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Multidimensional Engineering of CRISPR Systems—Synergistic Breakthroughs in Tool Innovation, Delivery Optimization, and Precise Regulation

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Abstract. The CRISPR-Cas system has emerged as a revolutionary gene-editing technology, yet its clinical translation is constrained by off-target effects, suboptimal delivery efficiency, and limited adaptability across therapeutic contexts. This article presents a "multi-dimensional engineering" framework, systematically integrating the collaborative strategies of tool innovation, delivery optimization, and precise regulation to break through existing technical bottlenecks. In tool innovation, various regulatory tools have significantly improved editing accuracy and safety, reducing off-target activities. In the field of delivery systems, new universal delivery platforms have achieved the efficient and safe delivery of CRISPR components and enhanced tissue targeting. At the clinical translation level, CRISPR technology has been successfully applied in POC detection of infectious diseases, cancer immunotherapy, and the construction of genetic disease stem cell models. This article further explores the self-targeting repair mechanism of archaeal CRISPR systems to provide insights into precise regulation and proposes future directions to achieve a leap from the laboratory to the clinic.

Keywords: CRISPR-Cas, precise editing, delivery optimization, multi-dimensional engineering, clinical translation

1. Introduction

CRISPR-Cas technology has emerged as a pivotal gene editing tool, but its limitations include potential chromosomal abnormalities, immunogenicity, and in vivo delivery hurdles. Researchers are working on tool development, delivery optimization, and regulatory mechanisms to improve precision, safety, and generalization of CRISPR technology in complex clinical scenarios. In terms of tool innovation, base editing and Prime editing achieve single-base substitution and small fragment editing by avoiding DSB, significantly reducing off-target risks [1]. Concurrently, CRISPR-derived tools—such as Cas13-mediated RNA editing and epigenetic regulatory systems—expand their utility in transcriptomic and epigenetic manipulation [2]. In the delivery system field, non-viral vectors (such as the phase separation peptide HBpep-SP) achieve the universal delivery of pDNA, mRNA, and RNP through the liquid-liquid phase separation mechanism, with higher efficiency than commercial reagents while the optimization of viral vectors (such as AAV) solves the

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problem of large fragment delivery [3]. Regarding precise regulation, the anti-CRISPR protein AcrIF25 provides new ideas for designing gene editing switches by decomposing the Csy complex, and the self-targeting repair mechanism of the archaeal CRISPR system reveals the potential application value of gene compensation in symbiotic relationships [4, 5]. This article systematically reviews the collaborative innovation of CRISPR technology within a multidimensional engineering framework, spanning four key modules: tool development, delivery optimization, regulatory mechanisms, and clinical translation. The aim was to provide a cross-disciplinary integrated perspective for future research and accelerate the transformation of CRISPR from basic research to clinical application.

2. Tool innovation of CRISPR: precision and multi-functionality

2.1 Breakthrough of precision editing tools

The evolution of CRISPR tools has been pivotal in addressing off-target risks and expanding their functional versatility. Traditional Cas9-mediated double-strand break (DSB) editing systems are increasingly complemented by DSB-free technologies. Base editors and prime editors now enable single-nucleotide substitutions or small insertions/deletions with efficiencies ranging from 30-60%. Prime editing, guided by pegRNA templates, enable scarless editing. Epigenetic tools like CRISPRoff/on modulate DNA methylation and histone modifications for long-term gene regulation, particularly in cancer research. Cas13a's collateral RNA cleavage activity enables ultrasensitive detection of viral RNA, as demonstrated by the SHERLOCK platform, which achieves zeptomolar (10^-21 M) sensitivity for SARS-CoV-2 RNA [6]. Integrated with microfluidic technologies, such CRISPR-based point-of-care (POC) systems align with WHO ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid, Equipment-free, Deliverable), positioning them as essential tools for resource-constrained settings.

2.2 Specific optimization strategy

Off-target effects are a critical barrier to its clinical adoption. Hybrid RNA-DNA guides (chRDNA) introduce 2′-deoxyribonucleotides (dnts) to distort Cas9-DNA heteroduplexes, slowing cleavage kinetics and promoting off-target dissociation [7]. In primary T cells, chRDNA reduced off-target activity by 42-fold compared to traditional crRNA, outperforming high-fidelity Cas9 variants, such as HiFi-Cas9, at single-mismatch sites. SStructural analyses reveal that chRDNA induces conformational rearrangements in Cas9 REC2 and REC3 domains, sterically inhibiting HNH nuclease activation. This mechanism is particularly advantageous in editing immune cells, where unintended edits could trigger autoimmunity [8]. Parallel advancements include anti-CRISPR proteins (Acrs), such as AcrIF25, which disassemble the Csy surveillance complex in a non-enzymatic, ATP-independent manner. By binding Cas7 subunits, AcrIF25 destabilizes the CRISPR-Cas complex, offering a reversible "off-switch" for gene editing [4]. This innovation is critical for applications requiring temporal control, such as CAR-T cell therapy, where transient CRISPR activation minimizes genotoxic risks.

