CRISPR system-mediated gene therapy: how flexible it used in genetic diseases

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Abstract：In the 1960 of the 20th century, scientists firstly advanced the concept of using gene therapy to cure the human genetic disease, which aimed to carry out precisely site-specific modification on the genome for obtain long-term therapeutic effect in patients. In the next 50 years, scientists have developed many new gene editing technologies including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR), etc. These new technologies have brought milestone breakthroughs in gene therapy era. In this review, we focus on how flexible the CRISPR system used in human genetic disease-focused research, gene therapy strategies, clinical trials and discuss some of the major challenges and significant benefit for its future use in patients.

Key words: CRISPR; gene therapy strategy; gene-editing technology; genetic diseases

1 Introduction

With the development of the medical research, we have identified and named human monogenic disease over 10000 and the overall incidence rate is about 1/100. The

quality of life of patients and their families is seriously reduced (World Health Organization). Drugs have many limitations in the treatment of hereditary diseases, which mainly improving the clinical symptoms and the quality of life of patients. Wilson disease (WD) is an abnormal copper metabolism disease caused by autosomal recessive mutation. Excess copper accumulates in important organs of human body such as brain, liver, cornea and kidney, which impairs the normal function of organs and can be life-threatening. At present, the disease is mainly treated by low-copper diet, copper chelating agents such as d-Penicillamine, trientine and zinc salt (Patil, Sheth, Krishnamurthy, & Devarbhavi, 2013). Surgery is common in the treatment of hereditary diseases. This therapy is consistent with drug therapy, which can only improve clinical symptoms and cannot cure diseases. Hereditary spherocytosis (HS) is a hemolytic disease caused by defects of erythrocyte membrane proteins. Its clinical symptoms are anemia, jaundice and hepatosplenomegaly. The red blood cells of patients are easily destroyed when passing through spleen, resulting in hemolytic anemia. Splenectomy is usually performed for treatment. Although the abnormal structure of red blood cells cannot be changed after splenectomy, it can prolong the

life of red blood cells and obtain therapeutic effects (Farias, 2017; Manciu, Matei, & Trandafir, 2017). With the concept of gene therapy proposed and developed, scientists have brought hope to patients with hereditary diseases.

Lederberg J, an American molecular biologist, who firstly proposed a preliminary concept of gene therapy in the 1960s, established a foundation for the development of gene therapy(Lederberg, 2008). Later, gene therapy was officially proposed and recognized as a treatment for human genetic diseases in 1972(Ta & Roblin, 1972).Since the 1990s, gene therapy has experienced three heatwaves because of its continuous innovation of gene editing technology. In 1996, Chandrasegaran team of Department of Environmental Health Sciences and Johns Hopkins University in the United States constructed ZFNs technology based on fusion of Fok I enzyme and zinc finger protein(Y. G. Kim & Chandrasegaran, 1994; L. Li, Wu, & Chandrasegaran, 1992). Based on the work of Chandrasegaran, Dana Carrol et al used ZFNs to inject Drosophila embryos and realized that gene can be modified in animals for the first time(Bibikova, Beumer, Trautman, & Carroll, 2003; Bibikova, Golic, Golic, & Carroll, 2002). Later, scientists used ZFNs technology to edit target genes in animal,

plant and human cells(Sundar et al., 2005). However, the design of zinc finger protein is time-consuming and laborious, and the cost is relatively high, which limits the large-scale application of this method(Hatada & Horii, 2015; J.-S. Kim, 2016).In 2009, Adam J. Bogdanove's team from the Department of Plant Pathology and Bioinformatics of Iowa State University and Ulla Bonas's team from the Institute of Biology of Martin Lu Se University in Germany respectively discovered the interaction between transcription-activator-like effector (TALE) and DNA from the genus Phytopathogenic Xanthomonas(Boch et al., 2009; Moscou & Bogdanove, 2009). A new generation of nuclease editing technology, TALENs, was constructed by combining tale protein with FokI enzyme region(J. Miller et al., 2011).Athough, the design of TALENs is much simpler than ZFNs, but a TALE recognition module is required for each base of the target sequence, the workload of TALENs construction is still large(Ramani, Shendure, & Duan, 2016). Miller et al edited NTF3 and CCR5 gene in human cells by TALENs for the first time in 2011, proving the regulation and modification of endogenous targeting genes by TALEN nuclease(J. Miller et al., 2011). The reconstruction of CRISPR/Cas9 in vitro in 2012 and its gene editing

