STEM CELLS AND DEVELOPMENT
Volume 20, Number 1, 2011

DOI: 10.1089/scd.2009.0424

Pluripotent Reprogramming of Fibroblasts by Lentiviralmediated Insertion of SOX2, C-MYC, and TCL-1A

Virgínia Picanço-Castro,[1,2] Elisa Russo-Carbolante,[2,3] Luiza Cunha Junqueira Reis,[1,2] Ana Maria Fraga,[2,4] Danielle Aparecida Rosa de Magalhães,[1,2] Maristela Delgado Orellana,[1,2] Rodrigo Alexandre Panepucci,[1,2] Lygia Veiga Pereira,[2,4] and Dimas Tadeu Covas[1,2,5]

Reprogramming of somatic cells to pluripotency promises to boost cellular therapy. Most instances of direct reprogramming have been achieved by forced expression of defined exogenous factors using multiple viral vectors. The most used 4 transcription factors, octamer-binding transcription factor 4 (OCT4), (sex determining region Y)-box 2 (SOX2), Kruppel-like factor 4 (KLF4), and v-myc myelocytomatosis viral oncogene homolog (C-MYC), can induce pluripotency in mouse and human fibroblasts. Here, we report that forced expression of a new combination of transcription factors (T-cell leukemia/lymphoma protein 1A [TCL-1A], C-MYC, and SOX2) is sufficient to promote the reprogramming of human fibroblasts into pluripotent cells. These 3-factor pluripotent cells are similar to human embryonic stem cells in morphology, in the ability to differentiate into cells of the 3 embryonic layers, and at the level of global gene expression. Induced pluripotent human cells generated by a combination of other factors will be of great help for the understanding of reprogramming pathways. This, in turn, will allow us to better control cell-fate and apply this knowledge to cell therapy.

Introduction

Embryonic stem cells (ESCs), derived from the inner cell mass of mammalian blastocysts, have the ability to indefinitely grow in culture while maintaining pluripotency [1,2]. These properties have led to expectations that human ESCs (hESCs) will be useful to understand disease mechanisms, to screen new drugs, and to treat patients with various diseases and injuries [3]. Although ESCs are promising resources for cell transplantation therapies, there are some important concerns related to ethical issues, safety, immune compatibility, and availability that preclude their generalized use. The development of induced pluripotent cells (iPS) from differentiated somatic cells is an important step to overcome some of these concerns.

iPS cells can be obtained from somatic adult cells reprogrammed by the action of pluripotent inducer factors. The pluripotency triggering of somatic cells has been achieved by the introduction of 4 pluripotent-related genes, octamer-binding transcription factor 4 (*OCT4*), (sex determining region Y)-box 2 (*SOX2*), Kruppel-like factor 4 (*KLF4*), and v-myc myelocytomatosis viral oncogene homolog (*C-MYC*) (OSKM) [4]. This result was confirmed and expanded in a series of reports which established that mouse iPS cells are similar to ESCs in morphology, expression of pluripotent markers, ability to differentiate into cells of the 3 germ layers in vitro and in vivo, to form germ-line competent chimeras in vivo, and to generate viable, fertile live-born progeny by tetraploid complementation [5–8] This technique was reproduced with cells from other animal species and with human cells [9–11]. In humans, reprogramming has been achieved using different cells such as fibroblasts, mesenchymal cells, keratinocytes, liver cells, pancreatic β cells, neural progenitors, and other cell types [9–16], proving that the procedures were reproducible and able to generate pluripotent cells derived either from normal individuals or from patients. Although the original OSKM transcription factor set has been the most commonly used method, iPS cells can be

[1]Center for Cell Therapy and Regional Blood Center, Ribeirão Preto, Brazil.
[2]National Institute of Science and Technology in Stem Cell Therapy, Ribeirão Preto, Brazil.
[3]Department of Clinical, Toxicological and Food Science Analysis, Faculty of Pharmaceutical Sciences, Universidade de São Paulo, Ribeirão Preto, Brazil.
[4]Department of Genetic and Evolutionary Biology, Institute of Biosciences, Universidade de São Paulo, Ribeirão Preto, Brazil.
[5]Department of Clinical Medicine, Faculty of Medicine of Ribeirao Preto, Universidade de São Paulo, Ribeirão Preto, Brazil.

obtained using different combinations of those same transcription factors with or without the aid of small molecules [14,17–20], or using nanog homeobox (*NANOG*) and lin-28 homolog (C. elegans) (*LIN28*) in place of *KLF4* and *C-MYC* [21].

The completely reprogrammed iPS cells resemble ESCs in their properties and potential to differentiate into a large number of adult cell types [22–26]. However, the mechanisms that lead to the complete reprogramming of somatic cells are still not completely understood.

The fact that different combinations of transcription factors can induce pluripotency suggests that different mechanisms of induction exist. The specific contribution of each reprogramming factor and the most efficient combination of factors to induce pluripotency remain unknown.

Based on the hypothesis that there are different combinations of reprogramming factors able to generate iPS cells, in the current study, we delivered a set of 6 genes related to pluripotency (*OCT4*, *SOX2*, *NANOG*, T-cell leukemia/lymphoma protein 1A [*TCL-1A*], *C-MYC* and β-*CATENIN*) to a population of adult human dermal fibroblasts (HDFs) to generate iPS cells which are comparable to hESCs with improved efficiency. We showed that a new combination of 3 transcription factors (*SOX2*, *TCL-1A*, and *C-MYC*) can successfully reprogram human skin fibroblasts to pluripotent reprogrammed cells.

Materials and Methods

Cell culture

The fibroblast line human fibroblast from ATCC (CCD27SK) (ATCC CRL1475) was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 0.5% penicillin and streptomycin (Invitrogen). 293T cells were maintained in DMEM containing 10% FBS, 2 mM L-glutamine (Invitrogen), and 0.5% penicillin and streptomycin. ESCs and reprogrammed cells *TCL1*, *SOX2*, and *C-MYC* (TSM) were cultured in 6-well plates coated with 0.3 mg/mL Matrigel™ hESC-Qualified Matrix (BD Biosciences). Unbound Matrigel was aspirated off and washed out with DMEM/F12. Embryonic or TSM cells were seeded onto Matrigel-coated plates in ESC medium (mTeSR™; WiCell). The medium was changed daily.

Plasmid construction

The 6 transcription factors were amplified by pyrococcus furiosus DNA polymerase chain reaction (PCR) of cDNA from hESCs. The open reading frame of human *NANOG* (NM_024865.1), *SOX2* (NM_003106.2), *OCT4* (NM_002701.3), *TCL-1A* (NM_021966.1), β-*CATENIN* (NM_001904.2), *KLF4* (NM_004235.3), and *C-MYC* (NM_002467) was cloned into pCR2.1-TOPO (Invitrogen). The fragments were removed from pCR2.1-TOPO by *Sma*I/*Xho*I digestion and subcloned into the *Sma*I/*Xho*I site of pLenti-cPPT-CIGWS. This is a replication-defective self-inactivating human immunodeficiency virus-1-derived vector, which is a bicistronic vector coding for enhanced green fluorescent protein (EGFP); and additionally, it contains the central polypurine tract and the central termination sequence (cPPT/CTS) from human immunodeficiency virus-1 that was inserted 5′ from the internal cytomegalovirus (CMV) promoter [27]. All the cloned fragments were verified by sequencing.

Lentiviral production

Vector DNA was introduced into 293T cells by a triple cotransfection with the packaging construct pCMVΔR8.91 [28,29] and the pseudotyping construct pMD2.VSVG, which codes for vesicular stomatitis virus glycoprotein G [30,31]. Transfection of monolayers with the DNA mixture was done by calcium phosphate coprecipitation. Sixteen hours after transfection, the supernatants were replaced with fresh medium, and cells were incubated for an additional 48 h. Culture supernatants containing pseudovirions were then collected, and the virus preparations were snap frozen and stored at −80°C. At this temperature, the titers remain stable for at least 2 months. Virus titers were determined as follows: 293T cells were seeded onto a 24-well plate at a concentration of 5×10^4 cells per well. Twenty-four hours later, 293T monolayers were transduced with serial dilutions of the viral concentrates supplemented with 1 μg/mL Polybrene (Sigma-Aldrich). After 72 h of incubation, the cells were harvested using trypsin-ethylenediaminetetraacetic acid and counted, and the transduced cells were detected by expression of EGFP using fluorescence-activated cell sorting (FACS) on a FACSCalibur flow cytometer (BD Biosciences). Titers were expressed as 293T-transducing units per milliliter.

