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Review

The CRISPR Growth Spurt: from Bench to Clinic on Versatile Small RNAs

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Clustered regulatory interspaced short palindromic repeats (CRISPR) in association with CRISPR-associated protein (Cas) is an adaptive immune system, playing a pivotal role in the defense of bacteria and archaea. Ease of handling and cost effectiveness make the CRISPR-Cas system an ideal programmable nuclease tool. Recent advances in understanding the CRISPR-Cas system have tremendously improved its efficiency. For instance, it is possible to recapitulate the chronicle CRISPR-Cas from its infancy and inaugurate a developed version by generating novel variants of Cas proteins, subduing off-target effects, and optimizing of innovative strategies. In summary, the CRISPR-Cas system could be employed in a number of applications, including providing model systems, rectification of detrimental mutations, and antiviral therapies.

Keywords: CRISPR-Cas system, DNA repair, adoptive immunity, genome editing

Introduction

The clustered regulatory interspaced short palindromic repeats (CRISPR) that are found in the genetic loci of bacteria help in providing defense against foreign invaders, such as viruses and plasmids. Viruses integrate their genome as stowaways in the quarry genome to be manifold. On the other hand, owing to the expression of antitoxin factors that are present in plasmids, victims forestall plasmid disposal [1]. In line with this premise, most archaea (~90%) and bacteria (~40%) utilize the CRISPR system to defend themselves against invasive elements [2]. Even mimivirus uses a similar defense system against virophage infection [3]. For the first time, the CRISPR system was identified in prokaryotes during phage infection experiments of the *Streptococcus thermophilus* sponsored by a yogurt company. In fact, before the CRISPR manifestation in basic sciences, dairies had been depending on CRISPR power to withstand viral predations to obviate food waste. Microbes habitually cope and thrive in the presence of different biotic and abiotic stressors, in which the CRISPR system plays a pivotal role [4]. A successful defense system in bacteria needs to be multilayered in order to deal with mutational evasion strategies of viruses that have been evolved to escape the acquired immune arm of the host. Although it was discovered in the 1980s that the CRISPR system consists of DNA repeat arrays that are conterminous with the alkaline phosphate (*iap*) gene, the CRISPR acronym was coined in 2002 [5]. CRISPR loci are composed of direct repeats that are separated by non-identical stretches of spacers with similar length, corresponding to segments of entranced invasive elements of viruses and plasmids [6]. The CRISPR array is preceded by a low-complexity, long, noncoding AT-rich leader sequence of about 500 base pairs, and likely contains a promoter for the transcription of the array into the pre-crRNA (CRISPR transcript). The transcript is subsequently processed into a spacer or mature CRISPR RNA (crRNA) flanked by two partial repeats or trans-

activating crRNA (tracrRNA) [2]. However, the size of the spacer and repeat varies from 24 to 72 bp and 24 to 47 bp, respectively. CRISPR RNAs with potentially strong secondary structures are encoded from some repeats with palindromic sequences. Each RNA cluster is neighbored predominantly by groups of conserved CRISPR-associated genes (*cas*). The set of Cas protein is composed of various groups of RAMPs (repeat-associated mysterious proteins) and core proteins; that is, Cas1−6 [7].

The sequence in a virus or plasmid fragment that is taken on as a spacer is called the protospacer [8]. Studies have shown that spacers are homologous to captured foreign nucleic acids. This idea has led to the assumption that the CRISPR system could be implied as an adaptive immune system to invasive elements (Fig. 1). In viral genomes, the existence of a conserved CRISPR motif or proto-spacer adjacent motif (PAM) downstream of the protospacer was revealed by comparative analysis. The question arises of why CRISPR-Cas systems do not show any autoimmunity after incorporating foreign nucleic acids as a spacer into the array. The answer is likely because of the absence of the PAM within CRISPR arrays, which permits the CRISPR-Cas system to specifically act upon the foreign nucleic acids [9]. It was revealed that Cas protein uses crRNAs as guides to be acquired on invading viruses and plasmids to interfere with invading nucleic acid proliferation in *E. coli*. To overview the steps that are engaged by adaptive immunity, three distinct stages were depicted in the following order:

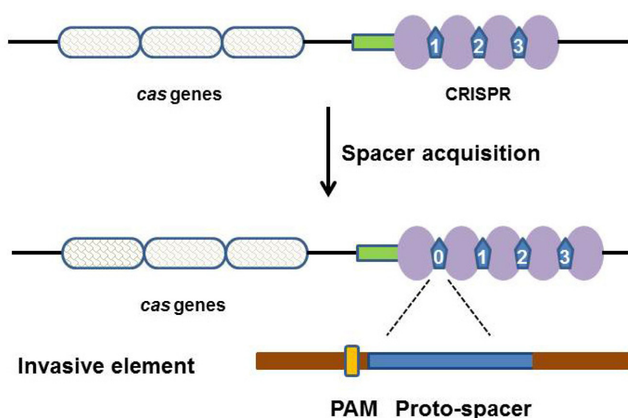

Fig. 1. Order of CRISPR/Cas genes, and the mechanism of spacer acquisition.
CRISPR array, including leader sequence (green rectangle), repeats (purple circles), and spacers (blue pentagons). The new spacer, which is numbered 0, always integrates at the 5′ site of the CRISPR array. The PAM site (yellow box) could be seated upstream or downstream of the protospacer (blue box) on the invasive element.

