

Highly Efficient microRNA-Enhanced mRNA Reprogramming of Diseased Human Fibroblasts in a Feeder-Free Culture System

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INTRODUCTION

Human induced pluripotent stem (iPS) cells hold great promise for advancing our understanding of human biology and medicinal research. It has been shown that ectopic expression of just a few key transcription factors in somatic cells can induce an embryonic stem cell phenotype hallmarked by the ability to differentiate into any cell of the three basic germ layers^{1,2}. Early reprogramming methods utilized retroviral (including lentiviral) vectors to introduce reprogramming-associated transcription factors to the target cells. While these pioneering experiments provided a fundamental proof-of-concept, these vectors require the integration of heritable, viral DNA sequence into the host cell's genome. The integration site of the provirus is highly random and can interfere with normal cellular processes in unpredictable ways. Since then, the focus has been to develop reprogramming technologies that reduce or eliminate the risk of insertional mutagenesis and the biohazards associated with viral vectors particularly those involved with oncogene delivery.

In efforts to generate iPS cells without genomic contamination (footprint-free) other methods have been explored including; adenoviruses, plasmid DNA, recombinant proteins, and small molecules. Although these systems do not require genomic transformations of the target cells during reprogramming, they are difficult to reproduce due to extremely low reprogramming efficiencies. Further developments of exogenous transcription factor-based reprogramming utilize Sendai virus (DNA-free) and transfection, or electroporation of episomal DNA. These methods are improvements, however still carry significant risk of either insertional mutagenesis or prolonged viral retention and persistent ectopic factor expression therefore necessitate screening of derived iPS cell clones.

The development of mRNA reprogramming technology provides a fast, safe and efficient means of generating clinical-grade human iPS cells from somatic tissue^{3,4}. Reprogramming human cells with mRNA factors produces reprogramming efficiencies greater than 1% without the use of virus or DNA vectors⁵. Today, mRNA-based reprogramming has proven to be the only non-viral, non-integrating method for reliable, safe and efficient generation of clinically relevant human iPS cell lines.

Ongoing research has demonstrated a role for microRNAs in the maintenance and retention of pluripotency for both embryonic stem (ES) cells and iPS cells^{6,7}. MicroRNAs are endogenous, small, non-coding RNAs (approximately 21 nucleotides) that target and down-regulate tens or even hundreds of mRNAs by blocking gene expression via translational repression or degradation of functionally transcribed mRNAs. In 2012, two groups demonstrated that delivery of specific microRNA combinations can functionally reprogram human fibroblasts to a pluripotent state in the absence of exogenous reprogramming factor expression^{8,9,10}. Subsequent efforts to validate these works have failed to demonstrate reproducible and efficient iPS cell colony generation. As a result, internal research at Stemgent is focused on the role of microRNAs in cellular reprogramming protocols utilizing mRNA for reprogramming factor delivery.

One of the key challenges in transcription factor facilitated reprogramming is efficient penetrance into recalcitrant, slowly-dividing cell populations common in older, diseased patient samples. Through global gene repression, pluripotency associated microRNAs induce self-renewal and proliferative networks, inhibit TLR-induced inflammatory cytokine expression, and promote a mesenchymal to epithelial (MET) transition^{11,12}. These combined elements support efficient transcript delivery and comprehensively influence the cellular transcriptome to facilitate reprogramming, which is particularly advantageous when working with difficult to reprogram patient samples.

This work has resulted in the development of a new RNA-based reprogramming method to generate high-quality human iPS cell lines. The microRNA-enhanced mRNA reprogramming system eliminates feeder co-culture during the reprogramming process while incorporating both the Stemgent Stemfect™ RNA Transfection kit (Stemgent Cat. No. 00-0069) and microRNA Booster Kit (Stemgent Cat. No. 00-0073). These modifications to the traditional feeder-based mRNA reprogramming protocol result in faster reprogramming kinetics (fully-reprogrammed iPS cells in as few as 12 days) and improved productivity across patient-derived cell lines when compared to other available reprogramming methods.

Additionally, the transient nature of mRNA expression provides a tunable reprogramming factor delivery system yielding stable, vector-free iPS cell clones in less than 2 weeks without the need for laborious single-colony sub-cloning and viral or DNA vector retention screening assays. The incorporation of the Stemgent Stemfect RNA Transfection Kit into the microRNA-enhanced protocol reduces daily workload by converting the arduous 4-hour transfections and medium-exchanges associated with using Invitrogen™ Lipofectamine® RNAiMax™ in the traditional feeder-based protocol, to a non-toxic overnight transfection. Furthermore, the microRNA-enhanced mRNA reprogramming system has been optimized for a feeder-free culture environment, excluding possible variability inherent in feeder cell co-cultures.

