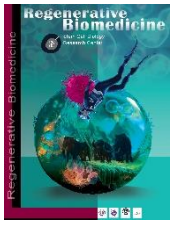

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CRISPR-dCas9 Tools for Precision DNA Methylation Editing in Regenerative Therapies

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Abstract

DNA methylation serves as a crucial epigenetic modification, influencing gene expression and determining stem cell fate along with its differentiation capabilities. Through the precise modulation of methylation patterns, it is possible to regulate cellular identity and differentiation potential, leading to notable advancements in cancer management, regenerative therapies, and personalized medicine. To this end, the emergence of the CRISPR-dCas9 system has facilitated accurate modifications of the epigenome. These tools signify notable progress in the precise and focused modification of the methylome, all the while preserving the integrity of the original genetic sequence. By leveraging this technology, the combination of nuclease-deactivated Cas9 (dCas9) with methylation-modifying enzymes like DNMT3A or TET1 enables targeted hypermethylation or demethylation at specific genomic loci. This specific epigenetic manipulation offers new avenues for disease modeling, cellular reprogramming, and the development of cutting-edge regenerative therapies. Notable applications include the activation of lineage-specific genes to guide stem cell differentiation and the correction of epigenetic abnormalities in disease models, including Rett syndrome and hematological malignancies. While the potential is significant, obstacles persist, especially concerning unintended effects, the efficiency of editing, and the in vivo delivery of these large complexes. Continuous improvements in sgRNA design, delivery mechanisms, and effector engineering are tackling these challenges. Ultimately, with the ongoing advancements in technology, CRISPR-dCas9-based DNA methylation editing presents significant possibilities for the fields of personalized and regenerative medicine.

Keywords: CRISPR-dCas9, DNA methylation, Epigenetic memory, Epigenome editing, Regenerative medicine

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Introduction

DNA methylation, an essential epigenetic modification, governs gene expression and is fundamental in regenerative medicine by influencing stem cell differentiation and tissue regeneration (1). Altered methylation patterns are associated with a variety of diseases, highlighting the need for accurate tools to manage these changes (2). CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) functions as a powerful instrument for precise genome editing through cellular repair processes. The capacity to precisely split DNA has transformed genetic engineering, enhancing the efficiency and programmability of gene knockouts and targeted modifications. This system introduces double-stranded breaks at specific DNA sequences utilizing a Cas endonuclease navigated by a single-guide RNA (sgRNA) (3). However, in the context of regenerative therapies and somatic cell reprogramming, the concern is more about erasing epigenetic memory to fully reprogram cellular identity (4). One variant of this system that has been designed for broader uses is CRISPR-dCas9. This variant is a catalytically inactivated version of Cas9 that retains precise site-specific binding but loses its ability to cut DNA (by mutations like D10A and H840A) (3,5). This characteristic positions dCas9 as an excellent tool for the recruitment of epigenetic effectors, facilitating precise modulation of gene expression while preserving the integrity of the DNA sequence (6) (Fig. 1).

The application of dCas9 alongside methylation-modifying enzymes enables the precise targeting of specific genomic regions. This configuration allows for alterations to the methylome while preserving the integrity of the DNA sequence. This leads to the emergence of novel possibilities for regenerative therapies (7,8). Specifically, studies have demonstrated that the fusion of dCas9 with enzymes such as ten-eleven translocation 1 (TET1) or DNA methyltransferase 3A (DNMT3A) enables targeted DNA methylation editing, thereby impacting gene expression and cellular states (9,10). For example, the specific demethylation of the *MyoD* distal enhancer has been demonstrated to enhance the reprogramming of fibroblasts into myoblasts, thereby aiding in myotube formation, which is significant for tissue repair and cellular reprogramming (11).

