Biochemical and Biophysical Research Communications 394 (2010) 189–193

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Reprogramming of human fibroblasts to pluripotent stem cells using mRNA of four transcription factors

Eduard Yakubov [a], Gidi Rechavi [b], Shmuel Rozenblatt [c], David Givol [a,*]

[a] Department of Molecular Cell Biology, Weizmann Institute of Science, 76100 Rehovot, Israel
[b] Cancer Research Center, Chaim Sheba Medical Center, Tel-Hashomer and Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel
[c] Department of Molecular Microbiology and Biotechnology, Tel-Aviv University, Tel-Aviv, Israel

ARTICLE INFO

Article history:
Received 21 February 2010
Available online 25 February 2010

Keywords:
Reprogramming
iPS
DNA integration
Malignancy
RNA transfection
Transcription factors

ABSTRACT

Reprogramming of differentiated cells into induced pluripotent cells (iPS) was accomplished in 2006 by expressing four, or less, embryonic stem cell (ESC)-specific transcription factors. Due to the possible danger of DNA damage and the potential tumorigenicity associated with such DNA damage, attempts were made to minimize DNA integration by the vectors involved in this process without complete success. Here we present a method of using RNA transfection as a tool for reprogramming human fibroblasts to iPS. We used RNA synthesized in vitro from cDNA of the same reprogramming four transcription factors. After transfection of the RNA, we show intracellular expression and nuclear localization of the respective proteins in at least 70% of the cells. We used five consecutive transfections to support continuous protein expression resulting in the formation of iPS colonies that express alkaline phosphatase and several ESC markers and that can be expanded. This method completely avoids DNA integration and may be developed to replace the use of DNA vectors in the formation of iPS.

1. Introduction

Induced pluripotent stem cells (iPS) are produced by reprogramming of somatic cells and are very similar to natural embryonic stem cells (ESC), showing the capacity to differentiate into any cell type and the ability to self renew. Potentially they hold great hope for personal regenerative medicine, cell therapy and disease models in addition to their value in the study of the process of cell fate determination. Since the pioneering work of S. Yamanka in 2006, iPS cells have been generated from a variety of somatic cell types by the transfection of DNA encoding four, or fewer, transcription factors (TF), Oct4, Sox2, Klf4, and c-Myc [1]. In human iPS cells, Lin28 and Nanog replaced Klf4 and Myc [2]. DNA was transduced into somatic cells by retroviral or lentiviral infection, by plasmids, or by other vectors [3–5]. iPS cells were obtained by various alternative methods of DNA insertions into cells, all of which resulted in multiple DNA integration sites in the genome of the generated iPS cells. Based on previous experience, particularly from gene therapy, it became obvious that genome integration by DNA is unpredictable and may lead to malignancy and other undesired results. Therefore attempts were made to minimize genome integration by combining the DNA of all four factors into one polycistronic vector [6,7] and more recently by Cre-loxP excision of the inserted genes after the reprogramming process was switched-on [5,8]. However some vector arms sequences remained at the integration site as a potential damaging element. Hence the danger of DNA damage was not eliminated completely from the procedures using DNA vectors.

We believe that avoiding DNA in the reprogramming process might be a necessary solution to the problem of genome integration, providing that it does not prevent the production of iPS. Recently the use of recombinant proteins of the same four factors, produced in bacteria, was reported with successful generation of PiPS (Protein-produced iPS) cells from mouse fibroblasts [9]. However proper post translation modification of the proteins is better processed inside the cell and not in bacteria.

Here we report the use of in vitro-produced mRNA encoding for Oct4, Lin28, Sox2, and Nanog for reprogramming of human foreskin fibroblasts (hFF) to generate RiPS (RNA-produced iPS) cells. This procedure avoids any DNA integration events and the associated genomic damage. RNA transfection resulted in protein expression level in less than eight hours and at least five consecutive transfections were needed to maintain protein expression levels that could switch-on the expression of endogenous stem cell pluripotent genes that drive the human fibroblasts to iPS.