(i) adaptation: integrating a protospacer sequence of the invading mobile genetic elements as new spacers into the CRISPR array; (ii) expression: transcription and maturation of individual crRNAs that are associated with the Cas protein set; and (iii) interference: making the cut on foreign nucleic acids at sites complementary to the crRNA spacer sequence by Cas proteins to neutralize the invader [10]. The latest categorization for the CRISPR-Cas system based on the *cas* genes collection, the resemblance of the Cas proteins, and locus organization and content, presented two classes that are diverged into six types and 19 subtypes [11]. The simplest and most broadly used form of CRISPR-Cas types is type II, which has been classified by the Cas9 key signature protein, a complex protein with power to make the cut on foreign nucleic acids for degradation, as well as bring forth crRNA [2]. This property is well documented to be enthusiastically useful for genome engineering application.

Engineered RNA-Guided Nucleases

The simplest form of the CRISPR-Cas system, type II, has been widely used as a genome engineering tool. In the type II CRISPR system, crRNA teams up with tracrRNA (together called guide-RNA or single guide-RNA (sgRNA)), which is crucial for the processing of the CRISPR array RNA transcript by ribonuclease III and Cas9 [12]. The Cas9 protein is recruited to the genomic target complex with gRNA by using 20 nucleotides at the 5′-end that is called protospacer [10]. Two essential features play a pivotal role in the functionality of the gRNA: the 20 bp spacer at the 5′-end and a double-stranded conformation at the 3′-end, to introduce Watson-Crick base pairing at the DNA target site and to stick to Cas9, respectively [13]. In line with this invention, by changing 20 nt at the 5′-end of the gRNA, any genomic target with a PAM recognition site is endowed to be targeted [10]. The target site of the *Streptococcus pyogenes* Cas9 (SpCas9) must lie upstream or downstream of the PAM sequence that rivals the canonical form 5′-NGG and the substitute form 5′-NAG, albeit at a lower frequency [14]. The SpCas9 protein has two putative nuclease domains, RuvC-like and HNH, which make a cut in ~3 bp upstream of the PAM at gRNA non-complementary DNA strand and the complementary DNA strand, respectively [15]. For regulating a double-stranded break (DSB), Cas9 needs an initial PAM recognition site [13], an RNA-DNA hybrid [2], and a proofreading mechanism that serves as an ultimate specificity checkpoint [16]. Furthermore, Cas9 is able to bind single-stranded RNA (ssRNA) targets complimentary

to the Cas9-associated gRNA sequence. When the PAM is introduced in *trans* as a distinct DNA oligonucleotide (PAMmer), it provokes site-specific cleavage of ssRNA targets, avoiding any alteration on corresponding DNA sequences [17]. Most recently, a type III-B CRISPR-Cas system was elucidated that has the ability to record RNA memories. This system contains a reverse transcriptase (RT) enzyme fused to Cas1, a nuclease together with Cas2, which catalyzes new spacer acquisition. After integrating RNA into the CRISPR array, the RT domain commences cDNA synthesis from the inserted RNA as a template and uses the opposite strand of the target CRISPR DNA as a primer. The new discovered system makes the CRISPR to draw defense against RNA invaders as well [18]. Moreover, CRISPR-Cas effector C2c2, as a RNA-guided RNase in the class II type VI-A, can be recruited by a single crRNA on ssRNA targets. By programming C2c2, specific knockdown can be introduced at the mRNA level [19]. It was elucidated that the HNH nuclease domain of Cas9 protein undergoes a conformational change after recognition of on-target DNA. This structural transition sparks the catalytic activity of the RuvC domain for guaranteeing the DSB formation [16]. Nickase variants (Cas9n) or nuclease protein with single–stranded DNA (ssDNA) cleavage ability can be generated by introducing a specific mutation at each nuclease domain; that is, an aspartate-to-alanine (D10A) mutation and a histidine-to-alanine (H810A) in the RuvC-like domain and HNH domain, respectively (Fig. 2) [20]. Moreover, simultaneously mutating both domains ensures an RNA-guided DNA binding protein [21]. The enzymatically inactive Cas9 (known as dead Cas9 (dCas9)) can be used to regulate gene expression at the transcriptional level by hindering RNA polymerase at the binding site, and initiation and elongation steps. This process is called CRISPR interference (CRISPRi) [22]. By fusing transcription repressor domains, the effect of repression can be heightened. In order to upregulate gene expression, the transcription inducer domain has been fused to dCas9, which is called CRISPR activation (CRISPRa). The distance between the CRISPRa binding site and regulatory element

