RNA-targeting CRISPR systems for transcriptional gene regulation and anti-viral countermeasure development

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Background

- CRISPR/Cas9 genome editing technology has created a new modality for treating viral infections indirectly by targeting critical host factors[1]
- Although the Cas9 endonuclease can target DNA viruses directly for degradation, many viruses of biodefense concern have RNA genomes that prevent direct targeting approaches by current CRISPR technology
- By developing programmable CRISPR systems that target RNA instead of genomic DNA, deleterious off-target concerns may be alleviated
- Additionally, to realize the full potential of CRISPR-based countermeasures, significant improvements in delivery are needed to reduce the risks associated with viral vectors
- Lipid-coated mesoporous silica nanoparticle (LC-MSN) technology can be adapted for large cargo delivery and provide advantages of both MSNs and liposomes, including controlled release, targeting specificity, colloidal stability, and biocompatibility[2]

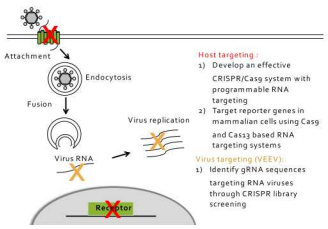

Figure 1. Approaches to CRISPR-based anti-viral countermeasures targeting host and virus

Objective: Develop safe CRISPR-based anti-viral countermeasures targeting host and viral genomes, packaged using nanoparticle technology for efficient and specific *in vivo* delivery

[1]Soppe JA. et al. *Trends in Microbiology* 2017; [2]Butler K. et al. *Small* 2016

Discovery and Characterization of an RNA-targeting Cas9

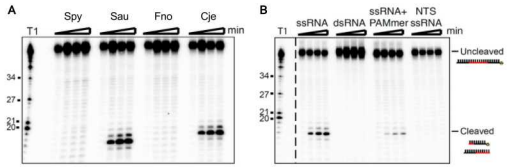

Figure 2. RNA-targeting Cas9 (R-Cas9) cleaves ssRNA without a PAMmer.[3] (A) *In vitro* cleavage of ssRNA by Cas9-sgRNA RNP complexes of several SpyCas9 homologs. (B) *In vitro* cleavage assay of various RNA substrates (PAMmer=DNA oligo).

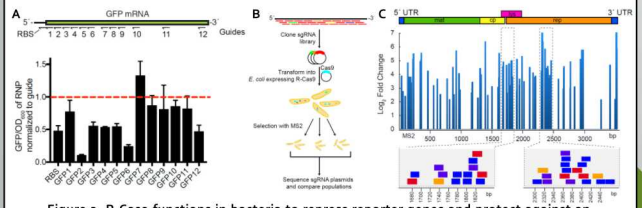

Figure 3. R-Cas9 functions in bacteria to repress reporter genes and protect against an RNA phage.[3] (A) Relative expression of GFP when targeted by RCas9 in sequences without PAMs (B) Overview of an MS2 phage CRISPR tiling library screen. (C) Log2 fold changes of guides with an FDR-corected p-value <0.05 mapped to the MS2 genome.

A subset of Cas9 enzymes recognize and cleave ssRNA substrates which can be exploited for programmable RNA targeting applications

[3]Strutt S, Torrez R, Kaya E, Negrete O, Doudna J; ***eLife*** 2018;7:e32724

RNA-targeting Cas9 library screening for gene suppression

Methods: A tiling library of guide RNAs targeting the complementary sequence of a mCherry reporter gene was introduced into cells stably expressing SaCas9. Guide RNA libraries were depleted of PAM adjacent sequences to eliminate DNA targeting activity. Reporter transfected cells with transduced libraries were sorted and sequenced to identify enriched gRNA sequences compared to controls.

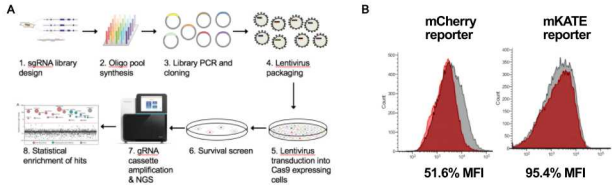

Figure 4. RNA targeting Cas9 library screening for identification of sgRNAs that reduce mCherry reporter gene expression. (A) Discovery of host and viral genetic targets for CRISPR-based anti-viral development involves screens with a pipeline illustrated above. (B) Population level analysis via FACS.

RCas9 library screening against a reporter gene identifies novel methods for gene suppression in mammalian cells

CasRx (13d) RNA targeting for gene suppression

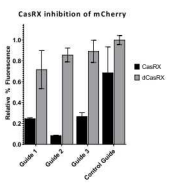

Figure 5. Evaluation of Cas13-based systems for host gene knockdown. (A) Published (Konermann et al, *Cell*, 2018) guide RNAs sequences targeting the mCherry gene were transfected into 293Ts along with CasRx and reporter expression plasmids. Flow cytometry analysis was performed 24hrs post-transfection and normalized values are shown. A comparison between catalytically active and inactive CasRx indicated HEPN dependent gene suppression.

