Knockout of microRNAs using the CRISPR-Cas9 system with paired synthetic crRNAs

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Introduction

The CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated 9) system derived from *Streptococcus pyogenes* uses a Cas9 nuclease directed by a guide RNA (gRNA) to create a DNA double-strand break (DSB) at the target site. The gRNAs can be dual synthetic molecules, like the native bacterial system containing a CRISPR RNA (crRNA) and a trans-activating CRISPR RNA (tracrRNA) **(Figure 1)**, or a single synthetic guide RNA (sgRNA). The DSB is most often repaired by either non-homologous end joining (NHEJ) or homology directed repair (HDR) through endogenous mechanisms within mammalian cells. NHEJ can result in insertions or deletions (indels) that produce functional gene knockouts through nonsense mutations or introduction of a stop codon. When using CRISPR-Cas9 components targeting coding genes, there are typically multiple protospacer adjacent motif (PAM) sequences (NGG for *S. pyogenes*) to choose from along the gene to design a gRNA. For most CRISPR Cas9 genome engineering experiments, one targeting gRNA is sufficient to generate the desired functional gene knockout. However, for some applications, it may be advantageous to use two gRNAs to generate a larger deletion and ensure gene knockout or to remove an exon, long non-coding RNA (lncRNA), or transcriptional regulatory element.

Synthetic crRNA and tracrRNA for functional gene editing

The use of a two-RNA system for programming of Cas9 nuclease enables rapid generation of ready-to-use guide RNAs.

- Ready to be delivered into cells
- Fast turnover to reduce off-target events
- High-throughput synthesis to enable arrayed screening
- Use with any source of Cas9

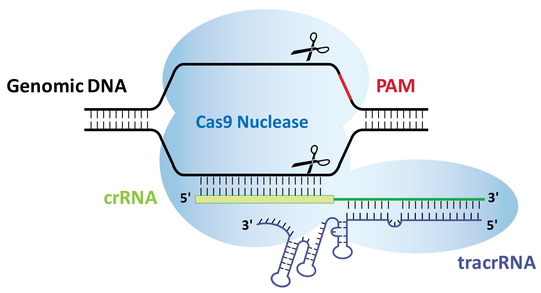


Figure 1. Gene editing with a three-component system: Cas9 nuclease (light blue), programmed by the crRNA:tracrRNA complex (green and blue, respectively) cutting both strands of genomic DNA 5' of the PAM (red).

Targeting microRNAs for knockout requires careful design

- Coding genes typically have hundreds to thousands of crRNA target sites
- microRNAs have a short gene length with limited sequence space which reduces number of potential crRNA target sites
- Potential off-targeting can further reduce design space
- Since microRNAs are a noncoding gene, knockout cannot rely on early stop codon for knockout
- Indel formation must disrupt microRNA expression or functional structure

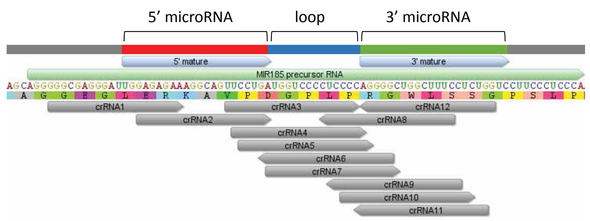
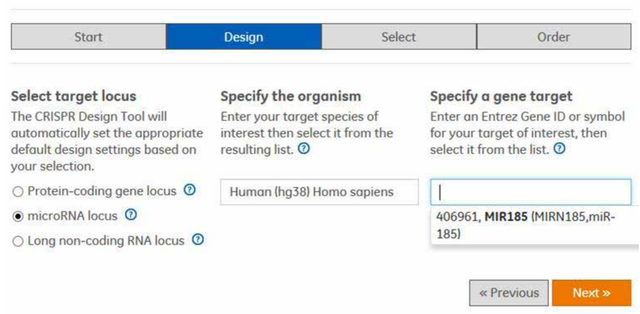


Figure 2. crRNAs can be designed to target any sequence that is upstream of a NGG PAM sequence (for *S. pyogenes*), but design space is very limited due to short microRNA gene length & elimination of designs due to potential off-targets predicted by specificity checking.

