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Induction of Pluripotency in Human Keratinocytes Through mRNA Transfection

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INDUCTION OF PLURIPOTENCY IN HUMAN KERATINOCYTES THROUGH mRNA TRANSFECTION. Robert D. Leone, Peter M. Rabinovich, Eugenie Cheng, Efim Golub, and Sherman M. Weissman. Department of Genetics, Yale University, School of Medicine, New Haven, CT.

Induced pluripotent stem (iPS) cells are epigenetically reprogrammed somatic cells that exhibit developmental and proliferative characteristics of embryonic stem (ES) cells. Other than alterations made during the reprogramming process, iPS cells are genotypically identical to donor tissue, giving them significant potential in regenerative medicine, basic biology of genetic disease, and drug development. Presently, iPS cell derivation largely relies on the introduction of reprogramming factors (eg, OCT4, SOX2, KLF4, c-MYC) directly into cellular genomes, leaving cells vulnerable to insertional mutagenesis and persistent expression of oncogenic transcription factors. This severely limits their use in clinical and research settings. Here we describe the reprogramming of human keratinocytes through the introduction of exogenous mRNA transcripts. mRNA presence within cells is transient and is unlikely to have permanent effects on the cellular genome, thus avoiding the pitfalls of present methods of iPS cell generation. Several benchmarks have been achieved toward this end, includ-ing: 1) successful expression in human cells of reprogramming factor proteins through the introduction of exogenous mRNA transcripts; 2) phenotypic transformation of human keratinocytes toward ES cell morphology by transfection with mRNA reprogramming factors; and 3) alkaline phosphatase activity (a well described early marker of pluripotency) in a small proportion of transformed cells. These results imply that mRNA transfection may be a viable method for reprogramming somatic cells towards pluripotency.

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This thesis is dedicated to my boys, William and Christopher, my love for whom is without bounds and to my late Grandmother, Marie, whose memory will always inspire the warmest feelings of love and joy.

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BACKGROUND

Human embryonic stem (hES) cells are characterized by two fundamental qualities: the ability to indefinitely self-renew, and the capacity to differentiate into any and all tissues of the body. Since first being isolated in 1998, hES cells have been the subject of significant attention from researchers as well as the lay public (1). Potential applications of these pluripotent and inexhaustible cells include studies of basic mechanisms of disease, screens for drug discovery, and the possibility of generating tissue for use in patients with degenerative diseases such as Parkinson disease, patients in need of bone marrow transplantation, or patients with infarcted cardiac tissue (2,3,4,5). One limitation of ES cells, however, is that they are generic in nature, and as such are unrelated to patients who may be the beneficiaries of such use. Consequently, tissues derived from hES cell lines will likely be the target of immune rejection in recipients (6,7). Also, because the only known source of hES cells is the inner cell mass of a human blastocyst, research into the basic nature and potential application of hES cells has been embroiled in ethical, religious, and political concerns. Spurred on by both the promise, as well as the limitations of hES cells, researchers have investigated alternative means of achieving pluripotency. These investigations have taken the form of nuclear transfer experiments (8,9), cell fusion experiments between somatic cells and ES cells (10,11), and the design of transformative cell culture conditions to attain pluripotency in testis cells (12,13).

Somatic cell nuclear transfer (SCNT), wherein a somatic cell nucleus is transplanted into an oocyte or ES cell and subsequently achieves pluripotency through epigenetic reprogramming, has been successfully demonstrated in a variety of non-human,

mammalian species (most notably in “Dolly” the sheep in 1996) (9,14,15). The products of successful SCNT experiments are pluripotent cells that are identical in genomic makeup to the starting cell nucleus (14). While this approach would theoretically produce patient-specific, rejection-proof pluripotent cells for therapeutic and analytical use, it is limited by several challenges. SCNT technology requires the use of unfertilized oocytes, which are difficult to obtain. Though it is possible that this limitation may be overcome by the use of fertilized zygote cells, as was recently shown in the mouse, SCNT is also a very technically challenging and inefficient technology that is not at present viable for routine use in the clinic (16).

Despite limitations, SCNT experiments have shown unequivocally that cellular differentiation is not necessarily a one-way street. By exposing a somatic nucleus to the appropriate cytocellular environment (oocyte or ES cell), epigenetic reprogramming is facilitated and pluripotency established. In the last several years, researchers have applied these concepts as they have attempted to more fully understand the specific elements present in oocytes and ES cells that affect nuclear reprogramming and maintain pluripotency. By carefully sifting through data examining transcription factors present in ES cells, researchers were able to identify a set of four such factors that were sufficient to reprogram somatic cells to pluripotency. In a biomedical milestone reported in 2006, Takahashi and Yamanaka successfully reprogrammed murine embryonic fibroblasts to de-differentiated states of pluripotency using retroviral insertion of gene sequences of four transcription factors: Oct4, Sox2, Klf2, c-Myc (7). These cells have been termed “induced pluripotent stem cells” (iPS cells). These results were extended to the

reprogramming of human fibroblast cells in the laboratory of James Thompson in 2007 using retroviral insertion of gene sequences for OCT4, SOX2, NANOG, and LIN28 (17). In the last few years iPS cells have been derived by various researchers from several different human cell types including keratinocytes, hematopoietic cells, cord blood, and hair follicle cells (18, 19, 20, 21).

Though the precise criteria for pluripotency is the subject of ongoing debate (22), iPS cells have been shown to be similar to embryonic cells in several important ways (23, 24). iPS cells show significant similarity to ES cells morphologically, epigenetically, and in their expression of key ES cell marker genes. iPS cells also demonstrate other important characteristics of pluripotency, including the ability to form teratomas when injected into mouse testes, the ability to contribute to tissues of adult chimeric mice that are germline competent, and the ability to form all tissues of a viable organism through tetraploid complementation assays (23, 24, 25). Though the most stringent of these assays—chimera formation and tetraploid complementation—are not ethically available for work involving human iPS cells, these cells have been successfully induced to form teratomas comprising three primary germ cell layers and have been subjected to several differentiation schemes to form specific human tissues, such as cardiomyocytes, hematopoietic stem cells, osteoclasts, hepatocytes, and neurons (18, 23, 24, 26, 27, 28, 29).

The ability to reprogram somatic cells to pluripotency has led to great excitement in the field of stem cell research and regenerative medicine. These reprogrammed somatic cells

have several important advantages over ES cells in biomedical application. First, iPS cell technology avoids ethico-religio-political complexities that have bridled embryonic stem cell research for much of the last decade. Second, iPS cells are derived in a patient-specific manner, and so, organs and tissue derived from these cells are likely to avoid immune rejection that would otherwise complicate application in regenerative medicine. Third, the patient-specific nature of iPS cells allows for the observation of developmental irregularities that are likely inherent in many disease states. For example, iPS cells derived from individuals with Parkinsons Disease or ALS can be differentiated into dopaminergic neurons and monitored developmentally for insight into sequential pathologic aberrations inherent in the disease process (30, 31, 32). Lastly, this technology holds great promise for evaluating medication-induced abnormalities in a tissue-specific, patient-specific manner. That is, although the nature of the toxicity of a particular drug on heart tissue may theoretically be ascertained by studies on cardiomyocytes derived from ES cells, the results could not take into account phenotypic and functional polymorphisms that may present in a given individual. iPS cell derivation could allow such tailored study (33).

