Nucleotide Modifications for Improved Messenger RNA Expression


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Abstract

In the last several years, in vitro transcribed mRNA has gained traction as a potential new therapeutic agent to deliver genetic information. mRNA provides several inherent benefits over traditional plasmid- and virus-based methods for gene therapy. One benefit is that mRNA is expressed in the cytoplasm and need only cross one membrane. This facilitates higher transfection rates and thus may be particularly useful for improving gene expression in intrinsically hard-to-transfect non-dividing cells. Additionally, in contrast to plasmid or viral vectors, there is no risk of insertional mutagenesis or subsequent oncogenesis upon mRNA transcription. Finally, the transient nature of mRNA is desirable for a number of applications, including cellular reprogramming, genome editing (ZFNs, TALENs, and CRISPR/Cas9) and vaccines.

Previously, a significant barrier to mRNA-based gene therapy had been mRNA induced innate immune responses in transfected cells. However, Kozak and colleagues showed that substitution of mRNA with pseudouridine and 5-methylcytidine dramatically reduces innate mRNA immune recognition and highlighted the potential for modified mRNA in the clinic. Activity and immunogenicity of mRNA likely depends on the chemical modification pattern, route of delivery, cell type/tissue transfected and possibly RNA structure. Here we investigate a series of base modifications in a variety of combinations in both EGFP and Firefly Luciferase mRNA. After assessing incorporation of each nucleotide by T7 RNA polymerase, we tested the translation potential of each modified mRNA in rabbit reticulocyte lysates. The activity was further evaluated in primary and immortalized cell lines transfected with mRNA.”

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

Applications
- Germline editing (Talos RNA, 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 therapies:
- Reduce innate immune responses and toxicity
- Maximize extent and duration of expression

Conclusion

- Several modified nucleotides enhance translation in a rabbit reticulocyte lysate in vitro translation system
- In the six cell types tested, several modifications had comparable or superior activity relative to pseudouridine
- Primary sequence influences activity of some modified mRNAs
- In most cases, adding a second modified nucleotide did not increase activity over single modification
- Pseudouridine modified RNAs produced the least stress granules
- Modifications that do not cause stress granules may still activate RIGI

Future Directions

- Test remaining modified RNAs in PKR-/- MEFs
- Test modifications in a Cas9 sequence context
- Assess whether mRNA purification influences performance
- Perform toxicity, dose response and cytokine measurements
- Conduct timetable of expression to examine mRNA stability
- Assess structure/activity relationship studies to improve NTP design
- Conduct single molecule mRNA/protein interaction studies to determine interaction with innate immune sensors TLR7/8/13, RIGI

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