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Review

Methods and applications of CRISPR/Cas system for genome editing in stem cells

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ABSTRACT

Genome editing technology holds great promise for genome manipulation and gene therapy. While widespread utilization, genome editing has been used to unravel the roles of specific genes in differentiation and pluripotency of stem cells, and reinforce the stem cell-based applications. In this review, we summarize the advances of genome editing technology, as well as the derivative technologies from CRISPR/Cas system, which show tremendous potential in various fields. We also highlight the key findings in the studies of stem cells and regeneration by genome editing technology.
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1. Introduction

Traditional methods for gene study include forward genetics and reverse genetics, of which reverse genetics shows more reliable and less time-consuming than forward genetics. However, the limitation of genome editing technology directly retards the study of gene and treatment of genetic disorders [1,2].

Genome editing technology has revolutionized the fields of genetics, and accelerated the study of functional genomics [3,4]. The power of genome editing technology allows the efficient manipulation of genome, thus provides with advanced methods to study genes and their interactions. Moreover, genome editing technology has been tested for treatment of genetic disorder that resulted from gene mutations, such as thalassemia. Hence, genome editing technology holds great promise for functional genomics and gene therapy.

Two genome editing technologies, ZFN (Zinc Finger Nuclease) and TALEN (Transcription Activator-Like Effector Nucleases), are developed by fusion of DNA binding domain and *Fok*I nuclease, and both of them utilize dimer *Fok*I nuclease to enable targeted DNA interference. Recent developed CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein) system shows superiority in convenience and versatility over ZFN and TALEN, thus is widely utilized [3,5–7].

Initially identified as defensive immune system against virus in archaea and bacteria, CRISPR system is divided into two classes according to the complexity of Cas effectors, of which single effector shows simplified assembly and efficacy, and current CRISPR/Cas system is mainly developed based on single effector, such as Cas9, Cas12 and Cas13 (Fig. 1) [6,8–12]. The derivate technologies of CRISPR/Cas system are applied in various fields, such as transcriptional regulation, epigenetic editing, base editing and cellular imaging [13–20]. In addition, the mechanisms of spacer acquisition of CRISPR/Cas system in bacterium and archaea are well studied and applied in molecular recording and storage of digital information, which represent the potential application of CRISPR/Cas system in information storage [21,22].

In this review, we summarize the development of CRISPR/Cas system and highlight the important CRISPR/Cas system, such as Cas9, Cas12 and Cas13, as well as the derivate technologies of CRISPR/Cas system. We also review the application of CRISPR/Cas system in stem cells and regeneration.

2. Development of genome editing technology

Heritable genome manipulation illuminates the gene therapy of genetic disorders, and the invention of genome editing technologies inspires the development of functional genomic studies [1,2]. Then ZFN, TALEN and CRISPR/Cas9 appear in turn with

