AbstractTitle: CRISPR/CAS9 GENE EDITING REVEALS NOVEL TERTIARY CONSTRAINTS IN CLUSTERED MIRNA PROCESSING

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CRISPR/CAS9, GENE EDITING, MICRORNA

Introduction: MicroRNAs (miRNAs) play an important role in the cellular function. They often form families, with members sharing high sequence homology, a property that hampers miRNA research as there is a lack of elegant tools for specific miRNA manipulation.
Objective: To establish a reliable workflow for miRNA inhibition using genome editing.
Methods and Results: This study focused on miR-195, a member of the miR-15 family and employed the CRISPR-Cas9 system. To this end we generated mouse vascular smooth muscle cells (VSMCs) stably expressing Cas9 nuclease. Cells were then transfected with an *in vitro* transcribed single guide RNA targeting the miR-195 stem loop. T7 endonuclease I assay (T7EI) and Sanger sequencing confirmed efficient editing. QPCR demonstrated effective decrease of miR-195 but not of miR-15a and miR-16, two highly expressed members of the miR-15 family in VSMCs. Surprisingly the expression of miR-497 was also decreased in edited cells. Noteworthy, miR-195 and miR-497 form a miRNA cluster and are co-transcribed as a primary miRNA. No gene editing was detected by T7EI and sequencing of the mir-497 genomic locus. Computational simulation predicted that mutations of the miR-195 stem loop led to changes in the three dimensional structure of the primary miR-497~195 transcript that could affect its processing to mature miRNAs.
Similar findings were obtained the miR-143~145 cluster that encodes miR-143 and miR-145a, two miRNAs that do not belong to the same family, show no sequence homology and play a pivotal role in vascular biology. Specific targeting of the mir-145a locus effectively inhibited the expression of both miR-143 and miR-145a while no genomic editing was observed for the mir-143 locus. Noteworthy, the expression of Carmn, a long non coding RNA in the vicinity of the miR-143~145 cluster that constitutes an independent transcription unit did not differ in miR-145a edited cells confirming that only the primary miRNA transcript is affected. On the contrary, gene editing in the miR-17~92 and miR-106b~25 clusters, two miRNA clusters with a key function in the cardiovascular system, resulted in targeted miRNA inhibition. MiR-18a and miR-25 were targeted on each cluster, respectively. Specific editing only for the intended miRNA locus was observed and QPCR quantification indicated inhibition of the edited miRNA. No effect on the expression of other miRNAs occurred, both for cluster miR-17~92 and miR-106b~25.
Detailed analysis of the gene editing in the four clusters revealed that the unintended inhibition of miRNA expression in the cluster coincides with disruption of sequence motifs of

the terminal loop of the targeted hairpin, suggesting that these elements are critical for the maturation not only of individual hairpins but the entire primary transcript in miRNA clusters.
Conclusions: CRISPR/Cas9 emerged as a powerful tool that can offer novel insights into the role of miRNAs in cardiovascular diseases.

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