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Review Article

Applications Utilizing CRISPR/Cas9

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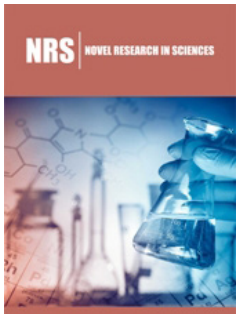

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Abstract

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Cas (CRISPR-associated) system is an adaptive immune system used by prokaryotes, which has been adapted for many laboratory applications. Here, we illustrate applications of CRISPR/Cas9 in 7 areas:

(i) Genome engineering

(ii) Edition of single-stranded RNA (ssRNA)

(iii) High throughput gene screening

(iv) Creating disease models

(v) Live labelling of chromosomal loci

(vi) Epigenome editing

(vii) Regulation of endogenous gene expression

Introduction

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Cas (CRISPR-associated) system is a novel technology originally discovered as a form of adaptive immunity in eubacteria and archaebacteria against intruding viruses and plasmids [1] by small RNA-based sequence-specific detection and silencing of foreign nucleic acids. CRISPR/Cas systems comprise cas genes organized in operon(s) and small DNA fragments of invading foreign DNA processed by Cas nuclease into spacers containing palindromic repeats (CRISPR array) [1,2]. These spacers act as transcriptional templates for producing guide RNA (gRNA), which guides Cas nuclease to identify and cleave DNA of foreign origins [2]. CRISPR/Cas systems are generally 3 types by their signature genes [3]; namely, Cas3 in type I systems, Cas9 in type II, and Cas10 in type III [4]. Specifically, the type II CRISPR/Cas9 system has been applied in various organisms; such as, microorganisms, fungi, plants, and animals. The Cas9 protein in type II system contains 1386 amino acids and encompasses a Recognition (REC) and Nuclease (NUC) lobe. The REC lobe mediates nucleic acid binding [5] while the NUC lobe contains a highly conserved RuvC nuclease domain that catalyzes the cleavage of a single strand complementary to the gRNA sequence, the target strand from foreign DNA [5-7]. In this review, we illustrate the application of CRISPR/Cas9 technology in 7 areas; namely, (i) genome engineering, (ii) editing single-stranded RNA (ssRNA), (iii) high throughput gene screening, (iv) biomedical modelling, (v) live labelling of chromosomal loci, (vi) epigenome editing, and (vii) regulating endogenous gene expression.

Genome engineering

Genetic modifications are facilitated through the generation of targeted Double Stranded Brakes (DSBs) in the genome [8] by synthesizing a single stranded gRNA to target the cleavage site [9]. A specific sequence of between 2 to 5 nucleotides, known as the Protospacer Adjacent Motif (PAM), must be present at the 3' end of the target DNA for a cut to be induced [9]. The exact sequence for PAM differs between different types of Cas9 nuclease [10,11]. To operate as a genome editing tool, Cas9 and gRNA ought to bind to a PAM sequence located at the 3' end of the target sequence [12]. The most frequently used Cas9 protein is obtained from *Streptococcus*

pyogenes, and it entails a PAM sequence of 5'-NGG-3'. Following the binding of Cas9 to the target, a DSB will be induced about three nucleotides upstream of the PAM. Hence, there is a need further work to expand the repertoire of PAM sequences [13]. The choice of instrument for the delivery of CRISPR components depends on the aim of the experiment and can vary from viral to non-viral vehicles [8]; including, DNA, mRNA, and Ribonucleoprotein Complexes (RNP). Viral vectors are promising vehicles and are considered a flexible tool with vast applicability mainly because their defined tropism can be retargeted though most tissues and cell types, and they can be administered locally or systemically.

a) Mammalian cells: Recent developments in genome engineering techniques have posed potential candidates for therapeutic applications [11], particularly for genetic disorders such as Sickle Cell Disease (SCD). SCD is an inheritable monogenic disorder brought about by the inheritance of two abnormal copies of the β-globin (HBB) gene, where at least one of which is the Hemoglobin S (HbS) variant, as a result of a single nucleotide mutation from adenine to thymine in the codon for the sixth amino acid in the β-globin gene. HbS is prone to polymerization under hypoxic or acidic conditions and aggregation, distorting red blood cells into rigid, sickle-shaped cells with shortened lifespans [11,14]. The presence of two HbS genes indicates the most severe case, sickle cell anemia. CRISPR/Cas9 gene editing approaches have displayed promising results in SCD preclinical studies [14]. These approaches include correction of the root mutation point of HBB in hematopoietic stem cells, induction of Fetal Hemoglobin (HbF) via gene-disruption of γ-globin repressors, and induction of HbF via introduction of advantageous hereditary persistence of HbF mutations on the β-globin locus.