Lentiviral infection and TSM generation

Human fibroblasts were seeded at 2×10^4 cells per 100-mm dish 1 day before transduction. The virus-containing supernatants were filtered through a 0.45 μm pore-size filter. Equal amounts of supernatants containing each of the 6 lentiviruses (Multiplicity of Infection [MOI] 10) were mixed, transferred to the fibroblast dish, and incubated overnight. Twelve hours after transduction, the virus-containing medium was replaced with DMEM 10% FBS. Six days after transduction, fibroblasts were harvested by trypsinization and replated at 1×10^4 cells per 6-well plate previously coated with Matrigel and ES medium supplemented with valproic acid (20 mM; Sigma-Aldrich). The medium was changed every day. Eleven days after transduction, colonies were selected and picked up, mechanically dissociated to small clumps by pipetting up and down, and re-plated onto a new 6-well plate to expand.

Detection of the lentiviral vector into the CCD27SK cells

To detect the lentiviral vectors integrated into the cell, we used specific primers for each lentiviral construction. The Genomic DNA was extracted from TSM reprogrammed cells using a DNeasy Tissue kit (Qiagen) according to the manufacturer's instructions. The PCR profile was 94°C for 2 min followed by 94°C for 40 s, 55°C for 40 s, and 72°C for 2.5 min for 35 cycles, with a final extension stage at 72°C for 5 min. The PCR products were then separated by 1% agarose gel electrophoresis and analyzed.

RNA isolation and reverse transcription

Total RNA was purified with Trizol reagent (Invitrogen) and treated with the Turbo DNA-free kit (Ambion) to remove genomic DNA contamination. One microgram of total RNA was used for reverse transcription reaction using the High Capacity cDNA Reverse Transcription Kit (Applied

Biosystems) according to the manufacturer's instructions. Quantitative PCR was performed by TaqMan (Invitrogen) and analyzed with the 7300 real-time PCR system (Applied Biosystems). Gene expression was normalized relative to that from the endogenous gene glyceraldehyde 3-phophate dehydrogenase.

Immunocytochemistry assay

Reprogrammed cells and CCD27SK cells (negative control) were fixed in 4% paraformaldehyde (Merck) for 20 min at room temperature, washed thrice with phosphate-buffered saline, and then permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 1 h at room temperature. The cells were blocked for 1 h with 5% bovine serum albumin and 2% goat serum in phosphate-buffered saline solution. Samples were incubated overnight at 4°C with primary antibodies against anti-stage-specific embryonic antigen 4 (SSEA4) (Stem Cell Technologies), tumor-related antigen (TRA-1-60; Chemicon), TRA-1-81 (Chemicon), OCT4 (Chemicon), NESTIN (Chemicon), β TUBULIN III, glial fibrillary acidic protein (GFAP) (BD Pharmingen), α-1 FETOPROTEIN (DAKO), CYTOKERATIN 18 (DAKO), and α-SMOOTH MUSCLE ACTIN (α-SMA) (Sigma-Aldrich) at room temperature overnight. The secondary antibodies used were AlexaFluor 594 rabbit antibodies (A-21207; Molecular Probes, Invitrogen) and AlexaFluor 594 mouse antibodies (A-21203; Molecular Probes, Invitrogen). The nucleus was stained with 4′,6-diamidino-2-phenylindole (VYSIS). Cells were visualized using a confocal laser scanning microscope (LSM 710; Carl Zeiss) with objective lens×63 in oil immersion, having a numerical aperture of 1.4. An Argon laser of 590 nm was used to excite the surface marked with primary antibodies, and the emission was measured at 617 nm. Fluorescence of eGFP was excited with an Argon laser at 488 nm, and the emission was measured at 525 nm. Visualization of the light scattering for each of the excitation wavelengths was recorded in multitracking mode using separate detection channels. Image analysis and co-
localization studies were carried out using the ZEN 2008 systems confocal software (ZEN, version 2.5). Control and test images were captured using identical settings.

In vitro differentiation of human reprogrammed cells

For spontaneous differentiation through embryoid body (EB) formation, human TSM reprogrammed cells were harvested by collagenase IV treatment. The cell clumps were transferred to a 0.6% agarose-coated dish in DMEM/F12 containing 20% FBS, nonessential amino acids, β-mercaptoethanol, L-glutamine, and penicillin/streptomycin. After 8 days in suspension culture, EBs were transferred to gelatin-coated plates (0.2%) and cultured in the same medium for 8 additional days.

Flow cytometry

The immunophenotypic characterization was performed by flow cytometry using the following monoclonal antibodies: CD45-fluorescein isothiocyanate, CD31-fluorescein isothiocyanate, CD90-phycoeritrin (PE), CD13-PE, and AC-133-PE (Pharmingen). Cells were incubated with the antibodies following the manufacturer's instructions. Nonspecific immunoglobulin G of the corresponding class served as the negative control. Cell suspensions were analyzed on FACSort flow cytometer (Becton-Dickinson) using CellQuest software.

Whole genome microarray experiments

Gene expression profiling was performed with the "Whole Human Genome Oligo Microarray Kit" (Agilent, G4112F) containing 41,000 probes. After DNase treatment and purification with the RNeasy kit (Qiagen), total RNA quality and concentration were assessed by agarose gel electrophoresis and spectrophotometry, using a NanoDrop 1000 instrument (NanoDrop). Target synthesis and hybridizations were performed with the "Quick Amp Labeling Kit, one-color" kit (Agilent, 5190-0442), following the manufacturers protocol. Briefly, 1 μg of the total RNA from each studied sample (CCD27SK fibroblasts and derived TSM reprogrammed cells) was mixed with a control RNA ("One Color RNA Spike-In Kit," Agilent, 5188-5282) and reverse transcribed using T7 oligo (dT) primers, followed by synthesis of the second complementary DNA (cDNA) strand and purification of double-strand cDNA with QIA quick columns (Qiagen). Cy3-labeled cRNA was then generated by an in vitro transcription reaction using T7 RNA polymerase and cyanine 3-cytosine triphosphate (CTP), and labeling efficiency was measured using a NanoDrop 1000 spectrophotometer. The cRNA was purified on RNeasy column, and 10 μg were fragmented and hybridized to microarrays overnight (17 h at 65°C and 4 rpm) in SureHyb chambers placed in a rotator oven incubator (Agilent, G2534A, G2530-60029 and G2545A), using the "Gene Expression Hybridization Kit" (Agilent, 5188-5242). After the post-hybridization washes ("Wash Buffers 1 and 2 Kit," Agilent, 5188-5327), arrays were dried and analyzed.

Data analysis

After processing, microarray slides were scanned at 535 nm and 5 μm/pixel resolution using a GenePix 4000B scanner and the "GenePix Pro 6.0" software (Molecular Devices). Images were analyzed with the "Agilent Feature Extraction 8.5" software, and the extracted expression values were normalized to the 75 percentile expression value of the whole array. Data were exported to Microsoft Office Access for further analysis.

The generated microarray expression data were compared with profiles that used the same platform as used here (Agilent 1 color Whole Genome) derived from 2 published reports and downloaded from the gene expression omnibus (GEO) database. The first data set consisted of the hESC lines H9, BG03, and ES01 [32] corresponding to GEO accessions GSM194390, GSM194391, and GSM194392, respectively. The second data set consisted of a dermal fibroblast line and an iPS cell line generated from it [33] and corresponded to GEO accessions GSM242095 and GSM241846, respectively.

Nonsupervised hierarchical cluster analysis was performed using the complete set of genes from the microarray and all the transcriptomes mentioned above. The Cluster 3 software was used to group the expression profiles by the average linkage method using a similarity metric based on an uncentered correlation coefficient [34]. Java TreeView was used to generate and visualize the dendrogram [35].

To further illustrate the shift toward an embryonic stem-like transcriptome, we selected a set of "consensus hESC signature genes" from genes showing a 2-fold up-regulation on the TSM reprogrammed cells generated by our group (as compared with CCD27SK fibroblasts) [10]. This set of genes was used to generate a heatmap depicting their expression levels on the reprogrammed cells, on fibroblasts from Takahashi's and our work [33], and also on the hESC lines characterized in Tesar's work [32].

The complete microarray data were submitted to Array Express and can be accessed at www.ebi.ac.uk/microarray-as/aer/login (ArrayExpress account username: Reviewer_E-MEXP-2422, password: 1256578647285; accession: E-MEXP-2422).

Teratoma formation assays

Approximately 1×10^6 TSM reprogrammed cells were subcutaneously injected into 6 NOD/SCID mice, and 2×10^4 cells were intra-ocularly injected into 2 NOD/SCID mice. After 12 weeks, the mice were sacrificed, and tissues were analyzed for tumor formation.

