Title

RNA Editing via Recruitment of Endogenous ADARs using Circular Antisense Guide RNAs

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RNA Editing via Recruitment of Endogenous ADARs using Circular Antisense Guide RNAs

A thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Bioengineering

By

James Jen Yen

Committee in charge:

Professor Prashant Mali, Chair
Professor Pedro Cabrales
Professor Ester Kwon

2021

The thesis of James Jen Yen is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

Dedication

This thesis is dedicated to my parents and brother for their unwavering support throughout my life. I would not be the person I am today without their love, support, and guidance; I thank them for the many sacrifices that they have made throughout their own lives in order to give me the greatest chance to succeed.

I would also like to dedicate this thesis to my partner, Lynze Tom. Lynze has been a pillar of support throughout my time working on this thesis. I thank her for the love, support, encouragement, and patience she has shown me during my time working on this thesis.

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I want to thank Amir Dailamy, Debbie Chen, Amanda Suhardjo, Yichen Xiang, and So-Yun Han for the close friendship we shared over the years; all of the meals and outings we shared together provided me much needed respite from hectic work days. I want to also thank everyone in the Mali Lab for creating a great work environment. It was inspiring watching everyone's ambition for their own work and this pushed me to work harder on my own projects in the lab.

Lastly, I want to thank my undergraduate mentor, Professor Eugene Nothnagel, for giving me the initial opportunity to work in his lab. Your passion for science and willingness to explain how to properly ask and answer scientific questions put me on the proper path to become the scientist I am today.

This work, in part, has been submitted for publication of the material as it may appear in Nature Biotechnology, 2021, Katrekar, Dhruva; Yen, James; Xiang, Yichen; Saha, Anushka; Savva Yiannis, Mali, Prashant. The thesis author was a researcher for this material.

ABSTRACT OF THE THESIS

RNA Editing via Recruitment of Endogenous ADARs using

Circular Antisense Guide RNAs

By

James Jen Yen

Master of Science in Bioengineering

University of California San Diego, 2021

Professor Prashant Mali, Chair

Genetic disorders collectively chronically affect 1 in 17 individuals in the world today. Currently, many of the treatments that exist for such disorders are palliative and only treat the

symptoms, not the underlying cause. The small amount of approved curative treatments that do exist utilize permanent genome editing tools that carry inherent risks of permanent off-target modifications. The aim of this thesis is to develop a platform for the safe and effective repair of genetic disorders using A-I RNA editing. It has been recently shown that delivery of long antisense guide RNAs can recruit endogenous adenosine deaminase acting on RNA (ADAR) enzymes to induce RNA editing in vitro. Importantly however, this approach is unable to induce RNA editing in vivo; we hypothesized this to be a result of the short half-life of linear guide RNAs resulting from vulnerability to exonuclease attack. By engineering and delivering highly stable circular guide RNAs via AAV8, we were able to induce robust RNA editing in mice livers: we observed 53% editing in the 3'UTR of the mPCSK9 transcript in C57BL/6J mice and 12% correction of a nonsense mutation in the IDUA-W392X mouse model for type mucopolysaccharidosis type I-Hurler (MPS I-H) syndrome. Furthermore, we were able to reduce the bystander editing profile of target transcripts by engineering loop secondary structures strategically placed throughout our circular antisense guide RNAs. Altogether, our platform paves the way for safe transcript-specific RNA editing for use in gene therapy.

Introduction

While generally rare, genetic disorders collectively affect 1 in 17 individuals worldwide arising from the immense possible variation of the human genome[1]. These genetic disorders typically manifest as a result of disruptive mutations to the genome that adversely affect the correct transcription and subsequent translation of key proteins in the body. Approximately 58% of these disorders are caused by pathogenic point mutations that change a codon in the mRNA sequence. This mutation can either lead to changes in the amino acid sequence that can result in a loss of protein function or in severe cases, result in premature termination of protein translation when a codon encoding for an amino acid is mutated into a stop codon[2]. Current available treatments for many of these rare chronic genetic disorders typically only treat the symptoms and not the underlying cause[3–5]. There is an increasing need for curative treatments to be developed, resulting in the rapid growth of the gene therapy field both in terms of new tools for genome modification as well as methods to deliver these tools.

Current methods used in the repair of pathogenic point mutations involve making permanent modifications to the faulty gene utilizing various tools such as transcription activator-like nucleases, zinc finger nucleases, as well as the widely known CRISPR-Cas systems[6–9]. Although certainly promising, there are risks associated with using DNA-based gene therapies that have yet to be completely addressed in the field. One of the primary drawbacks to using these tools is the possibility of introducing permanent off-target edits to the genome, potentially eliciting adverse effects, for example, increasing cancer risk by activating proto-oncogenes or inhibiting tumor-suppressor genes. Many of these genome editing tools are also have a prokaryotic origin, causing issues with severe immunogenic responses upon delivery[10,11].