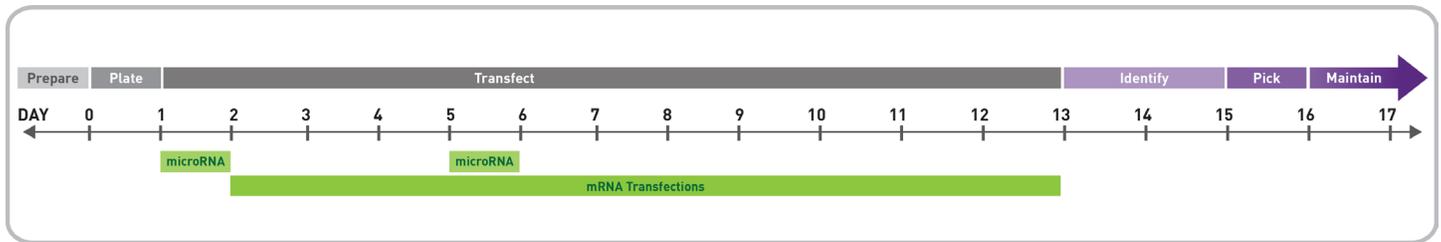


FIGURE 1. Reprogramming chronology: The above timeline shows the major steps in the feeder-free, enhanced mRNA reprogramming protocol.

METHODS

Note: Information about Stemgent products used in the experiment described within this Application Note can be found in Table 3. Any other materials used will be listed within the text of this publication.

Prior to reprogramming, Pluriton™ Medium was pre-conditioned with mitotically inactivated (γ -irradiated) human newborn foreskin fibroblasts, Stemgent® NuFF-RQ™ IRR (Stemgent Cat. No. GSC-3006G). To prepare NuFF-Conditioned Pluriton Medium (Pluriton CM); Pluriton™ Medium was incubated with NuFFs (24 hours after seeding) overnight at 37 °C, 5% CO₂. The medium was harvested from the NuFF cultures and replaced with fresh Pluriton Medium every 24 hours for 7 days and the collected media stored at -70 °C. After the 7th day of collection, the media fractions were thawed, pooled, and filtered for use during the reprogramming protocol.

For consistent systematic activity throughout the reprogramming process, all labile reagents were aliquotted prior to beginning the procedure. Pluriton Supplement, B18R recombinant protein, and the microRNA reprogramming cocktail were each aliquotted into single-thaw volumes and stored at -70 °C until use.

The mRNA reprogramming factor cocktail was prepared by combining the individual mRNA components according to the volumes in Table 1. The mRNA reprogramming cocktail was then aliquotted into single-thaw volumes and stored at -70 °C.

mRNA Factor	Volume
Oct4	400.0 μ L
Sox2	123.8 μ L
Klf4	162.0 μ L
c-Myc	153.5 μ L
Lin28	85.7 μ L
nGFP	115.0 μ L
Total mRNA Cocktail	1040.0 μ L

TABLE 1. Preparation of the mRNA reprogramming cocktail: The mRNA reprogramming cocktail was prepared by combining the 5 reprogramming factor mRNAs (Oct4, Sox2, c-Myc, Klf4, Lin28) plus nuclear GFP (nGFP) mRNA reporter at a stoichiometric ratio of 3:1:1:1:1 respectively.

The target cell cultures were pre-incubated with B18R recombinant protein, a soluble, type-1 interferon decoy receptor, prior to the first transfection (Day 1). Treatment of the cultures with B18R suppresses pathogen-associated molecular pattern or PAMP-mediated immune response to repeated delivery of long RNAs. Briefly: the fibroblast medium from each well was replaced with 1 mL of Pluriton CM containing Pluriton Supplement and B18R recombinant protein (300 ng/mL). The cells were transferred to a hypoxic (multi-gas) incubator and allowed to equilibrate to 37 °C, 5% CO₂, and 5% O₂ for 2 hours. Cells were maintained in the low oxygen incubator for the remainder of the reprogramming timeline.