CasRx may also support gene suppression of critical host factor genes

RNA-targeting Cas9 library screening for direct anti-viral inhibition

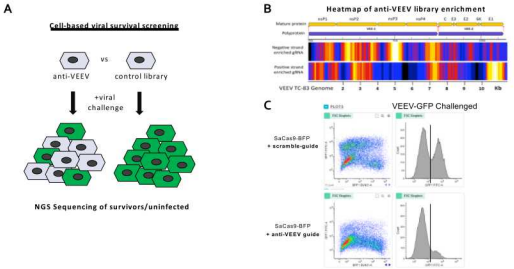

Figure 6. RCas9 library screening for identification of anti-viral gRNAs. (A) A tiling gRNA library targeting the genome and anti-genome of Venezuelan Equine Encephalitis virus (VEEV) was introduced into cells stably expressing SaCas9 and then infected with VEEV virus. Surviving cells were collected and sequenced to identify enriched gRNA sequences. (B) Bioinformatic analysis identified over 100 significant hits from the primary screen that were mapped to the genome/anti-genome and illustrated in a heatmap format. (C) One specific anti-VEEV guide validation experiment is shown to diminish infection levels as evidence by reduced reporter (GFP) expression.

RCas9 library screening against VEEV identifies potent guide RNA sequences for direct anti-viral countermeasure development

nanoCRISPR delivery technology

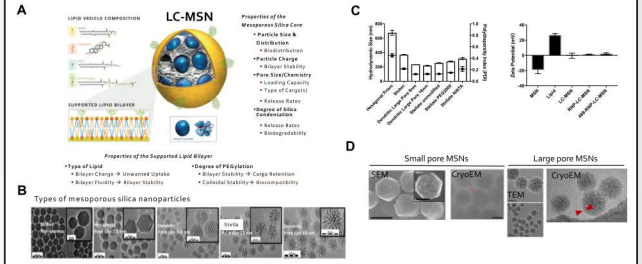

Figure 7. Lipid-coated mesoporous silica nanoparticle (LC-MSN) technology for *in vivo* delivery of Cas9/gRNA ribonucleoprotein complexes ("nanoCRISPR"). (A) Advantages of LC-MSN technology for therapeutic cargo delivery. (B) Various types of mesoporous silica nanoparticle cores with varying pore sizes. (C) Screening of nanoCRISPR-RNP formulations for colloidal stability and charge. (D) The ultrastructural assembly of the nanoCRISPR-RNP formulation compared to anti-viral delivery nanoparticles described by LaBauve et al, *Sci Rep*, 2018.

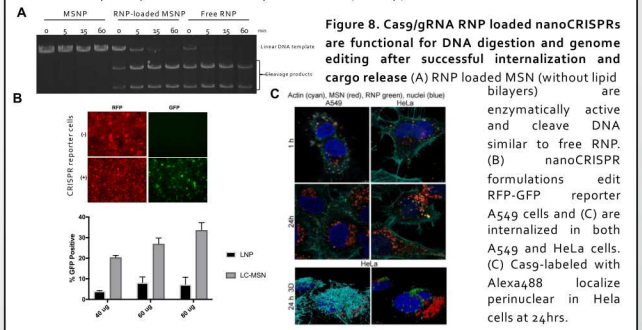

Figure 8. Cas9/gRNA RNP loaded nanoCRISPRs are functional for DNA digestion and genome editing after successful internalization and cargo release (A) RNP loaded MSN (without lipid bilayers) are enzymatically active and cleave DNA similar to free RNP. (B) nanoCRISPR formulations edit RFP-GFP reporter A549 cells and (C) are internalized in both A549 and HeLa cells. (C) Cas9-labeled with Alexa488 localize perinuclear in Hela cells at 24hrs.

LC-MSN technology adapted for Cas9/gRNA cargos (nanoCRISPR) provides a novel approach to non-viral CRISPR delivery

Conclusion

In this work, we highlight the use CRISPR technology for RNA targeting and novel anti-viral applications. CRISPR-based approaches can modify host genes involved in viral infection or target viral RNA directly using newly discovered functions in SpyCas9 homologs. For applications towards a host-directed therapeutic, we demonstrated preliminary data for reporter gene suppression by Cas9 and Cas13 systems in mammalian cells. As a direct defense against RNA viruses, we performed a tiling library screen using RNA-targeting Cas9 against Venezuelan Equine Encephalitis virus and identified several guide RNAs that protect cells from infection. Additionally, we demonstrated the adaptation of lipid-coated mesoporous silica nanoparticle technology for cellular delivery of Cas9/gRNA RNP in an attempt to overcome a major hurdle in the clinical translation of CRISPR-based countermeasures.

Acknowledgments

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