RNA base editing using adenosine deaminase acting on RNA (ADAR) has emerged as a promising alternative to repair premature stop codons that evades the complication of permanent off-target genomic changes[12]. ADAR1 is an endogenous mammalian enzyme that is able to

deaminate adenosine residues found on double-stranded RNA (dsRNA) into inosine (Figure 1). Inosine residues are recognized by the cell's endogenous translational machinery as a guanosine, which holds specific therapeutic relevance in the context of repairing pathogenic adenosine mutations or premature stop codons[13,14]. An increasingly common strategy to induce targeted ADAR-mediated RNA editing is to exploit the presence of double-stranded RNA binding domains found on ADARs by expressing antisense guide RNAs complementary to the transcript of interest that binds to form double-stranded RNA[15]. Editing specificity within the target region is then partially achieved by including a cytidine mismatched to the adenosine of interest. In particular, by expressing long antisense ADAR gRNAs (adRNAs), endogenous ADARs are able to be recruited to edit transcripts in vitro with efficiencies ranging from 30-80%[15,16].

Recently, ADAR-mediated RNA editing was shown to be possible in vivo for the first time using a platform that involving the overexpression of exogenous ADARs[16]. While exciting, this method has a major drawback in the form of transcriptome-wide off-target editing mainly due to ADAR overexpression. In order for ADAR-mediated RNA editing to become a robust tool capable of being used in the clinic, two major pitfalls of the technique need to be addressed: first, the current necessity of exogenous ADAR overexpression for in vivo editing and second, off-target editing in the transcriptome as well as within the transcript itself. Recruitment of endogenous ADARs has been demonstrated to be simple and effective in vitro by expressing long antisense adRNAs complementary to a transcript of interest[15,16]. However, usage of this method has not yet been shown to be effective in vivo and we hypothesize that this is partially due to the stability and corresponding residence time of the adRNAs.

To improve adRNA stability and residence times, we have developed a platform using a circularization technique described by Litke et al. wherein the adRNAs are flanked by autocatalytic twister ribozymes[17]. Upon cleavage, the ribozymes generate a 2',3'-cyclic phosphate group and a 5'-OH group on respective ends of the adRNA. These groups are then recognized and

circularized by RtcB, an endogenous RNA ligase (**Figure 1a**). Using our circular adRNA (cadRNA) platform, we demonstrate robust RNA editing in vitro as well as for the first time, in vivo using the recruitment of endogenous ADARs (**Figure 1b, 1c**). Further, we apply our platform to the well characterized IDUA-W392X mouse disease model of mucopolysaccharidosis type I-Hurler syndrome (MPS I-H) and confirm partial restoration of alpha-L-iduronidase (IDUA) activity[18–20].

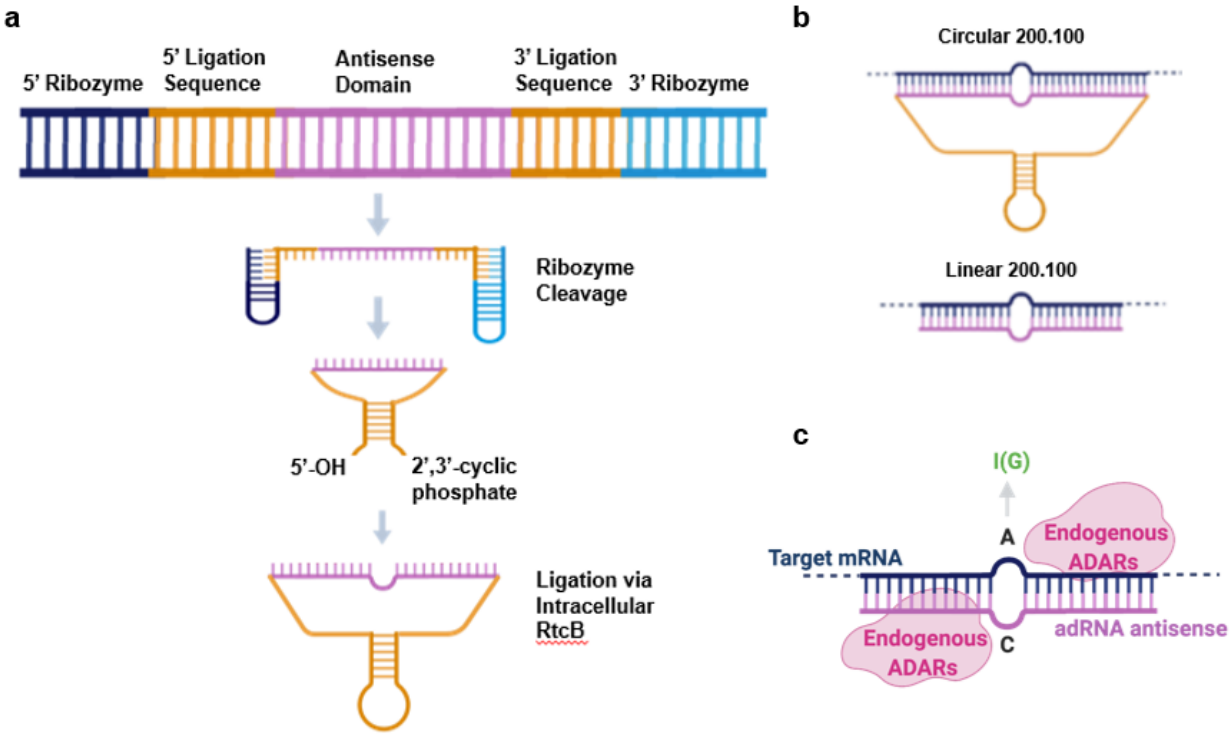


Figure 1: Schematic of endogenous ADAR recruitment and RNA editing via circular adRNAs
(a) Circularization scheme of cadRNAs through self-catalytic twister ribozyme cleavage and subsequent ligation via RtcB. **(b)** Schematic of linear and circular adRNA binding to transcript of interest to form a dsRNA complex. **(c)** Schematic of ADAR recruitment to dsRNA complex and subsequent deamination of adenosine into inosine.

