Stem Cells International

Advances in Induced Pluripotent Stem Cell Technologies

Guest Editors: Rajarshi Pal, Mahendra Rao, Mohan C. Vemuri, Paul Verma, and Andras Dinnyes

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Editorial Board

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Editorial

Advances in Induced Pluripotent Stem Cell Technologies

Rajarshi Pal,[1] Mahendra Rao,[2] Mohan C. Vemuri,[3] Paul Verma,[4] and Andras Dinnyes[5]

[1] *Manipal Institute of Regenerative Medicine, Manipal University Branch Campus, Bangalore 560 071, India*
[2] *NIH Center for Regenerative Medicine, Bethesda, MD 20892, USA*
[3] *Life Technologies, Frederick, MD 21704, USA*
[4] *Centre for Reproduction and Development, Monash Institute of Medical Research, Monash University, Clayton, VIC 33168, Australia*
[5] *BioTalentum Ltd., Godollo 2100, Hungary*

Correspondence should be addressed to Rajarshi Pal, rajarshi.pal@manipal.edu

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Progress in studying induced pluripotent stem cell (iPSC) has been extremely rapid. Ever since the remarkable discovery of iPSCs by Takahashi and Yamanaka, the field has continued to evolve with exciting discoveries furthering our understanding of early development, the process of cellular reprogramming, acquisition and maintenance of pluripotency, determination of cell fate, and enhancing our ability to model diseases in vitro. These advances and the possibility of generating patient or disease specific pluripotent cells placed the field on a trajectory that may lead to personalized cell therapy in the near future.

Although there have been significant advances in iPSC-based research, significant challenges remain and these will need to be addressed before tangible outcomes can be realized. Such challenges include developing robust strategies to reprogram cells free of a viral/genetic foot print, resolving the immune and potential tumorigenicity issues, understanding better the genome and epigenome status of iPSC, and developing techniques to predict the quality of iPSC clones. Understanding the basic biology of iPSCs well enough to allow for successful transition of iPSCs into the clinic someday will also require additional studies.

To further discussion of the barriers in the field of iPSC biology, the guest editors of this special issue have compiled select original research findings and review articles describing important issues in iPSC research. These include manuscripts that describe the role of recombinant Sox-2 (M. Thier et al.) and cMyc (C. Heffernan et al.) protines in reprogramming of fibroblasts. Other manuscripts describe the development of better methods of iPSC derivation such as the use of small molecules (S. K. Mak et al.), HDAC inhibitors (A. Kretsovali et al.), nonintegrating sendai virus-based generation (C. C. MacArthur et al.), microRNA mediated reprogramming (C.-H. Kuo and S.-Y. Ying). Addition articles on developing iPSC lines from nonhuman primates (Y. Wu et al.), and other large animal models such as equines (K. Khodadadi et al.) highlight the importance of this aspect in translational efforts. Yet other papers deal with the prospects of iPSC-based genetic repair (J. A. Pawitan), techniques for high-resolution genomic profiling (K. H. Elliott et al.), the promise of iPSC in dental problems (T. C. Srijaya et al.), using iPSCs for drug discovery and for developing models of toxicity (R. S. Deshmukh et al.). Elliot et al. describe resolution genome profiling that will facilitate assessment of the quality of iPSC.

We are enthused to see the rapidity with which the field has advanced and how international in scope the field has become. It was gratifying for us to see papers from Asia, Europe, Australia, and America and it is perhaps appropriate given the title of the journal Stem Cells International.

We hope these latest review articles and original research articles in the field of iPSC research will promote and enable better understanding of the basic biology of iPSC that is critical for successful translation of iPSC for patient specific cell therapies in future.

Rajarshi Pal
Mahendra Rao
Mohan C. Vemuri
Paul Verma
Andras Dinnyes

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Research Article

Laser-Based Propagation of Human iPS and ES Cells Generates Reproducible Cultures with Enhanced Differentiation Potential

Kristi A. Hohenstein Elliott,[1] Cory Peterson,[1] Anuradha Soundararajan,[2] Natalia Kan,[3] Brandon Nelson,[3] Sean Spiering,[3] Mark Mercola,[3] and Gary R. Bright[2]

[1] *Intrexon Corporation, Cell Engineering Unit, 6620 Mesa Ridge Road, San Diego, CA 92121, USA*
[2] *Cyntellect, Inc., 6620 Mesa Ridge Road, San Diego, CA 92121, USA*
[3] *Sanford-Burnham Medical Research Institute, 10901 Torrey Pines Road, La Jolla, CA 92037, USA*

Correspondence should be addressed to Kristi A. Hohenstein Elliott, khohenstein28@gmail.com

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Proper maintenance of stem cells is essential for successful utilization of ESCs/iPSCs as tools in developmental and drug discovery studies and in regenerative medicine. Standardization is critical for all future applications of stem cells and necessary to fully understand their potential. This study reports a novel approach for the efficient, consistent expansion of human ESCs and iPSCs using laser sectioning, instead of mechanical devices or enzymes, to divide cultures into defined size clumps for propagation. Laser-mediated propagation maintained the pluripotency, quality, and genetic stability of ESCs/iPSCs and led to enhanced differentiation potential. This approach removes the variability associated with ESC/iPSC propagation, significantly reduces the expertise, labor, and time associated with manual passaging techniques and provides the basis for scalable delivery of standardized ESC/iPSC lines. Adoption of standardized protocols would allow researchers to understand the role of genetics, environment, and/or procedural effects on stem cells and would ensure reproducible production of stem cell cultures for use in clinical/therapeutic applications.

1. Introduction

Human embryonic and induced pluripotent stem cell (ESC, iPSC) lines have been derived and maintained in a variety of ways, creating extensive variability and inconsistency from laboratory to laboratory. Currently, ESC and iPSC lines are cultured under diverse conditions, involving numerous methods of expansion, both feeder-dependent and feeder-independent matrices, and a variety of medium formulations [1–8]. Large-scale practical utilization of human ESCs and iPSCs for drug discovery applications, developmental and disease models, and regenerative therapeutic applications, will require more consistent and scalable culturing methods. Likewise, generation of GMP-quality stem cell lines will require standardized, traceable methods for stem cell derivation and expansion [9].

Manual passage, using specialized stem cell knives, razors, or pipettes to physically section stem cell colonies, is widely accepted as the best method for propagation of human ESC and iPSC lines. Manual propagation of stem cell lines does not involve the use of enzymes and therefore is thought to better maintain genetic stability of human ESCs and iPSCs in long-term culture [10–14]. Other benefits of manual expansion of stem cell cultures include passage of similar sized cell clumps, low cellular trauma, and selective transfer of specific undifferentiated colonies [7, 15]. However, scale-up of multiple stem cell lines using these methods is unattractive because of the high labor cost, inconsistency of output associated with varying expertise, risk of contamination, and the inability to effectively automate. Due to these technical demands associated with manual passage, routine propagation of most human ESC and iPSC lines is often performed using enzymatic passage [5, 6]. Enzymatic methods (e.g., using accutase, collagenase, dispase, trypsin, or TrypLE) are usually used for large-scale expansion and are well suited for automated platforms [16]. However, these methods are highly problematic as enzymatic dissociation results in variable-sized colonies leading to significant

inconsistency among cultures [7]. Also, human ESCs/iPSCs do not survive well as single cells which limit the utility of enzymatic propagation [17]. While chemical compounds (e.g., ROCK inhibitor) may be used to promote survival of dissociated stem cells, these compounds do not alleviate the heterogeneity associated with enzymatically passaged human ESC and iPSC cultures, nor is the full impact of routine usage of such compounds known [18]. The large heterogeneity in colony size also limits the usefulness of high density plates, such as 96-well and 384-well plates, for higher throughput applications.

This study reports a novel approach for the expansion of human stem cell lines using laser-based propagation. Laser-mediated passaging was performed by precise cutting of human stem cell cultures by a laser into specific sized cell sections. These cell sections were transferred by simple pipetting to new culture dishes for propagation. The cell sections were of uniform size leading to greater uniformity of the resulting colonies. Additionally, enzyme-free conditions were maintained throughout and all processing occurred within a sterile, closed environment. Operating within standard format multiwell plates enable incorporation of automated robotic systems for scalable delivery of standardized stem cell cultures.

Laser-mediated propagation maintained the quality and pluripotency of ESC/iPSCs and led to enhanced differentiation potential. This approach removes the variability associated with passaging stem cells, which should greatly improve the evaluation of gene expression signatures, genetic/epigenetic profiles, and differentiation capabilities/efficiencies of stem cell lines. Laser-mediated ESC/iPSC passage significantly reduces the expertise, labor, and time associated with manual passaging techniques and provides the basis for reproducible propagation of GMP-quality human stem cell lines.

2. Materials and Methods

2.1. Human ESC and iPSC Culture. Four human iPSC lines (BIMR 6, P15–40, and BIMR 14, P25–40 generated from adult fibroblasts, BIMR A, P20–50 and BIMR L, P30–45 generated from fetal fibroblasts, all transduced with retroviruses containing *Oct4*, *Sox2*, *Klf4*, and *c-Myc* genes, Burnham Institute for Medical Research (BIMR)) were cultured in KODMEM supplemented with 20% KnockOut Serum Replacement, 1% GlutaMax, 1% nonessential amino acids, 0.1 mM 2-mercaptoethanol, and 8 ng/mL bFGF (all from Invitrogen). iPSCs were expanded on PMEF-CFs (Millipore) or Matrigel (BD Biosciences, in medium conditioned using PMEF-CFs), medium was changed every day and cells were passaged at 1 : 2–1 : 8 every 5–7 days. H9 human ESCs (P35–65) were cultured using the same medium on PMEF-CFs and were passaged 1 : 2–1 : 4 every 5–7 days. Experiments involving H9 human ESCs were performed at the BIMR Stem Cell Core.

iPSCs were passaged by several methods including manual passage using a pipette tip or using StemPro EZPassage Disposable Stem Cell Passaging Tool (Invitrogen), enzymatic passage using collagenase IV (Invitrogen) or 0.05% trypsin (Invitrogen), and laser-mediated passage using the LEAP Cell Processing Workstation (Intrexon Corp).

Stem cell colony size was determined by measuring the longest diameter of colonies from brightfield images and by manual counting of Hoechst (Invitrogen) stained nuclei from fluorescent images. Whole well brightfield images of stem cell cultures were acquired on LEAP (Intrexon Corp) and Celígo (Brooks Automation, Inc). To generate ESC/iPSC growth curves, 0.25% trypsin (Invitrogen) was used to produce a single-cell suspension of ESC/iPSCs which were then counted using a hemocytometer on days 0–5 after passage.

Transfer efficiency after laser-mediated passage was determined by manual counting of the number of ESC/iPSC sections per well after sectioning (prior to section removal by pipetting), after removal of sections from the processed well, and after transfer of sections to new culture plates using whole well brightfield images. Two days after passage, passage efficiency was determined by manual counting of the number of alkaline phosphatase positive colonies in cultures containing transferred sections.

2.2. Laser-Mediated Passage. Optimal laser processing conditions were established assessing laser power, laser spot size, and density of laser spots for cutting stem cell colonies into sections with minimal loss of cells. Assessment was performed empirically by testing the ability of a given condition to consistently cut typical stem cell cultures across 96-, 12-, and 6-well plates. Photothermal laser processing was chosen to minimize cell loss during processing [19]. Laser pulse powers from 3–10 μJ and laser spot sizes from 10–25 um were systematically evaluated for sectioning through stem cell cultures of varying thickness. It was determined that ~8 μJ laser power delivered in a 10 um spot size was sufficient to section cultures of all thicknesses. To create a continuous sectioning line, laser pulses were positioned ~ 16 μm apart in a line to effectively cut stem cell colonies. After processing, samples were washed, sections were dislodged by pipetting using normal iPSC/ESC medium, and all sections were transferred to fresh culture plates containing PMEF-CFs or Matrigel. Cells were passaged at 1 : 2–1 : 8 every 5–7 days.

2.3. Human ESC/iPSC Differentiation. Embryoid bodies (EBs) were generated using collagenase IV treatment of day 5 human iPSC cultures for 0.5–1.0 hour to remove colonies from culture dishes. Colonies were grown in differentiation medium in suspension culture using Ultra Low Attachment plates (Corning). The longest diameter of resulting EBs was manually measured using brightfield images acquired on LEAP (Intrexon Corp) on day 4 or 5 of suspension culture.

Human iPSCs were induced to spontaneously differentiate in medium composed of KODMEM supplemented with 20% KnockOut Serum Replacement, 1% GlutaMax, 1% nonessential amino acids, and 0.1 mM 2-mercaptoethanol (Invitrogen). EBs were grown in suspension culture for 8 days and then plated onto gelatin-coated plates and allowed to differentiate for an additional 8 days. Medium was

changed every other day. Cultures were fixed on day 16 for immunocytochemical analyses.

Human iPSCs were induced to form cardiomyocytes by culturing EBs for 4 days in suspension culture in medium composed of KODMEM (Invitrogen) supplemented with 20% fetal bovine serum (FBS, Hyclone), 1% Glutamax, 1% nonessential amino acids, and 0.1 nM 2-mercaptoethanol (Invitrogen). On the 4th day, EBs were plated onto gelatin-coated plates and allowed to differentiate for an additional 18 days (22 total days). Medium was changed every other day. RNA was collected for QRT-PCR analyses on day 16, the number of contracting EBs was counted on days 16 and 22, and cultures were fixed for immunocytochemical analyses on day 22.

Human iPSCs were induced to form neural rosettes by culturing EBs for 7 days in suspension culture in medium composed of 50% DMEM/F12, 50% Neurobasal medium supplemented with glutamax, 0.5x N2 supplement, 0.5x B27 supplement (Invitrogen), 0.5 mM ascorbic acid, 0.1% albumin, 4.5×10^{-4} M MTG (Sigma), and bFGF (20 ng/mL, Peprotech). On the 7th day, EBs were plated onto gelatin-coated plates in the medium described above supplemented with EGF (20 ng/mL, Peprotech) and allowed to differentiate for an additional 4 days. On day 11, the number of EBs containing ≥ 1 neural rosette was manually counted.

2.4. Immunocytochemistry. Cells were fixed in 4% paraformaldehyde in PBS for 15 min, permeabilized in 0.1% Triton-X100 in PBS for 5 min, and then blocked for 1 hour in blocking buffer (10% serum of the same species as the secondary antibody, 0.05% Triton X-100 in PBS). Cells were washed and incubated with primary antibodies in 1% serum (same species as the secondary antibody) in PBS for 2 hours at room temperature or overnight at 4°C. Human iPSC/ESCs were characterized using the following antibodies: Oct4, Sox2, and Nanog (R & D Systems), SSEA4 (Developmental Studies Hybridoma Bank), and TRA1-60 and TRA1-81 (Santa Cruz Biotechnology). Apoptosis was analyzed using the following antibodies: caspase-3 and cleaved PARP (BD Biosciences) with staurosporine treatment of iPSC cultures used as a control (10 μM staurosporine, 4 hour treatment). Differentiated iPSCs were characterized using the following antibodies: Nestin, Map2, ANP (NPPA), and Troponin I (Millipore), Brachyury and Sox17 (R & D Systems), AFP and α-actinin (Sigma), and α-MHC (Developmental Studies Hybridoma Bank). Cells were then washed and incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen) for 2 hours. All antibodies were diluted according to manufacturer's instructions. Cell nuclei were stained with Hoechst (Invitrogen). All images were acquired using the LEAP system (Intrexon Corp).

2.5. Alkaline Phosphatase Staining. Cells were fixed in 4% paraformaldehyde in PBS for 15 min and stained with Fast Red TR hemi (zinc chloride) salt (Sigma) and Naphthol, AS-MX phosphate alkaline solution (Sigma) in H_2O for 15–30 min. All images were acquired using the LEAP system (Intrexon Corp).

2.6. Quantitative RT-PCR Analysis. RNA was prepared using the RNeasy Micro/Mini Kit (Qiagen) and cDNA synthesis was performed using the ABI High Capacity cDNA Reverse Transcription Kit. QRT-PCR was performed in triplicate for each primer set and in each cell sample using an ABI 7900HT Sequence Detection System. Amplification was performed using the Taqman Univeral PCR Mastermix (ABI). Specific primers and probes for stem-cell-associated genes, differentiation-associated genes, and cardiomyocyte genes were obtained from ABI. Stem-cell-associated genes included *Pou5f1* (Hs00999632_g1), *Sox2* (Hs01053049_s1), *Nanog* (Hs02387400_g1), *Tert* (Hs99999022_m1), *Zfp42* (Hs00399279_m1), *Dppa2* (Hs00414515_m1), and *Esg1* (Hs00988349_g1). Cardiomyocte-associated genes included *Nkx2.5* (Hs00231763_m1), *TnnI3* (Hs00165957_m1), *Actn1* (Hs00998100_m1), *Mef2C* (Hs01554599_m1), *Myh6* (Hs00411908_m1), and *Nppa* (Hs00383230_g1). Expression levels of all genes were normalized to Eukaryotic 18s rRNA (Hs99999901_s1), and then analyzed using the $2^{\Delta\Delta Ct}$ method [20].

2.7. Karyotype Analysis. Live cell cultures were analyzed by Cell Line Genetics. Cytogenetic analysis was performed on twenty G-banded metaphase cells.

2.8. aCGH Analysis. Genomic DNA was collected and purified using the Gentra Puregene Cell Kit (Qiagen). Hybridization was conducted with the 44 K Human StemArray (Ambry Genetics), with a resolution of ~24 kb over the entire genome and high resolution exonic coverage in known stem-cell-associated genes, tumor suppressors, and oncogenes. Samples were hybridized to a sex-matched pooled normal reference DNA (Promega). Data was analyzed by Ambry Genetics using DNA Analytics (Agilent) and reported using genome build HG18.

2.9. Statistical Analysis. Statistical analyses were performed using GraphPad Prism with a P value of ≤ 0.05 considered to be significant. One way analysis of variance (ANOVA) with Bartlett's test for equal variances was performed to evaluate resulting colony sizes generated after passage by five techniques ($n = 20$ colonies/sample, Figure 2(c)) and resulting EB sizes generated from laser-mediated, collagenase, and trypsin-passaged cells ($n = 30$ EB/sample, Figure 5(b)). Statistical analysis of QRT-PCR data ($n = 3$, Figures 4(c) and 5(d)) was performed using a two-tailed t-test.

3. Results

3.1. Optimization of Laser-Mediated Passage. Laser-mediated passaging conditions were optimized using four human iPSC lines and one human ESC line. Human iPSC/ESC cultures were initially passaged by standard methodology (i.e., collagenase treatment plus manual scraping of cultures) into plates containing mitomycin c-treated murine embryonic fibroblasts and cultured for 5 days in iPSC/ESC medium. Laser-mediated cutting of stem cell colonies into clumps or sections of cells was facilitated by addition of a reagent to

increase photothermal absorption of the laser's energy by the culture medium [19]. Laser processing conditions were optimized with respect to laser power, laser spot size, and number of laser shots required to effectively cut cultures with minimal loss of cells.

To determine the impact of section size on resulting colony size, stem cell cultures were cut into square cell sections ranging from 75 to 300 μm in size and transferred to new culture dishes by gentle pipetting (Figure 1(a), hiPSCs (top, middle), hESCs (bottom)). Sections below 75 μm contained very few cells (<8 cells/section), whereas 300 μm sections were too large to easily remove from the plate by gentle pipetting alone. Stem cell colony size and number of cells per colony were assessed by brightfield imaging and fluorescent staining of nuclei, respectively, after processing cultures into 100–250 μm sections (Figure 1(b)). Human iPSC cultures sectioned into 100, 150, 200, and 250 μm sizes resulted in sections containing 12, 25, 47, and 68 cells, respectively (Figure 1(b), top). Three days after passage human iPSC colonies measured 306, 367, 493, and 693 μm in diameter with 62, 119, 184, and 283 cells per colony, respectively. Similar results were obtained with all iPSC cell lines (data not shown) and with human ESCs (Figure 1(b), bottom) using the same laser processing conditions.

For routine propagation of iPSC and ESC cultures, section sizes of 200 μm were used, which allowed consistent splitting every 7 days. Notably, other groups have identified 200 μm (50–100 cells) as the optimal clump size for passage [1, 5, 15, 21]. Propagation of human iPSCs using 200 μm sections was highly efficient with an average transfer efficiency of 91 ± 2% with 93 ± 5% of the transferred sections forming viable colonies for an overall passage efficiency of 85 ± 3%. Passage of human ESCs resulted in similar data (overall passage efficiency of ~82%, data not shown). Laser-mediated passage with the current system required a total of ~50–90 min to process an entire plate of stem cells (depending on plate type using 200 μm sections; with the majority of time (>90%) spent for laser processing and only a few minutes spent by the user). iPSCs and ESCs cultured under feeder-free conditions were also successfully propagated using the same laser-mediated passage conditions. These data demonstrate that multiple stem cell lines can be propagated by laser-based passage and that the size of resulting colonies can be easily controlled by varying the input section size.

3.2. Improved Consistency of Stem Cell Cultures. Laser-mediated passage was compared with traditional passaging techniques, both manual and enzymatic. iPSC cultures (BIMR 6) were passaged by (1) laser-mediated passage using 200 μm sections, (2) manual passage using the StemPro EZPassage Disposable Stem Cell Passaging Tool (Invitrogen), (3) manual passage using a pipette tip (performed by an individual with 6-years experience), (4) enzymatic passage using collagenase, and (5) enzymatic passage using trypsin. Manual passage approaches generated significantly more uniform colonies than the enzymatic methods. Comparing laser-mediated passage with collagenase-based passage, image analysis of laser-passaged cultures revealed more homogeneous colony formation than collagenase passaged cultures (Figures 2(a) and 2(b). Stem cell cultures passaged by all methods were analyzed with respect to colony diameter and cells per colony (Figure 2(c)). Laser-mediated passage resulted in the most uniform colonies measuring 240 ± 43 μm (18% CV) in diameter containing 45 ± 7 (16% CV) cells per colony one day after passage. Enzymatic passage by collagenase or trypsin resulted in significantly variable sized colonies measuring 365 ± 177 μm (48% CV) and 172 ± 97 μm (56% CV) in diameter containing 90 ± 42 (47% CV) and 25 ± 19 (76% CV) cells per colony, respectively. Manual passage techniques using a pipette tip or the EZPassage tool resulted in more similar sized colonies measuring 214 ± 9 μm (37% CV) with 34 ± 3 (37% CV) cells per colony and 226 ±65 μm (29% CV) in diameter with 37 ± 9 (25% CV) cells per colony, respectively. However, the EZPassage tool does not allow colonies growing at the edge of each well to be propagated, leaving >25% of the culture unsectioned (data not shown). Statistical analysis of variance showed that the stem cell cultures propagated by laser-mediated passage varied significantly less than (P value < 0.0001) cultures passaged manually or by enzymatic methods, demonstrating that laser-mediated passage results in more consistent stem cell cultures than all other methods. Comparable results were also obtained using the BIMR A iPSC line (data not shown).

3.3. Pluripotency, Quality, and Stability of Stem Cells after Laser-Mediated Passage. The effect of the laser on human iPSC and ESC quality and pluripotency was examined immediately following laser-mediated sectioning of stem cell cultures into 200 μm sections. As shown in Figure 3(a), pluripotency markers such as Oct4, Sox2, Nanog, SSEA4, TRA1-60, and TRA1-81 were highly expressed in sectioned iPSC cultures. Image analysis demonstrated that all markers were expressed homogeneously across sections, even in cells right next to the laser sectioning lines. In addition, cells right next to the laser cutting lines did not show any significant increase in apoptosis, as measured by immunocytochemical analysis of activated caspase-3 and cleaved PARP four hours after laser-mediated sectioning (Figure 3(b)). Incubation of replicate cultures overnight (i.e., cultures were sectioned into 200 μm sizes and then given fresh medium) resulted in significant growth of cells into the areas previously sectioned using the laser. Morphological and immunocytochemical analysis of these cultures (using the same pluripotency and apoptosis markers above) indicated that cells regrown into the laser sectioning area were indeed undifferentiated human iPSCs. The laser was then used to section a wider area (~ 1000 μm) into cultures for analysis of growth over several days. Again, these cultures showed no change in morphology, apoptosis, and pluripotency marker expression, indicating that laser processing did not affect stem cell self-renewal or pluripotency (Supplemental Figure 1 of the supplementary material available online at doi:10.1155/2012/926463).

In addition, laser-mediated passage of human iPSCs and ESCs did not alter cell growth as the cells exhibited equivalent growth rates as compared with collagenase passaged cells after multiple rounds of expansion (Figure 3(c)).

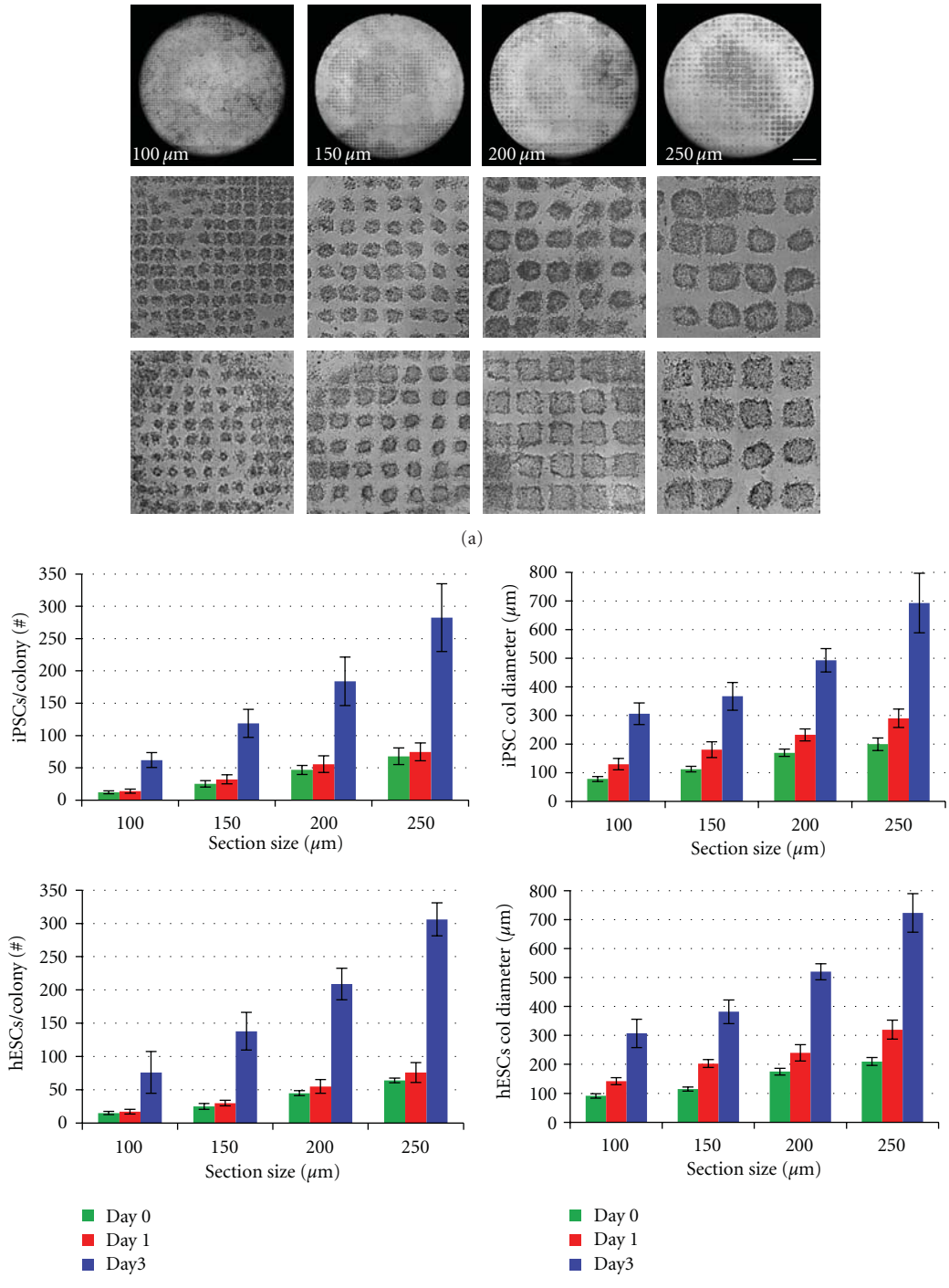


Figure 1: Stem cell colony size was controlled by section size using laser-mediated passage. (a) Brightfield images of human iPSC (top, middle) and ESC (bottom) cultures cut into 100–250 μm sections. Scale bar, 1 mm. (b) Colony size over time following propagation of 100–250 μm iPSC sections (top) or ESC sections (bottom) by laser-mediated passage. Number of cells per colony were manually counted using Hoechst stained cultures (left, $n = 15$ colonies per data point). Longest diameter of each colony was manually measured using brightfield images (right, $n = 15$ colonies per data point). Data are shown as mean $\pm$ s.d.

To further assess the potential laser effects on human iPSC and ESC stability and pluripotency, stem cell cultures were propagated using laser-mediated passage over long-term culture (two iPSC lines were maintained for >5 passages (5 weeks), one iPSC line was maintained for >10 passages (2.5 months), and BIMR 6 iPSCs and H9 ESCs were maintained for >24 passages (>6 months)) and compared with replicate cultures passaged using collagenase. Image

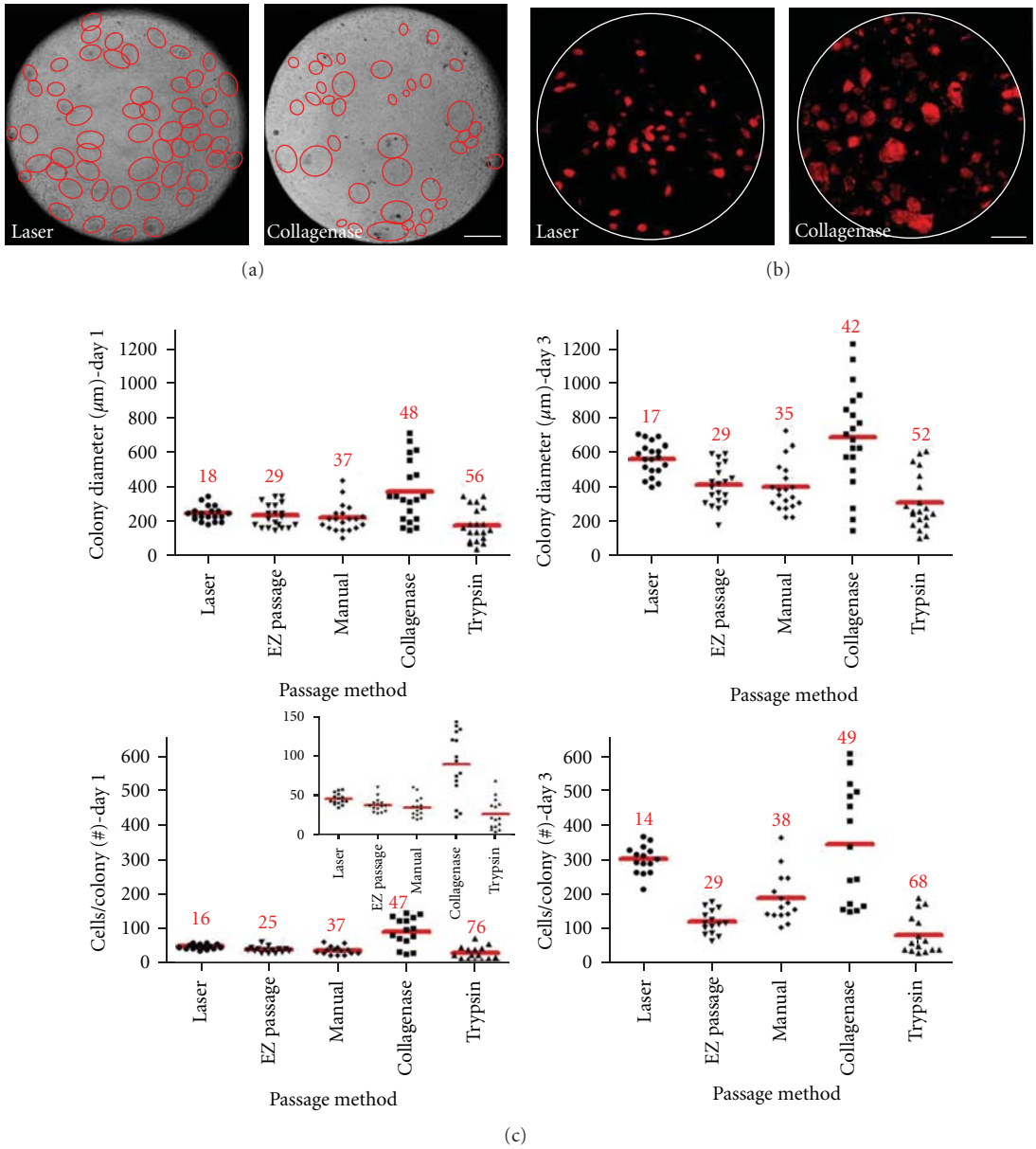
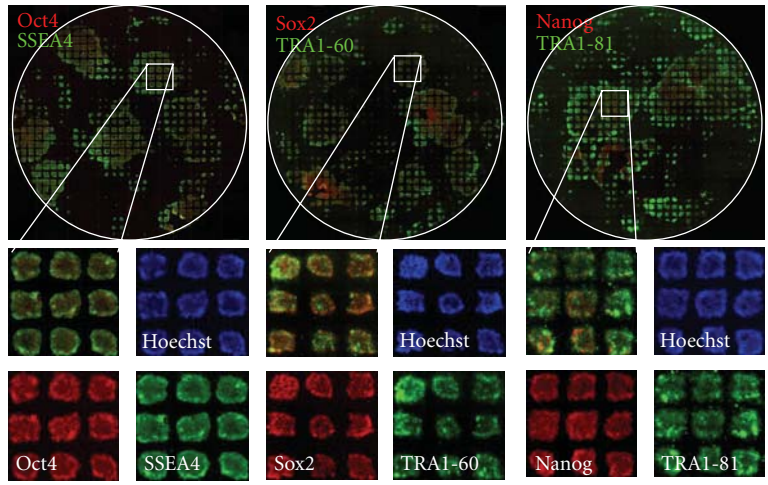


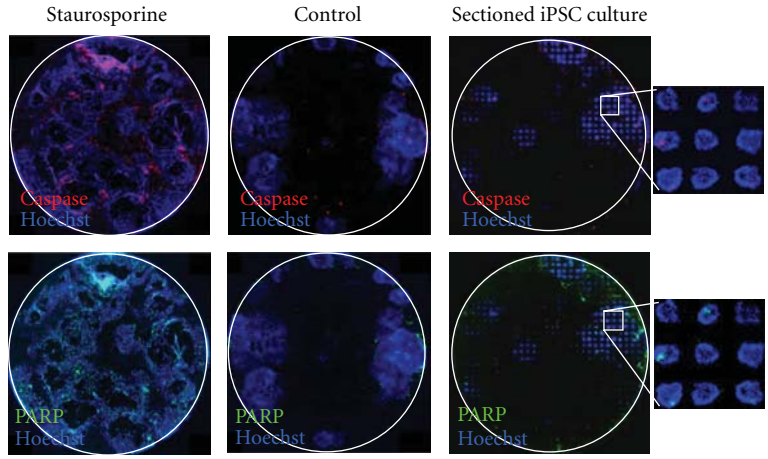
Figure 2: Improved uniformity of stem cell cultures by laser-mediated passage. (a) Brightfield images of iPSC cultures 2 days after laser-mediated passage (200 μm sections, left) or collagenase passage (right). Colonies are shown by red outline. Scale bar, 1 mm. (b) Alkaline phosphatase (AP) staining of iPSC colonies 1 day after laser-mediated passage (200 μm sections, left) or collagenase passage (right). Scale bar, 1 mm. (c) Colony size of iPSC cultures on days 1 and 3 after laser-mediated passage (200 μm sections), StemPro EZPassage Disposable Stem Cell Passaging Tool (EZ Passage), manual passage using a pipette tip, collagenase treatment, or trypsin dissociation of cells. Longest diameter of each colony was manually measured using brightfield images (top, $n = 20$ colonies per data point). Number of cells per colony was manually counted using Hoechst stained cultures (bottom, $n = 15$ colonies per data point). Data are shown as scatter plot with red line indicating mean. The CV is shown in red text above each sample. Asterisks (*) indicate variances that are statistically significant when compared to laser-mediated passage using ANOVA, with a $P \leq 0.05$ considered significant.

analyses of cultures propagated by laser-mediated passage showed no change in morphology with all cells exhibiting a high nuclear to cytoplasmic ratio typical of pluripotent stem cells (Figure 4(a)). Immunocytochemical analyses of these cultures demonstrated iPSCs and ESCs continued to express characteristic pluripotency markers including alkaline phosphatase (AP), Oct4, Sox2, Nanog, SSEA4, TRA1-60, and TRA1-81 (Figure 4(a)).

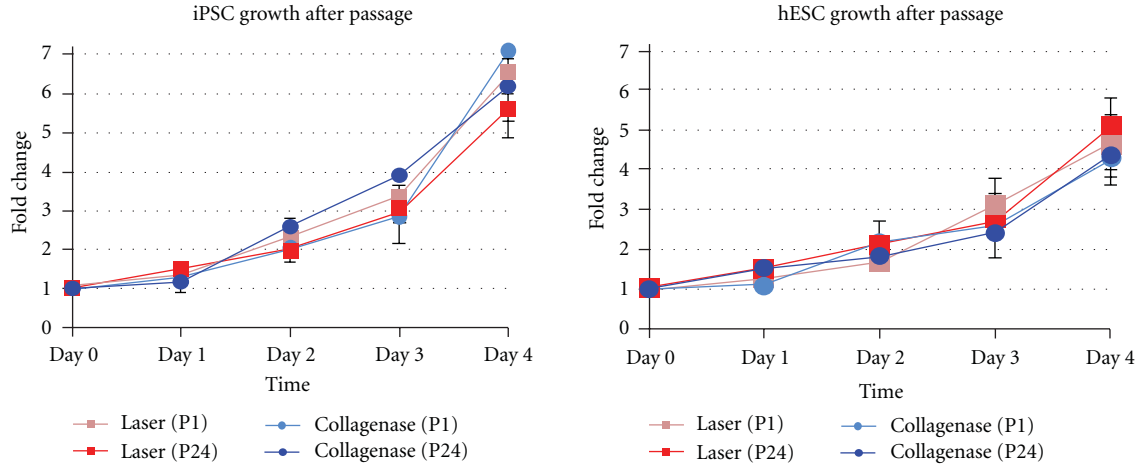
Visual comparison showed that human BIMR 6 iPSC cultures propagated using laser-mediated passage were of higher quality over time than those propagated using collagenase. Cultures propagated by laser-mediated passage contained many more undifferentiated, compact stem cell colonies with clear discernible colony borders compared to cultures propagated using collagenase. Collagenase passaged cultures had a greater tendency for spontaneous differentiation



(a)



(b)



(c)

FIGURE 3: Quality of iPSC cultures after laser-mediated passage. (a) Immunocytochemical analysis of Oct4, Sox2, Nanog, SSEA4, TRA1-60, and TRA1-81 expression immediately following laser-mediated sectioning of iPSC cultures (BIMR L) into 200 μm sections. Hoechst was used as a nuclear counterstain. Note that all markers are expressed homogeneously across iPSC clumps. Scale bar, 1 mm. (b) Immunocytochemical analysis of apoptosis markers, caspase-3, and cleaved PARP, following laser-mediated sectioning of iPSC cultures. Hoechst was used as a nuclear counterstain. Scale bar, 1 mm. (c) Analysis of iPSC (BIMR 6, left) and ESC (H9, right) growth following propagation using laser-mediated passage or collagenase passage. *P* indicates passage number. Data are shown as mean $\pm$ s.d. ($n = 3$).

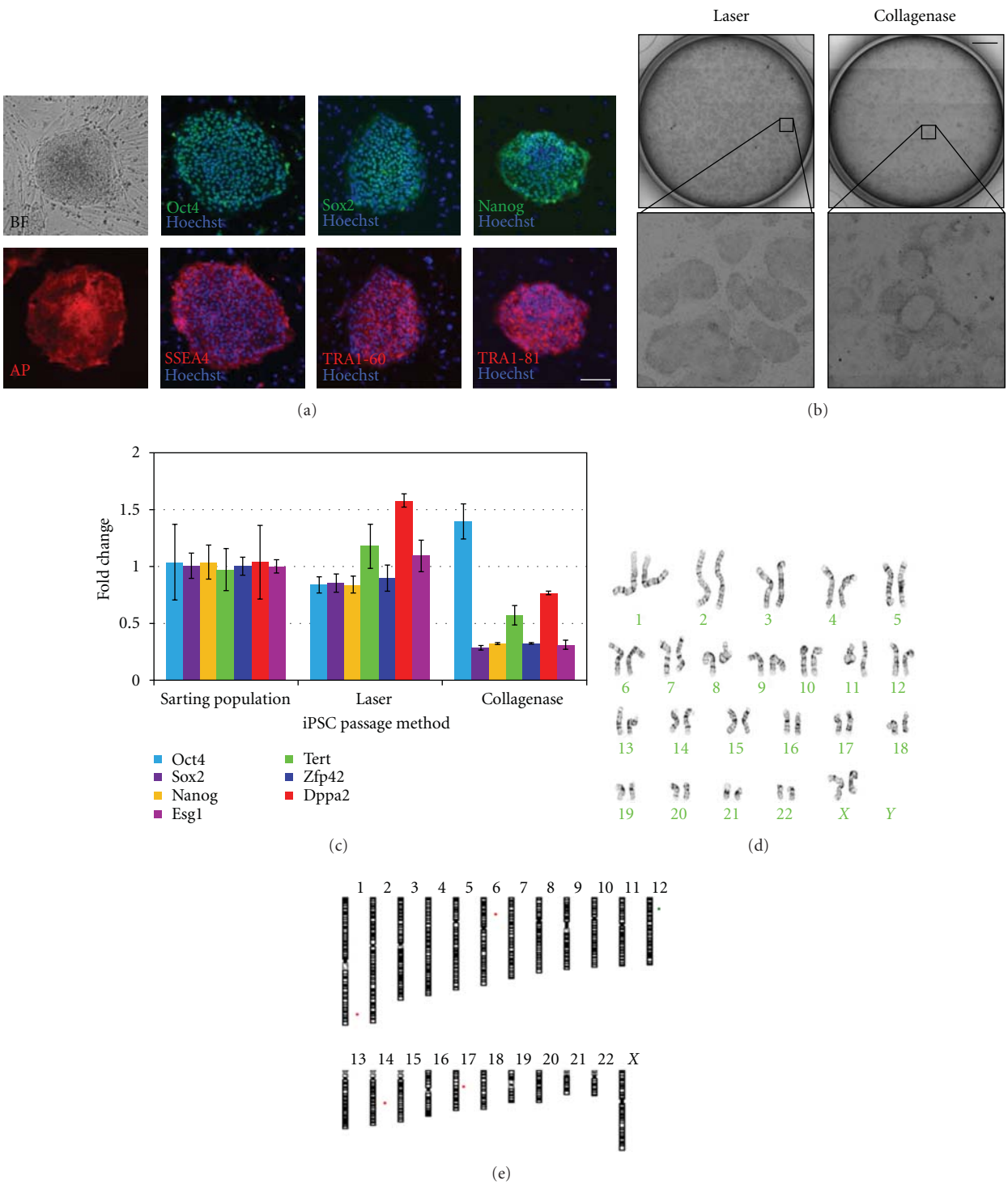


Figure 4: Pluripotency and stability of stem cells after laser-mediated passage. (a) Colony morphology (brightfield, BF) and immunocytochemical analysis of Oct4, Sox2, Nanog, alkaline phosphatase (AP), SSEA4, TRA1-60, and TRA1-81 expression in human ESCs (H9) after 24 consecutive laser-mediated passages. Hoechst was used as a nuclear counterstain. Scale bar, 250 μm. (b) Whole well brightfield images of human iPSC (BIMR 6) cultures after 10 consecutive laser-mediated passages or collagenase passages. Scale bar, 5 mm. (c) QRT-PCR analysis of stem-cell-associated gene expression in iPSCs (BIMR 6) after 10 consecutive laser-mediated passages or collagenase passages. The asterisks (*) indicate values that are statistically significant compared with the starting population of iPSCs. The data are presented as mean ± s.d. ($n = 3$). Statistical analysis was performed using t-test, with a P value ≤ 0.05 considered to be significant. (d) Normal karyotype of H9 human ESCs after 24 laser-mediated passages (6 months). (e) Schematic depicting genomic abnormalities of H9 ESCs at P35 (starting population) and at P59, after 24 laser-mediated passages (6 months), as determined by aCGH. No new subkaryotypic abnormalities were detected after 24 passages. Red bars indicate a deletion. Green bars indicate an amplification. See Table 1 for complete list of aberrations found in these cells.

(Figure 4(b)). To quantify these phenotypic observations, QRT-PCR analyses were performed using known markers for undifferentiated stem cells. As shown in Figure 4(c), iPSCs propagated using laser-mediated passage continued to express high levels of stem cell-associated genes, including *Oct4* (*Pou5f1*), *Sox2*, *Nanog*, *Tert*, *Zfp42* (*Rex1*), *Dppa2*, and *Esg1* (*Dppa5*), similar to the starting population of iPSCs (no statistical difference, P value ≥ 0.1). In contrast, iPSCs propagated using collagenase resulted in a significant decrease in expression of *Sox2*, *Nanog*, *Tert*, *Zfp42*, and *Esg1* when compared to the starting population of iPSCs (P value < 0.05, Figure 4(c)). Comparison of human ESC cultures passaged by both methods did not show any significant morphological changes or differences in gene expression (Supplemental Figure 2), although human ESC cultures were more established, later passage cultures than human iPSC cultures used in these experiments.

Data from multiple groups have shown that H9 human ESCs maintain a stable karyotype over long-term culture [1, 17, 22]. These cells were therefore used to examine the genetic stability of stem cells after long-term propagation using laser-mediated passage. Karyotype analysis of H9 ESCs after 24 consecutive laser-mediated passages showed no change in karyotype with all cells having a normal diploid karyotype (6 months, Figure 4(d)). To detect subkaryotypic alterations, array comparative genomic hybridization (aCGH) was also performed using the Stemarray (Figure 4(e), Table 1). No subkaryotypic alterations were detected in human ESCs propagated for 24 consecutive passages (P59) by laser-based passaging relative to the starting population (H9, P35), suggesting that the genome of laser-mediated passaged cells is both normal and stable. It is important to note that both subkaryotypic and karyotypic alterations were observed in H9 ESCs after consecutive passaging by collagenase for 4 and 6 months, respectively (data not shown). In addition, no subkaryotypic changes were detected in human iPSCs (BIMR 6) propagated for 10 consecutive passages by laser-mediated passage relative to the starting population (data not shown). Although genetic stability of iPSCs was only analyzed after 2.5 months, taken together with 6-month human ESC results, these data suggest that the genome of laser-mediated passaged stem cells is and stable.

3.4. Improved Differentiation Potential of EBs Generated after Laser-Mediated Passage. To test the differentiation potential of these cells, in vitro differentiation assays of human iPSCs were performed after propagation using laser-mediated passage (160 μm sections) or enzymatic passage. iPSCs passaged by either methodology efficiently formed well-defined embryoid bodies in suspension culture which could differentiate into derivatives of all three primary germ layers including endodermal cells (Sox17, Afp), mesodermal cells/cardiac muscle cells (brachyury, α-MHC), and ectodermal cells/neurons (Nestin, Map2, Supplemental Figure 3). Morphological analysis of the resulting EB populations showed that EBs generated from laser-passaged iPSCs were more uniform in size than those generated from enzymatically passaged iPSCs (Figure 5(a)). To quantify these observations, the diameter of resulting EB populations was measured manually using images acquired on day 4 of suspension culture. As shown in Figure 5(b), laser-mediated passage resulted in significantly more uniform EBs ($374 \pm 56\,\mu$m; 15% CV) than enzymatic passage by either collagenase ($336 \pm 145\,\mu$m; 43% CV) or trypsin ($158 \pm 85\,\mu$m; 54% CV). Statistical analysis of variance showed that EBs generated using stem cell cultures propagated by laser-mediated passage were significantly more uniform (P value < 0.0001) than EBs generated using enzymatically passaged cultures, demonstrating that laser-mediated passage results in more consistent EB cultures than other methods.

Several studies have shown that heterogeneity in human ESC colony size and resulting EB aggregate size results in variability in differentiation experiments and significant decreases in differentiation yields [23–26]. The effect of EB homogeneity on differentiation potential of human iPSCs into cardiomyocytes was examined. EBs were generated using iPSC colonies formed 5 days after laser-mediated passage (160 μm sections) or enzymatic passage. All EBs were differentiated using a standard multistage protocol, growing EBs in suspension culture for 4 days followed by adherent cell culture for an additional 18 days [27]. Cardiomyocyte differentiation potential was analyzed on day 22 of differentiation by manual counting of contracting EBs. EBs produced using enzymatically passaged iPSCs yielded a small proportion of beating EBs (~7%), whereas laser-mediated passaged iPSCs resulted in a significantly higher proportion (~60%) of contracting EBs (Figure 5(c)). QRT-PCR analyses confirmed these results with EBs generated from laser-mediated passaged cultures showing 3- to 51-fold higher expression of cardiomyocyte genes, *Nkx2.5*, *Actn1* (*α-actinin*), *Mef2C*, *Myh6* (*α-Mhc*), *TnnI3*, and *NppA* (*Anp*), than EBs generated from collagenase passaged cultures (Figure 5(d)). Similarly, immunocytochemical analyses demonstrated that EBs produced from laser-mediated passaged iPSCs contained substantially more cardiac cells within each EB (i.e., EBs contained more cells staining positive for known cardiomyocyte markers) than EBs produced from collagenase passaged cells (α-MHC and α-actinin shown in Figure 5(e)). EBs generated from all populations stained positive for all markers tested including α-MHC, α-actinin, cardiac troponin, and NPPA (data not shown).

To further analyze the effect of EB homogeneity on differentiation potential, EBs generated from iPSC colonies formed 5 days after laser-mediated passage (160 μm sections) or enzymatic passage were differentiated into neural rosettes using a modified multistep protocol. Ability to differentiate into neural rosettes was analyzed on day 11 of differentiation by manual counting of EBs containing ≥ 1 neural rosette. EBs produced using laser-mediated passaged iPSCs resulted in 95% of EBs containing neural rosettes, while EBs generated by trypsin or collagenase passaged iPSCs yielded only 29% and 32% of EBs containing neural rosettes, respectively (data not shown). These data indicate increased homogeneity in human iPSC colonies and resultant EBs result in significant increases in differentiation yield of iPSCs.

To investigate the effect of EB size on differentiation potential of human iPSCs were examined. iPSCs were

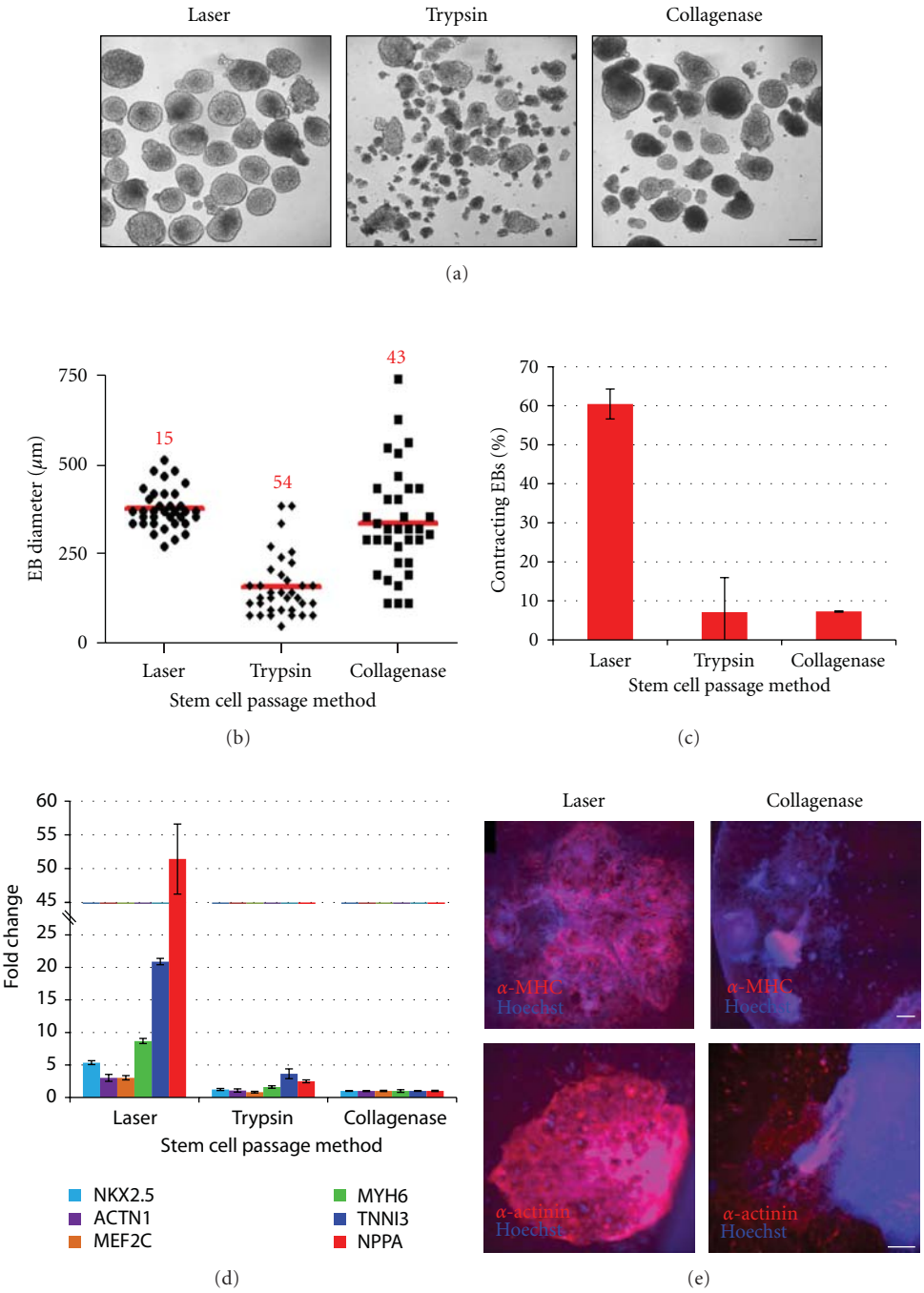


Figure 5: iPSCs propagated by laser-mediated passage differentiated more efficiently into cardiomyocytes. (a) Brightfield image of day 4 EBs generated from iPSC cultures (BIMR A) propagated by laser-mediated passage, trypsin dissociation, or collagenase treatment. Scale bar, 250 μm. (b) Size of EBs generated from iPSC cultures propagated by laser-mediated passage, trypsin dissociation, or collagenase treatment ($n = 35$ EBs per data point). Data are shown as scatter plot with red line indicating mean and CV shown in red text above each sample. The asterisks (*) indicate variances that are statistically significant when compared to laser using ANOVA, with $P \leq 0.05$ considered significant. (c) Percentage of EBs containing contracting areas. Data are shown as mean $\pm$ s.d. ($n = 2$ independent experiments containing 75 EBs/sample in each experiment). (d) QRT-PCR analysis of cardiomyocyte-associated gene expression in EBs generated using iPSC cultures propagated by laser-mediated passage, trypsin dissociation, or collagenase treatment. The asterisks (*) indicate values that are statistically significant as compared with EBs generated from collagenase passaged iPSC cultures. The data are presented as mean $\pm$ s.d. ($n = 3$). Statistical analysis was performed using t-test with $P \leq 0.05$ considered significant. (e) Expression of cardiomyocyte markers, α-MHC and α-actinin, in EBs generated from iPSC cultures propagated by laser-mediated passage or collagenase treatment on day 22 of cardiac differentiation. Hoechst was used as a nuclear counterstain. Scale bars, 250 μm.

Table 1: Regions in H9 human ESCs with genomic abberations as determined by aCGH (corresponding schematic is shown in Figure 4(e)). No subkaryotypic alterations were detected in hESCs propagated for 24 consecutive passages (H9 P59) relative to the starting hESC population (H9 P35), suggesting that the genome of laser-mediated passaged cells is both normal and stable. Data is reported using genome built HG18. $\log_2$ ratios ≥ 0.6 are amplifications (amp) or ≤ -1.0 are deletions (del) found in all cells. $\log_2$ ratios < 0.6 or > -1.0 represent mosaicism within the culture.

Chromosome: region	Cytoband	Size (Mb)	# Probes	Amp/Del	H9 P35 $\log_2$ ratio	H9 P59 $\log_2$ ratio	Annotations
Chr1: 224141493-224195678	q42.12	0.054	14	Amp	0.528	0.632	LEFTY1, PYCR2, LEFTY2
Chr6: 31663619-31691605	p21.33	0.028	3	Amp	0.686	1.220	LST1, NCR3, AIF1
Chr12: 21580165-22105263	q12.1	0.282	12	Del	−0.495	−0.480	GYS2, LDHB, KCNJ8, ABCC9, CMAS
Chr14: 62486603-62852257	q23.2	0.366	7	Amp	0.560	0.432	KCNH5, RHOJ, GPHB5
Chr17: 35097815-35153082	q12	0.055	15	Amp	0.469	0.485	PERLD1, ERBB2, C17orf37, GRB7

propagated by laser-mediated passage at varying section sizes (80, 160, and 240 μm sections). Five days after passage, EBs were generated and differentiated into cardiomyocytes as described above. Homogeneous EB populations ($\leq$15% CVs) of varying sizes, 278, 418, and 528 μm in diameter, were produced from 80, 160, and 240 μm section sizes, respectively (Supplemental Figure 4). Analysis of cardiomyocyte differentiation potential showed that 55% of EBs generated from 160 μm sections were contracting, while only 38% and 21% of EBs generated from 240 μm and 80 μm sections were contracting. Taken together, these data indicate that increased homogeneity in human iPSC colonies and resultant EBs, as well as EB size, significantly increase the differentiation yield of iPSCs. The ability to reproducibly generate uniform, size-specific colonies which subsequently result in more uniform, size-specific EB populations decreases variability in differentiation experiments and enhances differentiation yields of both ESC and iPSCs into specialized cell types.

4. Discussion

The lack of standardization in passage techniques for stem cell derivation and propagation is a major limitation within the stem cell field. Because universal protocols for human stem cell cultures have not been adopted, it is currently difficult to compare and interpret scientific data from cells cultured in different conditions. Passage method differences have significantly confounded the understanding of intra- and interline differences in gene expression data, expression of stem cell- and lineage-associated markers, miRNA signatures, and epigenetic profiles [28–31]. Although human ESC lines have distinct genotypes, it is unlikely that reported differences in cell lines (e.g., up to 65% variation in gene expression data across two ESC lines) can be attributed to genetic variation alone, as <2% variation in gene expression has been found in adult human tissues of different individuals [28, 32, 33]. Likewise, discrepancies associated with differentiation protocols and reported differentiation capabilities and efficiencies of stem cells into specialized cell types may be due to the lack of standardization [34]. Adoption of standardized protocols should greatly improve determination of the role of inherent genetic variation, environmental niche, and/or procedural effects on stem cell quality, self-renewal, pluripotency, and differentiation potential.

Laser-mediated passage provides a novel method for expansion of human ESCs/iPSCs which can be used to create standardized, traceable procedures for the production of GMP-quality stem cell lines without requirement for enzymes. This method combines the benefits of both manual and enzymatic passage techniques, allowing efficient, automated passaging of undifferentiated stem cell cultures into uniform-sized stem cell sections within a sterile closed environment. Laser-mediated passage is compatible with a variety of culture methods including animal-free, feeder-free-based conditions, and serum-free defined media conditions. Notably, this approach is not susceptible to inter-individual variation reducing the need for skilled technicians to create high-quality stem cell cultures.

Laser-mediated passage does not involve the use of enzymes and therefore should better maintain the genetic stability of human ESCs and iPSCs in long-term culture (3–12 months, [10–14]). The results show that H9 ESCs maintained a stable karyotype over six months (>24 passages). More importantly, laser-mediated passage did not induce subkaryotypic alterations over time in H9 ESCs (6 months, and iPSCs (2.5 months)) as monitored by aCGH. The more sensitive aCGH data suggests that laser-mediated passage maintains genetic integrity of human ESCs/iPSCs. Importantly, genetic abnormalities were detected in H9 ESCs after consecutive passaging by collagenase during the same time period. Results also showed that human iPSCs and ESCs propagated using laser-mediated passage maintained a normal stem cell morphology and continued to express high levels of stem-cell-associated genes and proteins. Although teratoma analyses were not performed on these cells, in vitro differentiation analyses of laser-mediated passaged iPSCs demonstrated the cells could spontaneously differentiate into derivatives of all three primary germ layers and could differentiate into cardiomyocytes and neural rosettes. In addition, iPSCs propagated by laser-mediated passage have been differentiated into motor neurons, RPE cells, endoderm progenitors, and hepatocytes-like cells (data not shown); taken together these data indicate that laser-mediated passage does not affect stem cell pluripotency. Likewise, laser-mediated passage did not alter the growth rate of stem cells or increase expression of apoptotic markers, all supporting that the laser sectioning did not affect stem cell quality, self-renewal, or pluripotency.