Despite the enormous promise of iPS cell technology, the field is presently limited in clinical application due to the manner of iPS cell derivation. Present methods of somatic cell reprogramming rely largely on viral vectors to introduce genetic material permanently into the genome. Inserted genetic material includes both the viral backbone, as well as transgene sequences coding for the transformative factors (OCT4, SOX2, KLF4, c-MYC, NANOG, LIN28) (7,17). Such an approach engenders several significant

pitfalls that at present limit clinical application. First, virally introduced material can produce insertional mutagenesis, disrupting the normal function of endogenous genetic material and leading to unintended phenotypes and possibly malignant transformation (34). Mutagenetic effects may also have significant effects on the differentiation potential of established iPS cells. Second, inserted transgenes may continue to be active after pluripotency has been established. This may also have implications for the differentiation potential of iPS cells, but, perhaps more importantly, has the potential to activate a tumorigenic phenotype, which is especially concerning considering the association of several of the induced factors (c-MYC, KLF4) with malignant transformation (17, 35).

In order for the full promise and clinical viability of iPS technology to be realized, pluripotency must be achieved by means that minimize or eliminate unanticipated and undesired phenotypes such as malignant transformation. This problem has been approached by several researchers in a variety of ways, including using adenoviruses or episomal vectors to provide nonintegrating transfer of genetic material (36, 37), Cre/LoxP recombination and Piggy-Bac technology to remove inserted genetic material after cellular transformation has been achieved (38, 39, 40), and protein-based schemes have been reported. Unfortunately these approaches also have significant drawbacks that limit their usefulness in the clinical setting, including very low yield, long turn-around time, and complicated procedures. In addition, many of these approaches continue to rely on the introduction of exogenous DNA into host cells, failing to completely remove the possibility of genetic recombination at the level of the genome. Post-transformation excisional schemes using Cre/LoxP recombination has the additional limitation of

imprecise excision of integrated transgenes, thus failing to circumvent concerns of insertional mutagenesis. The use of DNA constucts, such as episomal material, for transient transfection is problematic because of the inherent toxicity of DNA which limits the concentration range that can be used effectively (41). DNA also requires nuclear entry which is limited in some cell types (41). Such methods can also lead to low-frequency integration into host genome (42).

In an attempt to address many of the limitations of current procedures in deriving human iPS cells, we have investigated the viability of using mRNA transfection of somatic cells to induce pluripotency. The most important aspect of this approach lies in the fact that mRNA, unlike DNA, is unlikely to have a permanent effect on the cellular genome. mRNA is completely and irreversibly degraded, eventually leaving transfected cells absent of any transgene expression. Also, translation of mRNA into protein, unlike DNA-based methods, does not require nuclear entry. Rabinovich et al. have recently developed a fast and effective approach for *in vitro* synthesis of mRNA that can be introduced into human cells (41). These studies showed that mRNA transfection of human cells can be achieved at efficiencies much higher than that of DNA transfection, with a more uniform introduction of material across cell populations. This approach has successfully been employed to reprogram different types of primary cells and cell lines, including T lymphocytes, NK cells, B cells, and several cancer cell lines. T lymphocytes, and natural killer cells have been reprogrammed with chimeric immune receptor (CIR) constructs that direct these cells towards various surface targets (43). For example, by using anti-CD19 CIR and anti-8H9 CIR, a variety tissue types have been targeted by the

reprogrammed killer cells, including B cells, Daudi lymphoma, primary melanoma, breast ductal carcinoma, breast adenocarcinoma, and rhabdomyosarcoma (43). In the present study we have adapted these methods of mRNA transfection to develop an approach for the reprogramming of somatic cells to pluripotent states.

Recently, many researchers have addressed the poor efficiency and slow kinetics of iPS cell generation. Various methods have been successful in this regard. First, the choice of starting cell type bears heavily on the speed and efficiency of the reprogramming process. Although fibroblasts have been the standard starting tissue for most iPS cell studies, several investigators have reported that keratinocytes reprogram faster and more efficiently in both mouse and human systems (18, 44). There have also been mouse studies showing that early progenitors and hematopoietic stem cells are more easily and more quickly reprogrammed than more well-differentiated hematopoietic cells (45). Second, small molecule epigenetic modifying factors, such as valproic acid (histone deacetylase inhibitor) and 5'-azacytidine (DNA-hypomethylating agent), have also been shown to enhance the success of reprogramming (46). It is thought that these agents aid in the chromatin remodeling that must occur to produce fully transformed cells. Valproic acid has been shown to increase efficiency of reprogramming up to 40 times and has allowed reprogramming with the use of only two exogenous factors, OCT4 and SOX2 (46, 47). Third, recent studies have demonstrated that knock-down of P53 activity can enhance reprogramming. The process of reprogramming has been shown to activate the P53 pathway which in turn acts as a barrier to full reprogramming (48, 49). Lastly, the use of five factors (OCT4, SOX2, KLF4, c-MYC, NANOG) instead of the standard four

factors has shown promise in increasing yield and kinetics of iPS cell formation, especially in systems that do not employ retroviruses as the means of reprogramming (37, 50).

In the present work, we have optimized mRNA electroporation protocols for keratinocytes, performed *in vitro* mRNA synthesis of factors required for iPS reprogramming (OCT4, SOX2, KLF4, c-MYC, and NANOG) and demonstrated that, upon transfection, these transcripts produce detectable protein. In an effort to optimize the yield of transformed cells, we have also synthesized a truncated form of P53, known as P53DD, which has been shown to have a dominant negative effect on P53 activity in human cells (49, 51). We have investigated the capacity of both human keratinocytes and human fibroblasts to transform under various conditions, including 4 and 5 factor mRNA transfection, in the presence of valproic acid, and with concomitant transfection with a P53 dominant negative construct. The presence of iPS cell generation has been assessed by the presence of alkaline phosphatase activity of formed colonies, which is well known as an early marker of pluripotency. We have observed the generation of a small number of alkaline phosphatase-positive cells by electroporating human keratinocytes with OCT4, SOX2, KLF4, c-MYC, and NANOG.

MATERIALS AND METHOD

Cell Culture. Neonatal human epidermal keratinocytes (nHEK) and nenotal human foreskin fibroblasts (nHFF) were obtained from the Yale Cell Culture Core Facility. Keratinocytes were cultured in serum-free low calcium medium (Epilife, Invitrogen); fibroblasts were cultured in DMEM medium in 10% heat-inactivated fetal bovine serum (Gibco). For viral reprogramming, keratinocytes, passage 5-6, were infected with a 1:1:1:1 mix of retroviruses with OCT4, SOX2, KLF4, and c-MYC in the presence of 1μg/ml polybrene and centrifuged for 45 minutes at 750g on 2 consecutive days. For reprogramming with mRNA constructs, keratinocytes were electroporated with mRNA transcripts corresponding to reprogramming factors as described below. For initial experiments mRNA corresponding to each of four transcription factors (OCT4, SOX2, KLF4, c-MYC) were used in a 1:1:1:1 ratio respectively. For experiments using high OCT4 concentrations, the same four factors were used in a 3:1:1:1 ratio. For experiments using the initial four factors plus either NANOG or P53DD, the five mRNA transcripts were present in a ratio of 1:1:1:1:1. After viral infection or electroporation, keratinocytes were grown in fresh serum-free, low calcium medium at 37C and 5% CO2 for 2 days, after which they were trypsinized and seeded onto multi-well plates containing irradiated mouse fibroblasts. Transfected cells were seeded 2.5 x 10^6 cells/cm^2 and cultured with ES cell medium (DMEM/F12 containing 20% KOSR (vol/vol), 5–10 ng ml^{-1} bFGF, 1 mM L-GIn, 100 μM nonessential amino acids, 100 μM 2-mercaptoethanol, 50 U ml^{-1} penicillin and 50 mg ml^{-1} streptomycin**)**. Neonatal human foreskin fibroblasts were used to confirm the expression of individual mRNA constructs of the various reprogramming

factors and were cultured in DMEM with 10% heat inactivated fetal bovine serum. All cell culture procedures were carried out by Robert Leone.

