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Commentary

Boosting CRISPR-Cas9 efficiency through enhanced homologous recombination

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The clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 system is a versatile and precise tool for genome editing, applicable across a broad range of species due to its ability to target specific DNA sequences with remarkable accuracy.[1] The underlying mechanism involves a guide RNA (gRNA) that directs the Cas9 nuclease to a predetermined genomic locus, where it induces double-strand breaks (DSBs) in the DNA duplex.[2] These DSBs are subsequently repaired via two major DNA repair pathways, predominantly non-homologous end joining (NHEJ) or homologous recombination (HR). The choice between these pathways is primarily determined by the cell cycle phase and the presence of key proteins that facilitate either HR- or NHEJ-mediated DSB repair.[3] NHEJ, a template-independent repair mechanism, directly ligates the broken DNA ends but is inherently error prone, often resulting in unintended deletion mutations at the site of repair.[4,5] Consequently, NHEJ is more suitable for gene knockout experiments than precise gene knockin applications across various eukaryotic systems. In contrast, the HR pathway, which relies on homologous chromatids as templates, allows for high-fidelity genome editing.[5,6] However, HR is a more complex process, involving multiple steps, including Rad51-mediated homology search and strand invasion, which are crucial for accurate gene insertion and deletion. Despite the potential for precise editing, many cell types preferentially utilize the faster but less accurate NHEJ pathway, leading to a higher incidence of mutations and suboptimal editing outcomes.

Cas9 recognizes a specific protospacer-adjacent motif sequence proximal to the target site, facilitating the separation of duplex DNA and its hybridization with the gRNA to form an RNA-DNA hybrid known as an R-loop.[2] R-loops, which consist of an RNA-DNA hybrid duplex and a displaced single-stranded DNA, are unstable three-stranded nucleic acid structures that significantly influence genome stability and the regulation of gene expression.[2] The formation of the R-loop activates Cas9's nuclease domains, resulting in DSBs within the duplex DNA. Despite the significant advantages of Cas9-mediated genome editing, several technical challenges persist, including inefficiencies due to premature Cas9 dissociation from the R-loop prior to DSB formation and off-target effects that lead to unintended editing at non-specific genomic loci.

In a recent study published in *Molecular Therapy Nucleic Acids*, Park et al. explored strategies to enhance the efficiency of HR in CRISPR-Cas9-mediated genome editing.[7] A key focus of their research was the modulation of RAD51, a protein integral to HR-mediated DSB repair. The authors demonstrated that upregulating RAD51 not only increases the proportion of HR during DNA repair but also significantly improves the efficiency of CRISPR-Cas9-mediated gene knockin and knockdown (Figure 1). RAD51, along with its auxiliary factors, plays a critical role in the search for homology and strand invasion, processes essential for the formation of the displacement loop, a crucial recombination intermediate.[5,8] Moreover, the study showed that elevating RAD51 expression combined with inhibiting the NHEJ pathway, through the DNA ligase IV inhibitor SCR7, markedly enhances HR efficiency, resulting in more precise genome editing outcomes. The research introduced an innovative all-in-one CRISPR-Cas9-RAD51 system designed to boost HR efficiency by increasing RAD51 levels.[6] The proposed model suggests that the synergy between RAD51 expression and SCR7 treatment stabilizes the R-loop structure, enabling Cas9 to induce DNA breaks more effectively, thereby enhancing CRISPR-Cas9 genome editing efficiency. Additionally, the authors conducted molecular functional analyses and RNA sequencing to investigate the transcriptomic changes associated with altered RAD51 expression. The findings revealed that RAD51 depletion disrupts cell cycle progression, particularly inhibiting the G2/M transition, which is pivotal for HR activity. This disruption leads to inefficient DNA replication and potential cell death, underscoring the necessity of balanced RAD51 expression for successful HR-mediated genome editing. The stable formation and resolution of Cas9-RNA-DNA complexes within R-loops are essential for effective genome editing, and RAD51-mediated HR may facilitate this process by stabilizing these complexes. Therefore, the authors hypothesize that the observed enhancement in Cas9 editing activity with RAD51 expression and SCR7 treatment may be attributed to improved R-loop stabilization (Figure 1).

