Optimization of Cas9 RNA sequence to reduce its unexpected effects as a microRNA sponge

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Highlights: Cas9 RNA functions as a miRNA sponge. Let-7 is the dominant regulated miRNA by Cas9 RNA. RNA sequence optimization of Cas9 by synonymous mutation improves its safety.

Keywords: CRISPR–Cas9, miRNA sponge, Let-7, RNA sequence optimization

CRISPR–Cas9 gene-editing technology has a wide range of potential future applications, including cancer treatment [1]. Currently, the main safety concerns about CRISPR–Cas9 technology are the host immune response to its components [2, 3] and off-target modifications [4]. However, safety concerns at the RNA level have not been evaluated.

A wide range of interactions between microRNAs (miRNAs) and mRNAs occur, not only in the 3′ untranslated regions but also in the amino acid coding sequences (CDSs) of mRNA [5, 6]. An increasing number of research groups [7, 8], including our team [9–11], recently found that a long RNA can bind and adsorb partially complementary miRNAs through a “miRNA sponge” mechanism, upregulating other target genes inhibited by these miRNAs. In this study, we investigated whether Cas9 RNA, an exogenous long-chain nucleic acid substance, could affect host cell gene expression through a miRNA sponge mechanism and developed an RNA sequence optimization strategy to improve the safety of this gene-editing system.

Cas9 RNA could affect intracellular gene expression through a miRNA sponge mechanism

To analyze the regulatory effect of Cas9 on its predicted binding microRNAs and their target genes, we first mined a database with the transcriptome characteristics of 331 cell lines overexpressing a Cas9 RNA and their parental control counterparts [12]. We also analyzed
the binding possibilities between this Cas9 RNA and all the known human miRNAs using miRanda software [8]. The 51 miRNAs that were predicted to have the highest likelihood of binding Cas9 RNA were named “Cas9-miRNAs” (Fig. 1A-B and Table S1), while the 69 miRNAs predicted not to bind Cas9 were named “non-Cas9-miRNAs” (Fig. 1A and Table S2). According to the microRNA sponge hypothesis, the expression levels of a certain miRNAs targeting genes should be increased in cells into which Cas9 is introduced, and these cells could be identified as this miRNA related Cas9-sensitive cells, and vice versa. For most Cas9-miRNAs in most cell types, the change trends of their target genes were consistent with this hypothesis (Fig. 1C, Fig. S1). For example, more of the target genes of let-7i were upregulated after Cas9 introduction in 186 cell types, which should be identified as let-7i-related Cas9-sensitive cells, such as the T98G, TE-5 and 786-O cell lines (Fig. 1E, G, Fig. S1). Noteworthy, for the overall feature of 51 Cas9-miRNAs, their related Cas9-sensitive cells were significantly more than the Cas9-insensitive cells ($p = 3.9e-11$) (Fig. 1C).

In contrast, the non-Cas9-miRNAs, such as miR-1-3p (Fig. 1D, F, H), miR-215-5p, and miR-340-5p (Fig. S1), did not show such a trend. We therefore suggest that Cas9 RNA may act as a microRNA sponge, specifically influencing Cas9-miRNA target genes.

Among all the Cas9-miRNAs, let-7i was the mainly expressed Cas9-miRNA in most cells (Fig. S2). As let-7 family members are well-known tumor suppressor miRNAs [13, 14], the possible “microRNA sponge” effect of Cas9 on this family is worthy of further study. We also found by analysis with miRanda software that Cas9 RNA can bind to many other let-7 family members (Fig. S3). Moreover, through qPCR and Western Blot experiments, we confirmed that some validated let-7 target genes were significantly upregulated after the introduction of Cas9 in more types of cells (U251, 786-O, T98G v.s. MCF7) (Fig. 1I and J, Fig. S4).