Retroviral production. Following the method of Park, et al., retroviruses containing OCT4, SOX2, KLF4, and c-MYC were formed using pMIG vectors (Addgene) which also contained sequence for GFP expression (52). The production of retroviruses was carried out by Eugenie Cheng in the laboratory of Haifan Lin.

RNA synthesis. As described in Rabinovich et al. (41, 43), green fluorescent (GFP) mRNA constructs were produced *in vitro* with T7 polymerase and were based on the *Pontellina plumata* GFP sequence of plasmid pmaxGFP (Amaxa Biosystems, Cologne, Germany) (41,43). OCT4, SOX2, KLF4, c-MYC, P53DD, and NANOG constructs were created by replacing the GFP coding region in pmaxGFP with the appropriate open reading frame from each gene. DNA templates were produced by polymerase chain reaction (PCR) using AccuPrime *Pfx* DNA polymerase (Invitrogen) according to manufacturer's protocol. Twenty-five to 30 cycles of PCR were performed in a standard 50-µl reaction using 0.1 mg of template DNA. The forward primer contained the T7 RNA promoter and an anchoring sequence in the proximal part of the gene expression cassette. The reverse primer, with anchoring sequence in the distal part of the gene expression cassette, contained a stretch of 100 dT residues. Design of DNA inserts for OCT4, KLF4, SOX2, c-MYC, and NANOG was carried out Peter Rabinovich. Design of the DNA insert sequence for the P53 dominant negative construct (P53DD) was carried out by Robert Leone. PCR was carried out by Efim Golub.

mRNA synthesis with T7 RNA polymerase has been described by Rabinovich et al. (41, 43). This was performed with an mMESSAGE mMACHINE T7 Ultra kit (Ambion, Austin, TX), using the procedure recommended by the manufacturer. One hundred to 200 ng of DNA made by PCR with no further purification was used for the standard 20-µl transcription reaction. The product was treated with Escherichia coli poly(A) polymerase (from the same kit) in the presence of 1mM ATP according to the Ambion polyadenylation protocol. The yield of mRNA was 20 to 60 mg of mRNA per reaction. The final product was treated with DNase I (Ambion) and purified by LiCl precipitation. RNA was stored at -80C. mRNA production was performed by Robert Leone.

mRNA transfection of human cells

Electroporation of human keratinocytes and fibroblasts.

Electroporation was performed with an Amaxa Nucleofector II (Amaxa, Gaithersburg, MD) in accordance with manufacturer recommendations. Keratinocytes were electroporated with ‘‘Human Keratinocyte Nucleofector Kit Solution’’ and programs T007, T018, and T024. Human fibroblasts were electroporated with ‘‘Human Dermal Fibroblast Nucleofector Kit Solution’’ using program U020. For GFP transfection experiments, keratinocytes were electroporated with DNA (20µg/ml) or mRNA (30-200µg/ml) per sample. Cells were used at a concentration of 10–250 million/ml. In this interval of values the efficiency of transfection does not depend on cell density (Rabinovich et al., 2006). The efficiency of transfection was determined by flow cytometry 18 hours after transfection. Cell viability post-transfection was assessed by tryptan blue staining hemocytometry. In standard reprogramming experiments, OCT4,

SOX2, KLF4, and c-MYC were used in a 1:1:1:1 ratio with 30μg/ml final concentration of each factor. In experiments containing high OCT4, a 3:1:1:1 ratio was used. In experiments using NANOG, a 1:1:1:1:1 ratio (OCT4:SOX2:KLF4:c-MYC:NANOG) was used. In experiments using P53DD (P53 dominant negative construct), a 1:1:1:1:1 ratio (OCT4:SOX2:KLF4:c-MYC:P53DD) was used. All electroporation procedures reported on in this work was performed by Robert Leone.

Cationic liposomal transfection of human keratinocytes and fibroblasts.

Cationic-liposomal transfection experiments were carried out using the TransIT®-mRNA Transfection Kit (Mirus). Conditions were optimized for keratinocyte and fibroblast transfection according to the manufacturer's recommendations using both GFP plasmid as well as GFP mRNA transcripts produced by the above methods. Transfection of both keratinocytes and fibroblasts was performed in cell culture conditions on a feeder layer of irradiated mouse embryonic fibroblasts as described above. Optimization of transfection conditions for keratinocytes was performed by Robert Leone. Optimization of transfection conditions for fibroblasts was performed by Eugenie Cheng. Mirus transfection experiments using reprogramming factors was carried out by Eugenie Cheng.

Flow Cytometry. Flow cytometric analysis of cell subpopulations was performed at the Yale Cancer Center Flow Cytometry Shared Resource (New Haven, CT), using a FACSCalibur (BD Biosciences). Fluorescence signals were collected using a 488-nm laser on a logarithmic scale. Unless otherwise noted, at least 10,000 events were acquired for each sample. Data were analyzed with FlowJo software (TreeStar, Ashland, OR).

Flow cytometric analysis was carried out by Robert Leone with assistance from Eugenie Cheng and Peter Rabinovich.

Electrophoresis. All protein expression assays were performed on cell lysates from transfected neonatal human foreskin fibroblasts, passage 7-12. In the presence of protease inhibitors, whole cell extracts were obtained by lysis using either RIPA buffer and standardized against untransfected control cells using BCA protein quantitation (c-MYC, NANOG, P53DD) or laemmli buffer and standardized against untransfected controls by cell concentration measurement (KLF4, SOX2, OCT4). Protein extracts were analyzed by Western blot analysis using specific antibodies against c-MYC (StemGent 09-0032 at 1:100 dilution); NANOG (Abcam ab80892 at 1:100 dilution); P53 (Calbiochem Pab421 at 1:10 dilution), KLF4, SOX2, OCT4. Alkaline phosphatase-conjugated goat anti-mouse antibody (sc-2005, Santa Cruz) and goat anti-rabbit antibody (sc-2004, Santa Cruz) were used as secondary antibodies at 1/500-1/2000 dilution range. Blots were developed using SuperSignal *West Pico* Chemiluminescent Substrate System (Pierce). Electrophoresis analysis of OCT4 and SOX2 was performed by Eugenie Cheng. All other electrophoresis experiments were performed by Robert Leone.

Alkaline phosphatase analysis. Direct alkaline phosphatase (AP) activity was assessed using Alkaline Phosphatase Staining Kit **(**Stemgent, Inc, Cambridge, MA) according to the manufacturer's recommendations. Alkaline phosphatase analysis was performed by Robert Leone.

RESULTS

mRNA and DNA transfection. Transfection experiments were performed on human neonatal keratinocytes and fibroblasts. Keratinocytes have been reported to reprogram to iPS cells with faster kinetics and greater efficiency than human fibroblasts (18, 44). In unpublished data from our lab (Peter Rabinovich), it was demonstrated using rt-PCR that GFP mRNA transcripts maintain a half-life of approximately 4-5 hours in human lymphocytes, leaving only 10% of material after 15 hours. Because transfected mRNA transcripts are transient in cells and because translated proteins are diluted two-fold with each cellular division, the kinetic advantage of keratinocytes in reprogramming was thought to be an important attribute of our system. mRNA transfection was attempted by both electroporation using the Amaxa Nucleofector II as well as the Mirus system of cationic liposome-mediated transfection. To determine optimal electroporation parameters using Amaxa Nucleofector II, we first transfected keratinocytes with a green fluorescent protein (GFP) reporter gene from *Pontellina plumata,* using mRNA and DNA transgenes. Using keratinocyte electroporation solution proprietary to Amaxa we tested several electroporation protocols and determined that Nucleofector II program T-024 gave the highest efficiency of transfection when assessed by geometric mean (fig. 1). Transfection experiments using liposomal reagents on cells in fibroblast and keratinocytes in cultures were assessed by similar methods and attained similar efficiencies of transfection (data not shown).

