Generating Optimal Pseudouridine and 5-Methylcytidine Modified Messenger RNA for iPS Cell Reprogramming

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Objectives

Develop gram scale synthesis and purification methodologies that yield highly expressed, persistent and non-toxic messenger RNA (mRNA) for cGMP studies, stem cell reprogamming and gene therapy applications.

Abstract

There is significant interest in mRNA for gene therapy applications as well as for the generation and manipulation of stem cells. Several groups have shown that mRNA is an attractive vehicle for therapeutic gene expression in mammals, including Kormann et al. (2011), Kariko et al. (2012) and Wang et al. (2013). Additionally, Warren et al. (2010, 2012) demonstrated highly efficient induced pluripotent stem (iPS) cell generation by transfection of mRNA encoding reprogramming factors. mRNA has no risk of insertional mutagenesis and subsequent oncogenesis. Thus, the authors suggested that an iPSC generated using mRNA should be safer than an iPSC derived by plasmid transfection or viral transduction.

Pioneering work by Kariko et al. (2008) showed that substitution of uridine and cytidine residues with pseudouridine (pseudo-U) and 5-methylcytidine (5-Me-C) reduced innate immune recognition of mRNA . They also showed that pseudo-U modified mRNA was translated more efficiently and had increased nuclease resistance.

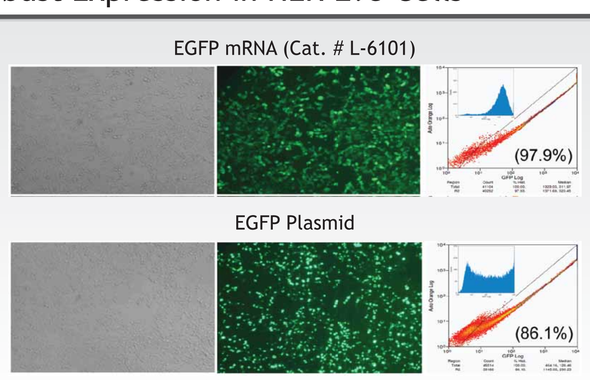
Herein, capped pseudo-U and 5-Me-C modified mRNA expressing Oct4, Klf4, Sox2, c-Myc, Lin28 (OKSML), FLuc and EGFP were synthesized at 10 mg scales. Fluorescence activated cell sorting demonstrated >95% transfection in HEK-293 cells. Expression in BJ fibroblasts, human CEM T-cells and CD34+ hematopoietic stem cells (HSC) was also achieved. Transfection efficiency and toxicity associated with repeat transfection of fibroblasts with RNAi MAX™ and *Trans*IT®-mRNA are compared. Repeated transfection with OKSML produced robust stem cell colonies without genetic abnormalities.

Therapeutic applications of mRNA will require scalable purification methods that yield gram quantities. Kariko et al. (2011) have shown that purification of mRNA by HPLC dramatically reduces innate immune response. Herein, mRNA purified by classic silica membrane chromatography and by HPLC are compared. Immunogenicity of HPLC fractions were evaluated.

Transcriptions

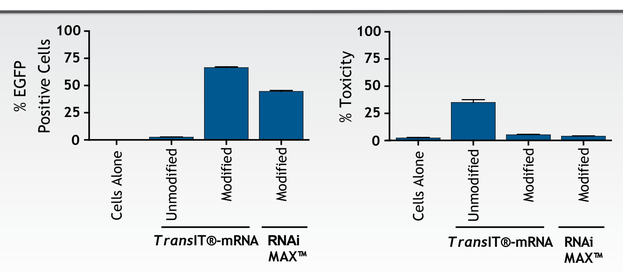
Transcriptions with T7 RNA polymerase were carried out in the presence of ARCA (TriLink Cat. # N-7003), pseudo-UTP (N-1019) and 5-Methyl-CTP (N-1014). After removal of free NTPs, the mRNA was phosphatase treated to remove residual 5' triphosphate.