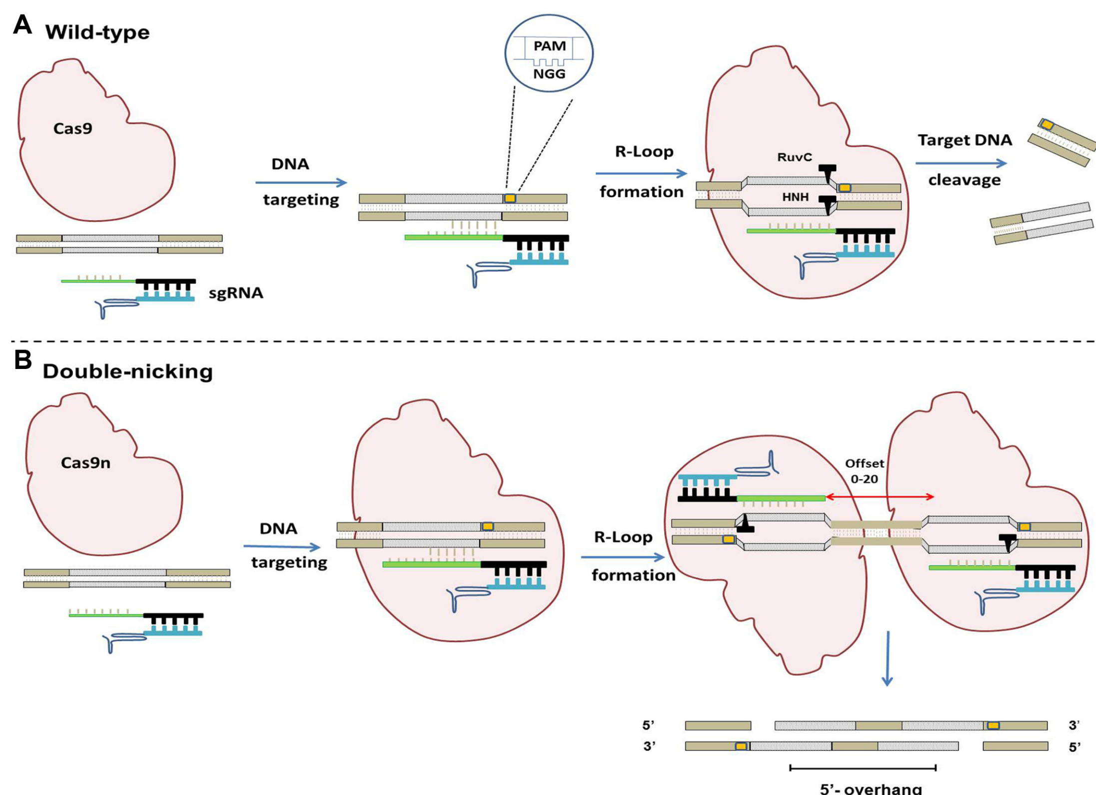

Fig. 2. Detailed schematic illustration of the CRISPR-Cas9-mediated DNA cleavage.
(**A**) Wild-type CRISPR-Cas9 DNA cleavage activity results in double-strand breaks. (**B**) A pair of sgRNAs guide Cas9n D10A nickase (Cas9n), which introduces a nick in the strand complementary to the sgRNA, on the target site. Double-nicking strategy by utilizing the sgRNAs in a specific orientation; PAM is distal from the cleaved spacer sequence, and sgRNA offset between 0 and 20 bp shows the highest efficiency to introduce 5′ overhangs. sgRNA = spacer 20 bp (green) + tracrRNA (black, light blue, dark blue).

Table 1. Pros and cons of the CRISPR-Cas9 system.

Issues	Pros	Cons
gRNA design	Target recognition is based on simple RNA:DNA base pairing; therefore, gRNA can be designed efficiently	The specificity requires a 2–5 nt PAM sequence immediately at 3′-site of non-complementary strand
Off-target	Designing gRNA based on powerful online software, controlling the expression of the components of the CRISPR system; availability of novel variants with high fidelity	Large genomes often contain highly homologous sequence to the target site
gRNA production	The artificial ribozyme-flanked gRNA (RGR) design allows efficient transcription of gRNA from any promoter and its subsequent cleavage [96]	Commonly used U3 and U6 snRNA promoters lack cell or tissue specificity, while RNA polymerase II promoters cannot be applied for gRNA expression in conventional design
Multiple on-target mutations	Applying useful strategies to induce HDR such as Cas9n [20], SpCas9-Gem [45], RS-1 [42], *etc*.	NHEJ dominates DNA repair during G1, S, and G2 phases [44]
Biallelic mutation	Strategies such as inducible CRISPR, inducible gene knock-out (KO), and conditional KO can induce generation of biallelic mutation	CRISPR-Cas9 may not necessarily cut the DNA at the one-cell stage of embryonic development