function in human cells in 2013 marked the beginning of a new generation of gene editing era(Cho, Kim, Kim, & Kim, 2013; Jinek et al., 2012). CRISPR/Cas9 system is derived from the naturally acquired immune system of bacteria and archaea, and it resists the invasion of exogenous DNA through the complex composed of CRISPR RNA (crRNA), trans-activating crRNA (tracrRNA) and Cas9 protein. Compared with ZFNs and TALENs, CRISPR/Cas9 uses a guide RNA matched with the target DNA fragment to guide nuclease to identify the target site, thus improving the specificity of Cas9 nuclease. At the same time, Cas9 begin to work under the guidance of gRNA, unlike FokI enzymes of ZFNs and TALENs which have the activity of cutting targeted DNA only by dimerization, avoiding the need of exquisite and complicated protein design or assembly(Cho, Kim, Kim, Kweon, et al., 2013).

CRISPR systems are divided into three types (I ~ III). Type I and III are found in bacteria and archaea and contain multiple Cas proteins and class II only exists in bacteria and contains only one Cas protein(Chylinski, Le Rhun, & Charpentier, 2013).CRISPR/Cas9, which is widely used in gene editing, is classified to type II CRISPR system and consists of Cas9 protein and sgRNA (single guide RNA) and

sgRNA combines with Cas9 nuclease protein to guide Cas9 to recognize and edit the target sequence. PAM (protospacer adjacent motif) containing NGG or NAG must exist near the target sequence(Bao et al., 2019).So far, CRISPR system has developed many different subtypes, such as CRISPR/SpCas9, CRISPR /FnCas9, CRISPR/Cas12a, CRISPR/dCas and so on. The following table is a comparison of different CRISPR system (table 1), and we also compared the advantages and disadvantages among the gene editing technologies of ZFNs, TALENs and CRISPR systems (table 2).

From the first in vitro validation of ZFNs in 1996 to the emergence of CRISPR/Cas9 technology in 2012, gene editing technology has developed rapidly (Fig 1). The editing efficiency and accuracy have been continuously improved and application fields have been continuously widened. CRISPR technology is simple to operate and low in cost, and it is possible to realize personalized treatment for clinical or preclinical studies of genetic diseases, cancers and other diseases. Next, we will focus on how CRISPR system can be flexibly applied in different genetic diseases(Kc & Steer, 2019; Mourad & Gianello, 2017; Shalem, Sanjana, & Zhang, 2015).

2 Correction strategy

2.1CRISPR system-mediated homology directed repair

Studies have shown that a large proportion of genetic diseases are caused by point mutations of specific exons of related pathogenic genes. However, in the transcription process, point mutations of genes will produce special shear or termination signals, resulting in the reduction or functional insufficiency of corresponding protein products. Therefore, the introduction of CRISPR/Cas9 system near the mutation site breaks DNA, and subsequent homologous directed repair (HDR) can be theoretically used to treat family genetic diseases caused by mutations (Fig 2a).

