Gene Expression from Pseudouridine and 5-Methylcytidine Modified Messenger RNA

Jiehua Zhou[1], Maggie L. Bobbin[1], Julie R. Escamilla-Powers[2], Anton P. McCaffrey[2], and John J. Rossi[1].

[1]Department of Molecular and Cellular Biology, Beckman Research Institute, City of Hope, Duarte, CA 91010. [2]TriLink BioTechnologies, Inc. San Diego, CA 92121.

Objectives

Develop methodologies for gram scale synthesis of messenger RNA (mRNA) for gene therapy applications, as well as scalable purification methods that yield highly expressed, persistent and non-toxic mRNA.

Abstract

Recently, there has been significant interest in the use of mRNA based expression systems for gene therapy applications and for the generation and manipulation of stem cells. Several groups have shown that mRNAs are attractive vehicles for therapeutic gene expression in mammals (Kormann et. al. Nat. Biotechnol (2011) 29, 154; Kariko et. al. Molecular Therapy (2012) Epub ahead of print). Additionally, Warren et al. demonstrated highly efficient induced pluripotent stem cell (iPSCs) generation by transfection of mRNAs encoding reprogramming factors (Warren et al. Cell Stem Cell (2010) 7, 618). The authors suggested that iPSCs generated in this manner should be safer than iPSCs derived by plasmid transfection or viral transduction as there is no risk of insertional mutagenesis and subsequent oncogenesis.

A key insight for the development of an mRNA expression system was the recognition that mRNAs induce innate immune responses in transfected cells. Kariko et al. showed that substitution of uridine and cytidine residues with pseudouridine (ΨU) and 5-methlycytidine dramatically reduced innate immune recognition of mRNA (Kariko et al. Molecular Therapy (2008) 16, 1833). In addition, pseudouridine modified RNA was translated more efficiently and had increased nuclease resistance.

These studies highlight the importance of development of stable, non immunogenic mRNA to support these applications. In this study, capped pseudouridine and 5-methlycytidine modified eGFP mRNAs were synthesized at milligram scales. Fluorescence activated cell sorting (FACS) demonstrated >95% transfection in HEK-293 cells. Expression in human CEM T-cells and CD34+ hematopoietic stem cells was also examined. Transfected mRNAs showed a surprisingly long duration of expression (8-10 days post-transfection).

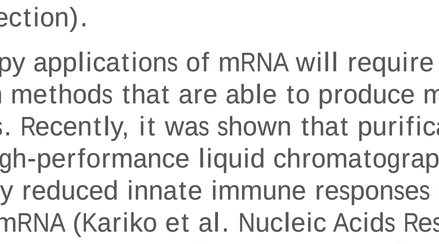
Gene therapy applications of mRNA will require scalable purification methods that are able to produce mRNAs at gram scales. Recently, it was shown that purification of mRNA by high-performance liquid chromatography (HPLC) dramatically reduced innate immune responses relative to unpurified mRNA (Kariko et al. Nucleic Acids Research 39, e142). Here we compare mRNA purified by classic silica membrane chromatography to HPLC purified materials.

Transcriptions

Transcriptions with T7 RNA polymerase were carried out in the presence of anti reverse cap analog (ARCA), pseudouridine and 5-methylcytosine. Transcription with traditional cap analog (mCAP) results in incorporation of the cap in the wrong orientation ~50% of the time. In contrast, transcription with ARCA results in insertion of the cap in proper orientation 100% of the time. After removal of NTPs, RNAs were phosphatase treated to remove residual 5' triphosphate.