determines the level of induction [23]. The other putative type II CRISPR system, which was discovered in several bacterial genomes and provisionally assigned to type V, is embracing Cpf1 protein instead of Cas9 [24]. Three features were claimed for Cpf1-containing CRISPR: (i) no need for the presence of the tracrRNA during the production of mature crRNAs [25], (ii) recognizing a short T-rich PAM in contrast to the G-rich PAM for Cas9 protein, and (iii) generating a DSB with a 4 or 5 nt 5′ overhang. The Cpf1 protein contains a single nuclease domain, the RuvC-like domain. It has been shown that the depletion of this nuclease results in abolishing the ability of the enzyme to generate DSB. The cleavage mechanism underlying the endonuclease activity of the Cpf1 proteins has been elucidated [26]. Identification of Cpf1 as a novel CRISPR-Cas system has tremendously broadened the genome editing applications [27]. Notwithstanding the broad potential of the CRISPR-Cas system in manipulating genomes, multiple pros and cons ought to be considered before utilizing this system in translational medicine (Table 1).

Taming off-Target Mutagenesis

The range of applications of RNA-guided nucleases (RGNs) in genome engineering and their targeting preciseness have gathered significant curiosity. Several studies have shown that the CRISPR-Cas9 system is a promiscuous tool, because RGNs can tolerate up to five mismatches in the sgRNA sequence [28]. A number of strategies have been adopted to fine-tune components of the CRISPR-Cas9 system, such as sgRNA, PAM, and Cas9, which play a pivotal role in cutting the genomic target and hence eliminating off-target effects. The sgRNA sequence was segregated into two parts: seed sequence and nonseed sequence [15]. An approximately 10−12 bp sequence abutting the PAM and 3′-end of the crRNA is considered as the seed sequence, which has a great impact on Cas9 specificity as compared with the rest of the crRNA [2, 29]. It has been revealed that the effectual concentration of the Cas9-sgRNA complex is controlled by the seed sequence as well [30]. It has also been reported that only 10 bp adjacent to the PAM is adequate to intervene in the Cas9 binding [31]. Furthermore, mismatches at the distal part of the PAM could be better tolerated than those at the proximal part, and depending on the position of the mismatches along the sgRNA, single and double mismatches are tolerated [32]. In the same way, a low or high GC content in sgRNA seems to be less active. The GC content at the proximal region to the PAM site has a positive association with mutagenesis efficiency. It has been claimed that selection of the effective sgRNA with appropriate GC content at the proximal sequence to PAM has a heritable mutation rate of over 60% [33]. gRNA with only a 30% GC content has a high rate of mutagenesis at off-target sites, showing the thermodynamic stability of the Watson-Crick base pairing and regulating Cas9-sgRNA efficiency at on-target and off-target sites [34]. Choosing a sgRNA with favorable base preferences (*e.g.*, guanine as the first base nearby to the PAM, cytosine at the fifth position proximal to PAM, adenine in the middle of the gRNA, and cytosine not preferred at the eighteenth position) are critical for Cas9 activity in vivo and effective genome editing [30, 33]. Moreover, it was revealed that G-rich sequences are able to entwine into constant G-quadruples in vivo, which in turn furnishes to sgRNA

stability. The tail sequence of the tracrRNA and the target sequence are critical elements for Cas9 activity as well [35]. The exertion of sgRNA also depends on the sequence of the PAM. Although in the beginning, NGG (where N can be G, C, A, or T) was considered as the canonical sequence for the PAM, recent findings have propounded that NRG (R is A or G) can also be applied as PAM for the type II CRISPR system [32]. For unraveling and cleaving of the target site, Cas9 must bind to NGG PAM sites, preferably with high frequency to GGG sequence [31]. If the PAMs, NGG or NRG, do not exist in the target genome for the widely used CRISPR-Cas9 system, Spcas9, then the different PAMs of the Cas9 orthologs, *Staphylococcus aureus* Cas9 Sacas9 and *Streptococcus thermophilus* Cas9, can be applied [36]. Modified gRNAs have also shown promising evidence to increase CRISPR-Cas9 specificity and taming off-targets effects. The most common alterations on the sequence of the gRNA that is complimentary to genomic target are (i) shortening the sequence at the 5′ end of the gRNA up to 3 nt (truncated gRNA or tru-gRNA), (ii) truncating the 3′ end of sgRNA, which is derived from the tracrRNA scaffold that interacts with Cas9, and (iii) adding two guanine nucleotides to the 5′ end of the sgRNA (exactly upstream of the 20th nucleotide). Scores of published studies have revealed that using tru-gRNA has reduced the chance of undesired off-target mutagenesis approximately 5,000-fold, along with on-target activities [37]. Conversely, chemical modifications of sgRNAs have enhanced the effectiveness of genome editing in human CD34^{+} HSPCs and primary T cells to a greater extent. Chemically modified sgRNAs were synthesized by incorporating 2′-*O*-methyl 3′thioPACE, 2′-*O*-methyl 3′phosphorothioate, or 2′-*O*-methyl at 3-terminal nucleotides of both the 5′ and 3′ ends. Chemically altered sgRNAs furnish some advantages over expressed or in vitro transcribed sgRNAs, such as (i) higher effectiveness, (ii) elasticity in the sgRNA design, (iii) preparation of highly pure sgRNAs, and (iv) enabling of a highly active CRISPR platform with curtailed cytotoxicity in primary cells in contrast to DNA plasmid-based systems [38]. More common CRISPR-Cas9 design tools are listed in Table 2.