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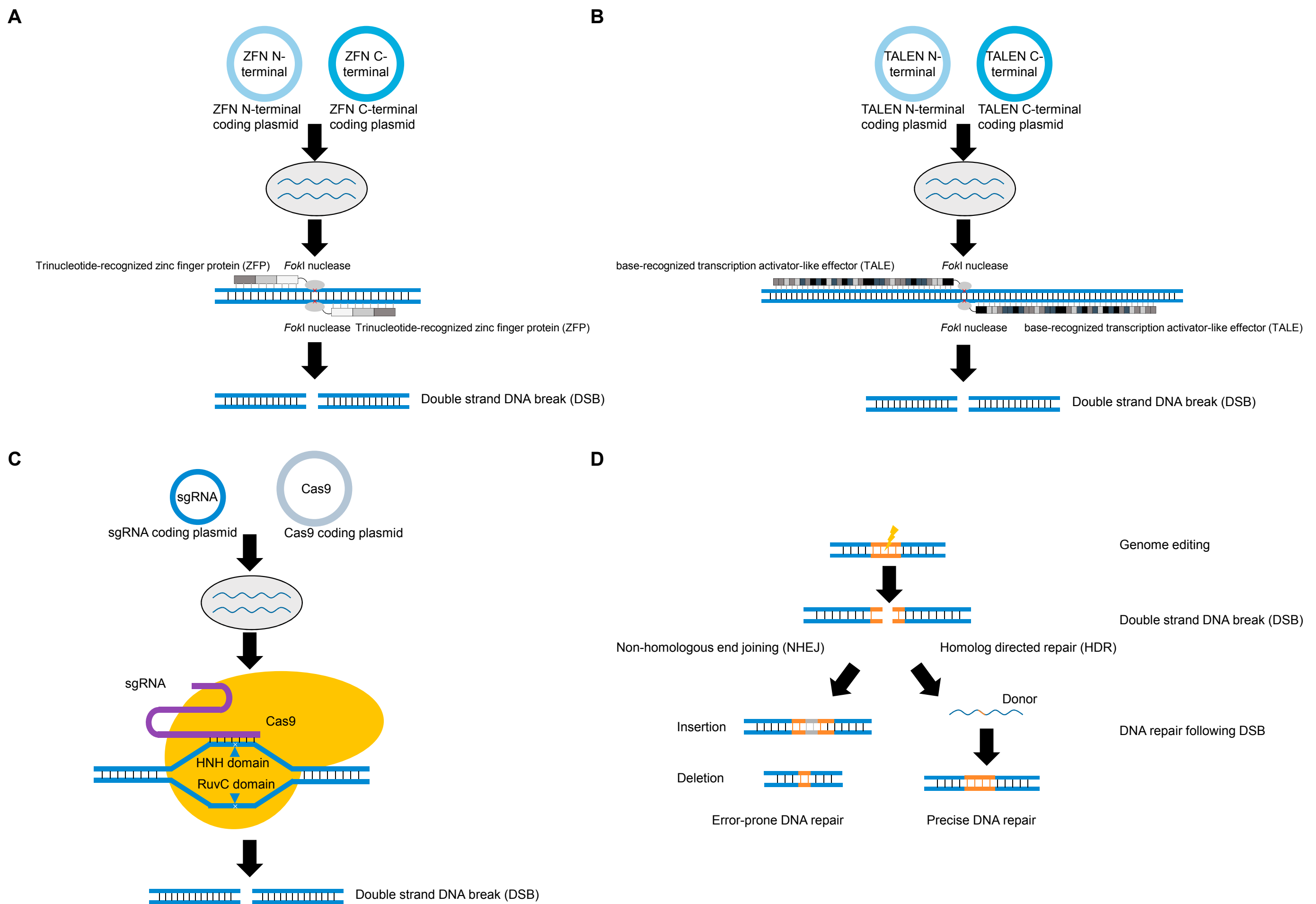

Fig. 1. The mechanisms of different genome engineering technologies. (A)–(C) The mechanisms of ZFN, TALEN and CRISPR/Cas9 in genome engineering. (D) The mechanisms of DNA repair following double strand DNA breaks (DSB) released by genome engineering.

advanced efficiency and precision [3,5–7]. ZFN and TALEN both act as nuclease by dimer *Fok*I nuclease, and genomic locator by the *Fok*I-adjacent DNA binding components, TALE or ZFP, which are different in the sequence and mechanisms of recognition. Both ZFN and TALEN induce double strand DNA breaks (DSB), and error-prone DNA repair following DSB results in the frame shift of CDS (coding sequence) of genes, thereby achieving gene knockout. TALE can perform single nucleotide resolution recognition while ZFP can only recognize trinucleotide. Moreover, the interactions between DNA and DNA binding motifs of ZFN are more complex than TALEN, which affect the genome engineering outcomes (Fig. 2A, B). Therefore, TALEN shows increased targeting efficiency and precision, and heritable disease models by TALEN have been generated [3,23,24].

In contrast to ZFN and TALEN, CRISPR/Cas system functions as nuclease by Cas9, which harbors robust nuclease activity, and genomic locator by chimeric targeted DNA-complementary sgRNA (single guide RNA), thus enabling efficient genome engineering (Fig. 2C) [6,7]. Because of the simplicity and efficacy over ZFN and TALEN, CRISPR/Cas9 is widely utilized in plants, animals, primates, and even human embryos [25–32]. Gene therapy by knockout of pathogenic mutant genes using CRISPR/Cas9 has also been reported in mice and dogs, indicating its tremendous potential in clinical application [33–36].

Furthermore, CRISPR/Cas9 functions by targeted DSB, which results in DNA repair by NHEJ (non-homologous end joining) or HDR (homolog directed repair). The error-prone NHEJ would lead to the sequence insertion or deletion (indels), thus enabling gene knockout, while HDR performs precise DNA repair by using donor as template, therefore, providing optional methods for genetic disorders (Fig. 2D) [37,38]. It is notifying that genetic screening by CRISPR/Cas9-mediated gene knockout also offers an efficient strategy to identify essential genes in functional genomics such as immunotherapy target [39,40].

In addition, the DNA binding trait of CRISPR/Cas system makes it possible to perform further genome manipulations beyond DNA interventions [13–20,41–43]. Meanwhile, Cas9 orthologues such as SaCas9, FnCas9, Cas12a that recognize different PAM sequence are also developed to enrich the genome editing toolbox, and Cas13 that can edit RNA is utilized for virial detection [12,44–46]. A interesting method derived from spacer acquisition of CRISPR system is also applied in information storage [21,22]. Taken together, CRISPR/Cas and its derivate technologies significantly extend the genome editing and enrich the genomic manipulation for functional genomics study.