2. Materials and methods

2.1. Cell culture, cells transfection

Human foreskin fibroblasts (hFF, Sigma) were cultured on gelatin-coated dishes (100,000 cells per well of six-well plate) with hFF

* Corresponding author. Fax: +972 89344125.
E-mail address: david.givol@weizmann.ac.il (D. Givol).

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.02.150

growth media containing DMEM (Invitrogen), 10% FBS (Beit-Haemek, Israel), 4 mM L-glutamine, 10 mM HEPES and 1:100 penicillin–streptomycin (Invitrogen). Transfection was in 1 ml of antibiotic-free hFF medium supplemented with 4 μg of RNA mix (1.0 μg of each of the 4 TFs) and 2 μl of Lipofectamine 2000 (Invitrogen) per well of six-well plate according to the manufacturer's instructions. After 5 h of incubation the medium was changed to fresh hFF medium without RNA and Lipofectamine while containing antibiotic. Usually five consecutive transfections were performed 24 h apart. After the last transfection the hFF cells were transferred to irradiated murine embryonic fibroblasts (iMEF) and cultured with hES medium prepared as DMEM/F12 (Invitrogen) supplemented with 20% knockout serum replacement, 2 mM L-glutamin, 1:100 nonessential amino acids, 1:100 penicillin–streptomycin (Invitrogen), 2 μg/ml Heparin (Sigma) and 10 ng/ml basic FGF (Peprotech). The medium was replaced daily. Colonies of iPS were observed between 1 and 2 weeks after the last transfection.

After 10 days on iMEF the hES medium was replaced with 50–75% conditioned medium [10]. Primary colonies were picked onto new iMEF plate 3 weeks after the start of the procedure.

2.2. Plasmids, primers, IVT with T7 RNA polymerase

Plasmid pTM1 was described previously [11] and was modified to yield pTMA, by the addition of a fragment containing polyA(82A) between the XhoI and NsiI sites after the 3′ end of GFP (Supplementary Fig. 1). To clone each of the cDNAs of the TF (Oct4, Lin28, Sox2, or Nanog), into pTMA the GFP coding region was excised from pTMA by EcoRI and either MscI or NcoI and was replaced by the coding sequences of Oct4, Lin28, Sox2 or Nanog, respectively. The DNAs of were amplified by PCR from the respective plasmids (Addgene Cat# 16579, 16580, 16577, 16578, 17219) using high fidelity DNA polymerase (Invitrogen, Cat#11304-011) and primers that include the appropriate restriction site sequences for cloning into pTMA after GFP excision. To prepare the RNA of the inserted TFs or of GFP, the pTMA was linearized by SalI after the polyA site, followed by in vitro RNA transcription (IVT) using the mMESSAGE mMACHINE T7 kit (Applied Biosystems/Ambion, TX, USA) that allows 5′cap formation. The IVT produced RNA includes also the IRES and the polyA. After DNaseI treatment, the transcribed RNA was recovered on RNeasy columns (Qiagen) according to the RNA Cleanup protocol of the manufacturer. RNA integrity was monitored by agarose gel electrophoresis and then with RT-PCR using dT_{25} and gene specific primers for each TF or GFP.