We then investigated why the microRNA sponge effect of Cas9 varies greatly among different cell lines. Again, we used let-7 as a representative microRNA. Based on a joint analysis of the transcriptome data of 331 cell lines and the basic miRNA and mRNA expression data and gene mutation data in the Cancer Cell Line Encyclopedia (CCLE) dataset, we identified the top 35 types of cells in which Cas9 induced the greatest increase in let-7i target
genes as “let-7i-related Cas9-sensitive cells” and vice versa. We found that the basal expression of let-7 family members, especially let-7i, was lower in “let-7i-related Cas9-sensitive cells” rather than the control cells (Fig. 1K, Fig.S5). At the same time, the basal expression levels of let-7 target genes were also lower in “let-7i-related Cas9-sensitive cells” (Fig. 1L, Fig. S6). We also investigated the gene mutation frequency in the two groups of cells. Interestingly, we found almost all high-frequency mutations of let-7 target genes are distributed in the “let-7i-related Cas9-insensitive” cells (Fig. 1M, Fig. S7). These results suggested that, if some key let-7 target genes are mutated, the effect of Cas9 on up-regulating let-7 target genes might be weakened. All these data indicate that let-7 could bind this Cas9 RNA directly (Fig. 1N).

Moreover, RNA pulldown experiments confirmed binding the let-7 family, especially let-7b (Fig. S8A). RNA used in our lab also had a very high possibility of types of Cas9 RNA. We found that another popular Cas9 members, especially let-7i, was lower in “let-7i-related Cas9-sensitive cells” (Fig. S8C-D). Several reported let-7 target genes were found to be upregulated in some tumor cells after this Cas9 introduction, such as CDK6, KRAS, FN1, and HMGA2 (Fig. 1O, S8E and S9A-B). However, the corresponding changes were not obvious after the introduction of excess let-7 (Fig. 1P), suggesting that Cas9 affects let-7 target genes through a “miRNA sponge” mechanism depending on the appropriate let-7 expression level, which is consistent with the results of previous bioinformatics analysis (Fig. 1K, Fig. S5). Furthermore, we found that dCas9-VP64, which was used for gene activation [15], could also increase the expression of some let-7 target genes in U251 cells (Fig. S9C).

Moreover, in Cas9-transgenic mice, KRAS and CDK6 expression levels were also found to be mildly increased in the testis tissues (Fig. 1Q), although they were not significantly changed in most of the tissues we tested (Fig. S10), which might also be associated with the basic expression levels of let-7 and its target genes. These results suggest that Cas9 RNA could regulate let-7 target genes through the miRNA sponge mechanism in some types of cells, indicating that Cas9 itself may affect cells through mechanisms other than DNA cleavage.

**RNA sequence optimization of Cas9 to reduce its effect on cell proliferation and let-7 downstream genes**

The majority of let-7 downstream target genes are oncogenes [13, 14]. Since Cas9 can regulate the expression of some let-7 target genes, we speculate that exogenously introduced Cas9 may affect the biological characteristics of cells. We transduced the Cas9 viral vector, as well as the control virus, into the human prostate cancer cell line DU145 and found that Cas9 slightly promoted cell proliferation in a Cell Counting Kit-8 (CCK-8) experiment (Fig. S11A), cell cycle assay (Fig. S11B), and cell colony formation assay (Fig. 2A). In an in vivo experiment, we also found that the tumor growth rate and tumor volume were slightly higher in the group with stable Cas9 expression (Fig. 2B).

In addition, we introduced Cas9 into several other types of tumor cells, such as the human renal carcinoma cell line 786–O and the human colon carcinoma lines HT-29 and HCT116. Cas9 had no significant effect on HT-29 cells (data not shown) but slightly promoted the proliferation of HCT116 and 786–O cells (Fig. 2C, Fig. S11C). These results indicate that Cas9 does not have a significant effect on all cells but could slightly promote the proliferation of DU145 cells, 786–O cells and HCT116 cells.

Moreover, Cas9 could also increase some let-7 target genes in normal human cultured primary bone marrow mesenchymal stem cells (bMSCs) (Fig. S12 A-E) and the human normal epithelial cell line HaCaT (Fig. S12F-G) and could promote the proliferation of HaCaT cells (Fig. 2D-E).

