mRNA-based Methods for Cell Fate Manipulation

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ABSTRACT

The ability to manipulate cell fate has significant potential to provide useful tools in regenerative medicine and drug screening applications. Current methods primarily rely on media additives or viral induction to induce the transformation of one cell type to another. However, these methods are limited by a number of factors, most notably low efficiency and genomic insertion. More robust cell fate manipulation methods would lead to a higher yield of target cell types; non-viral methods would lead to more clinically relevant cells. Here, we present results demonstrating the utility of mRNA-based methods for the generation of induced pluripotent stem (iPS) cells and the transdifferentiation of fibroblasts to myoblasts. We also introduce two new tools that enable these mRNA methods to be successful: 1) Stemgent Pluriton™ Reprogramming Medium, which allows for efficient cellular reprogramming of human fibroblasts to iPS cells, and 2) Stemfect RNA Transfection kit, a new lipid-based formulation for highly efficient mRNA delivery with titratable control over protein expression. These methodologies allow for the reproducible and efficient alteration of cell fate without insertional mutagenesis.

INTRODUCTION

Several recent publications have demonstrated the potential of mRNA to reprogram somatic cells to induced pluripotent stem (iPS) cells, albeit with varying levels of efficiency, reproducibility and colony stability [1, 2, 3]. Messenger RNA reprogramming is a desirable method for iPS cell generation for a number of reasons. First, it eliminates the need for DNA-based reprogramming factor delivery, thereby eliminating any risk for insertional mutagenesis of the parental cell genome. Second, published methods have demonstrated an increased yield in the number of iPS cell colonies generated per defined starting cell population, thereby potentially reducing the number of target cells required to initiate the reprogramming process. Third, this method has generated iPS cell colonies in as little as over two weeks and in doing so has minimized the primary cell culturing time during the reprogramming process when compared to other DNA based delivery methods. Efforts within our lab to develop a more robust and reproducible method for iPS cell generation using this integration-free technology led to a revised protocol and a new medium, Pluriton™ Reprogramming Medium. Together, these improvements have enabled a number of research groups to succeed with this reprogramming method on both control and patient-derived cell lines.

While these methods have advanced the field of iPS cell generation, improvements remain to be made in other areas of cellular differentiation. Currently, standard approaches for directed differentiation involve recapitulation of the molecular cues encountered by a cell during development. These molecular cues are provided *in vitro* in the form of small molecules and cytokines that regulate major signaling pathways, as well as by viral-mediated gene overexpression. The challenge in the field has been that these approaches are either plagued by low efficiency or safety concerns. To overcome these limitations, we sought to extend the mRNA-based methods to specific differentiation protocols. Weintraub and colleagues first demonstrated over 20 years ago the concept of transdifferentiation, whereby forced expression of a single gene, the transcription factor MyoD, could convert fibroblasts directly into myoblasts.[4,5] DNA or viral-based methods to express MyoD led to the formation of myotubes and upregulation of muscle-specific markers. Here, we show that transdifferentiation can be accomplished in both mouse and human cells using mRNA encoding for MyoD. This protocol required repeat transfections of the mRNA to achieve sustained expression of the protein, and led to the upregulation of the muscle specific markers MF-20, Myogenin and Desmin.

In concert, to enhance this process, we have developed a new RNA delivery method, based on leading edge lipid-based delivery systems, that allows for fine-tuned control over the amount of mRNA delivered and expressed. This reagent, Stemfect™ RNA transfection kit, has been shown to deliver mRNA at >95% efficiency in a broad range of cell types and could enable the reprogramming or differentiation of cell lines that are normally refractory to transfection.

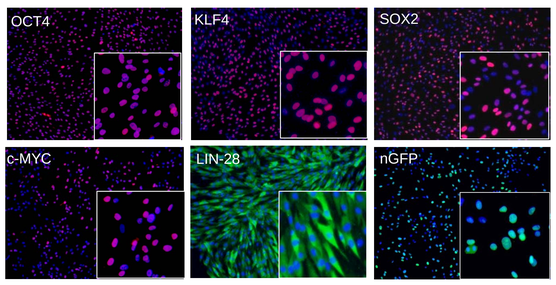

Figure 1. Expression of reprogramming factors using mRNA
BJ fibroblasts were transfected with mRNA encoding the transcription factors OCT4, SOX2, KLF4, c-MYC, LIN-28 or nGFP and fixed with 4% paraformaldehyde after incubation for ~18 hours. The cells were then stained with the appropriate antibody and DAPI for visualization. Merged images are shown.