2.3. RNA isolation and RT-PCR

Twenty four hour (including 5 h of transfection) after the first transfection about ~100,000 hFF cells were harvested using standard trypsinization procedure and frozen in liquid nitrogen. Total RNA was purified with Rneasy Mini kit (Qiagen). On-column DNase treatment (Qiagen) was performed to remove genomic DNA contamination. One microgram of total RNA was used for reverse transcription reaction with SuperScipt II (Invitrogen) and dT_{25} primer, according to the manufacturer's instructions. The resulting cDNA mix was five-fold diluted and 2 μl was then used as template for PCR with ReddyMix PCR Mater Mix (Thermo Scientific). The set of primers used for the PCR was for Oct3/4 (Forward 5′-CAGT GCCCGAAACCCAC; Reverse 5′- GGAGACCCAGCAGCCTCAAA), for-Lin28 (Forward 5′-AAGCTGCCACCCCAGCCCAAGA; Reverse 5′-TTC TGTGCCTCCGGGAGCAGGGTA), for Sox2 (Forward 5′- AGCTACAGCA TGATGCAGGA; Reverse 5′-GGTCATGGAGTTGTACTGCA), for Nanog (Forward 5′-CAGAAGGCCTCAGCACCTAC; Reverse 5′-ATTGTTCCAG GTCTGGTTGC), for GFP (Forward 5′- CACTACCAGCAGAACACCCCCA; Reverse 5′-ACAGCTCGTCCATGCCGAGAGT), and for GAPDH (Forward 5′-GTGGACCTGACCTGCCGTCT; Reverse 5′- GGAGGAGTGGG TGTCGCTGT). PCRs consisted of 21–27 cycles of 95 °C for 1 min, 55 °C for 45 s, and 72 °C for 1 min.

2.4. Alkaline phosphatase staining and immunocytochemistry

Alkaline phosphatase (AP) staining was performed using the Vector Red Substrate Kit (Vector Laboratories) according to the manufacturer's protocol. The AP staining was recorded as red color or by fluorescence. For Immunostaining cells were fixed in 4% paraformaldehyde in PBS and immunostained according to standard protocols using the following primary antibodies: GFP (rabbit,