In 2014(Yin et al., 2014), researchers reported a study of using CRISPR/Cas9-mediated HDR to treat hereditary tyrosinaemia type I (HTI). HTI is a fatal hereditary disease caused by mutation of Fah. The mutation is generally originated from G→A point mutation on exon 8 of Fah gene of HTI patients, resulting in unstable FAH protein. The defective FAH protein further causes accumulation of toxic metabolites in liver cells, leading to liver injury and systemic toxicity(Azuma et al., 2007; Paulk et al., 2010). Researchers designed a number of gRNA targeting exon 8 of Fah gene and

single-stranded DNA (ssDNA) donor and delivered them to Fah5981SB mice (carrying the same point mutation as HTI patients). In order to evaluate the gene editing effect in vivo, researchers selected a series of methods including gene sequencing, cellular immunohistochemical analysis, quantitative PCR and body weight monitoring for verification. After treatment, the body weight of mice was successfully maintained, showing that the treatment effectively protected the liver. The sequencing confirmed the accurate repair of mutation(Yin et al., 2014). This innovative study demonstrates the ability of CRISPR/Cas9 technology to successfully repair gene point mutations in vivo and provides strong evidence for CRISPR/Cas9 to treat gene deficiency diseases.

Similar treatment strategies have also been applied to other hereditary diseases. Yang et al(Yang et al., 2016), successfully repaired the G→A point mutation on exon 4 of mouse OTC gene in vivo by intravenous injection of CRISPR/Cas9 system and adeno-associated virus (AAV) carrying DNA template to realize effective treatment of mouse hyperammonemia. Likewise, this strategy has also been applied to other genetic diseases such as β thalassemia(Xie et al., 2014), Duchenne muscular

dystrophy (DMD)(Long et al., 2014a), Sickle Cell Anemia(Huang et al., 2015) and severe combined immunodeficiency (SCID)(Chang et al., 2015).

2.2 Targeting compensatory modifier genes

Neuromuscular disorders are often caused by heterogeneous mutations, structurally complex genes. Targeted compensatory modification genes may be beneficial to improve disease phenotype. Here we introduce a mutation-independent strategy to upregulate the expression of a disease-modifying gene associated with congenital muscular dystrophy type 1A (MDC1A) using the CRISPR activation system in mice.

MDC1A is a kind of autosomal recessive hereditary disease, which refers to the primary and progressive myopathy occurring at birth or within a few months after birth, with joint contracture occurring in the early stage. MDC1A is caused by LAMA2 gene mutation encoding laminin -α2 chain, producing nonfunctional laminin-α2 protein.laminin-211 is formed by the LAMA2, β1 and γ1 chains, and maintains the survival and stability of myotubes together with skeletal muscle and other related substances(Gawlik, Miyagoe-Suzuki, Ekblom, Takeda, & Durbeej, 2004).Muscle pathology shows characteristic changes of muscular dystrophy. Among

the patients with congenital muscular dystrophy, 40% are MDC1A, which is more common in European and American countries(Muntoni & Voit, 2004). Although scientists on the pathogenesis of MDC1A has been mature, there are still no effective drugs and surgery to cure it.

LAMA1 gene encodes a protein with similar structure to laminin-α2, called laminin -α1. Overexpression of LAMA 1 gene improves muscle atrophy and paralysis symptoms of MDC1A mouse model, proving its importance as a disease compensation regulator(Gawlik et al., 2004; Muntoni & Voit, 2004; Sunada, Bernier, Utani, Yamada, & Campbell, 1995). According to this idea, researchers used adeno-associated virus 9 (AAV9) to regulate the expression of Lama1 in a dy^{2j}/dy^{2j} mouse MDC1A model with obvious symptoms of hind limb paralysis and muscle fibrosis. The virus carries Cas9, VP64 trans-activators and sgRNA and the sgRNA was design to target the promoter of Lama1. The results showed that Lama1 in skeletal muscle and peripheral nerve was up-regulated, and malnutrition characteristics and disease progression of dy^{2j}/dy^{2j} mice were greatly improved and reversed. In general, the experimental data proved the feasibility ofCRISPR-dcas9 mediated Lama1

upregulation to improve MDC1A symptoms, which may make mutation-independent therapy possible for all MDC1A patients. The method has wide applicability to various disease modification genes and can be used as a treatment strategy for many genetic and acquired diseases(Kemaladewi et al., 2019) (Fig 2b 2c).