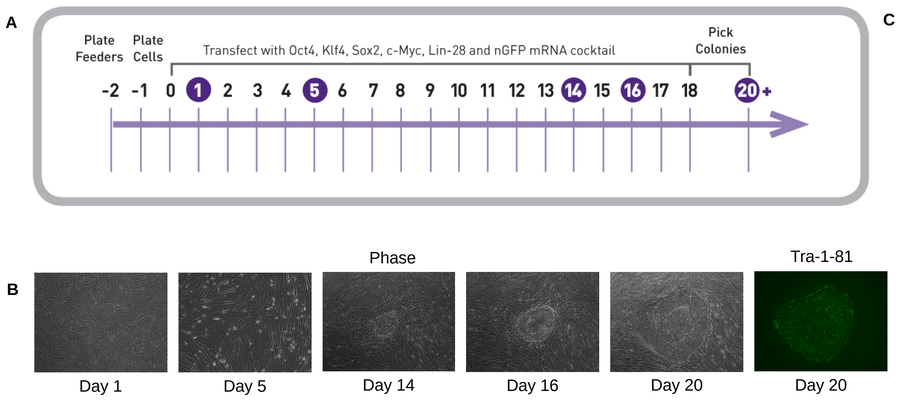

Figure 2. Modified protocol for mRNA reprogramming of human fibroblasts.
A) Timeline for the reprogramming of human fibroblasts by repeated mRNA transfection using Stemgent's optimized no-split mRNA Reprogramming protocol. **B)** Primary culture morphology progression (Phase - 10x) during the emergence of a primary iPS cell colony resulting from the reprogramming of Parkinson's disease patient dermal fibroblasts with Stemgent mRNA Reprogramming Factor Set in combination with Pluriton™ mRNA Reprogramming Medium. Last panel: Day 20 primary iPS colony identification using the Stemgent StainAlive™ Tra-1-81 antibody. **C)** Key adjustments/optimizations, by Stemgent, to the mRNA reprogramming protocol as published in Cell Stem Cell[1]. For the full protocol, please visit www.stemgent.com.

C Key Protocol Adjustments

1. Stemgent Pluriton™ Reprogramming Medium
2. No-split format
3. Conditioned Medium: Day 6 (post-transfection)
4. Feeder density: 250K/well
5. Target cell density: 10 - 50K/well