To assess the functionality of mRNA transcripts, transcripts for OCT4, SOX2, KLF4, c-MYC, P53DD, and NANOG were individually transfected into neonatal human foreskin

fibroblasts. Protein synthesis was assessed by Western blot analysis as shown in figure 2. Upon transfection each transcript allowed for significant protein production compared with untransfected control fibroblasts. (Sample quantities were standardized by determining pre-lysis cell quantity or by protein quantitation of cell lysates by bicinchoninic acid (BCA) protein assay). In all cases protein synthesis was evident above levels of untransfected control samples. Western blot analysis for NANOG showed detectable protein in untransfected controls fibroblasts. This is in concordance with a recent report of low-level basal NANOG expression in human fibroblasts (53).

Introduction of pluripotency factors into keratinocytes and fibroblasts.

Introduction of reprogramming factors using mRNA retroviruses.

For preliminary studies, retroviral transfection was performed to induce pluripotency in keratinocytes according to published methods (18, 52). Using a 1:1:1:1 mixture of retroviruses containing reprogramming factors (OCT4, SOX2, KLF4, c-MYC), keratinocytes (passage 5) were infected twice, 24 hours apart. Two days after the last infection, cells were trypsinized and seeded onto a feeder layer of irradiated mouse embryonic fibroblasts (iMEFs) in embryonic stem (ES) cell medium. Expression of retrovirally inserted genes was assessed by fluorescent microscopy and flow cytometric analysis of GFP expression one day post transfection. (fig. 3a-c) The fraction of cells expressing reprogramming factor ranged from 15% to 78% among the four samples. Despite rather low expression rates, induction of pluripotency was evidenced by formation of three colonies displaying typical ES cell morphology, each of which was positive for alkaline phosphatase activity, an early marker of pluripotency (fig. 3d).

Introduction of reprogramming factors by mRNA transfection.

Using optimized conditions, keratinocytes were transfected with mRNA transcripts coding for reprogramming transcription factors. Initially, keratinocytes were electroporated with OCT4, SOX2, KLF4, and c-MYC mRNA transcripts (day 0). After transfection, cells were grown in keritinocyte medium for 2 days without a feeder cell layer. On day 2, cells were trypsinized and moved to iMEF feeder cell layers in multi-well plates at a density of approximately 5000 cells/cm^2. Medium was changed to ES cell medium on day 3 after transfection. Thereafter, cells were grown in ES cell medium with or without 10 mM valproic acid (VPA) supplement. Medium was changed every other day. Two control populations of keratinocytes were treated to the same culture conditions, again with and without VPA. Transfected keratinocytes began to show evidence of transformation on day 4, at which time small colonies began to form that were particularly abundant in VPA-containing cultures. The colonies were tightly packed and mostly circular with relatively smooth edges (fig. 4b,d), bearing significant morphologic similarity to both human embryonic stem cell colonies as well as virally induced iPS cell colonies (fig. 4a). There were approximately 10-15 colonies/cm^2 in VPA-containing culture conditions and 6-10 colonies/cm^2 in cultures without VPA, and the rate of growth of individual colonies appeared to be slightly higher in VPA-containing conditions. However, the morphology of the colonies was not appreciably different between culture conditions (with or without VPA; fig. 4b,d). No colonies were observed in control cell cultures (no mRNA transfection performed) in either the presence or absence of VPA (fig. 4c,e). Colonies were assessed for pluripotency by determining alkaline phosphatase activity at day 10 and day 14. Colonies failed to show

alkaline phosphatase activity in this initial experiment. Human fibroblasts were transfected using a similar protocol, but showed no evidence of colony growth in either VPA treated or untreated culture conditions (data not shown). It was observed that keratinocytes survived the above transfection relatively poorly, demonstrating a viability only of about 3 percent.

We also attempted serial transfections of reprogramming factors into human keratinocytes using a liposomal delivery of mRNA transcripts. Three transfections were carried out on days 0, 3, and 6 on keratinocytes cultured on irradiated mouse embryonic fibroblast feeder cells. While cellular transformation was again achieved in a manner similar to results reported for electroporation experiments described above, all colonies were alkaline phosphatase negative (data not shown). Using GFP mRNA, it was discovered that keratinocytes were actually resistant to transfection of mRNA material after the initial transfection at day 0 (data not shown).

In subsequent experiments several additional approaches were attempted to affect successful transformation to pluripotency. In published reports, addition of NANOG to OCT4, SOX2, KLF4, and c-MYC increased the efficiency and the kinetics of reprogramming in human and mouse fibroblasts. This has been shown in both retroviral as well as episomal systems of transformation (37, 50). Particularly interesting about this approach is that the addition of NANOG seems to increase the kinetics of reprogramming in a fashion that is relatively independent of cellular proliferation rates (50). This is especially applicable to our system since mRNA transcripts in our system, unlike mRNA

produced from retrovirally-integrated genes, are not being actively produced or replenished by the cell during reprogramming. Thus, in addition to the loss in cytoplasmic mRNA concentration due to normal cellular control mechanisms, transcripts are diluted by half with each population doubling. NANOG mRNA was synthesized *in vitro* as described for the other four factors. Using Western blot analysis, we confirmed expression of NANOG protein by a transfection experiment using nHFF cells (fig. 5). The immunoblot showed increased levels over untransfected nHFF cells, however the evidence of endogenous NANOG expression in control sample was unexpected and likely due to contamination or nonspecific staining. In an attempt to induce pluripotency, nHEK cells were transfected with OCT4, SOX2, KLF4, c-MYC, and NANOG in a 1:1:1:1:1 ratio. In culture conditions without valproic acid (VPA), small colonies began to become evident on day 4 post-transfection. Two of these colonies showed steady growth over the first 11 days post transfection after which time their size stabilized. These colonies appeared smaller than colonies formed from the four factors alone, however they shared their general morphological attributes, being round with distinct borders. (No colonies were evident in culture conditions containing 1 mM valproic acid.) Cells were stained for alkaline phosphatase activity. We observed two alkaline-phosphatase positive cells that were round with smooth cell borders, distinct from both native keratinocyte morphology, as well as fibroblast morphology of the feeder layer cells (fig. 6). Beyond these findings of individual cells, cell colonies in this sample showed no alkaline phosphatase activity. Approximately 9×10^4 cells were plated in one well of a 24-well plate to achieve these results.

In another approach, we took advantage of recent reports showing that the stoichiometry of the expression of the four factors plays a role in optimal cellular transformation to pluripotency (54). Specifically, Papapetrou *et al.* have shown that reprogramming human fibroblasts to iPS cells is highly sensitive to levels of OCT4 and is optimized when the four proteins are present intracellularly in a ratio of 3:1:1:1 (OCT4:SOX2:KLF4;c-MYC). Accordingly, we transfected nHEK cells with the mRNA of the four factors in precisely this ratio. In both VPA-positive and VPA-negative culture conditions, cellular transformation was noted, showing one large colony per well, and many small round colonies that began to appear at day 4 post-transfection. These were similar in appearance to those produced through four factor transfection pictured in figure 4. On day 16 post-transfection, these colonies were stained for alkaline phosphatase activity as described. There was no significant alkaline phosphatase activity above negative control cells of untransfected nHEK.