2.3Knock out truncated protein

Mutations in genes sometimes result in early termination signals and transcription of truncated proteins. The accumulation of truncated proteins may lead to diseases and harm to health. It is necessary to prevent transcription of truncated proteins (Fig 2d).

Hutchinson–Gilford progeria syndrome (HGPS), firstly reported in 1886, is a congenital hereditary disease characterized by delayed development and progressive senile degenerative changes in infancy. Children suffering from this disease rarely survive to 13 years old(Gonzalo, Kreienkamp, & Askjaer, 2016; Worman, Ostlund, & Wang, 2010), and about one in eight million newborns suffer from this disease(Gonzalo et al., 2016). The main potential genetic cause is the substitution

mutation of laminin A coding gene, which results in a toxic protein called progerin(Scaffidi & Misteli, 2006).

Generally, LMNA gene encodes lamin A and lamin C proteins(Leeuw, Gruenbaum, & Medalia, 2017). The prelamin A interacts with a farnesyl group, and then farnesylated prelamin A is transported into the nucleus through the nuclear pores, making prelamin A temporarily adhere to the inner surface of the nuclear membrane. Next the FACE1 protease cleave the farnesylated prelamin A to remove the farnesyl group and several adjacent amino acids. Finally, prelamin A becomes mature laminA and lamin A, lamin B and lamin C together form a nuclear fiber layer, which provides structural support for the nucleus and regulates the structure of chromatin and gene expression(Al-Haboubi, Shumaker, Koeser, Wehnert, & Fahrenkrog, 2011; Andrés & Gonzalez-Granado, 2009; Schäpe, Prausse, Radmacher, & Stick, 2009). The majority of HGPS cases (~90%) result from a de novo c.C1824T (p.Gly608Gly) mutation that increases the usage of a cryptic splicing site and production of a truncated lamin A called progerin(Eriksson et al., 2003; Vidak & Foisner, 2016). However, progerin cannot be cleaved by protease ZMSPTE24(FACE1), which leads to its stable

combination with the inner nuclear membrane and accumulation,as a result, various cell defects caused, including abnormal nuclear structure, loss of heterochromatin, imbalance of DNA repair and redox homeostasis, etc(Reddy & Comai, 2011; Scaffidi & Misteli, 2006). Studies have found that mice without laminA live longer than wild-type mice, which indicates that HGPS is not due to lack of laminin A, but due to accumulation of progerin(Fong et al., 2006; Lopez-Mejia et al., 2014). Therefore, researchers guessed that HGPS can be treated with CRISPR-Cas9 targeted laminin A/ progerin destruction.

Researchers designed two gRNA to target LMNA gene to reduce the expression of laminA and progerin and the CRISPR/cas9 system was delivered into HGPS mice by AAV. Two months after the treatment, the mice not only became stronger, but also their cardiovascular health was significantly improved. After pathological examination, the degeneration of the main arteries in mice was reduced and the onset of bradycardia was delayed. However, the treatment of these two symptoms is precisely the difficult problem that premature aging and elderly patients often need to face! In general, the treated HGPS have similar activity levels as normal mice, and

their life span has increased by 25%! Not only that, the researchers also said that if the efficiency of infecting tissues in gene editing can be improved, they still have the confidence to further extend the life span of HGPS mice!(Beyret et al., 2019).

2.4 CRISPR in gene haploinsufficiency

The human genome contains two copies of each gene, one from the father and the other from the mother. Functional deletion mutation of one copy will lead to reduction of protein, leading to human diseases, which is called haploinsufficiency. However, it is currently estimated that more than 660 genes cause human diseases due to haploinsufficiency(Dang, Kassahn, Marcos, & Ragan, 2008; Landrum et al., 2015). Here we used CRISPR activation (CRISPRa) to target the remaining functional copy of a haploinsufficient gene to improve the phenotypes of these genetic diseases (Fig 2e).