Hoban et al. [15] demonstrated the sickle mutation correction in human CD34+ cells through CRISPR/Cas9 mediation where gRNAs were co-delivered with a homologous donor template containing 1.1kb of the human HBB gene compassing the cleavage site. Following that, CRISPR/Cas9 gRNAs demonstrated a 4.2 to 64.3% gene modification at the β-globin locus, resulting in the production of red blood cells containing normal hemoglobin proteins. HbS polymerization can be inhibited by HbF resulting in the alleviation of disease symptoms [16].

b) Plants: Production of plants with refined traits has been desired by the agricultural industry to enhance crop sustainability, yield, quality, nutritional value, and tolerance to biotic and abiotic stresses, among other commercially useful traits [17]. Hence, besides the applications of CRISPR/Cas9 as a genome-editing tool in mammalian cells, it has also been extensively applied in various plants species such as Arabidopsis, and crops, like rice, tobacco, wheat, etc. [12]. For example, to ensure co-segregation during the breeding process of major crop species, site-specific gene addition in major crop species can be used for 'trait stacking', whereby various coveted traits are physically linked [18]. Similar to genome engineering in other organisms, gene targeting in plants also involves coupling a foreign gene with a homologous sequence to act as a Homologous Recombination (HR) template. However, in plant systems, plant-specific RNA polymerase III promoters [AtU6 (Arabidopsis); TaU6 (wheat); OsU6 or OsU3 (rice)] are utilized to express Cas9 and gRNA [19]. For instance, Andersson et al. [20] demonstrated mutations in all four alleles in a single transfection in up to 2% of regenerated lines with the transient application of CRISPR/Cas9 mediated gene editing in protoplasts of tetraploid potato (*Solanum tuberosum*).

The transformation of plant cells to express guide RNAs and Cas9 following a process similar to these established for the generation of transgenic plants [17]. For example, *Agrobacterium tumefaciens* was utilized for the transportation of genes encoding Cas9, sgRNA, and a mutant GFP into Arabidopsis and tobacco [21]. The mutant GFP gene consisted of target sites in its 5' coding regions that were successfully cleaved by a Cas9/sgRNA complex. However, DSBs carry risks of genomic instability and unforeseeable outcomes of DNA repairment [12]. Hence, strategies to alter targeted DNA without the introduction of DSBs via a catalytically dead Cas9 (dCas9) variant have been explored. The dCas9 variant is created under the introduction of two silencing mutations into the RuvC and HNH domains of a Cas9 protein [22,23]. This dCas9 protein is fused with another effector protein that modifies the genome or epigenome by binding to target sequences without cleaving the double stranded DNA [12].

Editing single stranded RNA

Cas9 can also bind to targeted single-stranded RNAs (ssRNA) matching the Cas9-associated gRNA sequence when the PAM is present in trans as an isolated oligonucleotide [24]. Site specific endo nucleolytic cleavage of ssRNA targets can be stimulated by PAM-presenting oligonucleotides (PAMmers). The ability to target and edit RNA laid the foundation for the utilization of Cas9 as a tool for RNA investigation in cells [25]. This provides the potential of direct targeting of RNA viruses, such as West Nile and Dengue. Since most genetic alterations occur in the vast region of non-coding RNAs (ncRNAs), targeting the non-coding area with the CRISPR/Cas9 system provides a feasible approach toward cancer therapy [26]. There are various types of ncRNAs that modulate complex molecular and cellular processes besides protein coding RNAs [27]. Some of such ncRNAs include small nuclear RNAs (snRNAs), microRNA (miRNA), circular RNAs (circRNAs), etc. In general, ncRNAs have been identified as tumor suppressors and oncogenic drivers in various cancer types [26-28].

For example, Chang et al. [29] constructed CRISPR/Cas9 vectors containing the individual sgRNAs with complementary sequences to miR-17, miR-200c, and miR-141 genes respectively, and resulting in the levels of mature miR-17, miR-200c, and miR-141 declined up to 96% when compared to the control vectors. Furthermore, HT-29 cells with the miR-17 knockdown phenotype by CRISPR/Cas9 were injected into nude mice subcutaneously. Hence, demonstrating the generation of high efficiency downregulation of mature miRNA expression both in vivo and in vitro via the use of CRISPR/Cas9.

Similarly, Ho et al. [30] exhibited knockouts of miR-21, miR-29a, lncRNA-21A, UCA1 and AK023948 in diverse human cell lines, and Huo et al. [31] introduced indels into the precursor miRNA sequences using lentiviral CRISPR/Cas9 vectors.