Results

Kinetics of TSM reprogrammed colony generation

Induction of iPS cells from human fibroblasts requires a virus with high transduction efficiency [4]. We, therefore, optimized transduction methods in adult HDFs (CCD27SK) using a replication-deficient bicistronic lentiviral vector that encodes for EGFP, under control of the CMV promoter [27,30,36–38]. Transduction assays were done at MOI of 10 for each vector. Figure 1-I illustrates the time line of the reprogrammed generation using the 6 factors (*SOX2*, *OCT4*, *NANOG*, *TCL1A*, β-*CATENIN*, *C-MYC*). Approximately 9 days after reseeding the transduced cells on BD Matrigel, some cells began to change their morphology, becoming spherical cells with abundant cytoplasm (Fig. 1-II-C, D). In thirteen days, some granulated colonies containing cells of different morphology appeared (Fig. 1-II-E, F). Fifteen days later, the colonies were large, with about 200–300 cells per colony (Fig. 1-II-G, H). By day 17, we observed distinct colonies that were flat and resembled hESC colonies (Fig. 1-II-I, J). From 4×10^4 fibroblasts, we observed 18–23 hESC-like colonies (data from 2 independent experiments). At day 17, we picked hESC-like colonies, mechanically disaggregated them into small clumps without enzymatic digestion, and re-plated them onto matrigel-coated plates with ESC media. The hES-like cells expanded and formed tightly packed and flat colonies (Fig. 1-II-I, J). The cells exhibited morphology similar to that of hESC, characterized by large nuclei and scant cytoplasm (Fig. 1-II-I, J). After the first passage, the cells still exhibited morphology similar to that of hESC (Fig. 1-III-K–P).

To make sure that CCD27SK cells could be reprogrammed with the classical combination of transcription factors described by Takahashi and Yamanaka [4] (*SOX2*, *OCT4*, *KLF4*, and *C-MYC*) we also transduced these cells with lentiviral vector containing the transcription factors *SOX2*, *OCT4*, *KLF4*, and *C-MYC*. The first iPS-like colonies appeared 26 days after the OSKM transduction (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/scd).

Transcription factors integrated in reprogrammed cells

To determine the combination of exogenous transcription factors present in each isolated clone, we performed PCR with lentiviral vector specific primers. Our results showed that only the vectors containing *TCL-1A*, *SOX2*, and *C-MYC* were present in all the 5 colonies analyzed (Fig. 2A). To ensure that only the combination of *TCL-1A*, *SOX2*, and *C-MYC* was responsible for the generation of our reprogrammed cells, we transduced a new batch of CCD27SK fibroblasts with only these 3 factors. After 15–17 days of transduction, we obtained a total of 13 new iPS-like colonies, confirming our initial results (Fig. 2B). We confirmed the presence of the 3 exogenous factors in all clones analyzed (data not shown). We will refer to these 3-factor reprogrammed cells as TSM reprogrammed cells. Transduction with the individual factors *TCL-1A*, *C-MYC*, and *SOX2* and also with *TCL-1A*/*SOX2*, *TCL-1A*/*C-MYC*, and *C-MYC*/*SOX2* combinations was performed; but no colonies with hESC morphology were observed, indicating that only the 3 factors together can generate iPS-like colonies.

Given that the lentiviral transgenes contain a GFP marker co-transcribed with the reprogramming genes, transduced cells were initially GFP positive; but as previously described [39,40], we observed a decrease in GFP expression in the successfully reprogrammed colonies after the first passage (Fig. 2B), indicating that the expression of the exogenous factors was down-regulated.

Pluripotency of TSM reprogrammed cells

The TSM reprogrammed cells express the surface embryonic antigens SSEA-4, TRA-1-60 and TRA-1-81 and *OCT4* as demonstrated by immunohistochemistry (Fig. 2C).

Further, we used quantitative real-time-PCR to demonstrate that the TSM reprogrammed cells express the pluripotent genes *OCT4*, *SOX2*, and *NANOG* at levels higher than those observed in the parental CCD27SK fibroblasts but lower than those found in hESC. *C-MYC* in TSM reprogrammed cells is expressed in higher levels than those in hESC and CCD27SK. Interestingly, the levels of *KLF4* were higher in CCD27SK than those in the hESC samples, whereas TSM reprogrammed cells showed an intermediary *KLF4* expression level.

Our reprogrammed cells also showed a distinctive pattern of surface marker expression by flow cytometry as demonstrated in Fig. 2E. The TSM reprogrammed cells were negative for the markers CD45, CD13, CD31, CD90, and AC133 and positive for TRA-1-60, TRA-1-81, SSEA4, OCT4, and NANOG (Fig. 2E). It is important to note that the original fibroblast CCD27SK was positive for CD13 and CD90; whereas the reprogrammed cells were negative for these markers, showing that the TSM reprogrammed cells had changed the expression of some surface and intracellular markers.

Our reprogrammed cells have the ability to form EBs that subsequently differentiate into cells of the 3 germ layers (Fig. 3). Using immunofluorescence, we demonstrated the presence in culture of TSM reprogrammed-derived ectodermal cells that are positive for *NESTIN*, β-*TUBULIN III*, and *GFAP*; endodermal cells positive for α-*FETOPROTEIN*; and mesodermal cells positive for α-SMA. Moreover, we observed some cells weakly positive for *CYTOKERATIN 18*. We also analyzed

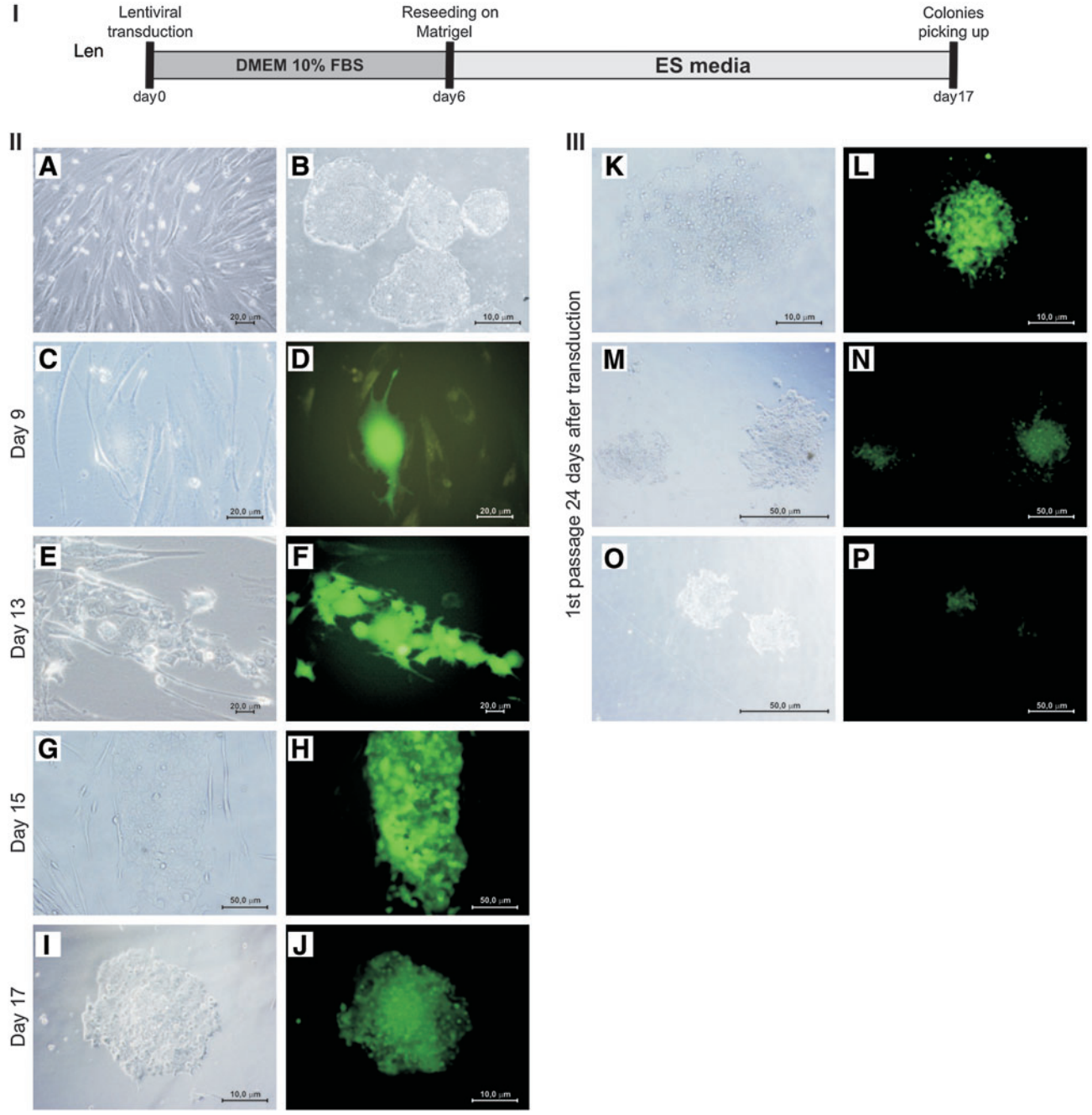
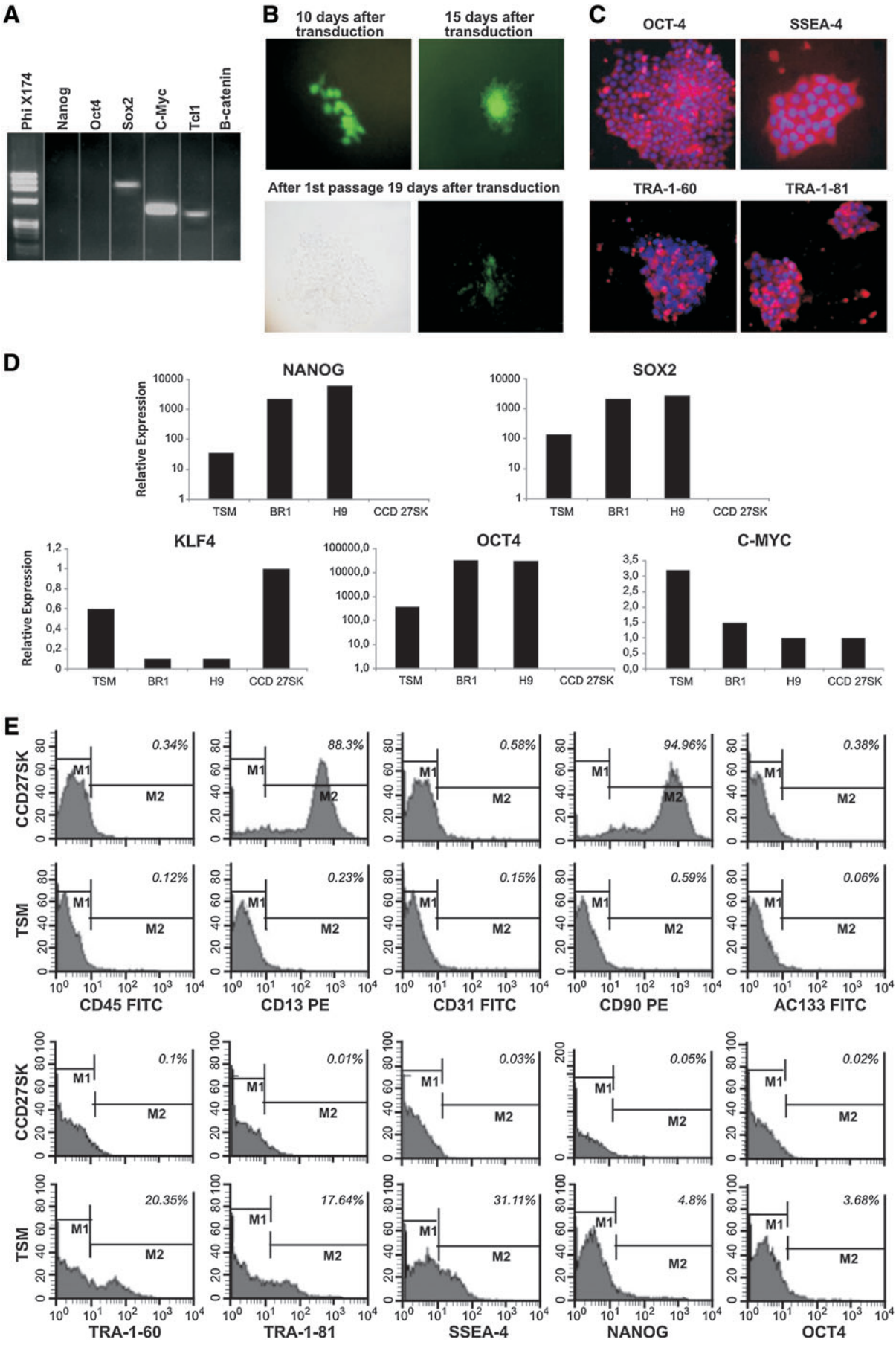