On the first day of transfection (Day 1), the cells were transfected with microRNA cocktail only. Each microRNA transfection complex was prepared by diluting 35 pmole of Stemgent microRNA reprogramming cocktail in 12.5 µL of Stemfect buffer. In a separate tube, 2 µL of Stemfect RNA transfection reagent was added to 10.5 µL of Transfection. The tubes were combined and the resulting 25 µL transfection complex was incubated undisturbed, at room temperature for 15 minutes before adding to each well to be reprogrammed. The plates were returned to the incubator at 37 °C, 5% CO₂, and 5% O₂ overnight. The entire transfection schedule was carried out using Stemfect RNA transfection kit.

Beginning on Day 2, the target cells were transfected with mRNA reprogramming cocktail every 24 hours through Day 12. Prior to each subsequent transfection the existing medium was exchanged with fresh Pluriton CM equilibrated overnight to the culture conditions (37 °C, 5% CO₂ and 5% O₂). B18R recombinant protein and Pluriton supplement were subsequently added to the equilibrated, Pluriton CM as before.

For mRNA delivery, 0.5 µg of mRNA cocktail was diluted in 12.5 µL of Stemfect buffer. In a separate tube, 2 µL of Stemfect RNA Transfection Reagent was added to 10.5 µL of Transfection. The tubes were combined and the mixture was allowed to complex undisturbed at room temperature for 15 minutes. The entire 25 µL volume was then added to each well to be reprogrammed. The plates were returned to the incubator at 37 °C, 5% CO₂, and 5% O₂ overnight.

On Day 5, both mRNA and microRNA reprogramming complexes were prepared as previously described and co-transfected sequentially.

One day after the completion of the transfection schedule (Day 14), primary colonies were identified via live-stain immunocytochemistry for pluripotency marker expression using StainAlive™ TRA-1-81 Antibody (Stemgent Cat. No. 09-0069). Reprogramming efficiency was quantified by counting TRA-1-81-positive colonies of each experimental well (Table 2). The cells were maintained in culture for an additional 1 to 3 days prior to clonal isolation. The colonies were then re-plated on new Corning® Matrigel®-coated plates and maintained in NutriStem® XF/FF Cell Culture Medium (Stemgent Cat. No. 01-0005) at 37 °C, 5% CO₂, and at atmospheric oxygen tension. During passaging, the cells were dissociated by EDTA chelation. After 5 passages, the cultures were harvested and cryopreserved in Stemgent CryoStem™ Freezing Medium (Stemgent Cat. No. 01-0013-50). Cell lines were later thawed and expanded in the same feeder-free culture conditions for further characterization.

RESULTS

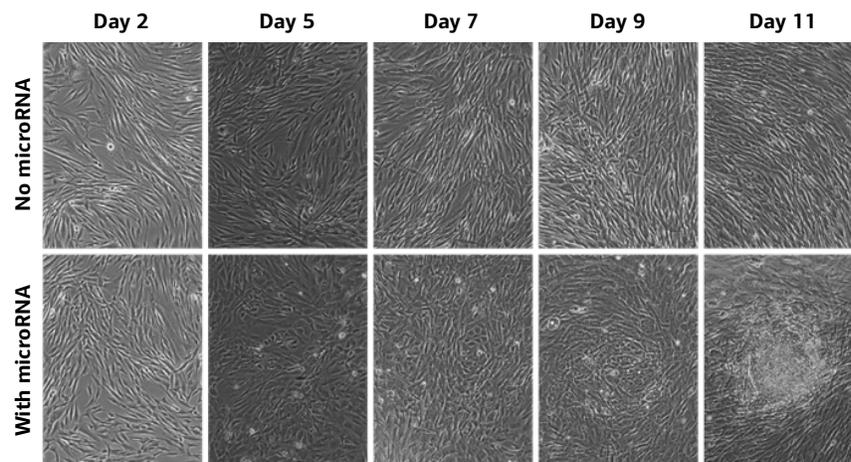


FIGURE 2. Morphological progression comparison of diseased patient fibroblasts during reprogramming with and without microRNA: The above panel demonstrates representative morphological progression of adult diseased patient fibroblasts with and without the Stemgent microRNA booster cocktail.