High throughput gene screening

Genetic screening enables identification of gene functions [32]. Screening of primary tumor cells aids the investigation of patient-specific responses, processes involving tumor stem cells, cell differentiation, and interactions with the tumor microenvironment. Additionally, CRISPR/Cas9 screens have provided insights to the molecular basis of gene essentiality, drug and toxin resistance, hypoxia response, and the role of flavivirus host factors in infection [33]. In combination, functional gene screening using the CRISPR system can help in the investigation of drug-gene interactions by adding perturbations; thereby, identifying novel targets for precise treatment and provide insights for disease development.

High-throughput synthesis of sgRNAs facilitates the generation of sgRNA libraries, enabling in vitro and in vivo gene function screening, providing an accessible platform for interrogation of genes involved in disease processes [8]. For example, Wang et al. [34] used a genome-scale lentiviral sgRNA library as an approach for a pooled, loss-of-function genetic screening that is appropriate for positive and negative selection. They performed screens in two human cell lines and brought about knockout collections using a library consisting of 73,000 sgRNAs. Each sgRNA would serve as a distinct DNA barcode used to enumerate the number of cells carrying it using high-throughput screening.

Creating disease models

CRISPR can be used to create disease models to investigate underlying molecular mechanisms of pathogenesis [8] and serve as potential platforms screening novel drug candidates. Cancer occurs due to accumulation of mutations that grant the transformed cells certain biological hallmarks distinctive of malignant phenotypes [35]. CRISPR-based cancer models allow recreation of genetic aberrations identified in a patient's tumor in experimental models. For example, Ng et al. [36] employed the CRISPR/Cas9 system to rapidly model mutations in target genes in a Small Cell Lung Cancer (SCLC) mouse model and demonstrated that the p107 gene functions as a tumor suppressor in SCLC. The utility of their system provides a more comprehensive understanding of the genetic factors contributing to SCLC progression. Large effort has been committed to defining the molecular and pathophysiological attributes of cardiovascular diseases over the last few decades [37]. Transgenic animals are used in the creation of models for human diseases and validation of new drugs [38]. For instance, Sano et al. [39] demonstrated the use of the CRISPR/Cas9 system in biomedical modelling, specifically murine models. They validated that employing the CRISPR system along with a lentivirus vector provided evidence suggests that the inactivation of DNMT3A mutations in hematopoietic cells contributes to cardiovascular disease.

Live cell labelling of chromosomal loci

The intranuclear location and dynamics of genomic loci are essential parameters for understanding the spatiotemporal regulation of gene expression [40]. CRISPR has been applied in live-cell labelling of chromosomal loci to facilitate the visualization of chromosomal dynamics in living cells and to explicate fundamental intra-nuclear processes [8,41]. For example, CRISPR has been used in the detection of endogenous genomic loci in mouse embryonic cells without the cleavage of underlying sequences [42] by co-transfection of a gRNA expression vector and the fusion of dCas9 and an Enhanced Green Fluorescent Protein (EGFP). The Characteristic Chromocenters (CCs) of MaSgRNA/dCas9-EGFP expressing cells displayed a bright EGFP signal, indicating the successful targeting of genomic DNA. With 3D-fluorescent in situ hybridization (3D-FISH), specific labelling by gRNA/dCas9-EGFP complexes was demonstrated. The Cas9 protein from *Staphylococcus aureus* (SaCas9) can be engineered for imaging endogenous genomic loci [41] with comparable efficiency and durability is the Streptococcus pyogenes Cas9 (SpCas9).

The combination of SaCas9 and SpCas9 demonstrated two-color CRISPR imaging can resolve genomic loci spaced by <300 kb, and the color-coding of more than two loci for simultaneous tracking. Combinatorial color mixing further allowed coding of multiple genomic elements in the same cell. Coupled expression of dCas9 from *Streptococcus pyogenes* (dSpCas9) with a fluorescent protein and the corresponding sgRNAs allowed labelling and imaging of repetitive and non-repetitive DNA sequences. This CRISPR imaging approach also enabled the live cell examination of telomere length, chromosome copy number, and gene dynamics in interphase and mitosis. Chen et al. [43] used an EGFP-tagged dCas9 protein and a structurally optimized sgRNA to display robust imaging of non-repetitive genomic sequences, and repetitive elements in telomeres and coding genes in living cells. Furthermore, multiple sgRNA were tiled along the target sequence, allowing the visualization of non-repetitive genomic sequences. In another study, Ma et al. [40] reported the design of multicolored variations of CRISPR using dCas9 from three bacterial orthologs used to achieve multicolor detection of genomic loci with high spatial resolution, presenting an approach for barcoding elements of the human genome in the living state.

