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Direct Comparison of DNA versus mRNA on the Efficiency of Induced Pluripotent Stem Cells Generation from Human Primary Fibroblasts

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Abstract

Background: Induction of pluripotency in somatic cells has opened a brand new spectrum on patient-specific stem cell therapies, therefore several approaches seek the optimization of the existing dedifferentiation processes. DNA and mRNA have been used to induce cells using different strategies, from diverse sets of pluripotency induction genes to variations of their delivery method. In this work we present a direct comparison between circular and linear DNA, as well as mRNA, capability to dedifferentiate human primary fibroblasts.

Methods and Findings: A single tricistronic vector harboring the previously successfully proven Oct4, Sox2 and Klf4 combination, along with two different IRES sequences that allow protein production regulation at the translational level was used. This approach enables the stoichiometry control of the translated proteins, as it is known it has a preponderant role on the methodology efficiency. Western Blotting made possible transcription factors detection and estimation of their relative translation rate. Alkaline phosphatase staining, flow cytometry and RT-PCR allowed cells with pluripotency potential identification. We found that, along with the non-integration benefits, the use of mRNA shows better yields and shortens the emergence time of AP positive colonies when compared to DNA approaches.

Conclusion: The results obtained suggest that iPSC can be generated with the same set of genes using circular and linear DNA, as well as mRNA, and that IRES sequences can enable ratios of protein production.

Keywords: Induced pluripotent stem cells; Primary human fibroblasts; MRNA; DNA; Non-integrative

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Introduction

The induction of pluripotency with defined transcription factors (TFs) in somatic cells was first proven by Takahashi and Yamanaka [1]. Distinct modifications to the original scheme seek the optimization of the procedure; within them are the genes used and the method to deliver them into the cell, as well as the cell type selected for induction. The classic dedifferentiation set includes Oct4, Sox2, Klf4 and c-Myc (OSKM). However, many different combinations have been analyzed [2-11]. Even though efficiencies are still low, promising results have been obtained with the Oct4, Sox2 and Klf4 (OSK) combination [6,12,13]. It is known that TFs stoichiometry influences dedifferentiation

efficiency as well as the epigenetic and biological properties of the generated iPSCs [14-16], nonetheless they are usually delivered individually. This makes difficult to control the quantity of each gene entering the cell and thus the ratio at which each could be transcribed and translated, and makes almost impossible to guarantee that every successfully transfected cell receives all the desired genes. To assure that all the required genes enter the cell, the use of polycistronic vectors is explored [15,17-20]. Also, TFs

delivering is commonly done via retroviruses or lenti viruses [21-25], deriving in transgenes' integration to the cell genome, hence non-integrative alternatives are being explored [11,26-30], Even though different cell types have been evaluated, the prevailing human cells used are fibroblasts [2,3,7,21,31,32] given their practicability of obtaining from donors and good dedifferentiation rates. Accordingly to the hypothesis that there is an optimal TFs intracellular ratio that might increase pluripotency induction efficiency, as each is expressed differently on embryonic and adult human stem cells [31,33], we designed a tricistronic vector harboring OSK. The set of genes is in between two Internal ribosome entry sites (IRES) from the Encephalomyocarditis virus (EMCV) [34], that working together with the 5' translation initiation site, allow simultaneous translation of the three TFs from a single mRNA [35-37] (**Figure 1 and Supplementary File 1**). We selected two different sequences that enable translation of each TF in different amount. The proposed structure of our vector (pFAPIS) is designed to produce Oct4 in higher quantity than Sox2, being Klf4 the lowest. Human primary fibroblasts were transfected with circular DNA, linear DNA, and mRNA to compare their total pluripotency induction efficiency. Alkaline Phosphatase (AP) staining was used to determine dedifferentiation capability, as it is one of the first pluripotency signs and an endpoint assay to monitor undifferentiated cells with self-renewal potential [38]. Other classic pluripotency markers were also detected using RT-PCR and flow cytometry, although it is known that not all the iPSC markers can be seen in the first days of dedifferentiation [39]. As it has been previously proved that OSK combination is sufficient to successfully induce pluripotency on human fibroblasts [6,12,13] the aim of this work is only to compare the efficiencies obtained with the three proposed induction methods, and demonstrate that IRES structures in between the genes arrangement allows different levels of protein production in either version of ribonucleic acid used. Then, cells that are easily obtained from patients and have reliable dedifferentiation capabilities are desirable for a non-invasive patient-specific approach. Thereby, the use of a set without the oncogene eliminates the risks of its reactivation, and the use of a polycistronic arrangement that

makes possible to control TFs stoichiometry, are advantageous features for clinical applications.