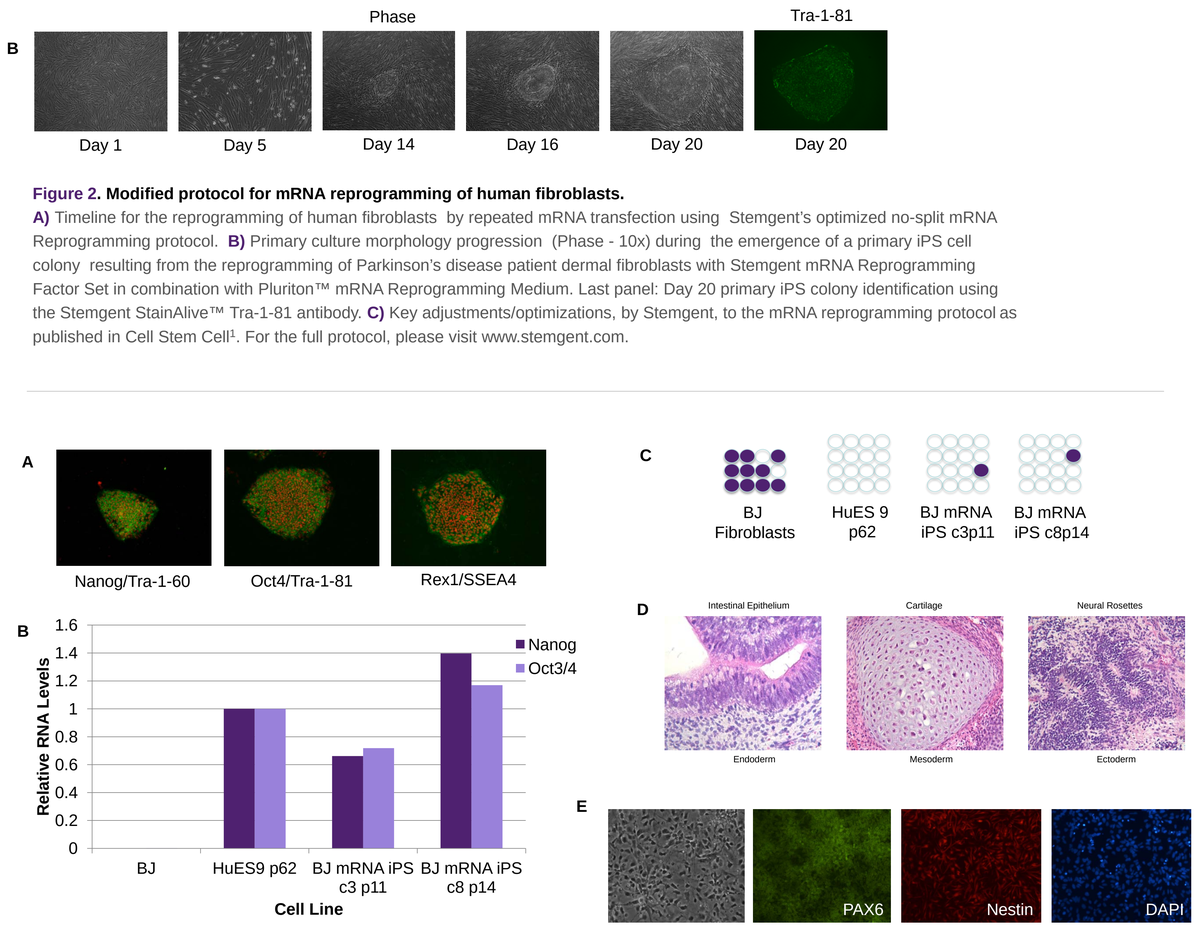

Figure 3. Characterization of BJ mRNA iPS cell lines.
A) Pluripotency immunocytochemistry for mRNA iPS cell line (c8) derived from BJ fibroblasts. Nuclear pluripotency for Nanog, Oct4, and Rex1 (Red). Cell surface pluripotency for Tra-1-60, Tra-1-81, and SSEA4 (Green). **B)** Relative endogenous mRNA expression level comparison for Nanog and Oct4. qRT-PCR calculations and data normalization against HuES9 p62 human ES positive control cell line. **C)** Bisulfite sequence analysis of Oct4 promoter for BJ mRNA iPS cell lines c3 p11 and c8 p14. Lines were also found to have a normal karyotype (data not shown). **D)** Hematoxylin and eosin staining and analysis of a teratoma resulting from the injection of the BJ mRNA iPS c8 cell line into SCID mice. **E)** Immmunocytochemical analysis of neural progenitor cells (NPCs) derived from BJ mRNA c8 cell line stained for PAX6 (green) and Nestin (red) expression.

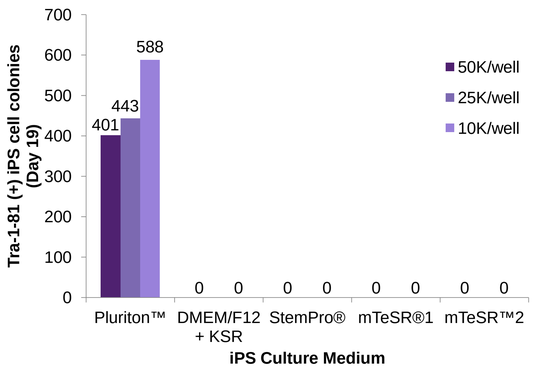

Figure 4. Comparison of Pluriton™ mRNA Reprogramming Medium and other common human ES culture media for iPS cell colony generation during mRNA based reprogramming.
Different target cell densities (50K, 25K, or 10K per well) of BJ fibroblasts were plated on human fibroblast feeders (250K/well) in a single well of 6-well plate. Each condition was incubated at 5% O_2 and transfected with 1.2 ug of mRNA reprogramming cocktail for 16 consecutive days without enzymatic passaging. Primary cultures were assayed with the Stemgent StainAlive™ Tra-1-81 antibody (1:100) on Day 19 and Tra-1-81 positive colonies were counted using a fluorescent microscope. Each bar in the graph is individually labeled with the number of iPS cell colonies generated.

