Exploring the Epitranscriptome: Properly Capped and Chemically Modified Cas9 mRNAs For Genome Editing as a Case Study

Dongwon Shin[1], Sriram Vaidyanathan[2], Krist T. Azizian[1], Jordana M. Henderson[1], Matthew H. Porteus[2], Richard I. Hogrefe[1], Anton P. McCaffrey[1] and Alexandre Lebedev[1]

[1]TriLink BioTechnologies, San Diego, CA 92121, USA.
[2]Department of Pediatrics, Stanford University, Stanford, California, USA.

Abstract

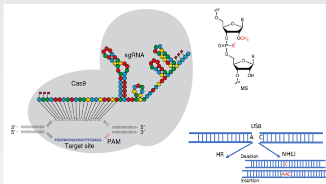
DNA genomes are regulated by epigenetic modification. Similarly, post-transcriptional messenger RNA (mRNA) modification mediates self/non-self recognition, translational regulation, decapping and mRNA stability. Still, our understanding of the "epitranscriptome" is in its' infancy. mRNA therapeutics have become popular for their ability to transfect non-dividing cells and because they cannot insert in genomes. As mRNAs enter the clinic for genome editing, gene replacement and vaccines, safe application requires understanding how cells and organisms interact with the epitranscriptome.

Transfected mRNAs must avoid detection by pattern recognition receptors (PRRs) that sense improperly capped or double stranded RNA. PRR activation leads to cytokine production, translational arrest and toxicity. mRNAs are post-transcriptionally modified [e.g. pseudouridine (Ψ) and 5-methylcytidine (5meC)] and these modifications reduce activation of PRRs by transfected mRNA.

Capped mRNAs (m^7GpppN = Cap 0) are methylated at the 2′ position ($m^7GpppNm$) to form Cap 1 to mark them as self mRNAs. mRNAs generated with commercial cap analogs are Cap 0 and may be recognized as viral pathogens. Capping enzymes used to make Cap 1 are costly and capping is variable. We recently developed a novel co-transcriptional capping method (CleanCap™) that yields Cap 1 with high efficiency and lower costs in a "one pot" reaction.

CRISPR/Cas9 allows facile gene inactivation or genome engineering. Both require delivery of Cas9 protein and a RNA guide to the nucleus. Often for clinical applications, a chemically synthesized guide RNA is co-transfected with Cas9 mRNA. We applied our knowledge of the epitranscriptome to generate more effective Cas9 mRNAs. First generation Cas9 mRNAs were modified with Ψ and 5meC and had Cap 0 structures. We prepared improved second generation Cap 1 mRNAs by sequence engineering and screening chemical modifications and significantly improved indel formation in primary CD34+ cells.

CRISPR/Cas9



- Cas9 endonuclease from S. pyogenes can be directed to induce double stranded breaks (DSB) at a specific location using a guide RNA (sgRNA)
- Chemical modification of three nucleotides at the ends of the sgRNA results increased DSBs (Hendel *et al.* Nat Biotechnol. 2015 (9):985-9)
- INDELS generated by the NHEJ pathway were quantified as a measure of Cas9 activity

Background: Why mRNA Therapeutics?

mRNA is a popular new tool for gene expression because it:

- Does not have a risk of insertional mutagenesis
- Can transfect difficult cells such as non-dividing cells
- Is transient

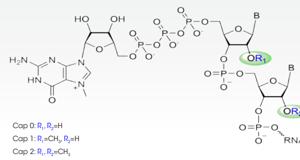
- Applications
  - Genome editing (Transposons, Cre, ZFNs, TALENs and CRISPR/Cas9)
  - Gene replacement
  - Vaccines
- Limitations
  - Innate immune response to unmodified mRNA
- Solutions
  - Proper capping
  - Chemical modification and sequence optimization of mRNA can prevent innate immune stimulation
  - Removal of dsRNA

Innate Immune Sensors (PRRs)

- Endosomal sensors
  - Toll-like receptors 3, 7 & 8 recognize different RNA forms
- Cytosolic sensors
  - Protein Kinase R (PKR): dsRNA | MDA5: dsRNA | IFITs: unmethylated cap structures | RIG-I: 5′ triphosphate | cGAS/STING | cytosolic DNA

Figure 1: Eukaryotic Cap Structures

- 100 % of eukaryotic mRNAs are Cap 1 and ~50% are Cap 2
- Traditional co-transcriptional capping with ARCA yields Cap 0 which is immunogenic
- mRNA cap structures are involved in modulating
  - Nuclear Export - Splicing - RNA Turnover - Translational Regulation
  - Cap 0 recognized as foreign
- Cap 1 and Cap 2 are important for self/non-self recognition by the innate immune system
  - IFITs recognize non-methylated caps
- Role of Cap 2 is largely unexplored