SIM1 gene plays a vital role in regulating hunger and satiety. Mutations in single copies of SIM1 genes are frequently observed in patients with severe obesity. When both copies of SIM1 work, people can control food intake. However, gene mutation can make one of the copies ineffective, which forces the human body to completely

rely on another normal gene copy. However, this normal gene copy cannot send a signal of satiety enough, which makes people eat continuously, eventually leading to severe obesity(Beckers, Zegers, Van Gaal, & Van Hecke, 2009; Holder, Butte, & Zinn, 2000). In 2019, researchers used CRISPRa system, targeting the functional copy of a haploinsufficient SIM1 gene, endogenous up-regulation of expression of SIM1, to reverse the obesity phenotype of $Sim1^{+/-}$ mice. The results showed that after $Sim1^{+/-}$ mice receiving CRISPRa treatment (at 4 weeks of age), the weight of the mice has been maintained at a healthy level and SIM1 expression has increased, which is comparable to that of normal mice. The untreated $Sim1^{+/-}$ mice whose body weight has been abnormal since 6 weeks of age, and by the 10th week, they are already severely obese. CRISPRa-treated $Sim1^{+/-}$ mice are 30%-40% lighter than control mice, and the effects are long-term. This means that scientists have found a way to control weight without changing the genome sequence. Although this study focuses on obesity, this method can be applied to other diseases caused by haploinsufficiency(Matharu et al., 2019).

2.5 Directly repaired mismatched bases

In the section 2.1, we have introduced the use of CRISPR-mediated HDR to repair point mutations. The followings we will introduce the directly repaired method of point mutations (Fig 2f).

β-thalassemia is a hereditary hemolytic disease, due to the reduction of β -globin caused by HBB gene mutation. The clinical symptoms are progressive hemolytic anemia, jaundice, hepatosplenomegaly, etc. Patients with severe illness will cause death. In the world, there are at least 200,000 newborns with β-thalassemia each year(Koren et al., 2014). The disease mainly occurs in sub-Saharan Africa, Mediterranean region, Middle East, Indian subcontinent and East and Southeast Asia, of which HBB − 28(A > G) mutations is especially common in China Southeast Asia patients with β-thalassemia(G. Li et al., 2017; Vichinsky, 2010). At present, blood transfusion and hematopoietic stem cell transplantation are still the main methods, which cost a lot and have unsatisfactory therapeutic effect(Nguyễn et al., 2017).

For HBB−28(A > G) mutations, researchers tried to use crispr/system to directly correct gene mutations instead of HDR repair. The effector protein consists of cytidine deaminase (rAPOBEC1), Cas9 and uracil DNA glycosylase inhibitor (UGI),

which deaminate cytidine (C) to uridine (U) without inducing DNA double strand break (DSB), and finally result in C-to-T (or G-to-A) conversion in the target DNA sequence, called BE technology. Researchers used this method to edit the point mutation of HBB in immature human ternary nuclear embryos. Although the repair efficiency is only 23%, this study opens a new window for the treatment of β-thalassemia and other genetic diseases(Liang et al., 2017).

At the same time, Chadwick et al also corrected the mutations of PCSK9 gene through BE technology to reduce the level of plasma cholesterol, this study confirmed the potential capability of BE technology in gene editing(Chadwick, Wang, & Musunuru, 2017).

2.6 Repair of gene fragment deletion

One of the important causes of hereditary diseases is the base deletion of fragments of different sizes in the genome, ranging from a single base to the entire exon. Such gene defects usually produce incomplete protein products, affecting their functions. Similar to the repair of point mutations, the HDR function of cells is utilized to combine with

appropriate DNA templates for repair. CRISPR/Cas9 gene editing technology has also been tried and tested in the treatment of this type of genetic diseases (Fig 2g).