Results

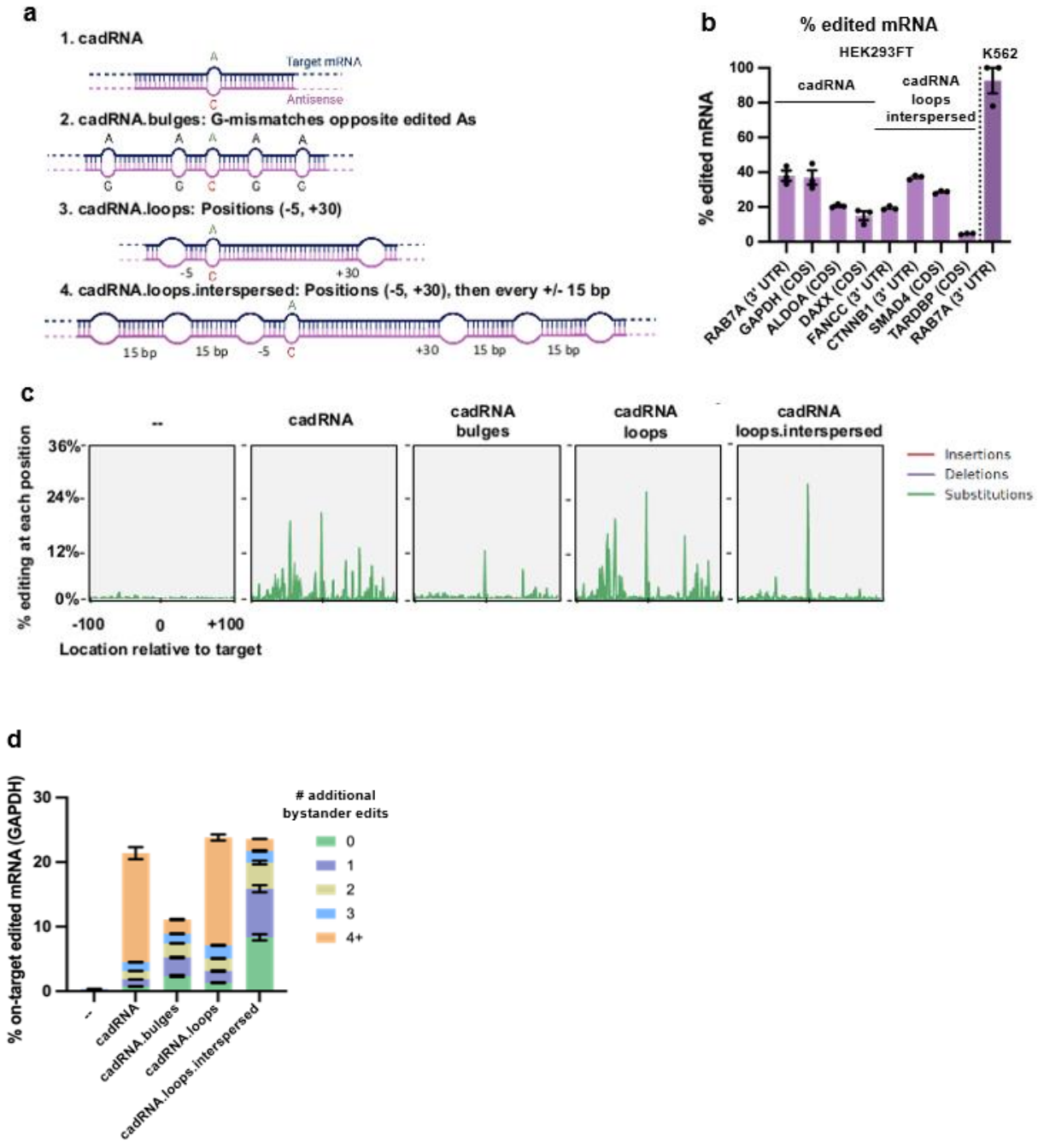
Validation of the *in vitro* efficacy of cadRNAs

In order to evaluate the ability of cadRNAs to robustly recruit endogenous ADARs for A-I editing, we decided to investigate their in vitro editing efficiency on eight different endogenous transcripts in HEK293FT cells. To accomplish this, we designed genetically encoded cadRNAs targeting a variety of different endogenous transcripts – RAB7A, GAPDH, ALDOA, and DAXX, and delivered them to HEK293FT cells using lipofection. The cells were harvested at a 48 hour timepoint and the corresponding transcripts were PCR amplified to be sent for Sanger sequencing. The observed editing efficiencies demonstrate that cadRNAs are indeed able to induce robust editing on target adenosines found both in the 3'UTR and CDS regions (**Figure 2b**).