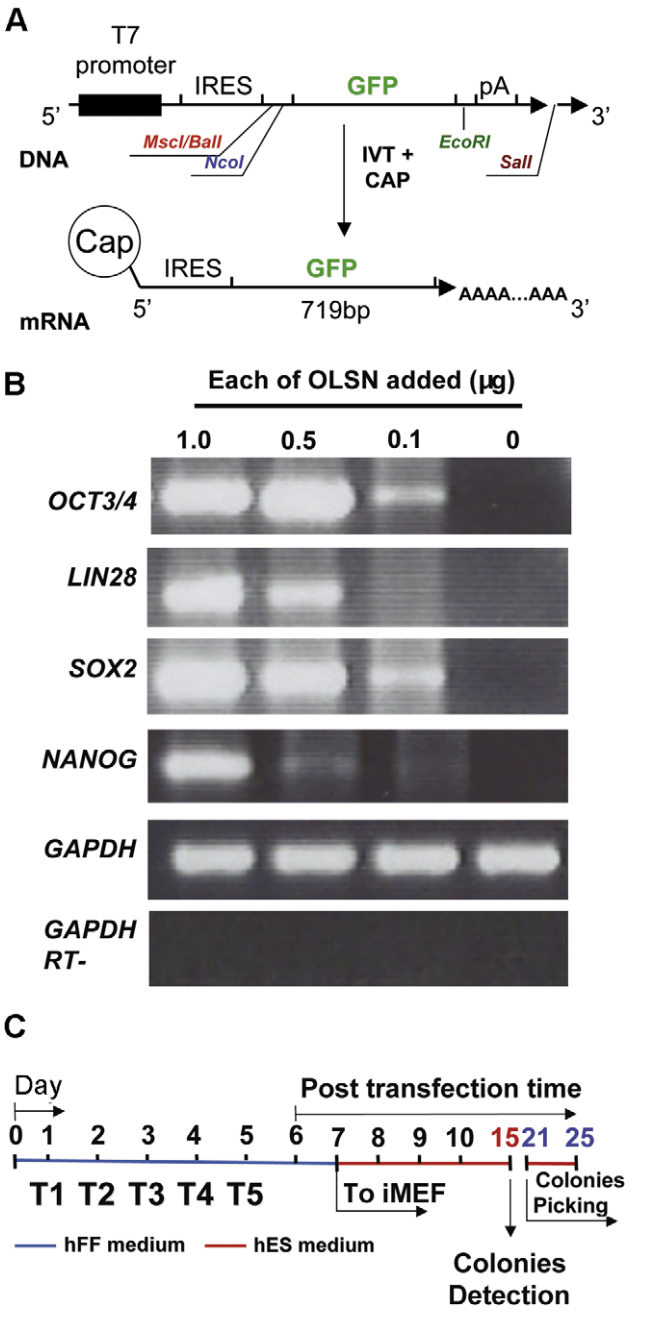

Fig. 1. Generation of specific mRNA used for production of iPS. (A) Structure of the DNA template in pTMA including the T7 promoter, IRES, cDNA, polyA, and SalI for linearization. The produced RNA is capped. (B) Determination by RT-PCR of the amount of each specific mRNA in hFF, following transfection with RNA mixture of four (OLSN) factors. (C) Schematic protocol for iPS generation by RNA transfection of hFF. T, transfection; OLSN, initials of the four TF.

Chemicon); hOCT4-3/4 (goat, Santa Cruz Biotechnology); hSOX2 (goat, Santa Cruz Biotechnology); hNanog (mouse monoclonal, Sigma): SSEA4 (mouse monoclonal, Millipore). Appropriate Cy2 or Cy3-conjugated secondary antibodies (Jackson lab) were used. Stained cultures were photographed in Olympus microscope.

3. Results

We used the pTMA plasmid (Supplemental Fig. 1) as a platform to prepare the mRNAs of each of the transcription factors by *in-vitro*-transcription (IVT) using T7 RNA polymerase. The plasmid pTMA contains the T7 promoter followed by IRES sequence and the GFP coding region followed by a polyA tail in the 3′ region (Fig. 1A). The coding sequences of human Oct4, Lin28, Sox2, or Nanog were amplified by high fidelity PCR from the respective plasmids (Addgene, Cambridge, MA) and used to replace the GFP in pTMA after appropriate restriction and ligation with the pTMA vector (Fig. 1A). The plasmid was linearized at restriction site SalI and subjected to IVT with T7 RNA polymerase which allowed for 5′cap formation (Ambion, Austin, TX). Supplemental Fig. 2 shows the resulting RNA as analyzed by gel electrophoresis. To determine the amount necessary for transfection, the RNA was transfected into hFF and the amount present in the cells 19 h post transfection was determined using RT-PCR on RNA obtained from these cells (Fig. 1B). It was concluded that 1 μg per well of six-well plate is optimal of each RNA for transfection. In the case of GFP, the encoding RNA obtained was used to follow the kinetics and stability of the intracellular expressed protein following the GFP fluorescence in the transfected cells. Protein expression was detected 8 h after transfection, reached a maximal level at 24 h and declined towards 72 h with lower expression that persisted longer than 72 h. Cells were transfected a second and third time at 24 h intervals and GFP expression persisted for more than 48 h after each transfection. On the basis of this result hFF cells were transfected with a mixture containing 1 μg RNA of each of the four factors according to the time-scale illustrated in Fig. 1C and protein expression was followed by immunocytochemistry with antibodies to the various factors. The results showed that whereas the expressed GFP was confined to the cytoplasm, the transcription factors were localized predominantly to the nucleus indicating the authenticity of the RNA and its translation process (Fig. 2). The GFP fluorescence of the transfected cells is shown in Fig. 2B whereas the staining with anti-GFP clearly shows the cytoplasmic localization of GFP (Fig. 2C and E). The staining with antibody to OCT4 (Fig. 2F–H) and to SOX2 (Fig. 2I–K) depicts nuclear localization of the expressed TF