Laser-mediated passage provides control of stem cell colony size. Regular passage schedules can be established by selection of section size. A section size of ~200 μm has enabled routine splitting of all ESC/iPSC lines every 7 days, allowing for more efficient planning of experiments. An overall passage efficiency of 85% combined with more uniform section sizes (20% CV), enables a larger proportion of ESC/iPSC colonies to contribute to culture expansion reducing the number of plates required for culture maintenance. Compatibility with conventional robotic systems enables scalability of culture needs. Additionally, the ability to control input section size, particularly smaller sizes, allows more effective creation of stem cell colonies in multi-well plates for large-scale experimentation and screening purposes. It is also likely that stem cell section size will affect cryopreservation and genetic modification efficiency of ESCs and iPSCs [35–38].

Laser-mediated passage involves sectioning the entire well systematically without respect for the boundaries of the colonies. Well-established undifferentiated stem cell cultures are easily propagated using this technique. For newly derived ESC/iPSC cultures, early passage ESC/iPSC lines, or less stable lines, which tend to have more spontaneous differentiation, a combination of manual selection or laser purification of colonies followed by laser-mediated passage would be recommended. One of the more important results of this approach showed that over time, stem cell cultures (in particular, early passage iPSCs which tend to be more susceptible to differentiation than later passage, more established ESCs) are of higher quality than those maintained by collagenase treatment. It is likely that passage of homogeneous sections is important for maintaining undifferentiated stem cells and limiting the differentiation of colonies. Therefore, potentially early passage ESCs/iPSCs will require less colony isolation before expansion using laser-mediated passage.

One of the more important outcomes of this study showed that uniform human iPSC colonies produced after laser-mediated passage resulted in a more homogeneous population of EBs, with respect to size and shape, with greater differentiation efficiency as compared with typical EB cultures derived from enzyme passaged cultures. EBs generated from laser-mediated passaged iPSCs resulted in a significant increase in cardiomyocyte yield, with up to 8.5-fold greater beating incidence than EBs generated from collagenase passaged iPSCs. The ability to reproducibly generate uniform colonies using laser-mediated passage resulting in EBs that are more uniform in size and shape will decrease variability in differentiation experiments and enhance differentiation yields of both ESC and iPSCs into specialized cell types. These yield enhancements could significantly reduce the cost of stem cell experimentation both in terms of labor and materials. Potentially, uniform colony formation will also augment differentiation yields of stem cells when performing direct differentiation procedures (i.e., without an EB intermediate).

5. Conclusions

In conclusion, proper maintenance of human stem cells is essential for successful utilization of ESCs and iPSCs as tools in developmental and drug discovery studies and in regenerative medicine. Standardization is critical for all future applications of stem cells and necessary in order to fully understand the potential of these cells and the differences observed among varying stem cell lines and between ESCs and iPSCs. Laser-mediated passage is an innovative method for maintenance and expansion of stem cell lines, without introducing genetic instability, which is generically applicable to all cell lines and to all technicians regardless of skill. This approach provides an efficient, standardized protocol for the propagation of human ESCs and iPSCs, which should significantly reduce the inconsistency and variability within the stem cell field. Laser-mediated passage allows for traceability and ensures reproducible production of stem cell lines according to standard operating procedures, all of which are necessary to manufacture stem cells for use in clinical/therapeutic applications.

Author's Contribution

C. Peterson and A. Soundararajan contributed equally to the paper.

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Research Article

Cellular Reprogramming Employing Recombinant Sox2 Protein

Marc Thier, Bernhard Münst, Stephanie Mielke, and Frank Edenhofer

Stem Cell Engineering Group, Institute of Reconstructive Neurobiology, University of Bonn-Life & Brain Center and Hertie Foundation, Sigmund-Freud Straße 25, D-53105 Bonn, Germany

Correspondence should be addressed to Frank Edenhofer, f.edenhofer@uni-bonn.de

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Induced pluripotent stem (iPS) cells represent an attractive option for the derivation of patient-specific pluripotent cells for cell replacement therapies as well as disease modeling. To become clinically meaningful, safe iPS cells need to be generated exhibiting no permanent genetic modifications that are caused by viral integrations of the reprogramming transgenes. Recently, various experimental strategies have been applied to accomplish transgene-free derivation of iPS cells, including the use of nonintegrating viruses, episomal expression, or excision of transgenes after reprogramming by site-specific recombinases or transposases. A straightforward approach to induce reprogramming factors is the direct delivery of either synthetic mRNA or biologically active proteins. We previously reported the generation of cell-permeant versions of Oct4 (Oct4-TAT) and Sox2 (Sox2-TAT) proteins and showed that Oct4-TAT is reprogramming-competent, that is, it can substitute for Oct4-encoding virus. Here, we explore conditions for enhanced Sox2-TAT protein stabilization and functional delivery into somatic cells. We show that cell-permeant Sox2 protein can be stabilized by lipid-rich albumin supplements in serum replacement or low-serum-supplemented media. Employing optimized conditions for protein delivery, we demonstrate that Sox2-TAT protein is able to substitute for viral Sox2. Sox2-piPS cells express pluripotency-associated markers and differentiate into all three germ layers.

1. Introduction

Pluripotent cells represent a most attractive source for both cell repair in regenerative medicine and disease modeling in basic biomedical research since they are able to differentiate into every cell type of an adult organism. Until recently, early embryonic stages of development represented the main source of pluripotent cells, and thus, those cells were designated as embryonic stem (ES) cells. Nowadays, the artificial derivation of pluripotent stem cells from somatic cells becomes increasingly important. Induced pluripotent stem (iPS) cells were first generated by retrovirally induced ectopic expression of four transcription factors Oct4, Sox2, Klf-4, and c-Myc in somatic cells [1]. Human iPS cells represent an attractive option for the derivation of pluripotent patient-specific cells as no embryos are required for their generation. However, crucial safety issues have to be addressed in order to generate human iPS cells that are clinically useful. Soon after identification of the viral reprogramming protocol in mouse cells [1] and its adaptation to human cells [2, 3], unwanted side effects such as tumorigenesis [4] became apparent.

Since the cause of tumor formation was ascribed to random integration of the retroviral vectors and sustained expression of transgenes after reprogramming, optimized protocols were explored to circumvent the permanent integration of foreign DNA into the genome. One strategy involves the excision of reprogramming transgenes employing DNA recombinases [5, 6] or transposases [7–10]. After iPS derivation, transgenes can be deleted by a second round of recombinase/transposase activation. However, further laborious and cumbersome genetic methods are needed to identify and confirm transgene-free iPS clones. An alternative strategy is to utilize less-invasive genetic vectors that do not integrate into the host genome. Repeated plasmid transfection has been used for iPS induction albeit with a very low efficiency [11]. Minicircle vectors lacking bacterial DNA and thus enabling high transfection efficiency and long ectopic expression were reported to reprogram as well [12]. Moreover, transduction employing viruses that do not integrate their genome into host cells such as adenovirus [13] or Sendai virus [14] were applied. Small molecules that are able to translocate into cells and interfere

with key signaling pathways have been identified to either enhance the process of reprogramming [15, 16] or replace [15, 17] single viral factors (for review, see [18]). The repeated transfection of synthetic mRNA [19–21] or the direct delivery of reprogramming proteins [22, 23] represents a straightforward but technically challenging approach to achieve nongenetic iPS derivation.

Protein transduction technology has been used to directly deliver numerous biologically active proteins into mammalian cells by modifying them with so-called cell-penetrating peptides (CPPs) or protein transduction domains (PTDs). These relatively small peptides confer cell permeability when linked to cargo molecules (for review, see [24–26]). A highly basic CPP derived from the *human immunodeficiency virus type 1* (HIV-1) Tat (transactivator of transcription) protein is often applied for cellular delivery (TAT) [25–28]. PTDs have been used to generate cell-penetrating versions of various transcription factors that play major roles in cell differentiation including HoxB4 [27], Pdx1 [28], Scl [29], Nkx2.2 [30], and Notch-ICD [31]. We previously reported the derivation of cell-permeant versions of reprogramming factors Oct4 and Sox2 [22]. Oct4-TAT and Sox2-TAT were shown to specifically bind to DNA such as the Oct4/Sox2 combined element in the Nanog promoter, and both proteins compensate the RNAi-induced loss of function after direct delivery into ES cells [22]. Moreover, employing Sox2-TAT, it has been demonstrated that Sox2 has an essential function in the preimplantation mouse embryo by facilitating establishment of the trophectoderm lineage [32]. Zhou et al. used fusion protein derivatives of reprogramming factors from *E. coli* for the derivation of mouse ES-like cells, albeit with very low efficiency [23]. Kim et al. reported the use of cell extracts from transfected HEK293 cells for the reprogramming of human newborn fibroblasts [33]. The recently reported use of ES cell extracts to induce pluripotency in murine fibroblasts [34] needs to be explored whether it can be adapted to human cells. In conclusion, a robust, standardized, and efficient protocol for the generation of protein-induced iPS cells from human adult cells still needs to be developed.

Further optimization of protein transduction for cellular reprogramming greatly depends on overcoming a major bottleneck associated with protein transduction: stability of recombinant factors under cell culture conditions. We recently established optimized stabilization conditions for Oct4-TAT and demonstrated the efficient substitution for Oct4-encoding virus by recombinant Oct4-TAT [36]. Here, we explore conditions for enhanced Sox2-TAT protein stabilization and delivery into somatic cells. We show that cell-permeant Sox2 protein can be stabilized by lipid-rich albumin supplements in serum replacement or low-serum-supplemented media. Employing these conditions for protein delivery, we demonstrate that Sox2-TAT protein is able to substitute for viral Sox2.

2. Materials and Methods

2.1. Protein Expression and Purification. The pSESAME-Sox2NTH expression plasmid [22] was transformed into *E. coli* BL21 (DE3) gold strain (Stratagene, La Jolla, USA) by heat shock at 30°C and incubated for 1 h in SOC medium at 30°C. Transformed bacteria were inoculated overnight at 30°C with shaking at 140 rpm in LB medium containing 50 mg/mL carbenicillin. For protein expression, the overnight culture was pelletized and resuspended in fresh TB medium (terrific broth)/50 mg/mL ampicillin, 0.5% glucose and incubated at 37°C with shaking at 110 rpm until an OD600 of 1.5 was achieved. Protein expression was induced by IPTG at a final concentration of 0.5 mM. Cells were harvested by centrifugation, and cell pellets were stored at −20°C.

For purification of His-tagged proteins, cell pellets were thawed and resuspended in 20 mL lysis buffer (50 mM Na_2HPO_4, 5 mM Tris, pH 7.8, 500 mM NaCl, and 10 mM imidazole) per 1 L of expression culture. Cells were lysed by application of 1 mg/mL lysozyme (Sigma, Deisenhofen, Germany), 10–15 U/mL Benzonase (Novagen, Darmstadt, Germany), and sonication. After centrifugation (17 200 g, 20 min), the cleared lysate was incubated with Ni-NTA agarose beads (Qiagen, Hilden, Germany) (1 mL of slurry for 1 L of bacterial expression culture) for 1 h with rotation at 4°C. The slurry was packed into a column and washed with 8 column volumes of wash buffer (50 mM Na_2HPO_4, 5 mM Tris, pH 7.8, 500 mM NaCl, and 80 mM imidazole). The Sox2-TAT protein was eluted with 3 column volumes of elution buffer (50 mM Na_2HPO_4, 5 mM Tris, pH 7.8, 500 mM NaCl, and 250 mM imidazole).

2.2. Preparation of Transduction Media. Sox2-TAT eluate fraction was supplemented with 7.5% serum replacement and dialyzed against DMEM F12 over night at 4°C. The next day cell culture supplements were added to the dialyzed fraction to a final concentration of 2% FCS, 2.5% Albumax II (200 mg/mL), 7.5% serum replacement, 1% ITS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.5 mM β-mercaptoethanol, and 1000 U/mL LIF. The mixture was preconditioned in a water bath for 1 h at 37°C and cleared by centrifugation (5 min at 2500 g) and sterile filtration.

2.3. Cell Culture. Oct4-GiP MEFs [38] were cultured in high-glucose DMEM (Invitrogen) with 10% FCS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 2 mM L-glutamine. MEFs were trypsinized at 70–80% confluence and reseeded on tissues culture dishes coated with gelatin. For reprogramming assays, MEFs were used to a maximum of passage 4.

mESCs were cultured in DMEM F12 (Invitrogen) supplemented with 2% FCS, 2.5% Albumax II (200 mg/mL), 7.5% serum replacement, 1% ITS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.5 mM β-mercaptoethanol, and 1000 U/mL LIF. Cells were split every 3 days and cultured on inactivated feeder cells.

2.4. Retroviral Infection and iPS Induction. Plasmids of pMXs-Oct3/4, pMXs-Sox2 (positive control), pMXs-c-Myc and pMXs-Klf4 were obtained from ADDGENE. The retroviruses were generated by the plat E packaging cell line as

previously described [39]. Target cells were seeded at 10×10^4 cells per well in six-well plates. 24 hours after transfection, the supernatant comprising the viruses was collected and filtered through a 0.45 μm cellulose acetate filter. For substitution experiments and for negative controls, Oct4, Klf4, and c-Myc were mixed in equal shares and supplemented with polybrene (Millipore) to a final concentration of 4 μg/mL. Positive controls additionally contained pMXs-Sox2 virus and were treated alike. Oct4-GiP MEF cells were incubated with viruses for 16 hours. Protein transduction experiments began after the virus-containing supernatant had been removed. After 5 days, cells were split onto irradiated feeder cells. 11 days later, cells were fixed with 4% PFA and analyzed by fluorescence microscopy. For the purpose of generating stable iPS cell lines, reprogramming assays were cultured for 21 days under designated conditions. Subsequently, colonies were picked and expanded monoclonally.

2.5. In Vitro Differentiation. Cells were harvested by trypsinization and transferred to bacterial culture dishes. Next, cells were grown in ES medium lacking LIF for 3 days. The derived embryoid bodies were transferred to gelatine-coated tissue dishes afterwards and incubated for another 3 days. In order to detect key marker expression specific for all three germ layers, immunostainings with antibodies against β-3-tubulin (TUJ1), smooth muscle actin (SMA), and α-fetoprotein (AFP) were conducted.

2.6. RT-PCR. Total RNA was extracted using the RNAeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Subsequently, reverse transcription of 1 μg RNA per sample was performed using the iScript cDNA Synthesis Kit (Bio-Rad). In order to detect viral transgene expression, the following primer pairs were used:

Oct4TGforw: CCCCACTTCACCACACTCTAC,

Oct4TGrev: TTTATCGTCGACCACTGTGC,

Klf4TGforw: AGGCACTACCGCAAACACAC,

Klf4TGrev: TTTATCGTCGACCACTGTGC,

Sox2TGforw: GCCCAGTAGACTGCACATGG,

Sox2TGrev: CCCCCTTTTTCTGGAGACTA,

c-MycTGforw: CAGAGGAGGAACGAGCTGAAGCGC,

c-MycTGrev: TTTGTACAAGAAAGCTGGGT.

For the detection of endogenous Oct4, Sox2, and Nanog, the following primer pairs were used:

Oct4forw: TCTTTCCACCAGGCCCCCGGCTC,

Oct4rev: TGCGGGCGGACATGGGGAGATCC,

Sox2for: TAGAGCTAGACTCCGGGCGATGA,

Sox2rev: TTGCCTTAAACAAGACCACGAAA,

Nanogfor: CAGGTGTTTGAGGGTAGCTC,

Nanogrev: CGGTTCATCATGGTACAGTC.

PCR-program: 95°C 2 min, 95°C 30 sec, 60°C 30 sec, 72°C 1 min, 72°C 10 min. Steps 2–4 are repeated 35 times.

3. Results

3.1. Purification of Reprogramming-Competent Sox2 Fusion Protein from Bacteria. We have previously shown that recombinant Sox2 can be purified from *E. coli* as a TAT-modified cell-permeant version, designated Sox2-TAT [22]. In particular, this fusion protein comprises an additional exogenous nuclear localization sequence (NLS), a cell-penetrating peptide TAT, and a carboxy-terminal Histidine-tag for single-step purification (Figure 1(a)). Sox2-TAT was shown to specifically bind to DNA and to compensate for the RNAi-induced loss of activity in ES cells [22] and preimplantation embryos [32]; however, its capability to reprogram somatic cells has not been assessed. In order to study the reprogramming activity of Sox2-TAT, we decided to combine the purified recombinant Sox2-TAT together with retroviruses encoding for Oct4, Klf4, and c-Myc to convert mouse embryonic fibroblasts (MEFs) into iPS cells (Figure 1(b)). Sox2-TAT-transformed bacteria were lysed and subjected to Ni-affinity chromatography. Immunoblotting of purification fractions employing a His-specific antibody revealed that Sox2-TAT is highly expressed in bacteria although the majority of the recombinant protein remains in the insoluble fraction (Figure 1(d)). However, the estimated 20% of protein solubilized and detectable in the supernatant turned out to be sufficient for further purification. The elution from the Ni-affinity chromatography column yielded a Sox2-TAT-containing fraction of about 70% purity (Figure 1(c)).

3.2. Defining Optimal Conditions to Stabilize Sox2-TAT Protein. Poor solubility and limited stability of recombinant proteins under cell culture conditions represent a significant hurdle to the application of protein transduction technology. On the one hand, serum components stabilize recombinant proteins in cell culture media, but on the other hand, they are known to inhibit interaction of transducible proteins with cells and by this decrease the cellular uptake. In some experimental settings, this can be overcome by applying the transducible protein in serum-free media. We assessed the stability of Sox2-TAT employing various cell culture conditions. In serum-free media, Sox2-TAT precipitates almost completely within 1 hour (Figure 2(a)). Serum components have been shown to execute a positive effect on the stability of recombinant proteins [36, 40]. Therefore, we aimed at stabilizing the protein by supplements like FCS, serum replacement [35], and lipid-rich albumin fractions (Albumax). Supplementation with either 5% FCS or 2.5% Albumax showed a strong stabilizing effect on Sox2-TAT in culture media, while Sox2-TAT exhibited major decrease in the presence of 7.5% SR. A combination of low FCS (2%) together with 7.5% SR resulted in a stabilization that was comparable to high FCS supplementation (5%) (Figure 2(a)).

Next, we set out to analyze to which extent the stabilizing supplements interfere with the protein transduction process. To that aim, we used a well-established transduction read-out system based on a cell-permeant version of the DNA recombinase Cre [40]. A major feature of this protein transduction system is that the efficiency of intracellular protein

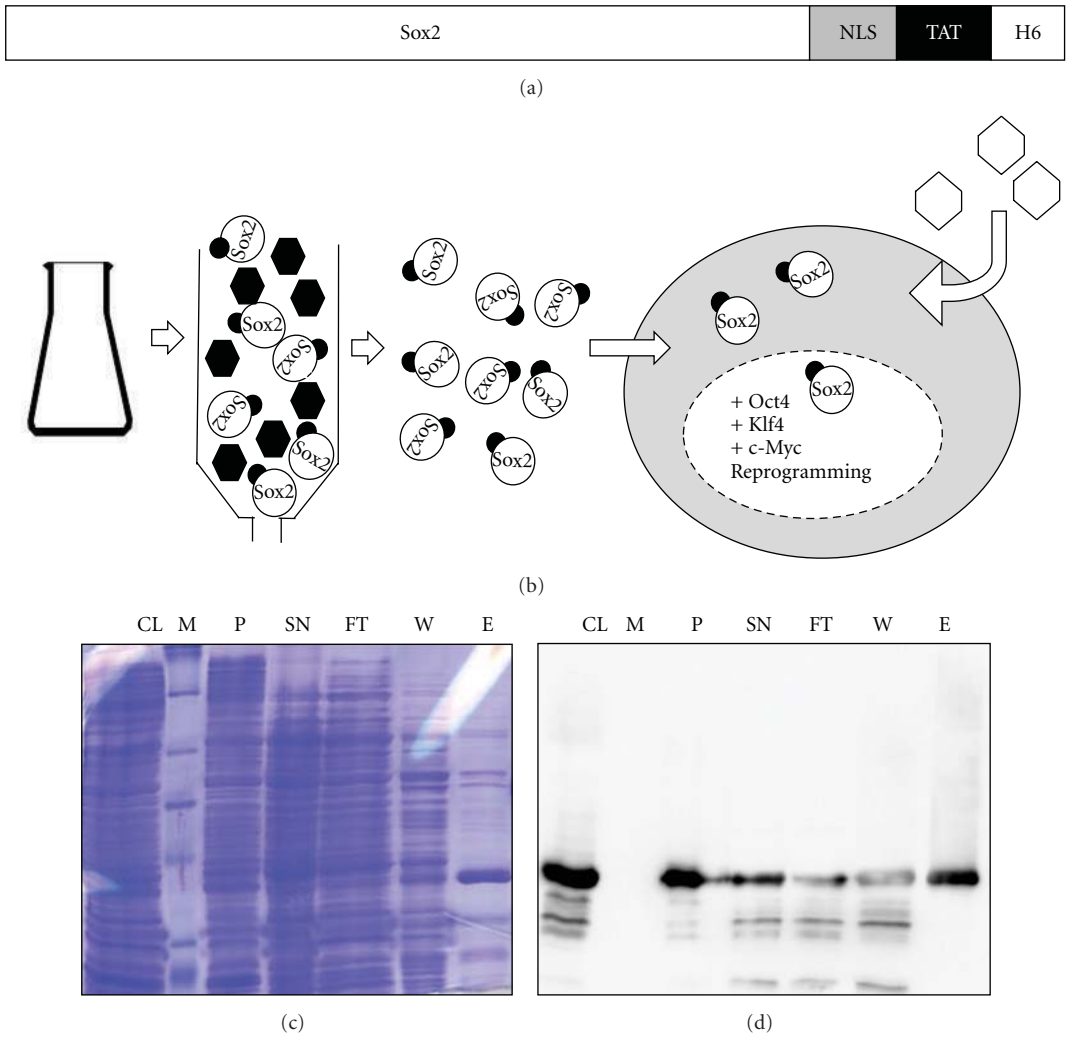

Figure 1: Purification of recombinant Sox2-TAT fusion protein and reprogramming setup. (a) The recombinant cell-permeant Sox2 fusion protein [22] consists of the full-length Sox2 protein and a carboxy-terminally fused sequence of tags consisting of a nuclear localization sequence (NLS), cell-penetrating peptide TAT, and a histidine-tag (H6) for single-step purification. (b) Schematic representation of the expression and purification procedure and the reprogramming setup used in this study. After expression in *E. coli*, Sox2-TAT-containing cell lysates are subjected to affinity column chromatography employing Ni-NTA resin. Purified recombinant Sox2-TAT protein is eluted from the column and its reprogramming competency assessed in combination with retroviruses encoding Oct4, Klf-4, and c-Myc. (c, d) Biochemical analysis of Sox2-TAT purification from *E. coli*. The following fractions were subjected to SDS-PAGE analysis: crude lysate (CL), marker (M), pellet (P), supernatant (SN), flow-through (FT), washing buffer (W), and elution fraction (E). SDS gels were either stained using Coomassie (c) or used for preparation of an immunoblot using anti-Sox2-specific antibody (d).

delivery can reliably be quantified by a Cre recombinase reporter assay. We used the CV1-5B Cre reporter cell line [37] that specifically expresses β-galactosidase after Cre-mediated recombination. Using this read-out system, we tested the influence of FCS and SR on the transduction efficiency (Figure 2(b)). 2 μM of TAT-Cre in 5% FCS-supplemented media induced recombination in approximately 35% of cells, whereas more than 90% of cellular targets were recombined in medium containing 15% SR. Application of 1 μM of TAT-Cre revealed a similar correlation: about 20% recombination in the presence of FCS and 70% with SR (Figure 2(b)). These data demonstrate that FCS in contrast to SR exhibits a strong inhibition on protein transduction. Based on these results, we established an optimized transduction protocol for iPS derivation employing Sox2-TAT. We decided to apply a two-step protocol to optimize both, protein stability and transduction capacity. In a first step, the eluate fraction was supplemented with 7.5% SR and dialyzed against DMEM/F12. Afterwards, the dialysis fraction was supplemented with FCS (2%) and Albumax (2.5%). The optimized medium showed protein stabilizing capacity during the dialysis and under cell culture conditions in the same range as compared to SR and FCS, respectively.

3.3. Reprogramming OKC-Infected Cells with Cell-Permeant Sox2 Fusion Protein. We then assessed the reprogramming activity of Sox2-TAT employing the optimized media conditions. For that, we used a modification of the classical

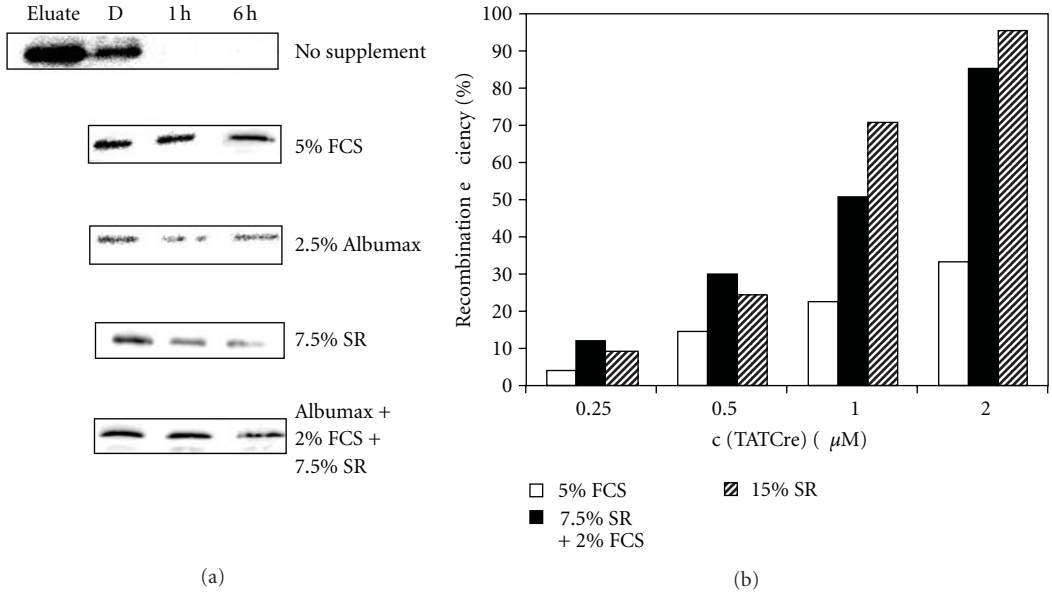

Figure 2: Effect of media supplements on the stability of cell-permeant Sox2-TAT fusion protein and efficiency of protein delivery. (a) Ability of media supplements to stabilize Sox2-TAT. Fetal calf serum (FCS), Albumax, and serum replacement (SR) were added to the eluate fraction and subsequently dialyzed against DMEM-F12 media. Depicted are anti-Sox2-immunoblots of the dialysis fraction (D) and a stability test of samples being taken after 1 hour (1 h) and 6 hours (6 h), respectively. (b) Influence of FCS, SR, and a combination of both on the transduction efficiency. Protein transduction efficiencies were analyzed by quantifying the recombined cells after delivery of cell permeant Cre-protein (TAT-Cre) into the CV1-5B Cre reporter cell line. Cells were treated with different concentrations of TAT-Cre (0.25 μM–2 μM) in transduction media supplemented with either 15% serum replacement, 5% FCS, or mixture of 2% FCS and 7.5% SR. To determine the recombination activity, cells were fixed and stained for β-galactosidase activity after 48 hours. Cre protein transduction and quantification of recombination in Cre reporter cells was performed as described previously [35].

four-factor viral reprogramming paradigm and aimed at substituting the Sox2-encoding virus by Sox2-TAT protein (Figure 3(a)). We transfected Oct4-GiP-transgenic MEFs [38], which enable GFP-based monitoring of reprogramming by reactivation of the Oct4 promoter, with viruses encoding for Oct-4, Klf4, and c-Myc (OKC). OKC-MEFs were initially cultivated for five days in medium containing either 200 nM or 400 nM Sox2-TAT. During the whole reprogramming procedure, we changed the protein-supplemented media every day to ensure a continuous delivery of the recombinant reprogramming factor. At day five, cells were split onto freshly plated feeder cells and either cultured in normal media or further exposed to Sox2-TAT-containing media for five more days (Figure 3(a)). First, iPS-like structures appeared after 9 days and formed well-defined GFP-positive colonies (Figure 3(b)). The viral transduction of the three factors Oct4, Klf4, and c-Myc without Sox2-TAT protein did not yield any GFP-positive colony (Figure 3(b)). GFP^+ colonies were quantified at day 16. We counted eight GFP^+ colonies in wells containing cells treated with 400 nM Sox2-TAT for five days. Prolonged incubation with Sox2-TAT until day 10 did not result in a marked increase of colony numbers. Instead, the number of colonies slightly decreased eventually due a strict time window required for Sox2 application. The application of 200 nM Sox2-TAT for five and ten days yielded no and just one GFP^+ colony, respectively (Figure 3(c)), indicating that the Sox2-TAT concentration is a limiting factor. We aimed at further increasing the Sox2-TAT concentration by either dialysis against glycerol-containing concentration buffer or ultrafiltration; however, beyond 400 nM, the protein strongly precipitated in culture media and interfering with cellular growth (data not shown).

3.4. Sox2-piPS Cells Exhibit Pluripotency. Two Sox2-protein iPS colonies were isolated and expanded for further characterization, yielding Sox2-piPS-1 and Sox2-piPS-2 cell lines, respectively. Both could be proliferated for at least 20 passages, and they maintained their Oct4 promoter-driven GFP activity. Moreover, they stained positively for the pluripotency-associated cell surface marker SSEA-1 (Figure 4(a)). Sox2-piPS-1 and Sox2-piPS-2 were subjected to PCR analysis in order to assess transgenic integrations. This analysis revealed that both lines carry integrated viral transgenes, but no exogenous Sox2 (Figure 4(b)). Transgene silencing represents a major hallmark of successful iPS derivation. Thus, we applied RT-PCR analysis to detect the transcripts of the transgenic reprogramming factors as well as endogenous stemness factors. Both clones analyzed exhibit no detectable transgenic Oct4, Sox2, Klf4, or c-Myc (Figure 4(c)). The mRNA of endogenous Oct4, Sox2 and Nanog, in contrast, was found as abundant as in the ES control cells, indicating complete silencing of exogenous factors and reactivation of the endogenous grid of stemness. Finally, we set out to confirm the pluripotent status of Sox2-piPS-2

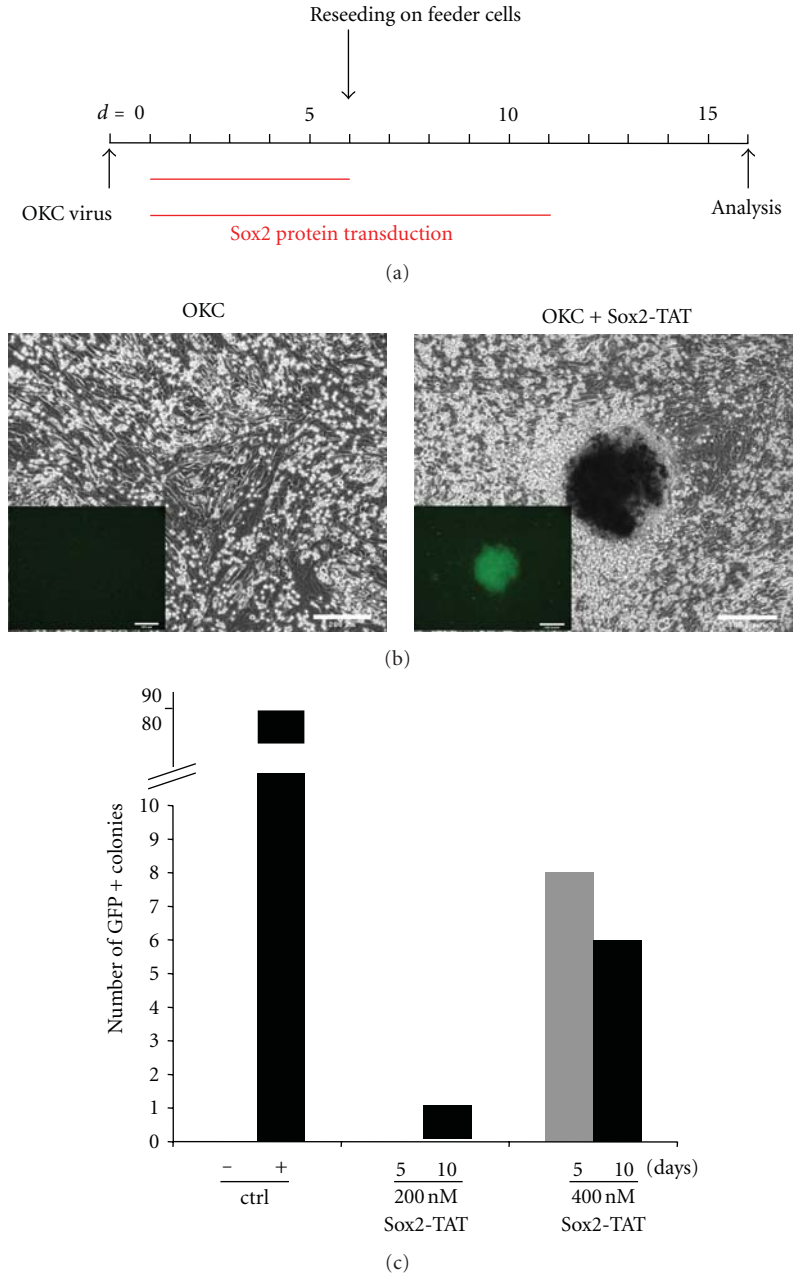

Figure 3: Reprogramming of MEFs using cell-permeant Sox2-TAT protein. (a) Schematic presentation showing the timeline of reprogramming setup. Oct4-GiP MEFs were infected with viruses encoding Oct4, Klf4, and c-Myc (OKC) at day 0. Starting at day 1 post infection (p.i.), cells were incubated with Sox2-TAT for 5 and 10 days, respectively, changing the Sox2-TAT-supplemented media daily. (b) Representative pictures of cells transduced with 200 nM Sox2-TAT protein (right panel) displaying phase contrast and GFP channel (inset) 14 days p.i. OKC-infected cells treated with medium only served as controls (left panel). Scale bar = 100 μm. (c) Quantification of GFP-positive colonies at day 16 p.i. OKC-infected cells (−) as well as SOKC-infected cells (+) served as controls.

by spontaneous differentiation into embryoid bodies (EBs). 5-day old EBs were plated and analyzed for the appearance of specific germ layer marker by staining against β-3-tubulin (TUJ1), smooth muscle actin (SMA), and α-feto-protein (AFP) (Figure 5). According to this analysis, Sox2-piPS-2 cells differentiated into all three germ layers, demonstrating an unrestricted *in vitro* differentiation potential.

4. Discussion

In this study, we elaborated an optimized protocol for the delivery of cell-permeant Sox2-TAT protein into mammalian cells. Poor stability of Sox2-TAT under cell culture conditions represents a major bottleneck of Sox2 protein transduction. Media supplements Albumax, SR, and FCS were analyzed for their stabilizing effect on Sox2-TAT. FCS was found

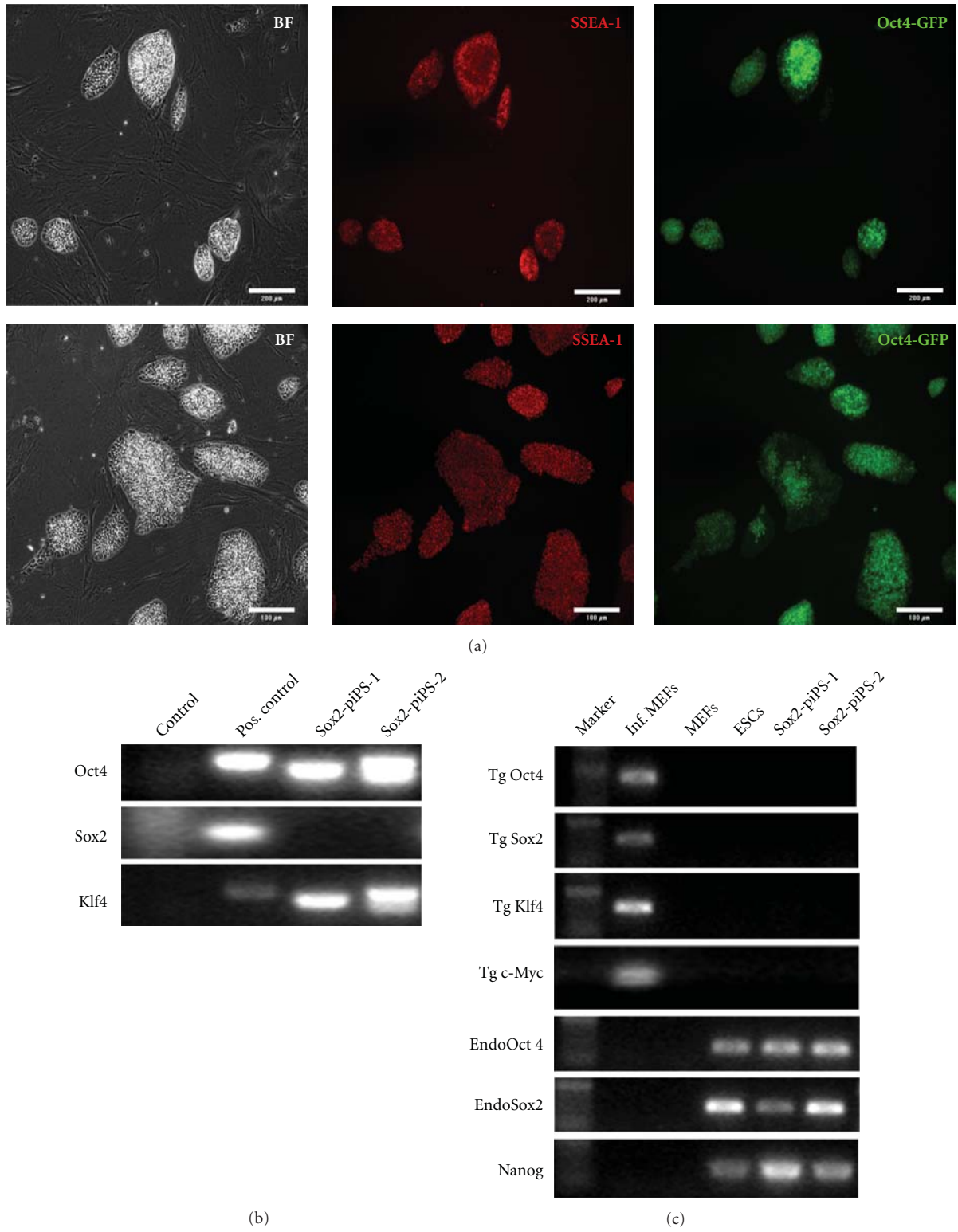

Figure 4: Cellular and molecular characterization of iPS clones derived by Sox2 protein transduction into OKC-MEFs. (a) Pictures of isolated cell lines Sox2-piPS-1 (upper row) and Sox2-piPS-2 (lower row) exhibiting brightfield (BF), staining against pluripotency-associated marker SSEA-1 and native GFP fluorescence. Sox2-piPS-1 cell line was clonally isolated from 400 nM Sox2-TAT treatment from day 1 to 5, and Sox2-piPS-2 was derived from 200 nM condition (day 5 to 10) Scale bar = 100 μm. (b) PCR analysis of genomic DNA demonstrating genomic integration of Oct4 and Klf4 transgenes. As expected, no transgenic Sox2 was detected in Sox2-piPS clones excluding possibility of contamination. (c) RT-PCR analysis demonstrating transgene silencing in Sox2-piPS cells. Primers specific for transgenic Oct4, Sox2, Klf4, and c-Myc were used. Additionally, we analyzed endogenous Oct4, Sox2, and Nanog transcripts. RNA preparations from infected (Inf.) and uninfected MEFs as well as ES cells served as controls.

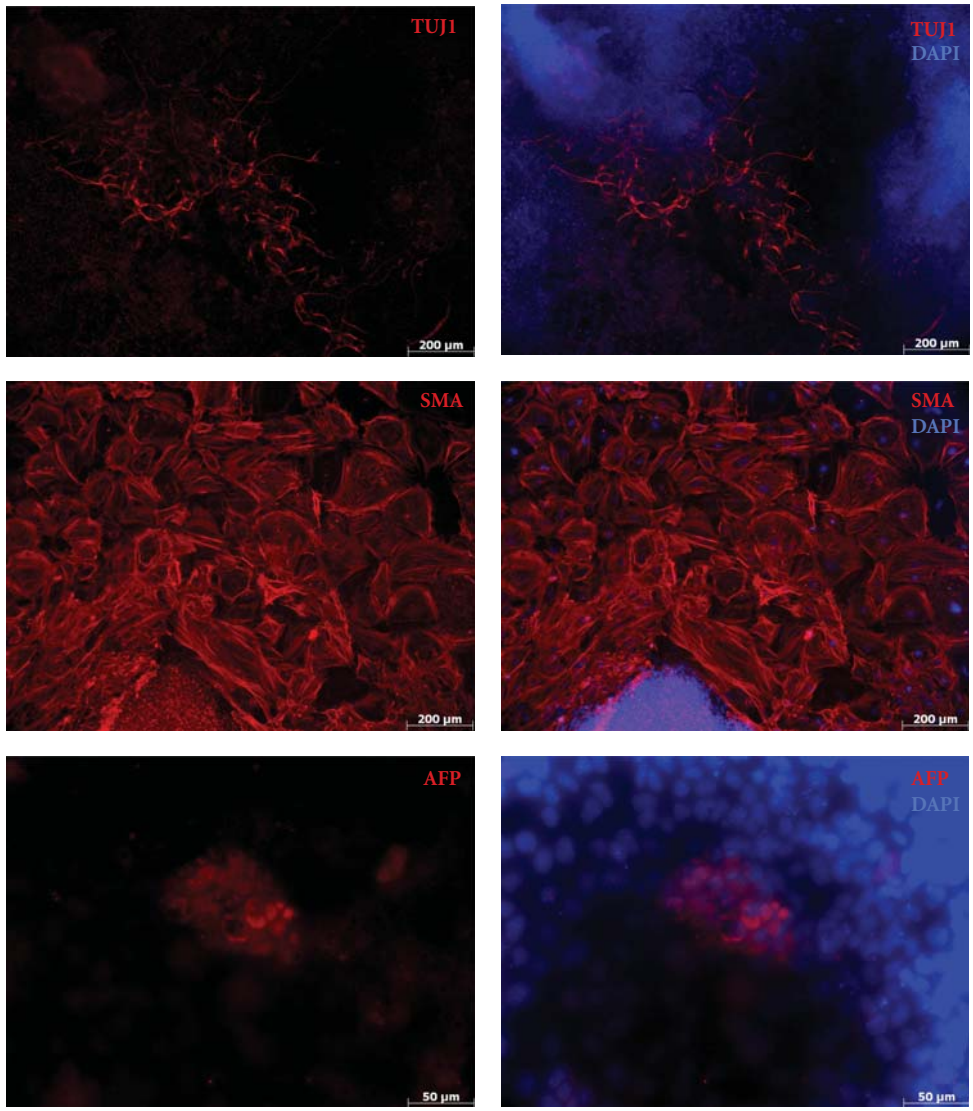

Figure 5: *In vitro* differentiation of Sox2-piPS cells. Spontaneously differentiated Sox2-piPS cells were stained for β-3-tubulin (TUJ1, ectoderm) smooth muscle actin (SMA, Mesoderm), and α-fetoprotein (AFP, endoderm) as indicated. DAPI co-staining was performed in every condition.

to stabilize Sox2-TAT, whereas the recombinant protein rapidly precipitates in protein-free media. We used the Cre protein transduction system [40] to assess the effects of media supplements SR, FCS, and Albumax on protein transduction in a quantitative manner. It turned out that SR has no deleterious effect on cellular delivery, whereas FCS strongly reduces the cellular uptake. Thus, in terms of protein delivery, supplementation of SR is preferred over FCS. However, since in contrast to ES and iPS cells fibroblast cells do not tolerate SR we employed a mixture of 7.5% SR and 2% FCS. These media conditions represent an optimal compromise for cultivation during protein-induced reprogramming of fibroblasts.

Employing these optimized conditions, we demonstrate that Sox2-TAT is able to substitute for a Sox2-encoding virus during the OKC-viral induction of pluripotency in fibroblast cells. Stable Sox2-piPS cell lines could be generated exhibiting major hallmarks of ES cells such as pluripotency-associated marker expression and full differentiation potential *in vitro*. We found that proliferation of Sox2-piPS cells does not depend on the continuous expression of the OKC transgenes as judged by RT-PCR analysis. Notably, the transduction of 200 nM Sox2-TAT from day 5 to day 10 resulted in the induction of one GFP^+ colony (data not shown), whereas treatment from day 1 to 5 did not give rise to any colony, albeit the same concentration of protein was used. Whether this observation hints at a specific time dependence of Sox2 during the reprogramming process or is a result from stochastic variation remains to be investigated. In general, the efficiency of Sox2-piPS derivation is at least one order of magnitude lower as compared to our previously reported generation of Oct4-piPS cells [36]. From this observation, we conclude that although we provide proof-of-principle data, that recombinant Sox2-TAT is reprogramming-competent,

sufficient delivery of biologically active Sox2 protein into the right cellular compartment represents a bottleneck for protein-induced iPS derivation.

Further investigations are needed to accomplish robust reprogramming of human adult cells such as fibroblasts or keratinocytes employing recombinant proteins. Optimized expression and purification protocols are needed to be established that, for example, exploit the insoluble fraction of recombinant Sox2-TAT by purification under denaturing conditions. Moreover, alternative expression hosts including eukaryotic cells might enhance the derivation of soluble native Sox2-TAT protein. Sox2 protein transduction might not only be instrumental for the derivation of factor-free iPS cells but also for the analysis of the reprogramming mechanism by providing a tool to precisely determine the duration and dose of Sox2 induction.

Acknowledgments

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Review Article

Cell Reprogramming, IPS Limitations, and Overcoming Strategies in Dental Bioengineering

Gaskon Ibarretxe, Antonia Alvarez, Maria-Luz Cañavate, Enrique Hilario, Maitane Aurrekoetxea, and Fernando Unda

Department of Cell Biology and Histology, Faculty of Medicine and Dentistry, University of the Basque Country (UPV/EHU), 48940 Leioa, Spain

Correspondence should be addressed to Fernando Unda, fernando.unda@ehu.es

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The procurement of induced pluripotent stem cells, or IPS cells, from adult differentiated animal cells has the potential to revolutionize future medicine, where reprogrammed IPS cells may be used to repair disease-affected tissues on demand. The potential of IPS cell technology is tremendous, but it will be essential to improve the methodologies for IPS cell generation and to precisely evaluate each clone and subclone of IPS cells for their safety and efficacy. Additionally, the current state of knowledge on IPS cells advises that research on their regenerative properties is carried out in appropriate tissue and organ systems that permit a safe assessment of the long-term behavior of these reprogrammed cells. In the present paper, we discuss the mechanisms of cell reprogramming, current technical limitations of IPS cells for their use in human tissue engineering, and possibilities to overcome them in the particular case of dental regeneration.

1. Cell Dedifferentiation and Pluripotency

Cellular dedifferentiation underlies important issues in biology, such as tissue regeneration and cloning, and signifies the withdrawal of cells from a given differentiated state into a stem-cell-like state that confers pluripotency. Pluripotency *in vivo* pertains to the cells of early embryos that can generate all of the tissues in the organism. Embryonic stem cells (ESC) are preimplantation embryo-derived cells having three properties: self-renewal, pluripotency, and primary chimera formation [1]. ES cells represent invaluable tools for research into the mechanism of tissue formation. *In vitro* pluripotency may be maintained in ES cells, which are harvested from the inner cell mass of the blastocyst stage embryo. ES cells have demonstrated longevity in culture by maintaining their undifferentiated state for at least 80 passages. Moreover, if ES cells are cultured with the appropriate nutrients at their disposal, these cells can potentially give rise to all cell types of the body, including pluripotent germinal cells, and their offspring can become integrated in a tissue, adopting the character and behavior of the cells in this new tissue environment. However, there are also significant problems associated with the use of human ES cells.

(i) Their obtention involves manipulation of human embryos and therefore serious legal and ethical issues [2].

(ii) If the transplanted cells differ genetically from the cells of the patient, the immune system of the latter may reject and destroy these cells and the patients would be on life-long immunosuppressants.

(iii) Pluripotent stem cells (SCs) present a safety concern because of their potential to form tumours. When these cells are transplanted in the undifferentiated state, they form teratomas, tumours derived from all three germ layers. Currently, the only way to ensure that teratomas do not form is to differentiate the ES cells, enrich for the desired cell type, and screen for the presence of undifferentiated cells [3].

The first two of these problems could be avoided using dedifferentiation of somatic cells as a means to obtain

autologous "patient specific" pluripotent stem cell lines. The capacity for dedifferentiation is retained in mammalian somatic cells, and the reprogramming technology has provided two strategies for the generation of pluripotent SCs from adult differentiated cells.

(1) The denominated somatic cell nuclear transfer (SCNT) also called "therapeutic cloning" or nuclear cloning (NC): one hallmark in this field of research took place in 1996 with the birth of *Dolly*, the cloned sheep conceived by transfer of an adult differentiated cell nucleus to an enucleated unfertilized oocyte [4]. This groundbreaking discovery made evident that even somatic highly differentiated cells retain the intrinsic ability to revert to a zygote state and thus provide a potentially inexhaustible source of ES cells.

(2) The generation of pluripotent cells from differentiated reprogrammed animal cells, known as induced pluripotent SCs, or IPS cells: one decade after the birth of *Dolly*, another decisive discovery brought the advent of IPS cells by transgenic expression of merely four transcription factors in adult somatic mouse cells, namely, *Oct3/4, Sox2, Klf4,* and *c-myc* [5]. This protocol also worked with adult human cells, using *OCT3/4, SOX2, KLF4,* and *c-MYC* [6] or *OCT-4, SOX-2, LIN28,* and *NANOG* [7].

Somatic cell nuclear transfer (SCNT) entails the removal of an oocyte nucleus followed by its replacement with a nucleus derived from an adult somatic cell [8]. SCNT has limitations; in addition to the serious ethical issues surrounding the cloning of human embryos created for research [9], the scarcity of fresh donated mature human metaphase-II oocytes of high quality available for research is a significant obstacle [10]. Currently, the efficiency of the overall cloning process is quite low as the majority of embryos derived from animal cloning do not survive after implantation [11]. At present, the medical applications of SCNT have been halted on account of the inefficacy of the process, the lack of knowledge of the underlying mechanism, and ethical concerns [12]. Nevertheless, nuclear transfer has shown that all genes required to create an entire organism are present in the nucleus of the differentiated cell and can be activated on exposure to reprogramming factors present in the oocyte [13]. In addition, SCNT is a powerful tool to probe the developmental potential of a cell, and the major conclusion from these findings was that development imposes reversible epigenetic rather than irreversible genetics changes on the genome during cellular differentiation [14]. However, generation of embryos directly from embryonic stem cells by tetraploid embryo complementation has become a popular means as an alternative to SCNT.

Somatic cells can be reprogrammed by fusion with ES cells, and Takahashi and Yamanaka concluded that ES cells contain factors that induce pluripotency, and these factors were also likely involved in the maintenance of pluripotency in ES cells. Based on this hypothesis, they showed that ectopic expression of defined transcription factors was sufficient to reprogram mouse embryonic fibroblasts and adult fibroblasts to pluripotent ES-like cells after retroviral-mediated transduction of the four transcription factors *Oct3/4*, *Sox2*, *c-myc,* and *Klf-4*, under ES culture conditions [5]. These cells, designated IPS cells, exhibit the morphology and growth properties of ES cells and express ES marker genes. Subcutaneous transplantation of IPS cells in nude mice resulted in teratomas. Injection of iPS cells into blastocyst contributes to chimaeras. IPS cells showed unlimited proliferation *in vitro* maintaining their pluripotency. Overexpression of the four factors generated cells capable of forming adult chimaeras and generating functional germ cells [15–18]. Human IPS cells, produced by expression of either *Oct-4, Sox-2*, *c-myc,* and *Klf-4* or *Oct-4*, *Sox-2, Nanog,* and *Lin-28*, are remarkably similar to human ES cells [19]. However, controversy exists with regard to the differential gene expression profiles (genetic signatures) in ES and IPS cells [20, 21]. Consistent with this, IPS cells show attenuated potential differentiation in comparison to ES cells [22, 23].

A major limitation of reprogramming strategies is the use of potentially harmful genome integrating viruses to deliver reprogramming factor transgenes. Most IPS cells are prepared by viral vectors, such as retrovirus [5] and lentivirus [24], that integrate the reprogramming factors into host genomes, increasing the risk of tumor formation. The residual presence of integrated transgenes following the derivation of IPS cells is highly undesirable. The four factors used to induce reprogramming are strictly speaking oncogenes, thus implying a risk of transformation to a cancer phenotype. There are substantial grounds to state that the process of nuclear reprogramming by virus-assisted factor insertion in the cell genome increases the risk of carcinogenesis [25]. Supporting this, the efficiency of reprogramming increases largely in cells where the p53 tumor suppressor gene is knocked-out [26, 27]. This high risk of carcinogenesis is largely, but not exclusively, related to the integration of c-*MYC* transgenes [28, 29].

Alternative gene factor delivery systems include nonintegrating adenoviruses [30], plasmid transfection [31], doxycycline-inducible excisable *piggyBac* (PB) transposon system [32], and nonintegrating episomal vectors [33]. Another different strategy consists of delivery of recombinant proteins rather than genes into the cells to be reprogrammed [34, 35]. Others have explored the induction of reprogramming by chemical stimulation and screening/selection of effective small molecules, thus reducing the amount of factors delivered to cells [36]. Using the latter approach, there have been successful trials to generate IPS cells with the introduction of only one reprogramming factor (*OCT-4*) in multipotent neural SC [37] and dermal papilla cells from hair follicles [38]. The four factors that were initially identified can now be substituted with different factors or with certain small molecules, but the original finding—that a set of factors is required—holds true, and certain key gene factors such as *OCT-4* cannot be omitted.

The possibility to obtain patient-specific IPS cells has brought big hope on the prospect of future tissue engineering regenerative therapies by cell transplant since these new

pluripotent cells circumvent two of the main problems traditionally associated with the sources of human pluripotent SCs: the serious ethical issues arising from the need to manipulate human embryos, and the possibility of rejection of the transplanted cells by the host immune system. However, the induction of carcinogenesis remains a pending threat. The potential of IPS cell technology is tremendous, but it will be essential to improve the methodologies for IPS cell generation and to precisely evaluate each clone and subclone of IPS cells for their safety and efficacy. It will be necessary to perform a detailed cellular and molecular study of somatic cells during their progression to pluripotent state [39]. Many aspects still remain to be clarified, given that the efficiency of the cell reprogramming process is certainly very low [40].

2. Tissue Engineering and IPS Cells: Definition and General Concepts

Tissue engineering aims to generate living tissues that could be used for the restoration of the function of several organs [41]. Tissue engineering has progressed from the use of biomaterials, which may repair or replace diseased or damaged tissue, to the use of controlled three-dimensional scaffolds in which cells can be seeded before implantation [42]. Organ shortage and suboptimal prosthetic or biological materials for repair or replacement of diseased or destroyed human organs and tissues are the main motivation for increasing research in the emerging field of tissue engineering in regenerative medicine [43–45]. While tissues such as bone or skin can effectively repair a small injury given sufficient time, many tissues such as myocardium, cartilage, and neural tissues do not regenerate properly without intervention [46].

The availability of sources of pluripotent SC has increased immensely the potential of cell therapy in medicine and opens up new perspectives in the treatment of diseases [47, 48]. IPS cells can proliferate and be induced to differentiate to a particular cell type, and the selected cells can be seeded in a specific mould or scaffold and cultured *in vitro*. Scaffolds (natural or synthetic) may be composed of polymers, metals, ceramics, or composites [49, 50]. Bioreactors are used to grow the cells on the scaffolds until the tissue or the organ is fully developed [41]. The cells can be expanded in culture and then reimplanted in the patient [51, 52] (Figure 1). The cells can come from the same individual (autologous) or the same species but from a different individual (allogeneic) or even can originate from different species (heterologous).

Nevertheless, because of the limitations inherent to the cell reprogramming process, it is advised that research on IPS cells in the field of tissue engineering is carried out in appropriate tissue and organ systems that permit a safe assessment of the long-term behavior of these reprogrammed cells. As we will discuss in the last part of the paper, the dental system may constitute a very good choice as a testing ground for IPS cells applied to tissue engineering, owing to several specific features of dental cells and tissues. We will proceed by briefly describing endogenous sources of dental SC, the process of normal tooth development and its associated structures, and finally we will discuss how these features may constitute a decisive advantage to investigate future applications of IPS cells in full dental regeneration.

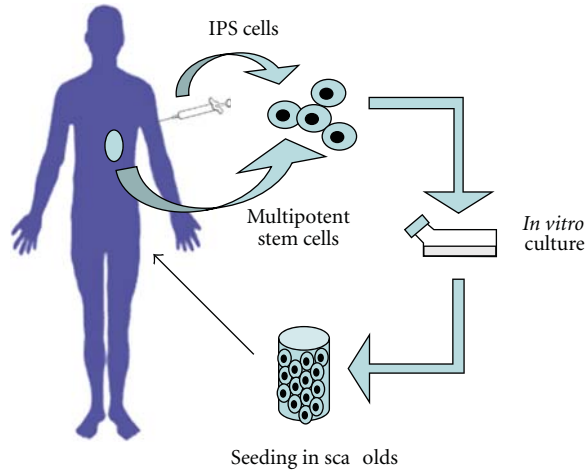

Figure 1: Basic scheme of tissue engineering. A biopsy is carried out to extract cells from the patient. These can be endogenous organ-specific multipotent SCs, or alternatively they can be adult differentiated somatic cells, reprogrammed to IPS cells. SCs are isolated, expanded and differentiated to the cell type of interest in an appropriate culture medium, seeded in a scaffold, and cultured *in vitro*. At this point, the new tissue is implanted in the patient.

3. Are Teeth the New Golden Mine of SC?

Teeth are nonvital organs that, remarkably, have proven to be a surprisingly rich source of multipotent ectomesenchymal SC (EMSC). The majority of live adult tooth tissues derive from the neural crest, and therefore all dental SCs considered here are collectively termed EMSCs. Teeth are easily amenable for extraction in the dental clinic, precluding the need of complex chirurgical care and invasive isolation methods. Owing to their amount and accessibility, dental tissues constitute one of the most consistent sources of human SCs that can be found nowadays. Human teeth are extracted and disposed of by thousands in dental clinics worldwide, the majority of them corresponding to third-molars (wisdom teeth) of young patients, which are usually removed for orthodontic reasons.

There are five different types of dental EMSCs that have been isolated and characterized: dental pulp SC, or DPSC [53], SC from human exfoliated deciduous teeth, or SHED [54], periodontal ligament SC, or PDLSC [55], SC from the apical papilla, or SCAP [56], and SC from dental follicle [57]. All these display stem cell features such as multilineage differentiation potential to various cell types including odontoblasts, cementoblasts, osteoblasts, chondroblasts, adipocytes, muscle cells, and neurons [58]. Notably, due to their neural crest origin, dental SCs are considered to be a good stem cell choice to generate neural and glial cell derivatives. Some of these cells, such as SHED, express early immature glial and neuronal cell markers

in basal conditions, even in the absence of neurogenic stimulation [54].

Very recent research has described a population of *OCT-4+*, *NANOG+*, *LIN28+*, and *SOX-2+* cells in the dental pulp that is argued to constitute an endogenous dental source of pluripotent SCs [59]. These cells can be induced to differentiate to endoderm and mesoderm cell derivatives. If this finding got confirmed by other research teams in the near future, the field of dental stem cell research will no doubt receive a definite boosting. Other tested strategies to obtain pluripotent cells out of dental cells consist of reprogramming dental multipotent EMSC or adult gingival and periodontal fibroblasts to IPS. This has been successfully carried out by different research groups [60–62].