Although we largely observed robust editing efficiencies in vitro using cadRNAs, we also observed significant bystander editing or hyperediting in many of the transcripts. To tackle this issue, we investigated the bystander editing profiles of the transcript GAPDH and engineered strategically placed secondary structures onto our cadRNAs (**Figure 2a**). We placed guanosines opposite of all adenosines in order to form secondary structures in the form of bulges that interfere with bystander editing (cadRNA.bulges)[14]. On a separate construct, we engineered 8 bp loops known to improve ADAR editing specificity and spaced these loops 36 bp apart from the adenosine in interest (cadRNA.loops)[21,22]. Delivery of cadRNA.loops to HEK293FT cells partially improved the bystander editing profile but did not completely eliminate hyperediting outside of the 36 bp region enclosed by the engineered loops (**Figure 2c**). Taking into account that the region enclosed by the loops experienced the greatest reduction in bystander editing, we hypothesized that placing loops alongside the entire transcript spaced 15 bp apart from the central loops (cadRNA.interspersed) would afford the greatest chance at eliminating hyperediting across the transcript. Excitingly, delivery of cadRNA.interspersed dramatically reduced bystander editing

across the entire transcript in comparison to delivery of unmodified cadRNA (**Figure 2c**). We further investigated the percentage of amplicons that were perfectly edited containing no bystander edits via Next-Generation Sequencing and found that approximately 9% of all reads were perfect using cadRNA.interspersed designs in comparison to almost 0% for the unmodified cadRNA design (**Figure 2d)**. In order to ensure that our modified design works on multiple transcripts, we transfected HEK293FT cells with cadRNA.loops.interspersed targeting four additional transcripts – FANCC, CTNNB1, SMAD4, and TARDBP, and observed robust editing efficiencies in all transcripts excluding TARDBP (**Figure 2b)**.

Figure 2: In *vitro* efficacy and transcript-level specificity of cadRNAs
(a) Schematic of various cadRNA designs with engineered secondary structures for the reduction of bystander adenosine editing. cadRNA: unmodified 200 bp long antisense complementary to transcript of interest. cadRNA.bulges: bulge secondary structures are created by positioning guanosines positioned opposite commonly hyperedited adenosines. cadRNA.loops: loop secondary structure created by mismatching 4 bp to the transcript of interest at positions -5 and +30 relative to the target adenosine of interest. cadRNA.loops.interspersed: loops placed in 15 bp intervals alongside the entire antisense on both sides of the central loops flanking the target adenosine in the cadRNA.loops design. **(b)** RNA Editing efficiencies of various endogenous transcripts in HEK293FT and K562 cells after 48 hours post-transfection of cadRNA or cadRNA.loops.interspersed designs in Figure 2a. Values represent mean +/- SEM (n=3). **(c)** Plots of the bystander editing profiles of the indicated cadRNA design showing degree of hyperediting in a specific position relative to the target adenosine (n = 1 for plots shown representative of each design). **(d)** Plot shows percentage of reads containing the indicated amounts of bystander edits within the 200 bp antisense region. Values represent mean +/- SEM on-target editing quantified by NGS (n=3).