2.3 Tool adaptability expansion

CRISPR tools have improved their adaptability through improvements in PAM compatibility and multi-target editing. Traditional systems like SpCas9 are constrained by the NGG PAM sequence, but SpRY—a PAM-agnostic Cas9 variant—enables editing at nearly any genomic locus. This

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breakthrough has been transformative for epigenome editing and gene activation in non-coding regions, with preclinical studies demonstrating its therapeutic potential in glioblastoma models [9].

Cas12a (Cpf1) expands CRISPR versatility by processing multiple crRNAs from a single transcript, facilitating simultaneous editing of several genes. This feature is useful for modeling polygenic diseases like cancer, as it can generate triple-mutant colorectal cancer organoids, mimic tumor evolution, and test combination therapies targeting co-occurring mutations. Cas12a's staggered DNA cleavage patterns improve homology-directed repair efficiency and enhance the precision of large gene insertions. Beyond DNA editing, CRISPR tools are being integrated with emerging technologies, such as prime editing and base editing, to address complex genomic landscapes. For instance, combining SpRY with prime editors enables scarless corrections in PAM-free regions, whereas Cas12a's multiplexing capability synergizes with base editors to model allele-specific oncogenic variants. Challenges remain, such as optimizing delivery for large Cas12a complexes and minimizing off-target effects in multiguide systems [7]. Future advances may focus on AI-driven design tools for optimal crRNA configuration and hybrid systems merging PAM-agnostic editing with epigenetic modulation—further establishing CRISPR as a cornerstone of precision genome engineering.

3. Delivery system breakthrough: versatility and targeting

3.1 General delivery platform design

The HBpep-SP system, a redox-responsive peptide-based platform, exemplifies the progress in non-viral CRISPR delivery. Leveraging liquid-liquid phase separation (LLPS), HBpep-SP self-assembled into coacervates capable of encapsulating diverse CRISPR cargo, including plasmid DNA (pDNA), mRNA, and ribonucleoprotein (RNP) complexes. Remarkably, this system achieves encapsulation efficiencies of 66.8%, 73%, and 99.8% (Fig3C)for pDNA, mRNA, and RNPs, respectively, surpassing those of commercial reagents such as Lipofectamine. The coacervates remain extracellularly stable but disassemble intracellularly upon exposure to glutathione (GSH), which cleaves disulfide bonds in the peptide, triggering payload release. This mechanism minimizes cytotoxicity while ensuring efficient drug delivery. In HeLa cells, HBpep-SP achieved a 23.3% editing efficiency at the HPRT1 locus, outperforming electroporation-based methods. Its versatility extends to challenging cell types, such as primary T cells, where it maintains a high editing efficiency without compromising cell viability. The platform's universal compatibility with all CRISPR formats (pDNA, mRNA, RNP) eliminates the need for customized formulations, streamlining workflows for research and therapeutic applications [3].

3.2 Targeted delivery strategy

For in vivo delivery, adeno-associated viruses (AAVs) remain dominant owing to their natural tissue tropism and low immunogenicity. However, their packaging limit (~4.7 kb) necessitates the use of compact Cas9 variants (e.g., SaCas9) or split delivery systems. Innovations like split-intein technology enable delivery of larger constructs (e.g., base editors) by dividing Cas9 into reconstitutable fragments. For example, a dual-AAV split-intein system delivered a 5.2-kb base editor to mouse liver, achieving therapeutic gene correction [1]. Non-viral approaches, particularly lipid nanoparticles (LNPs), show promise for organ-specific delivery. LNPs encapsulating Cas9 mRNA have demonstrated exceptional liver-targeting efficiency in murine models of hereditary transthyretin amyloidosis, reducing mutant protein levels by 80%. Tissue-specificity is enhanced via

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surface modifications—galactose ligands for hepatocytes or neuron-targeting peptides for brain delivery [2]. The SLICE protocol, which uses electroporation and microfluidic devices, achieves over 90% editing efficiency in primary T-cells, generating PD-1 knockout CAR-T cells with enhanced antitumor activity. Microfluidic platforms enhance precision by enabling single-cell electroporation and combining viral and nonviral advantages. For instance, virus-like particles (VLPs) loaded with CRISPR RNPs exploit viral capsids for cell entry but avoid genomic integration risks. Similarly, biomimetic nanoparticles coated with cell membranes derived from target tissues improve homing and immune evasion [5].

4. Precise regulation mechanism: dynamic control and self-repair

4.1 Anti-CRISPR Proteins as Molecular Switches

The discovery of anti-CRISPR (Acr) proteins has revolutionized spatiotemporal control of CRISPR-Cas systems, with AcrIF25 standing out for its unique mechanism. Unlike traditional Acrs that sterically block DNA binding or enzymatic activity, AcrIF25 dismantles the Type I-F Csy surveillance complex through non-enzymatic, stepwise disassembly. Structural analyses show AcrIF25 binds Cas7 subunits, disrupting inter-Cas7 interactions and crRNA scaffold stability. This "peeling-off" mechanism destabilizes the entire Csy complex, leading to crRNA degradation and irreversible inactivation of CRISPR-mediated immunity. In Pseudomonas aeruginosa, the introduction of AcrIF25 completely abolished CRISPR-based phage resistance, demonstrating its potent inhibitory capacity [10].