4. Potential of the Dental System for IPS Technology in Full Organ Bioengineering

Dental SCs have been successfully tested in tissue engineering research, where full generation of dentin pulp complexes and even whole teeth out of isolated cells (complete organ restoration) has proven to be possible [63]. The two cell types that take part in the generation of teeth come from different embryonic origins: surface epithelial (ectoderm) and ectomesenchymal (neural crest). Those tissues are precursors of the enamel organ and dental papilla, which will generate tooth enamel and the dentin-pulp complex, respectively. Tooth development takes place over different morphogenetic stages (placode, bud, cap, bell, appositional) and is governed through a complex series of epithelial-mesenchymal cellular inductions. As a consequence of continuous reciprocal signaling, the precursors of ameloblasts and odontoblasts, the two key mineralizing adult dental cell types, will elongate, polarize, and differentiate at the epithelium-mesenchyme interface. These ameloblastic (enamel producing) and odontoblastic (dentin producing) cells will terminally differentiate at the late bell-early appositional stage transition, and this will mark the beginning of secretion and deposition of hard enamel and dentin tissues, starting by the tooth cusps [64].

Importantly, once the deposition and maturation of tooth enamel is complete, ameloblastic cells will undergo a drastic regression, losing their elongated size and polarized state and mingling with adjacent epithelial cells to form the so-called "reduced enamel epithelium," a transient coating structure that will end up disappearing at the moment of tooth eruption. The only epithelial cells that will remain in adult tooth structures are the epithelial cell rests of Malassez (ECRM), deriving from Hertwig's epithelial root sheath (HERS), another transient structure involved in dental root formation. ECRMs play no known role in the adult tooth and appear as little cell clusters in the periodontal ligament. On the contrary, ectomesenchyme-derived odontoblasts and dental pulp tissues will persist throughout the tooth life well into adulthood (Figure 2).

De novo generation of fully functional dentin-pulp complexes and periodontal tissues has been successfully accomplished by transplantation of endogenous EMSC to experimental animals, in combination with mineralized hydroxyapatite/tricalcium phosphate scaffold carriers [65]. Transplanted cells eventually assimilate and remodel the scaffold to create completely biological structures. Under these conditions, it is also possible to design a biological tooth root that supports the placement of a synthetic tooth crown, as shown by elegant studies [66]. With improvements and adaptations of this technique, in the future it may be possible to replace synthetic implants by biocompatible engineered tooth root tissues in humans. Therefore, endogenous EMSCs hold a big potential for their use in regenerative dentistry.

Even more striking was the reported generation of functional mouse teeth generated exclusively from dissociated dental SCs. In a sound study, Tsuji et al. [67] isolated dental epithelial cells from E14.5 cap stage mouse teeth and recombined them together with ectomesenchymal DPSC in a collagen gel, thus creating a bioengineered molar tooth germ. Remarkably, the bioengineered tooth proceeded normally through all the different morphogenetic stages and could be eventually transplanted into the jawbone of a host mouse, to create a fully functional adult tooth that integrated well into surrounding tissues, presented a correct occlusion, supported masticatory forces, could perform orthodontic movements, was normally innervated, and responded adequately to pain stimuli. This positive experience holds great promise in the field of full dental organ regeneration, which would no doubt revolutionize future dentistry.

However, obviously much experimentation is required and major issues need yet to be solved before an approach like tooth germ engineering by dental stem cell recombination can be translated to the dental clinic. Probably the most important limiting factor is the absence of consistent sources of epithelial SC with odontogenic potential in the adult human individual, to be recombined with endogenous dental mesenchymal SCs. There has been substantial progress in the identification of possible epithelial substitutes, using PDL-derived ECRM [68], and postnatal oral mucosal epithelial cells [69]. Both these cell types can be cultured *in vitro* and induced to differentiate to ameloblastic cell lineages. Another realistic possibility, exclusively for research purposes, would constitute the rodent incisor, which contains an epithelial stem cell niche [70]. However, although the sources of endogenous dental epithelial SC seem to be scarce, an appealing alternative would be to obtain them from autogenic IPS cells, properly differentiated *in vitro*. Once this step is accomplished, the remaining process of recombination into collagen scaffold matrices, *in vitro* organ culture, and *in vivo* transplantation should not pose extreme technical difficulties. The final outcome would be a fully developed bioengineered human tooth obtained from dissociated autogenic EMSC and IPS cells, in which the latter would almost completely disappear after tooth eruption (Figure 3).

Thus, there are several arguments that point to teeth as a very attractive system to test IPS cells in a context of full organ restoration therapy.

(i) First, tooth development by epithelial (IPS derived) and ectomesenchymal (endogenous) autogenic or allogeneic cell recombination can be performed and

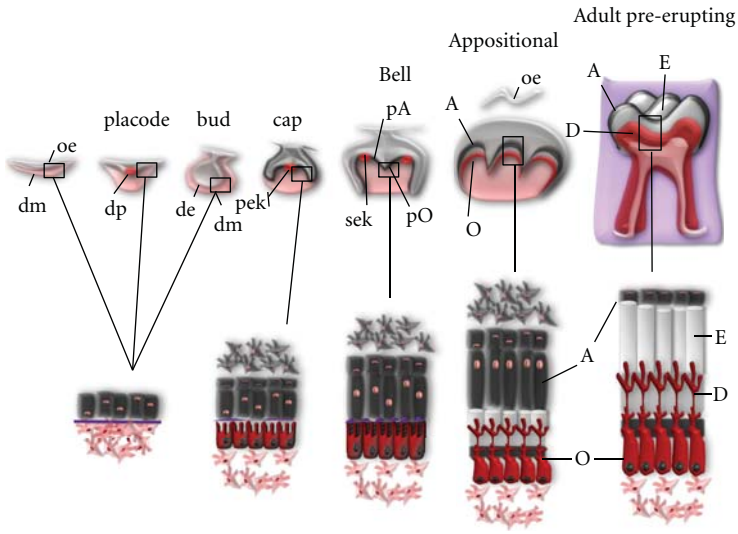

Figure 2: Stages and events of molar tooth development. Tooth morphogenesis is carried out by complex epithelium-ectomesenchyme interactions. Epithelial cells are depicted in gray and ectomesenchymal cells in red. As a consequence of sequential induction events, ameloblast (A) and odontoblast (O) cells start to differentiate at the interface between dental epithelium (de) and dental mesenchyme (dm) at the end of bell stage. Enamel (E) and dentin (D) tissues are secreted during the appositional stage, when the developing dental organ appears separated from the oral epithelium (oe). When enamel mineralization is completed, ameloblasts undergo regression, whereas odontoblasts will be maintained during the whole life of the tooth. The areas covered by squares are represented magnified below. Signaling centers during tooth morphogenesis are drawn as red circles: dental placode (dp), primary enamel knot (pek), and secondary enamel knot (sek). pA: preameloblasts; pO: preodontoblasts.

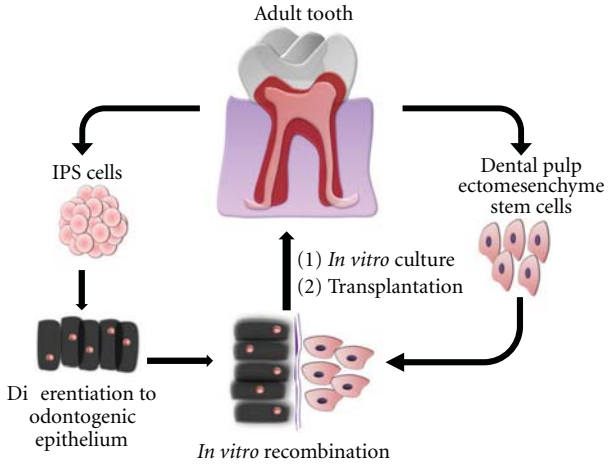

Figure 3: Theoretical design of a dental engineering process involving IPS cells. Tooth tissues already present well-characterized populations of ectomesenchymal SCs, that can generate *de novo* a complete dentin-pulp complex and periodontium. The hard enamel tissue constituting the remaining part of the tooth must be formed by dental epithelial cells. In this context, autogenic IPS cells could be used as a source of new dental epithelium, to be recombined with ectomesenchymal cells, thus creating a bioengineered tooth germ that can be cultured *in vitro* and transplanted to the jawbone/maxillary bone of a recipient host to form a fully functional tooth. Almost all IPS-derived epithelial cells will disappear after tooth eruption, as a consequence of normal dental development.

followed up *in vitro* during early developmental stages up to two weeks, therefore permitting selection of the most appropriate or best-looking bioengineered teeth before transplant.

(ii) Second, IPS cells in the context of tooth engineering are mostly needed as a source of dental epithelial cells and eventually enamel producing ameloblasts. These epithelial cell derivatives will be present only transiently and disappear after tooth eruption occurs, with the sole exception of ECRM. Therefore, the risk of IPS-induced tumorigenesis should be greatly reduced in the dental system.

(iii) Third, it is possible to generate autogenic (patient-specific) IPS cells from dental cells and tissues to minimize the chance of immune rejection.

(iv) Fourth, long-term outcomes of biological teeth can be readily followed during routine dental and periodontal check-ups, even by visual exploration.

(v) Finally, should complications arise because of the use of SC or IPS cells (tumorigenic or other), extraction of the tooth piece can be performed with relative simplicity, by noninvasive procedures and with no life-threatening risk to the patient.

At the present time, we have no means to predict what will be the future of IPS technology to treat human diseases by cell therapy, but the recently discovered process of adult cell reprogramming still continues to fascinate the research community. No doubt that development of safe

nontumorigenic IPS cell lines will have a great deal to do with their eventual success [71, 72]. Time will tell whether teeth will become an important testing ground for applications of IPS cells in a complex context of tissue regeneration, with multiple cells of different lineages, IPS and host, that will need to coordinate and communicate with each other. It seems clear that we will learn a lot about IPS cell integration on tissues over the next decades.

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Review Article

The Promise of Human Induced Pluripotent Stem Cells in Dental Research

Thekkeparambil Chandrabose Srijaya,[1,2] Padmaja Jayaprasad Pradeep,[3] Rosnah Binti Zain,[3,4] Sabri Musa,[5] Noor Hayaty Abu Kasim,[2] and Vijayendran Govindasamy[5]

[1] *Research and Development Department, Hygieia Innovation Sdn. Bhd, Lot 1G-2G, Komplex Lanai, No.2, Persiaran Seri Perdana, Persint 10, Territory of Putrajaya, 62250 Federal, Malaysia*
[2] *Department of Conservative Dentistry, Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[3] *Oral Cancer Research and Coordinating Centre (OCRCC), Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[4] *Department of Oral Pathology, Oral Medicine and Periodontology, Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[5] *Department of Children's Dentistry and Orthodontics, Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia*

Correspondence should be addressed to Thekkeparambil Chandrabose Srijaya, sreejayamol@gmail.com

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Induced pluripotent stem cell-based therapy for treating genetic disorders has become an interesting field of research in recent years. However, there is a paucity of information regarding the applicability of induced pluripotent stem cells in dental research. Recent advances in the use of induced pluripotent stem cells have the potential for developing disease-specific iPSC lines *in vitro* from patients. Indeed, this has provided a perfect cell source for disease modeling and a better understanding of genetic aberrations, pathogenicity, and drug screening. In this paper, we will summarize the recent progress of the disease-specific iPSC development for various human diseases and try to evaluate the possibility of application of iPS technology in dentistry, including its capacity for reprogramming some genetic orodental diseases. In addition to the easy availability and suitability of dental stem cells, the approach of generating patient-specific pluripotent stem cells will undoubtedly benefit patients suffering from orodental disorders.

1. Introduction

Human embryonic stem cells (hESCs) are pluripotent cells, which have remarkable proliferation ability to differentiate into any cell types of all three germ layers in a defined culture condition. Hence embryonic stem cells have been regarded as the most potent tool for experimental studies, drug screening, and regenerative medicine [1]. However, the ethical dilemmas regarding the donation or destruction of human embryos and the immunoincompatibility of hESCs have impeded its application in cell-based therapy [1]. In order to overcome these problems, reprogramming techniques have been introduced where somatic cells can be reversed into a pluripotent stem cell-like state. It is generally believed that induced pluripotent stem (iPSC) cells might demonstrate the potential for alleviating incurable diseases and aiding organ transplantation [2].

It has been shown that iPSCs can be derived efficiently from various human cell types [3–8]. An interesting observation is that the transcriptional and epigenetic features of iPSCs are reported to be similar to hESCs [9–11]. Nevertheless, further insights into the inherent similarities and differences between hESCs and iPSCs would be advantageous in understanding the reasons why the use of hESCs in clinical and translational applications has been held back [12, 13].

2. Generation of Induced Pluripotent Stem Cells

Induced pluripotent stem cells can be produced by forced expression of certain genes by reversing them to a pluripotent state similar to that of embryonic stem cells (ESCs). However, the generation of iPSC requires extremely safe and efficient approaches or strategies to decrease the risk of tumors that may result from the introduction of undifferentiated iPSCs

into patients. Though such constraints prevail, the approach of generating patient-specific pluripotent cells will undoubtedly benefit regenerative medicine in many ways [14]. The first iPSCs generation was reported by Takahashi and Yamanaka [15] in 2006. They generated the iPSCs through simultaneous overexpression of a group of transcription factors using cell lines derived from mice. A similar genetic manipulation approach was used to generate pluripotent stem cells from human fibroblast cells [11, 13, 16]. In addition to this approach, other modes have also been devised for iPSCs generation including; single polycistronic vector [17], nonintegrating adenoviral APS approaches [18, 19], the PiggyBac transposon system, which removes the transgenes from established iPSC lines after inducing pluripotency [20, 21], the Cre/lox Precombination system [22], and nonintegrating "episomal" vectors that create iPSCs free of vector and transgene DNA [23]. As these methodologies depended solely on foreign DNA transfer into target cells, protein-based methods have been introduced to address safety issues. In these methods various reprogramming proteins are delivered into cells by conjugating them with a short peptide that mediates protein transduction, such as HIV tat and polyarginine [24, 25]. In addition, an alternative approach has been described which uses synthetic mRNA to induce pluripotency and differentiation [26]. This new approach showed superior conversion efficiency and kinetics than the earlier described protocols. This mode of cellular reprogramming is a holistic approach as this transfers all the regulatory components from a target cell to a donor cell. Moreover, the cellular reprogramming is achieved by manipulating the whole genome system rather than a small set of master genes.

Therefore finding a safe and efficient mode of iPSCs generation requires a better understanding of the biology of cellular reprogramming. Even though live cells are the phenotypic representations of their genomic state (gene-regulation, epigenetic modifications, and cellular physiology), they do not have a steady molecular state [27]. For this reason, it is possible for the cells to be switched or reprogrammed into a pluripotent state, even in their differentiated form.

3. Characterization of iPSC Lines

Generation of iPSC lines were always followed up by subsequent characterizations to ensure the purity and quality of the generated cells and their pluripotency potential. One of the most convenient and direct methods developed for detecting and isolating iPSC was by live immunocytochemistry [28]. Using this technique the characterization of pluripotency can be achieved using intracellular and cell-surface biomarkers such as SSEA-3, SSEA-4, Tra-1-60, and Tra-1-81 [29]. In addition, flow cytometry analyses helps to quantify the expression of these markers at the individual cell level.

In addition to live staining, auxiliary identification was demonstrated using alkaline phosphatase (AP) staining for the reprogramming factors, as AP is a universal marker in the identification of iPSCs [29]. Further evaluation of pluripotency is performed through semiquantitative and quantitative polymerase chain reactions (PCRs) through the expression of both endogenous genes and transgenes [30]. This is followed up by the analysis of methylation status of the promoter region of pluripotent genes by bisulfite sequencing of the CpG islands [29]. Karyotyping analysis is also carried out using standard G-banding chromosome analysis to determine chromosome stability of iPS cell lines [29]. Further, *in vitro* differentiation of pluripotent stem cells is characterized by the formation of embryoid body followed by teratoma assays [30]. This assay is used to confirm formation of all three embryonic germ layers [30].

4. Advances in Disease-Specific iPSCs and Their Applications

Although most of the human-related disease studies are undertaken using rodent models, a genetic defect or disorder produced in human does not necessarily cause the same symptoms in rodents. Therefore, cell cultures from human tissues are considered to be the most suitable complement to animal models. The iPSC technology has made the production of disease-specific stem cells that carry the genome of the donor possible and it mimics the human diseases more reliably than animal models. Apart from generating an *in vitro* model, disease-specific iPS cell lines from different individuals also allow better understanding of the nature and complexity of a disease. At present the most immediate requirement of such a human disease model is to explore the progression of a disease in different tissues of the human body and also to compare the variability among patients [2].

5. Existing Types of Disease-Specific iPSC Lines

A number of studies have been conducted on disease-specific iPSC lines and some of them have provided understanding of the disease mechanisms. Table 1 summarizes the up-to-date literature in which human disease-specific iPSC lines have been generated. The most convincing fact for commencing these studies using iPSC technology was that disease-specific pluripotent cell lines could be generated successfully from patients with a variety of genetic disorders where the iPSC lines had similar characteristic capacity, equivalent to those from a normal individual [11]. Moreover, these iPSC lines were able to differentiate into required cell types of relevant diseases and recapitulate disease-specific effects *in vitro* which may not be detectable in animal models [31, 32].

6. Perspective of iPS Technology in Dental Research

Initially the concept of utilizing iPSCs technology to model disease was mostly emphasized in neural degenerative diseases, which was then extended to other genetic disorders including immune system, muscular, blood, pancreas, skin, bone marrow, liver, lung, retinal, premature ageing, as well as other physical and intellectual disorders. However, the concept of utilizing iPSCs technology is still in its infancy for orodental disorders and diseases. Chronic degenerative dental diseases are widespread in human populations and represent a significant problem for public health. The iPS

Table 1: Disease-specific-induced pluripotent stem cells (iPSCs) lines from various human genetic disorders.

Disease category	Disease	References
Neural	Amyotrophic lateral sclerosis	[31]
	Parkinson's disease	[11, 22, 33, 34]
	Huntington's disease	[11, 35]
	Lesch-Nyhan syndrome	[11]
	Rett syndrome	[36]
	Familial dysautonomia	[37]
	Angelman syndrome	[38]
	Prader-Willi syndrome	[38, 39]
	Friedreich's ataxia	[40]
	Rett syndrome	[41]
	Schizophrenia	[42]
Immune system	ADA-SCID	[11]
	Scleroderma	[43]
	Primary immunodeficiency	[44]
Muscular	Duchenne muscular dystrophy	[11]
	Becker muscular dystrophy	[11]
	Spinal muscular atrophy	[25]
	Duchenne muscular dystrophy	[45]
Blood	Thalassemia	[46, 47]
	Sickle cell anemia	[43, 47]
	Chronic myeloid leukemia	[48]
Heart	Long QT syndrome	[49, 50]
Pancreas	Juvenile diabetes mellitus	[11]
	Shwachman-Bodian-Diamond syndrome	[11]
	Type I diabetes	[51]
Skin	Leopard syndrome	[52]
	Recessive dystrophic, Epidermolysis bullosa	[53]
Bone marrow	Fanconi anemia	[54]
	Myeloproliferative diseases	[54]
Liver	Liver diseases: a1-antitrypsin deficiency, familial hypercholesterolemia, glycogen storagedisease type 1a, Crigler-Najjar, tyrosinemia type 1	[55]
Lung	Lung diseases: cystic fibrosis, a-1 antitrypsin deficiency-related emphysema	[43]
Others (physical and intellectual limitations)	Down syndrome	[11]
	Hurler syndrome	[44]
	Gaucher disease	[11]
	Fragile X syndrome	[56]
Premature ageing	Dyskeratosis congenital	[57]
Eye	Retinitis pigmentosa	[58]
	gyrate atrophy	[59]
Dental	?	

technology and its application in treating orodental diseases could be a powerful therapeutic tool in dentistry.

Most of the diseases and disorders have a major genetic component. Human diseases and disorders may result from single-gene mutations, but more commonly they are complex as a consequence of multiple gene-gene or gene-environment interactions [60]. The cause of the majority of orodental diseases could be genetically related if infection and traumatic effects are not taken into account. The characteristic signs and symptoms of these diseases indicate genetic origin [61–63], although not all have been clearly identified.

Globally, every year an average of 7% of infants have some mental or physical defect. Among these, 75% are related to craniofacial defects or malformations [64]. Again

it is the dental anomalies that form an integral aspect of such genetic disorders, often representing important clinical clues to the true underlying disorders. Specific examples that are well documented include (1) ectodermal dysplasia [65] with dental manifestations of oligodontia and conical shaped teeth and (2) cleidocranial dysplasia with multiple supernumerary and unerupted teeth [66, 67]. Therefore, it is necessary for dentists to be aware of the clinical characteristics and the possible alterations that are part of the genetic syndromes, so that they can offer patients multidisciplinary and the best possible treatments. Some of the documented examples of these types of direct or indirect genetic alterations causing dental defects are listed in Table 2.

Possibly, iPSC possess the potential for treating such genetic orodental disorders, confining the availability of suitable disease-specific iPSCs from the diseased person which are able to multiply, cooperate and reform the missing or diseased part. Though, multiple types of stem/progenitor cells have been identified based on their ability to repair/regenerate and partially restore organ function in the human body, growing evidence illustrates that stem cells are primarily found in niches and that certain tissues contain more stem cells than others [94].

7. Dental Stem Cell Niches as a Potential Source for Human iPSCs Generation

The foundation of personalized medicine profoundly lies on procuring the most suitable cell sources. In the human body, various cell sources have been shown to be reprogrammed into iPSC. Among these are dermal fibroblasts, the first of the cell types to be reprogrammed into iPSC, followed by other sources like amniotic fluid-derived cells, skin keratinocytes, embryonic stem cell-derived fibroblasts (ESFs), CD34 blood cells, mesenchymal stem cells (MSCs), and dental pulp [7]. However, studies are showing that it is easier to reprogram more immature cells than somatic cells. Hence immense research was carried out to refine the methodology of iPS technology in terms of techniques, efficiency, and cell type choice. It has been reported that reprogramming efficiency for human fibroblasts is relatively low, while the reprogramming process for keratinocytes generates iPSC colonies 100-fold more efficiently and 2-fold faster as compared to human fibroblasts [95]. The probable cause for such efficacy difference is that keratinocytes have expression levels of stem cell-related genes more similar to ESC than fibroblasts [95].

A similar comparable study reported that dental tissue-derived mesenchymal-like stem cells can be reprogrammed into iPSCs more efficiently, when compared to other mature somatic cells from human body such as neonatal foreskin fibroblasts, adult MSCs, and adult dermal fibroblasts [7]. This is probably because of the timing and other factors required for reprogramming a somatic cell to iPS varies greatly depending on cellular context. For example, the reprogramming of MSCs from somatic cell sources mentioned above requires the addition of hTERT (telomerase reverse transferase) and SV40 large-T to turn into iPSCs, whereas dental tissue-derived cells are not confined the same way [7]. Perhaps this emphasizes the use of dental pulp as the most feasible and rich source of mesenchymal stem cells to be used in regenerative therapy, as they are easily available when compared to the tedious collection procedure of other somatic cells.

Dental stem cells can be easily obtained from the pulp of exfoliated primary teeth (SHED) or extracted primary (SCD) and permanent (DPSC), apical papilla (SCAP), tooth germs, and human periodontal ligament. In fact, all these cells can be successfully reprogrammed into iPS cells [94]. A recent report further strengthens the potential of dental-derived stem cells, where reprogramming of human immature dental pulp stem cells (hIDPSCs) was successful within a short-time frame as compared to human fibroblasts, SHED, and DPSC. Furthermore, primary hIDPSC-iPSC colonies were readily obtained even under feeder-free conditions eliminating the possibility of contamination from xenoenvironment [95]. The physiologically intact dental pulp stem cells could be successfully differentiated to advanced derivatives of all three primary germ layers (odontoblast, osteoblast, chondrocyte, myocyte, neurocyte, adipocyte, corneal epithelial cell, melanoma cell, iPSC) (refer review, [94]). Collectively, its multipotency, high proliferation rates, and accessibility make the dental stem cell an attractive source of mesenchymal stem cells for iPS generation. Hence dental-derived stem cells should be considered as a strategy in future regenerative therapies. A schematic representation of the human iPSCs generation from dental stem cells and its applications in various therapeutic approaches is shown in Figure 1.

8. Therapeutic Potentials of Disease-Specific iPSCs for Genetic Orodental Diseases/Disorders

Mutations have been shown to play a dominant part in most orodental diseases as tabulated in Table 2 and these genetically caused diseases are the ones that could benefit the most from iPS technology. One of the main focuses of the present stem cell therapy is genetic correction, which would be a permanent solution. For example the iPSCs has shown its therapeutic capability to treat diseases by correcting the underlying genetic defects, which was successfully demonstrated in mouse models of sickle cell anemia [96]. The defective gene was replaced by wild type β-globin by homologous recombination. Surprisingly, the genetically corrected iPSC-derived hematopoietic progenitor was effective in improving and restoring the physiological function of the diseased animal. This proof of principle was also introduced in human individuals with Fanconi anemia, a disease characterized by severe genetic instability [54]. Hence, this approach can be applied to any genetic disease underlying the human body. Recent studies have also shown the possibilities of developing human endoderm tissue-derived iPSC lines. This, along with other established human iPSCs lines, has provided a base to elucidate the mechanisms of cellular reprogramming and also to study the safety as well as efficiency of differentially originated human iPSCs [27]. Studies on liver pathogenesis using iPSCs technology

Table 2: Human genetic oral diseases/disorders causing dental defects.

Dental disease/disorder	Symptoms	Genetic cause	References
Orofaciodigital syndrome 1 (OFD1)	Malformations of the face, oral cavity, oral clefts, underdeveloped nose flaps, finger abnormalities, hydronephrosis, and variable involvement of the central nervous system.	Mutations in OFD1 gene; mutations in the Cxorf5 gene, located in the Xp22	[68–70]
Oculofaciocardiodental (OFCD)	Canine radiculomegaly; oligodontia, delayed eruption of the dentition, malocclusion, root dilacerations, macrodontia, and enamel defects; microphthalmia and, congenital cataracts with secondary glaucoma	Mutations in the BCOR gene located in the chromosome Xp11.4	[71, 72]
Amelogenesis imperfecta (AI)	Developmental abnormalities in the quantity and/or quality of tooth enamel, occasionally in conjunction with other dental, oral, and extraoral tissues	Mutations in any of the six genes AMELX, ENAM, MMP20, KLK4, FAM83H, and WDR72	[73]
Cherubism	Bilateral bone enlargement of the jaws in childhood; displacement or aplasia of teeth and tooth-germs	Mutations in the gene encoding the binding protein SH3BP2 on chromosome 4p16.3	[74]
Disorders of human dentin: (a) dentinogenesis imperfectas (DI, types I–III) (b) dentin dysplasias (DD, types I and II)	Discoloured teeth (brown-blue or opalescent brown) and structural defects such as bulbous crowns and small pulp chambers	Mutation in dentin sialophosphoprotein gene (DSPP, 4q21.3)	[75]
Periodontal disease	Inflammatory as well as recessive alterations of the gingiva and periodontium	Mutation in interleukin-1 (IL-1) gene	[76, 77]
Hypodontia	Missing one to six teeth (excluding the third molars)	Mutations in transcriptions factors of MSX1 gene in chromosome 4 or another transcription factor gene PAX9 in chromosome 14	[78, 79]
Cleidocranial dysplasia (CCD)	Affects the bones of the face causing a wide skull, a prominent forehead, a flat nose and a small upper jaw; delayed resorption and shedding of primary teeth, delayed maturation, and partial or absent eruption of the permanent teeth combined with ectopic position and development of cysts around the nonerupted molar	Mutation in the RUNX2 (CBFA1) gene found on chromosome six, 6p21.1	[80–82]
Some dermatological syndrome causing oral and dental manifestation			
Congenital erythropoietic porphyria	Hemolytic anemia, photosensitivity (manifested as blistering of the skin), skin fragility, mutilating scarring, hypertrichosis and hyperpigmentation, and deposition of red-brown pigment in the bones and teeth; oral mucosa is pale and the teeth have a red to maroon color	Mutations in the UROS gene which is located in the locus 10q25.2–q26.3	[83]
Ectodermal dysplasias	Characterized by the observation of anodontia and hypodontia of the temporal and permanent dentition, impacted teeth, pin-type dental malformations, enamel hypoplasia, multiples diastemas, and underdeveloped alveolar ridges	Mutation of Xq12–q13.1 (XLHED-gene) and also mutations in the *TP63* gene	[84, 85]
Epidermolysis bullosa	Repeated blistering, the formation of scars, limitation of oral aperture, ankyloglossia, disappearance of the oral and vestibular sulci, perioral stenosis, severe periodontal disease and bone reabsorption, atrophy of the upper maxilla with mandibular prognathism, an increased mandibular angle, and a predisposition to oral carcinoma	Mutations in either the keratin 5 (KRT5) or keratin 14 (KRT14) gene	[86, 87]

Table 2: Continued.

Dental disease/disorder	Symptoms	Genetic cause	References
Gardner syndrome	Epidermoid cysts, desmoid tumors, and other benign tumors; supernumerary teeth, compound odontomas, hypodontia, abnormal tooth morphology, and impacted or unerupted teeth	Mutation in the APC gene located in chromosome 5q21. High-resolution banding analysis showed an interstitial deletion of the long arm of chromosome 5 (q22.1 → q31.1)	[88]
Incontinentia pigmenti	Distinctive swirling pattern of the skin; defects of teeth, hair, and nails; ophthalmic, central nervous system, and musculoskeletal abnormalities	Mutations in the NEMO gene that completely abolishes expression of NF-kappaB essential modulator	[89]
Naegeli-Franceschetti-Jadassohn syndrome	Affects the sweat glands, skin, nails, and teeth; reticulated hyperpigmentation, hypohidrosis, palmoplantar hyperkeratosis, abnormal teeth, and nail dysplasia; abnormally shaped teeth, polydontia, yellow spotted enamel, caries, and early total loss	Mutations in the keratin 14 (KRT14) gene, located on chromosome 17q11.2–q21	[90]
Papillon-Lefevre syndrome	Palmoplantar hyperkeratosis and rapid periodontal destruction	Mutations of a gene that regulates production of an enzyme known as cathepsin C, located on the long arm (q) of chromosome 11 (11q14–q21)	[91]
Sjogren-Larsson syndrome	Congenital ichthyosis, spastic diplegia or quadriplegia, and mental retardation; white dots in the fundus, speech defects, epilepsy, dental problems, and skeletal abnormalities	Mutations in the FALDH (ALDH3A2) gene on chromosome 17p11.2	[92, 93]

have provided a more amenable system to generate liver disease-specific cell lines. The ability to develop such disease-specific stem cell models can be utilized for disease modeling which helps in the study of the complicated pathogenesis and drug screening purposes [27]. Similarly, studies have also been undertaken for neural degenerative diseases like Parkinson's disease and retinal disease (Retinitis pigmentosa; gyrate atrophy) (Table 1).

Most clinical therapies and treatments on disorders of neural, retinal, hepatic, diabetic, bone, and tissue aberrations are mostly focused on only particular tissue aspects of human body. However, some of these disorders have orodental manifestations. Moreover, most of the identified genetic orodental diseases are also encountered with similar problems as those of other disorders. In this context, the use of iPSC therapy for treating such disorders that were applied earlier can also be considered for orodental diseases. The promise of regenerative medicine in orodental disease is reinforced with the potential applicability of stem cell therapy in dentistry, which could provide an ideal solution to certain prevailing problems. For example, an immature tooth with extensive coronal and pulp damage could be reversible through regeneration of tooth tissues. Similarly, regeneration of resorbed root, cervical, or apical dentin, periodontal regeneration, whole-tooth regeneration, repair and replacement of bone in craniofacial defects can facilitate restoring the physiologic structural integrity [97]. For instance, the successful regeneration of periodontal tissue, alveolar bone, cementum, and periodontal ligament has been achieved using autologous periodontal ligament mesenchymal stem cells (PDL-MSCs), with no adverse effect when transplanted [98]. Considering the success of such attempts using tissue engineering techniques, by applying the advanced iPS cell technology, more fruitful advantage can be expected for their use in cell transplantation therapies and gene corrections in orodental disorders.

The regeneration of orodental tissues is dependent on four basic components. The appropriate signals, cells, blood supply, and scaffold that are needed to target the tissue at the site of defect [99]. These four elements play a fundamental role in the reconstruction and healing of lost tissues. The cells provide the machinery for new tissue growth and differentiation, whereas the growth factors modulate the cellular activity and stimulate the cells to differentiate as well as produce tissue matrix [99]. The new vascular tissues provide the nutritional base for tissue growth and the scaffolds guide and create a template structure in three-dimensions to facilitate the tissue regeneration process [99]. Tissue engineering strategies using this basic cell transplantation approach can be successfully applied for a wide variety of oral structures such as bone, periodontal ligament, oral mucosa, skin, and teeth. In addition, such cells can also be genetically modified *ex vivo* by using iPS technology and thereby merging stem cell technology and precision gene therapy, a new therapeutic approach for oral genetic disorders is possible. This impulses the possibility of their use in iPS technology, since they can be utilized not only for dental associated problems, but may facilitate the repair of nondental tissues such as bones and nerves [61, 100, 101].

Hence, if we could attempt the real possibility of *ex vivo* genetic manipulations, iPSCs will be the most powerful therapeutic tools for a variety of dental pathologies which have yet to be investigated. In this regard, it is valuable to establish disease-specific iPSC lines, preferably for the genetic

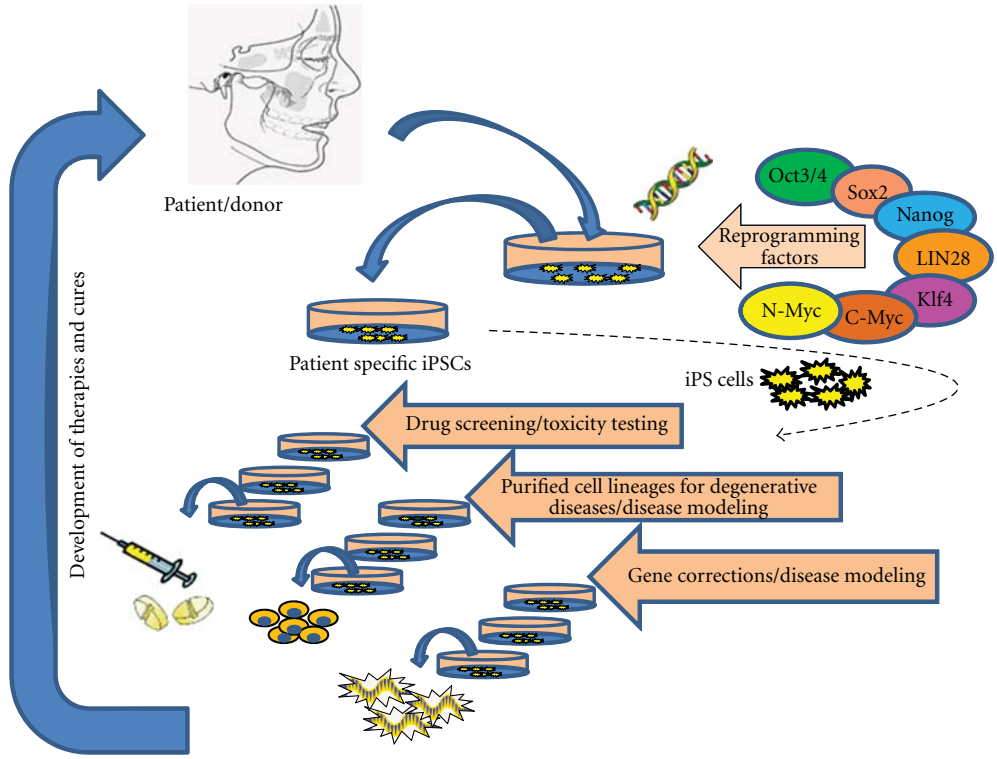

Figure 1: A schematic representation of the human iPSCs generation.

dental disorders to comprehensively evaluate their disease modeling potentials. Therefore fundamental research program is needed to ascertain the application of iPS technology in genetic orodental disorders, which requires extensive programs that can be directed to each aspect of dental diseases and its genetic cause.

9. Conclusion

Though studies have reported the successful generation of disease-specific iPSC lines from individuals with different diseases, effective disease modeling has been demonstrated only by a few studies. The development of iPSC models for orodental diseases is still a new concept. The availability of such iPSC models will lead to better understanding of the nature and behavior of orodental diseases. Possibly the opportunities for the exploration of iPS technology in treating orodental diseases will lead to a significant benefit for the population at large.

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Review Article

Drug Discovery Models and Toxicity Testing Using Embryonic and Induced Pluripotent Stem-Cell-Derived Cardiac and Neuronal Cells

Rahul S. Deshmukh,[1] Krisztián A Kovács,[1] and András Dinnyés[1,2,3]

[1] *BioTalentum Ltd., 2100 Gödöllö, Hungary*
[2] *Molecular Animal Biotechnology Laboratory, Szent Istvan University, 2100 Gödöllö, Hungary*
[3] *Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University 3584 CL Utrecht, The Netherlands*

Correspondence should be addressed to András Dinnyés, andras.dinnyes@biotalentum.hu

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Development of induced pluripotent stem cells (iPSCs) using forced expression of specific sets of transcription factors has changed the field of stem cell research extensively. Two important limitations for research application of embryonic stem cells (ESCs), namely, ethical and immunological issues, can be circumvented using iPSCs. Since the development of first iPSCs, tremendous effort has been directed to the development of methods to increase the efficiency of the process and to reduce the extent of genomic modifications associated with the reprogramming procedure. The established lineage-specific differentiation protocols developed for ESCs are being applied to iPSCs, as they have great potential in regenerative medicine for cell therapy, disease modeling either for drug development or for fundamental science, and, last but not least, toxicity testing. This paper reviews efforts aimed at practical development of iPSC differentiation to neural/cardiac lineages and further the use of these iPSCs-derived cells for drug development and toxicity testing.

1. Introduction

The history of induced pluripotency/cellular reprogramming dates back to the 1950s when Briggs and King developed a technique of nuclear transfer (NT) to test the developmental potential of late-stage blastula cells following transfer into enucleated *Xenopus* oocytes [1]. Later experiments [2, 3] with the transfer of nuclei of differentiated cells into the amphibian oocyte lead to the production of live offspring suggesting genetic/epigenetic reprogramming of the differentiated cells to the totipotent/pluripotent state. In mammalians, the first adult-cell-derived animal, Dolly the sheep, was produced using SCNT only in 1997 [4], strengthening the concept of epigenetic reprogramming of the differentiated mammalian cells to totipotent/pluripotent state mediated by the ooplasm. Several reports later proved that the oocyte contains certain factors which are responsible for the reprogramming of the transferred genome [5, 6].

Moreover the fusion of the somatic cells with pluripotent stem cells such as embryonal carcinoma cells (ECCs) derived from teratocarcinoma [7, 8] was able to induce pluripotency in the somatic cells [9]. This suggests that the pluripotent cells contain certain factors which are responsible for the conversion of the somatic cells into the pluripotent state. Embryonic stem cells (ESCs) derived from the inner cell mass of mouse [10] and subsequently from human blastocysts [11] are considered pluripotent. Mouse ESCs are able to contribute to all kinds of tissues of the live offspring after injection into the blastocysts. The pluripotency of human and mouse ESCs have been shown in vivo by teratoma formation and in vitro by embryoid body differentiation assays to form tissue of the three germ layers (endoderm, ectoderm, and mesoderm) [12]. The expression of key transcription factors such as OCT4, SOX2, and NANOG is mandatory to keep these cells self-renewing and pluripotent. Fusion of ESCs

with somatic cells has led to the conversion of somatic cells from differentiated to pluripotent state.

The conclusion from the above observations is that the pluripotent cells have certain regulatory pathways involving powerful transcription factors which are sufficient to revert the somatic cells to a pluripotent state. Screening of 24 different transcription factors by the Yamanaka group [13] surprisingly demonstrated that generation of induced pluripotent stem cells (iPSCs) required a combination of only four transcription factors: OCT4, SOX2, KLF4, and c-Myc (OSKM) [13]. These iPSCs were almost identical to ESCs at the molecular as well as morphological level. Generation of iPSCs has fundamentally changed stem cell research considerably. In the case of iPSCs derived from somatic cells, the ethical issues and immunocompatibility problems arising from use of ESCs for cell therapy can be avoided. Mouse and human iPSCs can therefore be used to study the early developmental process, disease mechanisms, cell therapy, drug discovery, and toxicity testing assays. Methods developed to produce the iPSCs with no/less genetic modifications are under examination for robustness and ease of use.

2. Methods to Produce iPSCs

Since the description of the first iPSCs from mouse [13] and human [14], several optimized methods have been developed to produce iPSCs from various tissues and species [15–20]. The c-Myc from the original OSKM factors was thought being responsible for tumorigenicity [21] and thus affecting the potential clinical use of the cells. Consequently, efforts were made to screen more transcription factors, and the iPSCs were produced using a set of transcription factors OCT4, SOX2, NANOG, and LIN28 suggesting alternative factors affecting the pathways needed for reprogramming [22–24]. iPSCs can also be generated with fewer transcription factors [25]. Initially the technology involved the use of retroviral or lentiviral vectors for the transduction of the reprogramming factors. This technique leads to the integration of the viral vectors in the genome causing insertional mutations, and these cells are not likely to be acceptable for the clinical purposes. This problem was partially overcome by the use of a single polycistronic lentiviral vector carrying all OSKM factors reducing the number of insertions in the genomes [26, 27]. Furthermore, the use of nonintegrative viral vectors has also been suggested [28].

Options towards delivering the reprogramming factors with less or no genetic modification include the use of LoxP sites and Cre-induced excision of the transgenes—this has been achieved successfully—while in case of transposon-mediated gene transfer the suggested excision by transposase of the integrated vector sequences has never been published [29–31].

Protein transduction can completely replace the need for gene delivery for the generation of iPSCs. The conjugation of proteins with the short peptides responsible for cell penetration can be used for delivery of the proteins into the cells. Mouse and human iPSCs were generated with this approach using purified polyarginine-tagged OCT4, SOX2, KLF4, and c-MYC proteins [32–34]. Yamanaka factors have also been introduced by an mRNA-transfection method, Although this approach is reported to be more efficient and is not based on integration into the host genome, there is still a very low but not negligible risk of genetic alteration given that the exogenous substance introduced into the cells is nucleic acid [35].

Though until recently the protein iPSCs was considered as the best approach, the use of microRNAs (miRNAs) with or without transcription factors has been shown to be an efficient method to generate these cells [36, 37]. The generation of iPSCs using only miRNAs without any transcription factors has created an interesting puzzle concerning the mechanisms of reprogramming [37]. Notwithstanding the enigmatic mechanism behind the miRNA-mediated reprogramming, presently this method offers the highest published yield of reprogrammed cells and does not modify the genome of the cells—qualifying miRNA-reprogrammed cells for potential clinical applications. Nevertheless, further evaluation and confirmations by independent teams will be needed to demonstrate the superiority of any given method—although all of them become commercially available (and patent protected) within months of their first publication, often without independent validation.

3. ESCs and iPSCs in Drug Discovery and Toxicity Testing

Developing reliable systems to study drug toxicity is a major challenge for developing new and safe drugs for the treatment of humans. Currently, toxicological testing is based on the established immortal cancer cells lines containing chromosomal abnormalities, primary explanted somatic cells, and laboratory animals. Immortalized cell lines, showing several features reminiscent of cancer, mimic neither the normal physiological status nor the diseased state of the organism in vivo. The heterogeneity of primary explant cultures leads to inconsistent results and low reproducibility in toxicity testing. Using live animal models for toxicity testing may not mimic the human physiology, can raise ethical/animal welfare concerns, and is rather expensive. Research on ESCs and iPSCs promises to enhance drug discovery and development by providing simple, reproducible, and cost-effective tools for toxicity testing of drugs under development and, on the other hand, for studying the disease mechanisms and pathways [38–40]. Modeling human disease in standardised cell culture and the opportunity for high throughput drug screening are potential advantages of using iPSCs [38]. Patient-specific iPSCs could improve the efficiency of drug discovery by helping the identification of drugs effective in specific patient populations.

The ESCs, and to some extent iPSCs, differentiated either into cardiac or into neural cell types have been used widely for drug discovery and toxicity testing. This application is the most advanced and practical use of pluripotent cells; however, the acceptance of major pharmaceutical companies to adopt new approaches and replace the well-established and FDA-approved test methods is a rather slow process [40–43]. Consequently, disease models developed from iPSCs have not been used for the development of drugs that have

reached clinical trials. Some experiments and ongoing efforts will be the focus of the remainder of this paper.

4. ESC- and iPSC-Derived Neurons in Drug Discovery

Neurodegenerative diseases including Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) constitute an increasing burden for society. The mechanisms of neurological disorders like AD and PD is not well known due to the limited accessibility of the diseased tissue. Several iPSC lines were derived from spinal muscular atrophy (SMA) [44], schizophrenia [45], familial dysautonomia (FD) [46], and Friedreichs ataxia (FA) patients [47] to study the disease progression. The differentiation potential of ESCs and iPSCs into functional neurons and glia is known, and the specific culture conditions needed for the differentiation of ESCs and iPSCs into neural lineage for optimization of drug discovery models are more or less established even though need further refinement.

The establishment of in vitro models of ALS by differentiation of ESCs into motor neurons was one of the pioneering examples [48–50] of neurological disease modeling. Transgenic mice carrying human superoxide dismutase mutation (G93A) responsible for ALS were used for ESC isolation. The major observation made by this study was the more rapid death of G93A-positive motor neurons than their wild-type counterparts. Moreover, the astrocytes in the G93A-positive cultures were involved in the secretion of toxic factors causing selective and increased death of motor neurons without having an effect on interneurons. Human ESC-derived motor neurons were also killed by the mutant mouse astrocyte-conditioned medium [49, 51]. This work has demonstrated the potential for successful disease modeling using ESC-derived neuronal and astroglial cells.

A group of neurodevelopmental defect related autism spectrum disorders (ASD) have also been studied using iPSCs [52], including the Rett syndrome caused by the X-chromosome linked MeCP-2 mutation. This mutation causes impaired neural development after one year of age and the affected individual shows symptoms similar to other ASDs. iPSCs generated from the affected individuals were differentiated into GABAergic inhibitory and glutamatergic excitatory neurons. Interestingly, erased X-inactivation of the MeCP-2 during reprogramming was reestablished in the differentiated neurons, an observation that highlights the great potential that exists in disease modeling by pluripotent cells, and also the need of producing the congruent cell-type by differentiation from pluripotent cells for the purpose of disease modeling. After 2 months—in culture no significant effect on survival of the MeCP-2 neurons was observed. However, the number of glutamatergic synapses decreased significantly as observed in the Rett syndrome. Interestingly, treatment of these neuronal cultures with the insulin-like growth factor 1 (IGF-1) has shown an increase in the number of synapses, pinpointing the fact that rescue experiments can successfully be designed based on cultures of neurons harboring a disease-specific mutation. Overall, this demonstrates the ability of patient specific iPSCs in helping understand complex neurodegenerative diseases and to serve as a model for drug discovery.

Another major neurological disorder PD, that affects a subset of midbrain dopaminergic neurons, is a sporadic and late-onset disease. Out of several identified mutations the most common PD associated mutation is in Leucine-rich repeat kinase-2 (LRKK2). iPSCs generated from patients carrying a LRKK2 mutation were differentiated down the neuronal lineage and physiologically active dopaminergic neurons were produced [53]. The comparative analysis of gene expression profile by microarray has shown that neurons produced from the patient iPSCs had higher expression of oxidative stress genes, compared to the control patient iPSCs. Moreover, the protein α-synuclein, responsible for formation of characteristic aggregates in PD was highly expressed [54]. These neurons were found to be susceptible to hydrogen peroxide and 6-hydroxydopamine like stressors. This experiment demonstrated that the patient specific iPSC-derived neurons can be used for neurological disease modeling.

Recently, FD, a genetic disease, caused by mutation in IκB kinase complex-associated protein (IKBKAP) was studied by Lee et al., [46]. The disease leads to the death of neural crest derived neurons in sensory and autonomic ganglia. iPSCs were generated from the affected individual and were subjected to neuronal differentiation. The neural crest precursors derived from these iPSCs showed low level of IKBKAP protein as well as defective migration and neuronal differentiation. The treatment of these cultures with kinetin, a plant hormone has resulted in relatively strong corrective effects on the FD neuronal cells, demonstrating that the iPSC-based drug discovery approach has good predictive value—although the full clinical proof-of-concept is still far away.

Thus, the patient-specific iPSCs offer a unique opportunity for studying and modeling the effects of specific gene defects on human neuronal development in vitro and for testing small molecules or other potential therapies for the relevant genetic disorders of the nervous system.

5. ESC and iPSC-Derived Cardiomyocytes in Drug Discovery

Cardiovascular disease is considered as one of the major and leading cause of death. Since adult cardiomyocytes have a limited regenerative capacity, their loss permanently compromises myocardial contractile function leading to loss of cardiac function and heart failure. Efforts are being made to develop different ways of treating cardiovascular diseases that involves not only perfecting the production of immunocompatible cardiomyocytes but also the establishment of more sophisticated cellular drug discovery and test systems.

Rodent models fail to mimic the basic physiological functions of heart due to their having a much faster heart beat than human; thus they make unreliable animal models to test the drugs for arrhythmias in human. Generation of iPSCs from patients suffering congenital heart disease and their differentiation into cardiomyocytes has been predicted to serve as a model system to study disease pathogenesis and

for drug discovery [55]. ESC- and iPSC-derived functional cardiomycytes can potentially improve the efficiency of the drug discovery and screening process [56–58]. Despite the immature sarcomeric and myofibrillar organization, the ultrastructural and morphological similarities between the in vitro derived cardiomyocytes and adult heart cardiomyocytes make them the better choice model for drug discovery [58–61]. Given that pluripotent cells can be differentiated into cardiac cells that form a functional syncytium in vitro within which action potential propagation is synchronized, previously unavailable models can be engineered for studying the effect of potential cardioactive drugs in the electric conduction in human cardiac tissue [62]. To build such models, which might substitute for whole animal experiments, differentiated cells have to be grown on the surface of multielectrode arrays (MEAs) [63]. The advantages of using in vitro differentiated cardiomycytes include their ability to keep the contractile function thereby providing the homogenous cell culture for screening which ultimately contributes to improved high-throughput drug discovery process. ESCs and iPSCs differentiated in vitro into cardiomyocytes are being used by pharmaceutical companies to screen compounds involved in several biological processes.

6. ESCs and iPSC-Derived Neurons in Toxicity Testing

Toxic effects of chemical compounds, environmental changes, and naturally occurring substances can lead to neurotoxicity which, in turn, leads to temporary or permanent harm to the central or peripheral nervous system. In case of excitotoxicity, a specific form of neurotoxicity, excessive stimulation of the neurons occurs due to spinal cord injury, stroke, or traumatic brain injury during which neurotransmitters like glutamate and similar substances are responsible for the damage and death of nerve cells. Environmental toxicants or pharmaceutical agents can influence such excitotoxic processes and can exaggerate their deleterious effect. Therefore, it is of utmost importance to develop more predictive cell-based models and powerful screening tools for assessing the neurotoxicity of chemical compounds, drug candidates, and environmental agents. Human neurons derived from ESCs and iPSCs can be attractive models to study the neurotoxicity. The ESC- and iPSC-derived neurons exhibit functionality and behavior of mature neurons and are available in large quantities. It is possible to develop live cell assays that allow characterization of neurons and neuronal networks for health and extent of the connectivity. The neurotoxicity test models will allow for studying on one hand the adverse effect of drug candidates on neuronal cells and on the other hand the general neurotoxicity in assays that are well suited for screening of lead compounds and potentially important for reducing animal experimentation and the cost of preclinical development.

One variable that can be used in neurotoxicity tests and can be easily turned into an assay endpoint is the amount and changes of intracellular calcium. Several toxicants have already been shown to have an effect on intracellular calcium homeostasis, which is a reliable indicator of neuronal health and undisturbed function. Developing a system to visualize intracellular calcium levels may shed light on similar toxic effects of previously uncharacterized substances. Currently, cell lines such as PC12 are typically used for the analysis of calcium signaling with the purpose of determining the complex cellular changes triggered by environmental and pharmacologic neurotoxicants [64]. However, scientific consensus exists that this immortalized cell line does not recapitulate the phenotypic features of neurons. Murine primary neuronal cultures could be more adequate to address such questions, but unfortunately the lifespan of such cultures is typically 2-3 weeks, and their establishment requires sacrificing pregnant mice and dissecting their embryos. Several studies have demonstrated the usefulness of calcium measurements in assessing the toxicity of various compounds. For instance, observing a decrease in the depolarization-elicited calcium elevations that accompanies the release of the specific neurotransmitter synthesized by a given neuron can provide valuable information about the toxicity of an uncharacterized compound. A similar approach has been used to reveal the toxic effects of hexabromocyclododecane (HBCD), a substance that is not biodegradable under aerobic conditions and is very toxic to aquatic organisms [64]. Calcium signals can also uncover excitotoxic effects that previously uncharacterized substances might have. Excitotoxicity is accompanied by an abnormally high level of neuronal activation and a concomitant increase in intracellular calcium, for instance, has been found to underlie the neurotoxic effects tributyltin, a substance used as a heat stabilizer, agricultural pesticide, and component of antifouling paints [65]. The relevance of calcium measurements for environmental toxicity is exquisitely exemplified by a study revealing that neurotoxicants such as manganese, lead, and benzo(a)pyrene (a product of incomplete combustion) alter the properties of intracellular calcium waves [66].

Neurotoxicity screens using test systems (TSs) such as two-dimensional neuronal culture with a defined percentage of neuronal subtypes or three-dimensional culture [67, 68] differentiated from pluripotent cells are under development. The TSs are studied by test methods (TMs) defining a set of variables (such as neurite length for example) to measure the changes occurring in response to the tested substance. Reference compounds are typically selected from a list of recommended compounds for developmental neurotoxicity test validation [69]. Comparing cytotoxic or apoptosis-triggering effects of a given compound at different stages of neuronal differentiation can also provide useful information about specific developmental neurotoxicity [70].

7. ESC and iPSC-Derived Cardiomyocytes in Toxicity Testing

Pluripotent stem cells can be spontaneously differentiated into beating cardiomyocyte-like cells. The current cardiac lineage differentiation system from pluripotent stem cells includes use of spontaneous embryoid body (EB) formation in suspension culture, coculture of pluripotent stem cells with mouse endoderm-like cells (END-2 cells), and directed differentiation towards cardiac lineage using defined growth

factors (such as BMP-4 or activin) either in suspension or in monolayer culture (REFs).

Cardiotoxicity can lead to the formation of reactive oxygen species (ROS), apoptosis, altered contractibility, change in cardiac rhythm, and altered cardiac gene expression, which can be life threatening or may lead to long-term alterations of cardiovascular functions. Of the 40% of drug failures during the clinical trials [71, 72], 19% drug withdrawal has been observed due to cardiotoxicities—thus it is extremely important to test the safety and efficiency of the drugs using an appropriate model system [73]. Development of high-throughput technologies (where more than, 100 molecules can be tested) for drug screening, evaluation and toxicity testing can help to identify the best compound, and improve the efficiency of drug discovery, and save economic loss [71].

In many cardiotoxicity cases, a direct interaction of drugs with specific ion channels expressed by the cardiomyocytes leads to alteration in ion conduction through these specific channels. Drug effects on potassium currents could lead to QT-prolongation, potentially fatal arrhythmias and sometimes cardiomuscular damage without affecting ion channels [42, 74]. Cancer chemotherapies might cause cardiomyocyte apoptosis and dysfunction; however, different chemotherapeutics might have different toxicity mechanisms [75, 76]. In pharmaceutical industries, the cardiotoxicity test models are based on cell lines, animal cardiomyocytes, and small/large animal models [40, 41]. Failure of animal tissue model systems to respond to the drugs in a similar manner as human tissue responds has motivated some of the pharmaceutical companies to use human ESCs and iPSCs derived cardiomyocytes for the cardiotoxicity testing [40–42]; it is expected that with time other companies will follow this example.

Several protocols and strategies have been reported for in vitro differentiation of cardiomyocytes from ESCs and iPSCs. These cardiomyocytes are functional in vitro and have responded to the drugs in similar way as fetal cardiomyocytes [77]. Usually, a subset of drugs with known and accepted arrhythmogenic properties can be used to treat the ESC- and iPSC-derived cardiomyocytes for functional characterization. Encouraging results have been obtained with the use of electrophysiology for studying the response of the ESC- and iPSC-derived cardiomyocytes to drug treatment. However, these results have limitations due to the different experimental setup and number of drugs in each study.

Human pluripotent stem-cell-derived cardiomyocytes have been used to study drug-induced QT interval prolongation [41, 78]. The drugs including quinidine D, L-sotalol, cisapride, and terfenadine used in this study were associated with QT prolongation and/or torsade de pointes in humans. Furthermore, drugs such as ketoconazole and verapamil were included as negative controls to demonstrate specificity [41]. A detailed dose-response analysis in which expected effects on QT interval overlapped with prolonged field potential duration demonstrated a good starting point, but to obtain a higher level of confidence in this system and to gain industry acceptance, a larger collection of drugs needs to be evaluated, and the cells have to be produced under standard operating procedures (SOPs) governed by an industry standard quality assurance and quality control system. Alternatively, for the comparison of the drug effects, two recent studies [79, 80] have demonstrated concordance between hiPSC-derived cardiomyocytes and conventional, well-validated, rabbit and canine ex vivo Purkinje fiber models, which are commonly used as follow-up assays. The transmembrane action potential of the ESC- and iPSC-derived cardiomyocytes has been studied using microelectrodes [80]. The drug-induced arrhythmic events were assessed by using reverse use dependence, triangulation of the action potential, and short-term variability of repolarization parameters. The results suggest that the rabbit Purkinje fibers and ventricular-like ESC- and iPSC-derived cardiomyocytes responded in a similar way with regard to the incidence of early afterdepolarization, increased triangulation, and short-term variability of repolarization in response to the human ether-a-go-go–related gene (hERG) channel blocking compound E-4031.

Another study demonstrated that ESC- and iPSC-derived cardiomyocytes provided exceptional pharmacological sensitivity in an action potential assay when compared with rabbit and canine Purkinje fibers [79]. Additionally, these cardiomyocytes resulted in reduced compound consumption, cost and time savings compared to the conventional Purkinje fiber assays. The study also reports that these cardiomyocytes are good detectors of proarrhythmic events and that soon after depolarization were induced by the reference compounds terfenadine, sotalol, cisapride, and E-4031. It is known that the compounds that do not interfere with ion channel functionality can also cause cardiotoxic insults. The ESC- and iPSC-derived cardiomyocytes are considered to be well suited to study the effects of compounds which do not interfere with the ion channel functions but still cause cardiotoxicity, an effect that cannot be revealed by using the conventional cell line and receptor overexpression-based approaches [41].

The ESC- and iPSC-derived cardiomyocytes have recently been used to study doxorubicin-triggered toxicity [81]. Two clinically decisive biomarkers of cardiac damage that are sensitive indicators for doxorubicin-induced toxicity were studied. The ESC- and iPSC-derived cardiomyocytes released detectable levels of cardiac troponin T and fatty acid-binding protein 3 in a dose-dependent manner after doxorubicin induction [82]. Based on the availability of very sensitive and rapid analytical tools for these biomarkers, the assay lends itself well to miniaturization and high-throughput formats. This strategy demonstrates that molecular mechanisms of cardiotoxicity after drug treatment can be studied.

8. Future Challenges and Perspectives

One of the important tasks in using ESCs and iPSCs for drug discovery and toxicity testing is producing large amount of cells with low heterogeneity that behave in a consistent way. The robotic and suspension culture methods for ESCs [83, 84] provide an extensively evaluated and standardized system for ESC culture. Although derivation of iPSCs is considered as interesting and superior model system for the development of drug discovery and toxicity testing model system,

several obstacles related to the reprogramming procedure have to be overcome. To increase the yield of reprogrammed cells, the choice of cells to be reprogrammed is of particular importance, given the variable reprogrammability of the different cell types [24, 85–87] (Tat et al. [20]). Furthermore the choice of reprogramming factors and gene delivery methods will constitute a cornerstone for each and every drug discovery or toxicity testing application.