a
1. cadRNA
Target mRNA
Antisense
2. cadRNA.bulges: G-mismatches opposite edited As
3. cadRNA.loops: Positions (-5, +30)
-5
+30
4. cadRNA.loops.interspersed: Positions (-5, +30), then every +/- 15 bp
15 bp
15 bp
-5
+30
15 bp
15 bp
b
% edited mRNA
HEK293FT
K562
cadRNA
cadRNA loops interspersed
% edited mRNA
RABTA (3' UTR)
GAPDH (CDS)
ALDOA (CDS)
DAXX (CDS)
FANCC (3' UTR)
CTNNB1 (3' UTR)
SMAD4 (CDS)
TARDBP (CDS)
RAB7A (3' UTR)
c
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cadRNA
cadRNA bulges
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Deletions
Substitutions
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36%
24%
12%
0%
-100
0
+100
Location relative to target
d
additional bystander edits
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2
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4+
% on-target edited mRNA (GAPDH)
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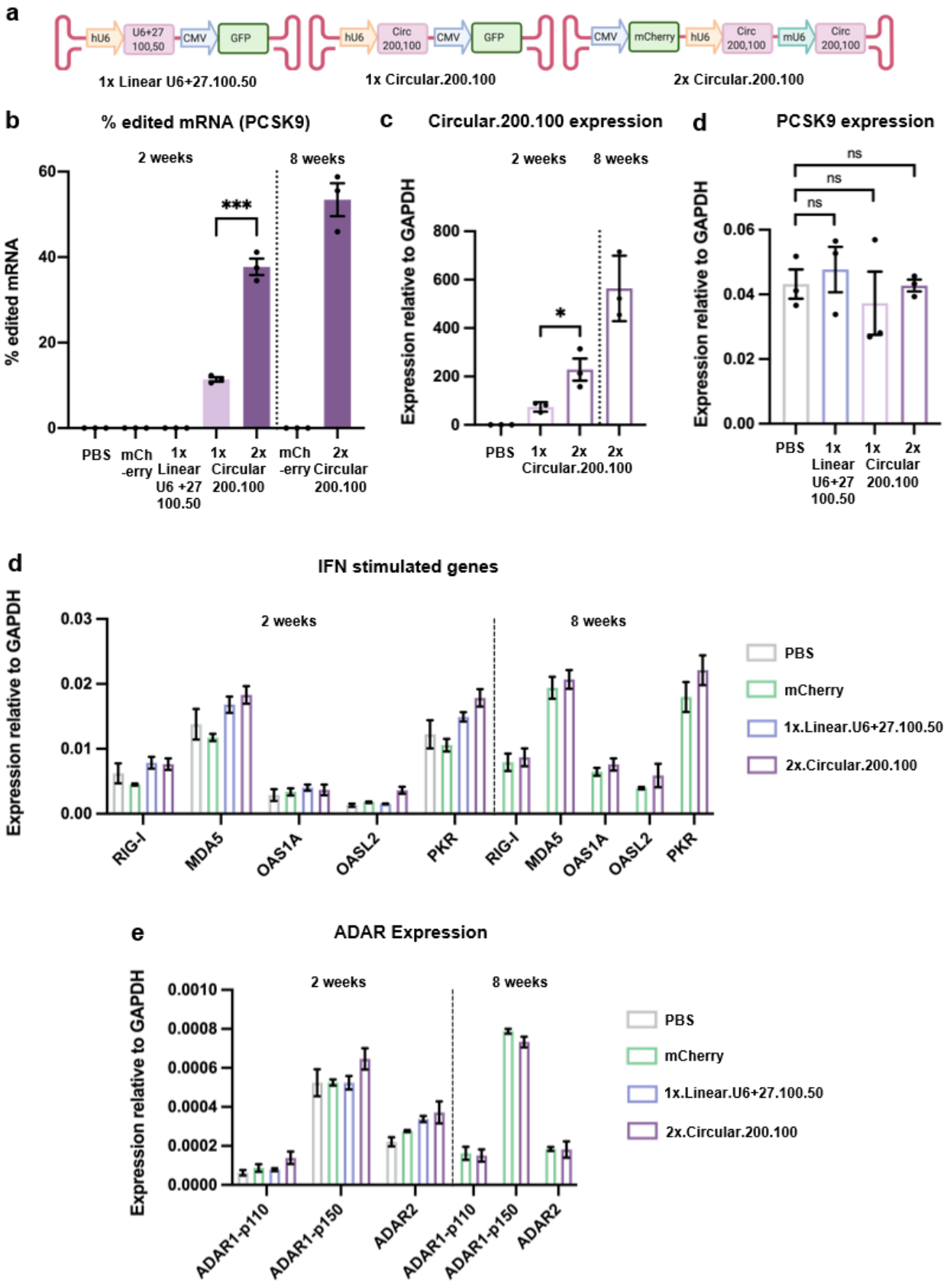
cadRNA
cadRNA.bulges
cadRNA.loops
cadRNA.loops.interspersed

Validation of *in vivo* efficacy and safety of cadRNAs

Having seen robust in vitro editing efficacy, we next investigated the potential of cadRNAs to induce in vivo editing. Towards this, we packaged single (1x) and dual (2x) copies of cadRNAs into AAV8 targeting an adenosine in the 3'UTR region of the mPCSK9 transcript and delivered our circular200.100 cadRNA retro-orbitally into mice. Two weeks post-injection, the livers of the mice were harvested and successful expression and circularization of cadRNA was confirmed via RT-qPCR using outward facing primers (**Figure 3c**). Encouraged by these results, we next investigated in vivo editing efficiency via Sanger sequencing and excitingly observed 11% and 38% target editing efficiencies for respective single and dual cadRNAs. Furthermore, we observed persistence of editing up to 8 weeks post-injection with average editing efficiencies of 53% (**Figure 3b**). Interestingly, cadRNA expression seemed to more than double over the course of 8 weeks, possibly as a result of the accumulation of the cadRNAs within the liver due to their higher stability and residence times. Finally, we confirmed via RT-qPCR that delivery of our cadRNAs did not significantly alter mPCSK9 expression levels (**Figure 3d**). We further investigated the credibility of our observed editing values in the circular200.100 treated group by looking at the expression of the ADAR1-p110, ADAR1-p150, and ADAR2 compared to an AAV8-mCherry control group. RT-qPCR results confirmed that delivery of circular200.100 did not significantly change the ADAR expression profile, supporting that our observed editing values are not an artifact of increased expression of ADARs (**Figure 3d)**. Lastly, we investigated the expression profile of a panel of IFN-stimulated genes[23] that are also involved in sensing dsRNA and found an increase in expression in MDA5 and PKR in the two week timepoint experiments (Figure X). Interestingly however, we did not observe significant upregulation in any of these genes in the longer term eight-week timepoint experiments (**Figure 3e**).