Each day, the cultures were examined under the microscope for proliferation, morphological changes, and nGFP expression. Wells treated with the combination of mRNA and microRNA cocktail displayed a marked increase in proliferative cell clusters expressing high nGFP reporter expression (Figure 3) compared to wells transfected with mRNA cocktail only. The prevalence of these early clusters was indicative of TRA-1-81-positive colony emergence by day 13 (Table 2). Also of note, the overall degree of mesenchymal-to-epithelial transition was greater in the microRNA treated wells compared to mRNA only treated wells. This transition reduced overgrowth and facilitated efficient delivery of reprogramming factors in the microRNA treated wells throughout the course of transfections whereas the less-epithelialized mRNA only treated wells grew progressively confluent and more refractory to transfection. Overall, the higher cellular confluence in the wells without microRNA treatment resulted in fewer, less-developed and more difficult to identify and isolate colonies.

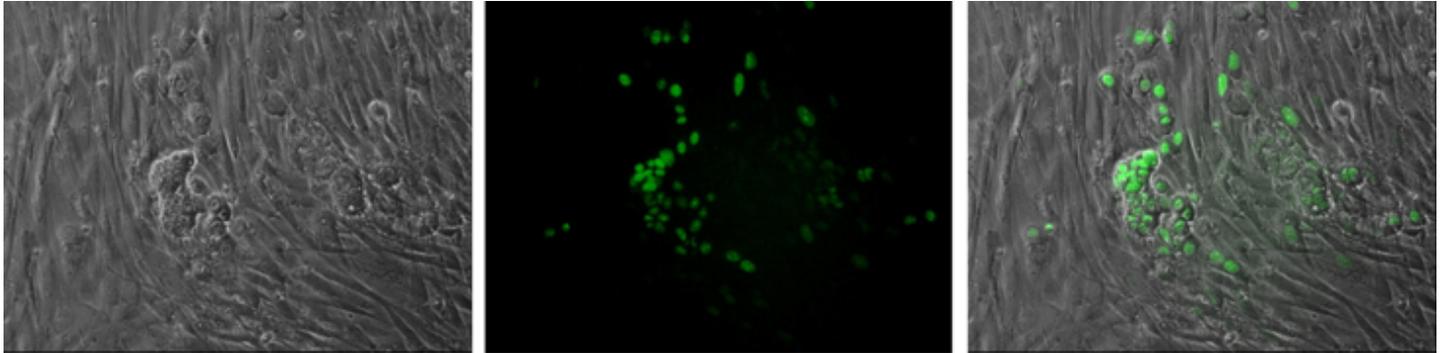


FIGURE 3. nGFP reporter allows for easy and rapid visualization of early reprogramming events: The above panel shows representative phase morphology (A), typical nGFP clustering (B), and associated overlay (C) with emergence of iPS cell colonies from dermal fibroblasts (20X).

Of the two diseased patient lines tested; patient sample #1 yielded three TRA-1-81 positive colonies with mRNA only, compared to 113 with the addition of microRNA treatment. TRA-1-81 positive colonies were not present when patient sample #2 was transfected with mRNA alone. However, with the addition of microRNA treatment, patient sample #2 yielded 68 TRA-1-81 positive colonies (Table 2).

Sample	microRNA	TRA-81+Colonies
Neonatal, Fibroblast Control	No	217
Adult, Human Diseased Fibroblast #1	No	3
	Yes	113
Adult, Human Diseased Fibroblast #2	No	0
	Yes	68

TABLE 2. Reprogramming productivity of diseased patient fibroblasts with and without microRNA: On day 0, 2.5×10^4 cells were seeded into Corning Matrigel-coated 12-well plates. On days 1 and 5 the appropriate wells were transfected with microRNA cocktail. On days 2-13 all wells were transfected with mRNA reprogramming cocktail. TRA-1-81(+) colonies were assessed and counted using Stemgent StainAlive TRA-1-81 Antibody (DyLight 488).

Upon resuscitation and extended culture (>20 passages) the iPSC clones maintained robust expansion, normal morphology, karyotype and uniform pluripotency marker expression by immunocytochemistry (ICC) (Figures 4 and 5).