Figure 1: mRNA Transfection Results in Very Robust Expression in HEK-293 Cells



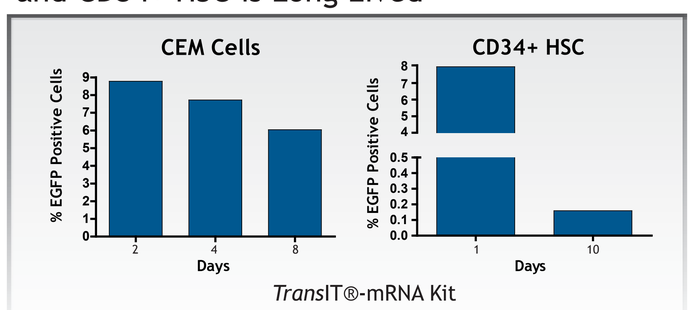
Lipofectamine™ 2000, 48 hr
Image courtesy of Jiehua Zhou, City of Hope

Figure 2: Pseudo-U and 5-Me-C Modified mRNA Results in High Expression, Low Toxicity in BJ Fibroblasts



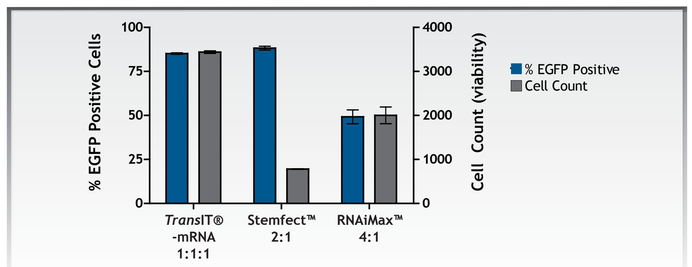
Data courtesy of Laura Juckem, Mirus Bio

Figure 3: EGFP mRNA Expression in CEM Cells and CD34+ HSC is Long Lived



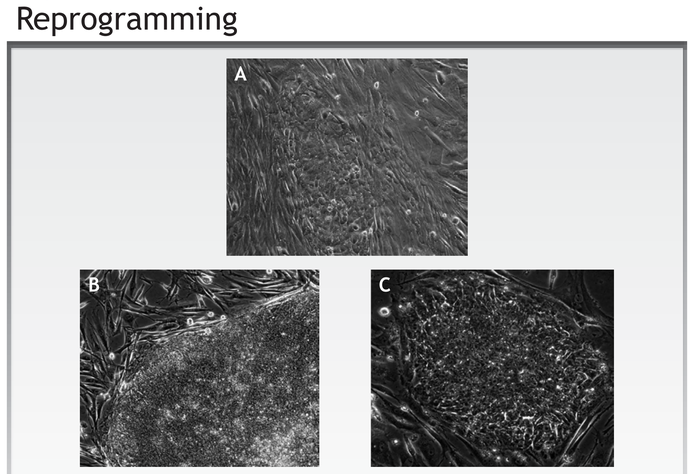
Data courtesy of Jiehua Zhou, City of Hope

Figure 4: High Expression, Low Toxicity After 14 Days of EGFP mRNA Daily Transfections in BJ Fibroblasts



Data courtesy of Laura Juckem, Mirus Bio

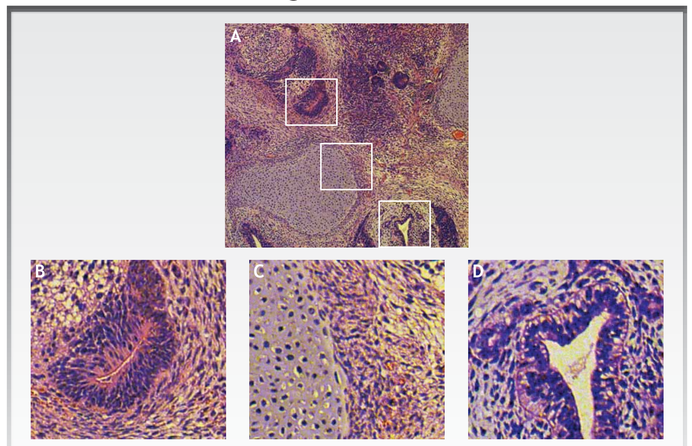
Figure 5: Generation of iPS Cells by mRNA Reprogramming



Patient derived fibroblasts were transfected with a cocktail of OKSML mRNA. (A) Emergence of iPS cell colonies after 19 days, phase contrast, magnification 100x. (B, C) Individual iPS cell colonies were picked and propagated on feeder cells in CDF12 stem cell media. Colonies are composed of many iPS cells with the typical morphology, e.g. characteristic nucleoli and the big nuclear cytoplasmic ratio. Magnification: (B) 100x (C) 300x.