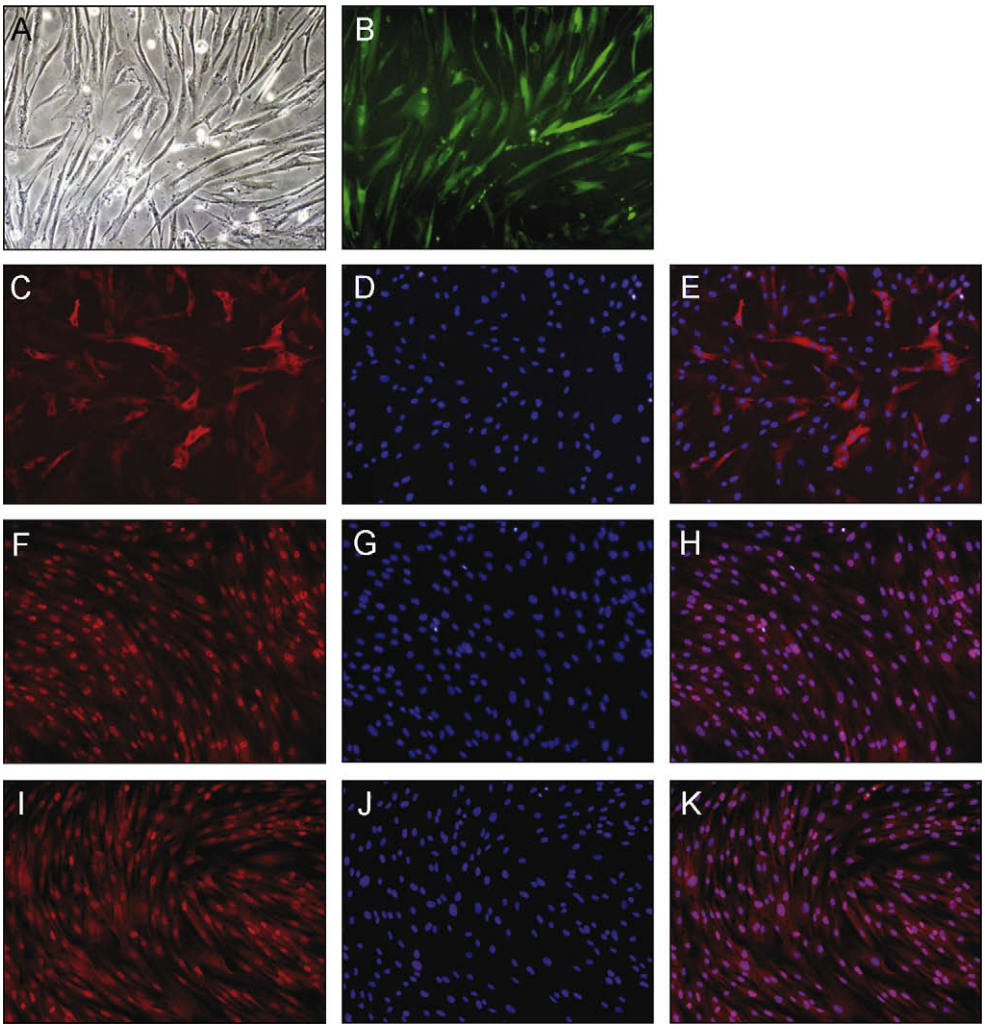

Fig. 2. Protein expression in transfected hFF cells. (A) Untransfected hFF cells. (B) Fluorescence of GFP detected 19 h after transfection. (C–E) Immunocytochemistry of hFF cells transfected with mixtures of four OLSN-mRNA or GFP and separately detected with specific antibody 24 h after transfection. (C–E) GFP, (F–H) OCT4, and (I–K) SOX2. The middle square shows DAPI stain and tight one is a merge of the previous two. Transfection was with 1 μg of each RNA. Magnification was ×100. Note the cytoplasmic localization of GFP and the nuclear localization of OCT4 and SOX2.

which is clear by merging with DAPI staining. Similar results were obtained also with IHC for Nanog and Lin28.

The four factors were expressed in high proportion (~70%) of the cells 24 h after first transfection. Later on the proportion of factors expressing cells decreased and only ~25% of the cells continue to express the proteins (data not shown). Nevertheless, this fraction of the cells showed stable expression since they retained the expressed transcription factor proteins even 7 days after the fifth transfection (Fig. 3).