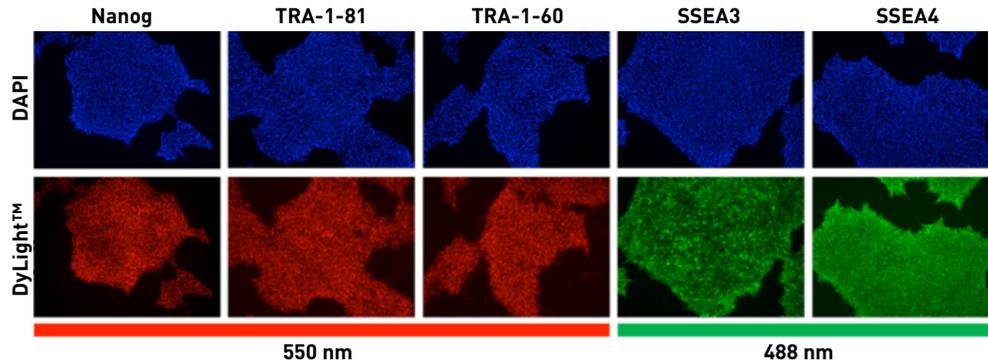


FIGURE 4. iPS cell clones retain pluripotency markers over extended passaging: Patient samples #1 and #2 displayed uniform pluripotency marker expression. Depicted above, is analysis of Nanog, TRA-1-60, TRA-1-81, SSEA3, and SSEA4 expression from patient sample #1 iPS cell clones isolated and expanded to passage 22. (Data not shown for patient sample #2)

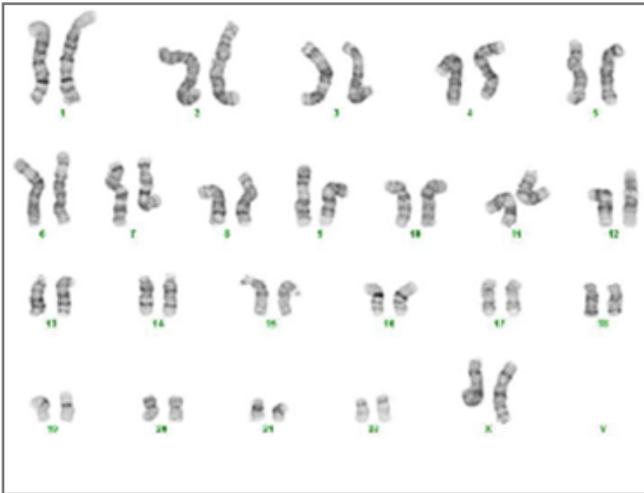


FIGURE 5. Human, diseased patient fibroblast clones display normal karyotype over extended passaging: Normal female karyotype was observed from both adult patient sample #1 and #2 iPS cell clones. Image depicted is karyotype analysis from patient sample #1 iPS cell clones isolated and expanded to passage 22. G-band analysis performed and analyzed by Cell Line Genetics, Madison, WI. (Data not shown for patient sample #2).

CONCLUSION

The hazards of viral and DNA-vector reprogramming methods limit the utility and suitability of resulting iPS cell in downstream applications. In addition, persistent expression of exogenous reprogramming factors can modulate an iPS cell-like phenotype characteristically similar to *de facto* iPS cells. Clonal attrition of these *pseudo*-iPS cell colonies over time necessitates the need for excessive initial colony isolation, maintenance, and screening resulting in added time and reagents.

Transient expression of non-integrative RNA reprogramming factors to induce reprogramming in somatic cells eliminates any potential of insertional mutagenesis in the genome of the target cells, producing stable, assay-ready iPS cell lines that are free of any genomic integration or viral manipulation. Moreover, when working with difficult to reprogram, refractory cell lines typical of geriatric and diseased patient lines, the incorporation of exogenous microRNAs significantly enhances reprogramming both in efficacy across adult patient lines and efficiency compared to mRNA alone. The defined nature of the system delivers reproducible results making microRNA-enhanced mRNA reprogramming amenable to high-throughput applications. Here we demonstrate an efficient, scalable platform to generate an abundant source of patient- or disease-specific iPS cell lines for pre-clinical applications and future therapeutic use.

For a thorough review of the major steps of Stemgent's RNA Reprogramming methods click on the links or scan the codes below:

microRNA Enhanced	mRNA only	Generating NuFF-Conditioned Pluriton™ Medium

PRODUCT ORDERING INFORMATION

Description	Stemgent Cat. No.
Nuff-RQ™ IRR	GSC-3006G
NutriStem™ XF/FF Culture Medium	01-0005
CryoStem™ Freezing Medium	01-0013-50
Stemgent® microRNA Booster Kit	00-0073
Stemgent® mRNA Reprogramming Kit	00-0071
Stemfect™ RNA Transfection Kit	00-0069
StainAlive™ TRA-1-81 Antibody (DyLight™ 488)	00-0969

TABLE 3. Consolidated Stemgent material list: See Stemgent's microRNA-Enhanced mRNA Reprogramming protocol for more information.

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