Using the Dharmacon CRISPR Design Tool to design crRNAs for microRNAs

- The design tool can design crRNAs to target microRNAs in over 30 species
- Rigorous specificity checking identifies designs with potential off-targets
- The CRISPR Design tool can assist with microRNA design and ordering (https://dharmacon.gelifesciences.com/gene-editing/crispr-cas9/crispr-design-tool/)



Experimental design

- Cas9-expressing cell line lipid transfected with synthetic crRNA and tracrRNA was tested for indel formation AND functional knockout of microRNAs
- Tested parameters that affect the functional knockout efficiency include:
  - Targeted region of the microRNA
  - Number of crRNAs

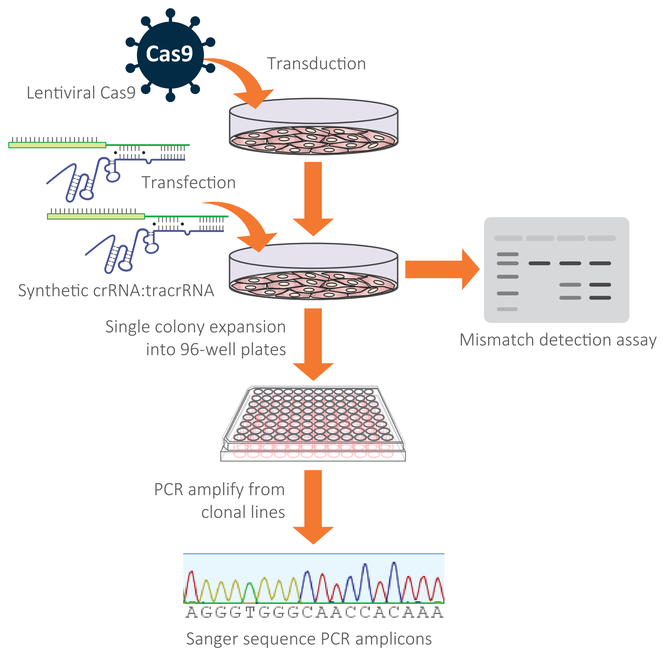


Figure 3. HEK293T and U2OS cells were transduced with Dharmacon Edit-R Lentiviral Cas9 Nuclease Particles (CAG-BlastR) and selected with blasticidin to generate cells stably expressing Cas9 nuclease. The selected cells were transfected with single synthetic crRNAs or paired synthetic crRNAs targeting microRNAs. After three days, the transfected cell populations were split into a 96-well plate for single colony expansion and tested for editing with a mismatch detection assay. After seven days, the expanded colonies were assessed for insertions and deletions by Sanger sequencing.

A single crRNA generates different deletions across clonal isolates

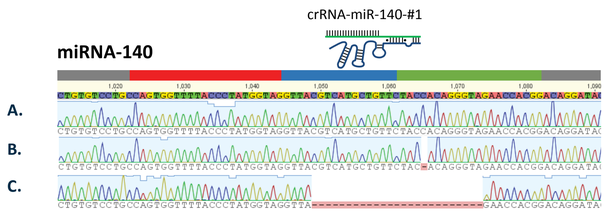


Figure 4. Sanger sequencing of clonal cells shows gene editing and indel formation in microRNA. The target region of the crRNA:tracrRNA is indicated by the relative position of the figure above the sequence. **A.** Wild type sequence of miR-140; untreated control. **B.** Homologous deletion of one base pair in the 3' microRNA region; may or may not disrupt functionality of the microRNA. **C.**Homologous deletion of 25 base pairs in the loop and 3' microRNA regions; expected to disrupt functionality of the microRNA.

Paired crRNAs can be used to create large deletions even if the microRNA sequence lacks PAMs

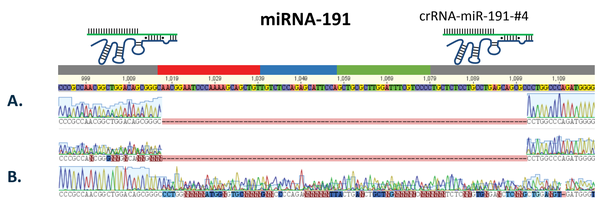


Figure 5. Knockout deletions can be generated even if the design space of the microRNA does not have any PAMs to design crRNA targeting sites. **A.** A large deletion of 85 base pairs induced by two different crRNA in regions flanking the microRNA gene cause almost complete deletion of the miRNA-191 sequence. **B.** Non-homologous deletions can also occur, with one chromosome being edited while the other remains unedited.