Methods

Vector construction

Oct4 [GenBank:NM002701.4], Sox2 [GenBank:NM003106.3] and Klf4 [GenBank:NM004235.4] sequences were optimized for human expression (DNA 2.0; California, USA). Each factor harbors a 6xHis tag to allow their identification with the same antibody. In between the TFs, two IRES sequences were included: IRES1 and IRES2 that correspond to wild type (MPS1-ECAT) and partially disabled (MPS1-ECAT811) [34]. The synthetic cassette was cloned into pTracer CMV/Bsd (Life Technologies; New York, USA).

DNA production

Top10F *E. coli* strain was transformed with pFAPIS. Cells were grown on LB broth (Difco; Maryland, USA) plus 150 μ g/mL carbenicillin (Sigma-Aldrich; Missouri, USA). DNA was purified with PureYield Plasmid Maxiprep System (Promega; Wisconsin, USA) and linearized using Sapl (New England Biolabs; Massachusetts, USA).

In vitro transcription

Ribomax Large Scale Production System T7 (Promega) was utilized for mRNA obtaining. The reaction product was cleaned using NAP-5 chromatographic columns (GE Healthcare, UK).

Cell culture

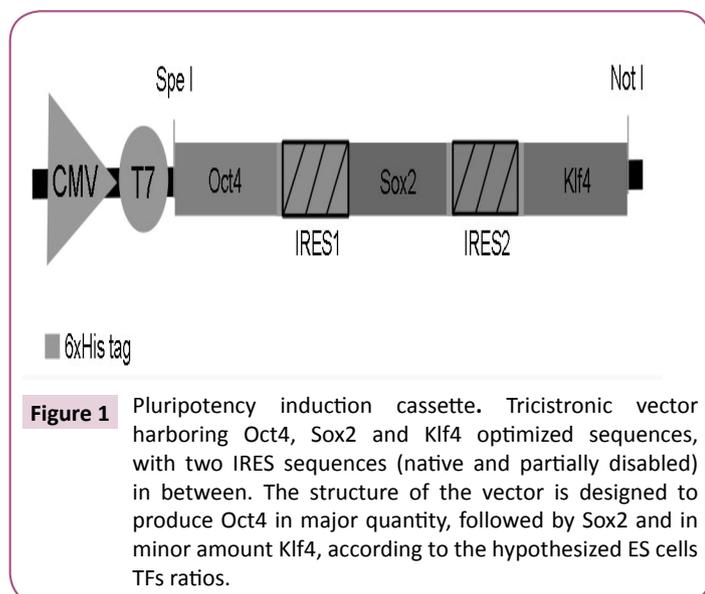
Human primary fibroblasts (ATCC; Virginia, USA) were grown on DMEM-F12 (Gibco; New York, USA) with 10% fetal bovine serum (FBS) (Gibco) and 1% Penicillin-Streptomycin (Pen-Stp) (Gibco). Derived iPSCs were grown with PluriSTEM Human ES/iPS (Millipore; Massachusetts, USA) on Matrigel (Corning; New York, USA). iPS cells were passaged using gentle cell dissociation reagent (Gibco). Both cell lines were maintained at 37°C, 5% CO₂.

Pluripotency induction

Fibroblasts were seeded at 4×10^5 cells/mL and transfected with Transit-2020 (MirusBio; Wisconsin, USA) using 1 μ g of DNA or 0.2 μ g of mRNA per mL of cell culture. When using DNA a single initial transfection was done (day 0), when using mRNA three total deliveries were given on days zero, two and four. In either case, cells were transferred to a Matrigel coated plate at day 5. Untransfected fibroblasts and transfected using pTracer CMV/Bsd backbone as mock plasmid (L1) and the same backbone with another non-relevant insert (pOne) were used as negative controls. They were transfected and maintained under the same conditions as the ones transfected with pFAPIS. Transfection efficiency was determined via fluorescent cells counting in all DNA cases. Transfection efficiency using mRNA could not be calculated.