In summary, we found that Cas9 RNA could slightly promote proliferation in some cells through a miRNA sponge mechanism. Although the effect is weak, especially in vivo, our observations suggested that it is necessary to improve the safety of CRISPR–Cas9 technology by modifying Cas9 itself in addition to reducing the off-target effect of the CRISPR–Cas9 system.

To further confirm the presence of this miRNA sponge mechanism and to optimize the Cas9 sequence at the RNA level, we constructed a synonymous mutant plasmid named Cas9-Mut. Cas9-Mut had mutations at the three let-7 binding sites; however, the mRNA transcript from Cas9-Mut could be translated into the same amino acid sequence as Cas9 (Fig. 2F). In fact, the RNA pulldown results in HEK293 cells showed that the binding of Cas9-Mut to let-7 was significantly reduced compared
to that of Cas9 (Fig. 2G). Moreover, although Cas9-Mut somewhat promoted colony formation in DU145 cells compared with the control cells, its activity was weaker than that of Cas9 (Fig. S13). EdU assays showed that the proliferation ability of Cas9-Mut-transfected DU145 cells was weaker than that of Cas9-transfected cells (Fig. 2H). The percentage of G1 phase cells increased and that of G2 phase cells decreased in the Cas9-Mut group compared with the Cas9 group (Fig. 2I). At the molecular level, we found that the ability of Cas9-Mut to upregulate the let-7 target gene was also significantly weaker than that of Cas9, not only in DU145 cells (Fig. 2J–K) but also in U251 cells (Fig. 2L). Additionally, in hepatic carcinoma cell line HepG2, the cell proliferation ability of Cas9-Mut-transfected cells was weaker than that of Cas9-transfected cells (Fig. 2M). Although the current mutants cannot completely prevent the binding of let-7 and Cas9 RNA at other sites with higher binding free energy nor can they prevent the binding of other miRNAs that may affect proliferation, such as miR-145-5p, the partial effectiveness of the mutation suggests that Cas9 RNA can be further optimized in the future by the method of synonymous mutation.

Theoretically, because the amino acid sequence in Cas9-Mut is not changed, the ability to cleave DNA is not changed (Fig. 2F). To determine whether the optimized Cas9-Mut still has a gene-editing function, we first tested the ability of Cas9-Mut to knock out the EGFP reporter gene. After the introduction of Cas9-Mut and the designed EGFP-gRNA into HEK293 cell line cells stably expressing EGFP, some EGFP-negative cells appeared, indicating that Cas9-Mut still has a gene-editing function (Fig. 2N). We further tested the gene-editing ability of Cas9-Mut by transducing the Cas9-Mut plasmid and gRNA targeting the UBXN4 gene into DU145 cells (Fig. 2O). Genomic DNA was extracted, and the sequence around the predicted cutting site was amplified by PCR (Fig. 2O). Sanger sequencing of this PCR product showed double peaks around the predicted cutting site, which suggested successful DNA cutting (Fig. 2O). Furthermore, UBXN4 protein was not expressed in the selected single clones of DU145 cells by Western blotting (Fig. 2P). Therefore, the gene-editing function of Cas9-Mut was confirmed, providing support for its potential use in practical applications.

Conclusion

Our bioinformatic analysis and experimental results suggest that Cas9 RNA could interact with endogenous miRNA through the miRNA sponge mechanism, and it is valuable to optimize CRISPR–Cas9 technology at the RNA level for safer application in the future. Specifically, the current widely used Cas9 RNA could bind and sequester let-7, thus upregulating some let-7 target genes and slightly promoting cell proliferation in some cell types. Through synonymous mutation, the RNA sequence optimization of Cas9, introducing modified let-7 binding sites, weakened the effect on the promotion of cell proliferation and the expression of some let-7 downstream genes (Fig. 2N).
Fig. 2 (See legend on previous page.)
The whole study design and protocols were approved by the Ethics Committee of Naval Medical University, China, in accordance with the instruction about Care and Use of Laboratory Animals published by the USNIH.

Consent for publication

The authors approval the publication of this manuscript.

Competing interests

The authors declare that they have no competing financial interests.

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