Making the Optimal Messenger RNA for Gene Therapy Applications: Evaluation of Novel Nucleotide Modifications for Improved Activity

Krist Azizian[1], Alexandre Lebedev[1], Dongwon Shin[1], Julie R. Escamilla-Powers[1], Brea Midthune[1], Hiroko Yokoe[2], Jonathan Kirschman[3], Philip Santangelo[3], Richard I Hogrefe[1], Anton P. McCaffrey[1] and Joel Jessee[2]
[1]TriLink BioTechnologies, San Diego CA 92121, USA. [2]Molecular Transfer, Gaithersburg MD 20877, USA. [3]Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University.

TriLink BIOTECHNOLOGIES

Abstract

Recently, there has been significant interest in the use of messenger RNA (mRNA) based expression systems for gene therapy applications. Several groups have shown that mRNA is an attractive vehicle for therapeutic gene expression in mammals (Kormann et al. Nat. Biotechnol (2011) 29, 154; Kariko et al. Molecular Therapy (2012) 20, 948). mRNAs are expressed in the cytoplasm of cells which may improve expression in non-dividing cells, which are difficult to transfect. Additionally, Warren et al. demonstrated highly efficient induced pluripotent stem cell (iPS cells) generation by transfection of mRNAs encoding reprogramming factors (Warren et al. Cell Stem Cell (2010) 7, 618). The authors suggested that iPS cells generated in this manner should be safer than iPS cells derived by plasmid transfection or viral transduction since mRNA poses no risk of insertional mutagenesis and subsequent oncogenesis. In addition, transient expression from mRNA is desirable for applications such as genome editing using zinc-finger nucleases, TALENs and Cas9/CRISPR. Lastly, there is considerable interest in using mRNA for vaccines (Geall et al. Expert Rev Vaccines (2015) 14, 151).

A key insight for the development of mRNA expression systems was the recognition that mRNA induces innate immune responses in transfected cells. Kariko et al. showed that substitution of uridine and cytidine residues with pseudouridine and 5-methylcytidine dramatically reduced innate immune recognition of mRNA (Kariko et al. Molecular Therapy (2008) 16, 1833). They also showed that pseudouridine modified RNA was translated more efficiently.

These studies highlight the importance of the development of stable, non immunogenic mRNA. Activity and immunogenicity of mRNAs likely depends on the chemical modification pattern, the route of delivery and cell type or tissue transfected. To date, however, there have been few studies to assess novel chemically modifications of mRNAs. Here we synthesized a panel of numerous novel nucleotide triphosphates (NTPs). The panel consisted primarily of 5-position-modified pyrimidines but also included other pyrimidine and purine NTPs. A large number of combinations of the modifications were used to synthesize GFP and luciferase mRNAs in order to evaluate the ability of different combinations of modifications to support transcription by T7 RNA polymerase. The translation potential of the mRNAs was evaluated in rabbit reticulocyte lysates. The activity of these mRNA was further evaluated in panels of different primary and immortalized cell lines. Comparison of in vitro translation with cell culture expression allows uncoupling of translation from cytotoxicity, RNA stability and innate immune stimulation in cells. While trends were seen, cell type specific differences in expression were also observed. These studies greatly expand our knowledge of the optimal chemical modification of mRNA required to achieve maximal expression in different cell types.

Background

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
- Chemical modification of mRNA can prevent innate immune stimulation
- Pseudouridine or 5-methylcytidine/pseudouridine are current industry standard

Objectives

To identify novel patterns of chemical modifications for optimal mRNA based therapeutics.
- Reduce innate immune responses and toxicity
- Maximize extent and duration of expression
- Identify novel modifications for use in aptamer selections