Colony appearance

Colonies could be seen as high-density cell arrangements with the naked eye, four days after the first transfection using mRNA induction and since day nine using either version of DNA. They



were analyzed using IX2-SLP microscope (Olympus; Ontario, Canada) with a 10X objective.

Alkaline phosphatase staining

mRNA transfected cells were analyzed at day 5 post-initial transfection and DNA transfected cells on day thirteen post-induction using the Alkaline Phosphatase Staining Kit (Millipore). AP positive structures were visible to the naked eye and analyzed using the DM IL microscope (Leica; Ontario, Canada).

Calculation of reprogramming efficiency

Image J software (NIH; Maryland, USA) was used to determine the colony count. Images for analysis were obtained before and after AP staining using the Perfection V370 scanner (Epson).

Flow cytometry

iPS Cell Characterization Kit (Millipore) was used to identify surface and intracellular stem cells markers, using Gallios Flow Cytometer (Beckman Coulter; Ontario, Canada), utilizing circular DNA induced cells at day 13 post-transfection.

RT-PCR

RNA extraction was performed at day 13 post-induction on cells transfected with circular DNA using Genelute mammalian total RNA (Sigma). OneStep RT-PCR kit (Qiagen; Maryland, USA) was utilized to execute the reaction. Primers for Oct4, Sox2 and Nanog were used (**Supplementary Table 1**). 1% agarose gel electrophoresis permitted reaction products analysis.

Protein extraction

At day thirteen post-transfection with circular DNA, cells were harvested according to Tansey [40]. In mRNA transfected cells case, two successive mRNA transfections were done 12 hours apart with sample collection 18 hours after the first delivery. The same collection protocol was used. Untransfected primary fibroblasts were used as negative control, and processed identically.

SDS-PAGE

Cell extracts were ran in a 10% SDS-PAGE [41], at 120 V for 1:20 hrs. Gels were loaded with 37 μ g of total protein of the corresponding cell extracts in all cases.

Western blotting

An HRP labeled anti-6xHis antibody (Roche; Québec, Canada) in a 1:500 dilution was used. SDS-PAGE proteins were transferred onto a PVDF membrane (Millipore) using the Fast Semi-dry Blotter (Thermo Scientific) for 35 minutes at 25 volts. Detection was made with Clarity Western ECL Substrate (BioRad Laboratories; California, USA) and an UltraCruz Autoradiography Film (Santa Cruz Biotechnology; Texas, USA). Total protein from untransformed fibroblasts, and circular DNA and mRNA induced cells was tested. A known molecular weight 6xHis-tagged protein was used as molecular weight marker.

Statistical analysis

Two-tailed Student's t-tests were performed with StatPlus Software (Analyst Soft) using data from circular and linear DNA induced groups, and from circular DNA and mRNA induced groups. One-way ANOVA enabled comparison of the three groups among them. P-values <0.05 were considered statistically significant.

Results

iPSCs induction using DNA and mRNA

When inducing pluripotency with DNA (**Figures 2a and 2b**) no time difference in colony appearance was seen with either of the two versions. In both cases AP staining was positive at day