CRISPR-dCas9 for DNA methylation editing

The advancements in CRISPR-based technologies have drastically altered the landscape of genome and epigenome editing (12). Research has demonstrated that employing dCas9 alongside the DNMT3A-DNMT3L single-chain effector domain facilitates precise methylation while preserving the original DNA sequence's integrity. This method, which uses the accuracy of the guide RNA system of CRISPR to enable exact epigenetic modifications, significantly reduces unwanted side effects when compared to conventional techniques (13). Unlike active Cas9, which breaks DNA, dCas9 is a DNA-binding platform under a sgRNA direction. This enables reversible epigenetic changes, which are crucial in dynamic cellular processes like regeneration (14). The combination of dCas9 with enzymes like DNMT3A allows for the addition of methyl groups, whereas the inclusion of

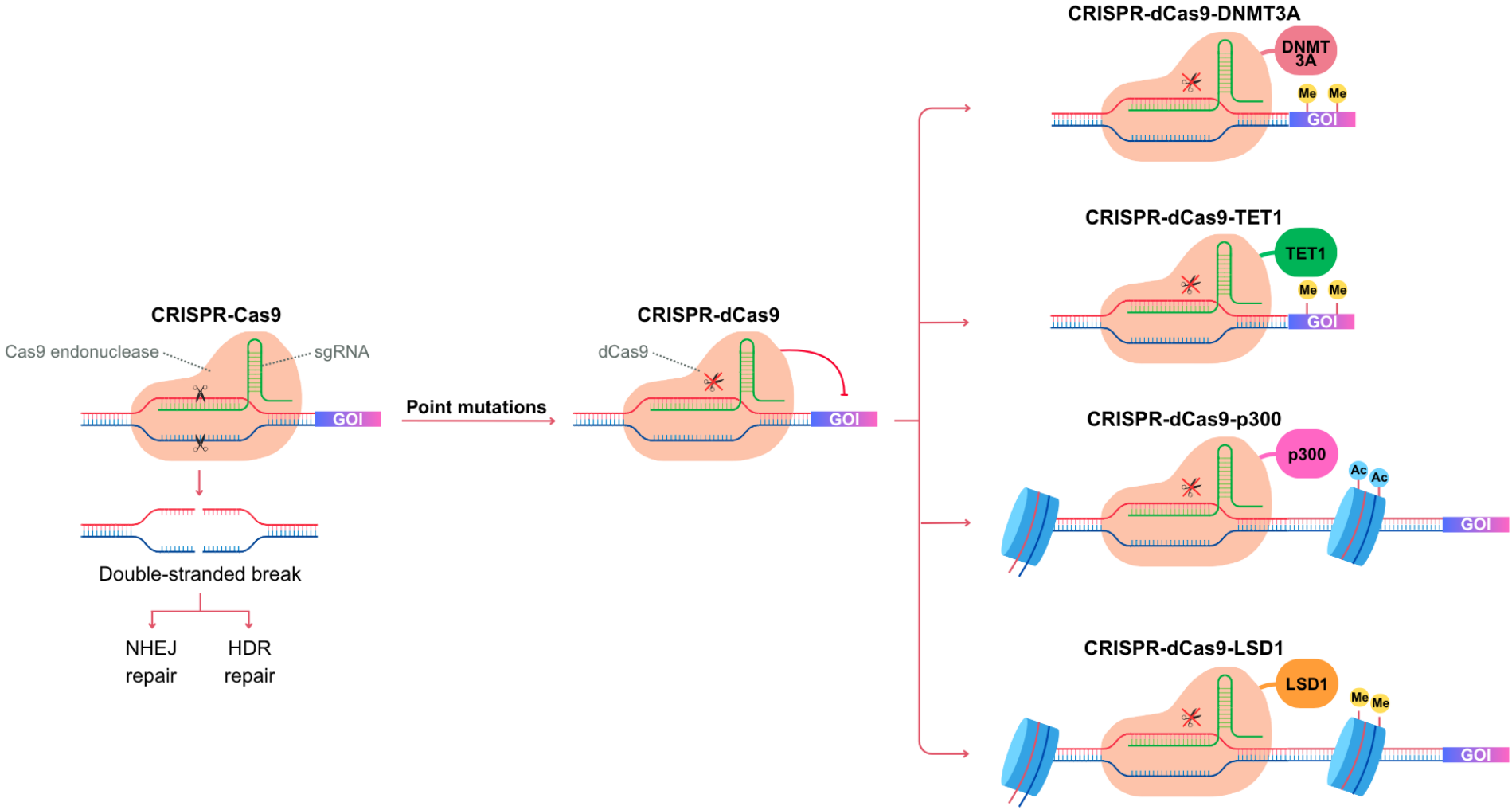

Figure 1. Schematic comparison of CRISPR-Cas9 genome editing and CRISPR-dCas9-mediated epigenetic modulation systems. The conventional CRISPR-Cas9 system (left panel) introduces double-stranded DNA breaks at designated sites using a Cas9 endonuclease guided by a sgRNA, thereby enabling targeted genome editing. The following repair mechanisms, such as non-homologous end joining (NHEJ) or homology-directed repair (HDR), lead to either gene disruptions or accurate sequence alterations. Through two point mutations (D10A and H840A), the CRISPR-dCas9 system (middle panel) employs a catalytically inactivated Cas9 variant (dCas9), eliminating its endonuclease activity while preserving DNA-binding capacity. This allows dCas9 to serve as a programmable platform for recruiting epigenetic effectors without altering the DNA sequence. The