Figure 1: Modified Bases Assessed

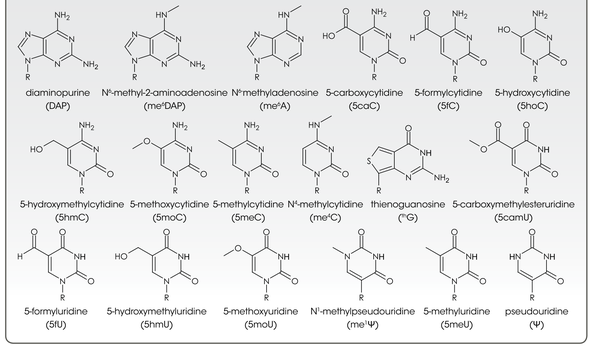


Figure 2: In Vitro Translation of Modified mRNA in Rabbit Reticulocytes

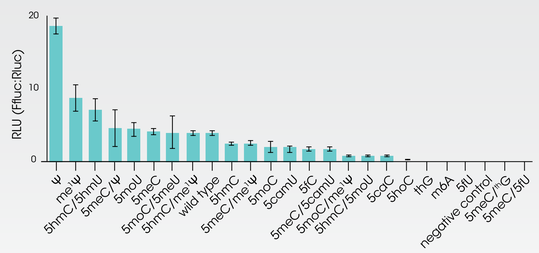
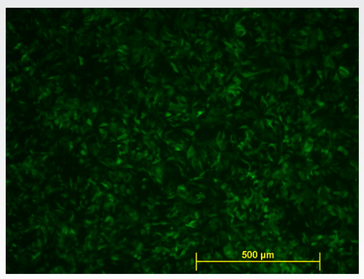


Figure 3: mRNA-In™ Transfection Reagent

- Product of Molecular Transfer Inc.
- Cationic lipophilic nanoparticle
- Transfects a variety of cell lines with as little as 2 pg/cell of mRNA



EGFP 24 hours post-transfection

Cell Culture Evaluation of Modified mRNA

Cell Lines Tested
- Adipose mesenchymal cells (AdMSC)
- Adult human dermal fibroblast cells (HDF)
- Human cervical carcinoma cells (HeLa)/
- Human umbilical vein endothelial cells (HuVEC)
- Human neural stem cells (NSC)
- Human neuroblastoma cells (SY5Y)

Some Modifications Had No Activity
- 5fC
- 5hoC
- N⁶-Methyl-ATP
- N⁶-Me-2-amino-ATP
- N⁴-Methyl-CTP

Figure 4: EGFP Fluorescence with Singly Modified mRNAs

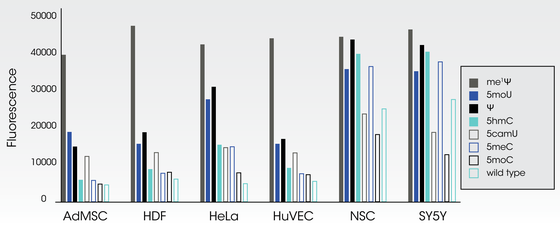


Figure 5: Combinations of Modifications are not Superior to Any Single Modification for EGFP mRNA Sequence Context

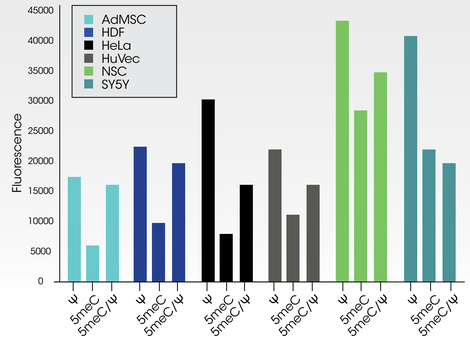


Figure 6: Rank Order of Modifications in FLuc mRNA Sequence Differ from EGFP mRNA Sequence

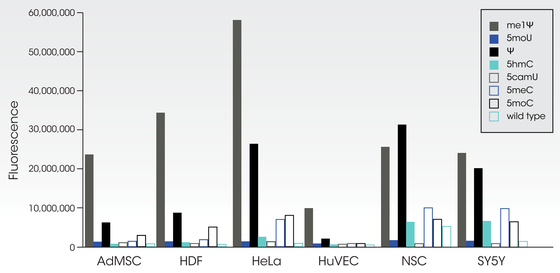


Figure 7: Combination of PseudoU/5meC is Superior to PseudoU Alone for FLuc Sequence Context