3. Mechanisms of CRISPR/Cas system

The class 2 CRISPR/Cas system that harbors single Cas effector shows different genome editing mechanism due to its different function domains [47]. The canonical type II CRISPR/Cas9 system that harbors HNH and RuvC domains could independently edit

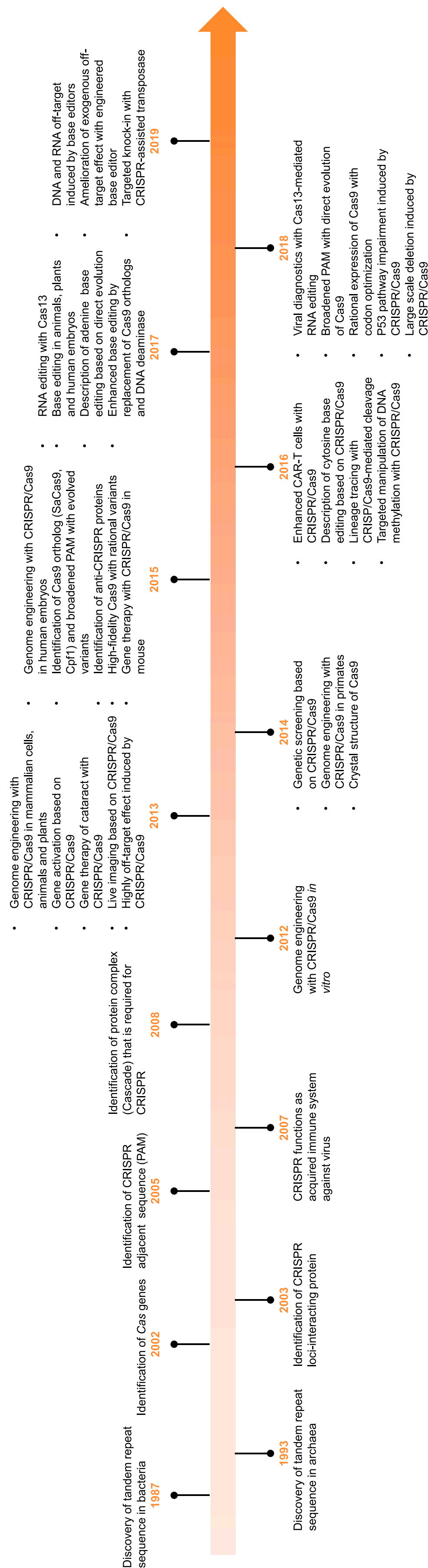

Fig. 2. Milestones of development and application of CRISPR/Cas system.

target strand and non-target strand, respectively (Fig. 1C) [48]. The crystal structure of Cas9 would change for domain activation upon formation of Cas9/sgRNA complex, and the DNA helicase activity unwinds the target double DNA, followed by the binding of Cas9 to target DNA with the guide of sgRNA. The change of DNA conformation offers the substrate for individual nuclease domains, and DNA-Cas9-sgRNA complex is then termed R-loop for its appearance (Fig. 1C) [48–50]. Different nucleases could be harnessed for different genome editing with different Cas9 variants possessing inactivated nuclease domains, and engineered Cas9 variants that show higher specificity are designed based on the regulation of interactions between DNA and nuclease domains [51–54].

In contrast to type II CRISPR/Cas9 system, type V CRISPR/Cas12a system only harbors RuvC domain, thus the outcome of genome editing by Cas12a is different from Cas9 system. Besides, Cas12a possesses crRNA biogenesis RNase and single strand DNase activity, therefore Cas12a could be harnessed for multiplex gene manipulation by a single sequence array. The trans-activated ssDNase confers Cas12a with the ability of detecting specific DNA sequence such as dsDNA virus by amplified signals [11,55–59].

Apart from the DNase activity, type VI CRISPR/Cas13 system that harbors HEPN RNase domain could target RNA and perform RNA editing, and trans-activated single strand RNase (ssRNase) activity induced by targeted RNA editing also perform substantial ssRNA editing *in trans*. Therefore, Cas13 could be used for RNA detection, such as RNA virus. It is worth noting that RNA detection based on CRISPR/Cas13 shows superiority in sensitivity and accuracy compared to traditional PCR methods [46,60,61].