FIG. 1. The TSM cells generated from adult human dermal fibroblast (CCDSK27). **(I)** Time schedule of TSM reprogrammed cell generation. **(II)** Development of TSM cells **(A)** Morphology of CCDSK27, **(B)** Morphology of hESC colonies, **(C, D)** The first changes in the morphology, 9 days after lentiviral transduction. **(E, F)** Colonies after 13 days of culture. **(G, H)** Colonies after 15 days of culture. **(I, J)** The formation of hESC-like colonies after 17 days of culture. **(III)** TSM cells culture after the first passage. **(A, C, E, G, I, K, M, O)**: phase contrast images. **(D, F, H, J, L, N, P)** Epifluorescence images of green fluorescent protein -positive cells containing the lentiviral vectors. TSM, reprogrammed cells *TCL1*, *SOX2*, and *C-MYC*; hESC, human embryonic stem cells; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CCDSK27, human fibroblast from ATCC. Color images available online at www.liebertonline.com/scd.

the expression of the differentiation markers in CCD27SK cells. Immunofluorescence analysis shows that CCD27SK is negative for the markers *CYTOKERATIN 18*, *NESTIN*, *GFAP*, *SSEA1*, and *SSEA3* and also shows a low level of α-*FETOPROTEIN*, α-*SMA*, *B-tubulin III*, and *SSEA4* (Fig. 4A). We performed flow cytometry analysis to quantify the expression of these markers, and only α-SMA is significantly expressed in CCD27SK (Fig. 4B). The cytometry results of our positive control, hES H1, is shown in the Supplementary Fig. S2.

Transcriptome of the TSM reprogrammed cells

We analyzed the complete transcriptome of the original CCD27SK fibroblasts and of the TSM reprogrammed cells by


A
Phi X174
Nanog
Oct4
Sox2
C-Myc
Tcl1
B-catenin
B
10 days after transduction
15 days after transduction
After 1st passage 19 days after transduction
C
OCT-4
SSEA-4
TRA-1-60
TRA-1-81
D
NANOG
SOX2
KLF4
OCT4
C-MYC
Relative Expression
TSM
BR1
H9
CCD 27SK
E
CCD27SK
TSM
M1
M2
0.34%
88.3%
0.58%
94.96%
0.38%
0.12%
0.23%
0.15%
0.59%
0.06%
CD45 FITC
CD13 PE
CD31 FITC
CD90 PE
AC133 FITC
0.1%
0.01%
0.03%
0.05%
0.02%
20.35%
17.64%
31.11%
4.8%
3.68%
TRA-1-60
TRA-1-81
SSEA-4
NANOG
OCT4