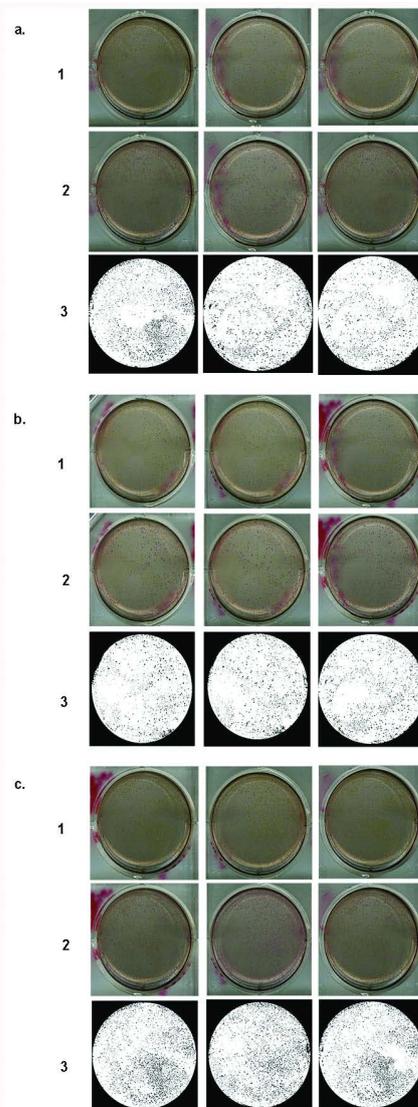


Figure 2 DNA AND MRNA INDUCED CELLS. REPRESENTATIVE TRIPLICATE OF WELLS FROM THE THREE INDUCTION METHODS OF CORRESPONDING COLONIES AP STAINING DAY. A. LINEAR DNA INDUCED CELLS B. CIRCULAR DNA INDUCED CELLS C. MRNA INDUCED CELLS. IN THE THREE CASES, IN ROW 1 THE WELLS BEFORE AP STAINING, ROW 2 SHOWS THE SAME WELLS AFTER AP STAINING, ROW 3 SHOWS WELLS' ANALYSIS USING IMAGEJ.

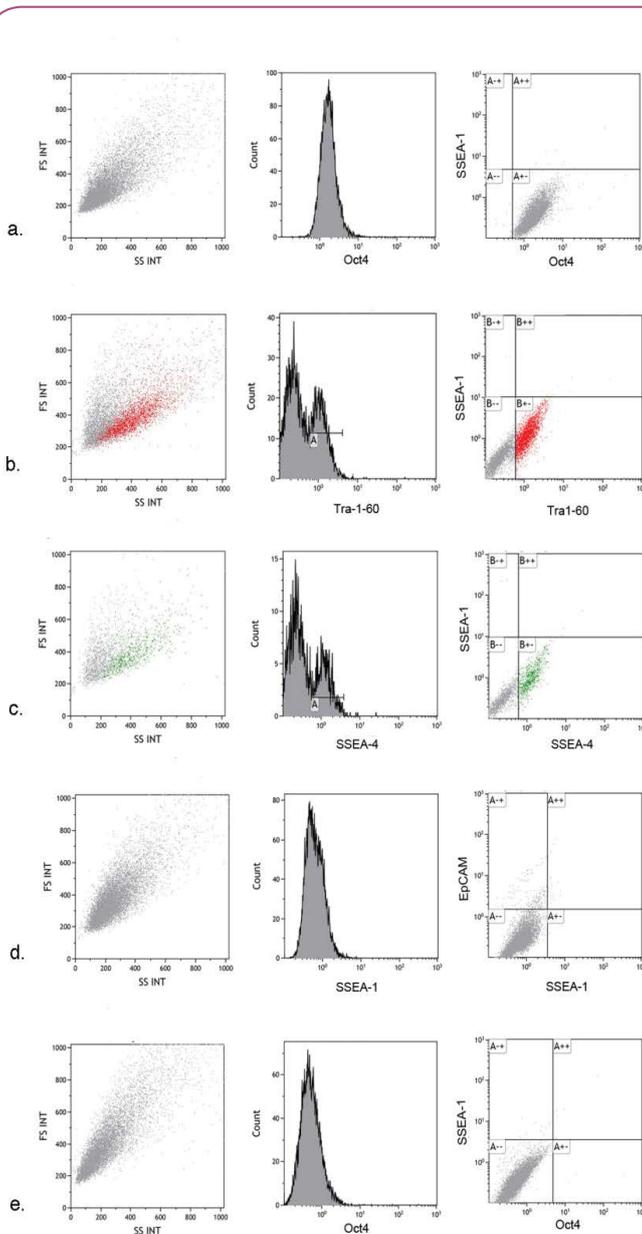


Figure 3 Flow cytometry histograms. Cells distribution graphs show that Oct4 (a.), Tra-1-60 (b.) and SSEA4 (c.) markers were present on cells populations on day 13 post-transfection, meanwhile SSEA1 (d.) could not be detected. Also none of the markers was present on the untransformed control (e.).