Duchenne muscular dystrophy (DMD) is an X chromosome hereditary disease characterized by progressive muscle weakness and emaciation. In 1987(Hoffman, Brown, & Kunkel, 1988), HOFFMAN et al. successfully identified dystrophin, the coding product of Dmd gene. Children with Dmd mutation will have myasthenia symptoms at the age of 5, and will completely lose walking ability at the age of 7-16 and need to rely on wheelchair movement, and eventually die of respiratory failure or heart failure.Dmd gene is the largest gene in human genome, including 2.4 million base pairs and 79 exons, with many mutation types, including small deletions, insertions, point mutations and repetitive sequences, and large fragment deletions. The occurrence rate of large fragment deletion accounts for about 70-80% of the mutations found. The hot spot region where mutations often occur is between exons 45-53(Bladen et al., 2015). Li et al. knocked the full-length sequence encoding exon 44 of Dmd gene into induced pluripotent stem cells (iPSCs) from patients to replace the original sequence in the genome to realize the restoration of protein coding region.

by analyzing skeletal muscle cells differentiated from edited iPSC, the researchers detected dystrophin mRNA containing complete and non-mutated exon 44 sequence, confirming that the inserted exon 44 and subsequent exon 45 can be normally expressed in sequence, showing the potential of this strategy in DMD treatment(Hongmei et al., 2014).

2.7 Non-homologous end joining and human induced pluripotent stem cell

There are different treatment strategies for the same hereditary disease, for DMD, that we can also use non-homologous end joining (NHEJ) and human induced plum stem cell (hiPSC) for treatment. Researchers first obtained skin cells from DMD patients and created induced pluripotent stem cells. Next, they used CRISPR/Cas9 technology to knock out mutations in hiPSC. During NHEJ, the 30 end of intron 44 and the 50 end of intron 55 join to create a ~1 kb chimeric intron and exon 44 joined with exon 56 to create a correct in-frame transcript. Researchers differentiated the edited hiPSC into myocardial and skeletal muscle cells, and then transplanted these

cells into DMD mouse models. The results showed that the transplanted mice could successfully generate human dystrophin protein(Young et al., 2016) (Fig 2f).

3 Clinical trial

On July 25, 2019, Editas Medicine founded by Gene Editor Feng Zhang and Allergan jointly announced that CRISPR Phase I/II clinical trial for Leber congenital amaurosis type 10 (LCA10) was officially started. This clinical trial is the world's first CRISPR gene editing study in human body and eighteen patients with LCA10would be recruited to verify the safety, tolerance and efficacy of CRISPR gene editing therapy.

Leber congenital amaurosis, most of which are chromosomal recessive hereditary diseases, is one of the most serious hereditary retinopathies. At birth or within one year after birth, cone cell function is completely lost, resulting in congenital blindness in infants and children. At present, various types of Leber congenital amaurosis caused by multiple gene mutations have been found. On December 10, 2017, FDA approved Spark's “AAV gene therapy”, which delivers the correct RPE65 gene to retinal cells through adeno-associated virus (AAV), to treating Leber congenital

334 melanoma type 2 (LCA2). This method is the first gene therapy approved by FDA
335 and costs as much as 850,000 US dollars per year. However, AAV therapy has no
336 effect on LCA10, because the coding sequence of CEP290 gene is 7.5kb, far
337 exceeding the packaging capacity limit of AAV virus (4.7kb), so LCA10 cannot be
338 treated by AAV delivering correctly coded CEP290 gene. In order to overcome this
339 limitation, Editas Medicine has developed a clever method based on CRISPR/Cas9
340 gene editing technology to solve the treatment problem. The research team delivered
341 saCas9 and CEP290 specific gRNA to photoreceptor cells through subretinal injection
342 using the AAV5 vector. The double gRNA targeted the mutant region respectively,
343 directly deleting or inverting the mutant region as a whole, restoring the normal
344 expression of CEP290 gene, and allowing the eyes to regain light. The results of this
345 preclinical study were published in the top medical journal *nature medicine* in January
346 2019 laying a foundation for gene therapy in human body based on CRISPR gene
347 editing.

348 On March 4, 2020, Allergan and Editas Medicine announced that CRISPR had
349 completed the first phase I/II clinical trial for the LCA10. Allergan and Editas

Medicine used milestones to describe the incident. The completion of drug administration for the first patient is a small step for Feng Zhang and Editas Medicine, but it is a key step for CRISPR gene editing therapy to conquer hereditary diseases. We look forward to the follow-up treatment results and the arrival of light.

