. Several problems remain that need to be resolved even though gene editing tools based on these two systems have shown effective gene modification in a variety of eukaryotic and prokaryotic organisms.

CONFLICT OF INTEREST

The author declares no conflicts of interest.

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