thirteen post-transfection and morphological iPSC characteristics appeared with no time difference between them. AP positive cell groups yield was slightly different, when induction was done with the linear vector an average of 1706 colonies per well were obtained, whereas when using circular DNA the average was 2109, conferring a dedifferentiation efficiency of 0.42 and 0.52% respectively. In order to verify that the AP positive colonies were also expressing other classic pluripotency markers, flow cytometry was performed using circular DNA transfected cells. Oct4, Tra-1-60 and SSEA4 positive cells were found, meanwhile SSEA1 was not detected. On untransformed cells no markers were found so it can be said that the generated cells present pluripotency potential (**Figure 3**). In the same fashion, an RT-PCR was run

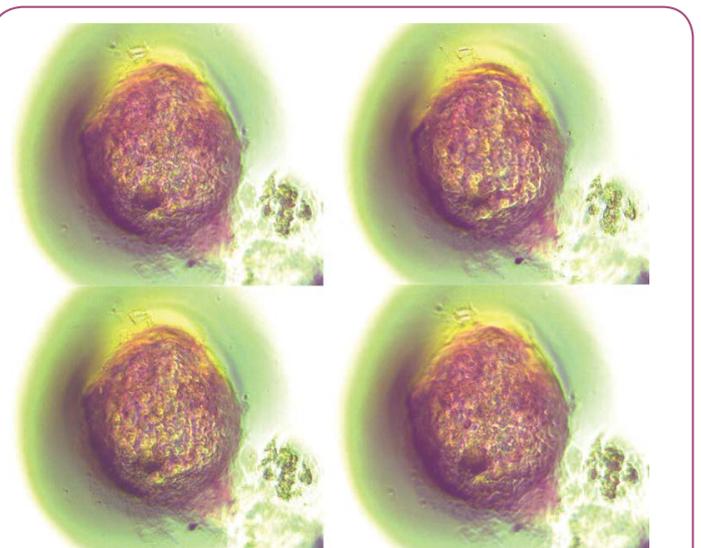


Figure 4 AP positive colony. Under a 10X magnification, an AP positive tridimensional colony can be seen after AP staining. Four different levels of the structure were focused in order to verify that the complete arrangement was positive for the staining.

using Oct4, Sox2 and NANOG primers. Bands corresponding to the three expected amplicons sizes were visualized, in contrast to the negative control (untransformed fibroblasts) lane, which did not show any amplification product (**Supplementary Figure S2**). A t-test analysis was performed between circular and linear DNA and the difference among them were statistically significant; therefore the first was used to run comparison experiments with mRNA induction. In the case of mRNA induction, colonies were seen since day four post-transfection with AP positive cell clusters at day five post-induction (**Figures 2c and Supplementary Figure S1**). mRNA yields were on average 3217 colonies, which corresponds to an efficiency of 0.8%. Negative control cells were unable to survive after 8 days from transfection (**Supplementary Figure S3**). Consequently, we decided that cell survival along with positive AP staining and Nanog expression was sufficient to presume that the identified cells showed pluripotent cells early characteristics and potential. DNA and mRNA induced cells behaved differently both in time of colonies appearance and in induction efficiency (**Supplementary Table 2**). The latter was confirmed to be statistically significant after a t-test was run between both inductions data and an ANOVA demonstrated that none of the treatments was similar among each other. Thus, it can be said that induction done with mRNA resulted 35% more efficient than the one with circular DNA, and the last is 19% more efficient than its linear counterpart. Also, both types of induction AP positive structures were analyzed under a 10X lens and they showed similar characteristics (**Figure 4**).

TFs ratio production analysis

In order to determine if our hypothesis of TFs production ratio was being fulfilled, we designed experiments that enable us to visualize each transcription factor at protein production level. Protein samples of circular DNA and mRNA induced cells were processed in Western Blot along with a molecular weight (MW)

marker (43.6 kD). In both samples, three bands in the calculated MW of each TF: Oct4 of 39.3 kD, Sox2 of 35.1 kD and Klf4 of 51.8 kD, could be identified. In opposition, on the negative control (Untransfected fibroblasts) lane, no proteins could be detected (**Supplementary Figure S4**). Each TF band presented different density that was alike in both types of induction, being the densest the one that represents Oct4, followed by Sox2 and then Klf4.