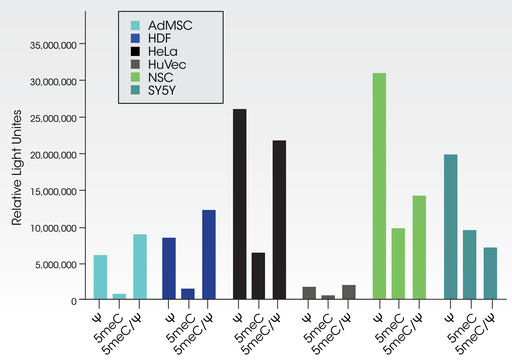
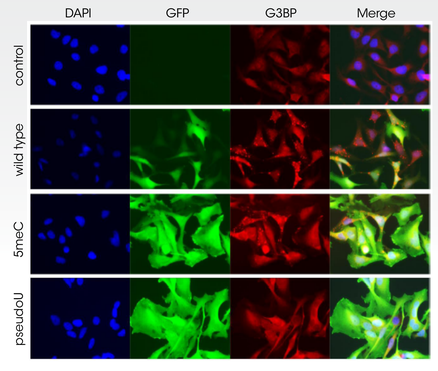


Figure 8: Modification of mRNA Can Attenuate Stress Granuales

Stress granules are dense aggregates of RNP complexes typically in a stalled translation state. G3BP (red) is used here as a marker for stress granules.



HeLa cells were plated to 100k cells per well in a 24 well plate for day of transfection. 200 ng of mRNA and 0.8 µl of Lipofectamine 2000 was used per well and media was changed at 5 hours. Cells were fixed at each timepoint in 4% PFA and stained for GFP, G3BP, and DAPI. Images taken on widefield microscope with 40X objective.

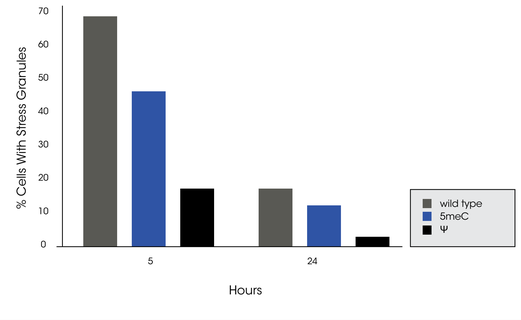


Figure 9: Novel Chemical Modifications For Aptamer Selection?

Chemical modifications that can incorporate with T7 RNA polymerase and amplify by RT-PCR may expand the universe of modifications available for aptamers.

mRNA Modification	RT-PCR Substrate
Unmodified	Yes
5caC	TBD
5fC	Yes
5hoC	Yes
5hmC	Yes
5meC/5fU	No
5meC/5moU	Yes
5meC/ thG	Yes
5moC	Yes
5meC/5camU	No
5meC	Yes
Ψ	Yes
5meC/Ψ	Yes
5moC/5moU	Yes
5moC/5meU	Yes
5hmC/5meU	TBD
me1Ψ	Yes
5meC/me1Ψ	TBD
5moU	Yes
5camU	Yes
m6A	Yes
5hmC/Ψ	Yes
5moC/Ψ	Yes
me6DAP	Yes
me4C	Yes
5fU	No

Conclusion

- Numerous novel NTPs were synthesized
- Individual modified NTPs or combinations of NTPs were used to transcribe EGFP and FLuc mRNAs
- Several modified nucleotides enhanced translation in a rabbit reticulocyte lysate *in vitro* translation system
- Modified EGFP and FLuc mRNAs were screened in 6 different cell types and several modifications had comparable or superior activity relative to pseudouridine
- Performance of different modified mRNA varied based on primary sequence
- Typically adding a second modified nucleotide did not increase activity over single modification
- Pseudouridine modified RNAs produced fewer stress granules
- Numerous modifications support RT-PCR, expanding the spectrum of chemical modifications available for *in vitro* selections

Future Directions

- Purity of crude mRNAs differs. Will HPLC purification change which modifications perform the best?
- Toxicity, dose response and cytokine measurements
- Timecourse of expression to examine mRNA stability
- Single molecule mRNA/protein interaction studies to determine interaction with innate immune sensors
  - TLR7, MDA5, RIGI
- Structure/activity relationship studies to improve NTP design

Contact

Dr. Anton McCaffrey, amccaffrey@trilinkbiotech.com

The Modified Nucleic Acid Experts™
www.trilinkbiotech.com