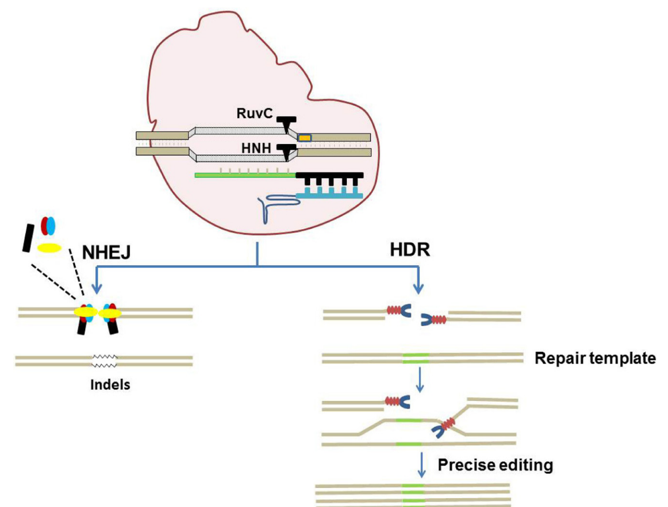

Fig. 3. Pathways for repairing double-strand breaks (DSB) introduced by CRISPR-Cas9.
Left: error-prone NHEJ pathway. Repair machinery processes the ends of DSB and rejoins them, which results in indel mutations. Indels in the coding region can cause frameshifting and gene knock-out. Right: homology direct repair pathway. Introducing repair template by plasmid or ssODN to DSB can force precise repair. Specific repair machinery by using repair template is able to leverage precise editing or even gene knock-in.

Subduing the concentration of the Cas9-sgRNA complex also is a critical step to restrict off-target activity. Decreased amount of the transfected DNA has strengthened the genome editing specificity and reduced on-target cleavage. Moreover, delivering RGN ribonucleoproteins (RNPs), because of having a shorter half-life, has increased the on-target mutations by 79% [39].

The DSBs induced by the CRISPR-Cas9 system were mended by applying homology directed repair (HDR) and non-homologous end joining (NHEJ) [40]. A controlled form of DNA repair leads to target of a specific site on the genome (Fig. 3). The fact that gene editing is more precise by using HDR, the pathway that can maximize the chance

Table 2. Common CRISPR-Cas9 design tools.

gRNA design tool	Website	Affiliation
CRISPR design	http://crispr.mit.edu/	Massachusetts Institute of Technology
ZiFiT	http://zifit.partners.org/ZiFiT/Disclaimer.aspx	Massachusetts General Hospital
COD	http://cas9.wicp.net/	Nanjing University
CHOPCHOP	https://chopchop.rc.fas.harvard.edu/	Harvard University
Cas-OFFinder	http://www.rgenome.net	Harvard Medical School
CCTop	http://crispr.cos.uni-heidelberg.de/index.html	Heidelberg University

of DNA repair, has drawn great attention. Since the error-prone NHEJ repair system competes with HDR to contend DSBs, inhibition of the NHEJ pathway will encourage the HDR pathway to rectify the DSB. Applying Scr7, the antagonist of DNA ligase IV, the principal enzyme in the NHEJ pathway, has enhanced the genome editing efficiency up to 19-fold [41]. RS-1, as a HDR enhancer, improves knock-in efficiency up to 5-fold at different genomic regions [42]. Coexpression of adenovirus E4orf6 and E1B55K proteins, which are suppressors of NHEJ key molecules, KU70 and KU80, has raised the efficiency of HDR up to 8-fold in mouse and human cell lines [43]. It has been reported that HDR efficiency is promoted in cell cycle synchronization in the late S and G2 phases, while NHEJ is the dominant repair pathway during the G1, S, and G2 phases [44]. Fusing human Geminin protein to SpCas9 (SpCas9-Gem) would encourage the degradation of the Cas9 protein during the G1 phase and would persist during the phases when HDR is active. Consequently, this variant can generate a cell line population free of introducing multiple alleles [45].