Discussion

When fibroblasts were transformed using DNA, colonies were visible later than the ones transfected with mRNA, this may be due to the fact that when the latter was delivered, it was ready to be translated. While DNA has to integrate to the cell's genome to be transcribed and translated, which delays protein availability. Efficiency differences could be consequence of the mRNA available for translation. When using DNA, its integration to the cells' genome is not always appropriate due to cell's vector random linearization and insertion direction, so not all integrated exogenous DNA would codify correctly for the proteins needed, hence the overall protein production efficiency decreases and ratios might not be the ones desired. The results obtained indicated that mRNA induction is faster and more efficient than the results obtained using DNA. As mRNA gave better outcomes than DNA, we contrasted those results with others with similar scope, in order to identify the strengths of this method over others previously reported. Comparing our findings with those from Warren et al. the method here shown shows some betterment. First, we discarded c-Myc, eliminating its risk of reactivation. Second, they used modified bases to synthesize the mRNA used, while we utilized regular nitrogen bases. They also used interferon inhibitor to overcome innate antiviral responses, however it has been reported that its reactivation enhances iPSCs generation [42], so we did not inhibit this natural response. With the technology here reported, we were able to see ES-like colonies 16 days before them with only three mRNA transfections of 1 µg each, in opposition to their 16 transfections of 600 ng each. Nonetheless our efficiency is lower than theirs, which might be correlated with the minor efficiencies reported when using OSK. Yoshioka *et al.* reported the utilization of OSKM in a single RNA replicon and B18R as innate immune response blocker. Utilizing up to five transfections, with efficiency around 0.025%, they obtained AP positive stained colonies in between days 25 and 30. In this case, the results here reported exhibit an efficiency 32X greater and a 4X times faster induction. Rais et al. used three rounds of OSKM and LIN28 mRNA transfections, ROCK inhibitor and Mbd3 siRNA, reporting pluripotent cells at day 8. We are able to generate AP positive colonies at day five post-transfection, using only three mRNA deliveries, without any modifications to the cells internal environment and with a simpler group of induction genes. Thereby, it can be said that

the technology here presented indicates that a single mRNA derived from a smaller set of dedifferentiation genes, is able to drive efficient pluripotency induction. The elimination of c-Myc fends off its reactivation, and as a non-integrative method is used, the integration-derived safety issues of the generated cells are overcome. These observations lead to the conclusion that our vector (on both DNA and mRNA versions) is able to drive the cell to produce the TFs in the proportion that was hypothesized, generating a stoichiometry that would correspond to human embryonic and adult stem cells distinctive concentrations, hence probably facilitating and hastening the dedifferentiation process. It can also be noticed that band intensities differ between DNA and mRNA lanes, this might be consequence of the mRNA availability for translation, as it could be greater in the latter than in the first. Together with the safety benefits of the non-integration given by mRNA, the reduction of TFs number that simplifies cells' external stimulus, and the ability to control the protein production ratios by the use of a single messenger, the system here presented exhibits some characteristics that might help improve pluripotency induction existing techniques. Even though previous evidence demonstrated that OSK is able to generate pluripotent cells, there are still tests to run in order to assure that the generated cells have all the properties that make them iPSC (such as the three germ layers generation capabilities and differentiation to specific cell lines potential), nevertheless the data obtained so far suggests that the proposed OSK vector that permits stoichiometry control is able to generate cells that show pluripotent characteristics and potential. Thereby, it can be stated that induction can be done with the same set of genes using two different procedures that enable protein level production control. As expected, differences between DNA and mRNA induced cells were seen. mRNA showed 35% better yields and almost half the time of AP positive cells emergency, when compared to circular DNA approach. So, in addition to the non-integration benefits intrinsic to the mRNA methodology, the aforementioned features may help to develop techniques with better qualities that could aid drive this technology outside the bench.

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Conflict of interests

The authors acknowledge that there is no conflict of interests.

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