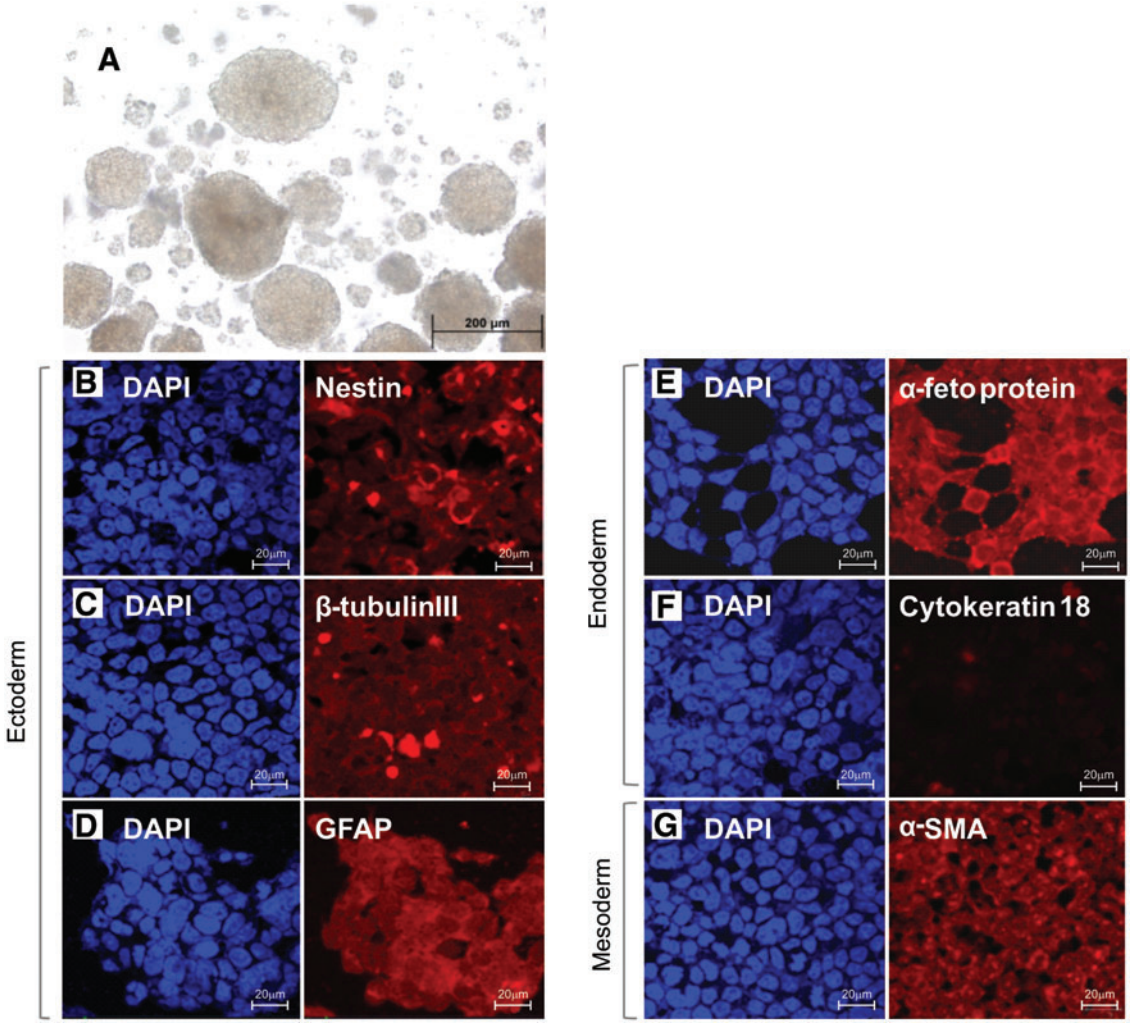


FIG. 3. Spontaneous differentiation through embryoid body formation **(A)**. **(B–G)**, In vitro differentiation experiments of TSM cells reveal their potential to generate cell derivatives of all 3 primary germ cell layers. Immunofluorescence analyses show expression of markers for nestin, β-tubulin III and GFAP (ectoderm), AFP, cytokeratin 18 (endoderm), α-SMA and vimentin (mesoderm). AFP, alphafetoprotein; α-SMA, α-smooth muscle actin. DAPI, 4′,6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein. Color images available online at www.liebertonline.com/scd.

microarray. The TSM cells profiles of gene expression were compared with the profiles of hESC (H9, BG03, and H1), with the profiles of the fibroblasts (HDF), and also with the derived 4 factors iPS^{HDF} cells generated by Takahashi et al. [33] (Fig. 5).

We demonstrated by hierarchical cluster analysis that the TSM reprogrammed cells clustered together with the iPS^{HDF} and the hESCs, whereas the fibroblasts (HDF and CCD27SK) formed a separated cluster (Fig. 5).

Additionally, we identified in our microarray data the genes that are considered by Lowry et al. [10] as "consensus hESC signature genes," which were expressed in TSM reprogrammed cells at levels at least 2-fold higher than in CCD27SK. We constructed a heatmap (Fig. 5) comparing data from all the cells used in the cluster analyses. The heatmap clearly shows that the TSM cells have an intermediate pattern of gene expression between the fibroblasts (HDF and CCD27SK) and the cluster formed by the iPS^{HDF} and the hESC.

FIG. 2. **(A)** Genomic DNA amplification of the TSM colony No. 1 for the detection of the integrated lentiviruses. **(B)** Gradual loss of green fluorescent protein 19 days after transduction. **(C)** Immunocytochemistry for SSEA-4, TRA-1-60, TRA-1-81, OCT4. Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). **(D)** Quantitative real-time polymerase chain reaction assay for expression of OCT4, SOX2, NANOG, and KLF4. Individual polymerase chain reaction assays were normalized against internal controls (glyceraldehyde 3-phophate dehydrogenase) and plotted relative to the expression level in the parent fibroblast cell line (CCD27SK). **(E)** Immunophenotyping of fibroblast CCD27SK and TSM. TRA, tumor-related antigen; FITC, fluorescein isothiocyanate; PE, phycoeritrin; TCL1, T-cell leukemia/lymphoma protein 1A; SOX2, (sex determining region Y)-box 2; C-MYC, v-myc myelocytomatosis viral oncogene homolog; SSEA-4, stage-specific embryonic antigen 4; OCT4, octamer-binding transcription factor 4; NANOG, nanog homeobox; KLF4, Kruppel-like factor 4. Color images available online at www.liebertonline.com/scd.

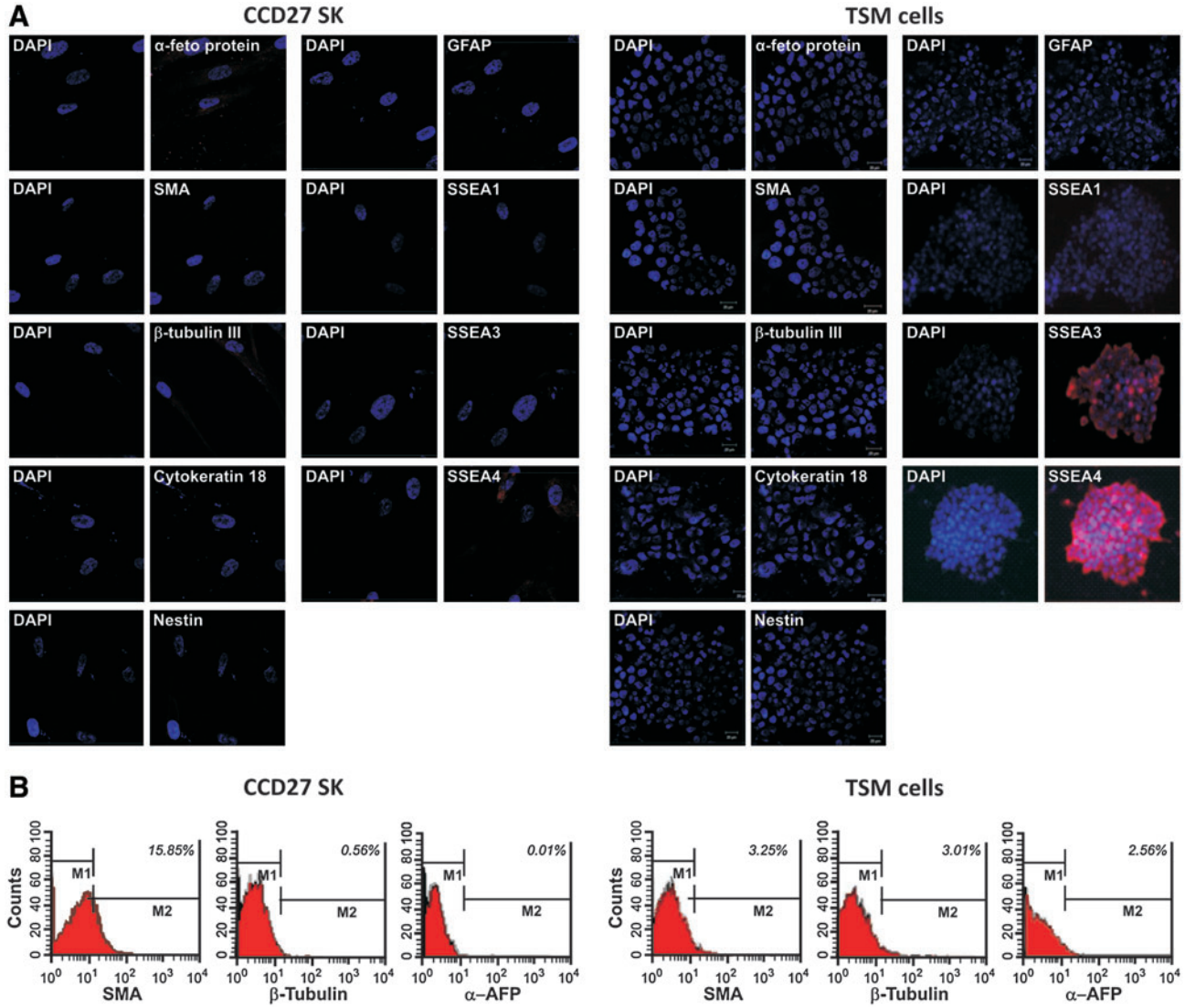


FIG. 4. **(A)** Immunofluorescence analyses of CCD27SK and TSM cells with antibodies for AFP, α-SMA, β-tubulin III, cytokeratin 18, nestin, GFAP, SSEA-1, SSEA-3, and SSEA-4. **(B)** Flow cytometry analysis of the markers α-SMA, β-tubulin III, and AFP. Color images available online at www.liebertonline.com/scd.

Discussion

We tested the hypothesis that the introduction of 6 pluripotent genes into the same cell population could reveal novel combinations of reprogramming factors able to reprogram cells. We found that the combined transcription factors *SOX2*, *TCL-1A*, and *C-MYC* are sufficient to reprogram HDFs into pluripotent cells. Although the generated reprogrammed cells (TSM reprogrammed cells) are not identical to hESCs or to iPS cells generated with the OKSM factors, they have a hESC-like morphology, express common hESC cell markers, and have a global gene expression profile similar to that from hESCs. In addition, the TSM reprogrammed cells also have the ability to differentiate into cells of the 3 embryonic germ layers in vitro. The TSM reprogrammed cell clones robustly proliferate and have been continuously cultured for 4 months. We also show that the fibroblasts used here can be reprogrammed with the classical transcription factors combination (OSKM).