4. Repurposing CRISPR/Cas system in versatile application

With its nuclease activity and DNA binding activity, CRISPR/Cas system can be further repurposed for more applications, and nuclease-inactivated Cas9 (dCas9) with mutant nuclease domain of Cas9 (D10A and H840A) would lead to the inactivation of Cas9 nuclease while retains the DNA binding activity, thus providing with further targeted DNA manipulation, including genetic screening, transcriptional regulation, epigenetic engineering, cellular imaging, base editing, etc. Moreover, the trans-activated nuclease activity of Cas12a and Cas13 also offers the promise for virus detection at small amount and faster than traditional PCR methods (Fig. 3) [13,39,62].

4.1. Genetic screening

Since the error-prone NHEJ repair of CRISPR/Cas9-mediated double strand DNA breaks (DSB) dominates the outcomes of DNA repair, CRISPR/Cas9 genome editing mainly leads to knockout [37]. Genome-wide gene knockout with sgRNA library covering whole genome can identify the key genes in specific signal pathways or other activities. A recent screen has identified key genes in immunotherapy checkpoint, tumorigenesis and development [40]. Intriguingly, CRISPR/Cas-based genetic screens have been upgraded with the genome-wide transcriptional regulations with CRISPR interference (CRISPRi) or CRISPR activation (CRISPRa) [13,63].

4.2. Targeted transcriptional regulation

Fusion of transcriptional activators or repressors to dCas9 enables targeted gene activation or repression with the RNA-guided CRISPR/Cas system, and shows highly specificity compared with miRNA-mediated RNA interference (RNAi) [63]. Various transcriptional factors have been utilized in dCas9 fusion [14,41,63]. Recent studies report the direct reprogramming of fibroblasts to neuronal cells by targeted transcriptional activation of potential