Eligible Cas9 variants are also believed to play a vital role in genome editing with higher specificity [46]. So far, seven variants of Cas9 have been introduced for genome editing approaches. Firstly, wild-type Cas9, which has two endonuclease active domains (*i.e.*, RuvC and HNH) for inducing DSB at target sites. Several previous reports elucidated that the wild-type Cas9 causes a high rate of off-target mutagenesis [47]. Secondly, Cas9n is paired with two distinct sgRNAs, each cleaving only one strand, thus making a DSB with 5′ overhang [48]. Two nicks are created by the double nickases strategy, one at the 5′ overhang, frequently preceding to the formation of indels, which are more than the one at the 3′ overhang [49]. The off-target activity was credibly reduced by 50−1,500-fold in cell lines by using the double nickases strategy, which paved the way for gene knock-out (KO) in mouse zygotes along with preserving on-target cleavage efficiency [50]. This versatile strategy boosted up the specificity of the CRISPR-Cas9 system, because unwanted nicks are generally recovered back by the high-fidelity base excision pathway [29]. Shen *et al*. [20] designed a high-quality paired sgRNAs framework, which was computationally foretelling the potential off-target sites for sgRNA pairs. Thirdly, fusing the FokI nuclease domain to dCas9 (RFN or dimerization-dependent RNA-guided FokI-dCas9) to increase the specifity. This variant (RFNs) has been shown to edit target DNA sites with >140-fold and >4-fold higher specificity than the wild-type Cas9 and paired nickases, respectively [51, 52]. Combination of tru-gRNA with RFN has reduced cleavage at off-target sites by 40% as compared with standard RFNs. This strategy improved the tool for genome editing applications to a greater degree with high precision in human cells [51]. In addition, the RFN efficiency was also used to generate KO mice via microinjection of dCas9 mRNA and sgRNAs into fertilized eggs. This experiment led to the development of a high efficiency animal model and decreased the risk of off-target effects [53]. Fourthly, the binding affinity of the SpCas9 protein, which is harboring an attenuated DNA-binding affinity, was improved by fusing a programmable DNA-binding domain (pDBD). Easily tuned-up specificity and affinity made this framework advantageous in making a flexible system and enabled it to precisely carry out genome editing at nearly any target sequence. However, a dichotomy between functional and inactive PAMs was rendered even for SpCas9-pDBD [54]. It is believed that Cas9 protein is tethered by pDBD and leads the effective concentration of the nuclease protein to act around target sites embodying a suboptimal PAM by rooting the Cas9-PAM interaction [13]. Fifthly, split-Cas9 is another variant of the wild-type Cas9 in which the bilobed architecture of the enzyme is split into two lobes, the catalytic nuclease lobe and α-helical lobe. Upon inclusion of the gRNA, the two lobes can be heterodimerized to regenerate an active enzyme complex, albeit at a reduced level relative to wild-type Cas9. It was elucidated that tru-gRNA is no longer able to congregate the split-Cas9 [55]. Hence, rather than gRNA, using peptide dimerization domains is more preferred to assemble the Cas9 lobes in return to small molecules or light [8, 56]. A split detaching of the C- and N-terminal Cas9 domains, conjugated to FK506 binding protein 12 (FKBP) and FKBP rapamycin binding (FRB) domain of the mammalian target of rapamycin (mTOR), was created that dimerizes upon the augmentation of rapamycin. In the absence of rapamycin, to eradicate background cleavage, the N-terminal part of the Cas9 was shuttled out into the cytoplasm with a nuclear export sequence that guarantees only the reconstituted complex would be brought back into the nucleus by supplying the C-terminal parts with two import signals [57]. The sixth feature is the enhanced specificity SpCas9 (eSpCas9) variant, in which cleavage activity at off-target sites was eliminated without decreasing on-target activity. In this system, mismatches along the sgRNA with target DNA would be less energetically auspicious by attenuating the helicase activity of SpCas9. By neutralizing positively charged residues such as alanine within a non-target strand groove, binding of the non-target strand would be dramatically undermined. In contrast, this

situation would strengthen re-hybridization between the non-target and target DNA strands. Therefore, more stringent Watson-Crick base pairing is needed between the target DNA strand and the gRNA [58]. The last introduced variant is high-fidelity SpCas9 (SpCas9-HF1) [59], harboring binding affinity alterations between the phosphate backbone of the target DNA strand and four SpCas9 residues (N497, R661, Q695, and Q926), which are framing direct hydrogen bonds [60, 61]. The energetics of the SpCas9-sgRNA complex is metamorphosed if one or more of these contacts is weakened. However, the retained binding energy is sufficient for robust on-target activity. GUIDE seq analysis and targeted next-generation sequencing revealed that there were no or just about no detectable off-target effects against the standard non-repetitive target sequences [62]. Utilizing tru-gRNA with SpCas9-HF1 has showed promising results in disrupting the targeted gene expression level [59]. Since no off-target mutations have been detected with SpCas9-HF1, this approach is especially applicable for atypical repetitive target sites [63].