The next challenge would be to optimize the protocols for the differentiation of ESCs and iPSCs into neuronal and cardiac lineages, though several protocols have been used to produce functional neurons and cardiomyocytes from the ESCs and iPSCs, albeit with varying efficiency. Understanding the different signaling pathways and factors responsible for cardiac and neuronal differentiation of these cells would help to improve the differentiation protocols. Development of more homogenous cell phenotypes after differentiation is a necessity for the development of reliable and well-standardized model system for drug discovery and toxicity testing. Moreover, production of more mature cell types from pluripotent stem cells can be beneficial for use as a model system for adult human organs in pharmacological and toxicological studies. Culture of the differentiated neurons and cardiomyocytes may help to produce more appropriate cell types for testing pharmacological compounds.

In conclusion, the use of ESC- and iPSC-derived neurons and cardiomyocytes for drug discovery and toxicity testing constitute an extremely promising tool when more sophisticated, efficient, and reproducible methods of differentiation and for scaling up are be achieved. The early "proof-of-concept" examples presented in this paper highlights the trend and the potential of the approach; however the current industrial and medical practice is still based on the properly validated "old" technologies. Further major efforts and commitment will be necessary to fulfill the expectations towards pluripotent stem cell-based systems—international efforts including the recent European Union 7th Framework Programme "Innovative Medicine Initiative" calls for human iPSC-based drug and toxicity testing systems hold good promise to finance and stimulate such developments.

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Research Article

Temporal Requirements of cMyc Protein for Reprogramming Mouse Fibroblasts

Corey Heffernan,[1] Huseyin Sumer,[1] Luis F. Malaver-Ortega,[1] and Paul J. Verma[1,2]

[1] *Cell Reprogramming and Stem Cells Laboratory, Centre for Reproduction and Development, Monash Institute of Medical Research, Monash University, 27-31 Wright Street, Clayton, VIC 3168, Australia*
[2] *South Australian Research and Development Institute, Turretfield Research Centre, Rosedale, SA 5350, Australia*

Correspondence should be addressed to Paul J. Verma, paul.verma@monash.edu

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Exogenous expression of Oct4, Sox2, Klf4, and cMyc forces mammalian somatic cells to adopt molecular and phenotypic characteristics of embryonic stem cells, commencing with the required suppression of lineage-associated genes (e.g., *Thy1* in mouse). Although omitting cMyc from the reprogramming cocktail minimizes risks of uncontrolled proliferation, its exclusion results in fold reductions in reprogramming efficiency. Thus, the feasibility of substituting cMyc transgene with (non-integrative) recombinant "pTAT-mcMyc" protein delivery was assessed, without compromising reprogramming efficiency or the pluripotent phenotype. Purification and delivery of semisoluble/particulate pTAT-mcMyc maintained Oct4-GFP$^+$ colony formation (i.e., reprogramming efficiency) whilst supporting pluripotency by various criteria. Differential repression of Thy1 by pTAT-mcMyc ± Oct4, Sox2, and Klf4 (OSK) suggested differential (and non-additive) mechanisms of repression. Extending these findings, attempts to enhance reprogramming efficiency through a staggered approach (prerepression of Thy1) failed to improve reprogramming efficiency. We consider protein delivery a useful tool to decipher temporal/molecular events characterizing somatic cell reprogramming.

1. Introduction

Forced expression of four key transcriptional regulators, Oct4, Sox2, Klf4, and cMyc, converts mammalian somatic cells to "induced pluripotent stem cells" (iPSCs), that satisfy all pluripotent criteria of embryonic stem (ES) cells [1–3]. The reprogramming of fibroblasts occurs sequentially, commencing with requisite suppression of lineage-associated genes [4, 5]; *Thy1* (CD90) is a glycosylphosphatidylinositol-anchored plasma membrane glycoprotein expressed in murine fibroblasts and commonly used as a lineage gene marker in reprogramming literature [5–7]. Although required for endogenous lineage gene suppression [8], exogenous cMyc expression is dispensable for the induction of pluripoten-cy, and its omission from the reprogramming cocktail favourable given its link to oncogenesis. However, fold-reductions in reprogramming efficiency commonly result (potentially due to maintenance of endoderm gene regulators and failure to activate microRNA clusters beneficial to reprogramming; [9–11]). Thus, application of nonintegrative cMyc conceptually circumvents risks of oncogenesis whilst utilising beneficial effects in regards to lineage gene suppression.

Application of fusion protein incorporating (i) a cationic polyarginine tag (for transduction across plasma membranes) and (ii) cMyc sequence, in combination with the other reprogramming proteins, to target cells *in vitro* successfully reprograms murine and human cells to pluripotency [12, 13]. These studies purified denatured protein from bacterial inclusion bodies before refolding and application to target cells (micromolar concentrations) [12], or applied unknown concentrations of whole protein extract from induced human cells without purification [13]. Initial attempts to purify recombinant proteins incorporating (i) reprogramming factor domains fused to (ii) a similarly arginine-rich basic domain (^{49}**R**KK**RR**Q**RRR**^{57}) of HIV transactivating transcriptional-activator (Tat) protein from

bacterial inclusion bodies under denaturing conditions encountered problems with restriction to endosomes in target cells following transduction [14]. Binding of the Tat transduction domain to plasma membrane-bound heparan sulfate proteoglycans initiates transduction through caveolar ("lipid raft") endocytosis; translocation to the nuclear compartment soon follows via an importin protein-independent mechanism [15–20]. However, subsequent studies have demonstrated cytoplasmic release of active recombinant protein [21].

Here, we implemented a similar strategy to dissect the early molecular mechanisms of iPSC derivation, namely, the contribution of each reprogramming factor in suppression of the murine fibroblast lineage gene Thy1 that characterizes reprogramming of transgenic (Oct4-GFP) mouse embryonic fibroblasts to iPSC [22] and exploited the ease of manipulation of protein delivery to attempt to maximize reprogramming efficiency through a staggered approach of reprogramming factor expression/exposure. Conceptually, advantages associated with utilizing protein delivery to dissect molecular mechanisms of reprogramming include its ready reversibility, allowing transient treatment of known and/or bolus quantities of protein, and the ability to circumvent the lag in transcription and translation inherent in constitutive and inducible proviral strategies. We further describe an alternative (fusion) protein purification approach, purifying and concentrating soluble (nondenatured) pTAT-mcMyc protein from induced bacterial cells, and deliver particulate/semisoluble protein of known concentrations.

2. Materials and Methods

2.1. Materials. All reagents were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise stated. All sequencing was performed at the Gandel Sequencing Facility, Monash Institute of Medical Research, Australia.

2.2. Methods

2.2.1. Mice and Animal Ethics. Experiments were approved by the Monash University Animal Ethics Committee and satisfied Australian National Health and Medical Research Council (NH&MRC) guidelines for animal experimentation. MEF were harvested from 13.5 dpc OG2 × OG2 mice harboring a GFP reporter expressed from Oct4 proximal and distal enhancers and the Oct4 promoter proper [23]. All experiments were conducted using MEF passage 3.

2.2.2. Construction of Tat Expression Vectors. PCR products were amplified (High Fidelity PCR protocol; Roche, Australia) from pMXs plasmid template encoding cDNA for mouse cMyc (mcMyc; Addgene, USA). Primers contributed restriction enzyme digest sites with adjacent linker DNA and stop sequences (where applicable) to PCR product (outlined by supplemental Figure 1C of the supplementary material available online at doi: 10.1155/2012/541014). Amplified PCR products and pTAT expression vector (generously provided by Dr Stephen F. Dowdy, University of California/Howard Hughes Medical Institute, USA) underwent overnight digestion (4°C) with restriction enzymes *EcoR1* and *Xho1* (Biolabs, Australia) before agarose gel purification and isolation (QIAgen Australia). Digested PCR products and pTAT plasmid were ligated with T4 DNA ligase (Promega, Australia) via manufacturer's conditions (overnight, 4°C), before electro-transformation to DH10B competent cells (BioRAD, Australia). Transformation preparations were spread to agar plates under 50 μg/mL kanamycin selection and clones screened for successful ligation by sequencing (T7 sequencing primer).

2.2.3. Expression, Purification, and Concentration of 6XHis-Tagged, pTAT-mcMyc Fusion Protein. Ligated pTAT-mcMyc plasmid, and native pTAT plasmid (control) were electrotransformed to BL21 (DE3) competent cells (Stratagene, USA) and spread on agar under kanamycin selection. Clones were expanded in LB Broth (made in house) containing kanamycin selection and sequenced again. Protein expression was performed by 0.1 mM isopropyl β-D-thiogalactosidase-(IPTG-)induced protein expression (230 rpm agitation overnight, 37°C). Initially, we purified soluble and insoluble (requiring purification of denatured protein) fractions of pTAT-mcMyc and control pTAT protein before analysis by Western Blot (purified Ni-NTA columns; QIAgen, Australia). We confirmed pTAT-mcMyc protein predominantly present in the soluble fraction by Western Blot (outlined below).

To purify soluble pTAT-mcMyc and pTAT control proteins, bacteria were lysed (1% Triton X-100, 0.1 mg/mL Lysozyme in 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and incubated at 37°C for 1 hour, and on ice for 30 mins. RNase (5 μg/mL; Invitrogen, Australia), DNase (2 units/mL; Promega, Australia) in 1 mM $MgCl_2$ and phenylmethylsulfonyl fluoride (1 mM) were added before sonication on ice. Cells were further homogenized through a 23-gauge needle before centrifugation (9000 xg, 30 mins, 4°C). The supernatant was collected for purification/elution through Ni-NTA columns via manufacturer's instructions.

To concentrate protein preparations, four-part ice-cold acetone was added to one part purified protein (v/v) and incubated at −20°C for 30–45 minutes. Following centrifugation at 6800 xg for 10 minutes (4°C), proteins pellets were resuspended in 50 μL sterile H_2O or PBS. Molar concentrations of proteins were calculated against known protein standards with the colorimetric BioRAD $\frac{D}{C}$ Protein Assay (for pTAT control protein; BioRAD, Australia), or by protein spectrophotometry (for pTAT-mcMyc).

2.2.4. Western Blot. Reduced and denatured pTAT-mcMyc and control pTAT protein was electrophorated (90 V, ~2 hour, 4°C) through 12–15% denaturing polyacrylymide gel and either (a) fixed in 10% methanol /7% acetic acid before staining with SYPRO Ruby whole protein stain (BioRAD, Australia) and visualization under UV light, or (b) blotted to PVDF membrane (Millipore, Australia). Blotted wet membrane was blocked with Odyssey blocking Buffer (Odyssey, Australia) and probed with anti-6xHis-tag primary antibody (1 : 2500; Sapphire/Abcam, Australia) or anti-mcMyc (target sequence CSTSSLYLQDLSAAASEC) primary antibody (1 : 50, Sapphire/Abcam, Australia). Primary antibodies were

detected with the anti-mouse Alexa fluor-680 secondary antibody (Molecular Probes; Invitrogen, Australia) for 1 h at room temperature. Test and negative control membranes (secondary antibody only) were visualized on an Odyssey InfraRed Imager (LI-COR Biosciences, Lincoln, NE, USA; intensity: 3–10, quality: medium, resolution: 169). Visualized protein bands were compared to predicted molecular weights for each protein calculated with EXpasy Software (http://web.expasy.org/compute_pi/).

2.2.5. Immunocytochemistry. To confirm translocation of pTAT-mcMyc and pTAT control protein to the nuclear compartment of treated cells, we performed fluorescent immunocytochemistry according to standard methods. 2×10^4 MEF were plated to coverslips before 100 nM pTAT-mcMyc or pTAT control protein was applied and incubated at 37°C for 1 hour [24]. Cells were extensively washed in PBS, fixed in 4% paraformaldehyde (prepared inhouse) and blocked in 2.5% skim milk/2.5% goat serum/PBS before labeling with 6xHis (1 : 1500; Sapphire/Abcam, Australia). For confirmation of expression of pluripotency markers, iPSCs were labeled with SSEA1 (dilution 1 : 100; Chemicon/Millipore, Australia). Bound primary antibody was detected with Alexa Fluor 488 or 555 (1 : 1500; Molecular Probes/Invitrogen, VIC, Australia). Cell nuclei were detected with 1 mg/mL Bisbenzimide Hoechst 33342. Images were taken using confocal microscope with FluorView software (version 1.5 or 4.5).

2.2.6. Induction of Pluripotency in MEF OSK ± pTAT-cMyc Protein Treatment. iPSCs were derived from OG2 MEF via established protocols, using pMXs retroviral vectors [1, 22]. Briefly, 3.8×10^4 MEF (12-well plates) were infected with retrovirus harboring mOct4, mSox2, mKlf4 (denoted OSK forthwith) ± mcMyc packaged in Platinum-E cell [25]. Parallel infection of MEF with a GFP-reporter transgene confirmed ≥80% infection efficiency. One-part FP media (10% (v/v) fetal bovine serum (FBS; JRH, Australia), 0.5% (v/v) penicillin/streptomycin in DMEM) replaced mcMyc retrovirus where applicable. After 24-hour incubation, virus containing media was replaced with standard ES cell culture media (designated day 0, see Figure 2(a); 15% FBS, 1% (v/v) Non-essential amino acids (Invitrogen, Australia), 1% (v/v) glutamax (Gibco, Australia), 0.1% (v/v) β-mercaptoethanol (Gibco, Australia), and mouse LIF (Millipore/Chemicon, Australia). ES media was changed daily for 12 days. At day 12, suitable clones of each condition were picked and expanded for future analysis.

To avoid pH-related protein denaturing events, ES media was equilibrated at 37°C/5% CO_2 for ≥1 hour before 100 nM semisoluble pTAT-cMyc or control protein was added to applicable conditions. On days 5, 7, 9, and 12 after infection (PI), Oct4-GFP$^+$ colonies were counted or cells collected for flow cytometry (outlined below).

2.2.7. Alkaline Phosphatase Expression Analysis. Alkaline phosphatase expression was confirmed in 4% paraformaldehyde fixed iPSCs colonies via standard protocols (Millipore, Australia).

2.2.8. Teratoma Formation Assay: Hind Leg Injection. Approximately 1×10^6 iPSCs (clone TATc1) were injected into the hind leg of 2x SCID mice. Teratomas were harvested 6–8 weeks after injection and sectioned for haemotoxylin/eosin staining (Histology Facility, MIMR) and visualization.

2.2.9. Flow Cytometry. Detached cells were blocked in blocking buffer (1-2% bovine serum albumin/PBS) for 15 minutes at room temperature before primary antibody was added (4.8 μg/mL Thy1-PE; eBioscience, Australia). Following 30–45 minute incubation, cells were washed 3 times in blocking buffer and resuspended in PBS (without Ca^{2+}/Mg^{2+}). Flow cytometry and analysis was performed on Becton Dickinson BDCanto II Flow cytometer (Becton Dickinson, Australia).

2.2.10. Embryo Aggregation. Zygotes (0.5 dpc) were isolated from ampullae of mated female F_1 mice and cultured in droplets of KSOM media (Chemicon/Millipore, Australia) until they developed to the compacted morula stage at 2.5 dpc. *Zona pellucidae* were digested from embryos with a short incubation in Acid Tyrodes solution (pH 2.5) before aggregation with 10–15 TATc1 iPSC in depressions in culture dishes formed with darning needles [26]. Embryo/cell aggregates were cultured until blastocyst stage (4.5 dpc) and assessed for contribution of GFP$^+$ cells to inner cell mass of aggregated embryos.

2.2.11. Mycoplasma Testing. Culture media was supplemented with 100 nM pTAT-mcMyc or pTAT-control and incubated for 24 hours at 37°C. Media was collected and tested for the presence of mycoplasma. Mycoplasma testing was performed with MycoAlert Mycoplasma Detection kit (Lonza Rockland Inc, ME USA) at MIMR Histology Core facility.

2.2.12. Statistical Analysis. Where variances of OSKM and other treatment groups were sufficiently different (Tukey's test), one-way ANOVA was performed on log-transformed data. Means of duplicate wells for experimental repeats of OSK and OSK + pTAT-mcMyc (Figure 2(d)) indicated within-day variability. One-way ANOVA was performed on raw data (equal variances) or log-transformed data (unequal variances) for normalized Thy1$^+$ cells of each treatment group (http://faculty.vassar.edu/lowry/t_ind_stats.html).

3. Results

3.1. Individual, and Combinations, of Reprogramming iPSC Factors Downregulate Thy1 to Varying Degrees. To highlight the effect of each combinations of reprogramming factor/s to Thy1 repression over 12 days, MEFs were infected with retrovirus harboring transgenes for individual or combinations of reprogramming factors before assessment for Thy1 expression by flow cytometry at day 12 ($n = 3$–5). Unsurprisingly, OSKM-expressing cells almost totally downregulated Thy1 over 12 days. All individual reprogramming factors, and combinations of ≤3 factors, suppressed Thy1 between approximately 50 and 75% of MEF over 12 days

(Figure 1), although insignificantly different from each other. Although OSKM expression almost entirely extinguished Thy1 expression over 12 days, the capacity of individual Klf4 or cMyc factors to suppress Thy1 was not significantly enhanced when both factors were combined with either Oct4 or Sox2. Similarly, no additive Thy1 repression was observed when Oct4 and Sox2 factors were combined, or when cMyc and Klf4 were expressed in the same MEF (Figure 1). This suggests that maximal Thy1 repression can only be achieved in the presence of all four reprogramming factors, and not ≤3 factors (Figure 1).

3.2. Construction of pTAT-mcMyc Expression Vector and Subsequent Protein Expression. Control over expression levels, as well as timing of expression, is limited using retroviral strategies. Therefore we adopted a recombinant protein strategy to dissect the molecular and temporal mechanisms of reprogramming. We amplified cDNA for mouse cMyc (mcMyc) flanked by an N-terminal EcoRI restriction enzyme digest site and a C-terminal XhoI site (linked by a single guanine to maintain in-frame mcMyc sequence; primers outlined in supplemental Figure 1(C)). Following direct restriction digestion of PCR products and subsequent DNA ligation, sequencing confirmed successful, in-frame ligation of mcMyc cDNA insert to digested pET28b TAT plasmid; denoted pTAT-mcMyc forthwith (Figure 2(a)). pTAT-mcMyc and empty vector (control) plasmid were transformed to BL21(DE3) cells for IPTG-induced protein expression. We initially purified pTAT-mcMyc protein under either soluble or insoluble (denaturing) conditions and employed SDS-PAGE to confirm which fraction the recombinant proteins (of predicted molecular weight) was present in (predicted pTAT-mcMyc 53.05 kDa; EXpasy software; http://web.expasy.org/compute_pi/). Protein concentration was equated by colorimetric protein assay or spectrophotometry before freezing aliquoted protein. Reduced and denatured pTAT-mcMyc protein (of both purifying preparations) was electroporated through 12–15% polyacrilymide gel and blots probed with antibodies recognizing either (i) the 6xHistidine leader sequence (Figure 2(b) and supplemental Figure 1(A)), or (ii) amino acids 186–203 of mouse/human mcMyc (Figure 2(b)). Contrary to previous reports, we detect-ed a primary band of purified, histidine-tagged pTAT-mcMyc protein close to predicted molecular weight primarily in the soluble fraction using antibody detecting the leader sequence (Figure 2(b)), with little detectable protein in denaturing conditions (supplemental Figure 1(A)). To confirm specificity of our anti-Histidine antibody, western blots were repeated using antibody directed against mouse/human cMyc; again, protein was detected at the same MW (Figure 2(b)). Expression of control protein of predicted molecular weight (6.099 kDa) was confirmed by His_6 leader sequence detection (data not shown). These results suggest successful construction, expression, and purification of semisolubilized, particulate pTAT-mcMyc and pTAT-control protein close to predicted molecular weight in our bacterial expression system.

Initial experiments confirmed the pH of culture media reduced in alkalinity to near neutral after 1-hour incubation at 37°C/5% CO_2. Therefore, to avoid denaturation of recombinant protein upon application to acidic culture media, neutrality was established prior to application of pTAT-mcMyc application by 1 hour incubation at 37°C/5% CO_2. To ascertain whether eluted pTAT-mcMyc protein (and pTAT control protein) can transduce to the nucleus of MEF, 100 nM of pTAT-mcMyc (or 100 nM control protein) was added to cultures of MEF grown on coverslips and incubated for 1 hour. A concentration of 100 nM was adopted from a survey of previous reports for TAT fusion protein delivery [27–30]. Fixed and permeabilized cells were labeled with antibody recognizing the 6xHistidine sequence. pTAT-mcMyc and pTAT-control proteins were detected primarily confined to the nuclear compartment of treated MEF, (compare "Nuclei" and "6xHis Detection" panels; Figure 2(c)). Minimal protein was observed in cytoplasm/cytoplasmic vesicles, perhaps reflective of recombinant TAT protein within cytoplasmic endosomes or in transit to the nucleus (red arrows, Figure 2(c); [15]). Detection of antibody binding in nontreated cells was minimal, suggesting minimal nonspecific binding of antibody to alternative histidine-rich proteins (data not shown). These results confirm rapid (≤1 hour) and efficient transduction of pTAT-mcMyc (and control pTAT protein) to nuclear compartment of MEF.

3.3. Addition of pTAT-mcMyc Protein to OSK Provirus-Expressing Cells Accelerates Oct4-GFP$^+$ Colony Formation over 12 Days (after Infection). A delayed and reduced efficiency of iPSC colony formation is observed when cMyc is omitted from the reprogramming factor cocktail [9]. We hypothesized that the addition of the pTAT-mcMyc recombinant protein to OSK-expressing MEF would result in a significant increase in Oct4-GFP$^+$ colony formation. In our hands, Oct4-GFP$^+$ iPSC colony formation in OSKM provirus expressing MEF was comparable to OSK-expressing cells up to 12 days, only surpassing OSK colony formation after 12 days (data not shown). Therefore, we infected Oct4-GFP transgenic MEF with retrovirus harboring transgenes for either OSK or mcMyc alone and examined OSK ± pTAT-mcMyc over 12 days. In four separate experiments, we added either 100 nM pTAT-mcMyc or pTAT control protein to duplicate wells of OSK-expressing MEF daily for 12 days and compared Oct4-GFP$^+$ colony counts to OSK-expressing MEF at days 5, 7, 9, and 12 after infection ($n = 4$; Figure 2(d)). The addition of pTAT control protein to OSK-infected cells did not elicit significant improvements in Oct4-GFP$^+$ colony formation over OSK-infected cells at any time point, suggesting the Tat and linker protein sequences had little effect on Oct4-GFP$^+$ colony formation (see green line, Figure 2(d)). MEF treated exclusively with 100 nM pTAT-mcMyc protein alone (Figure 2(d)) and mcMyc transgene-expressing MEF (data not shown) failed to generate colonies at any time points. Statistically significant improvements in Oct4-GFP$^+$ colony formation were observed at day 7 and 9 after infection ($P < 0.05$ and $P < 0.005$, resp.) in OSK-expressing cells treated with 100 nM pTAT-mcMyc protein. This suggests that nuclear-localized pTAT-mcMyc protein is biologically functional and can accelerate iPSC colony formation in OSK MEF.

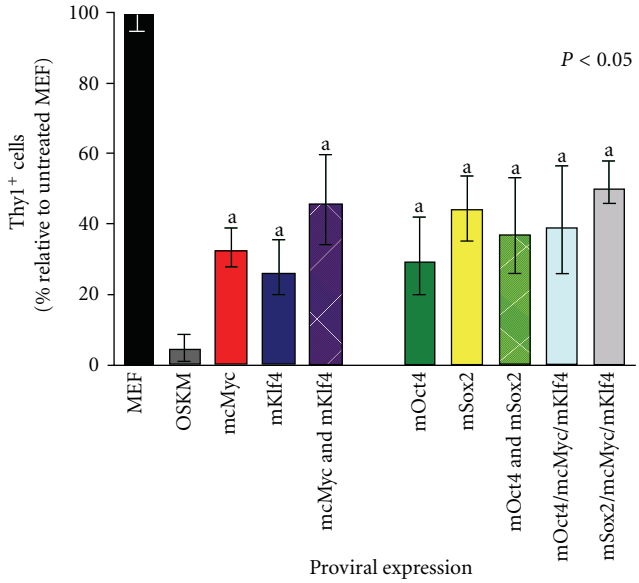


Figure 1: Contribution of individual reprogramming factors to repression of Thy1. MEFs were infected with either all four reprogramming factors (OSMK), individual reprogramming factors (mOct4 or mSox2 or mcMyc, or mKlf4), or combinations of reprogramming factors (mcMyc & mKlf4, OS, OMK, SMK). On day 12 after infection, the percentage of cells expressing the fibroblast marker Thy1 was assessed by flow cytometry in all treatment groups. Mean percentage Thy1$^+$ cells, normalized to untreated MEF controls (black bar) of $n = 5$ experiments ± SEM are shown. Statistical significance established by one-way ANOVA, with "a" denoting groups insignificantly different from each other.

3.4. Confirmation of Pluripotency of OSK$^+$ pTAT-mcMyc-Treated Cells. We expanded 3 × Oct4-GFP$^+$ clones from experiment outlined in Figure 2(d), of which one cell line is chosen on proliferative and morphological characteristics for further analysis (denoted TATc1 forthwith; Figure 3(B). We assessed the pluripotency of the TATc1 cell line by standard pluripotency criteria. We confirmed that colonies express alkaline phosphatase (Figure 3(C)). We confirmed this clone was not contaminated with mcMyc transgene by PCR using genomic DNA template ("T"gene" in Figure 3(D)) and confirmed expression of endogenous Oct4, Sox2, Klf4, cMyc, Rex1, and Nanog ("Endo" in Figure 3(D)). Histological inspection of teratomas formed from intramuscular injection of TATc1 into the hind leg of SCID mice highlighted regions of differentiation characteristic of all three cell lineages, cartilage characteristic of mesoderm, glandular tissue reminiscent of endoderm, and neural rosettes characteristic of ectoderm (Figure 3(E)). Immunocytochemistry confirmed that compact TATc1 (Oct4-GFP$^+$) colonies coexpress stage-specific embryonic antigen-1 SSEA1 (Figure 3(F)). TATc1 cells retained normal karyotype during the reprogramming period (passage 5, 20/20 counts 40XY; Figure 3(G)) and contributed to the inner cell mass of developing blastocysts when aggregated with diploid F_1 4–8 cell embryos (Figure 3(H)). Collectively, these results confirm that OSK + pTAT-mcMyc-treated cells share the hallmarks of pluripotency observed in OSKM- and OSK-induced cells [1, 9].

3.5. Addition of pTAT-mcMyc to OSK Provirus-Expressing Cells Augments Downregulation of Thy1 over 12 Days after Infection. All individual reprogramming factors can repress lineage gene expression (i.e., Thy1) over a 12 days period, with ≥5 days of initial cMyc expression required for efficient AP+ colony formation (Figure 1) [8]. Hence, we adopted a recombinant protein delivery approach to dissect the temporal and molecular mechanisms of somatic cell reprogramming, primarily repression of Thy1 [12, 31]. In an independent experiment, we collected experimental groups on days 5, 7, 9, and 12 ($n = 3$) to assess the percentage of cells expressing the Thy1 by flow cytometry, normalizing results to untreated MEF controls (39.2 ± 4.7%, mean ± SEM in our hands; Figure 4). A gradual downregulation of Thy1 was observed in OSK expressing cells over 12 days post infection (Figure 4). Application of 100 nM pTAT-mcMyc protein alone to MEF elicited a significant downregulation of Thy1 over the initial 5-day treatment period, which was maintained, from 5 to 12 days (Figure 4; $P < 0.05$). Improved (although not significant) Thy1 repression continued to 7 days in MEF expressing OSK and treated with 100 nM pTAT-mcMyc protein, with Thy1 repression maintained to 12 days after infection (Figure 4). Mycoplasma testing of protein-treated culture media confirmed Thy1 repression was not due to contaminating mycoplasma from protein purification (data not shown).

3.6. Five-Day Pretreatment of OG2 MEF with pTAT-mcMyc ± Exogenous mKlf4 Expression for Subsequent mOct4/mSox2-Mediated Reprogramming. Lineage gene repression early in reprogramming is a prerequisite for pluripotency gene activation late in reprogramming [4, 5]. The dispensability of cMyc from the initial 5 days of reprogramming suggests a role in lineage gene repression for this reprogramming factor [8]. Since significant Thy1 repression results from pTAT-mcMyc recombinant protein application (in presence or absence of Klf4 expression; Figure 4), we proposed a staggered approach to initiating reprogramming; a 5-day pretreatment (and therefore "prerepression" of Thy1) of MEF with Klf4 ± 100 nM pTAT-mcMyc recombinant protein could facilitate subsequent accelerated Oct4 + Sox2-mediated reprogramming (assessed by Thy1 downregulation, Figure 5(a); Oct4-GFP colony formation, Figure 5(b)). Therefore, we infected OG2 MEF with retrovirus harboring Klf4 transgene before incubation in the presence or absence of 100 nM pTAT-mcMyc for 5 pretreatment days (designated: 5 days; Figure 5(a)). Alternatively, MEF remained uninfected and untreated for the pretreatment period. After 5 days of Klf4 expression ± recombinant protein treatment (designated day 0), treatment groups and untreated MEFs were (i) collected and analysed by FACS for proportion of Thy1$^+$ cells and (ii) replated for a second infection of retrovirus for mOct4 and mSox2. As controls, nontreated MEF were plated for infection with retrovirus for (i) mOct4 and Sox2 only, (ii) mOct4, mSox2 and mKlf4 only, or (iii) mOct4, mSox2, mKlf4, and mcMyc (Figure 5). FACS analysis for Thy1$^+$ cells was performed

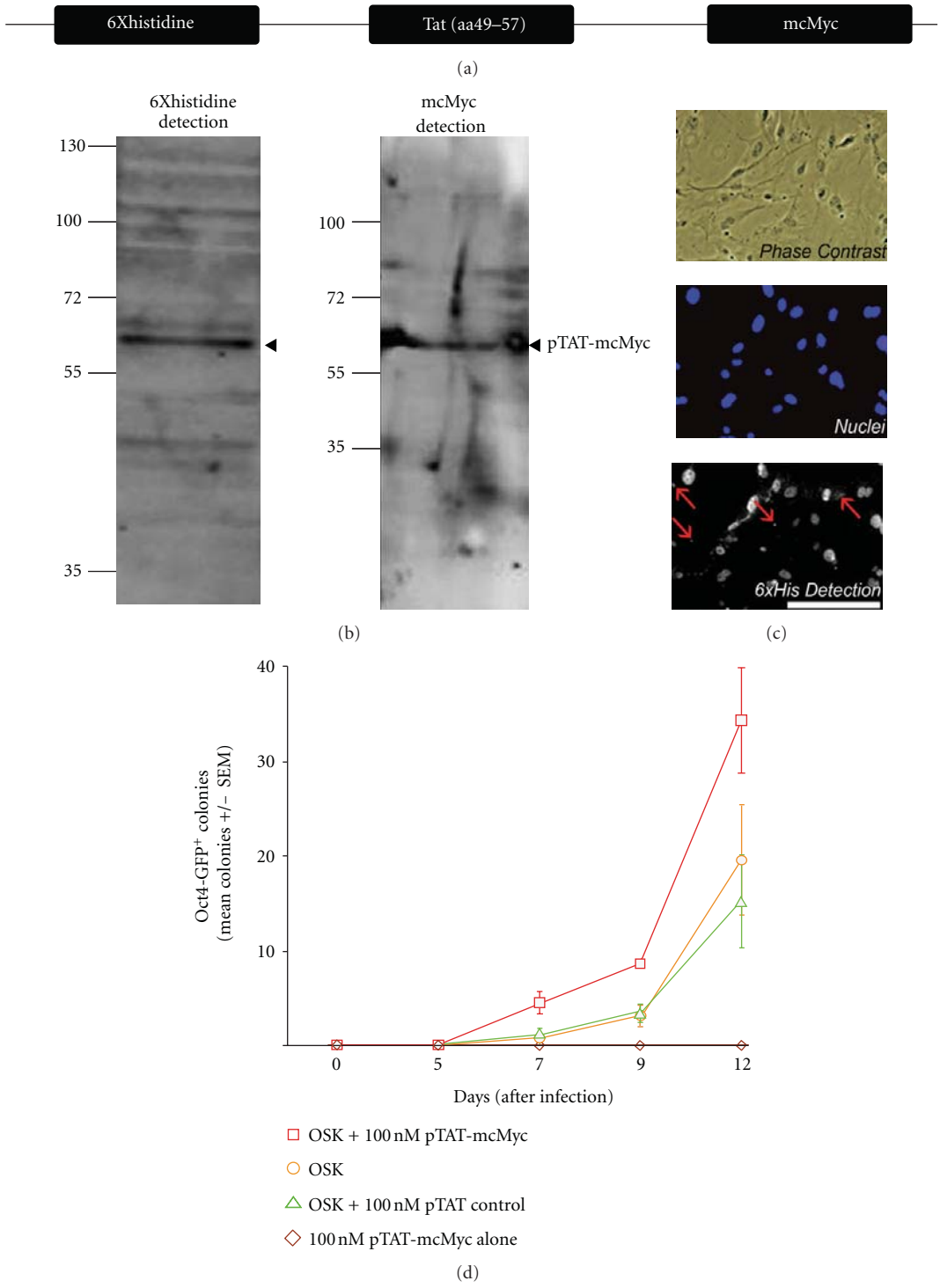


Figure 2: Construction and transduction of of pTAT-mcMyc recombinant protein to MEF *in vitro*. (a) Schematic representation of the pTAT-mcMyc construct. (b) Reduced and denatured pTAT-mcMyc recombinant protein was resolved on 12–15% SDS-polyacrylamide gel and transferred to PVDF membrane by electrophoresis (90 V, 2 hours, 4°C). Wet membrane was blocked for 1 hour in Odyssey blocking buffer (1 : 1 Tris-HCl buffer) and probed with either mouse anti-6xHistidine or mouse anti-mouse cMyc primary antibody before detection with goat anti-mouse Alexa Fluor-680 secondary antibody. Recombinant pTAT-mcMyc was detected at predicted molecular weight (57–60 kDa; arrow). (c) MEFs were plated to coverslips before 100 nM pTAT-mcMyc recombinant protein was applied to equilibrated culture media. Following a 1-hour incubation, MEFs were washed, fixed, blocked, and probed with antibody recognizing 6xHistidine (bottom panel). Cell nuclei were detected with Hoechst dye (middle panel). Scale bar: 200 μM. Red arrows shown detection of pTAT-mcMyc protein outside of detectable nuclei. (d) MEFs were infected with retrovirus harboring OSK or left uninfected. OSK-expressing MEFs were treated with 100 nM pTAT-mcMyc protein (red square) or pTAT-control protein (green triangle) daily for 12 days. Uninfected MEFs were left as a control, or treated with 100 nM pTAT-mcMyc protein (brown diamond) daily for 12 days. On days 5, 7, 9, and 12 after infection, Oct4-GFP$^+$ colonies were counted in duplicate wells. Mean ± SEM of $n = 4$ independent experiments shown; $P < 0.05$.

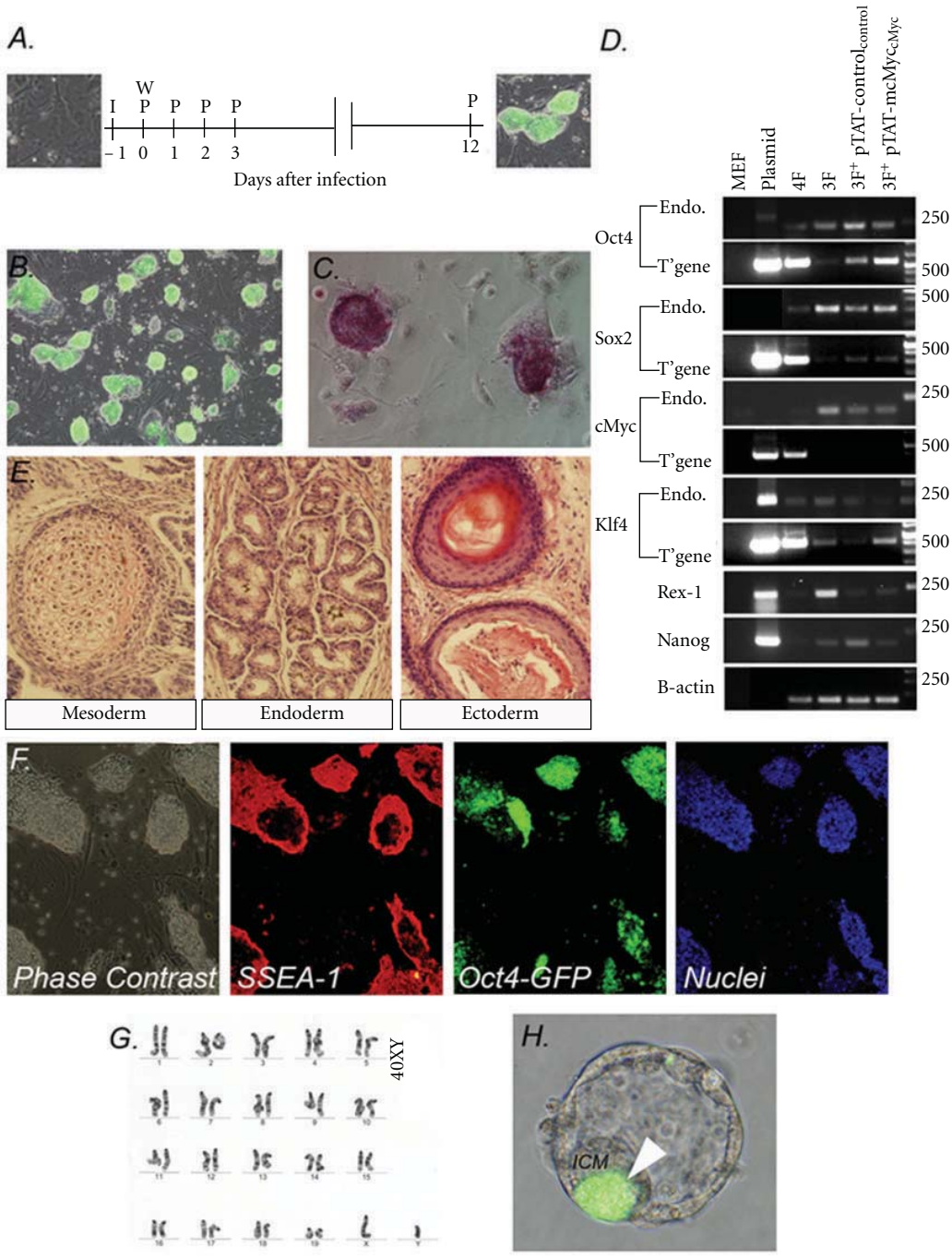


Figure 3: OSK + pTAT-mcMyc treated MEF display the hallmarks of pluripotency. (A) Schedule for pTAT-mcMyc treatment of somatic cells. MEF were infected for 24 hours ("I") before retrovirus was washed from cells ("W") and pTAT-mcMyc and control recombinant protein applied (day 0; "P"). Protein was applied daily for 12 days. (B) Oct4-GFP$^+$ clone Tatc1 from Oct4/Sox2/Klf4 + pTAT-mcMycs treated cells. (C) Confirmation of alkaline phosphatase expression in clone Tatc1. (D) RT-PCR confirms Tatc1 expresses endogenous markers Oct4, Sox2, cMyc, Klf4, Rex-1, and Nanog ("Endo"). We further confirmed this clone was not contaminated with mcMyc transgene by PCR using genomic DNA template ("T'gene"; all oligonucleotides outlined elsewhere) [1]. (E) Following injection into SCID mice, Tatc1 form teratomas with differentiated cells characteristic of the three germ layers (cartilage characteristic of endoderm, glandular tissue reminiscent of mesoderm, and neural rosettes characteristic of ectoderm). (F) Immunocytochemistry confirms Tatc1 expresses pluripotency marker stage-specific embryonic antigen-1 (SSEA1). (G) Reprogramming of MEF to Tatc1 maintained normal karyotype (40XY). (H) Following aggregation with 2.5 dpc embryos, Oct-GFP$^+$ Tatc1 cells contribute to the inner cell mass (labeled "ICM") of subsequent 4.5 dpc blastocysts (arrow).

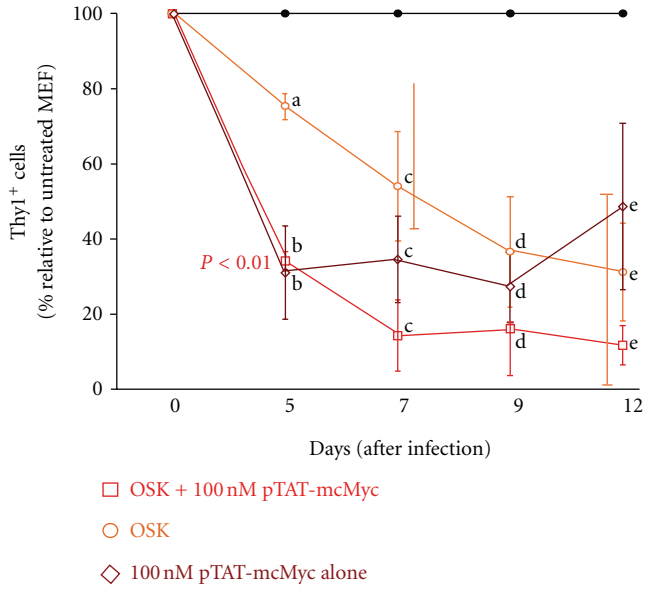
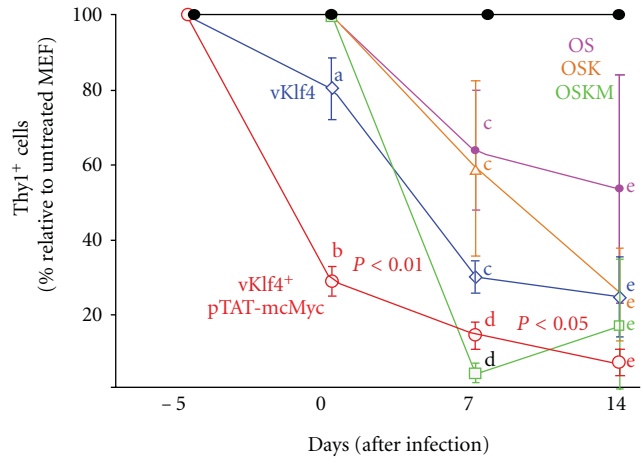
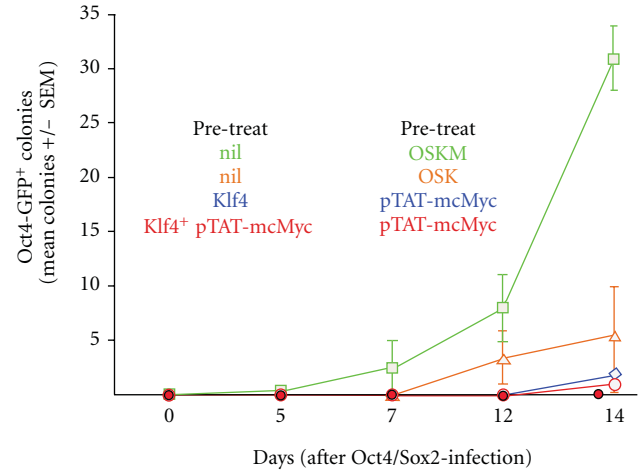


Figure 4: Thy1 downregulation by OSK, pTAT-mcMyc alone or OSK + pTAT-mcMyc at 5, 7, 9, and 12 days after infection. MEFs were infected with retrovirus harboring transgenes for mOct4, mSox2, and mKlf4 (OSK; orange line). 100 nM pTAT-mcMyc recombinant protein was applied to OSK expressing cells (red line), or to non-transduced MEFs (brown line). On days 5, 7, 9, and 12 after infection, percentage of cells expressing the fibroblast marker Thy1 was assessed in all treatment groups by flow cytometry with results normalized to untreated MEF controls (black line). Mean ± SEM of $n = 2$ experiments shown.



(a)



(b)

Figure 5: A staggered approach to application of reprogramming factors. (a) MEF were pre-treated with mKlf4 (provirus) ± pTAT-mcMyc protein (or untreated) for 5 days and infected with mOct4 and mSox2 at day 0. Alternatively, MEF were not pre-treated and infected with mOct4+mSox2, mOct4+mSox2+mKlf4 or all four reprogramming factors (OSKM). The percentage of Thy1+ MEF was ascertained relative to untreated MEF at days 0, day 7 and day 14 post-infection. Statistical significance in percentage of Thy1+ MEF was assessed by one-way ANOVA. Mean ± SEM of $n = 3$ experiments shown. (b) Oct4-GFP+ colony counts for experiments outlined in 6A were counted at days 0, 5, 7, 12, and 14 post-infection. Mean ± SEM of $n = 3$ experiments shown.

on days 0, 7, and 14 after mOct4/mSox2 infection with percentage Thy1+ cells in each treatment group normalized to untreated control MEF. Oct4-GFP colony counts were also counted at same time points to assess effect of pre/post-treatment on reporter gene+ colony formation (Figure 5(b)).

As highlighted in Figure 5, five days pretreatment/expression of (i) exogenous Klf4 and (ii) exogenous Klf4 + 100 nM pTAT-mcMyc resulted in repression of Thy1 in 20–70% of MEF, respectively (designated day 0, Figure 5(a)). Application of 100 nM pTAT-mcMyc significantly enhanced Thy1 repression in Klf4-expressing MEF ($P < 0.01$; Figure 5(a)). Untreated MEF were infected with OS, OSK, or OSKM (pink, orange, and green lines, resp.) and pretreated MEF were infected with OS only, with pTAT-mcMyc pretreatment continuing in pretreated MEF (red line).

After 7-day expression, Thy1 repression in OS- and OSK-infected (only) MEF was modest (in 35–40% of MEF). Thy1 expression was almost completely abolished in MEF infected with OSKM (i.e., not prereated) at day 0. Continued 100 nM pTAT-mcMyc treatment in Klf4 preinfected/Oct4/Sox2 postinfected MEF elicited continued Thy1 repression to day 7 to a level insignificantly different from MEF expressing all four reprogramming factors. Interestingly, preinfection with Klf4 (thus prerepressing Thy1), with subsequent Oct4/Sox2 infection, was equally effective at repressing Thy1 at 14 days than infecting cells concurrently with OSK (compare blue and orange lines; Figure 5(a)).

Concurrent OSKM (significant) and concurrent OSK (insignificant) infection still yielded more colonies than the staggered approach adopted above (Figure 5(b)). In fact, few Oct4-GFP+ colonies were observed for any of the Klf4 ± 100 nM pTAT-mcMyc pretreated/Oct4 + Sox2 postinfected groups. These results suggest that although increased Thy1 repression is achieved through pretreatment with Klf4 ± 100 nM pTAT-mcMyc, concurrent infection with all four reprogramming factors still yields most efficient reporter gene+ colonies at day 14. At 14 days, pretreatment of MEF with exogenous Klf4 + 100 nM pTAT-mcMyc before

Oct4/Sox2 infection at day 0, significantly repressed Thy1 than cells infected with Oct4/Sox2 alone. However, this effect is not reflected in GFP$^+$ colony counts at day 14.

4. Discussion

To highlight the contribution of each/combinations of reprogramming factors, and possible suppressive effects of each capability to repress Thy1, we infected MEF with individual reprogramming factors or combinations of factors and assessed Thy1 at day 12 after infection (Figure 1). Twelve-day expression of each individual reprogramming factors effectively downregulates Thy1 (Figure 1). We failed to observe an additive effect of expressing both Oct4 and Sox2 compared with expressing either factor alone, or expressing both cMyc and Klf4 rather than expressing either factor alone. The capacity of Oct4 or Sox2 to individually downregulate Thy1 was not significantly enhanced when (i) expressed concurrently, or when (ii) cMyc and Klf4 were also expressed in the same MEF (Figure 1).

We adopted a recombinant protein delivery approach to dissect the molecular mechanisms of somatic cell reprogramming. We describe a method for generating semisoluble, particulate recombinant mcMyc protein with an N-terminal linked 11-amino-acid, arginine-rich motif (^{49}**RKKRRQRRR**^{57}) of transactivating transcriptional-activator (Tat) of HIV (Figure 2(a)) [21, 32]. The TAT and mcMyc functional domains were coupled through a peptide linker sequence, reducing interference between these domains [14]. Detection of pTAT-mcMyc protein with antibodies recognizing either the 6xHistidine leader sequence or cMyc protein by western blot analysis confirmed expression and purification of in-frame recombinant protein of predicted molecular weight (Figure 2(b)). Initial experiments suggested pTAT-mcMyc protein was primarily soluble, and particulate when resuspended after acetone precipitation (data not shown). Binding of the Tat domain to heparan sulfate proteoglycan of cells *in vitro* initiates rapid (in the order of minutes) translocation of recombinant protein to the nuclear compartment (Figure 2(c)) [15–20, 33].

Notable differences in protein purification and concentration distinguish published reports from the present study, with recombinant proteins commonly purified from bacterial inclusion bodies before refolding [13, 14]. Kim et al. [12], expressed proteins in transfected human cells and applied unknown quantities of unpurified reprogramming factors in whole protein extracts to targets cells, with neither study concentrating recombinant protein fractions/preparations. Purification of soluble, un-denatured protein in the present study circumvents potential problems associated with misfolding of recombinant protein to numerous alternative (and potentially inactive) conformations, with acetone concentration removing nonprotein bacterial contaminants [14, 34, 35]. Previous attempts to utilize denatured Tat-fusion protein for reprogramming of human fibroblasts were hampered by restriction of protein to endocytotic vesicles [14]. Treatment of target cells in serum-free conditions may also restricted cytoplasmic vesicular release. Purification of semisoluble, particulate pTAT-mcMyc recombinant protein, and/or equilibration of culture media before recombinant protein application, may contribute to escape and/or evasion of our Tat-fusion proteins from such endocytotic vesicles [14].

We applied 100 nM pTAT-mcMyc protein to uninfected MEF or OSK-expressing MEF daily for 12 days and monitored Oct4-GFP$^+$ colony formation (Figure 2(d)) [32, 36, 37]. Contrary to previous reports demonstrating toxicity of 80 nM TAT-DsRED-Klf4 application in MEF [14], we failed to observe adverse effects in MEF cultured in 100 nM pTAT-mcMyc. This may be attributable to cytotoxicity observed in a number of red fluorescent protein (RFP) variants, or unexplained toxicity of Klf4 protein itself at these concentrations. Nonetheless, up to day 5 after infection, ES-like Oct4-GFP$^+$colonies were not observed in any treatment group (Figure 2(d)) [9]. Significantly accelerated Oct4-GFP$^+$ colony formation was observed in OSK-expressing cells treated with pTAT-mcMyc at days 7 and 9 after infection. Clone Tatc1 expressed (Oct4)-GFP and alkaline phosphatase, differentiated to all germ layers in teratomas, expressed pluripotency markers (as assessed by RT-PCR and immunocytochemistry), maintained normal karyotype and was capable of contributing to the inner cell mass of aggregated chimeric embryos (Figures 3(A)–3(H)). This confirms conversion of OSK + pTAT-mcMyc recombinant protein-treated MEF to a fully reprogramming phenotype.

We combined transgene expression ± pTAT-mcMyc recombinant protein delivery to highlight mechanisms of lineage gene repression. Thy1 (CD90) is glycosylphosphatidylinositol-anchored plasma membrane glycoprotein expressed in a variety of cell types (including fibroblast populations) implicated in cell proliferation and apoptosis, cytoskeletal organization, cell-cell/matrix adhesion, and a number of cytoplasmic signaling cascades [6, 7, 38]. Since constitutive expression of OSKM transgenes represses Thy1 in the majority of murine fibroblasts, we exploit the ability to apply controlled concentrations of proteins over defined periods to highlight mechanisms of Thy1 repression [5]. Five days of 100 nM pTAT-mcMyc recombinant protein treatment (±OSK expression) initiates considerable repression of Thy1 expression (Figure 4; as assessed by flow cytometry). OSK transgene expression also elicits downregulation of Thy1, but at markedly reduced efficiency. Surprisingly, combining OSK expression and pTAT-mcMyc delivery failed to elicit additive Thy1 repression on OSK alone. This result suggests that mcMyc primarily mediates Thy1 repression <5 days in the presence or absence of OSK, overriding or "saturating" the moderate OSK-mediated repression or is utilized preferentially when "expressed" in the same cell, thus suggesting Thy1 repression in the initial stages of reprogramming may be rate limiting. Perhaps OSK-mediated Thy1 repression is the default mechanism for early reprogramming, when sufficient concentrations of mcMyc are absent. It is unclear, yet possible, that cMyc directly or indirectly promotes recruitment of histone methyltranferase/s to the Thy1 promoter to initiate transcriptional repression, or disrupt cytoskeletal actin bundles to permit cellular morphological changes and adoption of pluripotent phenotype [38–40]. The transition from Thy1$^+$ to a Thy1$^-$ phenotype

in nasopharyngeal mucosa is a feature of carcinogenesis, and thus suggests that Thy1 represents a candidate as a tumor suppressor [41]. Given the similarities in genetic profile between embryonic stem cells and cancer stem cells, it is unsurprising that repression of tumour-repressor gene function/immortalization markedly increases the efficiency of somatic cell reprogramming [42–44]. Lung fibroblast populations lacking Thy1 have considerably reduced methyltransferase levels ascertained by real-time PCR, with chemically induced demethylation of Thy1$^-$ fibroblasts reinitiating Thy1 expression [39].

A gradual trend (although statistically insignificant) of Thy1 downregulation is observed in OSK expressing cells between 5 and 12 days (Figure 4). By contrast, pTAT-mcMyc protein alone fails to mediate significant improvements in Thy1 downregulation over the same period and is not a significant improvement on OSK-mediated Thy1 suppression. However, addition of 100 nM pTAT-mcMyc protein to OSK provirus-expressing MEF further promotes Thy1 suppression to 7 days after infection, an improvement approaching statistical significance on OSK alone ($P = 0.07$; Figure 4). A cooperative or additive mechanism between 100 nM pTAT-mcMyc and either O ± S ± K may accelerate Thy1 repression from 5 to 7 days.

The reversibility of recombinant protein delivery, as well as ability to control the temporal and concentration aspects of its application, permits the manipulation of the iPS reprogramming methodology with a view to improving reprogramming efficiency. cMyc is required for the initial days of reprogramming only (for AP$^+$ colonies), yet Oct4 is required throughout reprogramming [8]. Since lineage gene repression is an early prerequisite for reprogramming, we hypothesized a staggered approach, that is, prerepressing Thy1 through application of Klf4 ± pTAT-mcMyc with subsequent proviral expression of Oct4 and Sox2, could improve reprogramming efficiency, as assessed by Oct4-GFP$^+$ colony formation. This may also reduce the time required to expose target cells with reprograming factors, in particular Oct4 and Sox2. Although 5-day application of Klf4 ± pTAT-mcMyc significantly repressed Thy1 at the time of Oct4/Sox2 application (day 0), total Thy1 repression was comparable to consecutive 4-factor expression at 14 days (Figure 5(a)). Importantly, pre-repression of Thy1 did not improve Oct4-GFP$^+$ colony formation (Figure 5(b)). Although Thy1 expression is repressed with equal effectiveness over 7 and 14 days by (i) consecutive OSKM expression or (ii) staggered expression Klf4/pTAT-mcMyc and Oct4/Sox2 (Figure 5(a)), it does not translate to Oct4-GFP$^+$ colony formation at any time point (Figure 5(b)). In fact, Oct4-GFP$^+$ colony formation was also delayed in pretreated MEF (Figure 5(b)). These results suggest although Thy1 repression is a pre-requiste for subsequent pluripotency gene activation and successful reprogramming, with cMyc and Klf4 primarily responsible for mediating this early event (Figures 4 and 5) [4, 5, 8], early coexpression of Oct4 and Sox2 is still required for timely and efficient reporter gene$^+$ colony formation later in reprogramming (i.e., 14 days).

To conclude, we have exploited the endocytotic properties of Tat transduction peptide to rapidly transport key transcription factors to the nuclei of Oct4-GFP MEF. Experimental results outlined here suggest mcMyc regulates the initial (<5 days) suppression of Thy1 in MEF, followed by a cooperative mechanism driven by mcMyc/Klf4 from 5–9 days after infection (Figure 4). Protein delivery can be transduced to mitotically active or inactive cells, or cells that are difficult to infect with cDNAs (e.g., mesenchymal stem cells), does not permanently disrupt the host genome and can be transiently applied to drive reprogramming and is rapidly reversible. Therefore, we suggest application of additional Tat fusion constructs at varying concentrations and times will further highlight the events that characterize somatic cell reprogramming. As heparan sulfate proteoglycan display considerable sequence homology between mammalian species and are ubiquitously expressed throughout many cell types, the same Tat fusion constructs could potentially be used in many cells types within and between mammalian species and be used to generate protein-iPS cells in a controlled manner. We anticipate more studies dedicated to varying concentrations of recombinant proteins to highlight threshold levels of reprogramming factors to maximize somatic cell reprogramming.

Conflict of Interests

The authors report no conflict of interests.

Author Contributions

C. Heffernan contributed in performing experiments, intellectual input, and writing of manuscript, H. Sumer took part in intellectual input, writing the paper, L. Malaver contributed in performing experiments, and P. J. Verma participated in intellectual input, writing the paper, and funding.

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Review Article

Nonhuman Primate Induced Pluripotent Stem Cells in Regenerative Medicine

Yuehong Wu,[1,2,3] Anuja Mishra,[1,2] Zhifang Qiu,[1,2] Steven Farnsworth,[1,2] Suzette D. Tardif,[1,2] and Peter J. Hornsby[1,2]

[1] *Department of Physiology and Barshop Institute for Longevity and Aging Studies, University of Texas Health Science Center, 15355 Lambda Drive, San Antonio, TX 78245, USA*
[2] *Geriatric Research Education and Clinical Center, South Texas Veterans Healthcare System, San Antonio, TX 78229, USA*
[3] *Key Laboratory of Ministry of Education for Protection and Utilization of Special Biological Resources in Western China, and College of Life Science, Ningxia University, Yinchuan, Ningxia 750021, China*

Correspondence should be addressed to Peter J. Hornsby, hornsby@uthscsa.edu

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Among the various species from which induced pluripotent stem cells have been derived, nonhuman primates (NHPs) have a unique role as preclinical models. Their relatedness to humans and similar physiology, including central nervous system, make them ideal for translational studies. We review here the progress made in deriving and characterizing iPS cell lines from different NHP species. We focus on iPS cell lines from the marmoset, a small NHP in which several human disease states can be modeled. The marmoset can serve as a model for the implementation of patient-specific autologous cell therapy in regenerative medicine.

1. Induced Pluripotent Stem Cells in Regenerative Medicine

The aims of regenerative medicine are to restore healthy function to organs damaged by disease or aging. A major issue is the source of cells to be used in regenerative medicine. It is often thought to be desirable to use cells derived from the patient himself/herself, because this is hypothesized to avoid the need to administer drugs to suppress immune rejection of the transplanted cells. The possibility of using patient-specific cells in regenerative medicine was greatly expanded by the discovery of induced pluripotent stem cells (iPS cells) [1, 2]. iPS cells can be derived from any somatic cell, but have the properties of embryonic stem cells. Like embryonic cells, they can be used to generate any cell of the body that may be needed in regenerative medicine. It is widely thought that a form of autologous cell therapy will be possible, in which iPS cells would be derived from the patient's cells, in order to provide a source for cells that could be transplanted back to the patient to restore function to the heart, central nervous system, hematopoietic system, or other organs that are affected by disease or aging. The present experiments concern the development of nonhuman primate models for autologous cell therapy based on iPS cells.