Figure 3: *In vivo* RNA editing of mPCSK9
(a) Schematic of vectors used in the production of AAV8 for subsequent retro-orbital delivery into C57BL/6J. **(b)** In vivo RNA editing efficiencies of mPCSK9 transcript in mouse livers 2 weeks and 8 weeks post-injection for indicated constructs. Values represent mean +/- SEM (n=3; p=0.0305; unpaired t-test, two-tailed). **(c)** Expression of cadRNAs relative to mGAPDH. Values represent mean +/- SEM (n=3; p=0.6179, p=0.6125, p=0.9323; unpaired t-test, two-tailed). **(d)** Expression of mPCSK9 transcript relative to mGAPDH. Values represent mean +/- SEM (n=3; p=0.6179, p=0.6125, p=0.9323; unpaired t-test, two-tailed). **(e)** Expression profiles of IFN-stimulated genes involved in dsRNA sensing relative to mGAPDH 2 weeks and 8 weeks post-injection. Values represent mean +/- SEM (n=3; p-values for 2 week long experiment, 2x.circular.200.100 vs mCherry, for genes from left to right p=0.0721, p=0.0353, p=0.8082, p=0.0748, p=0.0303; p-values for 8 week long experiment, 2x.circular.200.100 vs mCherry, for genes from left to right p=0.7276, p=0.6020, p=0.3838, p=0.3491, p=0.2746; unpaired t-test, two-tailed). **(f)** Expression of ADAR1-p110, ADAR1-p150, and ADAR2 relative to mGAPDH 2 weeks and 8 weeks post-injection. Values represent mean +/- SEM (n=3; p-values for 2 week long experiment, 2x.circular.200.100 vs. mCherry, for ADAR variants from left to right p=0.3165, p=0.1885, p=0.2815; p-values for 8 week long experiment, 2x.circular.200.100 vs. mCherry, for genes from left to right p=0.8150, p=0.1440, p=0.9532; unpaired t-test, two-tailed).


a
hU6
U6+27 100,50
CMV
GFP
1x Linear U6+27.100.50
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GFP
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mCherry
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% edited mRNA (PCSK9)
2 weeks
8 weeks
% edited mRNA
***
PBS
mCh -erry
1x Linear U6 +27 100.50
1x Circular 200.100
mCh -erry
2x Circular 200.100
c
Circular.200.100 expression
2 weeks
8 weeks
Expression relative to GAPDH
*
PBS
1x Circular.200.100
2x Circular.200.100
d
PCSK9 expression
ns
ns
ns
Expression relative to GAPDH
PBS
1x Linear U6+27 100.50
1x Circular 200.100
2x Circular 200.100
d
IFN stimulated genes
2 weeks
8 weeks
Expression relative to GAPDH
RIG-I
MDA5
OAS1A
OASL2
PKR
PBS
mCherry
1x.Linear.U6+27.100.50
2x.Circular.200.100
e
ADAR Expression
2 weeks
8 weeks
Expression relative to GAPDH
ADAR1-p110
ADAR1-p150
ADAR2
PBS
mCherry
1x.Linear.U6+27.100.50
2x.Circular.200.100

In vivo RNA editing of IDUA-W392X mouse model of MPS I-H syndrome

To demonstrate the therapeutic potential of delivering cadRNAs, we applied our platform to the IDUA-W392X mouse model for MPS I-H, targeting the adenosine in a nonsense mutation that causes premature termination in the translation of alpha-L-iduronidase proteins. Lack of this key enzyme results in a buildup of glycosaminoglycan (GAG) sugar molecules in lysosomes. To repair the mutation resulting in a premature stop codon, two copies of circular200.100 cadRNA targeting the mutated IDUA site were packaged into AAV8 and injected retro-orbitally into IDUA-W392X mice. For the control mice, we packaged two copies of a circular200.100 cadRNA containing a scrambled sequence orthogonal to the transcriptome into AAV8 and delivered them in the same manner. Mouse livers were harvested two weeks post-injection and we observed an average of 12.5% on-target editing corresponding to repair of the premature stop codon (**Figure 4a)**. Excitingly, we observed approximately 33% less GAG accumulation in the treated mice compared to the scrambled control, indicative of partial restoration of enzymatic activity **(Figure 4c)**.