Shuttling CRISPR System Via Optimized Strategies

The major leftover impediment in genome editing and its practical applications is the efficient delivery of a genome editing platform into a target. “There are only three problems in gene therapy: delivery, delivery and delivery” as enunciated by Dr. Inder Verma [64]. Choosing the proper gene delivery strategy is a crucial step in achieving a comprehensive genome editing inside the target cells or organisms. Both viral and non-viral methods have been used to deliver Cas9-sgRNA into targets. Shuttling the CRISPR platform into the target is performed either by introducing the plasmid encoding nuclease and gRNA or by exposing their mRNA and protein directly. The real disadvantage of delivering a Cas9-sgRNA construct through plasmid-based vectors is excess dosing of the Cas9 protein and sgRNA mRNA, which in turn will scale up the cutting rate at off-target sites [65]. Although delivering RNPs has shown greater control over the intracellular concentration and editing timeframe of Cas9-sgRNA, the fragile tertiary structure, large molecular size of most proteins, and strong negative charges of RNAs limited their diffusion across cell membranes. Furthermore, RNAs are susceptible to endonuclease, which leads to their degradation. Luckily, different chemical medications strategies have been introduced to overcome this limitation. To date, several chemical and physical methods such as lipofection, nucleofection, electroporation, and cell penetrating peptide have been employed for delivering Cas9-sgRNA constructs into cultured cells. Certain features, including high reproducibility, simplicity, and enhanced gene expression, make these methods reliable carriers in the in vitro experiment. Yet, pathological and physiological conditions as well as physical delivery methods have brought more challenges in the in vivo applications [66].

Four major classes of viral vectors (lentiviruses, retroviruses, adenoviruses, and adeno-associated virus (AAV)) have been extensively used as vehicles for the Cas9-sgRNA delivery platform. SpCas9 cDNA is approximately 4.2 kb long, which has resulted in low titer production of AAV, albeit using the smaller size SaCas9 (3.3 kb) abolished the limitation to a greater extent [67]. Although viral delivery systems have demonstrated promising results in gene transfer and expression, their widespread use has been limited due to a number of issues, including their ability to induce carcinogenesis, immunogenicity, and random integration into the genome, and remain to be clarified [68].

A noteworthy bottleneck in the application of non-viral delivery systems is their reduced delivery efficiency. In this line, emergent nano-size carriers with physiochemical properties or surface modification have branched out aptitudes in targeted delivery of small molecules to peculiar sites [66]. Cationic nanocarriers, positively charged lipids or polymers, are extensively used in gene delivery. Thanks to negative charges on nucleic acids, they can be loaded and abridged starkly by electrical interaction on cationic nanocarriers. Recently, a biologically inspired yarn-like DNA nanoclew, which was synthesized by rolling circle amplification and had partial complementary to the sgRNA, was devised. It was loaded with the Cas9-sgRNA complex to deliver it into the target cell. In order to encourage endosomal escape, the particle was coated with a cationic polymer, polyethylenimine. This modification fine-tuned the gene editing phenomenon by providing a balance between binding and release of the Cas9-sgRNA complex [69]. The efficiency of the intracellular delivery methods often depends on the structure of the target molecule and cell type. Transient membrane disruptions that permit diffusion of biomaterials into the cytosol can be generated by rapid mechanical deformation of the cells. Methods to deform and shear cells for delivery have achieved high cell viability and high delivery efficiency, with the advantage of high-throughput delivery of siRNAs and plasmids into almost any cell type [70]. Microfluidic membrane deformation functions as a broad-based universal delivery platform because of exhibiting the advantages of

precise control over treatment conditions. CRISPR-Cas9 complex delivery via microfluidic membranes has executed genome editing with high efficiency. Achievement of high genome editing efficiency in non-adherent lymphoma cells suggested that the approach has also the potential to be use in clinical settings [71]. Despite the great efforts for developing novel efficient methods to deliver the components of the CRISPR system into cell or animal models, the apt in vivo delivery of CRISPR-Cas encounters big hurdles due to properties such as its large size, inferior ability to penetrate through membrane, feeble stamina for serum, and poor endosomal break-out (reviewed in [72]).