2. Autologous versus Allogeneic Cells in Cell-Based Therapies

Any consideration of the implementation of regenerative medicine for human subjects must assess the source of the cells used in the therapy [3, 4]. Following the discovery of iPS cells, it was almost immediately realized that this discovery opened the way to autologous cell therapy. A review in 2007 stated: "If this method can be translated to humans, patient-specific stem cells could be made without the use of donated eggs or embryos" [5]. It is assumed that if the cells are accepted as "self" then they would represent the best possible functional outcome of a transplant: cells that function in their natural environment, without eliciting chronic immune or inflammatory reactions, and without the problems that would result

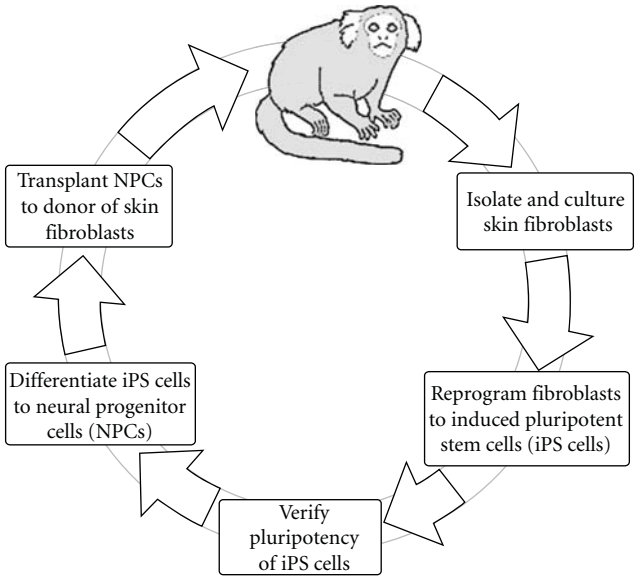

Figure 1: The marmoset as a preclinical model for patient-specific iPS cells in regenerative medicine. This scheme outlines progress to date and future studies of autologous cell transplantation using reprogramming and redifferentiation to a specific cell lineage. A skin biopsy is taken from an individual marmoset, and fibroblasts from the biopsy are grown in culture. Reprogramming factors are expressed in the cells; over a period of several weeks, clones of cells arise that may be iPS cells. Clones are isolated and screened to determine whether they are properly reprogrammed iPS cells; if so, they are expanded and cryopreserved. Neuronal progenitor cells (NPCs) are derived from these iPS cells via protocols described in the text. If the NPCs pass stringent tests of differentiation potential and safety, in the future they may be implanted into the CNS of the same individual from which the cells were originally derived.

from the use of immunosuppressive drugs. They would, in other words, be the "gold standard" for the best possible results of therapy based on cell transplantation. While allogeneic cells might produce an acceptable result for the patient, autologous cell transplants would provide the standard by which the results of allogeneic cells could be judged.

Shortly after the discovery of iPS cells, the technology was used in a tour-de-force study in which iPS cells were derived from a strain of mice that model human sickle cell anemia. The genetic defect was corrected in the iPS cells and they were transplanted back into mice of the same strain following differentiation to hematopoietic stem cells [6]. The symptoms in the treated mice were substantially ameliorated. This was the first demonstration of the potential power of iPS cell-based therapy. As these cells were derived from, and reintroduced into, mice of the same strain, they are an example of the use of syngeneic cells, rather than truly autologous cells. Subsequently, another study suggested that syngeneic iPS cells and their cell progeny may, in fact, elicit an immune response [7]. This unexpected finding has not yet received a satisfactory explanation. At the date of writing, the question of the immunogenicity of iPS cells and derivatives has only been addressed in mice, and not yet in more translationally relevant species, including primates.

Would therapeutic approaches based on the use of autologous cells be worth the considerable efforts of development and implementation that would be required? The answer at the moment is quite unknown. First, in the absence of suitable translational models, or actual clinical trials of iPS cell-based therapy, the advantages must remain theoretical. We do not know how much better, or not, therapy based on autologous cells would be in comparison to therapy based on allogeneic cells. Possibly, autologous cells will prove to be superior, but perhaps there will be little difference from allogeneic cells. In some therapies, the need for a very rapid treatment would preclude the use of autologous cells. For example, in stroke, due to the need for immediate treatment, "off-the-shelf" cells would be needed and iPS cells are unlikely to be useful. However, understanding whether immune-matched versus mismatched cells would have an advantage in a stroke model would be of great significance.

Second, it is extremely hard to predict how easily-implemented iPS cell-based therapy would eventually become. When iPS cells were first made from skin fibroblasts in 2006-2007, reprogramming was highly inefficient and laborious. Over the last 4 years, there has been astounding progress in terms of better, simpler protocols and increases in efficiency [8–11]. Given that there are no reasons to think that the process should not continue to undergo such improvement in efficiency, it is quite possible that the creation of iPS cells from a patient's cells would become quite routine and inexpensive at some time in the future. Similar dramatic improvements in efficiency and cost have been seen in other biomedical technologies, for example, DNA sequencing.

3. Importance of Nonhuman Primate Research in Regenerative Medicine

Before it would be possible to consider applying autologous cell therapy to human patients, the properties of iPS cells must be thoroughly explored in suitable animal models, in order to make sure that autologous cell therapy is both safe and effective. It has been generally recognized that clinically relevant experiments should be performed in a nonhuman primate (NHP) rather than a rodent. NHPs are thought be ideal for such preclinical trials because of their relatedness to humans and their similar physiology, particularly with respect to the central nervous system. Long-term studies of transplanted cell function (>3 years) will be possible in NHPs, but are impossible in rodents.

Thus there is a clear path from basic to translational studies in iPS cell-based regenerative medicine in NHPs. Of the various NHPs that could be used, the marmoset has several key advantages. The common marmoset (*Callithrix jacchus*) has the advantage of smaller size, more rapid breeding, and defined housing conditions. In contrast to humans, where uncontrolled environment and many comorbidities are confounding factors, marmosets can be housed

Table 1: Publications on nonhuman primate iPS cells.

Species	Title of publication	cDNAs used for reprogramming	Origin of cDNAs
Rhesus macaque (*Macaca mulatta*)	Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts [24]	*POU5F1, SOX2 KLF4* and *MYC*	Rhesus
Common marmoset (*Callithrix jacchus*)	Generation of induced pluripotent stem cells from newborn marmoset skin fibroblasts [25]	*POU5F1, SOX2 KLF4* and *MYC*	Human
Common marmoset (*Callithrix jacchus*)	Generating induced pluripotent stem cells from common marmoset (*Callithrix jacchus*) fetal liver cells using defined factors, including Lin28 [26]	*POU5F1, SOX2, KLF4, MYC, NANOG* and *LIN28*	Human
Rhesus macaque (*Macaca mulatta*)	Reprogramming Huntington monkey skin cells into pluripotent stem cells [27]	*POU5F1, SOX2* and *KLF4*	Rhesus
Pigtailed macaque (*Macaca nemestrina*)	Efficient generation of nonhuman primate induced pluripotent stem cells [28]	*POU5F1, SOX2 KLF4* and *MYC*	Human
Cynomolgus monkey (*Macaca fascicularis*)	Development of histocompatible primate induced pluripotent stem cells for neural transplantation [29]	*POU5F1, SOX2 KLF4* and *MYC*	Human
Rhesus macaque (*Macaca mulatta*)	Generation of pancreatic insulin-producing cells from rhesus monkey induced pluripotent stem cells [30]	*POU5F1, SOX2 KLF4* and *MYC*	Rhesus
Pigtailed macaque (*Macaca nemestrina*)	Safeguarding nonhuman primate iPS cells with suicide genes [31]	*POU5F1, SOX2 KLF4* and *MYC*	Human
Drill (*Mandrillus leucophaeus*)	Induced pluripotent stem cells from highly endangered species [32]	*POU5F1, SOX2 KLF4* and *MYC*	Human
Cynomolgus monkey (*Macaca fascicularis*)	Induction of retinal pigment epithelial cells from monkey iPS cells [33]	*POU5F1, SOX2 KLF4* and *MYC*	Human

The table lists the publications (in order of publication, up to September 2011) that have reported the derivation and characterization of nonhuman primate iPS cells. All used mixtures of retroviruses, carrying the indicated cDNAs.

in a defined environment and have few known comorbidities [12]. A variety of human diseases can potentially be modeled in marmosets [13–15]. A chemical-induced model of Parkinson's disease has also been developed in this species [16] and a stroke model [17] has been developed. Histological and MRI brain atlases are available [18]. The marmoset genome has been completed [19], and the marmoset is the first and so far only primate to have transgenic models that show germline transmission [20]. Although transgenics have also been created in the rhesus macaque, they have not passed the transgene to their offspring [21]. A genetic model of Parkinson's disease by overexpression of α-synuclein has been developed in the marmoset [20]. Finally, a spinal cord injury model in the marmoset has been used in tests of transplanted human neural stem cells for potential therapeutic effect [22, 23]. Our long-term goal is illustrated in Figure 1.

4. Progress in NHP iPS Cell Research

Despite the importance of NHPs in regenerative medicine, there has yet been relatively little work on iPS cells derived from NHPs, in comparison to the extent of work on iPS cells derived from mice and humans. The first NHP iPS cells were derived from the rhesus macaque [24]. At the present time (September 2011), iPS cells have been derived from five NHP species (Table 1); three species of macaque (rhesus macaque, pigtailed macaque, and cynomolgus monkey), the common marmoset, and an endangered primate, the drill [24–33]. Common features of all reports on NHP iPS cells are: derivation by mixtures of retroviruses carrying transcription factor cDNAs, principally *POU5F1*, *SOX2*, *KLF4,* and *MYC*; maintenance of pluripotent characteristics over long-term growth in culture; ability to differentiate into cells and tissues of the three germ layers; a lack of malignant properties, despite the ability to form benign teratomas in immunodeficient mice [24–33].

5. Marmoset iPS Cells: A Model for Autologous Cell Therapy

The eventual goal of our studies is to derive iPS cells from individual marmosets and implant the cells into the donor animal, following the directed differentiation of the iPS cells to specific cell lineages (Figure 1). Before such studies are possible, extensive in vitro investigations and studies in immunodeficient mice are needed.

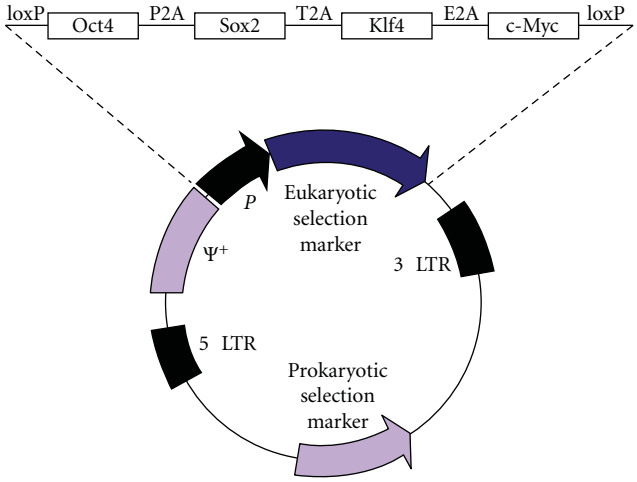

Figure 2: Retroviral reprogramming vector designed to deliver four reprogramming factors (Oct4, Sox2, Klf4, and c-Myc; OSKM) in a single virus using "self-cleaving" peptides, which support efficient polycistronic expression from a single promoter [8]. In this version, expression is driven by the 5 LTR. Additionally, loxP sites are present just before and just after the OSKM coding region, enabling excision of the vector from the genome of the reprogrammed cells. This vector was constructed by replacing the internal promoter (P) and eukaryotic selection marker of retroviral vector pLXSN by the OSKM sequence from FUW-OSKM [8].

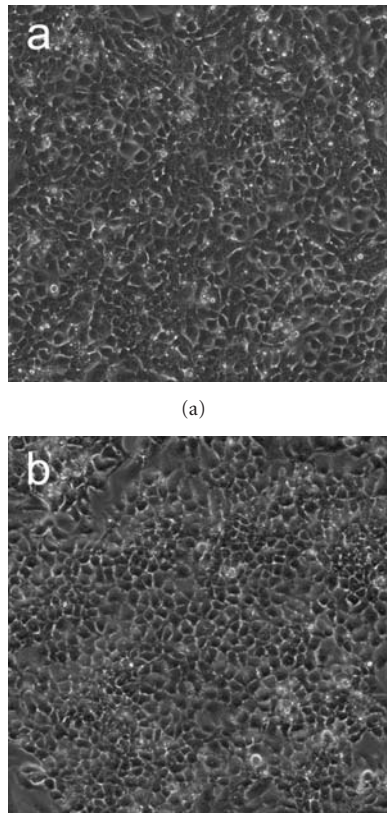

Figure 3: Marmoset iPS cells growing in feeder-free culture. (a) An iPS cell line derived by coinfection with four retroviruses (B8 cell line [25]). Cells are growing in defined xeno-free medium (Pluriton, Stemgent). (b) An iPS cell line derived by infection with a single retrovirus, encoding the OSKM reprogramming factors, illustrated in Figure 2.

We chose to derive marmoset iPS cells from skin fibroblasts because the fibroblast has been the most widely studied cell type for iPS cell generation, and because the use of small skin biopsies as a source of starting material is relevant to future clinical application of iPS cells and their derivatives. In initial experiments, we used fibroblasts derived from newborn marmoset skin [25]. Retroviruses encoding the human cDNAs for Oct4, Sox2, Klf4, and c-Myc [2] were prepared in Plat-A cells and were concentrated by Polybrene flocculation [34]. Following the infection of the cells with concentrated viruses, cultures were maintained in normal fibroblast growth conditions with the addition of valproic acid [35]. After 14–21 days, small colonies of altered morphology were noted in the confluent fibroblast cultures. These colonies comprised small rapidly dividing cells with high nuclear/cytoplasmic ratio and prominent nucleoli. When cultures containing such colonies were fixed and stained for alkaline phosphatase activity, most of the small colonies of altered morphology were found to be positive for alkaline phosphatase, a marker of pluripotency [36]. These colonies expanded rapidly, producing very dense patches of small cells. These cells have the morphological characteristics previously reported for human iPS cells [2].

Starting with a population of 4×10^5 marmoset fibroblasts, we obtained 100 colonies of cells with iPS cell-like morphology. Colonies were isolated and expanded on feeder layers. Of those colonies that were isolated from the fibroblast cultures, 30 showed sustained growth and were able to be expanded to the point where they could be cryopreserved. Of these, 8 were selected for further study. Karyotypes were investigated by G banding and were found to be normal [25]. Following the initial expansion of marmoset iPS cell clones on feeder layers, we investigated if the cells could be grown under feeder-free conditions. Cells were replated on Matrigel-coated dishes in medium containing 20% fetal bovine serum and 40% MEF-conditioned medium and continued to grow rapidly. Cell populations were expanded under these conditions for further studies.

Marmoset iPS cell clones expressed pluripotency markers at levels that were comparable to that in a human embryonic stem cell line (I6) or exceeded that level [25]. In all 8 marmoset iPS cell clones, NANOG and SOX2 mRNA levels were higher than those in I6 cells, and levels of OCT4 were comparable to that of I6 cells. Levels of OCT4 mRNA were >100-fold higher in iPS cell clones than in the fibroblasts used for reprogramming, and levels of NANOG and SOX2 were >50-fold higher. We assessed the relative levels of vector and total mRNAs for OCT4 and SOX2, two of the factors used for reprogramming. We used primer pairs specific for reprogramming vectors (vector sequence 5 primer and coding region 3 primer). Vector OCT4 mRNA was present at 0.01% to 0.1% of that of total OCT4 mRNA, while vector SOX2 mRNA was present at 0.1% to 1% of the total SOX2

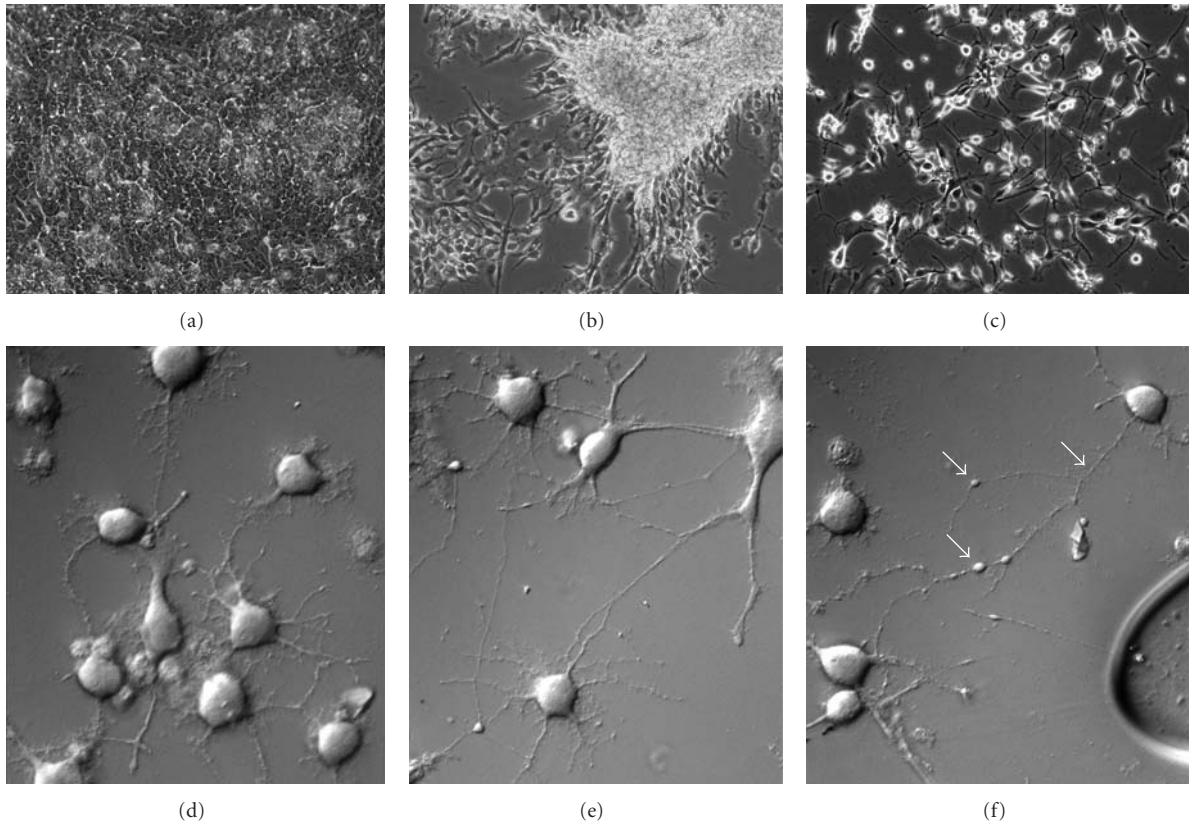

Figure 4: Derivation of neural progenitor cells (NPCs) from marmoset iPS cells and differentiation of NPCs to mature neurons. The series (a)–(c) shows the transition from undifferentiated iPS cells (a), to a line of NPCs (b), to mature neurons (c) (100x phase-contrast images). NPCs placed on a polylysine/laminin-coated glass surface stop dividing and form extensive axons and dendrites. Details of this further maturation are shown in series (d)–(f) (400x differential interference contrast images). Note particularly the varicosities of different sizes indicated by arrows in (f). These are sites of accumulation of cellular organelles and are precursors to the formation of synapses [37]. Their presence indicates the degree of maturity of these neurons.

mRNA. These findings indicate that the viral genomes are appropriately silenced [38].

In order to assess the potential of marmoset iPS cell clones to differentiate to cells of all three germ layers, cells were transplanted into immunodeficient mice (subcutaneous injection in 50% Matrigel: [39, 40]). Teratomas from marmoset iPS cells contained a variety of tissue structures representing derivatives of all three germ layers. Because it has been reported that teratomas derived from incompletely reprogrammed cells formed tissues of ectodermal and mesodermal origin but not of endodermal origin [38] we performed histological studies of the development of mature structures of endodermal origin; we observed endodermal tissues, including simple columnar and pseudostratified epithelia, epithelia with goblet cells, and exocrine glandular structures [25]. Immunohistochemical studies were also performed; ectodermal tissue (developing neural tissue) was demonstrated by presence of βIII tubulin; mesodermal tissue by smooth muscle actin; endodermal tissue by α-fetoprotein.

Subsequently, we investigated the potential of a polycistronic vector for reprogramming (Figure 2). This retroviral vector has the features that (a) because expression of the reprogramming factors is driven by the 5 LTR, expression is silenced during reprogramming, if cells have been properly reprogrammed [38]; (b) all factors are in one vector, thus avoiding the need for very high efficiency infection; (c) as a retroviral vector, only dividing cells are infected (this does not detract from the value of this type of vector, as iPS cells must arise from cells capable of cell division); (d) loxP sites enable future excision of the coding region when required. Marmoset iPS cells derived using this polycistronic retroviral vector exhibited the same characteristics of iPS cell clones derived by coinfection of the four factors. Therefore, cells derived by a 1 : 1 : 1 : 1 expression of the four reprogramming factors have properties that are basically the same as those derived by coinfection, in which the ratio of expression of the four factors is not necessarily equal and almost certainly varies from clone to clone.

Despite the advantages of such retroviral vectors, it is likely that the use of integrating forms of viral vectors for reprogramming will be made obsolete by nonviral reprogramming methods using modified mRNA or modified proteins [9]. These methods avoid any genetic modification of the target cells during the reprogramming process.

Successful long-term expansion of marmoset iPS cells is critical for any extensive studies of the properties of

the cells. Although we determined feeder-free conditions for growth of the cells, these conditions require fetal bovine serum and medium conditioned by a suitable cell type, such as mouse embryo fibroblasts. More recently, we have established that marmoset iPS cells can grow continuously and over long periods in defined medium without the addition of serum or of medium conditioned by another cell type. Several types of defined media support long-term marmoset iPS cell growth without loss of expression of pluripotency genes such as NANOG and OCT4/POU5F1. Both clones derived by coinfection and clones derived by infection with a polycistronic vector may be grown in defined medium (Figure 3).

In summary, by the criteria of morphology, growth requirements, expression of pluripotency factors, retroviral silencing, and the ability to generate teratomas with tissues of all three germ layers, we conclude that these lines of cells represent *bona fide* induced pluripotent stem cells.

6. Differentiation of Marmoset iPS Cells to Neural Progenitor Cells

In subsequent work, we investigated the potential of marmoset iPS cell lines to differentiate in vitro to cells of the neural lineage. Differentiation of iPS cells to neural progenitor cells (NPCs) has been extensively employed as a test of proper pluripotency; for example, this form of directed differentiation was used in a recent set of tests on a panel of well characterized human iPS cells [10, 11]. Protocols for NPC generation are of three general types: stromal cell-derived inducing activity (SDIA), a relatively poorly characterized mix of factors secreted by certain mesenchymal cells, such as the PA6 cell line [2, 41, 42]; embryoid body (EB) formation, followed by plating of the EBs on suitable surfaces in the presence of Neurobasal medium [43, 44]; and induction using small molecules, such as chemical inhibition of BMP/activin/nodal signaling via SMADs [45]. We have used each of these methods in marmoset iPS cells, and all of them produce NPC lines (Figure 4).

7. Summary

In summary, iPS cells from NHPs have a unique importance in preclinical research leading to the implementation of regenerative medicine in human patients. We have derived and characterized iPS cells from the marmoset, a small NHP that can serve as a suitable model for autologous cell therapy involving iPS cells. Future studies will test the principles of autologous cell therapy in individual marmosets.

Acknowledgments

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Research Article

Small Molecules Greatly Improve Conversion of Human-Induced Pluripotent Stem Cells to the Neuronal Lineage

Sally K. Mak, Y. Anne Huang, Shifteh Iranmanesh, Malini Vangipuram, Ramya Sundararajan, Loan Nguyen, J. William Langston, and Birgitt Schüle

Basic Research Department, The Parkinson's Institute, 675 Almanor Ave, Sunnyvale, CA 94085, USA

Correspondence should be addressed to Birgitt Schüle, bschuele@thepi.org

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Efficient *in vitro* differentiation into specific cell types is more important than ever after the breakthrough in nuclear reprogramming of somatic cells and its potential for disease modeling and drug screening. Key success factors for neuronal differentiation are the yield of desired neuronal marker expression, reproducibility, length, and cost. Three main neuronal differentiation approaches are stromal-induced neuronal differentiation, embryoid body (EB) differentiation, and direct neuronal differentiation. Here, we describe our neurodifferentiation protocol using small molecules that very efficiently promote neural induction in a 5-stage EB protocol from six induced pluripotent stem cells (iPSC) lines from patients with Parkinson's disease and controls. This protocol generates neural precursors using Dorsomorphin and SB431542 and further maturation into dopaminergic neurons by replacing sonic hedgehog with purmorphamine or smoothened agonist. The advantage of this approach is that all patient-specific iPSC lines tested in this study were successfully and consistently coaxed into the neural lineage.

1. Introduction

The advent of nuclear reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) for *in vitro* disease modeling also accelerated the field of differentiation into specialized cell types. Differentiation into specific lineages had its primary place to provide a resource for cell replacement therapies [1]. These specialized differentiated cells were in general derived from a small number of "approved" human embryonic stem cell lines [2, 3].

Patient-specific iPSC-derived differentiated cells have now become an attractive tool to study disease mechanisms on a human background and are a vanguard into a new era of science and potentially personalized medicine. In particular for monogenic forms of disease, patient-derived iPSCs have already been shown to recapitulate known disease mechanisms, as shown in spinal muscular atrophy [4], fragile X syndrome [5], progeria syndrome [6], and several genetic forms of Parkinson disease (PD) like LRRK2 [7], PINK1 [8], SNCA [9], and GBA [10]. This novel approach of disease modeling becomes very attractive for drug screening and discovery [11, 12].

One of the challenges is to differentiate these patient-derived iPSCs into the desired specialized cell type of interest. For neuronal differentiation, there were three main approaches developed in the last decade to derive dopaminergic neurons [13–15]. The first method is stromal-induced neuronal differentiation, termed stromal cell-derived inducing activity (SDIA) [16, 17]. The concept is that mouse stromal cells such as PA6 or MS5 or midbrain astrocytes were used to coax the regionalization of stem cells. The disadvantage of this method is the variability of stromal cells and unknown factors; furthermore, this protocol is overall lengthy and takes about 40–60 days *in vitro*. The second main approach is embryoid body (EB)/neurosphere-mediated differentiation [18, 19], caveats are clonal expansion of subgroup of cells and potential forebrain specification. However, the usefulness of neuronal precursors (NPCs) is that they can be expanded, cryopreserved, and be a starting pool for final maturation. NPCs are also important for scientific questions of developmental phenotypes related to disease. The third approach of direct neuronal differentiation utilizes high-density monolayer ESC/iPSC cultures via floor-plate formation which gives the promise of shortening the time for

neuronal development while reaching high differentiation efficiency of midbrain dopaminergic neurons [15] and show excellent survival and functional benefit which gives hope for regenerative therapies in Parkinson's disease [20].

Here, we describe a 5-stage EB differentiation approach using small molecules to enhance neural induction consistently in patient and control iPSC lines. In addition, we substituted sonic hedgehog (Shh) with purmorphamine (Pur) and/or smoothened agonist (SAG) to reduce cost for the final maturation and have shown comparable results between Shh and these small molecules. We have made considerable progress in consistency and reproducibility of this process; however, there is still a challenge of improving the overall yield of region-specific dopaminergic neurons.

2. Materials and Methods

2.1. Skin Biopsies of Patient and Control Subjects. Skin punch biopsies (4-mm circular) were taken from all individuals employing a standard punch biopsy [21]. All biopsies were taken from the upper inner arm, an area that is mostly unexposed to direct sunlight. We used a standard skin explant culture technique by cutting the biopsy tissue into 12–15 pieces and placed 2-3 pieces into one well of a gelatinized 6-well plate in 1 mL of high-glucose DMEM, 20% fetal bovine serum, 1x nonessential aminoacids (NEAA), 1x penicillin streptomycin (P/S), 1x L-glutamine (Glu) (all were purchased from Invitrogen, Carlsbad, CA). Outgrowth of keratinocytes was first noted 2–5 days after plating. Cells were expanded using standard tissue culture techniques, cells were passaged upon confluency using trypsin/EDTA (Invitrogen) and 15–20 million cells. Miocells were cryopreserved for banking. This study and protocol had Institutional Review Board approval and all subjects gave written informed consent for this study. Clinical information on the patients is provided in Supplementary Table 1 (see supplementary materials available at doi:10.1155/2012/140427).

2.2. Generation of iPSC. All iPSCs were derived using a retroviral system to deliver four genes encoding OCT4, KLF4, SOX2 and cMYC (from Addgene plasmids 17217, 17218, 17219, 17220, http://www.addgene.org/) using published protocols [22, 23]. All lines have been characterized for pluripotency, differentiation potential into three germ layers, are karyotypically normal and genotype-match the parental fibroblasts, (see Supplementary Table 2). IPSC line 1761 was previously described and characterized in Nguyen et al. 2011 [7].

2.3. iPSC Maintenance and Propagation. iPSCs were cultured and maintained on mitomycin C inactivated mouse embryonic fibroblasts (iMEF) (EMD Millipore Cat. No. PMEF-CF) in hESC media containing DMEM/F12, 20% knockout serum replacement (Invitrogen, Cat. No. 10828028), 1x NEAA, 1x P/S, 0.1% β-Mercaptoethanol (Invitrogen, Cat. No. 21985023), 0.5x L-Glu and 6 ng/mL of basic fibroblast growth factor (FGF2) (Cat. No. 233-FB, R&D Systems, Minneapolis, MN). Cells were split every week manually without enzymatic treatment.

2.4. Generation and Maintenance of Neural Progenitor Cells (NPCs). To derive NPCs, iPSC colonies were harvested using 1 mg/mL of collagenase IV (Invitrogen, Cat. No. 174104019). After about 1 hr, when all colonies lifted up completely from culture dish, colonies were transferred to 10 cm bacterial petri dishes (BD Bioscience, Bedford, MA). Forming embryoid bodies (EB) was cultured in suspension with agitation on rocker (Rocker II Model 260350, Boekel Scientific, Feasterville, PA) for 4 days in EB media, which consisted of hESC media minus FGF2 with or without 5 μM dorsomorphin (Dor) (Sigma, St Louis, MO, Cat. No. P5499) and 10 μM SB431542 (SB) (Tocris Bioscience, Ellisville, MO, Cat. No. 1614).

Next, EBs were cultured for additional 2-3 days with agitation in neural induction media (NIM) consisting of DMEM/F12 (Invitrogen, Cat. No. 12500, powder form), 1x NEAA, 0.5x L-Glu, and freshly made and sterile filtered N2, which contained 1.55 g/L glucose (Sigma, Cat. No. G7021), 2 g/L sodium bicarbonate (Sigma, Cat. No. S5761), 100 μM putrescine (Sigma, Cat. No. P5780), 30 nM sodium selenite (Sigma, Cat. No. S9133), 20 nM progesterone (Sigma, Cat. No. P8783), 0.1 mg/mL transferrin (Sigma, Cat. No. T0665), 0.025 mg/mL insulin (Sigma, Cat. No. I6634) and FGF2 (20 ng/mL). Cell culture plates were coated with Geltrex (Invitrogen, Cat. No. 12760021), media changed every day. Neural rosettes were formed in 2–5 days in adherent culture.

To obtain a pure population of NPCs, rosettes were manually isolated using No. 15 scalpel cutting in squares with distance to edges of colonies (Figure 3(F)). Dissected pieces of rosettes were lifted using a pipette, replated onto Geltrex-coated culture dishes and maintained in neural progenitor cell media (NPC media) containing neurobasal media (Invitrogen, Cat. No. 21103049), 1x NEAA, 1x L-Glu, 1x P/S, 1x B27 supplement (Invitrogen, Cat. No. 17504044), and FGF2 (20 ng/mL). Manual isolation of rosettes as described above was repeated once to obtain more pure population of NPCs. Approximately 5–10 pieces of rosettes were dissociated into single cells using Accutase (MP Biomedicals, Solon, OH, Cat. No. 0910004). Cells were treated with Accutase for 2–5 minutes until cells became round in shape, then the cells were collected, centrifuged, resuspended in NPC media, and plated onto one 96-well coated with Geltrex and cultured at 37°C and 5% CO_2. When confluent, NPCs were split at a ratio of 1 : 2 in single wells with larger surface area such as 48-well, 24-well, 12-well, and so forth in NPC media.

2.5. NPC Enrichment Using Anti PSA-NCAM Microbeads. For magnetic bead sorting, NPCs were treated with Accutase, collected, and passed through 30 μm nylon mesh (pre-separation filters, 30 μm, Miltenyi Biotec Auburn, CA, Cat. No. 130-041-407). The total cell number was approximately 10^7. Cell suspension was centrifuged at 300 $\times$g for 10 minutes. Supernatant was aspirated completely and cell pellet was resuspended in 60 μL of buffer (1x PBS, 2 mM EDTA (Ambion Inc, Austin, TX, Cat. No. AM9260G) and

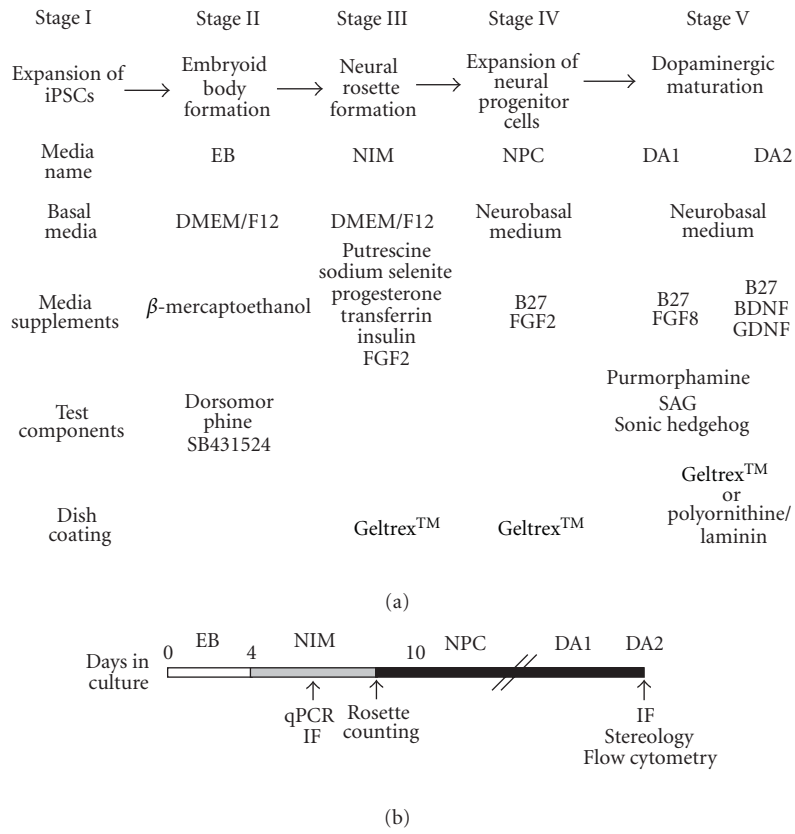


Figure 1: Schematic summary of the differentiation conditions used in the generation of dopaminergic neurons (a) Schematic diagram illustrating the different stages of NPC generation and dopaminergic neuronal differentiation. The abbreviations are. Dor/SB: Dorsomorphin and SB431542, EB: EB media, NIM: neural induction media, NPC: neural progenitor cell media, and DA1/DA2: medium for dopaminergic differentiation. (b) Timeline shows the medium used at different stages of NPCs and dopaminergic maturation and displays the sampling dates for performing gene expression studies, such as qPCR, marker characterization (immunofluorescence: IF), to analyze efficiency of rosette formation and for quantitative studies of TUJ1 and TH immunoreactivity using stereology and flow cytometry.

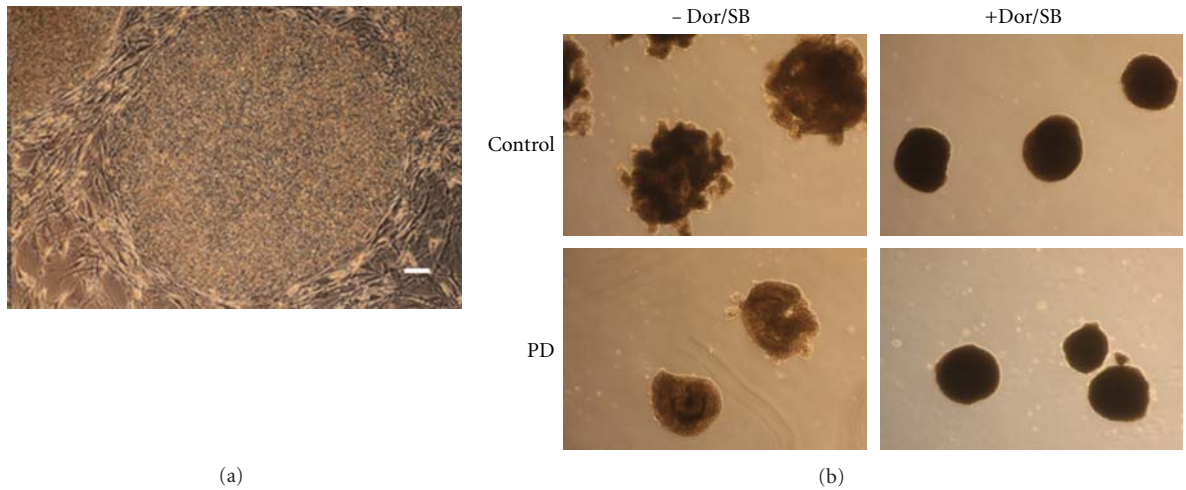


Figure 2: (a) Representative image of the quality of iPSC colonies used to produce EBs: distinct border with little to no differentiation. The recommended size for EB formation should be double the size as the depicted colony, approximately 2 mm in diameter. Scale bar 100 μm (b) In-control and PD-specific cell lines (Control and PD), EBs were lacking that compact structure and round borders when cultured without Dor/SB; EBs were found to be round, uniform in the presence of Dor/SB.

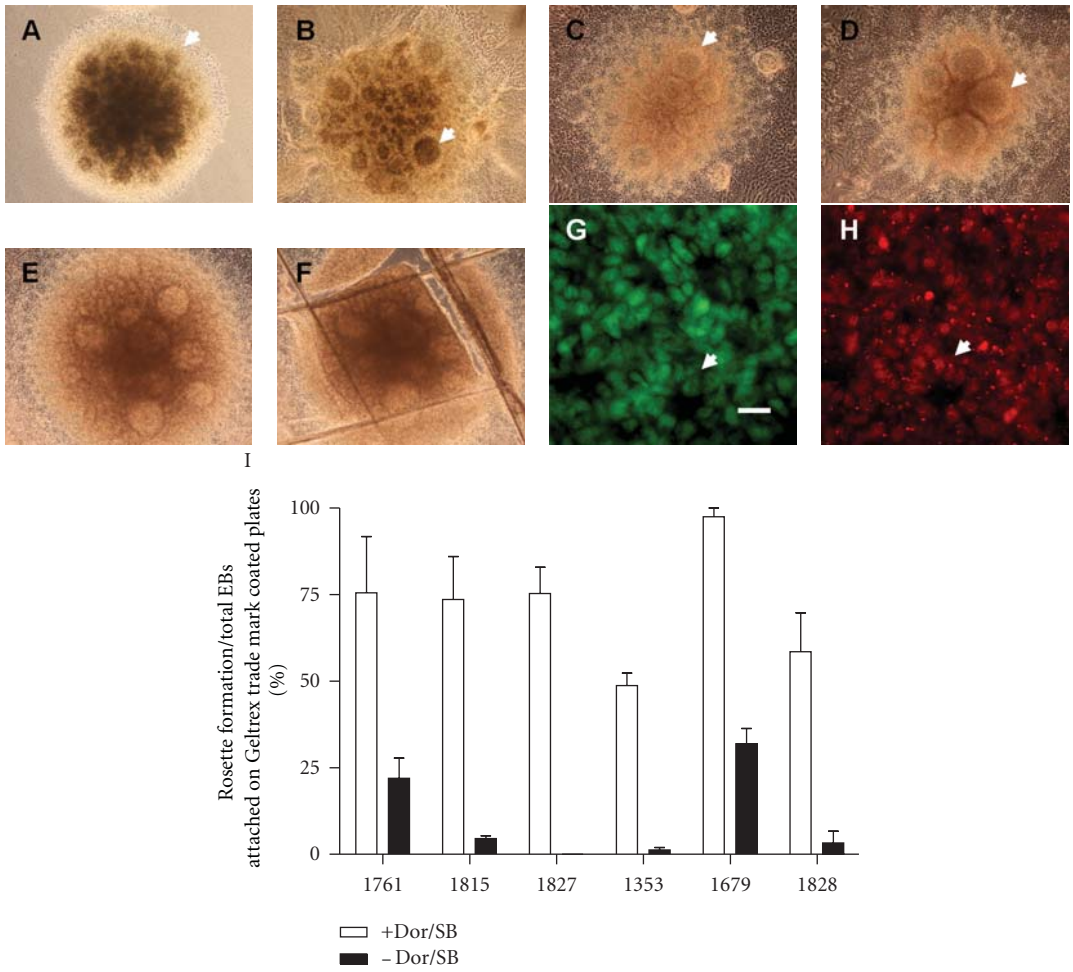


Figure 3: Representative images of neural rosettes at different stages (A–D). Neural rosettes after Dor/SB treatment at days 7–12, stage III of the differentiation protocol (5x magnification). Arrows indicate boundary lines in the early and late stages of rosettes. (E) and (F) Manual dissection of rosettes using scalpel are illustrated before (E) and after (F) cutting. (G and H) Arrows indicate the positions of neural rosettes that were immunoreactive with Pax6 (green) (E) and Sox1 (red) (F). Scale bar represents 20 μm. (i) There is a significant difference in neural rosette formation in all control and PD-specific EBs treated with Dor/SM compared to those with no Dor/SB treatment during stage II ($^*P < 0.01$). Data are presented as mean + standard error of the mean (SEM) compared to the controls ($n = 3$, except 1679, $n = 2$). P value of each study was assessed by one-way ANOVA along with Newman-Keuls post-*hoc* analysis.

0.5% albumin from bovine serum (BSA) (Sigma, Cat. No. A3294). Cells were mixed well and incubated for 10 minutes in the refrigerator (2−8°C). Then, 20 μL of anti-PSA-NCAM microbeads (Miltenyi Biotech, Auburn, CA, Cat. No. 130-092-966) were added to the mixture, mixed well with pipetting up and down, and incubated for 15 minutes in the refrigerator (2−8°C). Cells were washed by adding 2 mL of buffer and centrifuged at 300 ×g for 10 minutes. Supernatant was aspirated completely and cell pellet was resuspended up to 10^8 cells in 500 μL of buffer. A MS column (Miltenyi Biotec, Cat. No. 130-042-201) was placed in the magnetic field of a miniMACS separator (Miltenyi Biotec, Cat. No. 130-042-102), rinsed with 500 μL of buffer three times. Cell suspension was applied onto the column. The column was washed with 500 μL of buffer three times again. New buffer was added when the column reservoir was empty. The column was removed from the separator and placed on a 15 mL BD Falcon conical tube (BD Bioscience, 352097). One mL of buffer was added onto the column and magnetically labeled cells were flushed out by firmly pushing the plunger into the column. The eluted fraction was directly enriched over a second column and the magnetic separation procedure was repeated once by using a new MS column. One mL of NPC media was added onto the column to flush out the magnetically labeled cells. Then another 1 mL of NPC media was added and cell suspension was transferred to a 35 mm Geltrex-coated culture dish.

2.6. Dopaminergic Differentiation of NPCs. Dopaminergic differentiation was initiated by culturing NPCs on Geltrex-coated culture dishes or glass coverslips (Fisher Scientific, Pittsburg, PA, Cat. No. 12-545-80-12CIR-1) coated with poly-L-ornithine (20 μg/mL) (Sigma, Cat. No. P4957)

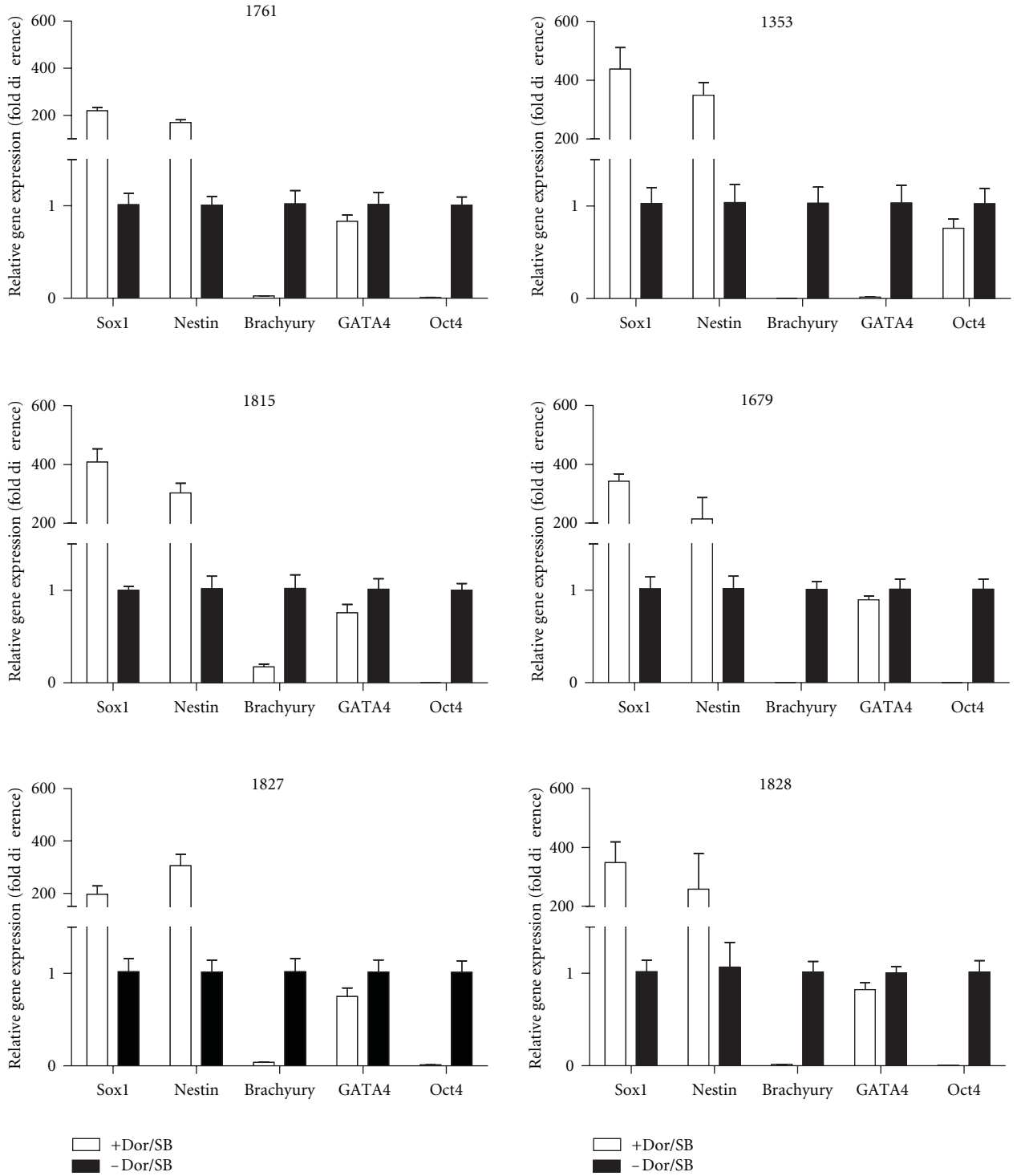


Figure 4: Quantitative gene expression analysis of PD and control lines with and without Dor/SB. Expression levels of neuroectoderm (Sox1 and Nestin), mesoderm (Brachyury), endoderm (GATA4), and pluripotent markers (Oct4) were assessed by quantitative PCR. The y-axis represents means + SEM of relative expression levels of each gene in the EBs with Dor/SB relative to no Dor/SB treatment ($^*P < 0.01$). Left panel depicts lines from healthy donors; right panels depicts cell lines derived from patients with PD. Data are presented as mean + SEM compared to the controls ($n = 3$, except 1679, $n = 2$). P-value of each study was assessed by one-way ANOVA along with Newman-Keuls post-*hoc* analysis.

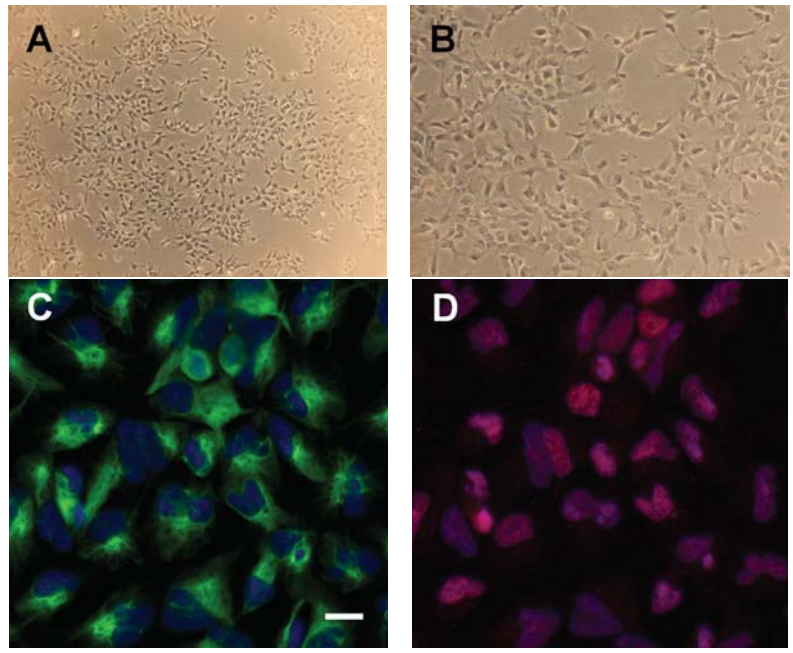


Figure 5: Characterization of NPCs. (A) and (B) NPC morphology was observed under phase-contrast microscopy with 5x (A and 10x (B) magnification. (C and D) NPCs expressed Nestin (green) (C) and Sox1 (red). (D) Nuclei were counterstained with DAPI (blue). Scale bar represents 20 μm.

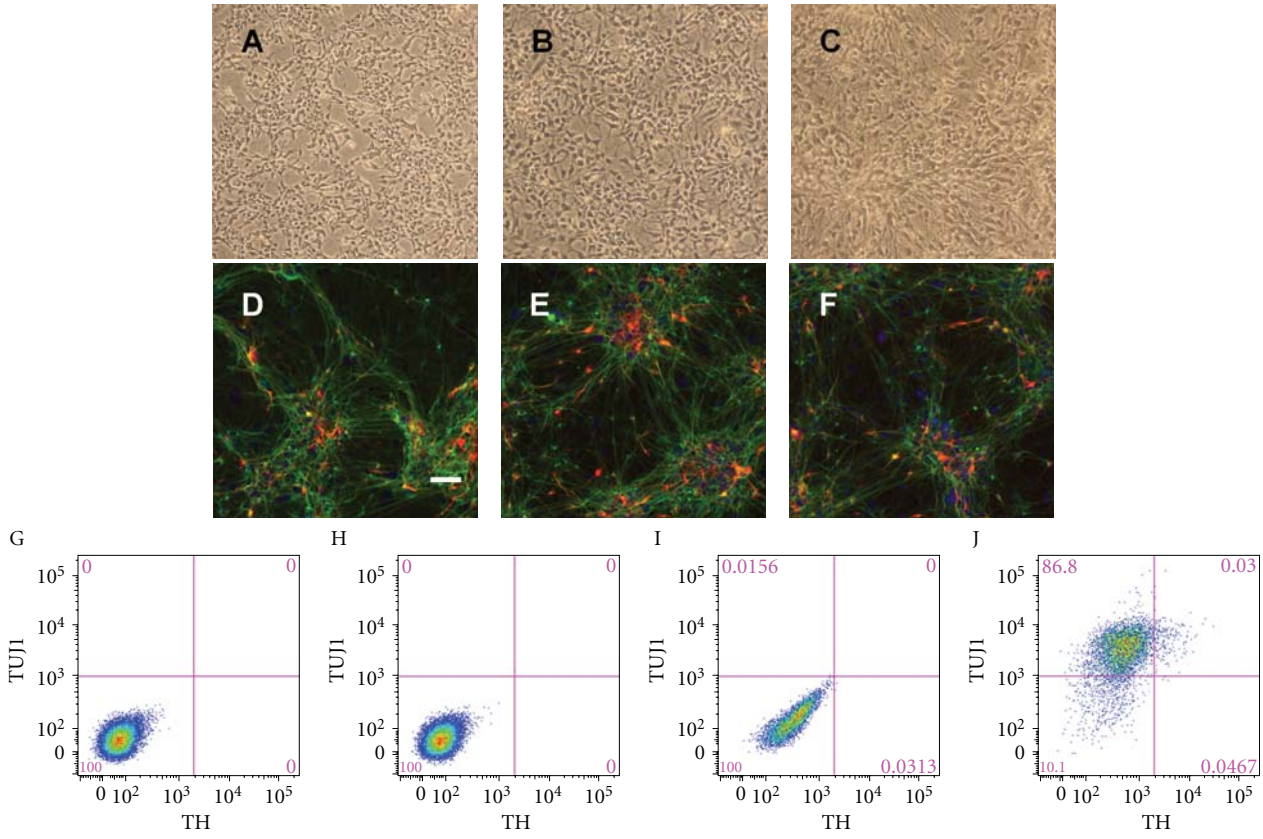
at 37°C for 4 hrs and laminin (Sigma Cat. No. L2020) (20 μg/mL) at 4°C overnight.

Dopaminergic differentiation in defined media was initiated by culturing ~0.5×10^6 NPCs (in 35 mm culture dish) in DA1 media for 10 days in Neurobasal media supplemented with 1x NEAA, 1x L-Glu, 1x P/S, 1x B27, FGF8b (100 ng/mL) (R&D Systems, Cat. No. 423-F8) and tested with either 2 μM Purmorphamine (Pur) (EMD Chemicals, Cat. No. 540220), Gibbstown, NJ), 0.4 mM SAG (Enzo Life Sciences, Farmingdale, NY, Cat. No. ALX-270-426-M001) or 200 ng/mL Sonic Hedgehog, C24II (Shh) (R&D Systems, Cat. No. 1845-SH). During these 10 days, when cells grew confluent, they were passaged with Accutase as described above and replated onto a Geltrex-coated plates at cell density of ~80%. Lower density led to cell death.

Final maturation into dopaminergic neurons was carried out in DA2 media containing neurobasal media supplemented with 1x NEAA, 1x L-Glutamine, 1x B27 supplement, 1x Penicillin-Streptomycin, 20 ng/mL BDNF (R&D Systems, Cat. No. 248-BD), 20 ng/mL GDNF (R&D Systems, Cat. No. 212-GD), and 1 mM dibutyryl cAMP (Sigma, Cat. No. D0627) for 20–30 days. Cells were not continued to passage in DA2 media when the processes formed. Cells were analyzed on day 30 of the maturation process.

2.7. Quantitative PCR (qPCR). EBs were collected on day 6 of EB formation before plating down for rosette formation. Total RNA was extracted using a RNeasy Micro kit (Qiagen, Valencia, CA) and 500 ng RNA was used for reverse-transcription into cDNA using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA). Total reaction volume was 20 μL; the resulting cDNA sample was diluted with 80 μL of ultrapure water, and 5 μL of the diluted cDNA sample was used as template for qPCR amplification. qPCR was performed using ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). All reactions were run in 20 μL reactions volume using SYBR Green PCR Master Mix (Applied Biosystems) and 30 pmol of each primer. qPCR parameters were as follows: 2 min at 50°C; 10 min at 95°C; 40 cycles at 95°C for 15 sec, and at 60°C for 1 min. Data were collected at 60°C. All data were normalized to *β-actin* expression and plotted as fold changes over samples from EBs without Dor/SB. Primers of genes used in this study: Sox1 (5′-GAGATTCATCTCAGGATTGAGATTCTA-3′ and 5′-GGCCTACTGTAATCTTTTCTCCAC-3′); Nestin (5′-TGCGGGCTACTGAAAAGTTC-3′ and 5′-AGGCTGAGGGACATCTTGAG-3′); Brachyury (5′-AGGTACCCAACCCTGAGGA-3′ and 5′-GCAGGTGAGTTGTCAGAATAGGT-3′); GATA4 (5′-GTCATCTCACTACGGGCACA-3′ and 5′-CTTCAGGGCCGAGAGGAC-3′); Oct4 (5′-TGGGCTCGAGAAGGATGTG-3′ and 5′-GCATAGTCGCTGCTTGATCG-3′) and β-actin (5′-CTGAACCCCAAGGCCAAC-3′ and 5′-TAGCACAGCCTGGATAGCAA-3′).

2.8. Immunocytochemistry. EBs and NPCs were fixed with 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA) at room temperature for 10 minutes. Neurons were fixed carefully by adding equal volume of 8% PFA into wells to equal volume of medium and incubated at room temperature for 10 minutes. Fixed cells were permeabilized with 0.3% Triton X-100 (Sigma, Cat. No. X100) for 5 minutes and were blocked in blocking buffer (5% normal



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	Stereology			Flow cytometry		
	TUJ1/ total cells	TH/TUJ1	TH/total	TUJ1/ total cells	TH/TUJ 1	TH/total
Pur	80.42 ± 2.21	8.58 ± 0.66	6.44 ± 1.12	71.30 ± 8.72	5.94 ± 1.55	4.47 ± 0.08
SAG	85.84 ± 3.10	7.00 ± 0.75	6.31 ± 0.63	80.52 ± 6.17	3.93 ± 0.88	3.28 ± 0.23
Shh	68.41 ± 12.49	7.58 ± 0.36	5.44 ± 0.55	68.89 ± 11.22	2.73 ± 1.32	1.81 ± 0.42

Figure 6: Analysis of number of TH+ and TUJ+ neurons (A)–(C). Image under phase-contrast microscopy showed plating density of NPCs at day 1 in DA1 media (5x magnification) (A), cell morphology at day 12 (arrows indicate the formation of processes) (B) 10x magnification) and neurons at day 30 during dopaminergic maturation (C) 10x magnification). (D)–(F), Neurons treated with Pur (D), SAG (E) and Shh (F) were characterized by immunostaining with TUJ1 (green) and TH (red) and counterstained with DAPI (blue). Scale bar represents 200 μm. (G)–(J) Flow cytometry: No TUJ1 and TH immunoreactive NPCs were detected (H), which is comparable to unstained NPCs (G). In contrast to the population of unstained differentiated neurons (I), differentiated cells showed positive immunoreactivity to TUJ1 and TH (j). (k) Table illustrates percentage of TUJ1 relative to total cells, TH immunoreactive neurons relative to TUJ1 immunoreactive neurons, and also relative to total cells quantified using stereology and flow cytometry in control NPCs after dopaminergic maturation.

goat serum, Vector Labs, Burlingame, CA) in 1x Phosphate buffered saline (PBS) (Sigma, Cat. No. P5493) and followed by incubation with the primary antibody at 4°C overnight in 5% normal goat serum and PBS. The following primary antibodies were used: Nestin (EMD Millipore, Billerica, MA, Cat. No. MAB5326), 1 : 200; Sox1 (EMD Millipore, Cat. No. AB15766), 1 : 100; Tyrosine Hydroxylase (TH) (PelFreez Biologicals, Rogers, AR, Cat. No. P40101-0), 1 : 300; Pax6 (Developmental Studies Hybridoma Bank, Iowa City, IA, Cat. No. Pax6), 1 : 20; neuronal class III β-Tubulin (TUJ1) (Covance, Princeton, NJ, Cat. No. MMS-435P), 1 : 500, and secondary antibodies were Alexa Fluor 488 Goat Anti-Mouse, Alexa Fluor 555 Goat Anti-Mouse, Alexa Fluor 488 Goat Anti-Rabbit, Alexa Fluor 555 Goat Anti-Rabbit (Invitrogen) at 1 : 300. Coverslips were mounted with Vectashield Mounting Medium with DAPI (Vector Labs). Fluorescent images were captured on an Eclipse Ti inverted fluorescence microscope (Nikon Instruments Inc, Melville, NY). Phase contrast images were taken with a Zeiss Axiovert 25 Inverted Microscope (Carl Zeiss AG, Oberkochen, Germany).

2.9. Stereological Analysis. Stereological analysis was performed using an Olympus BH2 microscope (Olympus, Center Valley, PA) with a motorized X-Y stage linked to a computer-assisted stereological system (Olympus America Inc.). This comprises a color video camera (CCD-Iris, Sony), a PC with a high-resolution SVGA monitor, a microcator (VRZ 401, Heidenhain), and Stereo Investigator (MBF Bioscience, Williston, VT). Immunostained coverslips were delineated at 4x magnification. From a random start position, a counting frame was superimposed on the image, and cells were systematically sampled using a 40x objective lens (Olympus), with DAPI stained nucleus used as the sampling unit. A minimum of 200 cells was sampled according to the rules of the optical dissector [24], and the coefficient of error for each stereological estimate was between 0.07 and 0.1 [25].

2.10. Flow Cytometry. After dopaminergic differentiation, cells were dissociated with TrypLE Express (Invitrogen, Cat. No. 12605-028) at 37°C for 5 minutes, washed with PBS, centrifuged, resuspended in PBS, and strained through a 70 μm cell strainer (BD Biosciences, Cat No. 352350), centrifuged, resuspended, and fixed in 4% PFA in PBS at room temperature for 10 minutes. Then they were centrifuged, resuspended, and permeabilized with 0.3% saponin (Sigma, Cat. No. 47036), incubated with TUJ1 (Covance, 1 : 100) and TH (Pel Freez Biologicals, 1 : 100) on ice for 30 minutes and washed with washing buffer (PBS and 0.03% saponin) once. Then cells were incubated with APC-conjugated anti mouse IgG antibody (BD Biosciences, Cat. No. 550826) and PE-conjugated anti rabbit IgG antibody (BD Biosciences, Cat. No. 558416) on ice for 30 minutes, washed with washing buffer, and resuspended in PBS. All sorting procedures were carried out using BD Digital Vantage (BD Biosciences) with a 80 μm nozzle. Data were analyzed by FlowJo flow cytometry software (Version 7.6.4, Tree Star Inc, Ashland, OR). We compared cell suspension of unstained NPCs and unstained differentiated cells as negative control to determine the threshold for detection of immunofluorescence.