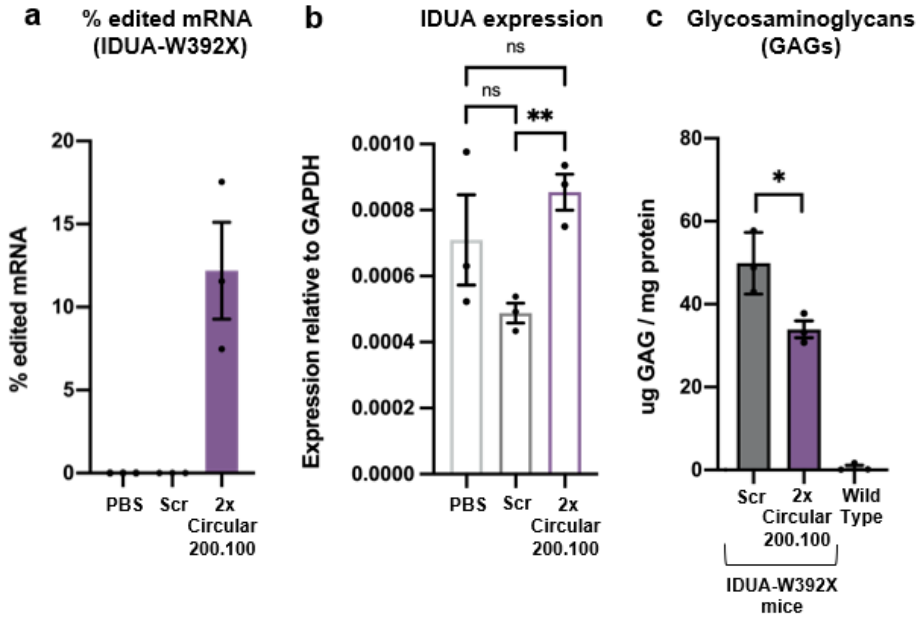

Figure 4: *In vivo* repair of IDUA-W392X nonsense mutation
(a) In vivo RNA editing efficiencies of mIDUA transcript in IDUA-W392X mice livers 2 weeks post-injection of PBS, AAV8 packaging two copies of a scrambled guide RNA, and AAV8 packaging two copies of circular.200.100 cadRNAs targeting mIDUA. Values represent mean +/- SEM (n=3). **(b)** Expression of mIDUA transcript relative to mGAPDH. Values represent mean +/- SEM (n=3; p=0.1185, p=0.3815, p=0.0042; unpaired t-test, two-tailed). **(c)** GAG content of IDUA-W392X mice livers 2 weeks post-injection. GAG content of wild-type C57BL/6J mice is included in the plot as a positive control. Values represent mean +/- SEM (n=3; p=0.0285; unpaired t-test, two-tailed).

Discussion

The recruitment of endogenous ADARs to repair pathogenic adenosine mutations presents a safe and efficacious alternative to the genome editing tools currently available in the gene therapy field. The experiments performed in this thesis, for the first time, provide the framework for high levels of transcript-specific RNA editing in vivo using the recruitment of endogenous ADARs. Importantly, we have shown that our platform can be applied in the context of a genetic disorder, partially restoring alpha-L-iduronidase activity in the livers of IDUA-W392X mice. Our circular guide RNAs can also be further engineered to include interspersed secondary structures in the form of loops that significantly reduce bystander editing within the transcript.

Our studies have demonstrated that delivery of circular antisense guide RNAs are an effective way to induce RNA editing in vivo, however there are many more aspects to the technique that need to be further improved and investigated. Although we are able to observe editing for a wide variety of different transcripts, interestingly, editing efficiencies often vary between transcripts. Understanding the mechanism behind this variation can possibly lead to improvement in cadRNA design and increased editing yields for transcripts that are currently not edited as frequently. Similarly, further modifications to the cadRNA can also be explored in the context of both increasing editing efficiency as well as reducing bystander adenosine editing. Well-known ADAR recruitment domains can likely be added to the delivered cadRNA to boost recruitment and correspondingly, editing efficiency[24,25]. In terms of further reducing bystander editing, engineering a combination of interspersed loops and mismatched guanosines opposite commonly hyperedited adenosines can likely further reduce the bystander editing profile while maintaining high levels of on-target editing efficiency[14,22,26]. Shortening the antisense domain length can similarly reduce hyperediting by decreasing the number of adenosines able to be edited, albeit likely at a detriment to editing yields.

Moving forward, an important issue that needs to be addressed when evaluating cadRNA delivery as a potential therapeutic to be used in the clinic is our current method of delivery. Delivery of cadRNAs via AAV8 does not allow for a secondary dose to be administered to a patient due to the development of neutralizing antibodies against AAV8 after initial dosing[27,28]. Although we observe persistent cadRNA expression in our studies, in the event that expression fades over time, inability to redose would likely result in a return of the disease phenotype due to the transient nature of RNA editing. Correspondingly, different methods of delivery should be examined that allow for redosing, for example lipid nanoparticles[29]. Altogether, the delivery of cadRNAs to induce transcript-specific RNA editing is a promising new tool that can be used in gene therapy.

This work, in part, has been submitted for publication of the material as it may appear in Nature Biotechnology, 2021, Katekar, Dhruva; Yen, James; Xiang, Yichen; Saha, Anushka; Savva Yiannis, Mali, Prashant. The thesis author was a researcher for this material.

Methods

Transfections

Unless otherwise stated, experiments were carried out in HEK293FT cells which were grown in DMEM supplemented with 10% FBS and 1% Antibiotic-Antimycotic (Thermo Fisher) in an incubator at 37 °C and 5% CO_2 atmosphere. HEK293FT cells were seeded in 24 well plates and transfected using 1000 ng adRNA plasmid or 48 pmol of IVT RNA and 2ul of commercial transfection reagent Lipofectamine 2000 (Thermo Fisher). Cells were transfected at 25-30% confluence. Plasmid transfection experiments were harvested 48 hours post transfections while IVT RNA experiments were harvested 24 hours post transfections. For 96 hour long experiments, cells were passaged at a 1:4 ratio, 48 hours post transfections. Cells after plasmid electroporation were harvested at 48 hours, while IVT RNA experiments were harvested 24 hours post electroporation.