Lastly, there have been several reports of the increased efficiency of reprogramming in the setting of P53 knockdown through use of either shRNA or a P53 dominant negative. P53 activity was more completely inhibited using a P53 dominant negative construct consisting of the first 14 N-terminus codons plus the C-terminus 302-390 originally described by Shaulian et al. (55). We synthesized this construct (P53DD) and confirmed expression by immunoblot analysis (fig. 5) with a primary antibody directed against the carboxy terminus of P53 (Pab421, Calbiochem). nHEK cells were transfected with OCT4, SOX2, KLF4, c-MYC, and P53DD in a 1:1:1:1:1 ratio. Very small colonies began

to appear on day 4 post-transfection. In contrast to experiments using the four factors and either NANOG or high OCT4 levels, these colonies remained very small without noticeable growth. Staining for alkaline phosphatase was done and yielded no significant results above negative control cells. There was no observable difference between VPA-positive and VPA-negative culture conditions.

Assessing keratinocyte viability in mRNA electroporation experiments. In carrying out the above experiments, it was observed that there was significant loss of cell viability after each mRNA transfection of keratinocytes. Cell loss ranged from 96-98% in experiments using all four factors, which used a total mRNA concentration of 120-150 µg/ml. This was significantly more than control keratinocytes that were electroporated without any mRNA in the transfection solution and which showed a more modest 60% loss in viability. It had previously been reported that mRNA showed very little toxicity in the electroporation of various other human cell types, including lymphocytes, fibroblasts and monocytes (41, 43, 64). The experiments described in these reports used mRNA at levels approximately equal to those used in our experiments. Initially, it was unclear whether the significant toxicity observed in our experiments was secondary to high mRNA levels or to the effect of the expression of the reprogramming factors themselves. To investigate this further, we compared viability data from nHEK electroporation experiments using increasing concentrations of GFP mRNA, expression of which has been previously shown not to be toxic in human cells (41, 43). We compared three groups of nHEK cells, including cells undergoing electroporation with no mRNA present, and two groups of cells electroporated with 30 µg/ml and 200µg/ml of

GFP mRNA respectively. Post-transfection all cells were incubated overnight in keratinocyte medium, after which they were trypsinized and anaylized for cell viability by manual counting with a hemocytometer (fig. 7). Data from this experiment show that increasing concentrations of GFP mRNA is toxic to human keratinocytes.

DISCUSSION

The use of retrovirally introduced transcription factors has been exceptionally and reproducibly successful as a general approach to the induction of pluripotency in somatic cells. We are indebted to researchers using these methods in the inception and early development of the field iPS cell technology. Beginning with the revelatory work of Yamanaka, the use of retroviruses to introduce transformative factors has produced great advances in our understanding of the capacity for differentiated cells to adopt pluripotent states. Using these methods researchers have unlocked the ability of somatic cells to be transformed into many tissues of the body, including cardiomyocytes, hematopoietic stem cells, osteoclasts, hepatocytes, and neurons (18, 23, 24, 56, 57, 58, 59). Researchers have also been successful in correcting genetic mutations in iPS cells derived from patients or mouse models with genetic mutations causing Duchenne muscular dystrophy, hemophilia, and sickle cell anemia, and subsequently differentiated these cells into disease-free phenotypes (60, 61, 62, 63). Work has also been accomplished, using similar methods of viral transformation, in transdifferentiating between somatic cell types, such as transforming pancreatic exocrine cells into insulin-producing beta islet cells (64). Importantly, all of this work has been accomplished—much of it in human systems—without the specter of ethical suspicion or political-legal regulation hindering advances. Indeed, the advances that have already been uncovered, along with the promise of things to come are truly historic and will likely have implications on many aspects of society.

Holding at bay future promise from becoming accepted advances, however, are current limitations of the technology of virally induced genetic material that has, to this point,

brought the field so far in such a short time. Insertional mutagenesis, transgene reactivation, and oncogenic transformation are all serious pitfalls in any reprogramming technology using DNA as the transformative element, especially those employing retroviruses. The potential of using mRNA transfection technology to induce pluripotency is an important advance in removing these last hurdles before clinical application of iPS technology.

In the current work we have accomplished important steps toward fulfillment of this goal. We have successfully synthesized mRNA constructs corresponding to the transformative factors OCT4, SOX2, KLF4, c-MYC, and NANOG and demonstrated their expression individually in human fibroblasts. We have also demonstrated that these factors can be used reproducibly to affect cellular transformation of human keratinocytes in culture. Lastly, although the efficiency was low, we have observed the presence of two alkaline-phosphatase-positive cells through keratinocyte reprogramming with OCT4, SOX2, KLF4, c-MYC, and NANOG in the absence of valproic acid. It remains to be seen if these cells are capable of forming viable colonies. The pluripotent potential of cells transformed in this way will be investigated in future work. Evaluation of expression of other markers of pluripotency markers such as SSEA-4, SSEA3, TRA-1-60, TRA-1-81 will be very important, as well as assessing the potential of these cells to form teratomas or differentiate into other somatic cell types by known methods.

We believe the most significant advantage of our approach—the transient nature of intracellular mRNA—is also its most challenging limitation. Previous experiments

showed GFP protein expression up to 2 weeks post-electroporation in human cells (unpublished data, Peter Rabinovich). However, in our examination of protein expression of OCT4 and SOX2 in mRNA-transfected human fibroblasts, transgene proteins were expressed for less than 6 days post-transfection (data not shown). Previous studies investigating the kinetics of reprogramming have found that at least 7 days of transgene expression is necessary for pluripotent transformation. We have attempted to address this issue by using approaches that have been reported to increase the kinetics of reprogramming, including the use of the four standard factors plus either NANOG or knockdown of P53 activity. (Our choice of keratinocytes as starting cells, which have been reported to have faster reprogramming kinetics, was also made with this in mind). Though it appears that the use of four factors plus NANOG has improved the reprogramming process (as evidenced by the generation alkaline-phosphatase positive cells), the efficiency of the process must be further optimized. This limitation may be further addressed in one of several ways. One approach would be to increase the half-life of mRNA in cells. It is possible that this could be achieved using constructs from negative RNA viruses, such as the Sendai virus, which enable the replication of mRNA strands in mammalian cytosol. Our lab is presently investigating the viability of this approach in several systems. Another approach would be to investigate the viability of repeated transfections of reprogramming factors performed in the course of cellular transformation. A similar approach was successfully implemented by Zhou et al. who achieved pluripotent transformation using recombinant protein reprogramming factors (65). In the present study we attempted serial transfections using a cationic-liposomal method. Using this approach, which allows for *in situ* transfection of cells in culture, we found that keratinocytes were unable to be transfected once they were exposed to ES

culture conditions (data not shown). While the reason behind this recalcitrance is unclear, it may be possible that optimization of transfection medium during reprogramming could produce a more favorable environment for transfection (66). While multiple electroporations of keratinocytes was an untenable idea in our present system considering the extremely low cell viability using the Amaxa Nucleofector II, there are other approaches to electroporation which may allow the possibility of multiple *in situ* transfections of these adherent cells (67, 68).

