Maximizing Translation of Cas9 mRNA Therapeutics by Sequence Engineering and Chemical Modification

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Abstract

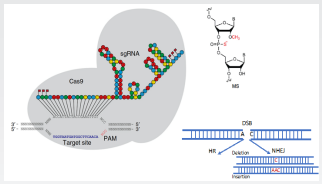
Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 is an exciting new system for facile gene inactivation or genome engineering. Both types of applications require delivery of Cas9 protein and a RNA guide to the nucleus of cells. Often for clinical applications, a chemically synthesized guide RNA is co-transfected with a Cas9 messenger RNA (mRNA) into cells or organisms.

For maximal expression in cells or target organs, transfected mRNAs must avoid detection by pattern recognition receptors (PRRs) that evolved to sense improperly capped RNAs and double stranded RNA. PRR activation leads to cytokine production, translational arrest and cell toxicity or death. Mammalian mRNAs are modified post-transcriptionally to contain modified nucleotides (e.g. pseudouridine and 5-methylcytidine). These modifications can reduce activation of PRRs and allow maximal translation of the transfected mRNA.

During RNA capping, Cap0 (m7GpppN) is formed as an intermediate. Methylation of the 2' position of the first nucleotide forms Cap1, which is found in ~50% of endogenous mRNAs. mRNAs generated with commercially available cap analogs (ARCA) contain Cap0 structures that can be immunogenic. Recombinant enzymes used to generate Cap1 mRNA are expensive, do not always go to completion and the RNA must be purified prior to capping. We recently developed a novel co-transcriptional capping method called CleanCap that yields Cap1 with high efficiency and lower costs in a "one pot" reaction.

First generation Cas9 mRNAs were modified with pseudouridine or 5-methylcytidine/pseudouridine and had Cap0 structures. We generated improved second generation Cap1 mRNAs through a combination of sequence engineering and screening of chemical modifications. In all, 18 combinations were tested with and without HPLC purification. These RNAs were assayed for double stranded RNA contamination, translation efficiency in wheat germ extracts, protein expression and interferon production in THP-1 cells and indel formation in primary CD34+ cells. Interestingly, wild-type, pseudouridine and 5-methyluridine containing mRNAs induced and interferon response in differentiated THP-1 cells. Through these studies we were able to dramatically increase indel formation in CD34+ cells relative to first generation mRNAs. Indel formation as high as ~85% was observed. By comparison, recombinant Cas9 protein complexed to guides gave ~65% indel formation. Interestingly, some modified RNAs did not require HPLC purification for maximal activity.

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

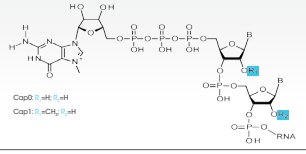
- Applications
  - Genome editing (Transposons, Cre, ZFNs, TALENs and CRISPR/Cas9)
  - Gene replacement
  - Vaccines
- Limitations
  - Innate immune response to unmodified mRNA
- Solutions
  - Proper capping
  - Chemical modification and sequence optimization of mRNA can prevent innate immune stimulation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

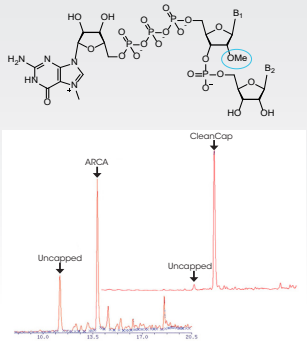


Cap 1 Does Not Activate PRRs

- 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 Cap1
  -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™

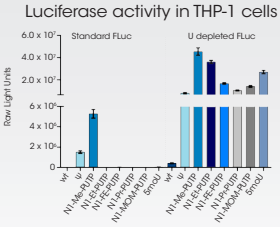
- Co-transcriptional capping with CleanCap trimer yields Cap1
- Capping efficiency much higher than ARCA



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: Uridine Depletion Using Synonymous Codons Increases Activity and Expands Allowable Bases: Luciferase Example

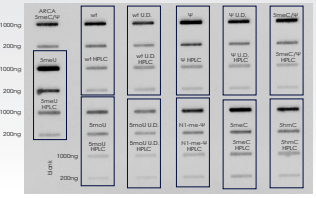


- Pseudouridine derivatives; H = pseudouridine, Me = N1-Methyl, Et = N1-Ethyl, FE = Fluoroethyl, Pr = Propyl, iPr = Isopropyl, MOM = Methoxy methyl, POM = Pivaloxy methyl, BOM = Benzyloxy methyl pseudouridine
- Will uridine depletion improve activity of Cas9?

Figure 4: Modifications Screened

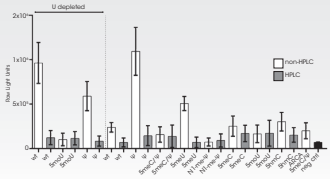
Abbreviation	Full Name	Uridine depleted	Cap Form	Screen
wt	wild type bases	No	Cap 1	Full
Ψ	pseudouridine	No	Cap 1	Full
5meC/Ψ	5-methyl cytidine/pseudouridine	No	Cap 1	Full
5moU	5-methyl uridine	No	Cap 1	Full
N1-me-Ψ	N1-methyl pseudouridine	No	Cap 1	Full
5meC	5-methyl cytidine	No	Cap 1	Full
5hmC	5-hydroxymethyl 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 5: Reverse Phase HPLC Decreases dsRNA as Measured by Slot Blot With a dsRNA Specific Antibody



Adapted from Weissman *et al.* Methods Mol Biol. 2013; 969: 43-54

Figure 6: Interferon Signaling in Differentiated THP-1 Dual Cells



2x10⁵ 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 7: CD34+ Transfection Graphic

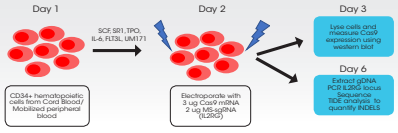


Figure 8: Indel Formation for Top Ten Candidates in CD34+ Cells

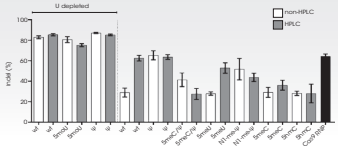
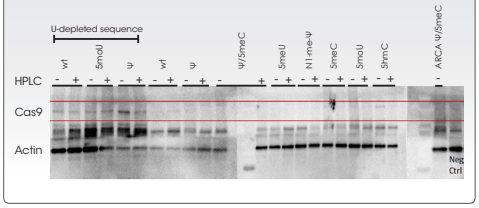


Figure 9: Cas9 Western Blot in CD34+ Cells



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 Cap1 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
- HPLC purification decreased the amount of double stranded RNA in mRNA preparations
- For a subset of modifications, HPLC purification:
  - Improved activity (WT and 5meU)
  - Lowered interferon stimulation in THP-1 cells (e.g. Ψ, 5meU, Ψ U.D. and WT U.D.)
- WT, Ψ and 5moU in U depleted Cas9 gave indel frequencies of ~80%
- In comparison, Cas9 delivered as a ribonuclear protein complexed with guide gave indel frequencies of ~65%

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