REFERENCES

1. Warren, L., Manos, P.D., Ahfeldt, T., Loh, Y.H., Li, H., Lau, F., Ebina, W., Mandal, P.K., Smith, Z.D., Meissner, A., Daley, D.Q., Brack, A.S., Collins, J.J., Cowan, C., Schlaeger, T.M., Rossi, D.J. (2010) Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell, 7:618-30.
2. Angel, M., Yanik, M.F. (2010) Innate Immune Suppression Enables Frequent Transfection with RNA Encoding Reprogramming Proteins. PLoS One 5:e11756.
3. Yakubov, E., Rechavi, G., Rozenblatt, S., Givol, D. (2010) Reprogramming of Human Fibroblasts to Pluripotent Stem Cells using mRNA of Four Transcription Factors. Biochem Biophys Res Commun. 394:189.
4. David, R.L., Weintraub, H., Lassar, A.B. (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell, 51:987-1000.
5. Weintraub, H., Tapscott, S.J., Davis, R.L., Thayer, M.J., Adam, M.A., Lassar, A.B., Miller, A.D. (1989) Activeation of muscle-specific genes in pigment, nerve, fat, nerve, and fibroblast cell lines by forced expression of MyoD. Proc. Natl. Acad. Sci. USA, 86:5434-5438.

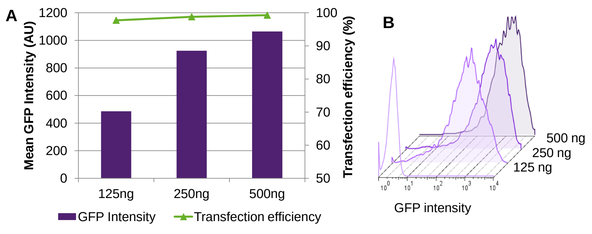

Figure 5. Titration of protein expression using the Stemfect RNA transfection kit. BJ fibroblast cells were seeded in a 24-well format and transfected with 125, 250 or 500 ng of eGFP mRNA. The cells were cultured at 37°C / 5% CO_2 and analyzed at 18-24 hrs. post transfection. **A)** Graph of mean fluorescence intensity and transfection efficiency as determined by flow cytometry. **B)** Representative histograms.

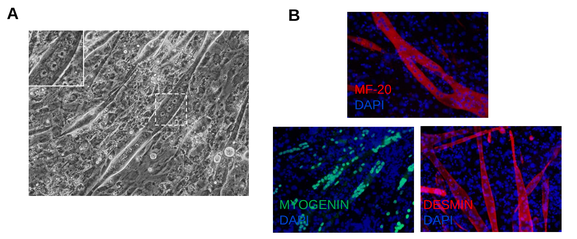

Figure 6. Transdifferentiation of mouse fibroblasts. 10 T1/2 mouse embryonic fibroblasts were seeded in a 12-well format and transfected with 500 ng of mRNA encoding "human" MyoD for three consecutive days in fibroblast media. The media was then switched to a low serum media and the cells allowed to differentiate for three days at 37°C / 5% CO_2 . **A)** Phase image of differentiated mouse fibroblasts showing clustered nuclei and myotube formation. **B)** Cells were fixed using 4% paraformaldehyde and stained for the muscle specific markers MF-20, Myogenin and Desmin.

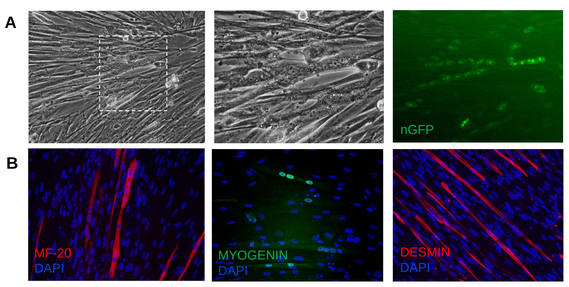

Figure 7. Transdifferentiation of human fibroblasts. IMR90 or MRC5 human embryonic fibroblasts were seeded in a 12-well format and transfected with 500 ng of mRNA encoding MyoD for five consecutive days in fibroblast media. The media was then switched to a low serum media and the cells allowed to differentiate for three days at 37°C / 5% CO_2 . **A)** Phase image of differentiated human fibroblasts showing clustered nuclei and myotube formation. The cells were cotransfected with nGFP to track nuclear clustering. **B)** Cells were fixed using 4% paraformaldehyde and stained for the muscle specific markers MF-20, Myogenein and Desmin.

SUMMARY

1. **Developed functionally validated mRNA reprogramming protocol**
   - **Integration-free**
   - **Increased reprogramming efficiency**
   - **Faster iPS cell colony generation – colony isolation within 3 weeks**
   - **Demonstrated on diseased patient fibroblasts**
   - **Validated at 5% O_2 and 21% O_2**
2. **Developed novel, xeno-free media essential for mRNA reprogramming success**
3. **Demonstrated the utility of mRNA for the transdifferentiation of human and mouse fibroblasts to myoblasts**
4. **Developed highly efficient RNA transfection reagent for broadened cell application**