In vitro transcription

Sense RNA fragments and circular adRNA were made by *in vitro* transcription using the HiScribe T7 Quick High Yield RNA Synthesis Kit (NEB) as per the manufacturer's protocol. DNA templates for the IVT reaction carried the T7 promoter sequence at the 5’ end and were created by PCR amplification of the desired sequence from plasmids or cDNA. PCR products were purified using a PCR Purification Kit (Qiagen) and then used for IVT.

Production of AAV vectors

AAV8 particles were produced using HEK293FT cells via the triple-transfection method and purified via an iodixanol gradient. Confluency at transfection was about 50%. Two hours before transfection, cell medium was exchanged with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100X Antibiotic-Antimycotic (Gibco). All viruses were produced in 5×15 cm plates, where each plate was transfected with 10 µg of pXR-8, 10 µg of recombinant transfer vector and 10 µg of pHelper vector using polyethylenimine (PEI) (1 µg/µl linear PEI in ultrapure water, pH 7, using hydrochloric acid) at a PEI:DNA mass ratio of 4:1. The mixture was incubated for 10 minutes at room temperature and subsequently applied dropwise onto the cell media. The virus was harvested after 72 hours and purified using an iodixanol density gradient ultracentrifugation method. The virus was then dialyzed with 1× phosphate buffered saline (pH 7.2) supplemented with 50 mM sodium chloride and 0.0001% Pluronic F68 (Thermo Fisher) using 50 kDA filters (Millipore), to a final volume of ~1 ml, and quantified by quantitative PCR using primers specific to the ITR region, against a standard (ATCC VR-1616): AAV-ITR-F, 5′-CGGCCTCAGTGAGCGA-3′; AAV-ITR-R, 5′-GGAACCCCTAGTGATGGAGTT-3′.

Animal experiments

All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. All mice were acquired from Jackson Labs. AAVs were injected retro-orbitally into both C57BL/6J mice and IDUA-W392X mice (B6.129S-Iduatm1.1Kmke/J), 6-8 weeks of age, at a dose of 1.0E13 vector genomes per mouse. Mice were monitored three times a week for the duration of the experiment (2 weeks).

GAG assay

The GAG assay was performed following the protocol described in[18]. Briefly, harvested mouse tissues were homogenized in 1 ml PBS with a syringe and 16 gauge (1.6 mm) needle. Tissue homogenates were then incubated on ice for 20 min with Triton X-100 added to a final concentration of 1%. Protein concentration in the supernatant clarified via centrifugation was estimated using the Bradford assay. Supernatants were digested in 1 mg/ml Proteinase K (Qiagen) for 12 h at 55 ◦C then boiled for 10 min to inactivate the enzyme. Nucleic acids were digested using Benzonase nuclease (Sigma) at 37 ◦C for 1 h followed by 10 min boiling to inactivate the enzyme. Total amount of GAG in each sample was measured using the Blyscan GAG assay kit (Biocolor).

RNA extraction and quantification of editing

RNA from cells was extracted using the RNeasy Mini Kit (Qiagen) while extraction from tissues was carried out using QIAzol Lysis Reagent and purified using RNeasy Plus Universal Mini Kit (Qiagen), according to the manufacturer's protocol. 500-1000 ng RNA was incubated with 1 µl of 5 µM of a target specific sense RNA (synthesized via IVT) at 95 ◦C for 3 minutes followed by 4 ◦C for 5 minutes. This step was carried out to capture the circular adRNA which if tightly bound to the target mRNA would block reverse transcription. cDNA was then synthesized using the Protoscript II First Strand cDNA synthesis Kit (NEB). 1 µl of cDNA was amplified by PCR with primers that amplify about 300-600 bp surrounding the sites of interest (outside the length of the antisense domain) using OneTaq PCR Mix (NEB). The numbers of cycles were tested to ensure that they fell within the linear phase of amplification. PCR products were purified using a PCR Purification Kit (Qiagen) and sent out for Sanger sequencing. The RNA editing efficiency was quantified using the ratio of peak heights G/(A+G). RNA-seq libraries were prepared from 250 ng of RNA, using the NEBNext Poly(A) mRNA magnetic isolation module and NEBNext Ultra II

Directional RNA Library Prep Kit for Illumina. Samples were pooled and loaded on an Illumina Novaseq 6000 (100 bp paired-end run) to obtain 40-45 million reads per sample.

qPCRs

1 µl of 1:4 diluted cDNA was used to set up a 10 µl qPCR reaction using iTaq Universal SYBR Supermix (Biorad). Primers were designed to keep the amplicon length within 300 bp. 2 technical replicates were carried out for each sample.

This work, in part, has been submitted for publication of the material as it may appear in Nature Biotechnology, 2021, Katekar, Dhruva; Yen, James; Xiang, Yichen; Saha, Anushka; Savva Yiannis, Mali, Prashant. The thesis author was a researcher for this material.

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