E-ISSN : 2181-4341
https://wordlyknowledge.uz
IMPACT FACTOR(SJIF) : 3,934

Gene Engineering News

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Annotation : Recent advances in genome engineering technologies based on the CRISPR-associated RNA-guided endonuclease Cas9 are enabling the systematic interrogation of mammalian genome function. Analogous to the search function in modern word processors, Cas9 can be guided to specific locations within complex genomes by a short RNA search string. Using this system, DNA sequences within the endogenous genome and their functional outputs are now easily edited or modulated in virtually any organism of choice. Cas9-mediated genetic perturbation is simple and scalable, empowering researchers to elucidate the functional organization of the genome at the systems level and establish causal linkages between genetic variations and biological phenotypes. In this Review, we describe the development and applications of Cas9 for a variety of research or translational applications while highlighting challenges as well as future directions. Derived from a remarkable microbial defense system, Cas9 is driving innovative applications from basic biology to biotechnology and medicine.

Key words: RNA , DNA , Cas9 .

The development of recombinant DNA technology in the 1970s marked the beginning of a new era for biology. For the first time, molecular biologists gained the ability to manipulate DNA molecules, making it possible to study genes and harness them to develop novel medicine and biotechnology. Recent advances in genome engineering technologies are sparking a new revolution in biological research. Rather than studying DNA taken out of the context of the genome, researchers can now directly edit or modulate the function of DNA sequences in their endogenous context in virtually any organism of choice, enabling them to elucidate the functional organization of the genome at the systems level, as well as identify causal genetic variations.

Broadly speaking, genome engineering refers to the process of making targeted modifications to the genome, its contexts (e.g., epigenetic marks), or its outputs (e.g., transcripts). The ability to do so easily and efficiently in eukaryotic and especially mammalian cells holds immense promise to transform basic science, biotechnology, and medicine

For life sciences research, technologies that can delete, insert, and modify the DNA sequences of cells or organisms enable dissecting the function of specific genes and regulatory elements. Multiplexed editing could further allow the interrogation of gene or protein networks at a larger scale. Similarly, manipulating transcriptional regulation or chromatin states at particular loci can reveal how genetic material is organized and utilized within a cell, illuminating relationships between the architecture of the genome and its functions. In biotechnology, precise manipulation of genetic building blocks and regulatory machinery also facilitates the reverse engineering or reconstruction of useful biological systems, for example, by enhancing biofuel production pathways in industrially relevant organisms or by creating infection-resistant crops. Additionally, genome engineering is stimulating a new generation of drug development processes and medical therapeutics. Perturbation of multiple genes simultaneously could model the additive effects that underlie complex polygenic disorders, leading to new drug targets, while genome editing could directly correct harmful mutations in the context of human gene therapy.

Eukaryotic genomes contain billions of DNA bases and are difficult to manipulate. One of the breakthroughs in genome manipulation has been the development of gene targeting by homologous recombination (HR), which integrates exogenous repair templates that contain sequence homology to the donor site.

HR-mediated targeting has facilitated the generation of knockin and knockout animal models via manipulation of germline competent stem cells, dramatically advancing many areas of biological research. However, although HR-mediated gene targeting produces highly precise alterations, the desired recombination events occur extremely infrequently (1 in 10^6–10^9 cells) presenting enormous challenges for large-scale applications of gene-targeting experiments.

To overcome these challenges, a series of programmable nuclease-based genome editing technologies have been developed in recent years, enabling targeted and efficient modification of a variety of eukaryotic and particularly mammalian species. Of the current generation of genome editing technologies, the most rapidly developing is the class of RNA-guided endonucleases known as Cas9 from the microbial adaptive immune system CRISPR (clustered regularly interspaced short palindromic repeats), which can be easily targeted to virtually any genomic location of choice by a short RNA guide. Here, we review the development and applications of the CRISPR-associated endonuclease Cas9 as a platform technology for achieving targeted perturbation of endogenous genomic elements and also discuss challenges and future avenues for innovation.

Programmable Nucleases as Tools for Efficient and Precise Genome Editing led to the realization that targeted DNA double-strand breaks (DSBs) could greatly stimulate genome editing through HR-mediated recombination events. Subsequently, Carroll and Chandrasegaran demonstrated the potential of designer nucleases based on zinc finger proteins for efficient, locus-specific HR Moreover, it was shown in the absence of an exogenous homology repair template that localized DSBs can induce insertions or deletion mutations (indels) via the error-prone nonhomologous end-joining (NHEJ) repair pathway .These early genome editing studies established DSB-induced HR and NHEJ as powerful pathways for the versatile and precise modification of eukaryotic genomes.

To achieve effective genome editing via introduction of site-specific DNA DSBs, four major classes of customizable DNA-binding proteins have been engineered so far: meganucleases derived from microbial mobile genetic elements , zinc finger (ZF) nucleases based on eukaryotic transcription factors transcription activator-like effectors (TALEs) from *Xanthomonas* bacteria and most recently the RNA-guided DNA endonuclease Cas9 from the type II bacterial adaptive immune system CRISPR.

Meganuclease, ZF, and TALE proteins all recognize specific DNA sequences through protein-DNA interactions. Although meganucleases integrate its nuclease and DNA-binding domains, ZF and TALE proteins consist of individual modules targeting 3 or 1 nucleotides (nt) of DNA, respectively . ZFs and TALEs can be assembled in desired combinations and attached to the nuclease domain of FokI to direct nucleolytic activity toward specific genomic loci. Each of these platforms, however, has unique limitations.