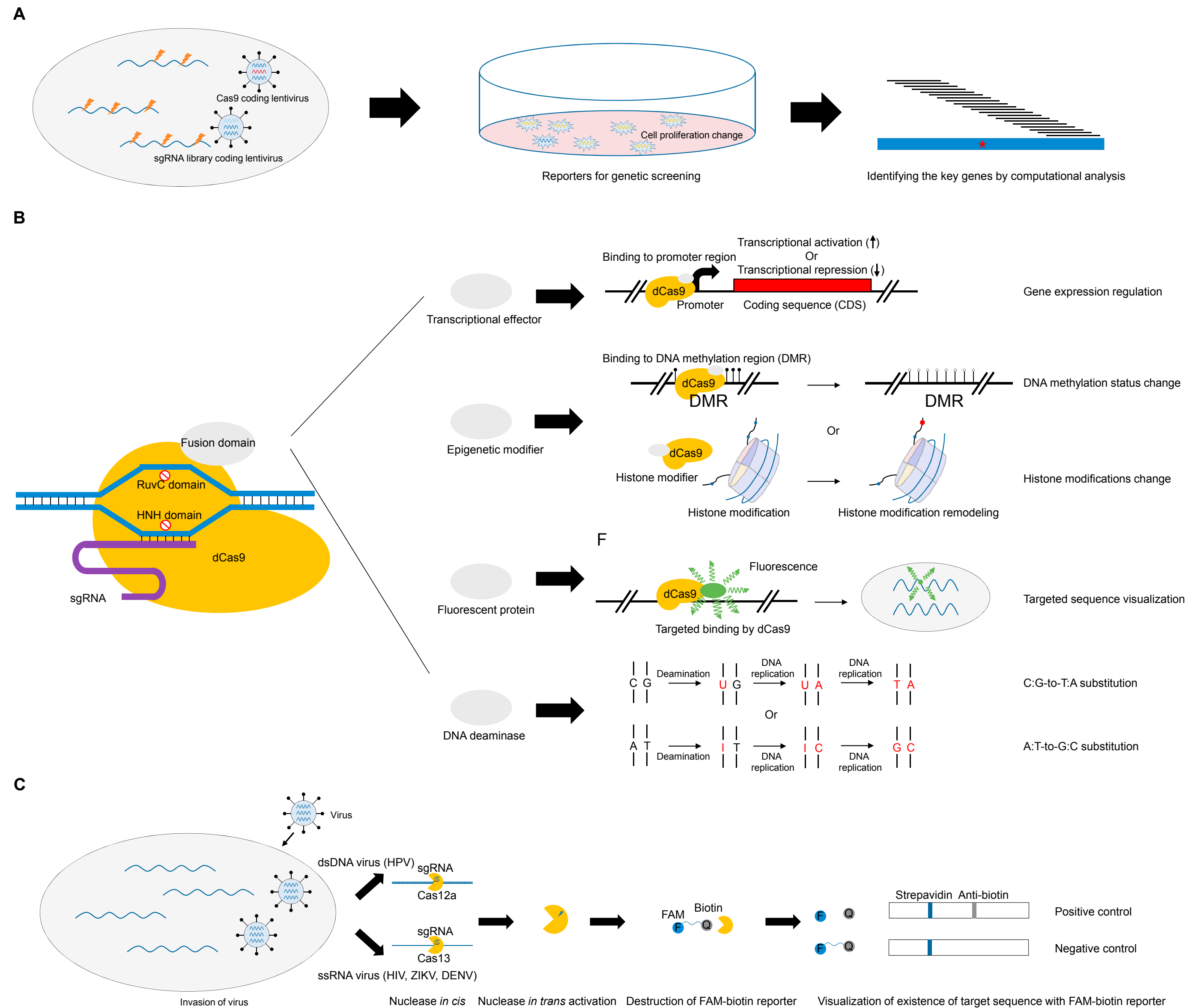

Fig. 3. Applications of CRISPR/Cas system. (A) Identifying the key gene with genetic screening. (B) Derivate technologies of CRISPR/Cas system. (C) Detection of dsDNA or ssRNA virus with CRISPR/Cas system.

transcription factors, and targeted transcriptional regulation combined with genetic screening has been successfully applied to identify the key genes in tumorigenesis [13,64]. Notably, targeted gene regulation has been used in gene therapy of obesity caused by gene haploinsufficiency [65]. However, current methods to active or repress gene expression cannot be precisely regulated, which may elicit side effect due to the over-expression or over-repression of the target genes.

It is of interest to note that targeted transcriptional regulation with fusion of dCas9 and transcriptional effectors can manipulate the gene expression notably without DNA sequence intervention, thus providing with additional tools for gene therapy from transcriptional level without inherited DNA change.

4.3. *Epigenetic engineering*

Apart from transcriptional effectors, DNA methyltransferase such as DNMT3a and DNMT3b can also be fused to dCas9 to enable targeted methylation or demethylation, which is critical for *in situ* epigenetic engineering and epigenetic studies [15,43]. Different combinations and linkers of DNA methyltransferase and dCas9 affect the outcomes of DNA methylation editing [15,42,66,67]. A recent study reports the rescue of neural defects of fragile X syndrome (FXS) neurons by targeted DNA methylation, demonstrating the tremendous potential of DNA methylation editing in gene therapy [68].

Histone modifications can also be engineered by fusion of dCas9 and histone mofidiers [69–71]. Recent studies show that, similar to the characteristics of histone modifiers themselves, fusion of dCas9 to histone demethylase LSD1 or histone acetyltransferase p300 can change the chromatin accessibility [72,73]. However, the changes of histone modifications status along with the global dramatic changes of gene expression which are difficult to regulate, therefore the therapeutic application of histone modification engineering by CRISPR/Cas system need further optimization.

4.4. CRISPR imaging

Fusion of fluorescent proteins such as green fluorescent protein (GFP) to dCas9 generates RNA-guided site-specific live imaging system that enables visualization of targeted DNA sequence within cells [18]. Recent studies have reported the generation of various CRISPR-derived cellular imaging systems to label different loci, including repetitive and non-repetitive genomic sequence [18,74,75]. Moreover, different fluorescent proteins have been used simultaneously to label different loci at the same time [19,20]. Notably, cellular imaging system has been used to unravel the DNA binding affinity of off-target and on-target of Cas9 during genome engineering, which advances the study of Cas9 dynamics within genome [75]. In addition, CRISPR imaging has been used to detect the movements of telomere during interphase [76]. One of the future clinical applications of CRISPR imaging may be the detection of tumor markers during tumorigenesis.

4.5. Base editing

The majority of CRISPR/Cas system-derived methods are related to the higher dimensional genome engineering beyond DNA sequence intervention, such as epigenome, transcriptome and live cell imaging. However, most genetic disorders are caused by gene mutations rather than the deletion of genes [1,2]. Therefore, more precise methods to correct the mutations of genetic disorders are required for gene therapy. Fusion of DNA deaminase to dCas9 can generate base editor (BE) that enables the base substitutions at single nucleotide resolution, thus offering an excellent tool to model or correct the point mutation of genetic disorder [16,17,77].

The core components of BE are DNA deaminases and Cas9 variants. The DNA deaminases act as effectors, which enable C:G-to-T:A or A:T-to-G:C substitution according to the types of DNA deaminases; while the RNA-guided CRISPR system functions as genomic locator of targeted locus. Because of the DNA binding characteristics of DNA deaminases, canonical BE system is composed of DNA deaminases and Cas9 nickase (Cas9n) which harbors D10A mutation, and single strand DNA (ssDNA) released by Cas9n provides substrates for DNA deaminases, which facilitates the activity of DNA deaminase, even though accompanying with relatively higher Cas9 nuclease activity. Inhibitors such as uracil DNA glycosylase inhibitor (UGI) that inhibit the cellular endogenous DNA repair system are also added to BE to enhance the efficiency and fidelity of base editors [16,17,77]. It is notable that UGI not only increases the efficiency of targeted C:G-to-T:A substitutions, but reduces the bystander of outcomes (indels and non-C:G-to-T:A substitutions), thus overexpression of UGI increases the targeting fidelity of cytosine base editor (CBE) [78,79].