A series of recent studies reported that the forced expression of varied combinations of 6 genes (*OCT4*, *SOX2*, *C-MYC*, *NANOG*, *LIN28*, and *KLF4*) is able to induce complete reprogramming of somatic cells to the pluripotent stem cell state, similar to the potency of ESC [4,5,7,21,41–45]. It has also been recently demonstrated that estrogen-related receptor beta (*ESRRB*) combined with *SOX2* and *OCT4* is able to reprogram mouse fibroblasts [46]. These remarkable studies demonstrate that the genetic program and epigenetic landscape of stem cells can be restored in differentiated cells. The use of the combination of *SOX2*, *C-MYC*, and *TCL-1A* has also the ability to induce pluripotency of somatic cells as demonstrated here for the first time. However, the TSM reprogrammed cells are not completely reprogrammed as indicated by the global gene expression profile and by the apparent

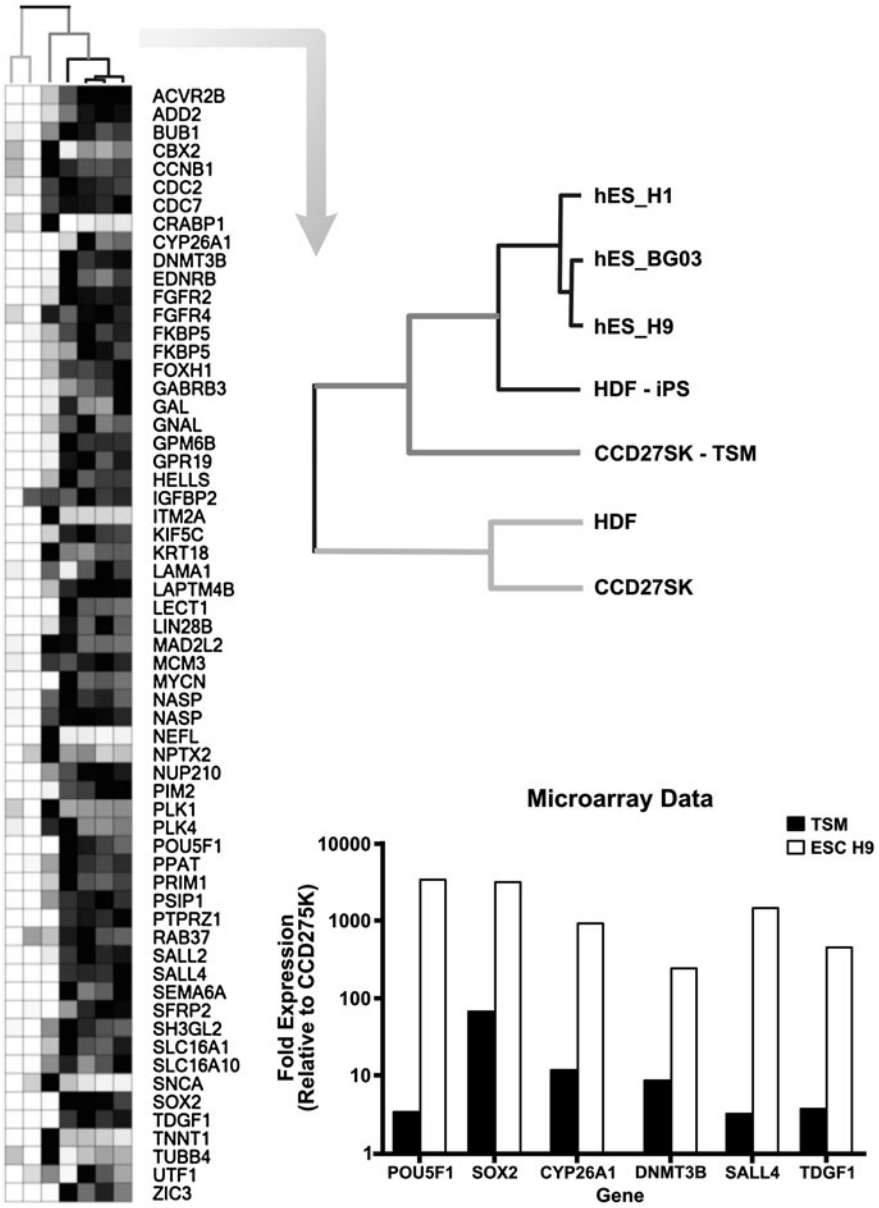


FIG. 5. Transcriptome shift of the generated TSM cells toward an embryonic stem-like transcriptome. The complete set of genes from the microarray was used in a hierarchical cluster analysis (uncentered correlation, average linkage) to group the expression profiles of fibroblasts, generated TSM cells, and hESC based on their overall similarities (top dendrogram). Genes deemed as "consensus hESC signature genes" [1], with at least a 2-fold increase in TSM cells (compared with CCD27SK fibroblasts), were used to generate a heatmap. Expression varies from absent or low (white and light gray) to higher levels (dark gray and black). The graph shows the heatmap using the Log2 transformed relative expression levels (obtained dividing microarray expression values of all cells by the value obtained for CCD27SK fibroblasts). This transformation results in numerical values with a reduced range that, in association with the default "row normalization" carried out by the Heatmap Builder software, allows more subtle differences to be visualized. HDF, human dermal fibroblasts; iPS, induced pluripotent cells; hES -H1, hES-BGO3 and hES-H9 are samples of human embryonic stem cells; HDF-iPS, iPS generated from human dermal fibroblast (HDF); CCD27SK-TSM, CCD27SK reprogrammed with TSM factors; HDF, human dermal fibroblast; CCD27SK, human fibroblast from ATCC.

incapacity to generate teratomas in immunodeficient mice (data not shown). Our reprogrammed cells show some chromosomal abnormalities. It has been shown that continuous passaging of reprogrammed as well as ESCs on Matrigel resulted in the appearance of chromosomal abnormalities [47,48]. Additional genomic alterations have also been identified in human iPS cells derived from fibroblasts [49]. To date, any study has solved this issue, which is essential for the application of iPS cells into regenerative medicine.

The pluripotency of ESC is regulated by a network of ESC-specific pluripotent transcription factors including *OCT4*, *NANOG*, *SOX2*, signal transducer and activator of transcription 3 (*STAT3*) and their effectors partners such as *TCL-1A*, embryonic stem cell expressed Ras (*ERAS*), sal-like 4 (*SALL4*), and undifferentiated embryonic cell transcription factor 1 (*UTF1*), which promote cell proliferation [50]. *OCT4*, *SOX2*, and *NANOG* have been found to regulate their expression in stem cells forming a core of auto-stimulating transcription factors [51–54]. *NANOG* also regulates T-box 3 (*TBX3*) and *TCL-1A* [55].

The *TCL1-A* gene is one of the regulator genes involved in the pluripotency pathway in both mouse and hESC [56]. One of the functions of *TCL-1A* is to repress a subset of neural crest genes [56]. *TCL-1A* and *ERAS* further stimulate the phosphoinositide-3-kinase/serine/threonine protein kinase Akt (Akt) signaling pathway to promote the cell cycle [50,57,58]. Other factors such as myb-related protein B (*B-MYB*), *C-MYC*, and the tumor suppressor gene P53 can affect the reprogramming of somatic cells. *B-MYB* and *C-MYC* activate the progression of the cell cycle, directly contributing to iPS generation. P53 induces growth arrest by holding the cell cycle at the G1/S regulation point on recognition of DNA damage. It was recently shown by several groups that p53 suppresses iPS cells generation [59,60] and

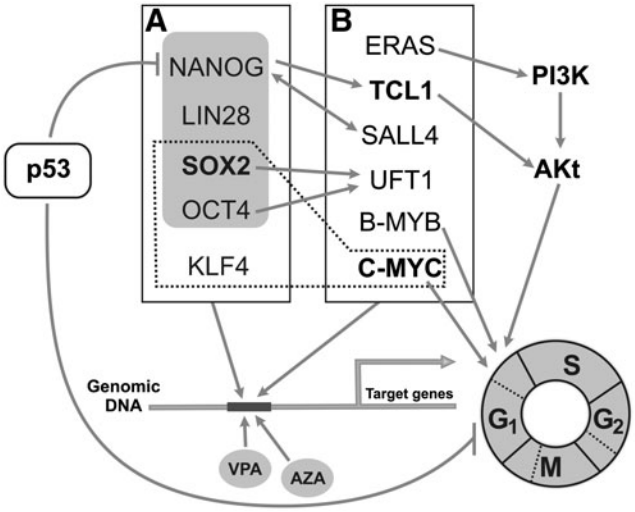


FIG. 6. Regulation of iPS cells generation. P53 suppresses NANOG expression and can halt progression of the cell cycle in G1. **(A)** Pluripotent factors drive the expression of certain factors **(B)** that control ES cell proliferation. Eras and Tcl1 can activate the PI3K/Akt pathway to promote the cell cycle. B-Myb and c-Myc directly stimulate the cell cycle progression. The gray rectangle represents the transcription factors used by Yu et al. [21], the genes marked by dashed line were utilized by Takahashi et al. [33], and the genes written in bold were used in this work. VPA and AZA induce demethylation of DNA, and they greatly enhanced the efficiency of reprogramming. Figure modified from Niwa [50]. PI3K, phosphoinositide-3-kinase; VPA, valproic acid; AZA, 5-azacytidine; UFT1, undifferentiated embryonic cell transcription factor 1; ERAS, embryonic stem cell expressed Ras; TCL1, T-cell leukemia/lymphoma protein 1A; Akt, serine/threonine protein kinase Akt; B-Myb, myb-related protein B; C-Myc, v-myc myelocytomatosis viral oncogene homolog (avian); SALL4, sal-like 4; KLF4, Kruppel-like factor 4; OCT4, octamer-binding transcription factor 4; SOX2, (sex determining region Y)-box 2; LIN28, lin-28 homolog (C. elegans); NANOG, nanog homeobox; p53, tumor suppressor protein 53.

can induce cell differentiation by suppressing *NANOG* expression [61] (Fig. 6).