Since stable expression was observed for only 72 h after the first transfection we speculate that the persistent expression of the factors 7 days after the fifth transfection may be due to activation of the endogenous genes of these factors rather than to residual transfected RNA. This contention is supported by staining with antibody to the stem cell surface marker SSEA4 which was not transfected at all but was very likely activated by the reprogramming process (Fig. 3). The expression of Nanog is shown in Fig. 3B and C and the expression of SSEA4 is shown in Fig. 3E and F. Note that Nanog is expressed mostly in the nucleus whereas SSEA4 is expressed in the cell membrane as is indicated by the merge with DAPI staining. After the fifth transfection the hFF cells were transferred onto iMEF cells and grown with the hES medium. Ten days after the last transfection we observed several small primary colonies. Fig. 4A depicts such colony in bright field and Fig. 4B shows their dye fluorescence. The general morphology of these colonies is shown in Fig. 4C and D at higher magnification. After further 10 days of growth a typical iPS colony after AP staining is shown in pink color in bright field (Fig. 4E) as well as in red fluorescence (Fig. 4F). In addition this colony is shown after DAPI staining (Fig. 4G) and after anti-Nanog staining whereas the MEF background was negative (Fig. 4H) indicating strong Nanog expression, likely from endogenous expression, since it was detected 20 days post transfection. We obtained approximately 50 colonies per 10^5 hFF cells and we are in the process of further analysis of the

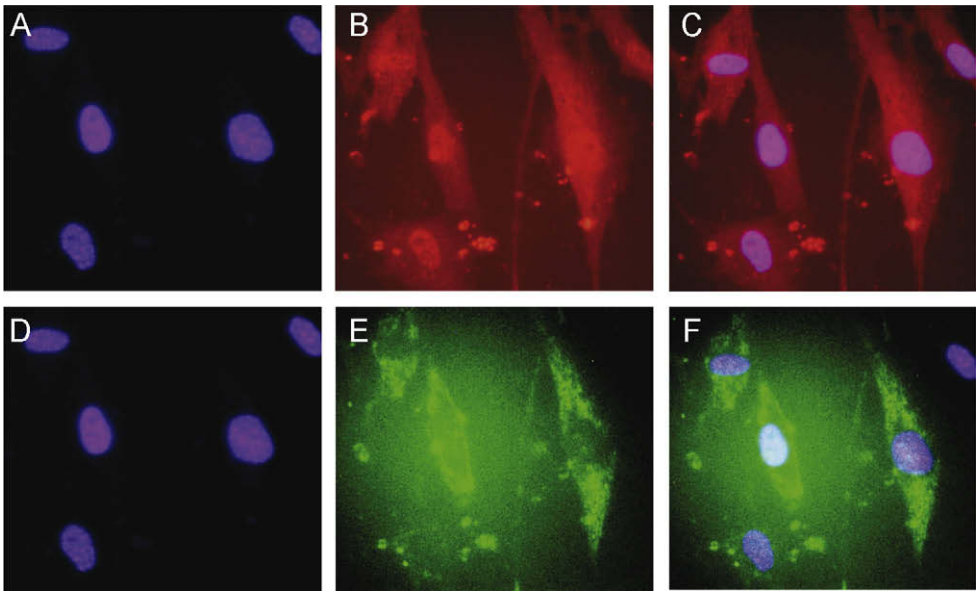

Fig. 3. Expression of Nanog and SSEA4 in hFF derived cells seven days after transfection as in protocol Fig 1. (A–C) Immunocytochemistry of Nanog using anti-Nanog at day 7 after the last transfection. (D–F) Immunocytochemistry of SSEA4 that was not included in the transfected RNA. In each line the first square was stained with DAPI the middle with specific antibody and the right one was a merge. Magnification ×400.