2.11. Statistical Analysis. Statistical analysis was performed using GraphPad Prism (Version 4, GraphPad Software, San Diego, CA). Data were analyzed by one-way analysis of variance (ANOVA). Newman-Keuls post hoc analysis was employed when differences were observed in ANOVA testing ($P < 0.05$). Data were presented as the means + standard error of the mean (SEM). All results were derived from at least three independent experiments, except results of cell line 1679 in Figure 1 and flow cytometry data in Figure 6 were derived from two independent experiments.

3. Results and Discussion

3.1. Neuronal Differentiation Using a 5-Stage Embryoid Body Approach. The majority of published neuronal differentiation methods describe selected human embryonic stem cell (hESC) lines such as H9 or I6 and these protocols were optimized around these cell lines. Despite general reproducibility across multiple hESC lines, in patient-specific human iPSCs consistent reproducibility has not been demonstrated, posing a challenge for disease modeling and drug screening. [26–29]. One recent publication points towards specific markers such as miR-371-3 and FoxA2 that could predict a priori the differentiation potential of iPSCs or ESCs into the neuronal lineage, which can be relevant for downstream applications [30].

Our goal was to develop a reliable protocol reproducible across various patient-specific iPSC lines. We tested a 5-stage protocol for neuronal dopaminergic differentiation that was originally introduced by Lee and Studer in mouse embryonic stem cells [18] and subsequently further developed [28, 29]. This protocol involves EB formation for four days, neural rosette formation, isolation of neural rosettes, and expansion and PSA-NCAM enrichment using magnetic bead sorting of neuroprogenitors. A final maturation stage utilizes FGF8 and sonic hedgehog (Shh) for the first ten days followed by BDNF, GDNF, B27, and dcAMP in Neurobasal media for another 20–50 days (Figure 1(a)). We reason that this 5-stage protocol generating EBs has several advantages in generating neural precursors that can be easily expanded without loss of differentiation potential [31]. Thus, this protocol is suitable for studying disease-mechanisms at the neuroprogenitor stage and maintaining potential for derivation of other CNS cell types [28].

In a control iPSC line, EBs incubated for 4 days in EB media showed a similar result of neural rosette formation as described by Swistowski et al., 2009 [28] (data not shown). When we attempted to derive NPCs from additional iPSCs derived from controls and patients affected with PD we observed very little neural rosette formation. Furthermore, these rosettes were not expandable as NPCs.

Small molecules have been reported to improve directing ESC/iPSCs into neural lineage [32, 33]. We tested a combination of small molecules: Dor and SB, both of which have been described for SMAD inhibition. The synergistic mode of action of inhibitors of SMAD signaling, Noggin and SB431542, has been reported to rapidly induce neural conversion of hESCs [15, 34]. Noggin, a bone morphogenetic protein (BMP) antagonist, and the small molecule Dor have similar activities which selectively inhibit the BMP type I receptors: ALK2, ALK3, and ALK6 and block SMAD1/5/8 phosphorylation [35]. SB has been shown to be a selective inhibitor of activin receptor-like kinase receptors ALK4, ALK5, and ALK7 [36].

For successful generation of NPCs, it is crucial to start with pristine, undifferentiated iPSC cultures. IPSC colonies should be densely packed show low nucleus to cytoplasma ratios and have discrete borders and no differentiation along the peripheries and/or in the centers of the colonies. In this protocol, we found that 2 mm diameter sized colonies yield the best results for neural rosette formation (Figure 2(a)).

3.2. Combination of Dorsomorphin and SB431542 Improved Neural Induction. IPSC colonies were enzymatically treated with collagenase. After detachment, half of the colonies in a dish were exposed to 5 μM Dor/10 μM SB. The other

half was left untreated. The colonies were then cultured for 4 days in EB media with or without Dor/SB. EBs cultured in EB media alone showed loose, less compact, and irregular shapes (Figure 2(b)) while the majority of EBs treated with Dor/SB demonstrated compact, solid and round shaped aggregates and had an average size of 350 μm in diameter (Figure 2(b)). On day 4, media was changed to NIM media containing N2 media which was freshly made of different individual components. None of the commercially available N2 supplements showed consistent results (data not shown). EBs were then plated onto Geltrex-coated culture dishes on day 6. During days 6–10, neural rosettes were detected by their characteristic morphology of radially arranged cells (Figures 3(A)–3(D)). In the early stages of NIM incubation (approximately days 8–10), neural rosettes showed darker centers of "flower-shaped" structures with indiscrete boundary lines (Figures 3(A) and 3(B)). In the latter incubation with NIM, "flower-shaped" morphologies were more distinct and edges more clearly defined, shown in Figures 3(C) and 3(D). Dissected rosettes (Figures 3(E) and 3(F)) that are replated and manually isolated a second time generate NPC populations of higher purity.

We evaluated neural differentiation of EBs on day 10 (Figure 1(b)) via immunocytochemistry. Neural markers Pax6 and Sox1 were used as well as the pluripotent cell marker Oct4. Pax6 and Sox1 showed positive staining in attached EB (Figures 3(G) and 3(H)), however, Oct4 showed no immunoreactivity (data not shown). At the same time point, the percentage of neural rosettes formed with and without addition of Dor/SB was quantified by manually counting the colonies containing neural rosettes divided by total colonies attached on the culture dish (Figures 1(b) and 3(I)). Without Dor/SB, we observed low rosette formation between 0% and 31.9%, and we were not able to derive expandable NPCs. The combination of Dor/SB, on the other hand, increased the neural rosette formation substantially to 48% to 97.5% of EBs in both control and PD-specific cell lines. Overall, we did not notice a difference in the efficiency of rosette formation between PD lines and control lines.

At day 6, we performed gene expression analysis of multiple markers in attached EBs. In all six lines we studied neuroectodermal markers Sox1 and Nestin, mesodermal marker Brachyury, endodermal marker GATA4 and pluripotent marker Oct4. Surprisingly, there was a striking difference of >150-fold in the gene expressions of neuroectodermal markers Sox1 and Nestin in Dor-/SB-treated EBs compared to EBs without small molecules (Figure 4). This suggests that the two small molecules very efficiently modulate the SMAD signaling pathway leading to this enormous increase in neuroectodermal markers. This increase was consistent in all six iPSC lines tested, and differences in neuronal differentiation were not observed between patient and control lines. Endo and mesodermal markers GATA4 and Brachyury as well as pluripotency marker Oct 4 were all lower compared to the untreated, normalized NPC lines.

Neural rosettes were manually cut and replated as pieces to produce a population of NPCs of higher purity. Rosettes were manually isolated once again, collected, enzymatically treated with Accutase, and plated and expanded in NPC media. Manual passaging and expansion of NPCs still yielded approximately 10% undifferentiated Oct4-positive cells in NPC cultures, which upon further expansion showed iPSC morphology (data not shown). Therefore, we used magnetic bead sorting with a neural cell adhesion molecule antibody against polysialic acid neural cell adhesion molecule (PSA-NCAM or CD56) (Figures 5(A) and 5(B)). We observed an approximately 20% cell loss after magnetic bead sorting. We characterized NPCs after sorting by immunocytochemistry with defined markers Nestin and Sox1. We detected >90% Nestin and Sox1 immunoreactive NPCs in all iPSC cell lines taken through this protocol (Figures 5(C) and 5(D)). NPCs were readily expandable at a passaging ratio of 1 : 2 to 1 : 3 with Accutase. Cultures grew well when media was prepared freshly every 2 to 3 days and B27 added freshly to NPC media, before media changes. We expanded NPC cultures for >15 passages after derivation and did not observe any changes in morphology or expression of Nestin and Sox1.

With this new approach for neural induction using small molecules, we have dramatically increased reproducibility and efficiency of neural rosette stage/NPC generation. This is invaluable when using patient-derived iPSCs for disease modeling, which may have an intrinsic disadvantage in culture when carrying potential disease-related deficiencies. Since NPCs can be easily expanded, this could become a suitable cell type for high throughput screening where a very large number of starting material is needed.

3.3. Substitution of Small Molecules Purmorphamine or Smoothened Agonist for Sonic Hedgehog Had Similar Effects on Neuronal Maturation. We investigated the substitution of sonic hedgehog (Shh) for small molecules purmorphamine (Pur) or smoothened agonist (SAG) during dopaminergic maturation. These chemicals that are considerably less expensive, have minimal lot-to-lot variabilities, and have longer shelf-life compared to recombinant proteins.

For final dopaminergic maturation, we used a 2-step approach. For the first ten days, we cultured NPCs in FGF8 and tested two small molecules SAG and Pur as substitutes for Shh in control and patient cell lines. At day 1 in DA1 media, the plating density of the NPCs should be approximately 60% to 70% (Figure 6(A)). During this 10-day protocol, cells were split at 100% confluency using Accutase and replated at a cell density of approximately 80%. When cells were plated at a lower cell density (<50%), we observed remarkable cell death and low rates of cell attachment. After ten days, we switched to Neurobasal media supplemented with BDNF, GDNF, dcAMP, and B27 every second day, but added B27 daily preventing cell death. Cells were split until they began growing out processes (Figure 6(B)). After day 30 of dopaminergic maturation, cells were fixed, immunostained with TUJ1 and TH, and counterstained with DAPI (Figures 6(C)–6(F)).

To measure the efficiency of the neuronal differentiation, we evaluated the percentage of TH and TUJ1 expressing neurons relative to total cells using two approaches: stereology with systematic random sampling and flow cytometry. Flow cytometry was employed to minimize bias. The challenges of

accurate counting of these cultures are the dense "patches" of neurons and the majority of TH immunoreactive neurons localized in these "patches" [37].

In the scatter plots for flow cytometry, (Figures 6(G)–6(J)), undifferentiated NPCs did not show immunoreactivity for TUJ1 and TH (Figure 6(H)) and had a similar pattern in the scatter plot to unstained NPCs (Figure 6(G)). Differentiated neurons were immunoreactive for TUJ1 and TH (Figure 6(I)) and were compared to the total number of unstained differentiated neurons (Figure 6(J)).

Both approaches, stereology and flow cytometry, showed no significant differences among the three different components Shh, SAG, or Pur used in dopaminergic differentiation in terms of the ratio of TUJ1/total, TH/TuJ1, and TH/total cells (Figure 6(K)). Data from flow cytometry was slightly lower than those from the stereological approach. We suspect that we lost neurons during the handling process such as dissociation and passaging through a cell strainer to filter clumps from cell suspension before flow cytometry was performed.

Some studies have shown that with an extension of culturing time by up to 60 days, more neurons convert to TH-positive as well as become electrophysiologically mature [37, 38]. Other studies showed a higher percentage of TH-positive neurons, however, different quantification approaches may have introduced bias toward a higher percentage of neuronal yields.

4. Discussion: Small Molecules for Efficient Neuronal Differentiation

Over the last few years, there has been an enormous push to optimize differentiation protocols with different small molecules and screens to identify new factors that would modulate and improve neuronal differentiation and maturation.

Other small molecules and compounds have been identified for the enhancement of neuronal differentiation. Glycogen synthase kinase-3 (GSK-3) inhibitors such as kenpaullone or SB-216763 have been shown to positively impact the neuronal differentiation of neural progenitor cells without changing cell cycle exit or cell survival [39]. Furthermore, GSK-3 inhibitors showed protection against excitotoxicity, mediated by NMDA and non-NMDA receptor agonists, in cultured rat primary cerebellar granule neuronal cultures from the cerebellum and hippocampus [40].

(+)-Cholesten-3-one but not cholesterol has been shown to effectively promote the activity of the TH promoter. (+)-Cholesten-3-one has also been shown to induce differentiation of neuroprogenitors into dopaminergic neurons monitored by expression of TH, dopamine transporter, dopa decarboxylase, and higher levels of dopamine secretion [41].

Neurosteroids are thought to affect neuronal survival, neurite outgrowth, and neurogenesis both *in vivo* and *in vitro* [42], that is, progesterone [43] and estradiol [44]. Progesterone added at the neural proliferation stage increased the number of dopaminergic neurons, whereas progesterone added during final differentiation did not induce significant changes in the number of dopaminergic neurons generated. Interestingly, this effect was not mediated by the activation of progesterone receptors because RU 486 did not block the effects of progesterone on dopaminergic differentiation [43]. It has also been shown that estradiol can increase the generation of dopaminergic precursors expressing Lmx1a and can induce formation of a higher percentage of mature dopaminergic neurons [44].

In addition, polyunsaturated fatty acids such as arachidonic acid (ARA) and docosahexaenoic acid (DHA) have been shown to have critical roles in brain development and function and can promote neurogenesis [45]. Specifically, DHA, a ligand for the RXR/Nurr1 heterodimer, can activate the Nurr1 gene in iPSCs. It has been shown that DHA facilitates iPSC differentiation into TH-positive neurons *in vitro* as well as *in vivo* [46].

Through a peptide library screen a novel small synthetic peptide Cripto BP was discovered to block Cripto, a glycosylphosphatidylinositol-anchored coreceptor. It has been shown that this receptor binds Nodal and the ALK-4 receptors and promotes cardiac differentiation. The deletion or inhibition of Cripto leads to a promotion of neuronal and midbrain differentiation of mouse embryonic stem cells. The synthetic peptide Cripto BP can mimic this effect [47].

5. Conclusion

Small molecules can enhance various steps of neuronal differentiation into dopaminergic neurons and can replace expensive recombinant proteins that were initially used in the pioneering protocols. However, there is still a need for improvement of differentiation protocols that increase the number and region-specificity of mature region specific dopaminergic neurons, but selective inhibitors and other small molecules might change the field and reduce the cost.

Conflict of Interests

The authors declare they have no conflict of interests.

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Research Article

High-Resolution Genomic Profiling of Chromosomal Abnormalities in Human Stem Cells Using the 135 K StemArray

Aaron M. Elliott, Kristi A. Hohenstein Elliott, and Anja Kammesheidt

Ambry Genetics, Departments of Genomic Services and R&D, 15 Argonaut, Aliso Viejo, CA 92656, USA

Correspondence should be addressed to Aaron M. Elliott, aelliott@ambrygen.com

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Culturing stem cells for an extended period of time can lead to acquired chromosomal aberrations. Determining the copy number variant (CNV) profile of stem cell lines is critical since CNVs can have dramatic effects on gene expression and tumorigenic potential. Here, we describe an improved version of our StemArray, a stem-cell-focused comparative genomic hybridization (aCGH) microarray, which contains 135,000 probes and covers over 270 stem cell and cancer related genes at the exon level. We have dramatically increased the median probe spacing throughout the genome in order to obtain a higher resolution genetic profile of the cell lines. To illustrate the importance of using the StemArray, we describe a karyotypically normal iPSC line in which we detected acquired chromosomal variations that could affect the cellular phenotype of the cells. Identifying adaptive chromosomal aberrations in stem cell lines is essential if they are to be used in regenerative medicine.

1. Introduction

Several studies have demonstrated that human embryonic and induced pluripotent stem cells (ESCs, iPSCs) acquire genomic abnormalities during prolonged culture [1–3]. These chromosomal aberrations can have dramatic effects on the survival, proliferative ability, and differentiation potential of the cells, which can result in unreliable experimental results and jeopardize their potential use in regenerative medicine. The most common method used by stem cell researchers to monitor the genomic stability of the cell lines is G-banded karyotype analysis. However, this method can only detect large variations over 5 megabases (Mb), and therefore, the majority of smaller chromosomal changes are missed. Recently, numerous groups have been employing other methods for stem cell characterization, including gene expression profiling and array-based comparative genomic hybridization (aCGH) microarray analysis [4–6]. Although gene expression profiling is beneficial to illustrate the true transcriptional state of the cells, the resolution of this technique is over 10 Mb in size [4]. aCGH is a technique which can detect unbalanced structural abnormalities at a typical resolution under 100 Kb. Studies using aCGH microarrays to detect copy number variations in stem cells have identified numerous subkaryotypic alterations acquired during cultural adaptation [2, 6]. However, the arrays used in these studies were nontargeted whole genome tiling arrays, which generally have low coverage of single genes and are relatively expensive for routine analysis.

We previously developed a stem-cell-targeted aCGH microarray which contains 44 K probes with increased probe coverage in targeted regions [7]. Here, we describe an updated and improved version of the StemArray that is currently used by a wide variety of stem cell laboratories to characterize the genomic integrity of their stem cell lines. The array contains 135 K probes to cover the entire genome at an average resolution of 15 Kb. In addition, the custom-targeted microarray has exon level resolution in over 270 stem cell and cancer-related genes. The use of the 12 × 135 K array platform, which allows 12 samples to be run per slide, significantly reduces the costs of the array and makes it competitive in pricing with karyotype analysis.

2. Materials and Methods

2.1. iPSC Line Culture. iPSC lines used in the study were generated from fibroblasts using standard retroviral transduction of *OCT4*, *SOX2*, *KLF4,* and *c-MYC*. Resulting iPSC lines were cultured on Matrigel (BD Biosciences) substrates in conditions described previously [7]. Genomic DNA was isolated using the Puregene DNA purification Kit (Qiagen) and the quality determined using an ND-1000 spectrophotometer (NanoDrop).

2.2. aCGH. The stem-cell-focused microarray was developed by Ambry Genetics (Aliso Viejo, CA) using Roche NimbleGen probe sets. The microarray contains 135,000 probes annotated against the human genome assembly build 37 (UCSC hg 19). Probe density was increased in over 270 stem cell and cancer-associated genes, with an average of in these regions (gene list available upon request). The remaining probes were tiled throughout the genomic backbone at a median probe spacing of 15 Kb. Following validation runs, only those probes with optimal performance were selected for the final array design. aCGH was performed according to the Roche NimbleGen protocol (V.8.0). Briefly, 500 ng of human stem cell DNA and 500 ng of pooled sex-matched reference DNA (Promega) were heat denatured at 98°C for 10 minutes and then labeled with Cyanine 3 Random Nonamers and Cyanine 5 Random Nonamers by Exo-Klenow fragment. The labeled DNA was then purified by isopropanol precipitation and the labeling efficiency determined using an ND-1000 spectrophotometer. Based on the concentration, 20 μg of the labeled sample and reference DNA along with 2X hybridization buffer, hybridization component A, and alignment oligo were added together and placed on the 135 K StemArray (Ambry Genetics). Microarrays were hybridized on the Maui Hybridization System (Roche NimbleGen) at 42°C for 72 hours. Slides were washed according to the protocol and scanned at 2 μM resolution on a NimbleGen MS200 high resolution scanner.

2.3. Data Analysis. Data was extracted and normalized using NimbleScan 2.6 software package (Roche NimbleGen). For aberration calling, normalized data sets were imported into Nexus Copy Number version 6.0 (BioDiscovery). To correct for GC content, a noise reducing systematic correction file was developed based on the genomic locations of the probes in the design. Aberrant regions were determined using the FASST2 segmentation algorithm with a significance threshold of 1.0E-6. The aberration filter was selected with the following parameters: minimum number of probes in the region 4, minimum absolute average $\log_2$ratio for one copy amplification was .3 and for a heterozygous deletion −.3, and a mean $\log_2$ ratio ≥1.0 represents a high copy gain and ≤1.1 a homozygous copy loss.

3. Results and Discussion

In an effort to identify smaller intragenic variations in genes important for stem cell maintenance, we have made significant improvements to the StemArray design published previously [7]. The probe content has increased from 44 K probes to 135 K probes, resulting in an overall increase in backbone resolution from 43 Kb to 15 Kb, respectively. The updated design also includes on average of 5 probes per exon in over 270 stem cell and cancer-related genes, enabling single exon resolution in these functionally important regions. For example, the 28 exons of the kinase BUB1, a key "stemness" gene essential for maintaining genomic stability, are covered at the exon level by 141 probes (Figures 1(a) and 1(b)) [8, 9]. In contrast, the standard catalog 135 K non-targeted array contains only one probe in the *BUB1* gene (Figure 1(c)).

iPSC lines are generally derived by transforming fibroblast cells with retroviral vectors containing *OCT4*, *SOX2*, *KLF4,* and *c-MYC* [10]. These genes provide good positive controls for iPSC aCGH data since multiple copies of these transgenes integrate into the DNA. Using our previous 44 K design, we were not able to detect transgene integration of *OCT4* due to lack of quality probes in the exons. However, with the new 135 K design, we are able to identify high copy amplifications of all four pluripotency genes (Figures 2(a)–2(d)).

To illustrate the importance of characterizing stem cell lines with a stem-cell-focused microarray, we monitored the genomic stability of a late passage iPSC line by both G-banded karyotyping and the custom focused 135 K StemArray. Karyotype analysis revealed no aberrations in this iPSC line (Figure 3(a)). Following this result, most stem cell researchers would consider this cell line normal and suitable for further research. However, high-resolution aCGH analysis revealed 9 subkaryotypic variations in the iPSC line ranging in size from 1.5 Kb to 595 Kb (Figure 3(b)).

When conducting aCGH on an iPSC line, it is recommended to first determine the genomic profile of the parental fibroblast line from which the cells were derived. Since all individuals genomic DNA contain copy number variations, performing this initial test will allow one to separate the chromosomal variations acquired during culture from those inherent to the parental fibroblasts. Moreover, it is not uncommon for fibroblast lines to acquire genomic alterations in culture similar to stem cells. Performing aCGH on these cells before one derives iPSC lines is good practice as one would not want to waste time and money developing stem cell lines from fibroblasts which already contain detrimental aberrations. By doing this, we could classify 7 of the identified variations as derived from either the integration of the 4 transgenes, or as copy number variations present in the parental fibroblast population. Therefore, the remaining 2 aberrations had been acquired by the iPSC line during reprogramming or prolonged culture.

The 595 Kb amplification at 3q13.13 contains 8 genes including the stem-cell-associated *DPPA2* and *DPPA4* genes (Figure 4(a)). Several studies have identified these tightly linked genes as specific markers for pluripotent cells [11–13]. The function of *DPPA2* and *DPPA4* in stem cells has been controversial. Madan et al. [14] created *DPPA2*/*DPPA4* double deficient mouse ES cells and concluded that these genes were dispensable to the ES cell phenotype, since they

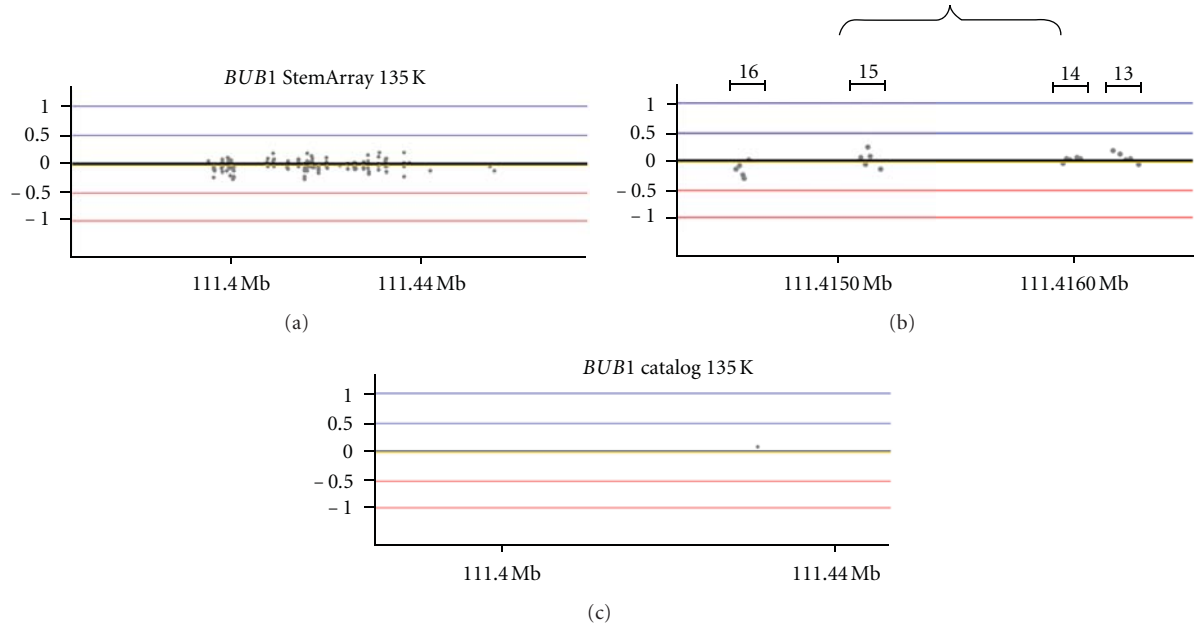

Figure 1: The 135 K StemArray has increased probe coverage in stem cell and cancer-associated genes. (a) Stemness gene *BUB1* contains 141 total probes resulting in single exon resolution. (b) Exons are covered with 5 probes/exon enabling the detection of a single exon deletion or amplification. (c) In comparison, the standard NimbleGen catalog 135 K array only contains a single probe in the *BUB1* gene and therefore does not have the resolution to detect a variation in this gene.

maintained their ability to self-renew and differentiate similar to wild-type ES lines [14]. However, a recent study by Du et al. [15] demonstrated that siRNA knockdown of *DPPA2* in mouse ES cells resulted in downregulation of marker genes *OCT4* and *NANOG*, accelerated differentiation, and decreased proliferation [15]. In support of these findings, several other knockdown screens have identified these genes as critical in mouse ES cell self-renewal, differentiation, and possible targets of *OCT4* and *SOX2* [16, 17]. It would be interesting to determine the function of *DPPA2* and *DPPA4* in human stem cells, as these stem cell markers have also been shown to be highly expressed in different types of human cancers [18]. Overall, the data suggests stem cells lines harboring amplifications of *DPPA2* and *DPPA4* may have a selective advantage, and one should be cautious using such lines in their studies.

The other abnormality acquired in the iPSC line during extended culture was a 285 Kb deletion at 16q23.3 spanning exons 4-5 of the *CDH13* gene (Figure 4(b)). *CDH13*, also known as H-cadherin, has been implicated in cell growth, survival, and proliferation [19]. Downregulation of *CDH13* has been observed in numerous cancer types and has been associated with increased tumor cell aggressiveness [19, 20]. Likewise, overexpression of *CDH13* in cancer cells results in reduced proliferation, increased susceptibility to apoptosis, and a reduction of tumor growth *in vivo* [21, 22]. Moreover, recurrent deletions encompassing *CDH13* have been observed in various cancers including lung cancer, ovarian cancer, and retinoblastoma [20, 23, 24]. This finding is in agreement with Baker et al. [25] who suggest there is a link between cultural adaptation and tumorigenic events that occur *in vivo* [25]. Chromosomal abnormalities such as these found in human stem cells during long-term culture raise obvious concerns about the safety of particular lines.

Although we could provide further examples of the utility of using our updated 135 K StemArray to monitor genomic stability, we believe the example provided here clearly demonstrates the benefits for such testing. Although karyotype analysis is still a popular technique to monitor the genetic integrity of stem cells, many stem cell researchers are beginning to realize the importance of using higher resolution methods to detect submicroscopic alterations. Using an Affymetrix 115,000 single-nucleotide polymorphism (SNP) microarray, Maitra et al. were able to identify an amplification of ~2 Mb on chromosome 8 encompassing the c-MYC oncogene in a high passage ESC line [1].

Several groups have identified the acquired duplication at 20q11.21 using a wide variety of microarray platforms from low-resolution bacterial artificial chromosome/P1-plasmid artificial chromosome (BAC/PAC) arrays to high-resolution 244,000 probe aCGH arrays [2, 5]. This alteration has been observed in both human ESC and iPSC lines and typically includes the stemness gene DNMT3B. Cell lines containing this duplication tend to grow better, have increased survival, and differentiate slower than wildtype lines. Interestingly, our group and others have also detected amplifications spanning this region which do not contain the DNMT3B gene but do include ID1 [7]. ID1 encodes a helix-loop-helix protein which interacts with the HLH transcription factors, altering their DNA-binding ability [26]. Recently, a study by Martins-Taylor et al. used 135,000 and 385,000 probe microarrays to identify recurrent copy number variations

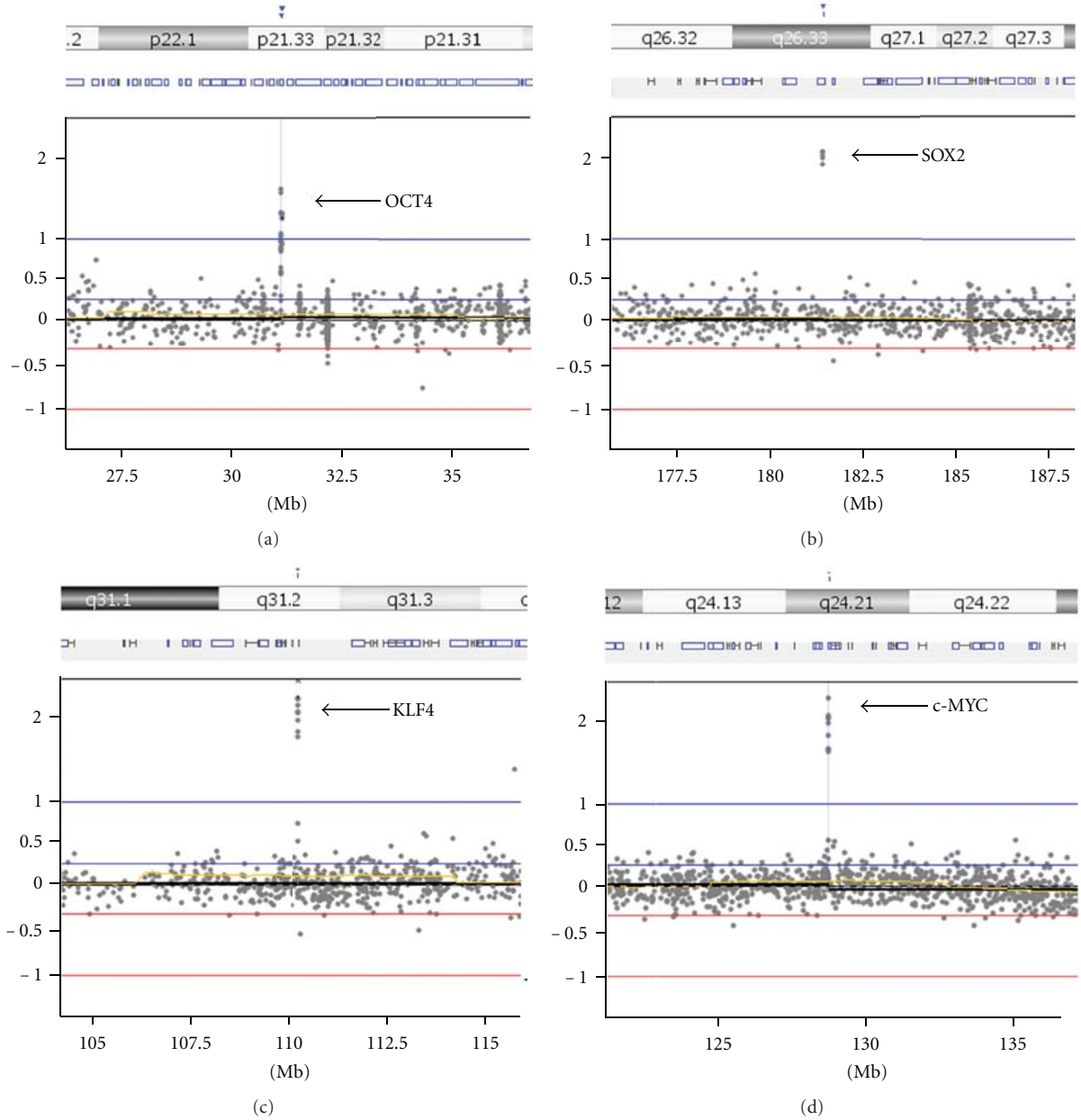

Figure 2: iPSC lines are typically derived by transforming fibroblast cells with retroviral vectors containing *OCT4*, *SOX2*, *KLF4,* and *c-MYC*. These reprogramming genes provide excellent positive controls for the microarray as multiple duplications for each transgene should be observed. The updated 135 K StemArray can detect multiple copy integrations of the iPSC transforming factors (a) *OCT4*, (b) *SOX2*, (c) *KLF4*, and (d) *c-MYC*.

in iPSC lines. Although several small regions commonly acquired in iPSC lines were discovered including 1q31.3, 2p11.2, and 17q21.1, there were no evident candidate genes in these segments with associated functions in stem cells [6].

Although these studies utilizing aCGH technology to characterize stem cell lines were informative, they were all conducted using nontargeted standard catalog microarrays. These microarray platforms are generally designed to tile the entire genome with the resolution dependent on the total number of probes used. Therefore, the majority of stem-cell-related genes in catalog microarrays have little to no coverage, and, as a result, small aberrations spanning these regions are typically missed. In accordance, we routinely detect causative aberrations when testing stem cell samples in our laboratory that had previously appeared normal with karyotype or catalog aCGH microarray analysis. It is important to note that karyotype analysis should not be disregarded completely, because it allows detection of balanced translocations, which is not possible with aCGH. For that reason, we believe both methods should be used in order to obtain a complete genetic profile of a stem cell line. As more data is generated with the 135 K StemArray,

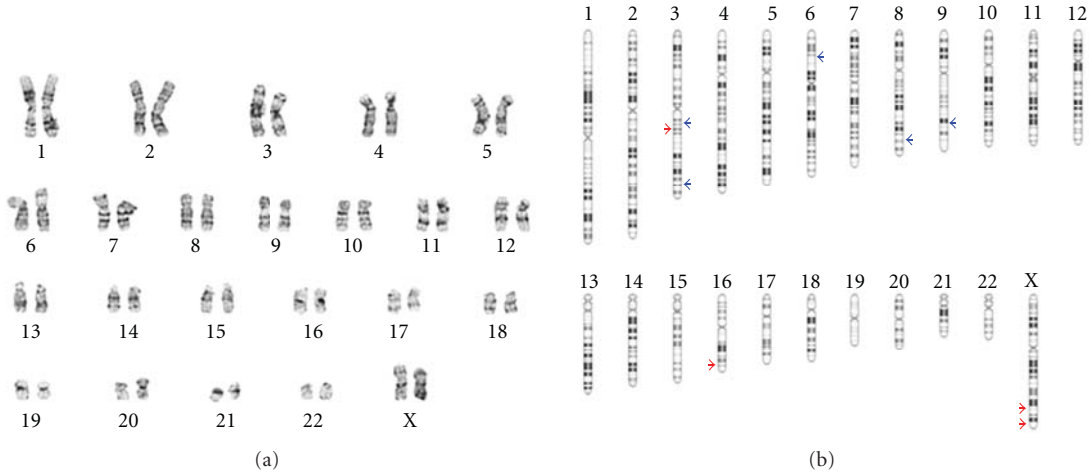

Figure 3: Genomic stability profiling of a human iPSC line by karyotype analysis and the StemArray. (a) The majority of stem cell researchers still characterize their cells by G-banding metaphase karyotyping which has a resolution of only 5 Mb. Testing our iPSC line with this method did not detect any aberrations. (b) aCGH with the custom 135 K microarray identified 4 deletions and 5 amplifications in the iPSC sample ranging in size from 1.5 Kb to 595 Kb.

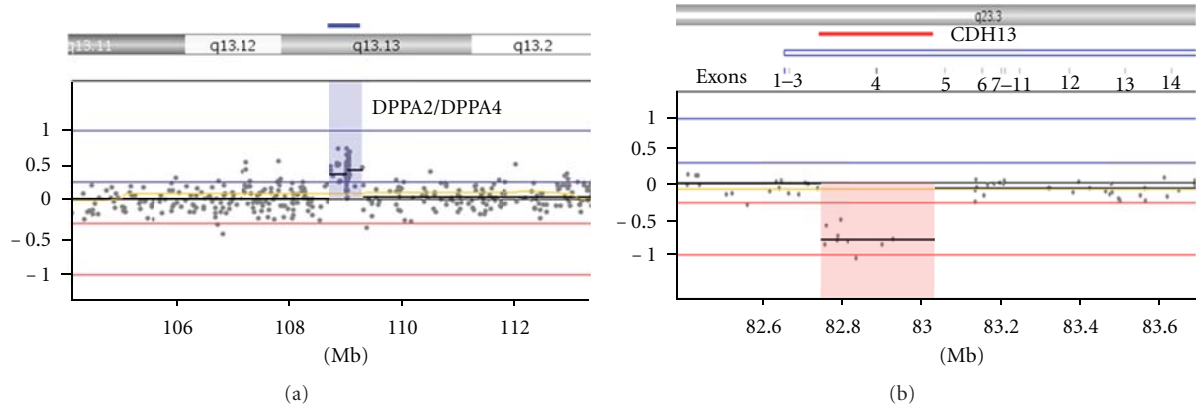

Figure 4: The stem-cell-targeted 135 K StemArray can detect causative aberrations in iPSC lines known to influence cell survival and proliferation. Detection of two acquired chromosomal abnormalities in stem-cell-associated and cancer-related genes in an iPSC line. (a) A 595 Kb amplification spanning the stem-cell-related DPPA2 and DPPA4 genes, and (b) a 285 Kb deletion covering exons 4-5 of the cancer associated CDH13 gene.

we expect to gain new insights into those regions important in stem cell maintenance. In addition, since microarray designs vary wildly in probe placement and gene coverage, it is important for stem cell researchers to agree on specific design parameters to monitor their cell lines if data is to be compared.

4. Conclusions

Human stem cell lines that are cultured for an extended period of time are susceptible to chromosomal aberrations. Obtaining a comprehensive genomic profile of these lines is essential, because the acquired structural variations can influence the proliferative ability of the cells. By using a stem-cell-focused microarray such as StemArray, researchers can identify causative aberrations that would otherwise be missed by karyotype analysis and standard catalog arrays. As ES and iPSC lines begin to be used for therapeutic purposes it will be necessary to assess the cells genomic stability with a high-resolution focused array to ensure safety and usefulness.

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Review Article

Advances in MicroRNA-Mediated Reprogramming Technology

Chih-Hao Kuo and Shao-Yao Ying

Department of Cell and Neurobiology, Keck School of Medicine, University of Southern California, BMT-403, Los Angeles, CA 90033, USA

Correspondence should be addressed to Shao-Yao Ying, sying@usc.edu

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The use of somatic cells to generate induced-pluripotent stem cells (iPSCs), which have gene characteristic resembling those of human embryonic stem cells (hESCs), has opened up a new avenue to produce patient-specific stem cells for regenerative medicine. MicroRNAs (miRNAs) have gained much attention over the past few years due to their pivotal role in many biological activites, including metabolism, host immunity, and cancer. Soon after the discovery of embryonic-stem-cell- (ESC-) specific miRNAs, researchers began to investigate their functions in embryonic development and differentiation, as well as their potential roles in somatic cell reprogramming (SCR). Several approaches for ESC-specific miRNA-mediated reprogramming have been developed using cancer and somatic cells to generate ESC-like cells with similarity to iPSCs and/or hESCs. However, the use of virus-integration to introduce reprogramming factors limits future clinical applications. This paper discusses the possible underlying mechanism for miRNA-mediated somatic cell reprogramming and the approaches used by different groups to induce iPSCs with miRNAs.

1. Introduction

In the 1980s, the first mouse embryonic stem cell line, which displayed both the capability for unlimited proliferation and the pluripotency and capacity to differentiate into three germ layers, was established, and since then, embryonic stem cells have shown great promise for advancing the fields of drug discovery, disease modeling, and regenerative medicine. It was believed that cells differentiated in a unidirectional manner until the invention of somatic cell nuclear transfer (SCNT) [1]. This technique replaces the nuclei of oocytes with those of somatic cells, resulting in the reversal of the fused cells from a differentiated to pluripotent status. Subsequently, many species of mammalian cells have been successfully cloned, including the famous cloned sheep Dolly [2]. However, this approach raises problems, such as the ethical concerns regarding the use of embryos and the technical challenges and low success rate associated with this method.

In 2006, Takahashi and Yamanaka successfully generated induced-pluripotent stem cells (iPSCs) with a novel approach using the viral integration of the four transcription factors, Oct4, Sox2, Kl4, and c-Myc [3]. This revolutionary approach has demonstrated the feasibility of using somatic cells to generate iPSCs. Patient-specific iPSCs can be derived from cells of the same patient, thereby avoiding the immune rejection that occurs with SCNT or human embryonic stem cells (hESCs). More importantly, use of somatic cells as the starting material circumvents the ethical issues associated with the use of human embryos. Subsequently, many iPSCs have been generated from various types of somatic cells, including keratinocytes, neuronal stem cells, and fibroblasts, among others [3–6]. These iPSCs display gene expression patterns, cell morphology, and the capacity of forming teratoma *in vivo* similar to those of hESCs [3, 6]. Nevertheless, the use of retroviral delivery hinders its application in clinical therapy because integration of viral genes into the iPSC genome may cause instability that leads to undesired mutations [7]. In addition, studies have shown that the ectopic expression of c-Myc, one of the original four transcription factors, correlates with increased tumorigenicity and further raises questions about the therapeutic potential of iPSC generated in this manner [8]. Although successful reprogramming has been reported using only 2 Yamanaka factors in the absence of c-Myc, this approach results in

greatly decreased efficiency [9]. In addition, synthetic modified mRNAs have been used to replace viral delivery for elevating expression of the four Yamanaka factors, and while these studies demonstrated the efficient reprogramming of cells in the absence of retrovirus, ectopic c-Myc was still present [10, 11].

In 2004, Suh and colleagues identified a set of embryonic stem cell-specific microRNAs (miRNAs), [12] which have subsequently been found to contribute to embryo development [13–15]. In fact, deficiency in theses miRNAs can cause detrimental defects in cell proliferation and differentiation [16, 17]. Among the highly expressed ESC-specific miRNAs, miR-302/367 is highly expressed in early embryonic development and then rapidly declines after differentiation [12, 13]. This information prompted several laboratories to investigate the role of miR-302/367 in reprogramming [18–20]. Numerous miRNA-mediated iPSC lines have been subsequently developed with either miR-302/367 or a combination of miR-302 and other miRNAs from mouse fibroblast, human dermal fibroblast, and human skin cancer cells [21, 22]. As this approach avoids the use of oncogene c-Myc, the possibility exists that miRNA-reprogrammed iPSCs would be more suitable for human use.

2. Proposed Mechanism of miR-302/367-Mediated Reprogramming

Currently, the mechanism by which somatic cells generate iPSCs remains unclear. Maternal materials rather than downstream transcription factors regulate the maintenance and renewal of fertilized oocytes [23]. A large portion of RNAs in mouse oocytes are transcribed from the maternal genome, and maternal miRNAs present in the oocyte rapidly decline during the oocyte-zygote transition. Therefore, it is reasonable to assume that these maternal miRNAs inhibit the developmental signal for maintaining pluripotency in the early embryonic stage through an RNAi effect. This, in combination with the observation of high miR-302/367 expression in the early embryo followed by a rapid decrease upon differentiation, strongly suggests that miR-302/367 serves as upstream pluripotency regulator to modulate the expression of Oct4, Sox2, Nanog, and other embryonic transcription factors. Given that miRNA can target several to hundreds of genes, the inhibition of multiple factors and pathways likely initiates miR-302/367 reprogramming.

miR-302/367 targets multiple epigenetic factors, leading to global demethylation. Global DNA demethylation occurs at the promoter binding site of several ESC-specific transcription factors during the 1–8 cell stages of early zygotes, resulting in preservation of imprinting. The same mechanism very likely happens during somatic cell reprogramming (SCR). MiR-302 silences lysine-specific histone demethylases 1 and 2 (AOF1 and AOF2) and methyl-CpG-binding proteins 1 and 2 (MECP1-p66 and MECP2). DNA methyltransferase 1 (DNMT1), an essential regulator in DNA methylation, is then silenced in response to the downregulation of AOF2, leading to genomewide demethylation and consequently coactivation of pluripotency-promoting genes [20, 24]. Indeed, silencing AOF2 enhanced global demethylation during reprogramming of human hair follicle cells by miR-302s [20].

miR-302/367 also directly targets NR2F2, a member of the nuclear orphan receptor family of transcriptional factors and a negative regulator of Oct4 [25]. In hESCs, NR2F2 expression begins with differentiation and conversely correlates with the expression of Oct4 and miR-302/367. Studies have also shown that Oct4, Nanog, and Sox2 bind to the promoter regions of miR-302/367 and increase its expression level [26]. Taken together, miR-302/367 expression induces global demethylation and suppresses NR2F2, two events that indirectly activate Oct4 expression, which in turn elevates miR-302/367 levels. This reciprocal cycle increases cellular levels of miR-302/367 and Oct4, which leads to the co-activation of other transcription regulators, such as Sox2 and Nanog. Studies from Lin and colleagues showed that overexpression of mir-302/367 (approximately 1.1- to 1.3-fold as compared with normal hESCs) leads to global demethylation and coexpression of Oct4, Sox-2, and Nanog in human iPSCs [20, 27]. A similar study using RUES2 cells confirmed that transfection with miR-302 elevates Oct4 and Nanog expression [14].

Additional targets may include the transforming growth factor beta receptor II (TGFBR2) and ras homolog gene family member C (RHOC) genes. A recent study has reported that a combination of miR-302b and miR-372 downregulates TGFBR2 and RHOC gene expression [19]. Furthermore, the inhibition of these two molecules correlates with increased efficiency of iPSC induction [19]. Evidence has shown that mesenchymal-to-epithelial transition (MET) occurs during the early reprogramming process in mouse fibroblasts as a result of blocking pro-epithelial-to-mesenchymal signals, such as TGF-beta [28]. The same process may also occur during reprogramming in human cells. We found that the miR-302 may also target TGFBR2 and RHOC, supporting the possibility that miR-302/367 has the same effect on MET as miR-302b/327. However, confirmation of this hypothesis requires further study.

miRNA-reprogrammed iPSCs display a decrease in tumorigenecity as compared with the iPSCs generated by conventional approaches [27], which is likely due to multiple factors. For example, miRNA-mediated somatic cell reprogramming does not require enhanced expression of c-Myc; therefore, no oncogene is involved in the process. Also, miR-302/367 targets several cell cycle regulators. During miRNA-mediated SCR, both cyclin E-CDK and cyclin D-CDK4/6 undergo downregulation. Consequently, this attenuates the G1 to S phase transition, resulting in decreased iPSCs tumorigenicity. In addition, two tumor suppressor genes, p16Ink4a and p14/19Arf, undergo upregulation through the silencing of BMI1 (B lymphoma Mo-MLV insertion region 1 homolog). Taken together, the cell cycle of microRNA-reprogrammed iPSCs is highly regulated and resembles the early mammalian zygote (20 to 24 hours) [27].

3. Different MicroRNA Approaches for SCR

In order to study the role of miRNAs in reprogramming as well as in other physiological events, it is crucial to develop an

artificial expression vector that can both generate functional mature miRNAs and maintain their expression *in vitro* and *in vivo*. Previously, several vectors have been established that use RNA polymerase III transcription activity to generate stable miRNA expression [29–32]. These vectors, however, have several drawbacks. First, the ubiquity of pol III makes it is difficult to target a specific population of cell types. Second, transcription by RNA pol III requires U6 and H1 promoters. Pol III activity could potentially generate the accumulation of large RNA transcripts (>25 bps), thereby producing interferon cytotoxicity [33, 34]. A recombinant gene expression system, mediated by RNA polymerase II and based on the mechanism of miRNA biogenesis, has also been developed [35–37]. The precise regulation of RNA splicing and nonsense-mediated decay of pol-II-directed RNA biogenesis ensures the degradation of excessive RNA accumulation and alleviates potential toxicity caused by high levels of long double-stranded RNAs [38, 39]. miRNAs transcripts are frequently located in an intron in proximity to the protein coding region; thus, this expression system uses an artificial intron (SpRNAi) placed between two exons that, together, encode a reporter, such as green or red fluorescent protein. Because the intronic miRNA of interest disrupts the functional structure of the reporter gene, expression will not occur without proper RNA splicing. Detection of the fluorescent signal provides a means to monitor the production of this intronic miRNA. The artificial intron (SpRNAi) consists of the following components: a 5′-splice site, a branch-point domain, a polypyrimidine tract, and a 3′-splice site, and the pre-miRNA insert is placed between the 5′-splice site and branch-point domain. This system has been shown to induce the RNAi effect in LNCaP, HeLa, and HCN-A94-2 cells as well as in mice [40, 41].

To date, viral transfection serves as the primary method to introduce reprogramming factors, either Yamanaka factors or miRNAs, into cells due to its high efficiency of delivery. However, as mentioned previously, this may result in the integration of exogenous genes into the host genome and as such is not ideal for clinical trials. Many studies have been conducted to find alternatives with higher transduction efficiency. A recent study examined mature miRNAs rather than vector-based delivery as a potential approach for SCR [18]. The use of mature miRNA bypasses the DNA-based plasmid and thus avoids any possible insertion of genes into genome of targeted cells. Further, higher efficiency (0.1%) has been achieved as compared with the retroviral delivery of the Yamanaka factors (0.01∼0.04%). Although this method seems promising, several problems remain. Depending on the cell type and experimental conditions, SRC requires sufficient amounts of cellular mature miRNAs over the course of days to weeks. Thus, repeated transfections may be necessary to maintain the appropriate levels over time. The need for ample amounts of synthetic nucleotides may greatly increase the cost for the large-scale production of iPSCs in a clinical setting. Moreover, instead of the miR-302 family alone, it has been argued that successful reprogramming by mature miRNAs always requires the combination of miR-302 s, miR-200c, and miR-369, while several miRNA-mediated iPSC lines have been generated with only miR-302 or miR-302/367 [20, 22].

4. Conclusions

Based on evidence of the successful establishment of iPSC lines using a miRNA-mediated strategy, it seems that ESC-specific miRNA, especially the miRNA-302/367 family, can induce reprogramming events similar to those of Yamanaka factors. The use of a miRNA expression vector, such as the intronic miRNA expression system, provides a simple and safe way to generate iPSCs due to the fact that no oncogene is required for successful reprogramming and, in the case of miR-302/367, only a single transcript is transfected rather than the simultaneous transfection of multiple genes, whose expression would be difficult to consistently maintain in iPSCs. In general, delivery of premade miRNAs provides a fast and direct way to recapitulate miRNA-mediated RNAi as compared to a vector-based approach. While its practicality for large-scale production of patient-specific iPSCs remains to be determined, the potential underlying mechanism of miRNA-mediated reprogramming suggests that it may represent an improved means to generate patient-specific iPSCs, with better quality and safety for regenerative medicine and transplantation therapy.

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Research Article

Long-Term Cultured Human Term Placenta-Derived Mesenchymal Stem Cells of Maternal Origin Displays Plasticity

Vikram Sabapathy,[1] Saranya Ravi,[1] Vivi Srivastava,[2] Alok Srivastava,[1,3] and Sanjay Kumar[1]

[1] *Center for Stem Cell Research, Christian Medical College, Bagayam, Vellore 632002, India*
[2] *Department of Cytogenetics, Christian Medical College, Bagayam, Vellore 632002, India*
[3] *Department of Hematology, Christian Medical College, Bagayam, Vellore 632002, India*

Correspondence should be addressed to Sanjay Kumar, skumar@cmcvellore.ac.in

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Mesenchymal stem cells (MSCs) are an alluring therapeutic resource because of their plasticity, immunoregulatory capacity and ease of availability. Human BM-derived MSCs have limited proliferative capability, consequently, it is challenging to use in tissue engineering and regenerative medicine applications. Hence, placental MSCs of maternal origin, which is one of richest sources of MSCs were chosen to establish long-term culture from the cotyledons of full-term human placenta. Flow analysis established bonafied MSCs phenotypic characteristics, staining positively for CD29, CD73, CD90, CD105 and negatively for CD14, CD34, CD45 markers. Pluripotency of the cultured MSCs was assessed by in vitro differentiation towards not only intralineage cells like adipocytes, osteocytes, chondrocytes, and myotubules cells but also translineage differentiated towards pancreatic progenitor cells, neural cells, and retinal cells displaying plasticity. These cells did not significantly alter cell cycle or apoptosis pattern while maintaining the normal karyotype; they also have limited expression of MHC-II antigens and are Naive for stimulatory factors CD80 and CD 86. Further soft agar assays revealed that placental MSCs do not have the ability to form invasive colonies. Taking together all these characteristics into consideration, it indicates that placental MSCs could serve as good candidates for development and progress of stem-cell based therapeutics.

1. Introduction

The term Mesenchymal stem cells (MSCs) was coined by Caplan in 1991 [1]. MSCs are defined as the class of stem cells that has the potential to self-renew and differentiate into multiple cell lineages [2, 3]. The presence of mesenchymal stem cells in the bone marrow was hypothesized by Cohnheim in 1860s [4]. In 1920s, Maximow postulated the importance of the marrow stromal tissue in supporting the development and maintenance of blood and hematopoietic organs [5]. In 1960s, Friedenstein was the first to demonstrate stromal cells could be isolated from whole bone marrow aspirate based on differentiation adhesion to tissue culture plastic dishes [6]. In addition, MSCs secrete proangiogenic [7] and antiapoptotic cytokines and possess immunosuppressive properties [8]. Bone marrow MSCs are most commonly used and primary source of MSCs [9]. However, due to invasive nature of bone marrow aspiration and limited proliferative capacity, efforts are underway to identify abundant and reliable sources of MSCs for clinical applications [9]. Mesenchymal stem cells can be broadly grouped into two different subgroups adult MSCs and fetal MSCs. Adult MSCs are isolated from bone marrow, peripheral blood. Fetal MSCs are isolated from Placenta, amniotic fluid, umbilical cord and umbilical cord blood [10]. Placenta provides one of the most reliable and abundant source of MSCs [11]. Term placental tissues are discarded after birth, hence these tissues can be effectively utilized for research as well as clinical application without much ethical concern. In this paper, we systematically characterize the term placental MSCs isolated from cotyledons and validated that the isolated MSCs fulfill the genotypic and functional

criteria laid out for a proper MSC [11, 12]. We have demonstrated that these MSCs have the ability to rapidly expand up to even 25–30 passages without compromising the chromosomal number, cell cycle or apoptosis pattern, phenotypic characteristics, pluripotency-associated endogenous gene expression profile, and differentiation capacity. Placental MSCs were able to transdifferentiate into other cell lineages thus exhibiting their inherent plasticity.

2. Materials and Methods

2.1. Collection of the Human Placenta Samples. The ethical committee of Christian Medical College (CMC), Vellore, approved the study. Following the written consent term placental samples were collected from donors after elective caesarean.

2.2. Cell Isolation. Term human placental MSCs were isolated from cotyledons present towards the maternal side of the placenta. The placental membrane from the maternal side of the placenta was cut open and about 80 g of cotyledons was exercised. The cotyledons was thoroughly washed with PBS and cut into small pieces. The blood clots present in the cotyledons were mechanically removed. The minced placental was once again washed with physiological saline and subjected to sequential digestion with trypsin and collagenase I. The tissues were incubated with 0.25% trypsin for 1 hour at 37°C. After trypsin digestion, the sample was filtered through 250 μm metal sieve. The retentate was collected and subjected to second digestion with12.5 U/mL collagenase I for 1 hour at 37°C. Collagenase I digested tissue sample was passed first through 250 μm metal sieve and filtrate collected was passed through 100 μm cell strainer. The filtrate containing cell suspension after dual filtration stages were subjected to centrifugation at 300 g for 10 minutes. The cell pellet was resuspended in RBC lysis buffer and centrifuged at 300 g for 10 minutes. Finally, the cell pellet was resuspended in Mesenchymal expansion medium (αMEM + 10% FBS + 50 u/mL penicillin + 50 μg/mL streptomycin + 1 mM L-glutamine) and plated into two 75 cm^2 flasks.

2.3. Antibodies. Information on primary and secondary antibodies used for flow-cytometry and immunostaining experiments is provided in Supplementary Table 1 is available online at doi: 10.1155/2012/174328.

2.4. Flow Cytometry. Cells after trypsinization was equally aliquoted (1×10^5 cells per reaction) into FACS tubes and stained on live cells with respective antibody. Unstained antibody and cells stained with isotype antibody acted as controls. Antibodies were added to the cells in dark to avoid bleaching. After addition of the antibody, the sample was incubated at room temperature in dark for 20 minutes. Cells were washed with 1 mL of DPBS without calcium and magnesium and centrifuged at 300 g for 5 min. The pelleted cells were resuspended in 300 μL DPBS w/o calcium and magnesium and analyzed with a flow cytometer (FACS Calibur; Becton Dickinson). A minimum of 10^4 gated events was acquired from each sample for analysis using cell quest.

2.5. Cytogenetic Analysis. Karyotyping of human placental MSCs was carried at Passages 5 and 25 to verify the chromosomal integrity. Metaphase chromosomal preparations were performed according to standard procedures at a 400–550 GTG band level. Zeiss axioplan microscope was used to identify and analyse the chromosomes. Images were analyzed with a photometrics charged coupled device camera and controlled with smart capture imaging software.

2.6. Immunostaining. The cells cultured in 6-well plates were blocked with PBS (without Ca^{2+} and Mg^{2+}) containing 0.1% BSA, fixed with 4% paraformaldehyde and permeabilized using 0.2% Triton X-100. If using unconjugated antibody, samples were first incubated with primary antibody, blocked with PBS containing 0.1% BSA and subsequently incubated with fluorescent dye conjugated secondary antibody. All cell samples were additionally counterstained with Hoechst 33342. Images were taken using leica DMI6000B (Leica) equipped with DFC360FX digital camera and analyzed with Lecia AF imaging software (Leica).

2.7. Total RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA isolation was carried out using Trizol (Invitrogen). cDNA was prepared with superscript III reverse transcriptase enzyme. The primer sequences and their respective annealing temperature are presented in supplementary entary Table 2. PCR conditions were initial denaturation at 94°C for 2 min, followed by denaturation at 94°C for 1 min, annealing for 1 min, extension at 72°C for 2 min for 35 cycles, and final extension was carried out at 72°C for 5 min. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) RNA was used as a control for normalization of RNAs. PCR products were analyzed using ethidium bromide stained 2% agarose gels. Analysis of the gel images was carried out (Supplementary Table 2).

2.8. QPCR. Total RNA was extracted with Trizol (Invitrogen) according to the manufacturer's protocol. cDNA synthesis was carried out using Superscript III First-Strand synthesis system (Invitrogen). qRT-PCRs were carried out with SYBR Green master mix and AB real-time thermocycler (AB 7500). Primer sequences for the analysis of endogenous pluripotency gene expression are mentioned in the table below. The expression levels of individual genes were normalized against β-Actin (Supplementary Table 2).

2.9. Cell Cycle Analysis. For cell cycle analysis [13], cells were fixed with cold methanol, treated with RNase A 10 μg/mL, stained with Propidium Iodide 50 μg/mL, and analyzed by flowcytometer.

2.10. Apoptosis Analysis. Apoptosis analysis was carried by following the manufacturer's instructions (BD Pharmingen Annexin V). The cells were subjected to live staining with Annexin V and 7-AAD and analyzed the cells through flowcytometer.

2.11. Oligo-Lineage Differentiation Analysis. Placental MSCs at various passages were subjected intra- and translineage differentiation protocol to analyze the plasticity of the cells. After differentiation, cells were stained with appropriate stains and examined microscopically under Leica microscope.

2.12. Adipogenic Differentiation. Placental MSCs at 5×10^4 cells were seeded onto 24-well plate (corning) containing adipogenic differentiation medium (Invitrogen) for 30 days, fresh medium added every 48 hours. Oil red O staining was carried out to visualize the presence of fat droplets. Cells were fixed with 4% paraformaldehyde, washed with sterile water, and incubated with 60% isopropanol at room temperature. Fixed cells were stained with 0.5% oil red O in isopropanol for 20 minutes at room temperature. After staining, cells were first washed with 60% isopropanol later rinsed with sterile water before observing under the microscope for imaging.

2.13. Chondrogenic Differentiation. Chondrogenic differentiation was carried out using falcon25 static cell culture system (specially fabricated in our lab for chondrocyte differentiation). Cells were subjected to micromass cell culture conditions to induce the chondrocyte differentiation under chondrocyte differentiation medium (Invitrogen) for 30 days. One million MSCs were pelleted at 300 g and chondrocyte differentiation medium was added without disturbing the pellet. Media was changed every 48 hrs. After differentiation, cells were fixed with 10% formalin, stained with merchrome, and embedded in paraffin. Staining on deparaffinized 5 μm sections staining for proteoglycans was carried out using saffranin O and 3% alcian blue. After staining, sections were rinsed with distilled water, air dried at room temperature, immersed in xylene, and mounted using DPX before observing under microscopy.