Many factors are involved in the establishment and maintenance of iPS cells, but little is known about how these factors work to maintain the pluripotent network. The development of iPS cells using new combinations of reprogramming factors will be of fundamental importance to understand and control the mechanisms of pluripotency and self-renewal of human iPS cells.

One proposed mechanism of iPS generation is that the reprogramming is achieved when, on the one hand, there is an equilibrated exogenous expression between pluripotent transcription factors (*NANOG*, *SOX2*, *OCT4*, *KLF4*, *LIN28*) and when, on the other hand, proliferative genes such as *C-MYC* are present, (Fig. 6). The role of the proliferative genes could be providing chromosomal access to the transcriptional factors mediated by cell cycle progression. In this model, the proliferation genes have no obligatory role, as it is possible to induce full reprogramming of somatic cells without the forced expression of these genes. However, in the presence of these genes, the efficiency of reprogramming dramatically increases [20]. Similarly, the presence of valproic acid and 5-azacytidine, which increase the chromatin accessibility, improves the efficiency of iPS generation [17,62].

The combination including *TCL-1A* does not seem to be universal. The contribution of *TCL-1A* to the process of cell reprogramming could be, similar to the proposed role of *B-MYB* and *C-MYC*, the stimulation of cell proliferation to allow access, in the case of TSM reprogrammed cells the transcription factor *SOX2*, to the control region of the pluripotent genes (Fig. 6). In favor of this model, it is of note that the fibroblast CCD27SK expresses high level of *KLF4* (Fig. 2D), and the TSM reprogrammed cells themselves express *NANOG* and *OCT4*. Thus, the induction of pluripotency with the forced expression of only 1 transcription factor (*SOX2*) and 2 cell cycle genes (*C-MYC* and *TCL1-A*) in a cell that already has higher levels of *KLF4* expression agrees with the general mechanism proposed for pluripotent reprogramming (Fig. 6) [50].

In conclusion, we demonstrated that the forced expression of 3 genes (*SOX2*, *C-MYC*, and *TCL-1A*) is capable to induce pluripotent reprogramming of human somatic fibroblasts. This achievement could contribute to the understanding of the mechanisms of pluripotent cell generation, and it could also permit the generation of cells that may be used in experimental protocols of drug screening, in disease models, or even in protocols of experimental cell therapy.

Acknowledgments

This work was supported by: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Financiadora de Estudos e Projetos (FINEP), Brazil.

Author Disclosure Statement

No competing financial interests exist.

References

1. Evans MJ and MH Kaufman. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156.
2. Martin GR. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA 78:7634–7638.
3. Thomson JA, J Itskovitz-Eldor, SS Shapiro, MA Waknitz, JJ Swiergiel, VS Marshall and JM Jones. (1998). Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147.
4. Takahashi K and S Yamanaka. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676.
5. Maherali N, R Sridharan, W Xie, J Utikal, S Eminli, K Arnold, M Stadtfeld, R Yachechko, J Tchieu, R Jaenisch, K Plath and K Hochedlinger. (2007). Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. Cell Stem Cell 1:55–70.
6. Okita K, M Nakagawa, H Hyenjong, T Ichisaka and S Yamanaka. (2008). Generation of mouse induced pluripotent stem cells without viral vectors. Science 322:949–953.
7. Wernig M, A Meissner, R Foreman, T Brambrink, M Ku, K Hochedlinger, BE Bernstein and R Jaenisch. (2007). *In vitro* reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448:318–324.

8. Zhao XY, W Li, Z Lv, L Liu, M Tong, T Hai, J Hao, CL Guo, QW Ma, L Wang, F Zeng and Q Zhou. (2009). iPS cells produce viable mice through tetraploid complementation. Nature 461:86–90.
9. Aoi T, K Yae, M Nakagawa, T Ichisaka, K Okita, K Takahashi, T Chiba and S Yamanaka. (2008). Generation of pluripotent stem cells from adult mouse liver and stomach cells. Science 321:699–702.
10. Lowry WE, L Richter, R Yachechko, AD Pyle, J Tchieu, R Sridharan, AT Clark and K Plath. (2008). Generation of human induced pluripotent stem cells from dermal fibroblasts. Proc Natl Acad Sci USA 105:2883–2888.
11. Stadtfeld M, M Nagaya, J Utikal, G Weir and K Hochedlinger. (2008). Induced pluripotent stem cells generated without viral integration. Science 322:945–949.
12. Eminli S, J Utikal, K Arnold, R Jaenisch and K Hochedlinger. (2008). Reprogramming of neural progenitor cells into induced pluripotent stem cells in the absence of exogenous Sox2 expression. Stem Cells 26:2467–2474.
13. Hanna J, S Markoulaki, P Schorderet, BW Carey, C Beard, M Wernig, MP Creyghton, EJ Steine, JP Cassady, R Foreman, CJ Lengner, JA Dausman and R Jaenisch. (2008). Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. Cell 133:250–264.
14. Kim JB, H Zaehres, G Wu, L Gentile, K Ko, V Sebastiano, MJ Arauzo-Bravo, D Ruau, DW Han, M Zenke and HR Scholer. (2008). Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. Nature 454: 646–650.
15. Maehr R, S Chen, M Snitow, T Ludwig, L Yagasaki, R Goland, RL Leibel and DA Melton. (2009). Generation of pluripotent stem cells from patients with type 1 diabetes. Proc Natl Acad Sci USA 106:15523–15524.
16. Stadtfeld M, K Brennand and K Hochedlinger. (2008). Reprogramming of pancreatic beta cells into induced pluripotent stem cells. Curr Biol 18:890–894.
17. Huangfu D, K Osafune, R Maehr, W Guo, A Eijkelenboom, S Chen, W Muhlestein and DA Melton. (2008). Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. Nat Biotechnol 26:1269–1275.
18. Nakagawa M, M Koyanagi, K Tanabe, K Takahashi, T Ichisaka, T Aoi, K Okita, Y Mochiduki, N Takizawa and S Yamanaka. (2008). Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol 26:101–106.
19. Shi Y, C Desponts, JT Do, HS Hahm, HR Scholer and S Ding. (2008). Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. Cell Stem Cell 3:568–574.
20. Wernig M, A Meissner, JP Cassady and R Jaenisch. (2008). c-Myc is dispensable for direct reprogramming of mouse fibroblasts. Cell Stem Cell 2:10–12.
21. Yu J, MA Vodyanik, K Smuga-Otto, J Antosiewicz-Bourget, JL Frane, S Tian, J Nie, GA Jonsdottir, V Ruotti, R Stewart, Slukvin, II and JA Thomson. (2007). Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917–1920.
22. Choi KD, J Yu, K Smuga-Otto, G Salvagiotto, W Rehrauer, M Vodyanik, J Thomson and I Slukvin. (2009). Hematopoietic and endothelial differentiation of human induced pluripotent stem cells. Stem Cells 27:559–567.
23. Hanna J, M Wernig, S Markoulaki, CW Sun, A Meissner, JP Cassady, C Beard, T Brambrink, LC Wu, TM Townes and R Jaenisch. (2007). Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science 318:1920–1923.
24. Raya A, I Rodriguez-Piza, G Guenechea, R Vassena, S Navarro, MJ Barrero, A Consiglio, M Castella, P Rio, E Sleep, F Gonzalez, G Tiscornia, E Garreta, T Aasen, A Veiga, IM Verma, J Surralles, J Bueren and JC Izpisua Belmonte. (2009). Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. Nature 460:53–59.
25. Tanaka T, S Tohyama, M Murata, F Nomura, T Kaneko, H Chen, F Hattori, T Egashira, T Seki, Y Ohno, U Koshimizu, S Yuasa, S Ogawa, S Yamanaka, K Yasuda and K Fukuda. (2009). *In vitro* pharmacologic testing using human induced pluripotent stem cell-derived cardiomyocytes. Biochem Biophys Res Commun 385:497–502.
26. Narazaki G, H Uosaki, M Teranishi, K Okita, B Kim, S Matsuoka, S Yamanaka and JK Yamashita. (2008). Directed and systematic differentiation of cardiovascular cells from mouse induced pluripotent stem cells. Circulation 118:498–506.
27. Picanco V, S Heinz, D Bott, M Behrmann, DT Covas, E Seifried and T Tonn. (2007). Recombinant expression of coagulation factor VIII in hepatic and non-hepatic cell lines stably transduced with third generation lentiviral vectors comprising the minimal factor VIII promoter. Cytotherapy 9:785–794.
28. Zufferey R, D Nagy, RJ Mandel, L Naldini and D Trono. (1997). Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol 15:871–875.
29. Mastromarino P, C Conti, P Goldoni, B Hauttecoeur and N Orsi. (1987). Characterization of membrane components of the erythrocyte involved in vesicular stomatitis virus attachment and fusion at acidic pH. J Gen Virol 68:2359–2369.
30. Follenzi A and L Naldini. (2002). Generation of HIV-1 derived lentiviral vectors. Methods Enzymol 346:454–465.
31. McGlynn LK, CR Mueller, M Begbie, CR Notley and D Lillicrap. (1996). Role of the liver-enriched transcription factor hepatocyte nuclear factor 1 in transcriptional regulation of the factor V111 gene. Mol Cell Biol 16:1936–1945.
32. Tesar PJ, JG Chenoweth, FA Brook, TJ Davies, EP Evans, DL Mack, RL Gardner and RD McKay. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature 448:196–199.
33. Takahashi K, K Okita, M Nakagawa and S Yamanaka. (2007). Induction of pluripotent stem cells from fibroblast cultures. Nat Protoc 2:3081–3089.
34. de Hoon MJ, S Imoto, J Nolan and S Miyano. (2004). Open source clustering software. Bioinformatics 20:1453–1454.
35. Saldanha AJ. (2004). Java Treeview—extensible visualization of microarray data. Bioinformatics 20:3246–3248.
36. Tonn T, C Herder, S Becker, E Seifried and M Grez. (2002). Generation and characterization of human hematopoietic cell lines expressing factor VIII. J Hematother Stem Cell Res 11:695–704.
37. Herder C, T Tonn, R Oostendorp, S Becker, U Keller, C Peschel, M Grez and E Seifried. (2003). Sustained expansion and transgene expression of coagulation factor VIII-transduced cord blood-derived endothelial progenitor cells. Arterioscler Thromb Vasc Biol 23:2266–2272.
38. Follenzi A and L Naldini. (2002). HIV-based vectors. Preparation and use. Methods Mol Med 69:259–274.
39. Loh YH, S Agarwal, IH Park, A Urbach, H Huo, GC Heffner, K Kim, JD Miller, K Ng and GQ Daley. (2009). Generation of induced pluripotent stem cells from human blood. Blood 113:5476–5479.