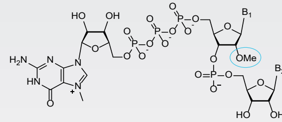


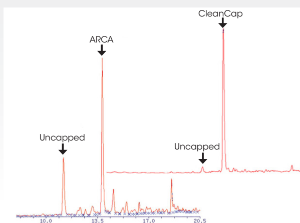
Cap 1 Does Not Activate PRRs

- IFIT-1 has reduced binding for Cap 1 and Cap 2
  - Abbas *et al.* Proc Natl Acad Sci U S A. 2017;114(11):E2106-E2115
- IFIT-5 binds 5′-p, 5′- ppp and Cap 0 but not Cap 1
  - Katibah *et al.* Proc Natl Acad Sci U S A. 2014;111(33):12025-30
- RIG-I is not activated by Cap 1 double stranded RNA
  - Schuberth-Wagner et al. Immunity. 2015;43(1):41-51

Figure 2: CleanCap™

- Co-transcriptional capping with CleanCap trimer yields Cap 1
- Capping efficiency much higher than ARCA





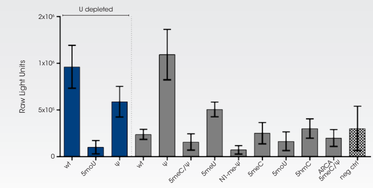
Chemical Modification of mRNA Hides It From Innate Immune Sensors

- Modification of mRNAs with pseudouridine:
  - Reduced binding to innate immune sensors *in vitro*
  - Reduced toxicity
  - Prolonged expression in cultured cells and *in vivo*
  - Pseudo U modification increased translation *in vitro*
  - Kariko *et al.*
    - Mol Ther. 2008 (11):1833-40
    - Immunity. 2005 (2):165-75
    - Nucleic Acids Res. 2010 38 (17):5884-92
- Can we identify chemical modifications of Cas9 that are similar or superior to pseudouridine?

Figure 3: Modifications Screened

Abbreviation	Full Name	Uridine depleted	Cap Form	Screen
wt	wild type bases	No	Cap 1	Full
Ψ	pseudouridine	No	Cap 1	Full
5meC/Ψ	5-methyl cytidine/pseudouridine	No	Cap 1	Full
5meU	5-methyl uridine	No	Cap 1	Full
N1-me-Ψ	N1-methyl pseudouridine	No	Cap 1	Full
5meC	5-methyl cytidine	No	Cap 1	Full
5hmC	5-hydroxymethyl cytidine	No	Cap 1	Full
N1-et-Ψ	N1-ethyl pseudouridine	No	Cap 1	Initial
5moU	5-methoxy uridine	No	Cap 1	Initial
me1-Ψ/5meC	N1-methyl pseudouridine/5-methyl cytidine	No	Cap 1	Initial
5moC	5-methoxy cytidine	No	Cap 1	Initial
5camU	5-carboxy methyl ester uridine	No	Cap 1	Initial
10% 5meC/2sU	5-methyl cytidine/2-thio uridine	No	Cap 1	Initial
25% 5meC/2sU	5-methyl cytidine/2-thio uridine	No	Cap 1	Initial
ARCA 5meC/Ψ	5-methyl cytidine/pseudouridine	No	Cap 0	Initial
wt U.D.	wild type bases	Yes	Cap 1	Full
5MoU U.D.	5-methoxy uridine	Yes	Cap 1	Full
Ψ U.D.	pseudouridine	Yes	Cap 1	Full

Figure 4: Interferon Signaling in Differentiated THP-1 Dual Cells



$2x10^5$ cells THP-1 Dual cells (Invivogen) per well were seeded in a 24-well plate format and allowed to differentiate in culture for 72 hours using 200 nM 12-Tetradecanoylphorbol-13-Acetate (Cell Signaling Technology)). Cells were transfected using 100 ng RNA and mRNA-In (MTI-GlobalStem). Lucia luciferase was assayed using QUANTI-Luc coelenterazine luciferase substrate (InvivoGen) as a readout for interferon responsive promoter activity.

Figure 5: CD34+ Transfection Graphic

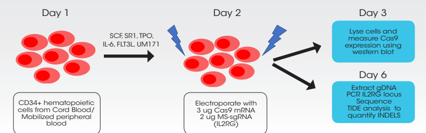
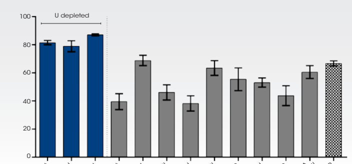


Figure 6: Indel Formation for Top Ten Candidates in CD34+ Cells



Conclusion

- mRNA is an attractive tool for expressing Cas9 in cells for genome editing
- Here we introduce a novel co-transcriptional capping method (CleanCap™) that produces Cap 1 mRNAs with high capping efficiencies
- Uridine depletion increased Cas9 activity
- Indel formation did not correlate with interferon stimulation
- Indel formation roughly correlated with Cas9 protein levels in CD34+ cells
- wt, Ψ and 5moU in U depleted Cas9 gave indel frequencies of ~87%
- In comparison, Cas9 delivered as a ribonuclear protein complexed with guide gave indel frequencies of ~67%

Matthew Porteus
mporteus@stanford.edu
Anton McCaffrey
amcaffrey@trilinkbiotech.com
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