4 Challenges and prospects of system-mediated gene therapy

Since CRISPR technology can efficiently and accurately edit mammalian genes, scientific reports on CRISPR have rapidly swept across all fields of life science. However, the accuracy and efficiency of CRISPR system still need to be solved. Gene therapy has many different methods to treat DMD, such as somatic cell editing(Long et al., 2016), germ cell editing(Long et al., 2014b), induced pluripotent stem cell editing(Young et al., 2016), etc. The gene repair rate of these different methods was less than 30%, and off-target effect was founded. I think we can improve the difficulties in the following areas: 1) The optimization and modification of viral vectors can further improve the transduction of foreign genes and reduce the immunogenicity of organisms; 2) The upgrading of gene editing tools can improve the

efficiency of targeted cutting and reduce the generation of miss effect. 3) The development of highly efficient and accurate targeted genome integration strategy will help the long-term stable integration of foreign genes and realize the long-term effective treatment of genetic diseases.

Gene therapy also has ethical problems. On November 26, 2018, He Jiankui announced that two gene editing babies was born in China. The twins' CCR5 gene has been modified so that they can resist the HIV virus after birth. The news quickly aroused discussions, shocking China and the world. It is the common wish of mankind to keep science to be benevolent. While enjoying the benefits brought by science and technology, we should not neglect its negative effects. We should maintain a cautious attitude, observe the moral bottom line and take into account the long-term interests of mankind.

Figure legends

figure 1 progress of the development of the gene-editing technology

figure 2 different gene-editing strategies

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CONFLICTS OF INTEREST

All the authors have no conflict of interest.

Data Availability Statement

All data can be used for online publication

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Table 1 comparison of different CRISPR system

Cas protein	target	Number components	Guide RNA	Large (bp)	PAM/PFS	references
Cas9	dsDNA	2	crRNA+ tracrRNA	1368	-NGG- GC-rich	(Hirano et al., 2016; Koonin, Makarova, &

						Zhang, 2017)
Cas12a	dsDNA	2	crRNA	1307	-TTN- AT-rich	(T. Li et al., 2019; Zetsche et al., 2015)
Cas12b	dsDNA	2	crRNA+ tracrRNA	~1300	AT-rich	(Hirano et al., 2016; Strecker et al., 2019)
SaCas9	ssRNA	3	crRNA+ tracrRNA	1053	-NNGRRT-	(Ran et al., 2015; Wang et al., 2019)
spCas9	ssRNA	3	crRNA	1368	-NGG-	(Hirano et al., 2016; Jia, Jin, Orbovic, Zhang, & Wang, 2017)
FnCas9	ssRNA	3	crRNA+ tracrRNA	1629	-NGG-	(Chen et al., 2017)

Cas13a	ssRNA	2	crRNA	1389	3’PFS: non-G	(Chaudhary, 2018; Khan, Amin, Hameed, & Mansoor, 2018)
Cas13b	ssRNA	2	crRNA	1127	5’PFS: non-C 3’PFS: NAN/NNA	(Slaymaker et al., 2019)

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641 Table 2 advantages and disadvantages among the gene editing technologies

Gene editing technology	advantages	disadvantages	references
ZFNs	High efficiency of targeted-gene delivery; High efficiency of targeted binding	Nuclease is difficult to design; High off-taget rate; Difficult for high-throughput targeting	(Klug, 2010)
TALENs	High specificity and easy design; High efficiency	Low efficiency of targeted-gene delivery;	(Boch & Bonas,

	of targeted binding	Repeated sequences may cause nonspecific cleavage;	2010; J. C. Miller et al., 2011)
CRISPR systems	The editing efficiency is higher than ZFNs and TALENs; Simple operation and low cost; No limitation on flux.	High off-taget rate; The efficiency of Homologous recombination is low.	(Gaj, Gersbach, & Barbas, 2013)

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