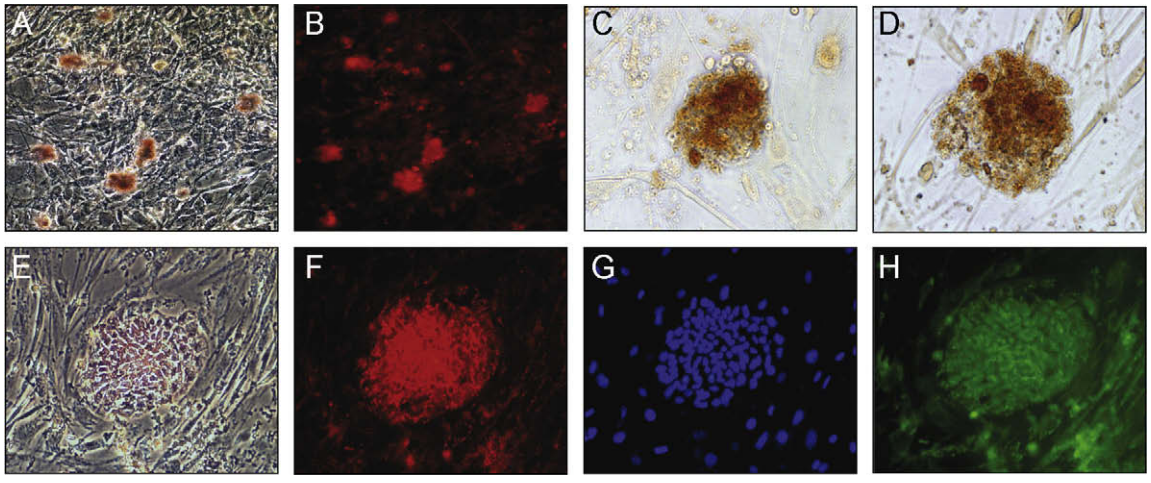

Fig. 4. Generation of mRNA induced iPS cells from hFF culture. (A–D) Primary (10 days from fifth transfection) iPS cells colonies visualized by AP staining. (A,C,D) bright field, (B) fluorescence. Primary (20 days from fifth transfection) RiPS cells colony. (E) AP staining, bright field, (F) AP staining, fluorescence, (G) DAPI, (H) anti-Nanog staining. Magnification (A,B) ×40, (C,D) ×400, (E–H) ×100.

properties of these colonies and their pluripotency. Further optimization of the reprogramming process by using chromatin modification by small molecules (e.g. valproic acid and others) is also being examined.

4. Discussion

In this paper we describe an experimental approach for the preparation of human iPS from foreskin fibroblasts using transfection of mRNAs encoding the four TF whose DNA constructs were used previously to prepare iPS. We established a convenient and efficient plasmid platform to prepare in vitro relatively large amounts of translatable mRNA, using T7 RNA polymerase and the linearized pTMA. The efficiency of transfection of this RNA was very high for all four factors and >70% of the cells expressed the proteins encoded by the RNA, peaking at 24 h post transfection and subsequently declining towards 72 h, probably through degradation. In the case of DNA transfection, the DNA has to enter the cell, penetrate the nucleus, integrate into the genome, where it is being transcribed to mRNA that crosses the nuclear membrane to the cytoplasm where it undergoes translation. In the case of the transfected RNA it is translated as it enters the cell and reaches the cytoplasm. This may explain the rapid expression and the high yield of the protein product since the entire RNA is ready for translation without dependence on the cellular transcription machinery. Furthermore, the transient nature of the transfected RNA can be regulated and used to switch the plutipotential process on and off. In the case of DNA transfection special precaution are necessary to shut off the expression of the integrated transcription factors.

Surprisingly, almost nothing has been reported on the use of functional RNA transfection as a tool to endow cells with a new function that can be regulated. For example it was possible to change the phenotype of differentiated neurons into that of astrocyte by transfer of the entire transcriptome of the astrocyte to neurons [12]. Very recently the RNA encoding the T cell receptor with anti-HER2 specificity was utilized to transfect T cells that transiently became cytotoxic for cancer cells expressing HER2. This activity was maximal at day 2 and persisted not later than day 9 [13]. In our system five transfections were needed in order to switch-on the stem cell specific gene repertoire and later the transfected RNA was eliminated without trace in the genome.