Despite the promising advancements in enhancing HR-mediated genome editing, significant challenges and limitations remain. Careful consideration is necessary when addressing the potential risks associated with RAD51 overexpression and NHEJ inhibition, as both may lead to chromosome abnormalities, gene mutations, and subsequent genome instability. Elevated levels of RAD51 could potentially trigger the expression of oncogenes, raising serious concerns regarding the risk of oncogenesis. Furthermore, the inhibition of DNA ligase IV with SCR7, while advantageous for promoting HR, may disrupt the immune system and result in growth impairment and genetic diseases. The regulation of RAD51 expression and achieving sufficient protein levels

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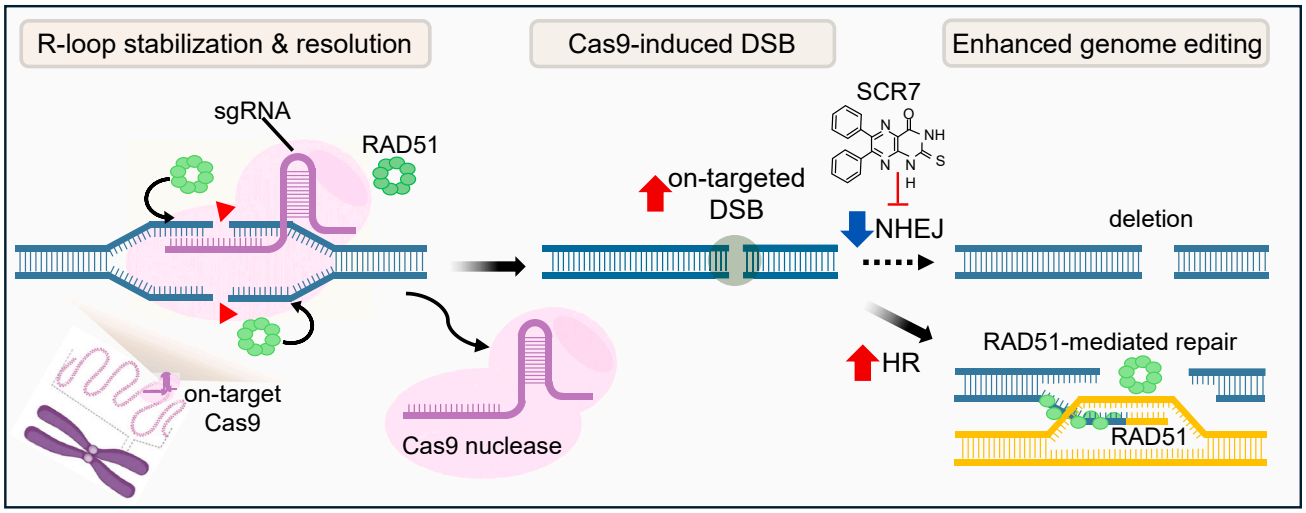

Figure 1. Enhancement of CRISPR-Cas9 efficiency through RAD51 upregulation and NHEJ inhibition

The study by Park et al. demonstrated that concomitant upregulation of RAD51 expression coupled with SCR7-mediated suppression of the NHEJ pathway markedly increases homologous recombination efficiency, thereby optimizing the overall efficacy of genome editing.

in vivo continue to pose substantial challenges. As an alternative approach, the study explored the use of small molecules, SCR7 and RS-1, to enhance gene editing efficiency. These molecules offer the critical advantage of temporal and concentration control, which is crucial for precise genome editing in therapeutic applications. The employment of small molecules to augment HR pathways represents a flexible and promising strategy for enhancing Cas9 activity. By utilizing a cocktail treatment of single molecules that inhibit NHEJ while promoting HR, researchers can achieve more efficient genome editing. This approach is particularly beneficial for *in vivo* applications, where controlling protein expression levels presents a significant obstacle. Therefore, this study holds profound implications for the future of Cas9-mediated gene therapy, potentially leading to more reliable therapeutic interventions for genetic disorders.

In conclusion, the findings of Park et al. provide compelling evidence that enhancing HR by increasing exogenous RAD51 levels and suppressing NHEJ can significantly improve the efficiency of CRISPR-Cas9 genome editing. Moreover, the use of cocktail small-molecule treatments presents a viable strategy for *in vivo* applications, resulting in more effective gene editing processes. A deeper understanding of the mechanisms driving these improvements will be essential in future studies to optimize the effectiveness of Cas9-mediated genome editing platforms and ensure their safety in clinical applications.

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AUTHOR CONTRIBUTIONS

S.H.K., S.Y., and K.P.K. wrote the manuscript. S.Y. and K.P.K. created the artwork.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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