BE shows widespread versatility across plants, rodents, fish, and human embryos, and it has been applied in modeling and correcting human disease [80–88]. Of note, genetic disorder with pathogenic mutation has been corrected in human embryos by BE, demonstrating its potential in precise gene therapy [89]. Besides, BE-mediated inducible pre-mature stop codons (iSTOP) that enables gene knockout without DSB has been applied in animals and T cells with multiplex gene knockout simultaneously, showing the promise of modeling human disease caused by multiplex gene mutations and reinforcement of chimeric antigen receptor (CAR) T cell immunotherapy with precise genome engineering technologies [90–93].

Recent studies to increase the targeting efficiency, scope and fidelity of BE mainly focus on utilization of DNA deaminases harboring higher activity, Cas9 orthologues that recognize different PAM sequence, deaminase variants that reduce the bystander mutation, codon optimizations that increase the mRNA translation rate of BE and optimization of nuclear localization signals (NLS) [94–101]. Further optimization and amelioration have been proposed according to the directly evolution of BE protein, which extends the targeting scope to an unprecedented level [102].

Not only base substitution of targeted DNA sequence, BE that consists of RNA-guided RNA targeting CRISPR effectors such as Cas13 and RNA deaminases also offer optional technologies that enable RNA base editing [12,103]. RNA base editor exhibits tremendous potential in RNA viral detection, such as ZIKV and DENV, and it shows superiority in cost and time over traditional methods, thus holding great promise for diagnosis of RNA virus during incubation periods [55].

4.6. Targeted knock-in with CRISPR-associated transposase

Recent studies have demonstrated the potential of CRISPR-associated transposase (CAST) in targeted knock-in, and this system shows comparatively equal efficiency with cargo size ranging from 0.5 to 10 kb, suggesting the tremendous potential of CAST system in targeted knock-in [104,105]. This system is composed of CRISPR-associated transposase and CRISPR effector (Cas12k) that harbors only DNA binding activity. RNA-guided Cas12k acts as genomic locator, while Cas12k-associated transposase enables targeted insertion with the recognition of long terminal repeats (LTR) of both ends. Therefore, this system makes it possible to perform large fragment insertion at targeted sites [104].

Although success of CAST system in prokaryote, it is unknown whether this system work in mammalian cells, which will contribute to the rescue of truncated genes induced by exon skipping mutations.

5. Methods and strategies to increase the safety of CRISPR/Cas system

Genome editing holds great promise for gene therapy. However, side effect of off-target that arises from the incorrect recognition of sgRNA and targeted sequence may generate pathogenic mutations during gene therapy, which affects its further clinical application [106]. Therefore, the safety concern of off-target should be seriously considered before clinical application.

Some amelioration has been developed to reduce the side effect due to off-target, including truncation of sgRNA, utilization of Cas9 nickase, engineered Cas9 variants that show high specificity and the usage of Cas9 protein (RNP) (Fig. 4) [51–54,107,108]. Truncated sgRNAs show more rigorous base pair compared to canonical sgRNA, and even 1 nt mismatch would lead to remarkably off-target activity, with slightly reduced on-target activity even [107]. Cas9 nickase with one of two mutant nuclease domains retains the on-target activity relatively with minimal off-target *in vivo* [51]. High-fidelity Cas9 variants show more dependence on sgRNA-DNA interactions, thus decrease the off-target while maintain the on-target activity [109]. Cas9 protein can work directly with sgRNA without translation phase of Cas9 mRNA, therefore, RNP works in a short time, and reduces potential nonspecific interactions due to long period incubation [75,108].

For derivations of CRISPR/Cas system, the off-target can be caused by over-expression of fusion domains. Because the interactions between Cas9 and fusion domain may be loose, then the fusion domain may escape from the complex to act independent of Cas9. Recent studies show that base editor generates substantial off-target mutations in mouse embryos and rice, while the major off-target sites do not resemble sgRNA sequence, demonstrating the off-target is induced by Cas9-independent DNA deaminases [110,111]. Moreover, transcriptional RNA mutations are also observed in BE-treated cells [112], indicating that there is a long