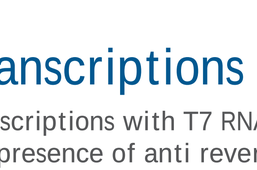
Figure 1

Nucleotide Structures

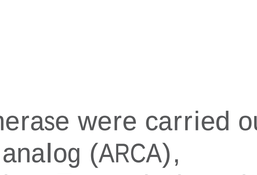


ARCA - Anti Reverse Cap Analog (TriLink Cat. # N-7003)

Modifications Reducing Immune Stimulation



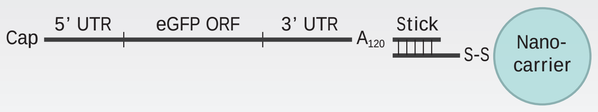
Pseudouridine Triphosphate
(TriLink Cat. # N-1019)



5-Methylcytidine Triphosphate
(TriLink Cat. # N-1014)

Figure 2

eGFP Stick mRNA

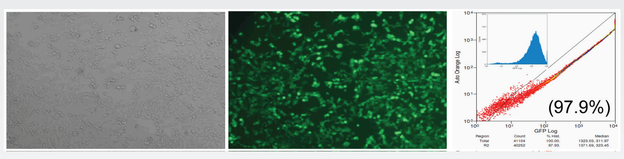


Stick allows hybridization to delivery vehicles such as nanocarriers or aptamers

Figure 3

mRNA Transfection Results in Very Robust Expression in HEK-293 Cells

eGFP-Stick mRNA with Lipofectamine 2000, 48 hr



GFP Plasmid with Lipofectamine 2000, 48 hr

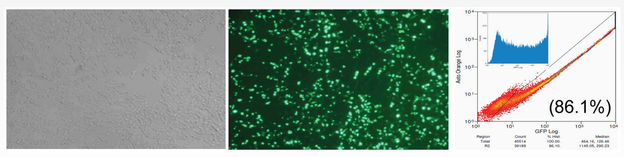
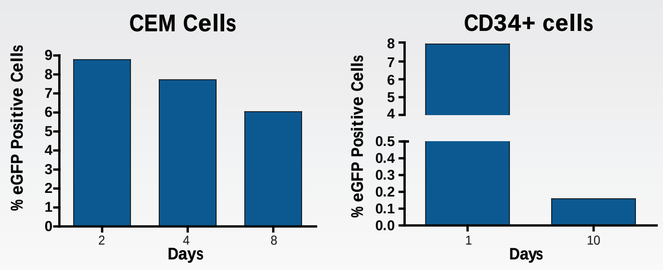


Figure 4

eGFP-Stick mRNA Expression in CEM Cells and CD34+ Hematopoietic Stem Cells is Long Lived



*Trans*IT®-mRNA Kit (Mirus Bio, LLC)

HPLC Purification of mRNA

Kariko, et. al. reported that relative to unpurified mRNA, HPLC purified mRNA was less immuno-stimulatory and expression was significantly higher in cultured cell and in vivo. However, a typical mRNA workup involves purification of the mRNA on a silica membrane spin column. Standard treatment was compared to HPLC purification.