Image courtesy of Karl-Dimiter Bissig, Baylor College of Medicine

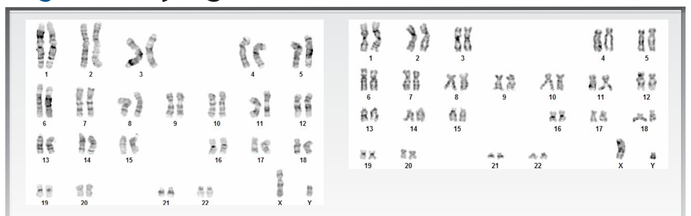
Figure 6: Analysis of Teratomas from iPS Cells Show All Cell Lineages



(A) Overview low magnification of subcutaneous teratoma, (B) neural tissue, ectoderm, (C) cartilage and muscle, mesoderm (D) intestinal epithelium, endoderm. (B), (C) and (D) are high magnifications of insets in (A).

Image courtesy of Karl-Dimiter Bissig, Baylor College of Medicine

Figure 7: Cytogenetic Evaluation of iPS Cells



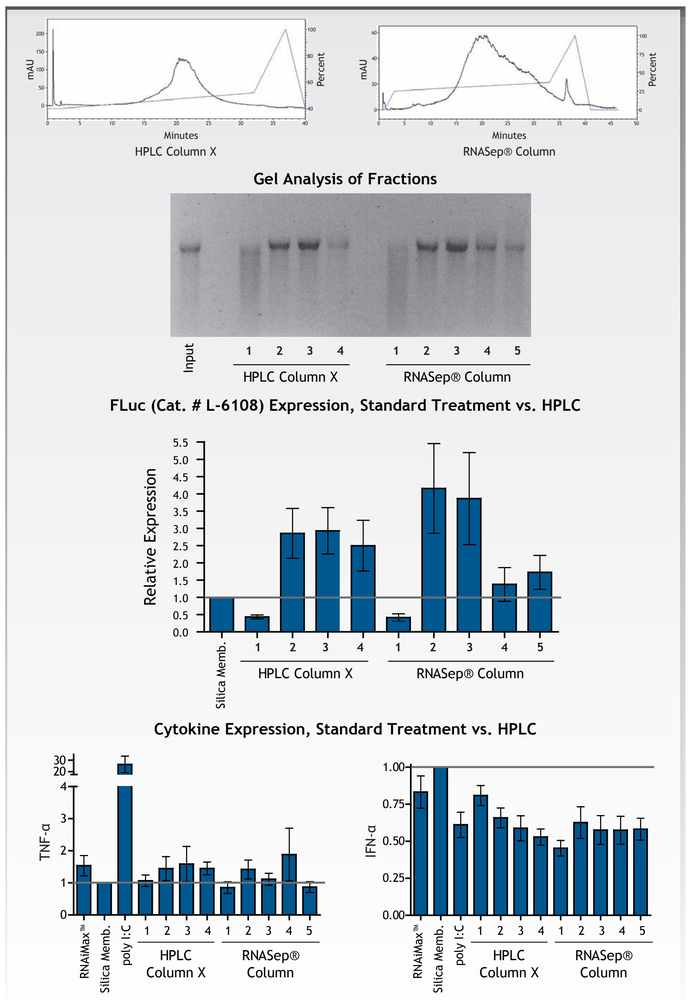
Cytogenetic evaluation of harvested cells derived from modified mRNA revealed a normal male karyotype in all analyzed metaphases.

Image courtesy of Karl-Dimiter Bissig, Baylor College of Medicine

HPLC Purification of mRNA

Kariko et al. (2011) reported that relative to unpurified mRNA, HPLC purified mRNA was less immuno-stimulatory and expression was significantly higher in cultured cells and *in vivo*. Standard mRNA workup involves purification on a silica membrane spin column. Standard treatment was compared to HPLC purification.

Figure 8: Effect of HPLC Fractionation on Expression and Cytokine Production in Primary Human Dendritic Cells



Data courtesy of Maggie Bobbin, City of Hope

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-Me-C dramatically increases mRNA expression and reduces toxicity.
- Repeat transfection with the correct transfection reagent results in low toxicity and high expression.
- Pseudo-U and 5-Me-C modified mRNA efficiently produces stem cells without genetic abnormalities.
- HPLC purification isolates functionally distinct mRNA populations.

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