An unexpected finding in our study was that of the apparent toxicity of high levels of mRNA on keratinocytes during electroporation. The implications of this finding are unclear at present. Several previous reports, including work done in our lab, had uncovered no toxic effect of similar levels of mRNA (GFP) on mammalian cells, including human fibroblasts, lymphocytes, peripheral blood mononuclear cells, and murine splenocytes (41, 43, 69). In these studies, the subsequent expression of GFP protein was also not found to be toxic to these cells. This led us to speculate that it was the increasing concentration of mRNA itself that was toxic to keratinocytes. It is known that keratinocytes are subject to a phenomenon known as the "ribotoxic stress response." This response involves the activation of stress-activated protein kinases (SAPK) and is typically triggered by damage to ribosomal RNA by toxins, antibiotics, UVB radiation, or other cellular stressors. Interestingly, the SAPK cascade, which often results in cellular apoptosis, can only be triggered by ribosomes that are actively translating (70, 71, 72). It is possible that such a mechanism, wherein cell death is correlated to the intensity of protein synthesis, may be involved in the results we have observed. That is, as a higher

concentration of mRNA is introduced into cells and translational machinery is more fully engaged, all during the stress of electroporation and concomitant recovery, cells become more highly susceptible to the mechanisms of ribotoxic stress response. Future investigations should be made into the benefit of delaying protein translation for some amount of time after electroporation. This could be accomplished by the use of a reversible inhibitor of protein biosynthesis such as cyclohexamide. If keratinocyte cell death is in fact related to the degree of ribosomal activity, a delay of such activity until after cellular recuperation might lessen the effect. Whatever the mechanism of cell death, it is clear that the electroporation process is selecting for a rather small subpopulation of keratinocytes. Whether these surviving cells are more or less likely to be reprogrammed is not known. The fact that they have demonstrated morphological transformation and some evidence of alkaline-phosphatase activity is quite encouraging, however. In the future, the issue of poor cell viability may be addressed by decreasing mRNA concentration in electroporation experiments, as well as investigating alternative transfection procedures. Using keratinocytes at lower passage number may also increase the health and subsequent viability of these cells.

Future work on this project will be directed in several directions. First, effort will be directed towards more fully elucidating the nature of the transformed cell colonies that have been produced from the reprogramming of keratinocytes in this work. Because of the poor cell viability after electroporation with mRNA, we did not have sufficient cells from experiments that produced alkaline-phosphatase activity to apply more stringent pluripotency assays. This will be of foremost importance in future work. The important

question is whether these cells (as well as cells that have been morphologically transformed and do not show alkaline phosphatase activity) show any further characteristics of pluripotency. This can be done by comparing molecular markers such as epigenetic patterns of CpG methylation and histone acetylation on promoter regions of key pluripotency genes such as OCT4 and NANOG. Microarray data could also be obtained and compared between transformed cells, iPS cells, ES cells, and known malignant phenotypes of keratinocytes. RT-PCR experiments would also be valuable in determining precise levels of transcripts that are known to be associated with pluripotency (SSEA-4, SSEA3, TRA-1-60, TRA-1-81), as well as levels of transcripts that specifically indicate a differentiated keratinocyte phenotype. Second, increasing the half-life of mRNA in cells will be an important step towards more effective reprogramming. This work is presently being engaged in our lab with emphasis on uncovering proteins or nanoparticles which have the ability to stabilize ectopic mRNA. As mentioned above, strategies that allow for repeated *in situ* transfections would be useful in prolonging the presence of mRNA and expressed protein factors in cells. Third, there will be continued effort directed toward improving the kinetics of reprogramming in our system. Possible approaches include using additional transformative factors such as Lin28, which has been shown to increase rate and efficiency of iPS cell formation (44). Lastly, using alternative starting cell populations may also allow faster and more efficient reprogramming. A recent study by Eminli *et al.* et al showed that less differentiated cells of the hematopoietic system are more amenable to reprogramming than mature cells (45). A trial using CD34+ cells as a starting population, for instance, would be an interesting experiment. In our experience, keratinocytes appear to be unusual in their sensitivity to high mRNA concentrations during electroporation. As such, we believe that cell types

other than keratinocytes will likely not encounter similar problems with post-electroporation viability.

FIGURES

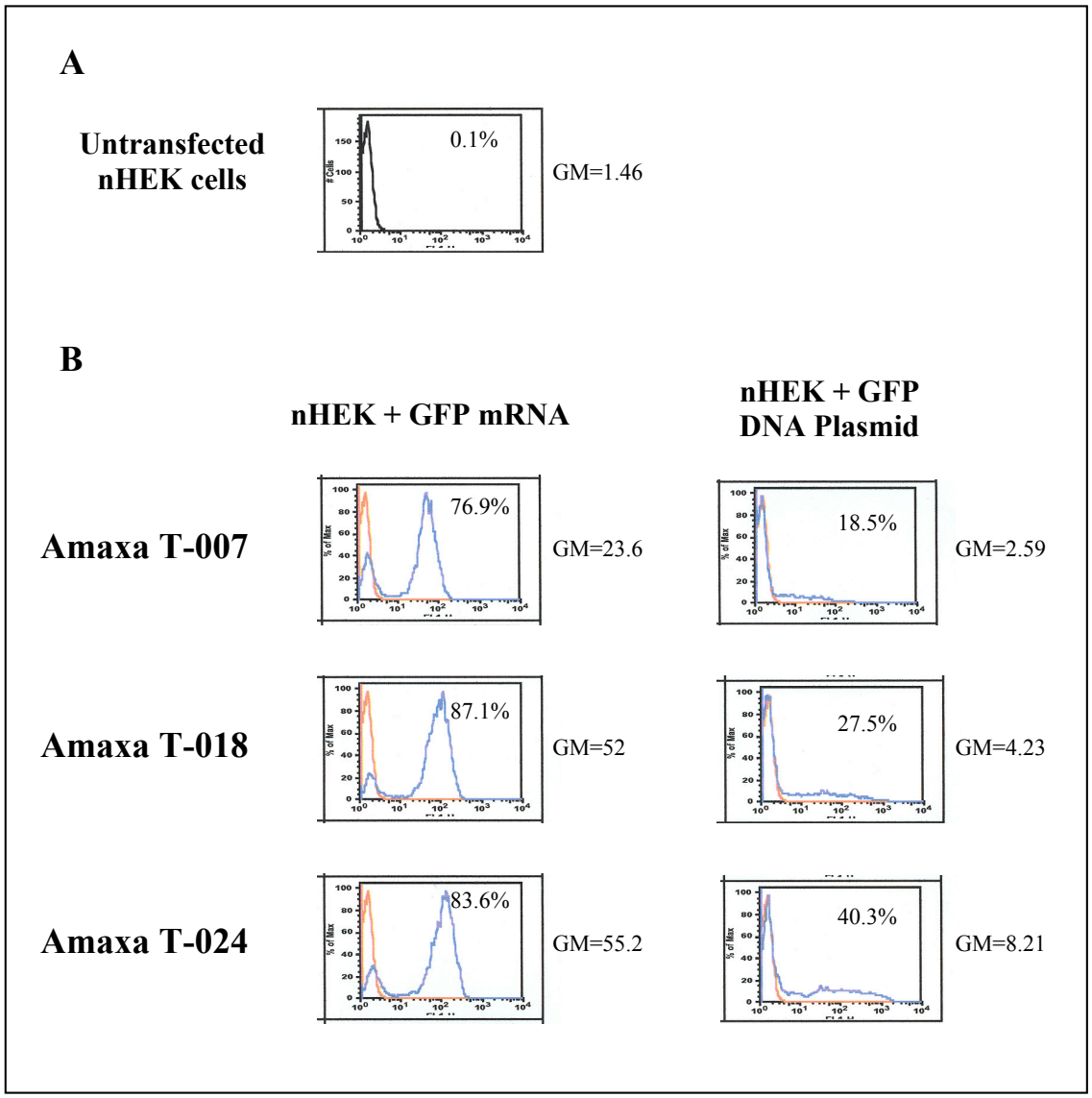

Figure 1. Flow cytometry analysis of keratinocyte (nHEK) transfection reactions using three Amaxa Nucleofector II programs. *(A)* Untransfected control keratinocytes show essentially no fluorescence. *(B)* Electroporation of keratinocytes with GFP mRNA compared with GFP DNA plasmid with fraction of GFP-expressing cells shown; GM=Geometric Mean; nHEK=neonatal Human Epidermal Keratinocytes; GFP=Green Fluorescent Protein..