Figure 5

mRNAs of Different Sizes Elute at Similar Times from HPLC

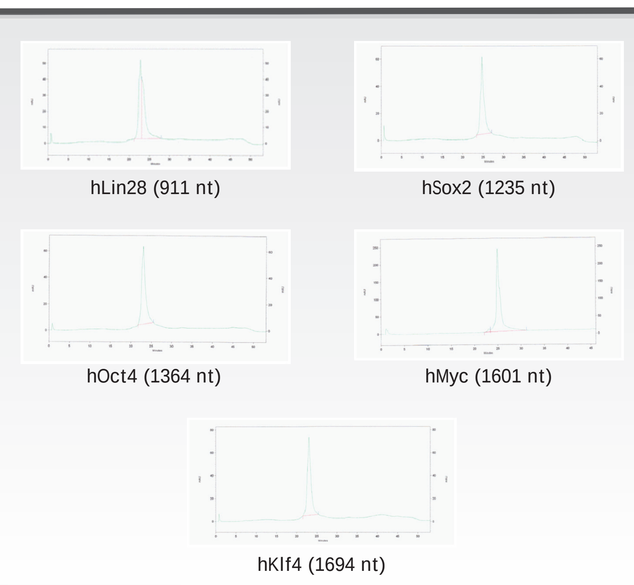


Figure 6

Transcript Processing

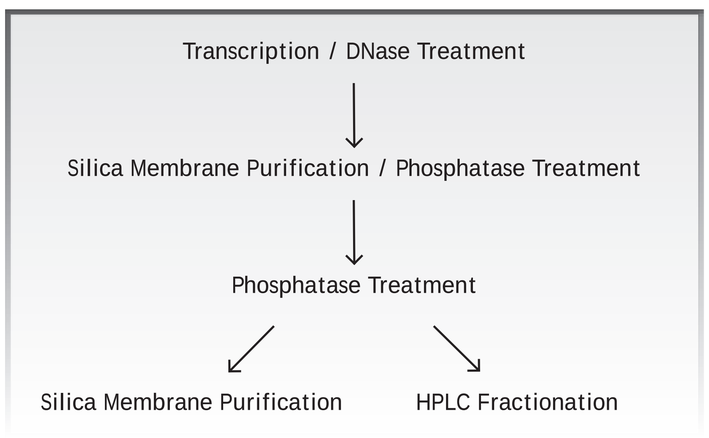
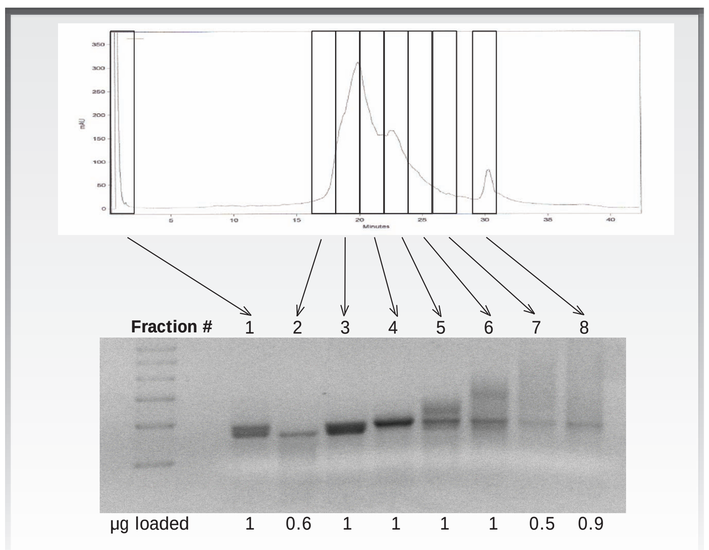


Figure 7

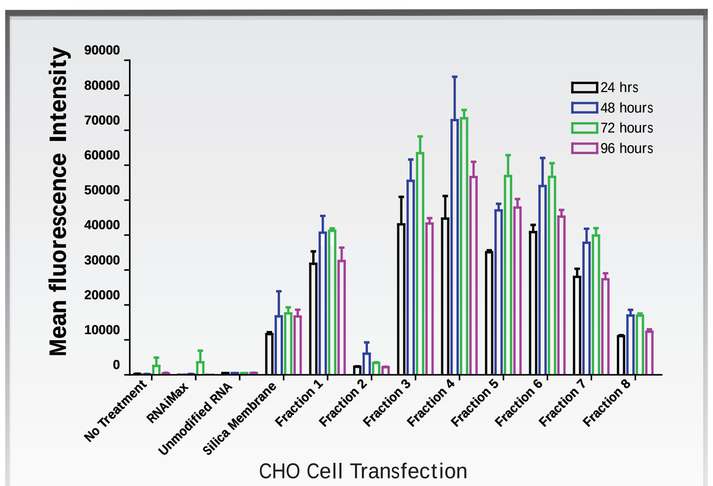
Effect of HPLC Fractionation on eGFP Intensity



RNASep™ Column (Transgenomic, Inc.)
Glyoxal Treated RNA on Agarose Gel

Figure 8

Mean eGFP Fluorescence Intensity



CHO Cell Transfection
100 ng mRNA
RNAiMax™ (Life Technologies)

Conclusion

- mRNA transfection results in high level gene expression without the risk of insertional mutagenesis.
- As reported by Kariko, et. al., substitution with pseudo-U and 5-methyl-C dramatically increases mRNA expression.
- HPLC purification isolates functionally distinct mRNA populations.

For further information, please contact Anton McCaffrey, amccaffrey@trilinkbiotech.com