40. Park IH, R Zhao, JA West, A Yabuuchi, H Huo, TA Ince, PH Lerou, MW Lensch and GQ Daley. (2008). Reprogramming of human somatic cells to pluripotency with defined factors. Nature 451:141–146.
41. Okita K, T Ichisaka and S Yamanaka. (2007). Generation of germline-competent induced pluripotent stem cells. Nature 448:313–317.
42. Huang J, T Chen, X Liu, J Jiang, J Li, D Li, XS Liu, W Li, J Kang and G Pei. (2009). More synergetic cooperation of Yamanaka factors in induced pluripotent stem cells than in embryonic stem cells. Cell Res 19:1127–1138.
43. Kim JB, B Greber, MJ Arauzo-Bravo, J Meyer, KI Park, H Zaehres and HR Scholer. (2009). Direct reprogramming of human neural stem cells by OCT4. Nature 461:649–653.
44. Utikal J, N Maherali, W Kulalert and K Hochedlinger. (2009). Sox2 is dispensable for the reprogramming of melanocytes and melanoma cells into induced pluripotent stem cells. J Cell Sci 122:3502–3510.
45. Zhou W and CR Freed. (2009). Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. Stem Cells 27:2667–2674.
46. Feng B, J Jiang, P Kraus, JH Ng, JC Heng, YS Chan, LP Yaw, W Zhang, YH Loh, J Han, VB Vega, V Cacheux-Rataboul, B Lim, T Lufkin and HH Ng. (2009). Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb. Nat Cell Biol 11:197–203.
47. Draper JS, HD Moore, LN Ruban, PJ Gokhale and PW Andrews. (2004). Culture and characterization of human embryonic stem cells. Stem Cells Dev 13:325–336.
48. Mitalipova MM, RR Rao, DM Hoyer, JA Johnson, LF Meisner, KL Jones, S Dalton and SL Stice. (2005). Preserving the genetic integrity of human embryonic stem cells. Nat Biotechnol 23:19–20.
49. Chin MH, MJ Mason, W Xie, S Volinia, M Singer, C Peterson, G Ambartsumyan, O Aimiuwu, L Richter, J Zhang, I Khvorostov, V Ott, M Grunstein, N Lavon, N Benvenisty, CM Croce, AT Clark, T Baxter, AD Pyle, MA Teitell, M Pelegrini, K Plath and WE Lowry. (2009). Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. Cell Stem Cell 5:111–123.
50. Niwa H. (2007). How is pluripotency determined and maintained? Development 134:635–646.
51. Storm MP, HK Bone, CG Beck, PY Bourillot, V Schreiber, T Damiano, A Nelson, P Savatier and MJ Welham. (2007). Regulation of Nanog expression by phosphoinositide 3-kinase-dependent signaling in murine embryonic stem cells. J Biol Chem 282:6265–6273.
52. Suzuki A, A Raya, Y Kawakami, M Morita, T Matsui, K Nakashima, FH Gage, C Rodriguez-Esteban and JC Izpisua Belmonte. (2006). Maintenance of embryonic stem cell pluripotency by Nanog-mediated reversal of mesoderm specification. Nat Clin Pract Cardiovasc Med 3 Suppl 1:S114–S122.
53. Wu H, KJ Kim, K Mehta, S Paxia, A Sundstrom, T Anantharaman, AI Kuraishy, T Doan, J Ghosh, AD Pyle, A Clark, W Lowry, G Fan, T Baxter, B Mishra, Y Sun and MA Teitell. (2008). Copy number variant analysis of human embryonic stem cells. Stem Cells 26:1484–1489.
54. Mitsui K, Y Tokuzawa, H Itoh, K Segawa, M Murakami, K Takahashi, M Maruyama, M Maeda and S Yamanaka. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113:631–642.
55. Hatano SY, M Tada, H Kimura, S Yamaguchi, T Kono, T Nakano, H Suemori, N Nakatsuji and T Tada. (2005). Pluripotential competence of cells associated with Nanog activity. Mech Dev 122:67–79.
56. Boyer LA, TI Lee, MF Cole, SE Johnstone, SS Levine, JP Zucker, MG Guenther, RM Kumar, HL Murray, RG Jenner, DK Gifford, DA Melton, R Jaenisch and RA Young. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122:947–956.
57. Takahashi K, M Murakami and S Yamanaka. (2005). Role of the phosphoinositide 3-kinase pathway in mouse embryonic stem (ES) cells. Biochem Soc Trans 33:1522–1525.
58. Sun H, R Lesche, DM Li, J Liliental, H Zhang, J Gao, N Gavrilova, B Mueller, X Liu and H Wu. (1999). PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc Natl Acad Sci USA 96:6199–6204.
59. Hong H, K Takahashi, T Ichisaka, T Aoi, O Kanagawa, M Nakagawa, K Okita and S Yamanaka. (2009). Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature 460:1132–1135.
60. Kawamura T, J Suzuki, YV Wang, S Menendez, LB Morera, A Raya, GM Wahl and JC Belmonte. (2009). Linking the p53 tumour suppressor pathway to somatic cell reprogramming. Nature 460:1140–1144.
61. Lin T, C Chao, S Saito, SJ Mazur, ME Murphy, E Appella and Y Xu. (2005). p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. Nat Cell Biol 7:165–171.
62. Mikkelsen TS, J Hanna, X Zhang, M Ku, M Wernig, P Schorderet, BE Bernstein, R Jaenisch, ES Lander and A Meissner. (2008). Dissecting direct reprogramming through integrative genomic analysis. Nature 454:49–55.

Address correspondence to:
Dr. Virgínia Picanço-Castro
Center for Cell Therapy and Regional Blood Center
Rua Tenente Catão Roxo 2501
14051-140 Ribeirão Preto
Brazil

E-mail: v.picanco@hemocentro.fmrp.usp.br

Received for publication October 27, 2009
Accepted after revision May 24, 2010
Prepublished on Liebert Instant Online May 26, 2010