arrow from dCas9 in the center panel represents the steric hindrance of RNA polymerase II, leading to transcriptional repression. Fusion constructs such as dCas9-DNMT3A (adds DNA methylation, depicted as yellow "Me" circles), dCas9-TET1 (removes methylation), dCas9-p300 (induces histone acetylation, shown as light blue "Ac" circles), and dCas9-LSD1 (catalyzes histone demethylation) enable locus-specific gene regulation (right panel). These tools offer a non-permanent, mutation-free approach to modulate gene expression and are particularly valuable in regenerative therapies, where erasing epigenetic memory and reprogramming cellular identity are critical. GOI, gene of interest.

Ten-Eleven Translocation (TET) catalytic domains permit the removal of methyl groups. This approach creates a robust mechanism for precise methylation editing at designated loci (8,15). For instance, dCas9-DNMT3A has been employed to inhibit tumor suppressor genes in cancer models, showcasing its accuracy (16,17). In this context, Vojta et al. investigated the targeted methylation of the *IL6ST* promoter in human cells, resulting in notable increases in methylation and a decrease in gene expression. Their study emphasized the tool's capacity to influence immune-related pathways pertinent to regenerative scenarios (13). Indeed, Liu and colleagues demonstrated that targeted demethylation of the *BDNF* promoter IV or the *MyoD* distal enhancer using dCas9-TET1 resulted in increased BDNF expression in post-mitotic neurons or activation of *MyoD* respectively, thereby promoting the reprogramming of fibroblasts into myoblasts. The findings highlight the adaptability of dCas9-based tools in effectively inducing both hyper- and hypomethylation with remarkable specificity (11).