2.14. Osteogenic Differentiation. For osteogenic differentiation, 5×10^4 cells were seeded per well in 24-well plate containing osteogenic induction medium (Invitrogen) for 30 days, with media change every 48 hrs. After differentiation, presence of extracellular calcium was confirmed by VonKossa staining. For vonkossa staining, the cells were fixed in precooled methanol. After fixing, the cells were washed with DPBS (W/O Ca^{2+} and Mg^{2+}), treated with 5% silver nitrate solution in water, and exposed to UV light for 1 hour under the laminar hood. Stained cells were washed with water and incubated with 5% sodium thiosulphate in water for 2 min at room temperature. Finally, sample was rinsed with sterile water and observed under the microscope for imaging.

2.15. Myotubule Differentiation. For myotubule differentiation [14], 5×10^4 placental MSCs were seeded in 25 cm^2 flask containing mesenchymal expansion medium with 3 μM 5-azacytidine. The cells were cultured for 21 days with media changes every 7 days. The cells were stained with Hoechst 33342 (5 μg/mL), incubated at 37°C for 30 minutes before observing under the microscope for imaging.

2.16. Tubular Assay. Matrigel (BD) was thawed at 4°C for overnight. 50 μL of matrigel was aliquoted per well of 96 well plate using precooled tips. The plate was centrifuged at 300 g for 5 min, 4°C. Allowed to polymerize at 37°C for 30 min. MSCs at 1×10^5 cells/well were seeded in mesenchymal expansion medium. Cells were incubated at 37°C under hypoxic condition for 6 hours before observing under the microscope for imaging [15].

2.17. Neural Differentiation. To induced neuronal differentiation [16], 5×10^5 placental MSCs were seeded onto serum-free α-MEM containing 5 mM β-mercaptoethanol and cultured for 6–9 hrs. The cells after induction were fixed for immunostaining analysis.

2.18. Retinal Cell Differentiation. For Retinal differentiation [17], 1×10^5 cells were seeded into media containing Mesenchymal expansion medium supplemented with 50 μM Taurine with 1 mM Beta-mercaptoethanol. The cells, were cultured for 4 days with media changes every 4 days. After retinal induction cells were collected in trizol for RT-PCR analysis or fixed for immunostaining.

2.19. Pancreatic Progenitor Cell Differentiation. For pancreatic differentiation [12, 18, 19], 25 cm^2 flasks were treated with gelatin and 5×10^5 cells were seeded onto gelatinized dish containing mesenchymal expansion medium with 10 mM nicotinamide and 1 mM β-Mercaptoethanol for 24 hrs. Following preinduction, cells were treated with Mesenchymal expansion medium without FBS but containing 10 mM nicotinamide and 1 mM β-Mercaptoethanol for 6 hours, and for following 18 hrs cells were treated with induction media containing FBS. After differentiation, cells were collected in trizol and subjected to RT-PCR analysis or fixed for immunostaining.

2.20. Soft Agar Assay. For Soft agar assay [20], 0.6% agar containing MEM was layered on the surface of 35 mm dish (corning) and incubated in laminar hood for 30 min. Later, 2×10^4 MSCs were mixed with 0.3% agar containing MEM and overlayed on the top of 0.6% agar layer. Plate was incubated in hood for 20 minutes. Following incubation, 500 μL of Mesenchymal expansion medium was added and incubated for 21 days. To the dish, 500 μL of fresh media was added every 7 days once. HeLa cells were used as a positive control.

2.21. Dithizone Staining. For Dithizone (STZ) Staining, the cells were incubated with DTZ solution 100 μg/mL in α-MEM media for 20 minutes at 37°C. After staining, the

cells were rinsed with twice with PBS and examined under microscope [21].

2.22. Cell Population Doubling Time (Gt). Population doubling time indicates the growth rate of the placental MSCs [22], population doubling (PD)

$$\mathrm{PD} = \frac{\ln(\mathrm{Nf}/\mathrm{Ni})}{\ln 2}, \quad (1)$$

where ln equals natural logarithm, Nf equals final cell count, Ni equals initial cell count

$$\mathrm{Gt} = \frac{t}{\mathrm{PD}}, \quad (2)$$

t = Time in hours after cell seeding.

Average Gt value was attained by adding the obtained Gt values for different experiments divided by number of experiments.

3. Results

3.1. Derivation of Adherent Fibroblast Like Mesenchymal Stem Cells (MSCs) from Maternal Side of Human Placenta and Immunophenotypic Characterization of Human Placental MSCs. Enzyme-mediated fractionation of human termed placenta resulted in derivation of fibroblast-like cells, which are generally term placenta-derived multipotent mesenchymal stem cells (PD-MSCs). Selection for MSCs rested on the classic adhesion method on tissue culture plastic. Placental MSCs from 8 term placental samples have been established from maternal side lobules of human placenta following trypsin digestion and collagenase-I treatment following which samples were passed through the 100 μ filter and were seeded in α-MEM containing 10% FBS, and adherent cell population was then characterized for their proliferation capabilities, cell cycle, apoptosis pattern, immunophenotypic features, and differentiation capabilities. The isolated MSCs formed a homogenous monolayer of adherent spindle-shaped fibroblast-like cells. The protocol proved successful in 8 of 8 placental tissues collection. Plating of cell suspensions from the first digest with trypsin did not produce any colonies, but cell suspensions produced from final collagenase I digest of placental tissue fragments typically produced MSC colonies of variable sizes that contained outgrowing fibroblast-like cells. After initial plating of the cells, the colonies became visible after 7 days. These MSC colonies in turn started to proliferate steadily, the flask was almost 60–70% confluent and ready for splitting by day 14. Typically, approximately 5–6 $\times$ 10^4 cells were obtained within 12–14 days after plating. Following the process of initiation the flasks were subjected to trypsinization in 1 : 2 or 1 : 3 ratio. The 75 cm^2 at 1 : 2 splitting was subconfluent by day 3, indicating these isolated cells had very rapid proliferating capacity. Outgrowing cells when harvested and replated in higher dilutions rapidly formed secondary colonies from single cells (Figures 1(a) and 1(b)). PD-MSCs were expandable up to passage 25–30 (as far as we cultured) without any changes in the morphological characteristics (Figures 1(c) and 1(d)) and were amenable to routine cryopreservation, thawing and differentiation protocols. The MSCs were characterized using flow-cytometry-based positive reaction for mesenchymal lineage surface markers $CD29^+$, $CD73^+$, $CD90^+$, $CD105^+$; and negative for hematopoietic marker $CD34^-$, $CD45^-$, also negative expression of $CD14^-$, HLA DR^-; was used to define MSCs (Figures 1(c), 1(d), and 1(e)). Flow cytometry revealed very little scatter in the phenotypic marker profile of placenta-derived isolates between all 8 cases, also population doubling time calculated were not significantly altered. The expression profile confirmed to the criteria generally defined for multipotent mesenchymal stem cells [23].

3.2. Plasticity of MSCs. Specific induction of differentiation was investigated with PD-MSCs, one early, one mid, and one late passage from all 8 subjects. This confirmed that the mesenchymal stemness profile by PD-MSC populations indeed associated with the ability to generate different mesodermal lineage cell types on their exposure to soluble growth and differentiation factors *in vitro*. At the same time, when subjected to translineage differentiation MSC shows remarkable plasticity to differentiate into ectodermal (neuronal cells, retinal cells) and endodermal lineage (pancreatic beta cells). Subconfluent culture was found critically important to maintain the stemness phenotype of PD-MSCs during expansion. The phenotypic profile of PD-MSCs when subcultured at 50–70% cell density remained unaffected, also maintained their initial marker profile and their ability to differentiate as well. MSCs can be differentiated into cells from all the three germ layers under suitable supplementary conditions *in vitro*. The figures display representative results of adipogenic (Figure 2 (a)), osteogenic (Figure 2 (b)), and chondrogenic (Figures 2 (c), and 2 (d)) differentiation assays, visualizing large lipid vacuoles, mineralized bone with calcium deposits and saffranin O positive collagen matrix respectively. These adipogenesis, osteogenesis, and chondrogenesis along with myotubule formation (Figure 2 (e)) and endothelial cells tubular assay (Figure 2 (f)) indicates the ability of the MSC to differentiate into mesodermal cell lineage. Moreover, reports are available on MSC culture in presence of the angiogenic growth factor VEGF induced expression of CD34, which is a marker of hematopoietic, as well as endothelial, precursors [35]. Figure also shows neurogenesis (Figures 2 (g), 2 (h), 2 (i), 2 (j), and 2 (k)) and retinal cell (Figure 2 (l)) differentiation which exhibits the ectodermal differentiation capacity of MSCs. Further, differentiation in pancreatic beta cells indicates (Figure 2 (m)) the endodermal differentiation capacity of placental MSCs. Also, RT-PCR amplification of calbindin2 and recoverin genes shows (Figure 2 (n)) retinal (ectodermal lineage) differentiation, and pancreatic amylase gene (Figure 2 (n)) was also amplified after pancreatic beta cell induction.

3.3. Extensively Passaged Placenta-Derived MSC Does Not Significantly Alter the Cell Cycle or Apoptotic Pattern While Maintaining the Normal Karyotype. In the next set of

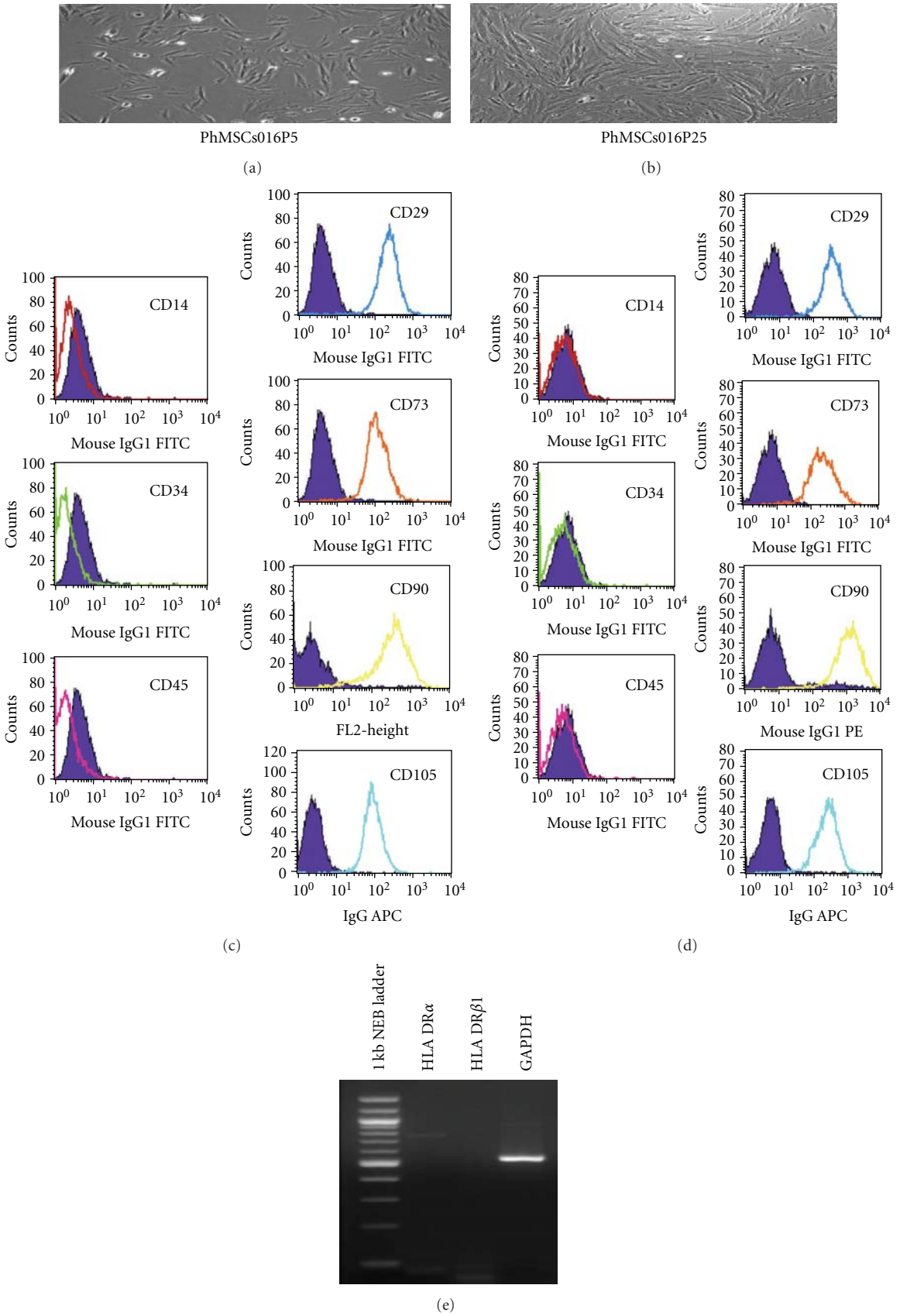

Figure 1: Morphology and characteristics of placental MSCs. (a) morphology of the placental MSCs at passage 5; (b) morphology of the placental MSCs at passage 25; (c) flowcytometric analysis of Placental MSCs at passage 5; (d) flowcytometric analysis of Placental MSCs at passage 25; (e) RT-PCR analysis of placental MSCs (PhMSCs 020P3) for MHC class II antigens.

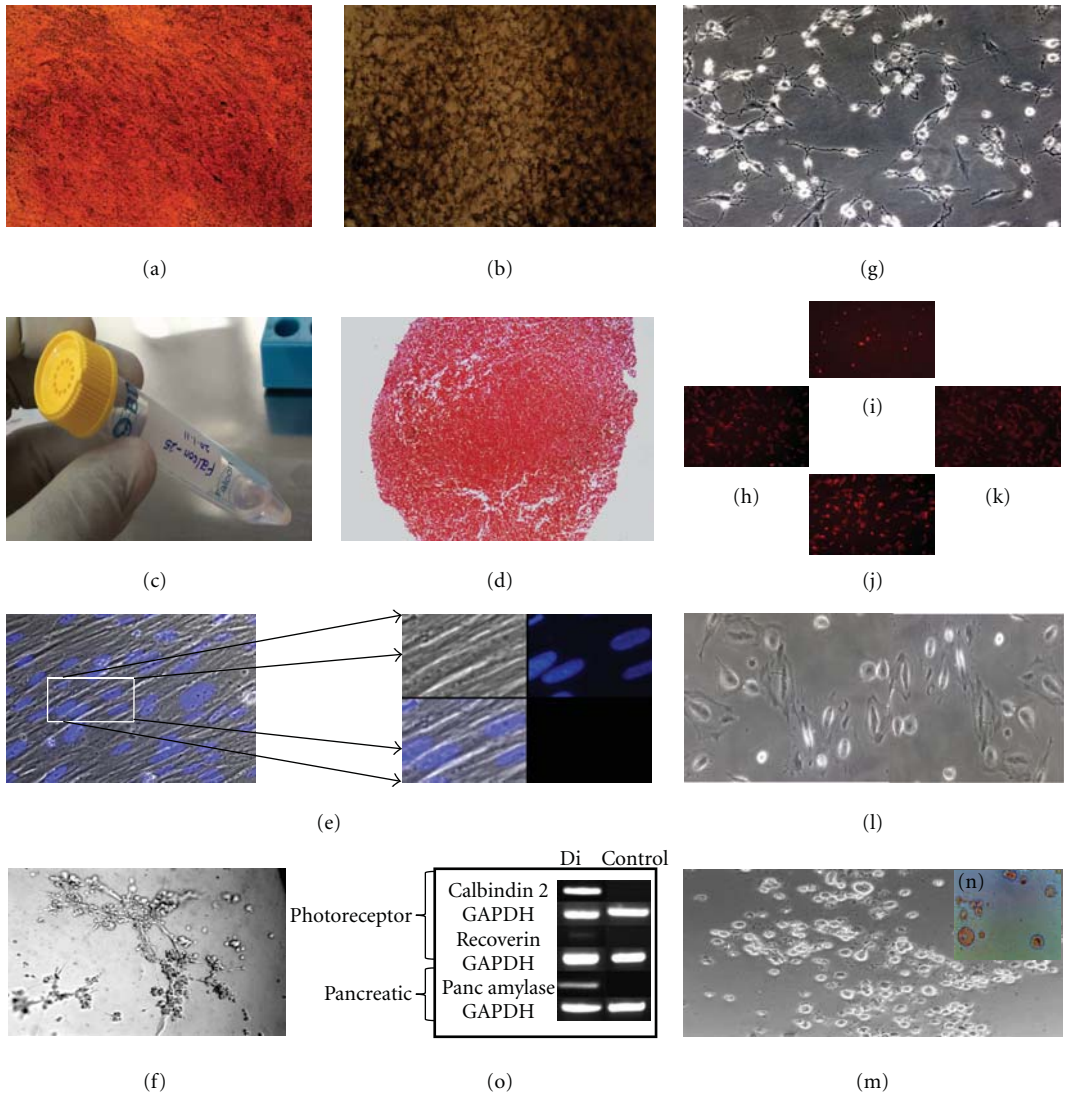

Figure 2: Pluripotency property of placental MSCs. (a) Oil red O staining (PhMSCsP5); (b) Von Kossa staining (PhMSCsP10); (c) "Falcon 25" static micromass cell culture system for chondrocyte differentiation; (d) saffranin O staining (PhMSCsP5); (e) hoechst 33342 staining of myotubules; (f) tubular assay; (g) neural differentiation of placental MSCs (PhMSCsP20); (h) map2 staining (PhMSCs021P15); (i) NeuN staining (PhMSCsP15); (j) GFAP staining (PhMSCs021P15); (k) Neural filament staining (PhMSCsP15); (l) Retinal cell differentiation of placental MSCs (PhMSCsP9); (m) Pancreatic progenitor cell differentiation of placental MSCs (PhMSCsP9) (n) dithizone (DTZ) positive pancreatic progenitor cells; (o) PCR analysis of ectodermal lineage (photoreceptor genes calbindin2 and recoverin) and endodermal lineage (pancreatic amylase gene).

experiments after propidium iodide staining, we tested MSC cell cycle status; Figure 3(a) shows during early and late passaging there was not significant change in the cell cycling process. As detailed in Figure 3(b), karyotypes were normal 46, XX in all test samples. Chromosome number was found normal in all analyzed PD-MSC isolates ($n = 8$). Looking at maternal origin, we found that PD-MSC isolates obtained with our isolation procedure were always of maternal origin. Also, it was important to document the apoptosis pattern of the each passage proliferating MSC; Annexin-V and 7AAD stainning did not show (Figure 3(c)) significant change in the percentage apoptotic cells (~5–7% cells).

3.4. Placental MSCs Displays Higher Endogenous Gene Expression of Oct4, Sox2 and Nanog Compared to BM-Derived MSC. FACS analysis by Oct3/4, Stro-1 antibodies did show positive reaction. Next, we wanted to analyze the pluripotency-associated endogenous gene expression profiles of PD MSCs and bone-marrow-derived MSC (BM-MSC). Figure 4 shows data from comparative real-time qPCR, which revealed higher expression levels of Oct4, Sox2, and nanog compared to BM-MSC. Reports are also available of flow cytometry and immunocytochemistry, which revealed that PD-MSCs were positive for stage-specific embryonic antigen SSEA-3 but negative for SSEA-4 [11].

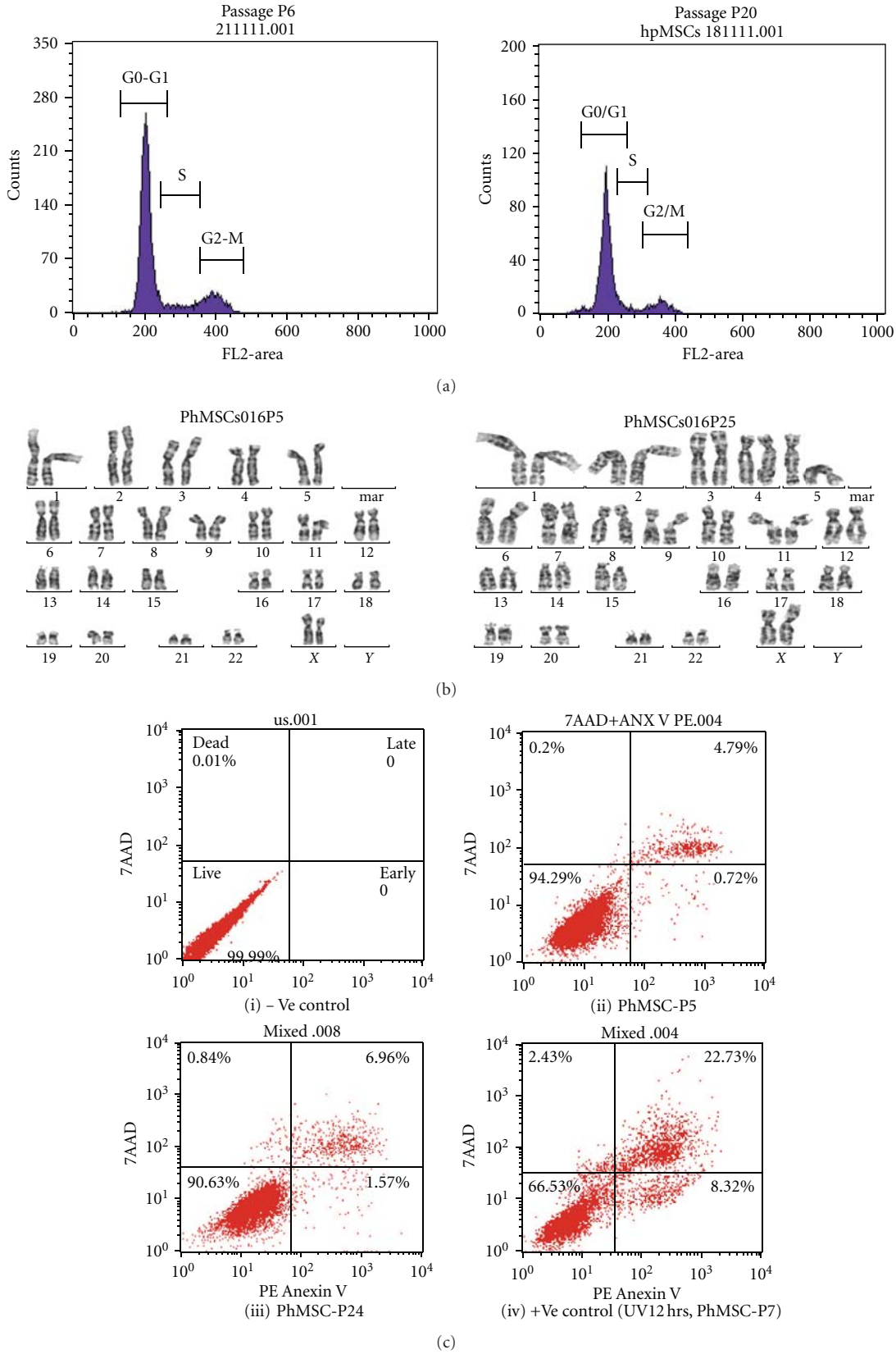

Figure 3: Cell cycle karyotype analysis: (a) cell cycle pattern of early (passage 5) and late passage (passage 20). PD-MSC were analyzed by FACS after propidim iodide staining. (b) Karyotype analysis was performed on early (passage 5) and late (passage25) passage MSC. (c) Apoptosis analysis was done by FACS using Annexin V and 7AAD. (i). negative control. (ii) Total % apoptotic MSC cells (Passage 5). (iii) % apoptotic cells (Passage 24) (iv).positive control.

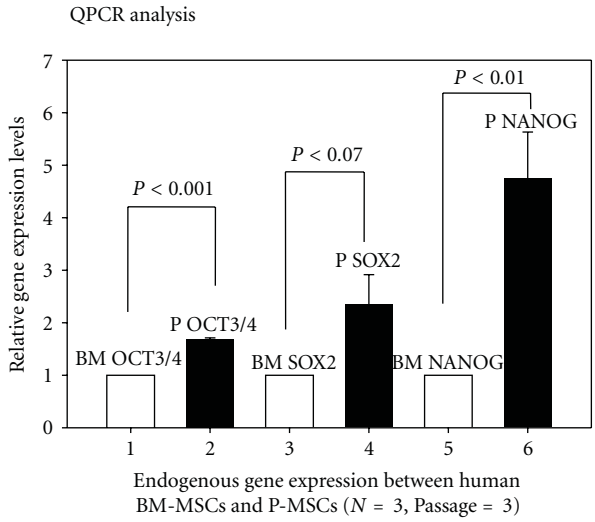

Figure 4: Comparative analysis of pluripotency associated endogenous gene expression between human marrow derived MSC and human placenta-derived MSC. Oct4. Sox2, and nanog gene expression profiles of bone-marrow-derived MSC and PDMSC analyzed by real-time qPCR analysis; error bars represent SE in five separate experiments.

3.5. In Vitro Tumor-Genesis Detection Assay. Placental MSCs when subjected to soft agar assay did not yield tumoroids even after 4 weeks of in vitro culture in soft agar assay (Figure 5). However, HeLa cells began to form aggregates within 7 days, and many bigger colonies were formed at the end of day 21 (Figure 5).

4. Discussion

The human embryonic stem cells (ESCs) have the potential to differentiate into all the three cell lineages [24]. However, some of the practical and ethical concerns render them in usable for day-to-day clinical applications. Nonetheless, extra embryonic tissues can be effectively used to isolate pluripotent stem cells. Placenta is one of the extra embryonic organs that has rich source of progenitor or stem cells [25]. Placenta has two sides; one is foetal side consisting of amnion and chorion and other is the maternal side consisting of deciduas [24]. Mesenchymal stem cells (MSCs) isolated from maternal side of human term-placenta represent an important cell type for stem cell research and clinical therapy not only because of their ability to differentiate into mesodermal lineage cells, such as osteocytes, chondrocytes, muscle, or endothelial cells [2], but also for their remarkable translineage differentiation capabilities like neuronal cells, retinal cells (ectodermal), and pancreatic beta cells (endodermal lineage). In addition, they secrete large amounts of proangiogenic and antiapoptotic cytokines [26] and possess remarkable immunosuppressive properties [27]. MSCs have been derived from many different organs and tissues [28]. Evidence has emerged that different parts of human placenta, umbilical cords and amniotic membrane, as well as umbilical cord blood harbor MSC [29–32]. These tissues are normally discarded after birth, avoiding ethical concerns [23] Mechanical, as well as enzymatic, methods for MSC isolation from different regions of human placenta of different gestational ages have been reported in literature [29, 33–47]. Knowledge about vitality, average population doubling time, stable karyotype, cell cycle and apoptosis pattern, phenotype, and expandability of such placenta-derived MSC isolates is a prerequisite for therapeutic application; however, systematic investigations into reliability of this MSC source and phenotypic stability did not get that much attention. Furthermore, reports on placenta-derived MSCs often lack information about the cell cycle, apoptosis pattern, progenitor-specific endogenous gene-expression profile, and karyotype of the cell isolates. In this paper, we describe enzymatic fractionation of term-human placenta that facilitates recovery of oligo-lineage, fibroblast-like cells, which generally are termed as placenta-derived mesenchymal stem cells (PDMSCs) with high fidelity. As demonstrated by cell cycle or apoptosis analysis of cells from early as well as late passages; with average unaltered population doubling time, PD-MSC did not shows significant variations in either cell cycle or apoptosis pattern. Also, genotypic analyses of cell isolates from most of placental tissues were of maternal, not fetal, origin. Our systematic characterization of cell isolates from multiple cases showed that these cell isolates reproducibly fulfill the general definition of MSCs by both phenotypic and differentiation capabilities criteria. [24]. We demonstrate that maternally derived PD-MSCs can be greatly expanded, do not alter significantly change their cell cycle or apoptosis pattern, show pluripotency-associated endogenous gene expression, and maintain their differentiation capacity and stable phenotype displaying unaltered kayotype up to passage 25–30 passages. In these experiments, the placental MSCs were isolated from the cotyledons present in the maternal side of the placenta. Our method of cell isolation by way of sequential digestion of the trophoblast cell layer with trypsin and following digestion of remaining placental tissue with collagenase I proved very effective for obtaining PD MSCs. Outgrowth of PD-MSCs from collagenase I digests was successful in 8 of 8 placental tissues and resulted in populations with remarkably little scatter in their MSC profiles, between subjects. As for propagation, we found out that PD-MSCs must be propagated in subconfluent culture to maintain their MSC profile, because confluent culture led to gradual loss of MSC identity. With proper subconfluent passage, PDMSCs maintained their phenotypic MSC profile up to 30 passages. The flow cytometry studies indicate there is significant similarity in surface marker characteristics from passage 1 till passage 30. Microscopic observations revealed that placental MSCs proliferate rapidly till passage 30 without compromising on the morphological features and quality of the mesenchymal stem cell properties like cell cycle and apoptosis pattern, pluripotency-associated endogenous gene expression, and normal karyotype.

The characteristic data beyond passage 30 has not been tested in this study. The MSCs had spindle shaped fibroblast morphology. The absence of HLA DRα and HLA DRβ1

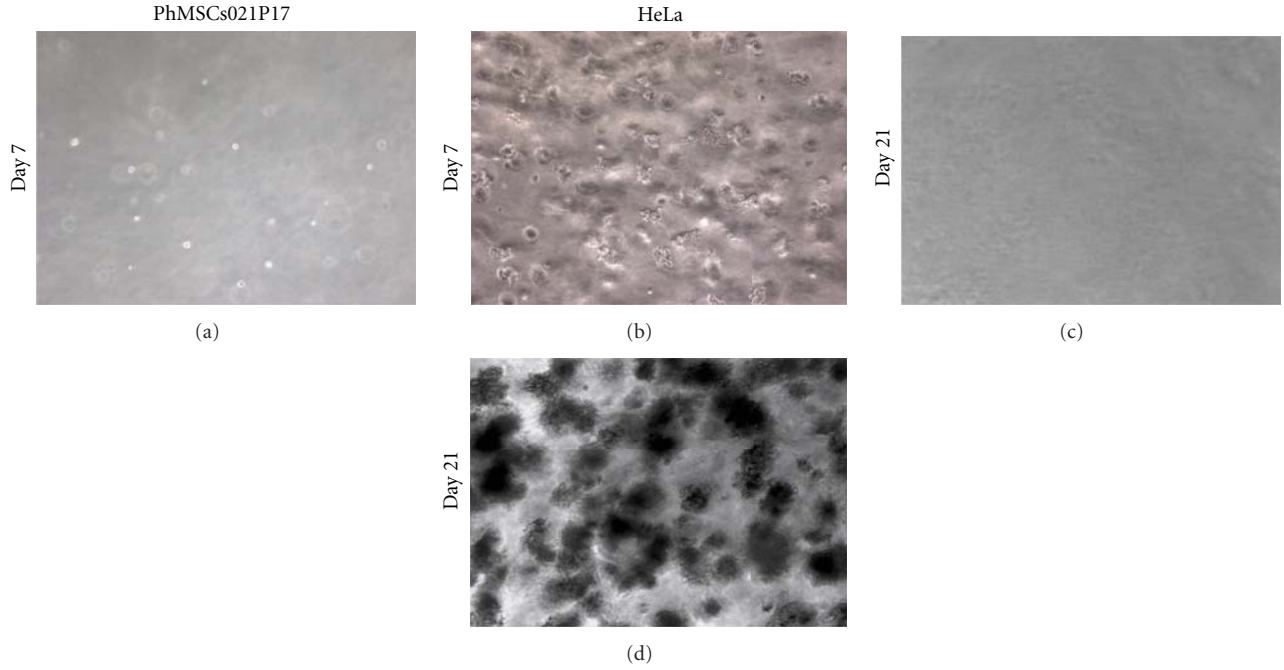

Figure 5: Soft agar assay. (a)Placental MSCs day 7; (b) HeLa cells day 7; (c) Placental MSCs day 21; (d) HeLa cells ay21.

expression, analyzed from RT-PCR, results indicate that placental MSCs could be effectively used for both autologous and allogenic transplantations. The rate of differentiation of MSCs is much quicker, efficient and scalable when compared to ES cells. The soft agar assay indicates that isolated placental MSCs do not possess any malignant property. Several animal as well as human trials have indicated that use of MSCs unlike ES cells does not lead to the formation of teratomas *in vivo* [24]. In addition, usage of term placental MSCs has fewer ethical concerns since they are isolated from foetal tissues that anyway would have been discarded.

5. Conclusion

The human term-placenta is relatively easily available and attracts less ethical concerns. Placental tissue constitutes a robust source of MSC. In this study, we investigated several parameters, namely, (1) chromosome number, (2) pluripotency associated gene expression, (3) maternal origin, (4) sequential enzymatic digestion (trypsin followed by collagenase) as methods of isolation, (5) cell propagation, cell cycle, and apoptosis pattern, that are important for their principal utility for cell-based therapy and could influence their proliferative, as well as differentiation, capacities. Based on the results, we conclude that the abundance of pluripotent cells, rapid proliferation, stable karyotype, plasticity and immunomodulatory property make placental MSCs ideal choice for clinical and tissue engineering applications. Nevertheless, the main drawback of using MSCs is that, a panel of surface markers are required for characterization of isolated MSCs for their homogeneity. Further, unlike the adult MSCs, where significant numbers of human clinical trials are underway, use of placental MSCs in clinical applications is relatively new. Additional studies are required to substantiate the use of placental MSCs in medical applications.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Generation of Human-Induced Pluripotent Stem Cells by a Nonintegrating RNA Sendai Virus Vector in Feeder-Free or Xeno-Free Conditions

Chad C. MacArthur,[1] Andrew Fontes,[1] Namritha Ravinder,[2] David Kuninger,[1] Jasmeet Kaur,[1] Matthew Bailey,[1] Antje Taliana,[2] Mohan C. Vemuri,[3] and Pauline T. Lieu[1]

[1] *Primary and Stem Cell Systems, Life Technologies Corporation, 5781 Van Allen Way, Carlsbad, CA 92008, USA*
[2] *Molecular and Cell Biology, Life Technologies Corporation, 5781 Van Allen Way, Carlsbad, CA 92008, USA*
[3] *Primary and Stem Cell Systems, Life Technologies Corporation, 7335 Executive Way, Frederick, MD 21704, USA*

Correspondence should be addressed to Pauline T. Lieu, pauline.lieu@lifetech.com

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The generation of induced pluripotent stem cells (iPSCs) from somatic cells has enabled the possibility of providing unprecedented access to patient-specific iPSC cells for drug screening, disease modeling, and cell therapy applications. However, a major obstacle to the use of iPSC for therapeutic applications is the potential of genomic modifications caused by insertion of viral transgenes in the cellular genome. A second concern is that reprogramming often requires the use of animal feeder layers and reagents that contain animal origin products, which hinder the generation of clinical-grade iPSCs. Here, we report the generation of iPSCs by an RNA Sendai virus vector that does not integrate into the cells genome, providing transgene-free iPSC line. In addition, reprogramming can be performed in feeder-free condition with StemPro hESC SFM medium and in xeno-free (XF) conditions. Generation of an integrant-free iPSCs generated in xeno-free media should facilitate the safe downstream applications of iPSC-based cell therapies.

1. Introduction

Takahashi and Yamanaka first demonstrated that induced pluripotent stem cells (iPSCs) can be generated from somatic cells by transducing four transcription factors using retroviral vectors [1, 2]. This breakthrough in creating induced pluripotent stem cells opened the door for personalized medicine using patient-derived iPSCs [3–6]. The major limitation for potential clinical application is the integration of viral transgenes into the host genome that can result in multiple insertions and risk of tumorigenicity [7, 8]. Another major disadvantage of reprogramming cells with integrating vectors is that silencing and activation of transgenes are unpredictable, which may affect terminal differentiation potential and increase the risk of using iPSC-derived cells. Multiple methods have been developed to address these issues, including reprogramming with episomal vectors, mRNAs, miRNAs, protein transduction, or treatment with chemical compounds [9]. The majority of these methods has one or more limitations, such as low reprogramming efficiency or requiring multiple rounds of transfections, or is effective only with specific cell types, such as skin fibroblasts. We have previously shown that sustained expression of reprogramming factors is required for at least 10–20 days [10], and often these reprogramming methods fail to sustain expression and are difficult to repeat. Further, due to poor efficiency of existing methods, reprogramming often has been performed in the presence of animal feeders (inactivated mouse embryonic fibroblasts) to maximize colony formation [11] along with the use of serum and xeno-containing products, which are not ideal for clinical applications [11, 12].

Here, we investigate the use of Sendai virus vector to generate transgene-free iPSCs in different conditions. Sendai virus vector is a negative-strand RNA virus that belongs to the *paramyxoviridae* family [13–15]. Unlike other RNA viruses, it replicates in the cytoplasm of infected cells and

does not go through a DNA phase that can integrate into the host genome [13]. In addition, Sendai virus vector can infect a broad host range and is nonpathogenic to humans. Sendai virus vector has been considered for clinical studies of gene therapy for cystic fibrosis [16, 17] and vaccine delivery [18]. Recent papers also demonstrated that Sendai virus vector can reprogram somatic cells with higher efficiency than other reprogramming methods [19]. Thus, the nature of Sendai virus vector makes it an ideal tool for cell reprogramming and stem cell research.

The current methods of reprogramming often require the use of inactivated feeders and animal-derived products during several steps to maximize reprogramming efficiency. However, exposure of human cells to animal origin products may increase the risk of nonhuman pathogen transmission and immune rejection [20], hence limiting their use in clinical settings. Given the reported high efficiency of Sendai virus vector in generating pluripotent cells, we investigated the generation of transgene-free iPSCs with Sendai virus vector in feeder-free conditions using StemPro hESC SFM medium and in xeno-free conditions. Several reports have demonstrated the generation of iPSCs in animal product-free culture media but their performance tends to be lower than knockout serum (KO-SR) based media [21]. Here, we report a robust and an efficient system to generate transgene-free iPSCs in different conditions, and its ease of use can be applied to a wide range of different cell types. Generation of transgene-free iPSCs under these conditions will be important to facilitate the safe clinical translation of iPSC-based therapies.

2. Materials and Methods

2.1. Cell Culture. Human neonatal fibroblasts (ATCC Cat. no. CRL-2252) were maintained in fibroblast growth media consisting of DMEM + GlutaMAX (Cat. no. 10569-010), supplemented with 10% ES cell-qualified fetal bovine serum (Cat. no. 16141-079), and 1% nonessential amino acids (Cat. no. 11140-050). Feeder-free iPSC cultures were maintained on hESC-qualified GelTrex (Cat. no. A10480-02) with chemically defined StemPro hESC SFM (Cat. no. A1000701) supplemented with 100 μM 2-mercaptoethanol (Cat. no. 21985-023) and 10 ng/mL recombinant human basic FGF (Cat. no. PHG0264). For xeno-free culture, human neonatal fibroblasts were maintained in StemPro MSC SFM Xeno-Free medium (Cat. no. A10675-01). Xeno-free iPSC cultures were maintained on KnockOut-DMEM/F-12 (Cat. no. 12660012) supplemented with 15% KnockOut-SR Xeno-Free CTS (Cat. no. A1099202), 1X KnockOut SR Xeno-Free Growth Factor Cocktail, 2 mM GlutaMAX (Cat. no. 35050-061), 1% nonessential amino acids, 100 μM 2-mercaptoethanol, and 20 ng/mL of bFGF. For feeder condition, human iPS cells were maintained on irradiated mouse embryonic fibroblasts (Cat. no. S1520-100) in DMEM/F-12 + GlutaMAX (Cat. no. 10565-018) supplemented with 20% KnockOut SR (Cat. no. 10828-028), 1% nonessential amino acids, 100 μM 2-mercaptoethanol, and 4 ng/mL of bFGF. iPSCs were passaged manually using a 26-gauge needle or StemPro EZPassage Disposable Stem Cell Passaging Tool (Cat. no. 23181-010). All reagents are from Life Technologies. Colonies were passaged every 3-4 days at a splitting ratio of 1 : 3. The Gibco Episomal hiPSC line was purchased from Life Technologies (Cat. no. A13777) and maintained on irradiated mouse embryonic fibroblasts in DMEM/F-12 + GlutaMAX supplemented with 20% KnockOut SR, 1% nonessential amino acids, 100 μM 2-mercaptoethanol, and 4 ng/mL bFGF.

2.2. Generation of iPSCs in Feeder-Free and Xeno-Free Conditions. For the generation of iPSCs in feeder-free condition, approximately 100,000 human neonatal fibroblasts were seeded per well in a 6-well plate and incubated at 37°C and 5% CO_2. Two days later, cells were transduced with the CytoTune iPS Reprogramming Kit containing Sendai virus vectors using the F gene-defective vector as described in [19, 22] (Life Technologies Cat. no. A1378001) in fibroblast growth medium at an MOI of 3, as described in the manufacturer's protocols. One day after transduction, the medium was replaced with fresh fibroblast growth medium and the cells were cultured in fibroblast growth media for 6-7 days. On day eight after transduction, cells were transferred to 10 cm Geltrex-coated tissue culture dishes at 5×10^5 cells per dish. After culturing overnight in fibroblast growth media, the medium was replaced daily with StemPro hESC SFM medium. The iPSC colonies were picked for expansion and characterization from days 17–21 of reprogramming.

For the generation of iPSCs in xeno-free condition, approximately 500,000 human neonatal fibroblasts were seeded in StemPro MSC SFM Xeno-Free medium per well in a 6-well plate and incubated at 37°C and 5% CO_2. The next day, cells were transduced with the CytoTune iPS Reprogramming Kit in StemPro MSC SFM Xeno-free medium at an MOI of 3. One day after transduction, the medium was replaced with fresh StemPro MSC SFM Xeno-Free medium and cells were cultured for 6-7 days. On day eight after transduction, cells were transferred to 10 cm tissue culture dishes plated with inactivated human feeders (NuFF, GlobalStem Cat. no. GSC-3001G) at a density of 2×10^5 cells per dish. After culturing overnight, the medium was replaced daily with xeno-free iPSC medium. The xeno-free medium is composed of KnockOut D-MEM CTS containing 15% KnockOut SR Xeno-Free CTS, 2 mM GlutaMAX, KnockOut SR GF Cocktail CTS (used at 1 : 50), and 8 ng/mL bFGF. Just prior to use, medium was equilibrated at 37°C and 5% CO_2 in humidified air, 100 μM 2-mercaptoethanol was added to the KnockOut SR Xeno-Free Complete Medium. The iPSC colonies were picked for expansion and characterization from days 21–25 of reprogramming.

2.3. Immunofluorescence and Alkaline Phosphatase Staining. For immunofluorescence staining, cells were fixed in 2% PFA for 30 minutes at room temperature, blocked for 30 minutes at room temperature in blocking buffer (5% normal goat serum, 1% BSA, and 0.1% Triton X100 in D-PBS), incubated with primary antibodies (diluted in blocking buffer) overnight at 4°C, washed 3X with D-PBS, incubated with secondary antibodies (diluted in D-PBS) for 30 minutes at room temperature, and washed 3X with D-PBS. For

live staining, cells were washed 1X with DMEM/F-12 and incubated with primary antibody at 1 : 100 dilution (in DMEM/F-12) for 60 min at 37°C. Cells were washed 3X with DMEM/F12 and incubated with secondary antibody at 1 : 500 dilution (in DMEM/F-12) for 60 minutes at 37°C and washed 3X with DMEM/F-12. Characterization was carried out using the following antibodies: Mouse anti-Tra1-60 antibody (Cat. no. 41–1000), Mouse anti-Tra1-81 antibody (Cat. no. 41–1100), Mouse anti-SSEA4 (Cat. no. 41–4000), Alexa Fluor 488 goat anti-mouse IgG (H + L) antibody (Cat. no. A11029), Alexa Fluor 594 goat anti-mouse IgG (H + L) antibody (Cat. no. A11032), Alexa Fluor 488 goat anti-rabbit IgG (H + L) antibody (Cat no. A11034), and Alexa Fluor 594 goat anti-rabbit IgG (H + L) antibody (Cat no. A11037). All reagents are from Life Technologies. Clones were screened for residual virus using rabbit anti-SeV antibody (MBL International Corporation, Woburn, MA; Cat. no PD029). Images were captured using a Zeiss Axiovision microscope and processed using AdobePhotoshop CS. Where applicable, on day 25 of reprogramming, cells were stained using VECTOR Red Alkaline Phosphatase Substrate Kit (Vector Labs Cat. no. SK 5100) and positive colonies were counted.

2.4. RNA Isolation, qRT-PCR, and End Point PCR for Gene Expression Analysis. Total RNA was extracted from cells using TRIzol LS reagent (Cat. no. 10296-010) with DNaseI (Cat. no. AM2222). RT-PCR reactions were carried out using 20x Reverse Transcriptase enzyme mix and 2x RT Buffer provided in the TaqMan Gene Expression Cells-to-CT Kit (Cat. no. 4399002) with 1 ug of total RNA per reaction. Gene expression assays were performed according to the manufacturer's protocols. Primers for gene expression can be found at http://www.invitrogen.com/site/us/en/home.html. All reactions were performed in triplicates and normalized to endogenous B-actin. End point PCR was performed with 10 uL of cDNA in 50 uL of AccuPrime Pfx Supermix (Cat. no. 12344040) with the following primers to detect for the presence of Sendai virus vector (SeV: Frw: GGATCACTAGGTGATATCGAGC, Rev: ACCAGACAAGAGTTTAAGAGATATGTATC) as described in [19]. The expected size of the PCR product is 181 bp. All reagents are from Life Technologies. PCR was performed with the following conditions: denaturation 95°C, 30 sec, annealing: 55°C, 30 sec, elongation: 72°C, 30 sec, for 30–35 cycles. PCR products were analyzed on 2% agarose gel electrophoresis.

2.5. MicroRNA Profiling. Total RNA was extracted from cells using TRIzol LS reagent (Cat. no. 10296-010) with DNASE 1 (Cat. no. AM2222). 500 ng of total RNA was subjected to cDNA synthesis using TaqMan MicroRNA Reverse Transcription Kit (Cat. no. 4366597) and TaqMan Human MicroRNA Array Card A (Cat. no. 4398965) to quantify microRNA expression levels, according to the manufacturer's protocol. All reagents are from Life Technologies. Real-time PCR was performed on an AB 7900HT Sequence Detection System with cycling conditions of 95°C for 10 min followed by 95°C for 15 sec and 60°C for 60 sec for a total of 40 cycles. The Ct values for all miRNAs were normalized to mammalian U6 snRNA levels by calculating their respective delta Ct values. Relative changes in miRNA expression levels between samples were compared using the delta Ct values.

2.6. TaqMan Protein Assays and Reagents. All TaqMan Protein Assays and associated reagents kits were obtained from Life Technologies. Cells were harvested, counted, and 50 K cells were incubated in 100 uL of resuspension and 100 uL of lysis buffer. TaqMan Protein Assays were carried out with cell lysate dilutions of 500 or 250 cells per reaction using Applied Biosystems StepOnePlus real-time PCR system. In parallel, the same cell inputs were also subjected to 18S genomic ribosomal DNA assays to obtain endogenous control Cq, described in [10].

2.7. In Vitro Differentiation of iPSCs. Undifferentiated iPSCs were harvested using collagenase to generate embryoid bodies (EBs) and were cultured for 4 days in suspension in differentiation medium containing DMEM-F12 with GlutaMAX, 20% Knockout Serum Replacement, 1% nonessential amino acid, and 55 μM 2-mercaptoethanol (Life Technologies). On day 5, EBs were seeded on Geltrex coated plates for an additional 17 days of differentiation in the same medium, then the cells were used for immunocytochemistry. Induction to neural lineages was performed as described in [23].

3. Results and Discussion

3.1. Generation of iPSCs with Sendai Virus Vector in Feeder-Free or Xeno-Free Condition. Generation of human iPSCs with Sendai virus vector encoding OCT3/4, KLF4, SOX2, and cMYC was performed on feeder-free conditions with StemPro hESC SFM and Geltrex coated plates, or xeno-free conditions on KnockOut-DMEM/F-12 supplemented with 15% KnockOut-SR Xeno-Free CTS, and 1X KnockOut SR GF Cocktail CTS on inactivated human feeders. Human neonatal foreskin fibroblast cells were plated onto a 6-well plate two days before transduction, and approximately 5×10^5 cells were transduced with OCT3/4, KLF4, SOX2, and cMYC Sendai virus vector at an MOI of 3. About 6-7 days after transduction, cells were collected and transferred to Geltrex-coated matrix plate (feeder-free condition) or onto inactivated human feeders (xeno-free conditions) at the density of 500,000 cells per 10 cm dish for feeder-free condition and 200,000 cells per 10 cm dish for xeno-free conditions. The workflow is outlined in Figure 1, and viable colonies were visible as early as day 10 after transduction, and these colonies could be readily picked by 21–25 days.

A majority of colonies generated with Sendai virus vector have ES-like morphology, and very few transformed colonies were observed. Clones from human fibroblasts were stained with pluripotent markers, SSEA4, Sox2, Nanog, and Tra1-81 or Tra1-60 shown on Figure 2(A) (feeder-free condition) and Figure 3(A) (xeno-free condition). Alkaline phosphatase staining demonstrated efficiency on feeder-free condition to be between 0.01 and 0.04%, and very few transformed or non-iPSC colonies were detected, shown on Figure 2(C). In addition, these cells were able to give rise to embryoid bodies (EBs) that could differentiate to all the three lineages: ectoderm, endoderm, and mesoderm (see Figure 2(B) for

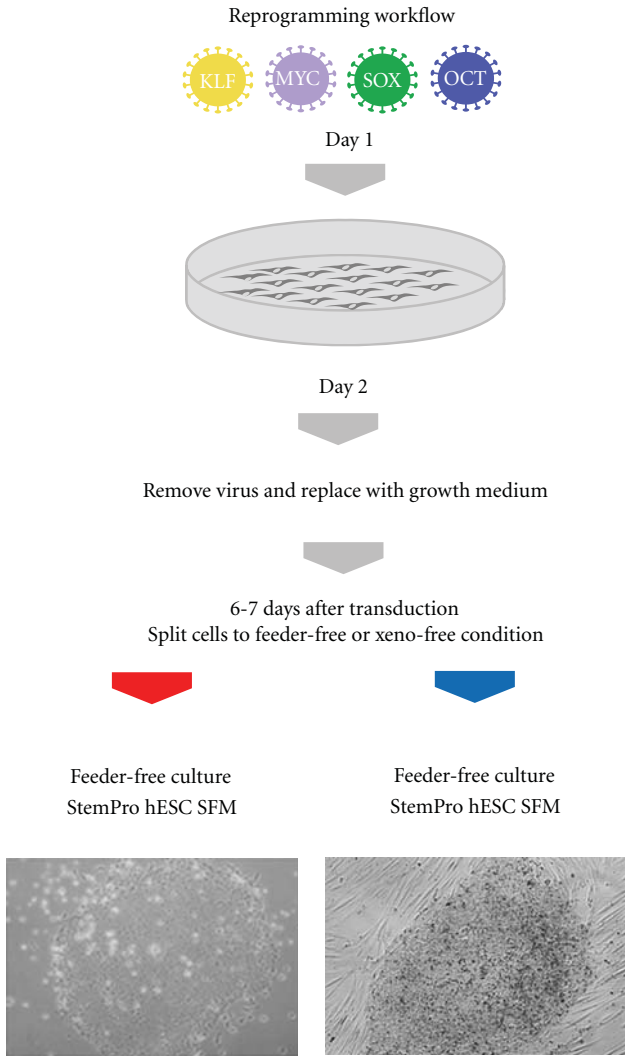

Figure 1: Reprogramming workflow on feeder-free and xeno-free conditions. (1) Transduce cells with CytoTune Sendai virus vector overnight. (2) Remove virus and replace with cell's growth medium. Cells are grown in cell's growth medium for 6-7 days after transduction. (3) Split cells onto Geltrex-coated plates or inactivated human feeders. Culture transduced cells in feeder-free conditions with StemPro hESC SFM medium and Geltrex-coated plates or xeno-free medium consisting of KnockOut-DMEM/F-12, 15% KnockOut-SR Xeno-Free CTS, and 1X KnockOut SR Xeno-Free Growth Factor Cocktail. Colonies can be picked between 17–25 days after transduction.

an example in feeder-free conditions and Figure 3(B) for xeno-free conditions). We further performed directed induction of clones from feeder-free condition to neural lineages using N2B27 supplements as described in [23] and stained cells with beta III tubulin Figure 2(D) (j) and nestin (k), or by spontaneous differentiation that gave rise to beating cardiomyocytes, Figure 2(D) (l). The differentiation profile was similar to that seen with cells reprogrammed with other methods, and little variation between different subclones was seen.

3.2. Expression of Pluripotency Markers in Different iPSC Lines by TaqMan Gene Expression and Protein Assays. We compared gene expression levels of OCT3/4, SOX2, NANOG, and LIN28 of iPSC lines generated by Sendai virus vector or by an episomal vector and found that expression profile was similar in all these lines as well as comparable to the WA09 embryonic stem cell line, shown on Figure 4(a). We previously demonstrated that TaqMan Protein Assays can be used to characterize different iPSC lines for the expression of pluripotent protein markers and have reported variations of protein levels among iPSC lines [10]. When iPSC lines generated from Sendai virus vector were compared to an episomal generated iPSC line [24] or the WA09 hESC line, there were subtle differences in the levels of protein expression for OCT3/4, SOX2, NANOG, and LIN28 between different iPSC lines. Noticeably, SOX2 levels of the Sendai-generated iPSC lines were lower than the episomal-generated iPSC line and the WA09 hESC line (Figure 4(b)). It is likely that the difference is due to the fact that the Sendai-generated iPSC lines were at earlier passages (P5–P10) compared to the episomal iPSC (>P20) or the WA09 hESC line. The variations in the levels of the markers were within the range we have observed in iPS lines generated with other methods [10], and in no case was the variation sufficiently large that the cells were not pluripotent as assessed by our *in vitro* assays. We noticed that the difference becomes less noticeable when the iPSC lines were at higher passages (data not shown). Recent publication by Chung et al. reports that human-induced pluripotent stem cells derived under feeder-free conditions display similar levels of gene expression as in hESCs [25]. In addition, the variability among iPSC lines is minimized when using a feeder-free culture system compared to on feeders [25].

3.3. MicroRNA Profiling. We performed miRNA profiling analysis with 384 unique miRNAs among three cell types, BJ fibroblasts, iPSCs generated with the Sendai virus vector, and the WA09 human embryonic stem cell (hESC) line. Data show poor correlation between BJ fibroblast and the WA09 human embryonic stem cell (hESC) line Figure 5 (R^2 = 0.3189). On the other hand, comparisons of the WA09 human embryonic stem cell (hESC) line and iPSC line generated from the Sendai virus vector were highly correlated though subtle differences in miRNA expression (R^2 = 0.9167), Figure 5, were observed. We also analyzed the "pluripotent" miRNAs identified previously [26, 27] in human iPSCs and hESCs, specifically miR-302, miR-367–371, mi-17–92, miR-299, and mi-Let 7 cluster, and found high correlation between the WA09 human embryonic stem cell (hESC) line and the iPSC line generated with Sendai virus vector (R^2 = 0.9662). These data demonstrate that the iPSC line generated with Sendai virus vector is very similar to the WA09 human embryonic stem cell (hESC) line as assessed by miRNA profiling by qPCR.

3.4. Detection of Virus Free iPSCs. Since Sendai virus vector is an RNA virus that only replicates constitutively in the cytoplasm of infected cells [13], it carries no risk of modifying the host genome and it gradually lost as the cells proliferate.

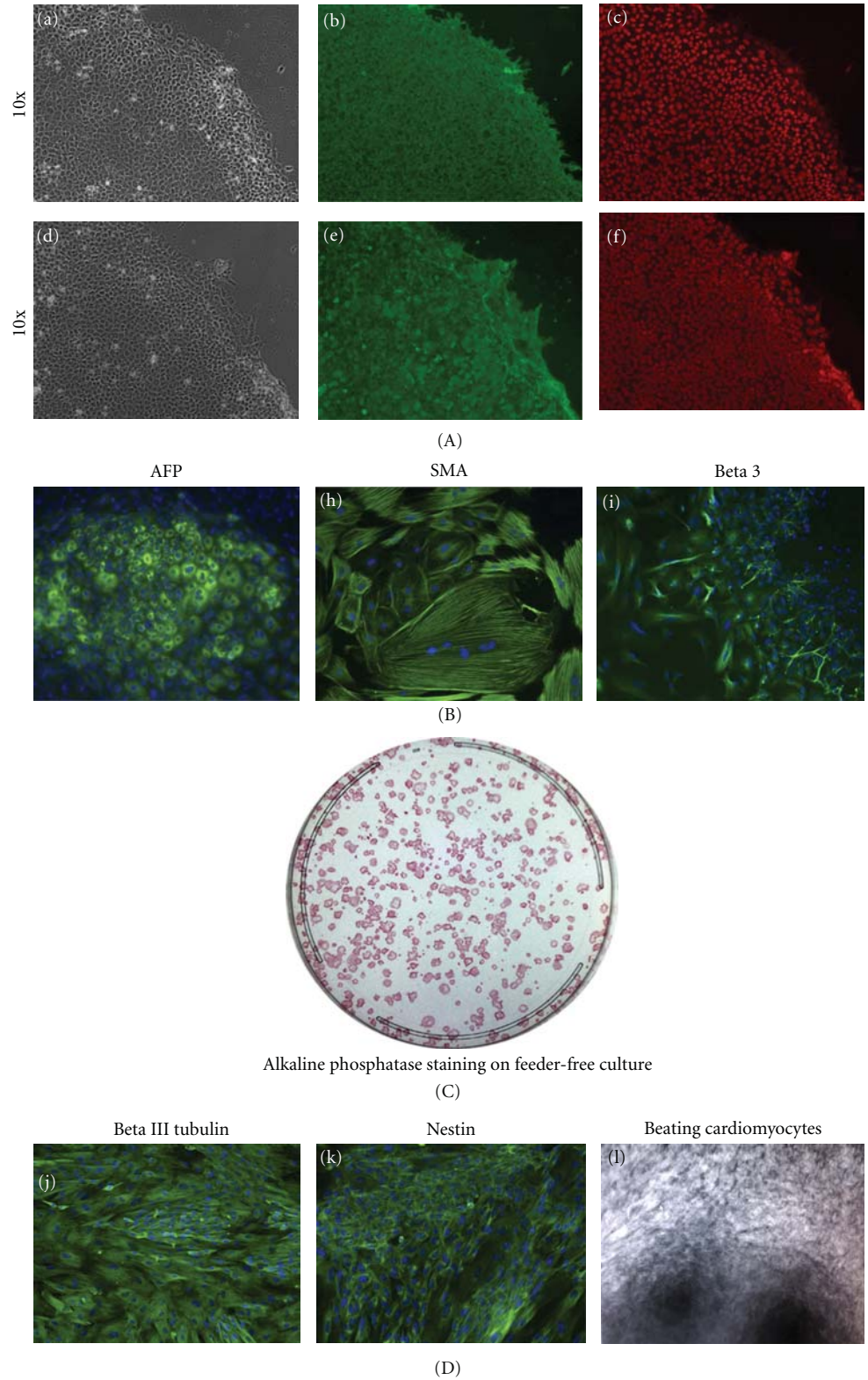

Figure 2: (A) Characterization and differentiation of iPSC colonies generated on feeder-free conditions. Colonies were stained for pluripotent markers: SSEA4 (b), SOX2 (c), Tra1-81 (e), NANOG (f), and phase ((a) and (d)). (B) These colonies were differentiated *in vitro* into embryoid bodies (EBs) comprising of the three embryonic germ layers: endoderm marker α fetoprotein (AFP) (g), mesodermal marker smooth muscle actin (SMA) (h), and ectoderm marker Beta 3-tubulin (Beta III) (i). (C) Colonies were stained with alkaline phosphatase staining 25 days after transduction. (D) Colonies were also subjected to directed differentiation to neural stem cells, stained with Beta 3-tubulin (Beta III) (j), Nestin (k), and random differentiation to beating cardiomyocytes (l).