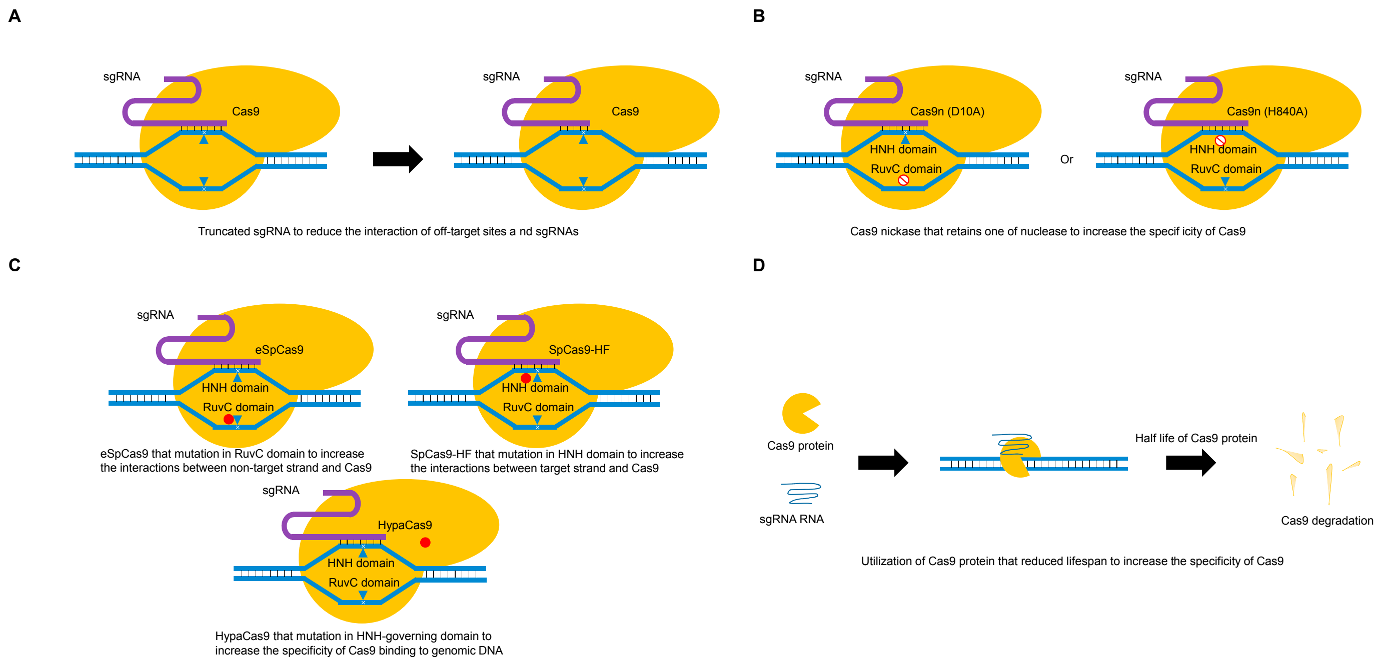

Fig. 4. Methods to increase the specificity of CRISPR/Cas system. (A) Truncating sgRNA to increase the specificity of CRISPR/Cas system. (B) Utilizing Cas9 nickase to increase the specificity of CRISPR/Cas system. (C) Engineering Cas9 to increase the specificity of CRISPR/Cas system. (D) Applying Cas9 protein and sgRNA (RNP) to increase the specificity of CRISPR/Cas system.

way to go for addressing the safety issue of CRISPR/Cas system before clinical application.

Another safety concern is the possible immunological response caused by Cas9 protein, which may result in severe immunological rejection [113]. Recent study has demonstrated the avoidance of immunogenicity of Cas9 by treatment at neonatal stage, without detectable immunological rejection [114].

Besides, unusual p53 response is also detected in Cas9-treated cells, which may come from the toxicity of Cas9 [115,116]. Considering the unique role of p53 signal pathway in tumorigenic potential and regulation between signal pathways, it is necessary to evaluate the safety and attenuate the potential immunological responses that arise from the toxicity of Cas9. Utilization of Cas9 protein and anti-CRISPR factors that decrease the half-life of Cas9 may be helpful [117–119].

6. Application of CRISPR/Cas system in stem cell and regeneration research

Pluripotent stem cells (PSCs) are derived from primary stem cells that harbor the potential of differentiating into specialized downstream cells, which recapitulate the specificity of different tissues, such as brain, and studies on differentiation of stem cells confirm the reliability and function of differentiated cells [120,121]. The limitations of genetic manipulation of PSCs hinder the development of study in PSCs [122–124]. The studies on stem cells reveal the key signal pathways of early embryonic development by genome editing, and the function of canonical signal pathway could be confirmed with genome editing, thus accelerates the functional study of stem cells. Besides, genome editing technology provides guidelines for therapeutic applications, and transplantation with PSCs can theoretically cure diseases elicited by impairments of specialized cells to some degree, especially neurodegenerative disease, such as Alzheimer's disease (AD) and Parkinson's disease (PD), which are induced by the neural dysfunction, along with cognitive impairments [125,126]. In addition, transplantation of inducible pluripotent stem cells (iPSCs) that are derived from reprogramming of somatic cells can avoid the ethic issue, and has been tried in PD patients [127,128].