AcrIF25's modularity has enabled innovative synthetic biology applications. Engineered Acr-Cas systems combining AcrIF25 with light-inducible Cas9 (e.g., LightCas9) achieve optogenetic gene editing control. In neuronal circuits, this hybrid system reduces off-target effects by 95% compared to conventional Cas9, enabling precise synaptic plasticity manipulation without collateral damage. Furthermore, Acr proteins are being explored as tools for modulating the microbiome dynamics. By transiently suppressing CRISPR activity in specific bacterial populations, AcrIF25 can facilitate the introduction of beneficial traits (e.g., antibiotic resistance or metabolic pathways) into complex microbial communities, offering new strategies for microbiome engineering.

However, several challenges remain. Although AcrIF25 and archaeal symbiotic mechanisms show potential, their clinical translation faces common challenges: in vivo delivery efficiency of Acr proteins, fitness of archaeal self-targeting in higher organisms, and immune responses triggered by exogenous components. Future optimization of delivery systems in conjunction with synthetic biology and development of endogenous repair modules to enhance safety are needed [4]. At the interface of Acr regulatory mechanisms and archaeal CRISPR self-evolution lies a fundamental question: how to achieve reversibility and safety in gene editing through dynamic molecular-level control. The "molecular switch" established by AcrIF25 through exogenous protein intervention demonstrates the capability of artificial design tools to precisely regulate CRISPR systems; meanwhile, the endogenous gene compensation mechanism triggered by archaeal self-targeting reveals the innate wisdom of life systems in achieving self-repair through CRISPR-mediated genetic information remodeling.

4.2 Archaeal self-targeting and symbiotic repair

The CRISPR system, traditionally understood as a defense mechanism, has revealed an unexpected function through studies of archaeal symbioses: self-targeting activity can drive metabolic

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interdependence. In the deep subsurface symbiosis between "Candidatus Altiarchaeum" (host) and "Candidatus Huberiarchaeum" (epibiont), the host's CRISPR system actively targets its own genome, deleting metabolic genes such as those involved in amino acid biosynthesis. Paradoxically, this self-destructive behavior enhances survival. Macrogenomic analyses indicate that it is due to host self-deletion of genes that symbiotic bacteria provide homologous genes through horizontal gene transfer to form metabolically complementary symbiotic dependencies. CRISPR-mediated "genetic outsourcing" stabilizes the partnership, as both organisms rely on each other for metabolic completeness. This discovery inspires a "safety net" strategy for eukaryotic gene editing. Mimicking archaeal symbiosis, researchers propose designing CRISPR edits to delete non-essential genes, paired with engineered backup systems (e.g., episomal vectors, synthetic circuits) for compensatory function. In cancer therapy, this could involve CRISPR targets oncogenes, such as MYC or KRAS, with backup systems expressing conditionally stabilized versions of these genes in healthy tissues to prevent unintended toxicity. Similarly, in gene therapy for monogenic disorders, CRISPR edits can be coupled with fail-safe mechanisms to rescue unintended large deletions or chromosomal rearrangements.

Translating this concept faces hurdles: low self-targeting efficiency in human cells, the need for precise gene expression control in backup systems, and immune responses to exogenous components (e.g., bacterial Cas9). Unlike archaea, human chromatin—particularly heterochromatic regions—may impede CRISPR targeting, while DNA repair mechanisms (predominantly error-prone NHEJ) limit editing fidelity. Future research could explore endogenous repair pathways or CRISPR-activated host genes as safer alternatives [5].

5. Conclusion

The multidimensional engineering framework enhances CRISPR's clinical translation by synergizing tool innovation, delivery optimization, and precise regulation. The introduction of base/prime editing, chRDNA-guided strands, and anti-CRISPR proteins addresses the core issues of editing accuracy and safety, and the optimization of phase-separated peptide delivery systems and viral/non-viral vectors breaks through bottlenecks in delivery efficiency and targeting. CRISPR technology has been successfully applied for infectious disease detection, cancer immunotherapy, and genetic disease model construction, demonstrating its integrated advantages across disciplines.The literature is insufficient, with only 10 references. Data support and case studies are lacking. Moreover, the limited scope of current validation methods and insufficient clinical sample analyses weaken the generalizability of conclusions. Thus, future research must prioritize establishing standardized multi-omics platforms integrating epigenomic profiling with long-read sequencing, alongside expanding multicenter clinical cohorts to systematically address efficacy and safety. Key challenges remain: standardizing off-target detection via single-cell sequencing integration, optimizing delivery system immunogenicity through low-antigenicity Cas variant development, and enhancing complex target editing efficiency with AI-assisted design tools. Additionally, insights from archaeal CRISPR self-repair mechanisms and Acr protein dynamic control strategies inform the development of next-generation controllable editing systems. Through multidimensional innovation, CRISPR technology promises to revolutionize laboratory discoveries into clinical precision medicine, benefiting global patient populations.

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