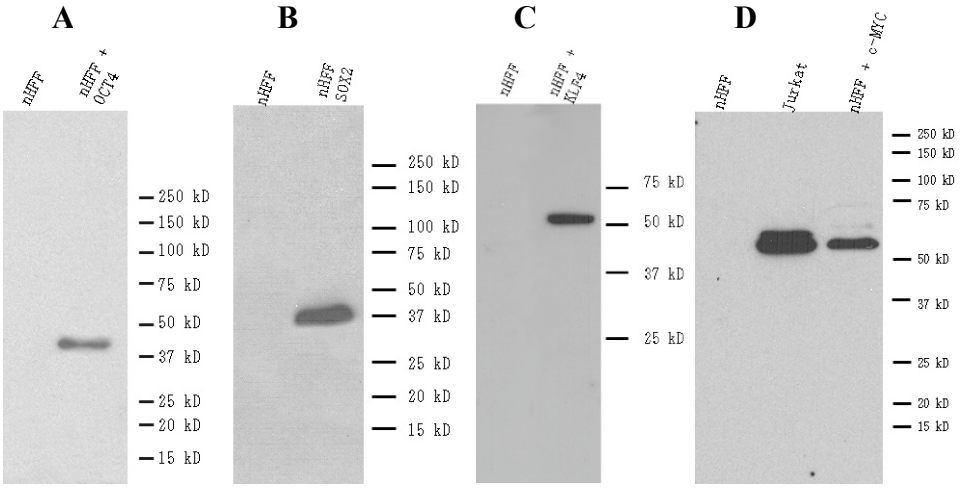

Figure 2. Immunoblots of neonatal human foreskin fibroblasts (nHFF) transfected with mRNA as shown. *(A)* First lane: untransfected nHFF; second lane: nHFF transfected with OCT4 mRNA transcripts. *(B)* First lane: untransfected nHFF; second lane: nHFF transfected with SOX2 mRNA transcripts. *(A)* First lane: untransfected nHFF; second lane: nHFF transfected with KLF4 mRNA transcripts. *(A)* First lane: untransfected nHFF; second lane: Jurkat cells as positive control; third lane: nHFF transfected with c-MYC mRNA transcripts. Samples were standardized either by cell counts (A-C) or direct protein quantitation (75 µg in each lane of gel pictured in D).

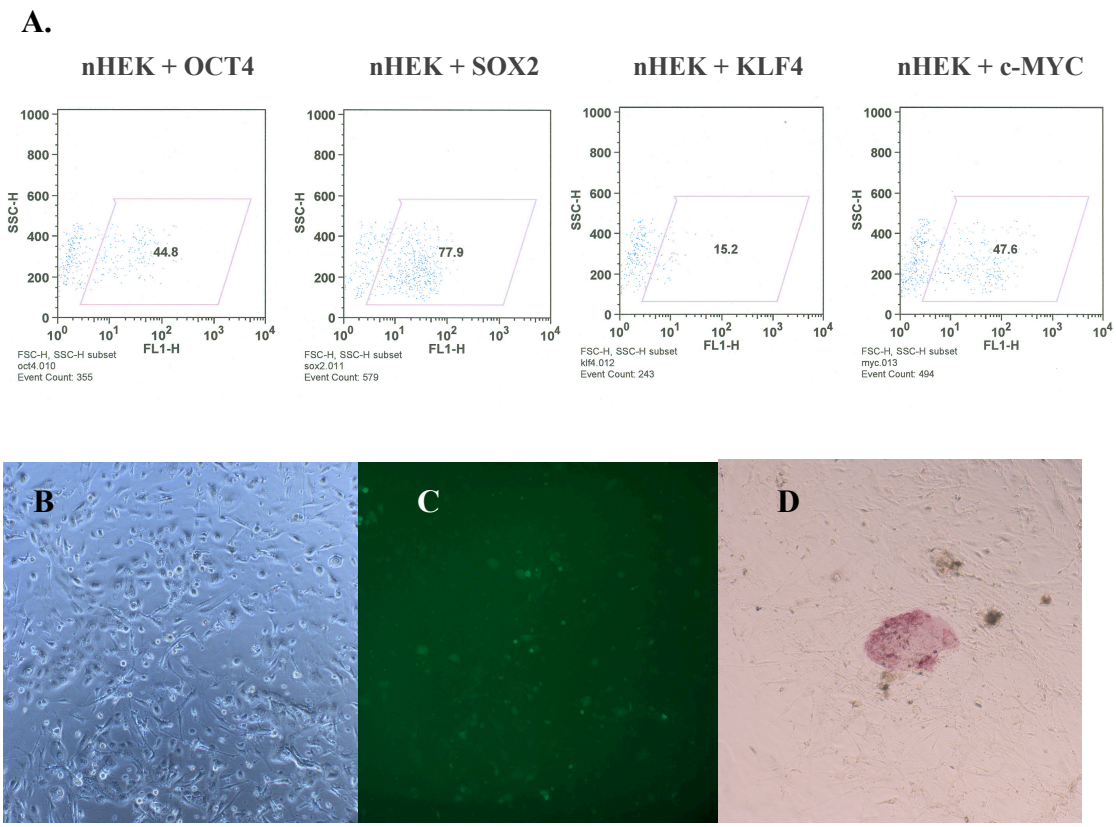

Figure 3. Neonatal human epidermal keratinocyes (nHEK) infected with four retroviruses containing reprogramming factors OCT4, SOX2, KLF4, and c-MYC. *(A)* Flow cytometry data for nHEK cells individually infected with retroviruses containing one of the four reprogramming factors. *(B)* Infected nHEK cells infected with all four reprogramming factors, three days post-infection at 100x magnification; *(C)* nHEK cells from *(B)* under UV microscopy and displaying production of GFP marker, confirming transgene expression; *(D)* Representative colony stained by alkaline phosphatase assay. nHEK=neonatal Human Epidermal Keratinocytes.