Paired crRNAs generate large indels regardless of crRNA directionality or DNA target strand

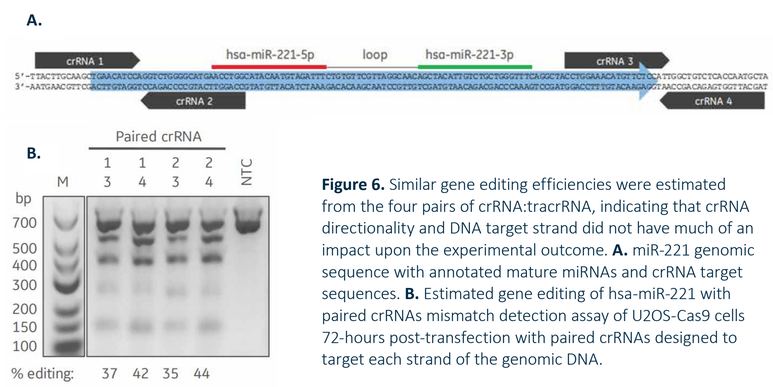
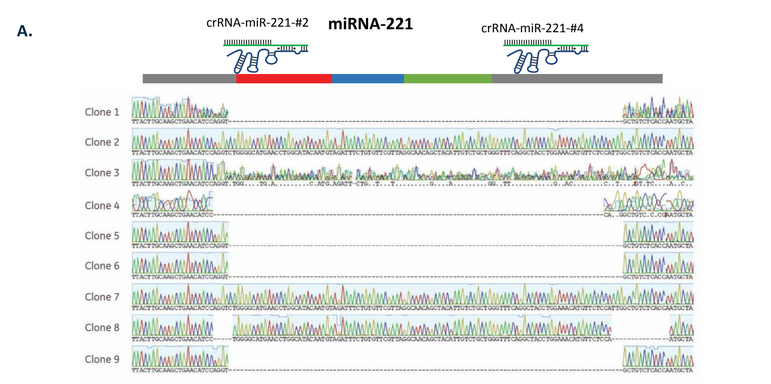


Figure 6. Similar gene editing efficiencies were estimated from the four pairs of crRNA:tracrRNA, indicating that crRNA directionality and DNA target strand did not have much of an impact upon the experimental outcome. **A.** miR-221 genomic sequence with annotated mature miRNAs and crRNA target sequences. **B.** Estimated gene editing of hsa-miR-221 with paired crRNAs mismatch detection assay of U2OS-Cas9 cells 72-hours post-transfection with paired crRNAs designed to target each strand of the genomic DNA.

Formation of large deletions in the microRNA result in functional knockout



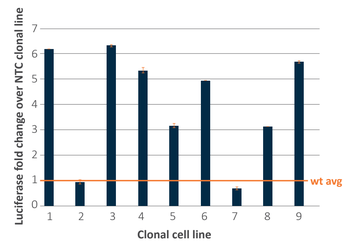


Figure 7. Large deletions as shown by Sanger sequencing result in a functional knockout of the microRNA. **A.** Sanger sequencing of U2OS hsa-miR-221 paired gRNA clonal cell lines. **B.** Use of a luciferase reporter plasmid to detect hsa-miR-221 functional knockout. U2OS hsa-miR-221 paired gRNA clonal cell lines were transfected with an has-miR-221 luciferase reporter plasmid and luciferase expression was measured (72-hours) after transfection. Unedited, wild-type U2OS cells were also transfected and are indicated by the wt avg solid line on the graph. Clones 2 & 7 show repression of luciferase and presence of the microRNA while all others show high luciferase indicating that microRNA function is knocked out.

Conclusions

- CRISPR-Cas9 gene editing is an effective strategy of knocking out microRNA function to study the biological role of microRNAs
- microRNAs can be more difficult to knockout with CRISPR-Cas9 technology than coding genes due to limited design space, and small indels may be tolerated by microRNAs due to the imperfect complementarity of the native structure
- By using the CRISPR design tool, we were able to quickly design highly functional synthetic crRNAs that were effective in functional knockout of microRNA
- Paired synthetic crRNA:tracrRNA shows an extension of the utility of CRISPR-based genome engineering to enable researchers to disrupt noncoding genomic targets

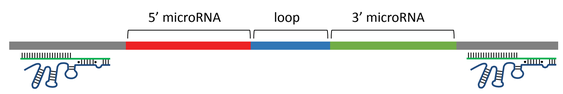


Figure 8. Utilization of two crRNAs targeting the immediate sequence upstream and downstream of a microRNA is a viable method for functional knockout of a microRNA.

References

More details can be found online in Application Note https://dharmacon.gelifesciences.com/uploadedFiles/Resources/edit-r-crispr-cas9-targeting-microrna-appnote.pdf

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