Epigenome editing

Epigenetics refers to inheritable changes of heritable information without perturbing DNA sequences [44]. Application of CRISPR/Cas9 in epigenome editing is a propitious technology to modulate gene expression to direct cell phenotype and analyze the epigenetic mechanisms of gene regulation [45]. The main strategy for epigenome editing with CRISPR/Cas9 is fusing dCas9 with a transcriptional repressor or activator domain, known as the epigenetic effector (epieffector) [44,46]. The Krüppel-Associated Box (KRAB) is the most frequently used effector for targeted gene silencing [45]. It is a naturally occurring transcriptional repression

domain involved in engaging a heterochromatin forming complex that mediates histone methylation [47]. The localization of dCas9-KRAB to a HS2 enhancer, a distal regulatory element that coordinates the expression of multiple globin genes, induced H3K9 tri-methylation (H3K9me3) at the enhancer and decreased chromatin accessibility of the enhancer and its promoter targets [45]. This targeted epigenetic alteration of HS2 repressed the expression of multiple globin genes with minimal off-target changes. In contrast, the activation of gene expression is possible via the fusion of a dCas9 to a Histone Acetyltransferase (HAT) enzyme that catalyses direct covalent modification of ε-lysine residues on histone tails [47,48]. In another study, Kang et al. [49] modulated methylation at specific CpG sites and induced gene expression through the utilization of the CRISPR/Cas9 knock-in and CRISPR/dCas9-Tet1 (ten-eleven translocation dioxygenase 1) systems by targeting the transcriptionally blocked murine Oct4 gene.

Regulating endogenous gene expression

Gene expression is a multistep procedure that entails harmonized control of transcription, translation, RNA processing, and protein and messenger RNA (mRNA) turnover [10,50]. Techniques that can accurately control gene expression contributes to cellular physiology understanding, which is essential for biomedical advancements [10]. CRISPR technology accomplishes streamlined and scalable disruption of gene expression for studying gene function, developmental pathways and disease mechanisms [8,51]. Particularly, the CRISPR interference (CRISPRi) system can efficiently repress the expression of targeted genes in *Escherichia coli* with no observable off-target effects and is able to be adapted for gene repression in mammalian cells [23]. Fusion of dCas9 with a repressor (CRISPRi) or activator domain (CRISPRa) can trigger gene silencing or activation [8]. A catalytically dead Cas9 (dCas9) lacking endonuclease activity generates a DNA recognition complex that can specifically interfere with transcriptional elongation, RNA polymerase binding, or transcription factor binding when expressed with a 20 Base Pair (bp) complementary region sgRNA [23]. This approach has been used to repress gene expression in various organisms and cell types, including plants and human induced Pluripotent Stem Cells (iPSCs) [8]. To produce a more efficient transcriptional interference, dCas9 was fused with the Krüppel-associated box (KRAB) [52].

The CRISPR system can be utilized as a modular and adaptable DNA-binding platform for recruiting proteins to a target DNA sequence [53]. The coupling of dCas9 to effector domains with definite regulatory functions enables efficient transcriptional repression or activation in human and yeast cells with the site of delivery determined by a co-expressed sgRNA. Furthermore, CRISPRi technology was used to efficiently repress genes to study early differentiation, and to model disease with iPSCs [52]. Contrastingly to CRISPRi, a transcriptional activator is one type of effector that dCas9 can be fused to for boosting gene expression [22]. The CRISPRa system is formed under the application of an sgRNA together with the fusion of dCas9 to a transcriptional activator [54]. For instance, Bikard et al. [55] achieved programmable transcription activation in *Escherichia coli* by directing the fusion of the omega subunit of RNA Polymerase (RNAP) and dCas9 to bind upstream promoter regions. Endogenous gene expression can also be manipulated using a light-activated CRISPR/Cas9 effector (LACE) system that induces transcription of endogenous genes in the presence of blue light [56] where light inducible heterodimerizing proteins CRY2 and CIB1 from Arabidopsis thaliana was fused with the VP64 transactivation domain and either the 5' or 3' end of dCas9. To exhibit the versatility of the system, multiple endogenous gene targets were activated by delivering the LACE system and a group of four gRNAs that target human promoters to HEK293T cells.

Conclusion

We illustrate the application of CRISPR/Cas9 technology in 7 areas: (i) genome engineering, (ii) editing single-stranded RNA (ssRNA), (iii) high throughput gene screening, (iv) creating disease models, (v) live labelling of chromosomal loci, (vi) epigenome editing, and (vii) regulating endogenous gene expression.

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