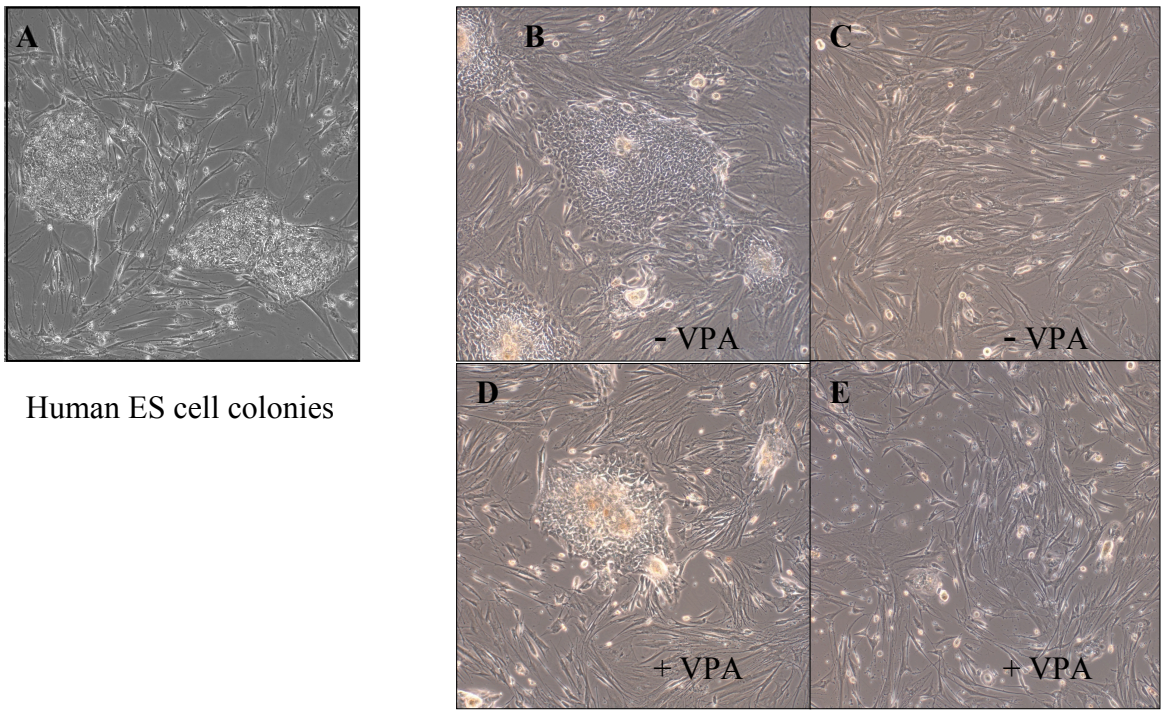

Figure 4. Representative morphology of transformed cell colonies 7 days post-transfection. *(A)* Representative colonies of human embryonic stem cells (hES H1 P60) grown on iMEF feeder layer pictured here for morphologic comparison; *(B)* Representative colony of transformed nHEK cells at day 7 post-transfection with no VPA exposure; *(C)* Control well seeded with untransfected nHEK cells and exposed to the same culture conditions (no VPA) as cells in *(B)*; *(D) (B)* Representative colony of transformed nHEK cells at day 7 post-transfection with 3 days of VPA exposure; *(E)* Control well seeded with untransfected nHEK cells and exposed to the same culture conditions (3 days in VPA) as cells in *(D)*.

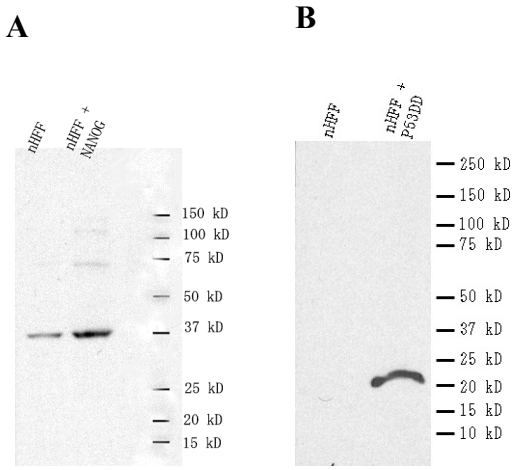

Figure 5. Immunoblot analyses of neonatal human foreskin fibroblasts (nHFF) transfected with mRNA as shown. *(A)* First lane: untransfected nHFF; second lane: nHFF transfected with NANOG mRNA transcripts. *(B)* First lane: untransfected nHFF; second lane: nHFF transfected with P53DD mRNA transcripts.

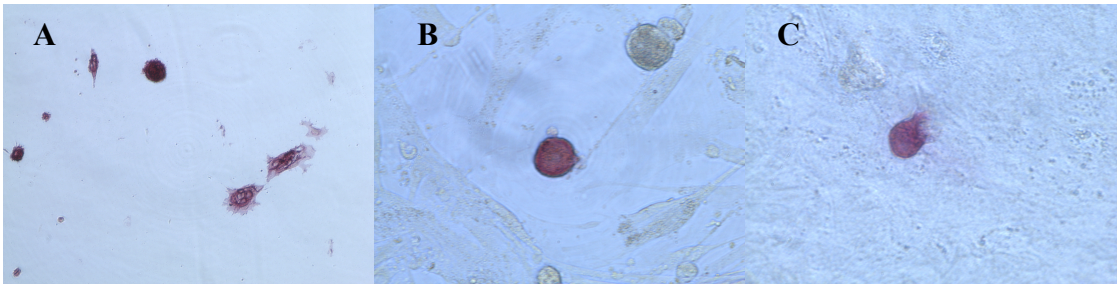

Figure 6. Alkaline phosphatase activity of human embryonic stem cells and transfected human keratinocytes *(A)* Representative colonies of human embryonic stem cells (hES H1 P60) grown on matrigel and stained for alkaline phosphatase activity; *(B-C)* Two small round cells produced from electroporation of five transcription factors (OCT4, SOX2, KLF4, c-MYC, and NANOG) into neonatal human epidermal keratinocytes demonstrating alkaline phosphatase activity at day 16 post transfection.

A

Condition	Cell survival at 18 hours	
Electroporation: no mRNA	6.4 x 10^4 cells	96
Electroporation: 30 µg/ml GFP mRNA	5.3 x 10^4 cells	109
Electroporation: 300 µg/ml GFP mRNA	6.2 x 10^3 cells	5

B

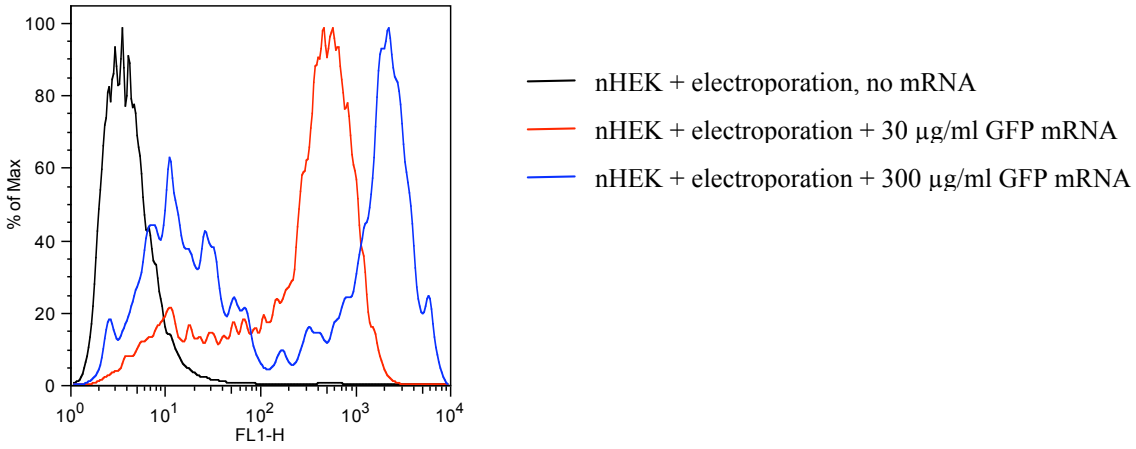

Figure 7. Keratinocyte viability after electroporation with increasing concentrations of GFP mRNA. *(A)* Cell counts 18 hours post-transfection show decreasing viability relative to increasing mRNA concentration used in transfection. Starting amount was approximately 2 x10^6 cells in each transfection. *(B)* Flow cytometry data showing showing increasing mean fluorescence with increasing mRNA concentration. A low-fluorescing population of cells make up a more significant percentage of cell population in the high-concentration mRNA transfection sample. nHEK=neonatal Human Epidermal Keratinocytes.

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