A recent report described the production of iPS without DNA vectors, by using bacterially synthesized recombinant proteins of the four factors slightly modified to allow cell membrane penetration [9]. In this procedure the proteins were made in bacteria (a potential problem for therapeutic use) as inclusion bodies and then were refolded and inserted into the cells. In our procedure using mRNA, the proteins fold in their normal cellular environment and most importantly, they undergo the natural post translational modifications. We point out that the procedure of RNA preparation in vitro is simple and easy to scale up and transfection is very efficient. The construction of plasmids that allow the synthesis in the test tube of capped RNA containing IRES and polyA was found to be superior in stability and protein expression over RNA that lacks these elements (data not shown).

The production of RiPS (RNA-induced pluripotent cells) cells by RNA overcomes an important hurdle on the way to personalized cell therapy. It eliminates the hazard of insertion mutations and genome damage that may be associated with the use of DNA vectors and may result in malignancy or genetic defects. It underscores the potential of using functional RNA for regulating cell functions without genome integration.

Acknowledgment

This work was supported in part by the Kahn Family Foundation. We thank R. Kaufman, Drs. J. Chebath and E. Ainbinder for advice and help and Dr. H. Gal for editorial assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.02.150.

References

[1] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, Cell 126 (2006) 663–676.
[2] J. Yu, M.A. Vodyanik, K. Smuga-Otto, et al., Induced pluripotent stem cell lines derived from human somatic cells, Science 318 (2007) 1917–1920.
[3] K. Okita, M. Nakagawa, H. Hyenjong, et al., Generation of mouse induced pluripotent stem cells without viral vectors, Science 322 (2008) 949–953.
[4] M. Stadtfeld, M. Nagaya, J. Utikal, et al., Induced pluripotent stem cells generated without viral integration, Science 322 (2008) 945–949.
[5] M. Wernig, A. Meissner, R. Foreman, et al., In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state, Nature 448 (2007) 318–324.
[6] B.W. Carey, S. Markoulaki, J. Hanna, et al., Reprogramming of murine and human somatic cells using a single polycistronic vector, Proc. Natl. Acad. Sci. USA 106 (2009) 157–162.
[7] C.A. Sommer, M. Stadtfeld, G.J. Murphy, et al., Induced pluripotent stem cell generation using a single lentiviral stem cell cassette, Stem Cells 27 (2009) 543–549.
[8] C.A. Sommer, A.G. Sommer, T.A. Longmire, et al., Excision of reprogramming transgenes improves the differentiation potential of iPS cells generated with a single excisable vector, Stem Cells 28 (2010) 64–74.
[9] H. Zhou, S. Wu, J.Y. Joo, et al., Generation of induced pluripotent stem cells using recombinant proteins, Cell Stem Cell 4 (2009) 381–384.
[10] J. McWhir, D. Wojtacha, A. Thomson, Routine culture and differentiation of human embryonic stem cells, Methods Mol. Biol. 331 (2008) 77–90.
[11] B. Moss, O. Elroy-Stein, T. Mizukami, et al., New mammalian expression vectors, Nature 348 (1990) 91–92.
[12] J.Y. Sul, C.W. Wu, F. Zeng, et al., Transcriptome transfer produces a predictable cellular phenotype, Proc. Natl. Acad. Sci. USA 106 (2009) 7624–7629.
[13] K. Birkholz, C. Krug, A. Hombach, S. Reuter, M. Kershaw, E. Kämpgen, G. Schuler, H. Abken, N. Schaft, J. Dörrie, Transfer of mRNA encoding recombinant immunoreceptors reprograms $CD4^+$ and $CD8^+$ T cells for use in the adoptive immunotherapy of cancer, Gene Ther. 16 (2009) 596–604.