CRISPR-dCas9 applications in egenerative medicine

The capability to accurately modify DNA methylation through CRISPR-dCas9 offers considerable potential for progress in regenerative medicine (18). A crucial application entails directing the differentiation of stem cells. By modulating the methylation status of genes involved in differentiation pathways, it is possible to precisely control cell fate decisions (19). For instance, it has been demonstrated that a human pluripotent stem cell line designed to express an inducible dCas9-SAM activator (H9-iCas9.SAM) can precisely activate endogenous genes, enabling directed differentiation into lineages including neural stem cells and so providing a potential platform for regenerative therapies (20). Furthermore, CRISPR-dCas9 tools enhance disease modeling by replicating epigenetic changes associated with conditions such as cancer and neurological disorders (21). For instance, using dCas9-TET1, Qian and colleagues demethylated the hypermethylated MECP2 promoter and regulatory regions in human embryonic stem cells (hESCs) and generated neurons from Rett syndrome patients. The dormant X chromosome strongly reactivated *MECP2* expression. This focused demethylation dramatically restored *MECP2* expression levels by reversing Rett syndrome-associated electrophysiological abnormalities and reduced neuronal soma size (8,22). Similarly, targeted hypermethylation of the *p15* (*CDKN2B*) promoter in human hematopoietic stem and progenitor cells (HSPCs) using CRISPR/dCas9 demonstrated stable, heritable changes that altered blood cell development. This approach linked epigenetic editing to functional changes in hematopoiesis, highlighting its relevance in modeling age-related and malignant blood disorders. These findings show that CRISPR/dCas9-mediated epigenetic editing could be a useful way to treat epigenetic abnormalities in hematological malignancies like acute myeloid leukemia. The results could have implications for clinical applications such as hematopoietic regeneration and disease modeling (23).

Challenges and future directions

Although they hold great potential, CRISPR-dCas9 tools aimed at DNA methylation editing encounter numerous obstacles. Unintended methylation changes pose a significant concern, as they have the potential to disrupt gene regulation. Recent developments in sgRNA design and specificity assays, including high-throughput sequencing, have successfully minimized off-target methylation in optimized systems, as demonstrated by Stepper et al. (15). A further challenge lies in attaining stable and efficient methylation alterations, given that the degree and durability of edits fluctuate depending on cell type and genomic context. Enhancing dCas9-effector constructs, including the implementation of SunTag systems to boost effector recruitment, has led to a remarkable increase in methylation efficiency by as much as 90%, as shown in the work of Huang et al. (24). The delivery methods for CRISPR-Cas9 tools present considerable challenges for *in vivo* applications, underscoring the necessity for safe and targeted carriers (25). An optimized protocol utilizing lipid nanoparticles for the delivery of CRISPR/dCas9-based epigenetic editors in the form of mRNA and guide RNAs facilitates efficient, nonviral in vivo targeting of solid tumors. This approach allows for precise gene regulation without DNA cleavage, effectively addressing the delivery challenges linked to large dCas9 fusion proteins (26). Anticipating future developments, the combination of CRISPR-dCas9 with approaches such as single-cell epigenomics has the potential to improve its accuracy and broaden its applications in regenerative medicine (27).

Regarding demethylation, systems such as glycosylase-based tools (e.g., ROS1CD) and dCas9-R2 show interesting substitutes for TET enzymes (28–30). Nonetheless, particularly for prolonged and *in vivo* applications, their effectiveness, specificity, and potential cytotoxic effects warrant further investigation. The early-stage development of glycosylase-based strategies indicates that further optimization is necessary for clinical viability. Moreover, although CRISPRa systems have effectively induced pluripotency and guided differentiation (31,32), their incorporation into regenerative workflows requires more dependable delivery methods and enhanced control over activation timing. Future efforts should prioritize the creation of inducible, reversible, and multiplexed editing platforms to facilitate dynamic epigenetic remodeling suited to therapeutic applications. Ultimately, enhancing delivery systems, refining editing accuracy, and confirming long-term impacts in preclinical models will be essential steps in moving these innovative tools from the laboratory to clinical application.

Conclusion

The utilization of CRISPR-dCas9 tools for precise DNA methylation editing signifies a significant advancement in the field of regenerative medicine. These tools provide new avenues for stem cell engineering, disease modeling, and therapeutic development through the facilitation of targeted epigenetic modifications. It is crucial to tackle current challenges by improving specificity, efficiency, and delivery to fully unlock their potential. As investigations progress, CRISPR-dCas9-based epigenetic editing presents the opportunity to revolutionize regenerative therapies, paving the way for personalized medical strategies and improved patient outcomes.

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