Tantalizing Application of CRISPR-Cas System

The use of CRISPR-Cas9 for genome editing can tantalize into a number of important approaches, such as development of model systems, rectification of detrimental mutations [73], and antiviral therapies. Cell and animal models delineate useful preclinical systems for studying complex diseases such as brain tumors and testing new compounds [74]. In vitro and in vivo disease modeling by CRISPR-Cas9 has been reported for a number of diseases, including but not limited to modeling colorectal cancer in human intestinal organoids [75], generation of rabbit models of cardiac diseases [76], and development of rat models of Duchenne muscular dystrophy (DMD) [77]. Applications of this technology in disease modeling have been reviewed in depth previously [78, 79]. The CRISPR-Cas9 system, as a robust technology, has also been utilized for genome editing in specific tissues, simultaneous generation of multiple gene modifications [8, 80], inducible or conditional KO strategies [81], as well as flexible manipulation in epigenomes to control gene expression [82] or even convert fibroblasts to neural cells as a novel method to reprogram cell fate by manipulating the epigenome of the endogenous genes (BAM factors) [83]. Likewise, trait-associated common genetic variants frequently restricted to regulatory sites, identified by genome-wide association studies (GWAS) and chromatin immunoprecipitation sequencing (ChIP-seq), may be valuable targets for genome editing therapy. GWASs for Fetal hemoglobin (HbF) level introduced variations at the second intron of *bcl11a*, the product of which is demonstrated to negatively direct HbF expression [84−86]. By employing in situ saturating mutagenesis by a pooled CRISPR-Cas9 gRNA library, the critical features of the human and mouse developmental stage-specific, lineage-restricted BCL11A were mapped precisely. Consequently, BCL11A erythroid enhancer was certified as a particularly promising therapeutic target for HbF re-induction. The first case in point that CRISPR, delivered by AAV, had in triumph targeted a genetic disease was inside an adult mouse model of DMD, with a strategy that has the ability to be rendered into human therapy. In this line, exon 23 deletion by CRISPR-Cas9 set off restoration of functional dystrophin protein moderately in cardiac muscle and skeletal myofibers, expression of the modified dystrophin gene, a boost of muscle biochemistry, and momentous improvement of muscle force [87]. Recently, single nucleotide polymorphism (SNP)-derived PAM emerged its potential to be targeted by CRISPR-Cas9 allele-specific genome editing of heterozygous missense mutation, both in vitro and in vivo. The personalized therapeutics, by using the advantage of SNP-derived PAMs, exhibits insightful strategies for dominant congenital conditions [88]. In addition, CRISPR-Cas systems undoubtedly have a drastic impact on the future of virology research as well as the remedy of viral diseases. The diverse roles of CRISPR-Cas9 were considered in targeting and eradicating HIV provirus infection. Excision and deactivation of the HIV-1 proviral sequence advocated that latent virus, which is nested in reservoirs, will be successfully abstracted. In addition, genome editing by the CRISPR-Cas9 system elucidated valuable results for the study of the gene functions in the Vaccinia virus [89]. Cell-line engineering strategies have been successfully utilized for the enhancement of recombinant therapeutic proteins in Chinese hamster ovary (CHO) cells [90]. As one of the main drivers for RGNs, next-generation CHO cells can be generated by elucidating the mechanistic basis behind achieving high protein titers. Regardless of the CHO culture mode (adherent or suspension), transfection strategy, or target locus (*FUT8, LDHA,* or *MGAT1*), using a multiplex CRISPR-Cas9 platform resulted in high indel frequencies. Generation of improved reference genomes for CHO cells by the advent of genomic sequencing technologies, together with the cost-effective CRISPR-Cas9 system, has led to innovative engineering platforms (including KO, knock-in, or gene expression and repression) for CHO cells as the predominant mammalian cell factories [91]. The ultimate aspiration in genome editing by CRISPR-Cas9 is how to fix genetic errors in germ line cells and deliver those genetic fixes to the next generations. The first hastily done experiment of CRISPR-Cas9 applied to human pre-implantation embryos, targeting the gene responsible for β-thalassemia, ran into serious obstacles. As a result, several debates sparked to forward a prudent path toward human embryo editing. The CRISPR growth spurt by introducing Cas9 variants, such as SpCas9-HF1, and the detailed

mechanism of CRISPR system's function, have resulted in the announcement of utilizing this system to treat a rare form of blindness in human [92].

Summary and Perspectives

The electrifying evolution of CRISPR-Cas9 (a bacterial adaptive defense system) technology inspires us to scrutinize the frontiers of the natural world. The simplicity and efficiency of the CRISPR-Cas9 system as a biological toolbox ensures to manipulate almost any cell type and organism. New high-fidelity variants of Cas9 protein, rational selection of gRNAs from a diverse range of target sites, as well as introducing effective and safe delivery systems, such as nanoparticles and microfluidic membranes, open up cutting-edge strategies to cultivate CRISPR-Cas system efficiency. Furthermore, evaluating how to scale up HDR efficiency, in addition to chemically or genetically inactivating components of the NHEJ pathway, could improve RGN specificity [93]. A good grasp of the structural and biochemical levels of the CRISPR-Cas systems could conquer confounding effects of off-target mutations. Hence, enhancement of bioinformatics tools for blueprinting of gRNA target sites and further advancements to abolish off-target mutations come across as being pivotal. The ethical issues such as feeble knowledge of the mechanism and undesirable consequences on the ecological balance, which are fostered through the epoch of straightforward germline editing, are obligated to be addressed to warrant utmost merit while curtailing risks. Despite the huge potential of the CRISPR-Cas9 system for genome editing, employing the system to manipulate human germline cells has raised serious controversy among scientists [94]. Nevertheless, the association of vigorous read-out methods and CRISPR-Cas systems empowers us to puzzle out complex diseases [95], especially neurological disorders, in the near future.

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