6.1. Genome editing diversifies the stem cell research

Embryonic stem cells (ESCs) are typical PSCs that derive from inner cell mass (ICM) of blastocysts, and ESCs show difficulty in efficient genome editing. The CRISPR/Cas system could efficient manipulate the genome without complicated procedures [129], which accelerates the stem cell study. Besides, combining CRISPR/Cas system and reverse system enables efficient and reversible genome editing for comprehensive gene dissection [130]. Also, derivate methods of CRISPR/Cas system promote the genome editing of stem cells up to an unprecedented level, with different dimensional manipulation or more precise gene manipulation. Recent study revealed the success of transcriptional regulation by CRISPR in stem cell, and direct differentiation by CRISPR-based transcriptional regulation has also been reported [64,131–133].

6.2. Screening for factors that are responsible for reprogramming

ESCs harbor the differentiation ability into all germ layers differentiated tissues, therefore provide a promising therapeutic application of genetic disorders. However, the application of ESCs are confront with serious ethics issue, and inducible pluripotent stem cells (iPSCs) that derive from individual somatic stem cells provides an ideal alternative for ESCs [134]. The iPSCs are originally generated by reprogramming of somatic cells with Yamanaka factors into ESCs-like cells that resemble their pluripotency and epigenetic status, thus iPSCs are considered as the ideal objects to perform stem cell therapy without the ethic concerns. It is worthy of remark, Yamanaka factors that introduce iPSCs are not sufficient to resemble the epigenetic status of natural ESCs entirely, and addition of transcription factors that play roles in pluripotency

maintenance of ESCs may further promote the reprogramming of somatic cells into ESCs-like state [127,134–138]. Traditional methods to identify reprogramming factors rely on the manual addition and remove of specific transcription factors, which are time-consuming, often ended with failure, while genetic screening with CRISPR/Cas system can accelerate the discovery of reprogramming factors [127,134,139]. In addition, transcriptional gene activation with CRISPR/Cas system also provides optional methods to induce the iPSCs [133]. Therefore, CRISPR/Cas system offers platform for identification of reprogramming factors.

Besides, small molecules that contribute to reprogramming are also reported, and genetics screening also facilitates the discovery of small molecules [140–142].

6.3. Modeling of neurodegenerative disease

CRISPR/Cas system simplifies the procedure of knockout, knock-in and transgene, etc., and reversible genome engineering has also been achieved in stem cells [71,130,132,143]. Therefore, genome editing with CRISPR/Cas system accelerates the generation of stem cell lines, especially disease models.

Neurodegenerative diseases are caused by neuronal impairments, with progressive onset-age, thus stem cell models offer appropriate insights into study and cure of neurodegenerative disease [124–126]. Recent studies show that modeling the clinical pathogenic mutations can recapitulate the specificity of neurodegenerative diseases, such as amyloid-β (Aβ) generation in Alzheimer's disease (AD) [38,144,145]. Therefore, modeling disease with stem cells provides insights into mechanical and therapeutic study of genetic disorders. Furthermore, direct gene correction with CRISPR/Cas system illuminates the validity and safety before clinical application [146,147]. Taken together, genome editing technology provides unlimited possibilities for treatment of neurodegenerative disease.

7. Conclusion

CRISPR/Cas system accelerates genome editing *in situ*. The precision and convenience traits of CRISPR/Cas system show superiority over other genome editing tools, like ZFN and TALEN. DSB released by CRISPR/Cas system induces further genetic modifications, such as NHEJ and HDR, resulting in targeted precise DNA modifications. Besides, the versatile CRISPR/Cas system can act as genomic locator to offer possibilities for targeted genomic manipulation with the fusion domains, which can be transcription factors, epigenetic modifiers, deaminases, fluorescent proteins, thereby conferring transcriptional regulation, epigenetic modification, base editing, and gene visualization, respectively.

Previous stem cell studies rely on the traditional genetic methods such as homologous recombination, which is costly and time-consuming. The invention of CRISPR/Cas system offers an excellent platform to perform efficient and convenient genome engineering, which facilitates the study of stem cells by generating conditional stem cell lines and modeling genetic disorders. Moreover, the targeted binding trait of CRISPR/Cas system also provides versatile derivate technologies, which perform higher dimensional genomic manipulations beyond DNA intervention. In summary, CRISPR/Cas system and its derivations significantly promote the genome engineering and facilitate the gene therapy.

Conflicts of interest

The authors declare that there is no conflicts of interest.

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