Meganucleases have not been widely adopted as a genome engineering platform due to lack of clear correspondence between meganuclease protein residues and their target DNA sequence specificity. ZF domains, on the other hand, exhibit context-dependent binding preference due to crosstalk between adjacent modules when assembled into a larger array.

Although multiple strategies have been developed to account for these limitations, assembly of functional ZFPs with the desired DNA binding specificity remains a major challenge that requires an extensive screening process. Similarly, although TALE DNA-binding monomers are for the most part modular, they can still suffer from context-dependent specificity and their repetitive sequences render construction of novel TALE arrays labor intensive and costly.

Given the challenges associated with engineering of modular DNA-binding proteins, new modes of recognition would significantly simplify the development of custom nucleases. The CRISPR nuclease Cas9 is targeted by a short guide RNA that recognizes the target DNA via

E-ISSN : 2181-4341
https://wordlyknowledge.uz
IMPACT FACTOR(SJIF) : 3,934

Watson-Crick base pairing. The guide sequence within these CRISPR RNAs typically corresponds to phage sequences, constituting the natural mechanism for CRISPR antiviral defense, but can be easily replaced by a sequence of interest to retarget the Cas9 nuclease. Multiplexed targeting by Cas9 can now be achieved at unprecedented scale by introducing a battery of short guide RNAs rather than a library of large, bulky proteins. The ease of Cas9 targeting, its high efficiency as a site-specific nuclease, and the possibility for highly multiplexed modifications have opened up a broad range of biological applications across basic research to biotechnology and medicine.

The utility of customizable DNA-binding domains extends far beyond genome editing with site-specific endonucleases. Fusing them to modular, sequence-agnostic functional effector domains allows flexible recruitment of desired perturbations, such as transcriptional activation, to a locus of interest.

In fact, any modular enzymatic component can, in principle, be substituted, allowing facile additions to the genome engineering toolbox. Integration of genome- and epigenome-modifying enzymes with inducible protein regulation further allows precise temporal control of dynamic processes CRISPR-Cas9: From Yogurt to Genome Editing

The recent development of the Cas9 endonuclease for genome editing draws upon more than a decade of basic research into understanding the biological function of the mysterious repetitive elements now known as CRISPR (Figure 3), which are found throughout the bacterial and archaeal diversity. CRISPR loci typically consist of a clustered set of CRISPR-associated (Cas) genes and the signature CRISPR array—a series of repeat sequences (direct repeats) interspaced by variable sequences (spacers) corresponding to sequences within foreign genetic elements Whereas Cas genes are translated into proteins, most CRISPR arrays are first transcribed as a single RNA before subsequent processing into shorter CRISPR RNAs (crRNAs), which direct the nucleolytic activity of certain Cas enzymes to degrade target nucleic acids.

The CRISPR story began in 1987. While studying the *iap* enzyme involved in isozyme conversion of alkaline phosphatase in *E. coli*, Nakata and colleagues reported a curious set of 29 nt repeats downstream of the *iap* gene.

). Unlike most repetitive elements, which typically take the form of tandem repeats like TALE repeat monomers, these 29 nt repeats were interspaced by five intervening 32 nt nonrepetitive sequences. Over the next 10 years, as more microbial genomes were sequenced, additional repeat elements were reported from genomes of different bacterial and archaeal strains. Mojica and colleagues eventually classified interspaced repeat sequences as a unique family of clustered repeat elements present in >40% of sequenced bacteria and 90% of archaea.

These early findings began to stimulate interest in such microbial repeat elements. By 2002, Jansen and Mojica coined the acronym CRISPR to unify the description of microbial genomic loci consisting of an interspaced repeat array.

At the same time, several clusters of signature CRISPR-associated (*cas*) genes were identified to be well conserved and typically adjacent to the repeat elements serving as a basis for the eventual classification of three different types of CRISPR systems (types I–III). Types I and III CRISPR loci contain multiple Cas proteins, now known to form complexes with crRNA (CASCADE complex for type I; Cmr or Csm RAMP complexes for type III) to facilitate the recognition and destruction of target nucleic acids . In contrast, the type II system has a significantly reduced number of Cas proteins. However, despite increasingly detailed mapping and annotation of CRISPR loci across many microbial species, their biological significance remained elusive.

A key turning point came in 2005, when systematic analysis of the spacer sequences separating the individual direct repeats suggested their extrachromosomal and phage-associated origins.

E-ISSN : 2181-4341
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This insight was tremendously exciting, especially given previous studies showing that CRISPR loci are transcribed and that viruses are unable to infect archaeal cells carrying spacers corresponding to their own genomes.

Together, these findings led to the speculation that CRISPR arrays serve as an immune memory and defense mechanism, and individual spacers facilitate defense against bacteriophage infection by exploiting Watson-Crick base-pairing between nucleic acids.

Despite these compelling realizations that CRISPR loci might be involved in microbial immunity, the specific mechanism of how the spacers act to mediate viral defense remained a challenging puzzle. Several hypotheses were raised, including thoughts that CRISPR spacers act as small RNA guides to degrade viral transcripts in a RNAi-like mechanism or that CRISPR spacers direct Cas enzymes to cleave viral DNA at spacer-matching regions.

Working with the dairy production bacterial strain *Streptococcus thermophilus* at the food ingredient company Danisco, Horvath and colleagues uncovered the first experimental evidence for the natural role of a type II CRISPR system as an adaptive immunity system, demonstrating a nucleic-acid-based immune system in which CRISPR spacers dictate target specificity while Cas enzymes control spacer acquisition and phage defense .

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