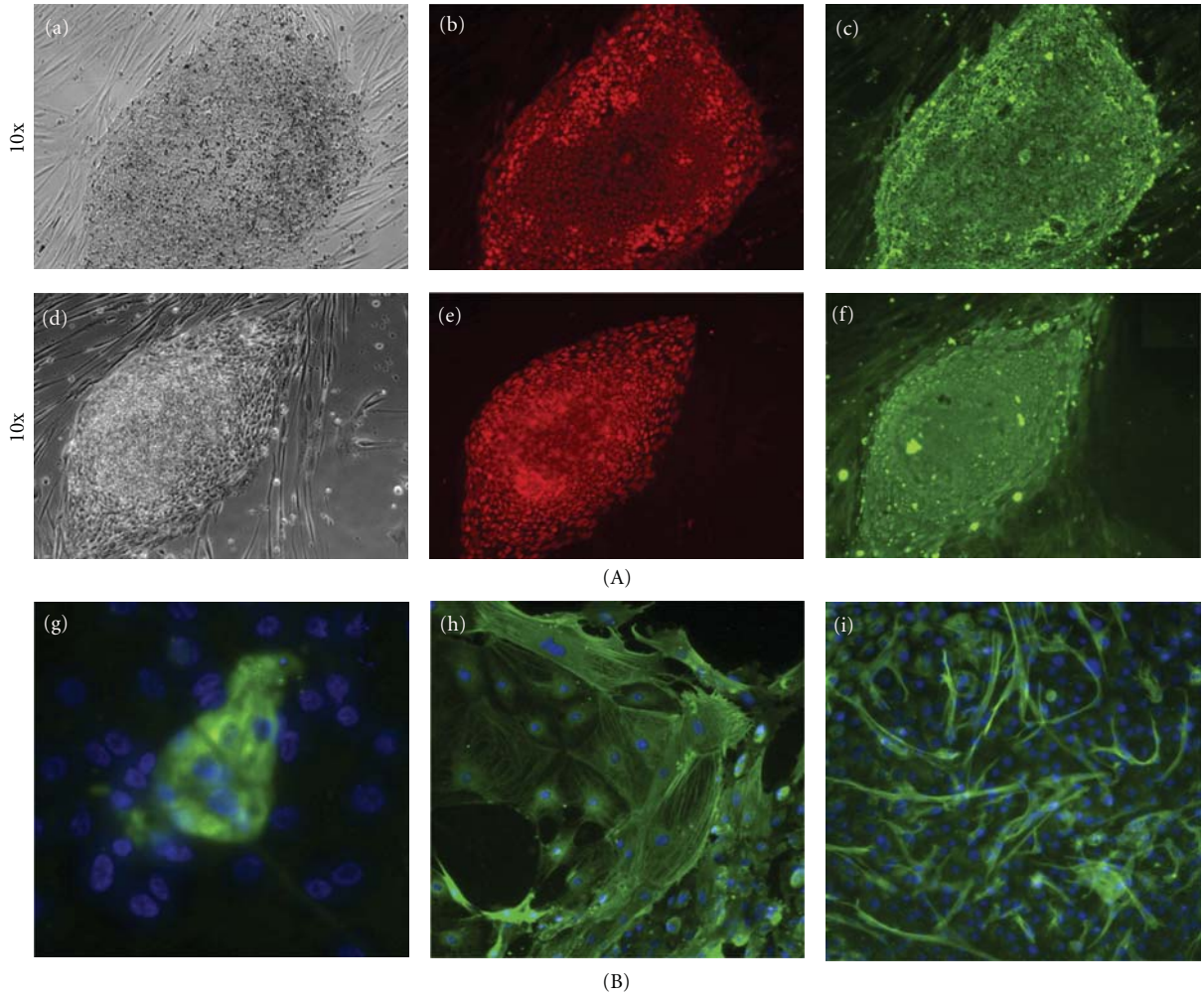

Figure 3: (A) Characterization and differentiation of iPSC colonies generated on xeno-free conditions. Colonies were stained for pluripotent markers: NANOG (b), SSEA4 (c), SOX2 (e), Tra1-60 (f), and phase ((a) and (d)). (B) These colonies were differentiated *in vitro* into embryoid bodies (EBs) comprising of the three embryonic germ layers: endoderm marker α fetoprotein (AFP) (g), mesodermal marker smooth muscle actin (SMA) (h), and ectoderm marker Beta 3-tubulin (Beta III) (i).

We used two different methods to detect loss of Sendai virus vector in cells. Using RT-PCR with Sendai specific primers, we can detect transgene-RNAs as early as 24 hours after transduction, Figure 6(a). The rate at which the transgenes disappeared varies between each clone, and transgene-free iPSC colonies were obtained by passage 10 using end point PCR method, Figure 6(b). The second method used to detect the presence of Sendai virus vector is by immunochemistry staining with anti-Sendai antibody. As early as passage 5, we can detect some colonies staining negative for Sendai virus vector, shown on Figure 7. Sendai virus vectors tend to dilute out and disappear as the clones are being carried out to further passages. By passage 10, residual Sendai transcripts were completely eliminated in three out of four clones tested. In addition, the Sendai backbone vector carries temperature-sensitive mutations that enable complete removal of the residual vector by shifting to nonpermissive temperature of 38-39°C [22]. Thus, removal of Sendai virus vector is less labor intensive compared to alternative methods that require subsequent excision of transgenes from the host genome [6, 28].

4. Conclusions

Sendai virus vector has been used as vectors for gene therapy since it has no risk of being integrated into the host genome and is nonpathogenic to humans. In addition, Sendai virus vector has been shown to generate transgene-free human iPSCs with high efficiency from fibroblasts and CD34+ cord blood cells [22]. In the present study, we demonstrated that the Sendai virus vector can be used to generate transgene-free human iPSCs in feeder-free and xeno-free conditions. Comparing the feeder-based and feeder-free systems, we noticed a decrease in reprogramming efficiency in feeder-free conditions, independent of which media we used. Since Sendai virus vector can reprogram with high efficiency, we are able to obtain sufficient number of colonies for further expansion in either condition. Another advantage of using

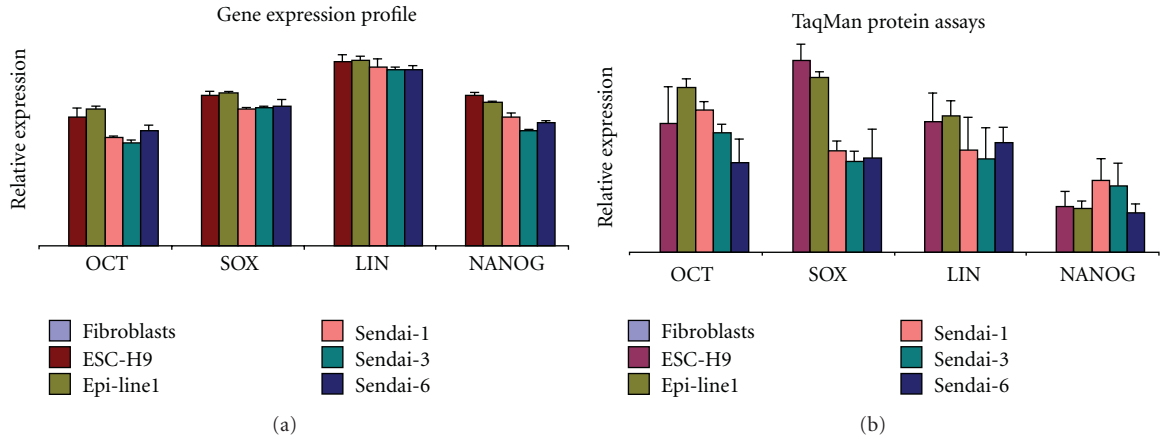

Figure 4: (a) TaqMan Gene Expression Assays of OCT4/POU5F1, SOX2, LIN28, and NANOG were compared among the WA09 hESC line, an episomally derived iPSC line (Epi-line 1), and three Sendai-derived iPSC lines (Sendai 1, 3, and 6). Samples were normalized against the endogenous reference B-actin. (b) TaqMan Protein Assays of OCT4/POU5F1, SOX2, LIN28, and NANOG are compared among the WA09 hESC line, an episomally derived iPSC line (Epi-line 1), and three Sendai-derived iPSC lines (Sendai 1, 3, and 6). Samples were normalized against the endogenous reference 18S ribosomal genomic DNA and plotted relative fold change to the expression level of untransduced human dermal fibroblasts.

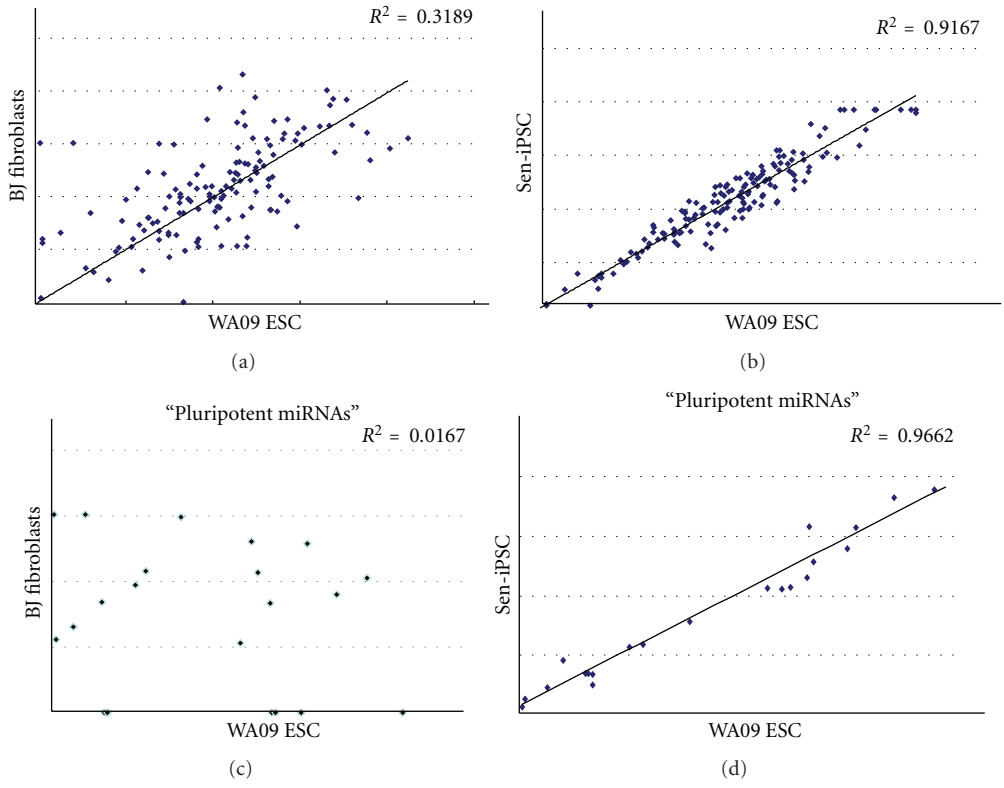

Figure 5: ((a) and (b)) Scatter plots comparing microRNA expression profiles using the TaqMan Human MicroRNA Array of BJ fibroblasts, iPSCs generated with the Sendai virus vector (Sen-iPSC), and the WA09 human embryonic stem cell (hESC) line. ((c) and (d)) Scatter plots comparing "pluripotent" microRNA expression profiles.

Sendai virus vector is the ease of use with single transduction comparing to other methods that require multiple transfections [29]. Finally, iPSCs depleted of virus from the cytoplasm can be easily obtained without further manipulation. Thus, the high efficiency and ease of use of the Sendai virus vector is ideal for reprogramming with different cell types on feeder-based or feeder-free conditions.

Gene expression of pluripotent markers and miRNA profiling data show that iPSCs derived from Sendai virus vector on feeder-free condition are similar to hESC line (the WA09

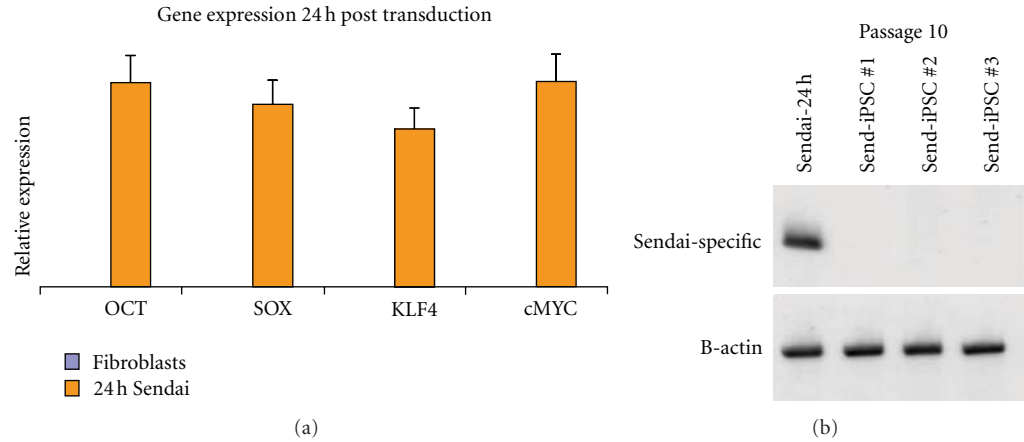

Figure 6: (a) Detection of viral gene expression at 24 hours after transduction with Sendai virus vector by TaqMan Gene Expression Assays. (b) Detection of virus-free iPSCs after 10 passages in culture by end-point PCR.

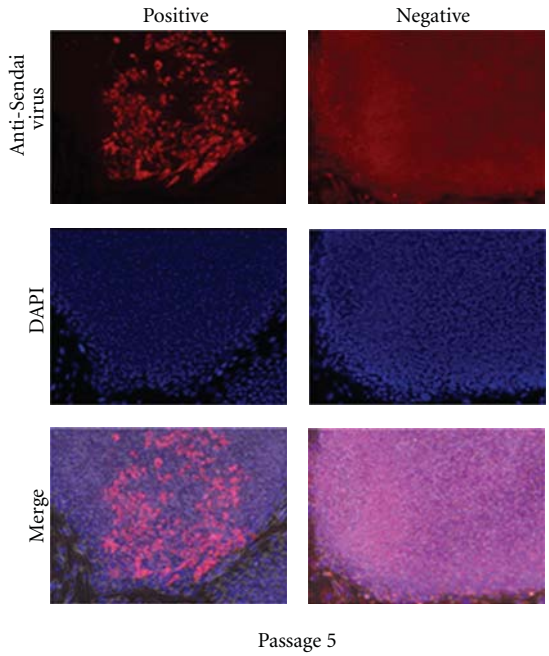

Figure 7: Immunofluorescence staining with anti-Sendai virus vector antibodies to detect virus-free iPSCs. iPSC clones were stained at passage 5.

embryonic stem cell line) and to another episomally derived iPSC line. Our data is consistent with the recent publication by Chung et al., which reports that feeder-free iPSCs have similar transcriptomic profiles and differentiation capacity to hESCs [25]. In addition, increasing evidence demonstrates the importance of mircoRNAs (miRNAs) for human embryonic stem cells self-renewal, pluripotency, and differentiation [27]. Previous publications reported a signature group of miRNAs that is upregulated in both iPSCs and hESCs, such as the miR-17–92 clusters, miR-290 clusters, miR-302, 367 cluster, miR-371/372/373, and let-7 family [26]. We further compared these "pluripotent" miRNAs and found high correlation between the human iPSCs generated by Sendai virus vector and the WA09 human embryonic stem cell (hESC) line. These data suggest that the nature of iPSCs generated with Sendai virus vector is similar to that of hESC line.

In summary, reports have shown that the use of integrating viruses to generate iPSCs is not ideal due to the potential of tumorigenicity and the exposure of iPSCs in animal containing products impedes downstream applications. It is important to develop a system that can generate iPSCs that are free of transgenes and in conditions that are free of animal-derived products. Exposure of human cells to animal origin products may increase immune rejection of grafted cells and make them unsuitable for the generation of clinical-grade iPSCs [11]. Here we have demonstrated that iPSCs can be generated in feeder-free or xeno-free conditions. Further, the iPSCs generated here are free of transgenes and behave similar to those generated on feeders or animal-derived products. The generation of a footprint-free iPSCs under these conditions should facilitate the safe clinical translation of iPSC-based therapies.

Acknowledgments

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Research Article

Induction of Pluripotency in Adult Equine Fibroblasts without c-MYC

Khodadad Khodadadi,[1] Huseyin Sumer,[1] Maryam Pashaiasl,[1,2] Susan Lim,[3] Mark Williamson,[4] and Paul J. Verma[1,5]

[1] *Centre for Reproduction and Development, Monash Institute of Medical Research, Monash University, Clayton, VIC 3800, Australia*
[2] *Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz 51666-14766, Iran*
[3] *Stem Cell Technologies i (SCTi), Gleneagles Medical Centre, Singapore 258499*
[4] *Gribbles Veterinary, Clayton, VIC 3168, Australia*
[5] *South Australian Research Institute (SARDI), Turretfield Research Centre, Rosedale, SA 5350, Australia*

Correspondence should be addressed to Huseyin Sumer, huseyin.sumer@monash.edu

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Despite tremendous efforts on isolation of pluripotent equine embryonic stem (ES) cells, to date there are few reports about successful isolation of ESCs and no report of *in vivo* differentiation of this important companion species. We report the induction of pluripotency in adult equine fibroblasts via retroviral transduction with three transcription factors using *OCT4, SOX2*, and *KLF4* in the absence of c-MYC. The cell lines were maintained beyond 27 passages (more than 11 months) and characterized. The equine iPS (EiPS) cells stained positive for alkaline phosphatase by histochemical staining and expressed OCT4, NANOG, SSEA1, and SSEA4. Gene expression analysis of the cells showed the expression of *OCT4, SOX2 NANOG*, and *STAT3*. The cell lines retained a euploid chromosome count of 64 after long-term culture cryopreservation. The EiPS demonstrated differentiation capacity for the three embryonic germ layers both *in vitro* by embryoid bodies (EBs) formation and *in vivo* by teratoma formation. In conclusion, we report the derivation of iPS cells from equine adult fibroblasts and long-term maintenance using either of the three reprogramming factors.

1. Introduction

Cartilage and tendon injuries are common features of tissue damage in both humans and horses. These two tissues have a poor vascular system with low mitotic ability and therefore a limited ability for self-repair. The reduced performance and reinjury create considerable attention for treatments [1].

Adult mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), and reprogrammed somatic cells such as induced pluripotent stem (iPS) cells can provide potential sources of cells for treatment of cartilage and tendon injuries.

MSCs can be isolated from different sources such as bone marrow aspirates [2], umbilical cord [3], and adipose tissue [4] and have the ability to differentiate into different cell types such as muscle, cartilage, and bone [5–7]. MSCs have been used for treatment of cartilage injuries in equines and humans. Although there were the early improvements in cartilage injuries, no significant or long-term recovery could be observed [8, 9]. In addition, MSCs are limited in bone marrow aspirates and need to be cultured after isolation for at least 4 weeks and have limited *in vitro* differentiation potential compared with ESCs [1, 10].

ES cells can overcome this limitation, as they can provide an inexhaustible supply of cell derivatives of all three germ layers. Despite tremendous efforts on isolation of ESCs, to date there are a few reports on isolation of equine ESCs, which had limited success and no investigation of *in vivo* differentiation of the isolated cells [11, 12]. Isolation of equine ESCs is difficult due to the shortage of oocytes and embryos, as well as complexity associated with oocyte collection, maturation, IVF, and *in vitro* culture in this species [13]. Even if ES cells can be successfully derived, a subsequent problem is the anticipated immune rejection of the derivatives of

ES cells by the recipient due to incompatibility of the major histocompatibility complex (MHC) antigens because of differences in genomic DNA compared with that of the recipient [14].

There are alternative methods to produce autologous cells lines via reprogramming of adult somatic cells to the pluripotent states such as somatic cell nuclear transfer (SCNT) [15, 16] and induced pluripotent stem (iPS) cells [17]; however, limitation with derivation of equine ESCs extend to SCNET-ESC isolation as well.

Takahashi and Yamanaka [17] reported the generation of pluripotent cells from adult mouse fibroblast following retroviral-mediated transduction of four transcription factors, *OCT4, SOX2, c-MYC*, and *KLF4*. A number of studies have shown that iPS cells are similar to ESCs in morphology and epigenetic status, expression of pluripotent markers, and ability to differentiate into derivatives of all three embryonic germ layers both *in vivo* and *in vitro* and contribute to the germ-line in chimeric mice confirming their true pluripotency [17–19]. Therefore, these cells could have therapeutic application in both human and animals.

Pluripotency has been induced in somatic cells from human [20], primate [21], rat [22, 23] pigs [24–27], sheep [21], and cattle [28].

More recently, the generation of equine iPS cell lines from fetal fibroblasts using transposon-based delivery of four factors has been reported [29]. In this study, we report the generations of equine-induced pluripotent stem (EiPS) cells by retroviral-mediated transduction of adult equine fibroblasts using three transcription factors: *OCT4, SOX2,* and *KLF4*, *(OSK)* without the protooncogene *c-MYC,* and the pluripotent characteristics of the resulting EiPS cells have been demonstrated both *in vitro* and *in vivo*.

2. Materials and Methods

Experimental procedures were carried out under the guidelines of the Monash University, Animal Ethics Committee, and conducted according to the International guidelines for Biomedical Research Involving Animals. All chemicals were sourced from Sigma (Castle Hill, Australia) unless otherwise stated.

2.1. Generation of Induced Pluripotent Stem (iPS) Cells from Adult Equine Fibroblasts

2.1.1. Transfection, Isolation, and Culture of iPS Cells. Equine iPS cells were generated as previously reported [28]. Briefly, for VSVG pseudotyped retroviral production 3×10^6 GP2 293 cells (Clontech; Scientifix, Cheltenham, Australia) were seeded in a 100 mm culture dish one day before transfection and incubated overnight at 37°C, 5% CO_2. pMX-based retrovirus vectors encoding human DNA sequence of *OCT4, SOX2*, and *KLF4* were transfected into packaging cells (GP2 293) by FuGENE 6 transfection reagent (Roche, Castel Hill, Australia), and the media were replaced by fresh media on the following day. Viral supernatant was collected 48 and 72 hours later and filtered through a 0.45 μm cellulose acetate filter. Viral supernatants were then mixed with polybrene to a final concentration of 8 ng/mL. Adult equine fibroblasts were plated one day prior to transduction at a density of 1×10^5 cells per 100 mm dish. The cells were incubated overnight with the viral supernatant including equal contributions of the factors and 8 ng/mL polybrene. The following day, transduction process was performed similar to the first day. A *pMX-GFP* and no-vector dishes were provided as a positive and negative control, respectively. Transduced cells were then cultured in conventional medium containing α-minimum essential medium (α-MEM) with deoxyribonucleosides and ribonucleoside (Invitrogen, Mulgrave, Australia), supplemented with 2 mmol/mL glutamax (Gibco, Invitrogen, Mulgrave, Australia), 0.1% (v/v) Mercaptoethanol (Gibco), 1% (v/v) nonessential amino acid (NEAA) (Gibco), 1% (v/v) ITS (10 μg/mL insulin, 5.5 μg/mL 125 transferrin, 6.7 ng/mL selenium; Gibco), 5 ng/mL human LIF (Millipore, North Ryde, Australia), 10 ng/mL βFGF (Millipore), 10 ng/mL EGF (Invitrogen), 0.5% (v/v) penicillin-streptomycin (Gibco), and 20% (v/v) FBS. The medium was changed every other day to maintain cell proliferation. After 12 to 16 days of iPS induction, the best colonies based on equine ES cell-like colony's morphology were picked and manually passaged onto mouse embryonic fibroblasts (MEFs) inactivated with 4 μg/mL of mitomycin C and plated in an organ culture dish. Colonies were manually cut into small clumps by insulin syringe needles and expanded on the freshly inactivated feeder layers to maintain the EiPS cell line. Seven cell lines were initially produced and maintained in culture, and one cell line was characterised in detail. The transduction efficiency of adult equine fibroblast was evaluated by expression of the *pMX-GFP* vector control, which was conducted in parallel with the iPS induction experiments. Seventy-two hours after *pMX-GFP* induction, cells were photographed under a fluorescence microscope, and the percentage of cells expressing GFP was quantified by flow cytometry. Reprogramming efficiency evaluated by correlation of *pMX-GFP* transduction efficiency with iPS cell colony numbers was established [17].

2.1.2. FACS Analysis. Cells were incubated in incubator (37°C, 5% Co_2) using 0.25% trypsin-EDTA (Invitrogen) for five min and dissociated through pipetting. After spinning at 400 g for 3 min, the pellet was resuspended and filtered through a 40 μm cell strainer (BD Falcon) and analyzed by a BD FACSCanto Flow Cytometer (BD).

2.2. Characterization of Equine iPS Cell Lines

2.2.1. Alkaline Phosphatase and Immunofluorescence Staining. Cells were fixed for 15 min in 4% (w/v) paraformaldehyde at room temperature and then stained. For alkaline phosphatase (ALP) activity, the cells were stained by histochemistry according to manufacturer's instructions using Alkaline Phosphatase Detection kit (Millipore). For OCT4 and NANOG staining, the cells were permeabilized in 0.2% Triton X-100 in 3%(v/v) goat serum in DPBS for 15 min. The cells were incubated with 3%(v/v) goat serum in DPBS at RT

Table 1: List of primers used for RT-PCR.

Markers	Primer F	Primer R	References
GAPDH	GATTCCACCCATGGCAAGTTCCATGGCAC	GCATCGAAGGTGGAAGAGTGGGTGTCACT	
OCT4	TCTTTCCACCAGGCCCCCGGCTC	TGCGGGCGGACATGGGGAGATCC	
NANOG	TCAAGGACAGGTTTCAGAAGCA	GCTGGGATACTCCACTGGTG	
SOX2	GGTTACCTCTTCCTCCCACTCCAG	TTGCCTTAAACAAGACCACGAAA	
STAT-3	TCTGGCTAGACAATATCATCGACCTT	TTATTTCCAAACTGCATCAATGAATCT	Li et al. [12]
β-Tubulin III	CAGAGCAAGAACAGCAGCTACTT	GTGAACTCCATCTCGTCCATGCCCTC	Li et al. [12]
GATA-4	CTCTGGAGGCGAGATGGGACGGG	GAGCGGTCATGTAGAGGCCGGCAGGCATT	Li et al. [12]
α-Fetoprotein	CTTACACAAAGAAAGCCCCTCAAC	AAACTCCCAAAGCAGCACGAG	Li et al. [12]
BMP4	TCGTTACCTCAAGGGAGTGG	GGCTTTGGGGATACTGGAAT	Pashaiasl et al. [30]

OCT4 and *SOX2* primers were based on primers specific for *Homo sapiens* primers. The sequences of these genes were blasted against horse nucleotide sequences that have 95% and 94% coverage with the coding sequence of Equus caballus. *BMP4* primers were designed on a bovine sequence that has 91% coverage with the coding sequence of Equallus equa. *STAT3, GATA4, β-tubulin III*, and *α-fetoprotein* primers have been applied by Li et al. [12].

for 1 hr to block nonspecific binding of the primary antibodies and then incubated with primary antibodies raised against mouse anti-human SSEA1 (Millipore, MAB4301), mouse anti-human SSEA-4 (Millipore, MAB4304), mouse anti-human OCT4 (Santa Cruz, sc-5279) and rabbit anti-human Nanog (Abcam-ab21603) diluted at 1 : 100 in DPBS containing 3% (v/v) goat serum overnight at 4°C. The next day the dishes were washed with DPBS three times and incubated with secondary antibodies (diluted in DPBS 1 : 1000, Alexa Flour 594 or 488, Invitrogen) for 1 hr at RT. After three washes with DPBS, the cells were counterstained with 1 μg/mL Hoechst 33342 in DPBS for 10 min at RT. Control cell lines were treated mouse ESD3 and human ES cells as well as negative control by omitting the primary antibodies (Supplemental Figures 1 and 2) (In Supplementary Material available on line at doi: 10.1155/2012/429160). Images were captured on an Olympus Ix71 microscope.

2.2.2. RNA Extraction and RT-PCR Analysis of Gene Expression. Gene expression was analyzed by RT-PCR. Total RNA was extracted from harvested cell samples using Dynabeads mRNA DIRECT Micro Kit (Invitrogen) or using the RNeasy kit (Qiagen, Doncaster, Australia) according to the manufacturer's instructions. RNA concentrations were determined using the nanoDrop ND-1000 (NanoDrop Technology, Australia). The extracted RNA was treated by RQ1 DNase (Promega, South Sydney, Australia) to remove any contaminating genomic DNA. cDNA was generated using the superscript III enzyme as described before [30]. The first strand cDNA was further amplified by PCR using forward and reverse primers for specific genes. All samples were checked for *GAPDH* to verify the success of the RT reaction and then for other specific genes with individual primers. PCR amplification was performed in 50 μL reaction containing 5 μL DNA polymerase 10x reaction buffer, 3 μL $MgCl_2$ (25 mM), 1 μL dNTP mixture (10 mM), 0.4 μL GoTaq DNA Polymerase, 1 μL (10 μM) from each forward and reverse primer, 1 μL sample and μL Milli-Q water (Promega). The PCR was processed in a MyCycler Thermal Cycler and run for 35 cycles: denaturation (95°C, 45 s), annealing (55–56°C), and extension (72°C, 45 s) steps.

All PCR samples were analyzed by electrophoresis on a 2% (w/v) agarose gel. The sequence of primers used for PCR and the product size are listed in the Table 1.

2.2.3. Chromosome Counts of Equine iPS Cell Lines. Chromosome counts were performed at P15 and P22. To estimate chromosome number, the cells were treated with 5-bromo-2-deoxyuridine (BrdU) overnight and then with Colcemid (Gibco) for a further 4 hours to suppress mitosis. After treating with TrypLE Express (Invitrogen) and hydrating in hypotonic KCL for 15 min, they were washed and fixed in methanol and acetic acid in a ratio of 3 : 1 and centrifuged. The fixation and centrifuge process were repeated three times. The fixed cell pellet was resuspended in 50 uL fixative and was dropped onto clean slides at RT. The slides were stained with a freshly made staining solution containing 3 mL of Leishman stain in 17 mL Gurrapostrophes buffer (Invitrogen) for 8 min. The Leishman stain was prepared by dissolving 2 g Leishman powder in 1 liter methanol. A coverslip was mounted on the slides with Histomount and slides viewed using a light microscope under oil immersion optics (Nikon C1) at 1000x magnification.

2.3. Differentiation Potential of Equine iPS Cell Line

2.3.1. Embryoid Body Formation. Equine iPS cells colonies were mechanically dissociated into clumps with needles and cultured on Petri dishes in medium containing α-MEM with deoxyribonucleosides and ribonucleoside supplemented with glutamax (Gibco), mercaptoethanol (Gibco), nonessential amino acid (NEAA, Gibco), ITS (insulin, transferrin, selenium; Gibco), penicillin-streptomycin (Gibco), and FBS [30] at 39°C in a humidified gas environment of 5% CO_2 in air. Culture medium was changed every 3 days. Samples from attached and nonattached EBs were collected at two weeks to check gene expression of ectodermal markers (β-tubulin

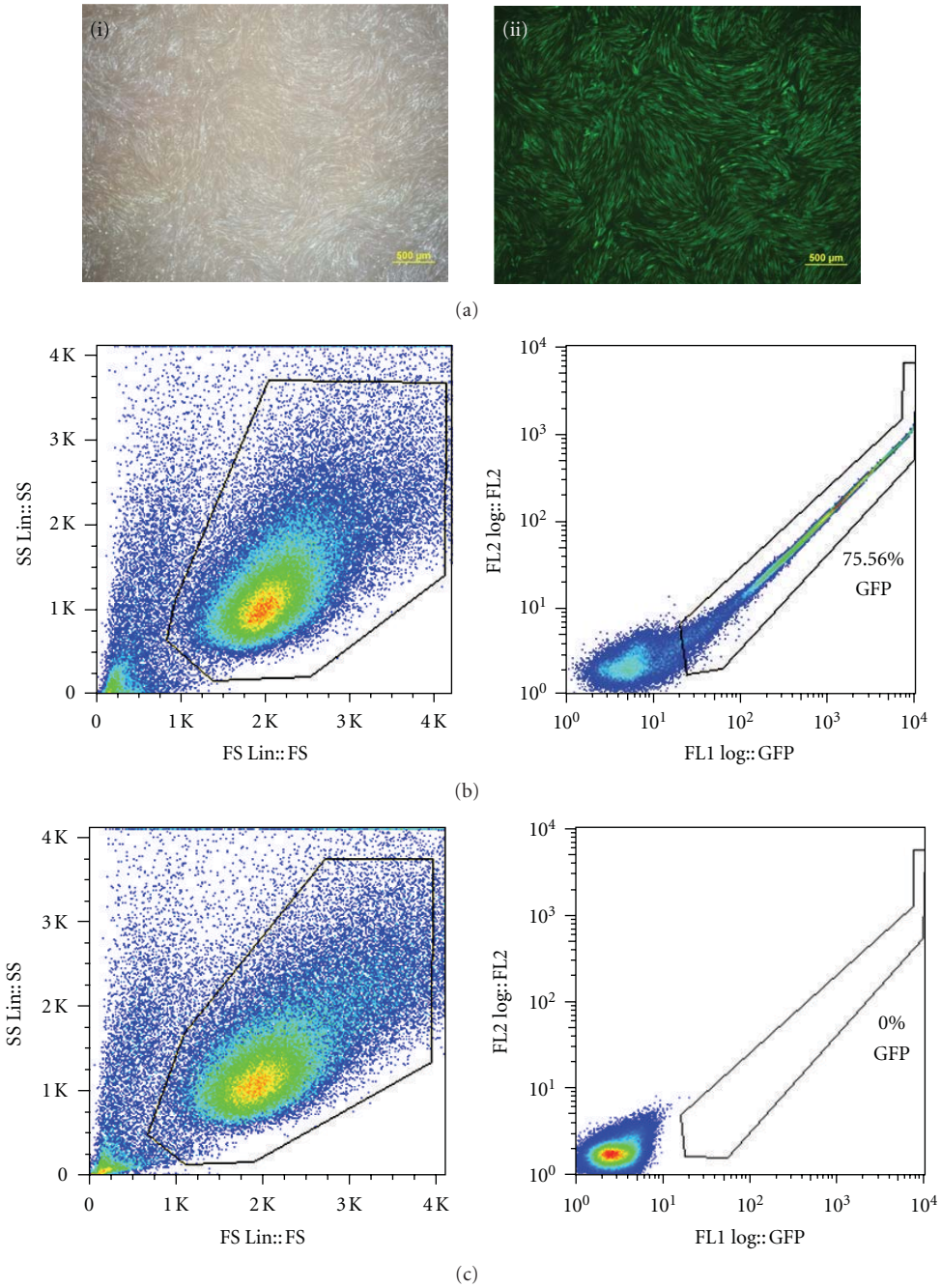

Figure 1: FACs analysis showing GFP fluorescence in adult equine fibroblasts following *pMX-GFP* viral transduction. (a) GFP fluorescence in AEFs following GP2293 mediated retroviral transduction, scale bar 200 μM. (i) Bright filed. (ii) Green filter. FACs profile of GFP fluorescence, (b) Retroviral transduction using GP2 293 packaging cell. (c) Control EAFs.

III), endodermal markers (Gata4), and mesodermal markers (BMP4) (Table 1) by RT-PCR as described before.

2.3.2. Teratoma Formation. Equine iPS colonies were dissociated into single cells and left on ice until preparation of mice for injection. Five-week-old male SCID mice were used for hind leg muscle injection of 2×10^6 EiPS cells. All procedures were performed with sterile materials in a biological safety cabinet. They were then monitored for well-being and teratoma formation. A growth in the hind leg was visible after approximately 8–10 weeks after injection. Mice were humanely sacrificed; the tumor was dissected out, washed in DPBS, fixed in HistoChoic,e and embedded in paraffin for histological analysis. The samples were sectioned at 4 μm thickness onto superfrost slides and allowed to dry overnight. After staining with hematoxylin and eosin, sections were observed using an Olympus Ix71 microscope.

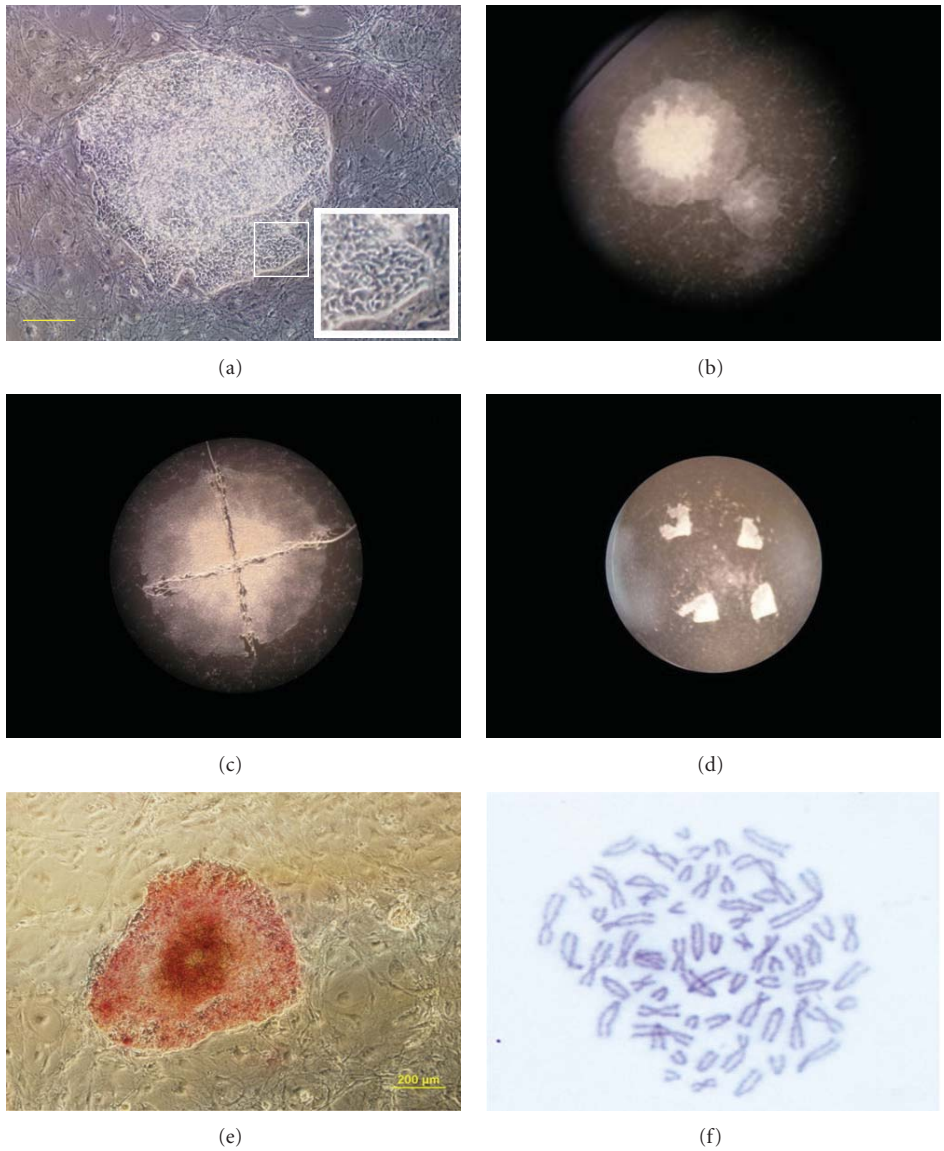

Figure 2: Generation of EiPS cells. (a) Morphology of EiPS single colony growing from individual fibroblast. (b) Typical colony of EiPS cells on the MEF and colony selection. (c) Cutting individual colony for manual passaging. (d) Passaged pieces on the MEF. (e) Alkaline phosphatase activity of EiPS cells scale bar 200 μM. (f) Chromosome spread of EiPS cells.

3. Freezing and Thawing

One hour before freezing the cells, a cryofreezing container containing isopropanol was equilibrated at 4°C. The colonies were dissociated into small clumps about 100 to 200 cells and collected into 15 mL falcon tube and washed by iPS cells medium and centrifuged for 3 min at 400 g. Supernatant was discarded, and clumps were resuspended in appropriate amount of EiPS cells medium. Freezing medium which consists of 80% FBS (JRH Bioscience, Australia) supplemented with 20% dimethyl sulphoxide (DMSO) was added to prepared 500 μL suspension including 80–100 clumps of putative EiPS cells in iPS medium in a cryovial (Nunc, Thermo Fisher, Scoresby, Australia). Then the vials were initially frozen to −80°C overnight and then transferred to a LN_2 tank at minus 196°C for long-term storage. The thawing process involved the placing of the cryovials containing the clumps of EiPS cells in a water bath at 37°C to be thawed, and cells were transferred to a 15 mL falcon tube, and then 10 mL iPS cells medium was slowly added. The cells were centrifuged for 3 min at 400 g, and then supernatant was discarded, the pallet was resuspended with EiPS medium, and clumps were implanted on fresh MEF in a culture dish using insulin syringe needle.

4. Results

4.1. Generation of Induced Pluripotent Stem (iPS) Cells from Adult Equine Fibroblasts. After two rounds of repeated transduction with the three factors (*OCT4, SOX2*, and *Klf4*)

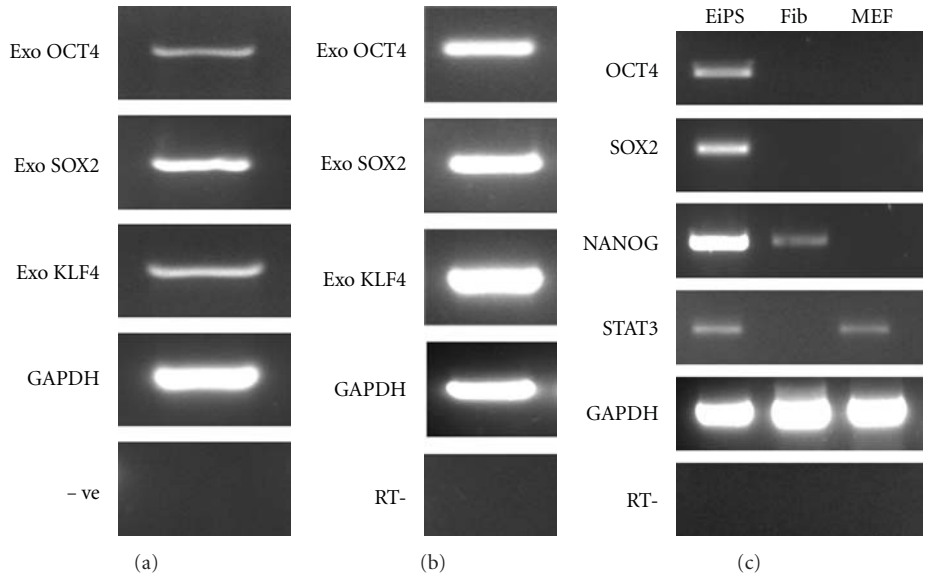

Figure 3: Gene expressions profile of EiPS cells and genomic DNA analysis. (a) Genomic PCR confirming the integration of the four transgenes. (b) Gene expression of exogenous reprogramming factors. (c) Gene expression profile of the EiPS cells compared to the parental EAFs and MEF as feeder cells.

into adult equine fibroblast, we achieved a transduction efficiency of greater than 60% on day two postinfection using *pMX-GFP* control plasmid, while negative control showed no *GFP*-marked cells (Figures 1(b) and 1(c)).

iPS cell colonies first appeared on the day 8–10 postinfection with the dome-like and tightly packed structure. They became large enough at around day 16 to be picked and expanded. Colonies were isolated mechanically and transferred onto prepared culture dishes containing MEF layer and equine ES cell medium (Figure 2).

4.2. Characterization of Equine iPS Cell Line. EiPS cells had a low cytoplasm to nuclear ratio and formed colonies to those observed in cattle [28]. The cell line was characterized by molecular analysis. The integration of reprogramming transgenes into the genome of the cells was confirmed by gDNA PCR analysis and expression of exogenous factor examined at passage 24 (Figures 3(a) and 3(b)). RT-PCR analysis showed mRNA expression of key pluripotent markers including *OCT4, SOX2, NANOG*, and *STAT3* (Figure 3(c)). Some expression of Nanog was detected in equine fibroblasts, and STAT3 was also detected in the mouse embryonic feeder cells using the primer pairs. The cell line expressed a high level of alkaline phosphatase activity (Figure 2(e)). They were positive for protein expression of OCT4, NANOG, SSEA1, and SSEA4 as determined by immunofluorescent staining (Figure 4). Moreover, chromosome spreads revealed a normal diploid chromosome count of 64 in metaphase spreads at passage 15 (data not shown) and 22 (Figure 2(f)). More than 90% of frozen EiPS cells clumps were recovered after thawing and formed colonies after implanting on fresh MEF feeder layer. Thawed cell lines survived and were maintained for more than four passages without losing iPS cell morphology.

4.2.1. Differentiation Potential of Equine iPS Cells. The EiPS cells formed embryoid bodies after 5 days in suspension culture, after which they were transferred to gelatin-coated dishes to attach and develop outgrowths (Figures 5(a) and 5(b)). RT-PCR results demonstrated mRNA expression of genes representative of the three embryonic germ layers [11, 12], endoderm (α-fetoprotein), mesoderm (*Gata4 and BMP4*), and ectoderm (β-*tubulinIII*) (Figure 5(c)). Equine iPS cells formed teratomas 8 to 10 weeks after injection containing cells of the three embryonic germ layers: endoderm (vessels), mesodermal cells (muscle), and ectoderm (epidermal cells) (Figure 5(d)).

5. Discussion

Due to similarity in size, physiology, and immunology, large animals are better models for human genetic or acquired diseases compared with rodents. In addition, they have a longer life span and have a heterogeneous genetic background which is similar to humans and unlike rodents; therefore, they can provide a good model for long-term experiments. Also about 95 equine genetic diseases share a high homology with human genetic defects [13]. Furthermore, equine can be an appropriate model for human diseases such as osteoarthritis as well as a model for musculoskeletal injuries as there are common features of the athletic injuries in human and equine. Limited capability for full functional repair of musculoskeletal injuries has limited treatments outcomes [1]. Joint injuries and related illnesses cost an estimated US$6.5 billion annually for the equine race industry [13].

MSCs, ESCs, and iPS cells are options for research and therapeutic applications regarding musculoskeletal injuries. Compared with MSCs and ESCs, iPS cells are better as they

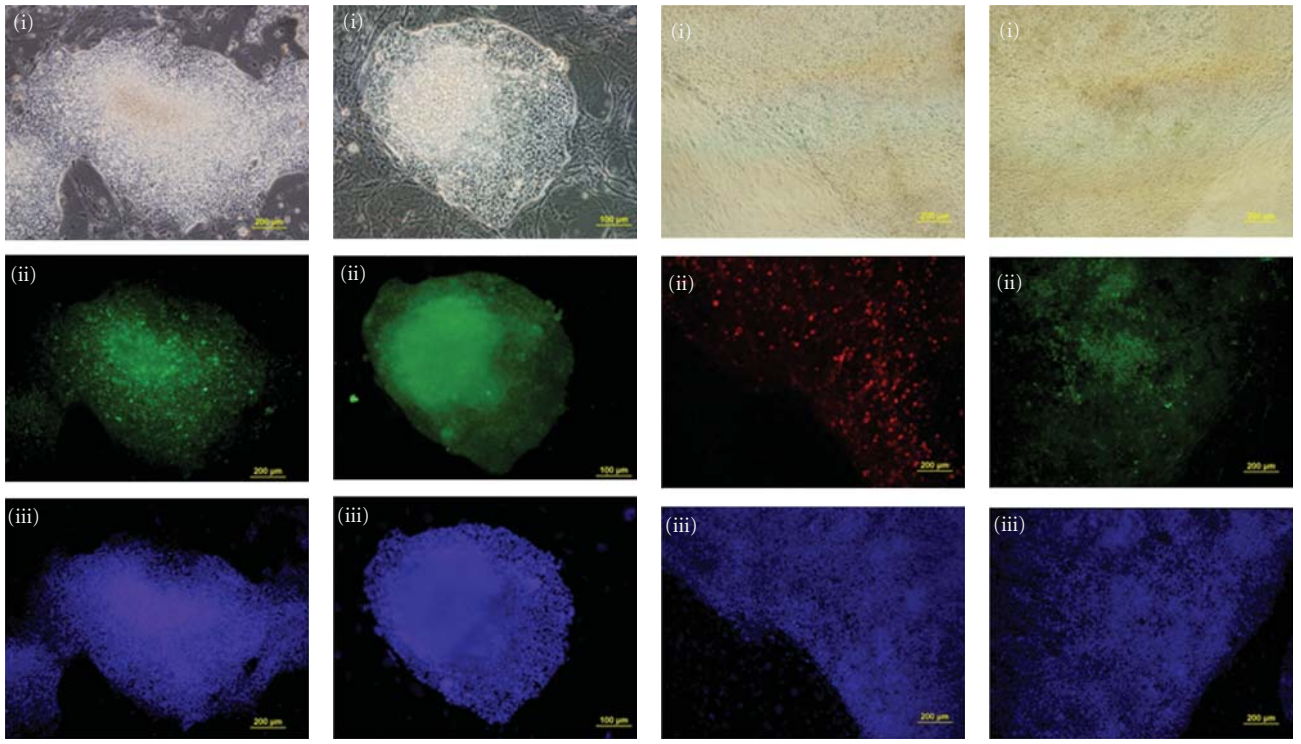

Figure 4: Immunoflourescence staining of pluripotent markers in EiPS cells. Immunostaining of EiPS cells for (a) *OCT4*, (b) *NANOG*, (c) *SSEA1*, and (d) *SSEA4*, counterstained with DAPI, scale bar 200 μM.

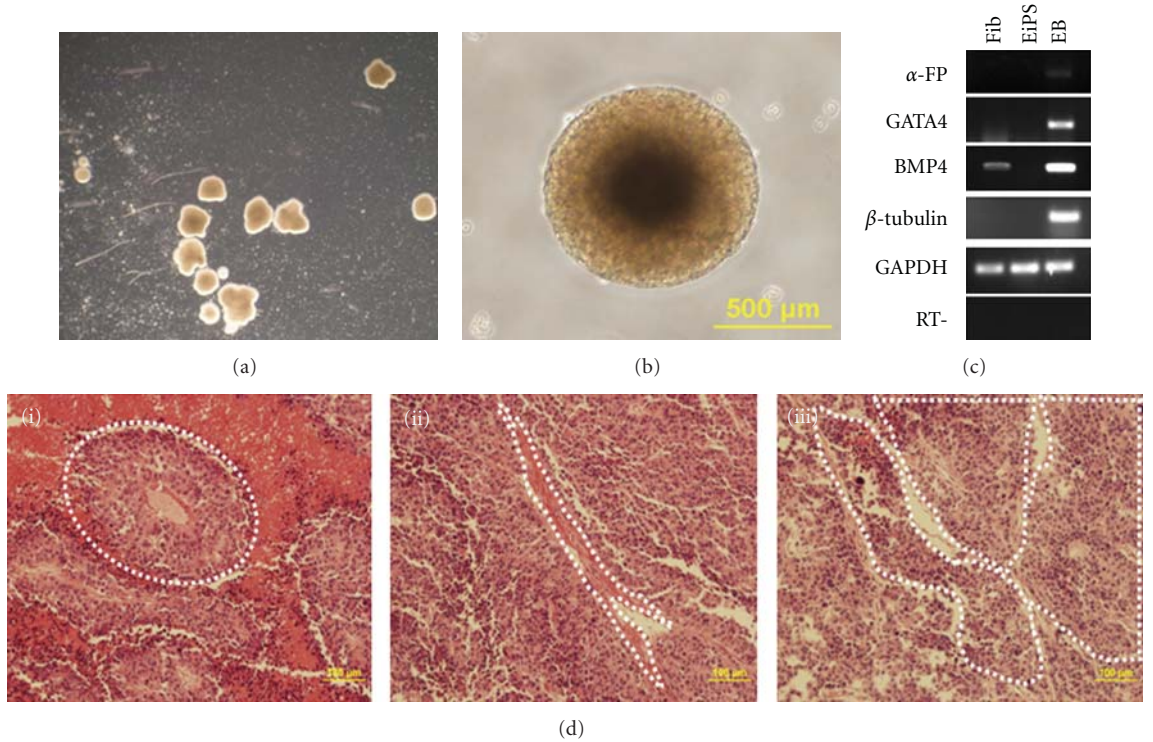

Figure 5: Differentiation potential of EiPS cells. (a) Embryoid body formation of EiPS cells grown in suspension medium in the absence of LIF. (b) Single EB, scale bar 500 μM. (c) Gene expression profile of EiPS cells following differentiation of embryoid body. (d) Histology of differentiated tissues found in the hind leg muscle of SCID mice following injection of EiPS cells included (i) endodermal differentiation, (ii) mesodermal differentiation, (iii) ectodermal (neuroblastic) differentiation, scale bar 100 μM.

provide pluripotent cells that can be immunocompatible to the recipient. There is one report on induction of pluripotency in equine [29], using the Yamanaka cocktail (OSKM) to generate iPS cells from fetal cells. In this study we report the generation of equine iPS cells from adult cells and without the use of the protooncogene c-MYC which opens the door for autologous transplantation in cartilage and tendon injury models. Similar to the finding of Nagy and colleagues the equine iPS cells generated required continuous expression of the transgenes to maintain pluripotency. Apart from one report in sheep [21], iPS cells generated in other domestic species have shown similar traits [12, 18, 24, 25, 28], suggesting that maintenance of pluripotency largely depends on the expression of the reprogramming transgenes.

We established the equine iPS cell line which proliferated in culture beyond 27 passages. The cells maintained ESC characteristics and expressed pluripotent markers including alkaline phosphatase activity and expression of pluripotency markers OCT4 and NANOG. Furthermore, the cells stained positively for SSEA1 similar to mouse pluripotent cells; as well as SSEA4 which is expressed on human pluripotent cells, similar findings have been reported in equine ES [11, 12] and iPS cells [29]. The EiPS cells expressed pluripotency genes *OCT4, SOX2, NANOG*, and *STAT3* by RT-PCR. The EiPS cells showed differentiation potential *in vitro* by EB formation and expressing genes indicative of the three embryonic germ layers. Some of the discrepancies in the markers are due to the difficulties in characterizing pluripotency in the horse as there is a lack of reliable pluripotency markers [1] and lack of suitable antibodies raised against equine cells for immunocytochemical analyses [29]. Therefore, *in vivo* differentiation by teratoma formation was used as further evidence of pluripotential of the cells as has been routinely conducted for iPS cells from most domestic species.

In summary, our findings indicate that adult equine fibroblast can be reprogrammed into pluripotent state via the retroviral delivery of transcription factors, *OCT4, SOX2*, and *KLF4*. The generated iPS cells are pluripotent as shown by expression of pluripotent markers and have capability to differentiate into cell types indicative of the three embryonic germ layers both *in vitro* and *in vivo.*

Acknowledgment

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Review Article

Histone Deacetylase Inhibitors in Cell Pluripotency, Differentiation, and Reprogramming

Androniki Kretsovali,[1] Christiana Hadjimichael,[1,2] and Nikolaos Charmpilas[2]

[1] *Institute of Molecular Biology and Biotechnology, FORTH, Heraklion, 70013 Crete, Greece*
[2] *Department of Biology, University of Crete, Heraklion, 71409 Crete, Greece*

Correspondence should be addressed to Androniki Kretsovali, kretsova@imbb.forth.gr

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Histone deacetylase inhibitors (HDACi) are small molecules that have important and pleiotropic effects on cell homeostasis. Under distinct developmental conditions, they can promote either self-renewal or differentiation of embryonic stem cells. In addition, they can promote directed differentiation of embryonic and tissue-specific stem cells along the neuronal, cardiomyocytic, and hepatic lineages. They have been used to facilitate embryo development following somatic cell nuclear transfer and induced pluripotent stem cell derivation by ectopic expression of pluripotency factors. In the latter method, these molecules not only increase effectiveness, but can also render the induction independent of the oncogenes c-Myc and Klf4. Here we review the molecular pathways that are involved in the functions of HDAC inhibitors on stem cell differentiation and reprogramming of somatic cells into pluripotency. Deciphering the mechanisms of HDAC inhibitor actions is very important to enable their exploitation for efficient and simple tissue regeneration therapies.

1. Introduction

Stem cells are distinguished from other cell types by their unique properties to self-renew and differentiate along multiple lineages [1]. These processes are regulated by extrinsic and intrinsic determinants that affect gene expression profiles, signal transduction pathways, and epigenetic mechanisms.

DNA methylation and histone modifications constitute major mechanisms that are responsible for epigenetic regulation of gene expression during development and differentiation [2–4]. Among other histone modifications, acetylation is very important in nucleosome assembly and chromatin folding. Acetylation favors an open chromatin structure by interfering with the interactions between nucleosomes and releasing the histone tails from the linker DNA. Chromatin regions that are marked by lysine acetylation catalyzed by Histone Acetyl-transferase (HATs) are generally actively transcribed, whereas regions that are bound by Histone Deacetylases (HDACs) bear deacetylated lysines and are inactive [5]. Accordingly, HATs and HDACs reside in multiprotein coactivatory or corepressory complexes, respectively. HATs and HDACs may act either in a site-specific manner, when they are recruited through binding to sequence-specific DNA binding activators or repressors, or in a broad manner whereby they function across large genomic areas.

There are up to date 18 genes coding for histone (or epsilon lysine) deacetylases in the mammalian genomes. They are grouped in four families. Group I (comprising HDACs 1, 2, 3, and 8). IIa (HDAC 4, 5, 7, 9), IIb (6, 10), III (SIRT 1–7), and IV (HDAC 11) [6]. In spite of their name, histone deacetylases have also nonhistone target proteins especially those belonging to group II which do not have histones as substrates. Class I HDACs participate in diverse repressory complexes via interaction with different cofactors such as the Sin3A, Nurd, and CoRest [7]. Contrary to their consideration as repressors, HDACs may act as coactivators of transcription as was reported in the interferon stimulated genes [8]. Genome-wide detection of HATs and HDACs of higher eukaryotic organism has revealed a highly complex situation, active

genes are bound by both enzyme types, whereas inactive genes are not bound by HDACs [9]. Inactive genes that were primed for activation by H3K4 methylation were transiently bound by both HATs and HDACs [9].

HDAC inhibitors (HDACis) are natural or synthetic small molecules that can inhibit the activities of HDACs. In spite of similarities in their enzymatic activities, loss of function experiments have attributed highly specific roles to individual members of HDAC proteins in the course of development and differentiation. In addition, HDAC inhibitors that have broad specificity towards their HDAC targets have shown highly specific effects depending on the target cell type [10].

The profound events that govern stem cell differentiation and somatic cell reprogramming to pluripotency are mainly epigenetic [11]. HDACis are epigenetic modifiers that can promote efficient and temporally regulated control of gene expression. This paper will discuss the role of HDACi in stem cell pluripotency and differentiation as well as in the reprogramming of somatic cells into pluripotency.

2. The Role of HDAC Class I and II Members in Mammalian Development and Differentiation

Analysis of knockout mice lacking HDAC genes has revealed their functions during mammalian development and differentiation [10]. HDAC1 gene deletion is embryonic lethal due to cell proliferation and growth defects [12]. The same proliferation defects were reported in HDAC1-null embryonic stem (ES) cells which overexpress the cell cycle inhibitors p21 and p27 [13]. This analysis has revealed a dual role for HDAC1 in both repression and activation of gene transcription. Tissue-specific deletion of HDAC1 in mice did not have significant effect due to functional redundancy with HDAC2 [14]. However, deletion of HDAC2 was reported to cause perinatal lethality in one publication [12], whereas it resulted in a failure to reactivate fetal gene expression programme under cardiac hypertrophic stress in another study [15]. Regarding cardiac growth and development, one allele of either HDAC1 or 2 is sufficient, whereas conditional deletion of both HDAC1 and 2 is lethal due to heart development failure [12].

Similar to the cardiac differentiation, HDAC1 and 2 have essential but redundant roles in the differentiation of neuronal precursors into neurons [12]. Deletion of both enzymes results in severe brain abnormalities and lethality at postnatal day 7 [12]. The roles of individual HDACs 1, 2, and 3 have been assessed in the differentiation of cortical stem cells using dominant negative mutants [16]. Specifically, all three of them inhibit oligodentrocytic differentiation, HDAC2 inhibits astrocytic, whereas HDAC1 is required for neuronal differentiation. On the other hand, specific deletion of both HDAC1 and 2 in oligodendrocyte lineage cells resulted in Wnt pathway activation, which in turn inhibited oligodendrocyte development by repressing Olig2 expression [17]. In agreement with these data, ablation of both HDAC1 and 2 in Schwann cells caused severe myelination deficiency due to NFkB deacetylation [18].

Finally, HDAC1 and 2 have important functions in hemopoiesis [19]. HDAC1 activity is required for erythroid, whereas it blocks myeloid differentiation [20].

HDAC3 deletion is embryonic lethal due to deficient gastrulation [21–23] that is connected to failure in DNA damage repair mechanisms [23]. Conditional tissue-specific deletions of HDAC3 have pointed to an involvement in liver [22] and heart [21] function.

Although class I HDACs are widely expressed, members of the IIa group show tissue-restricted expression. HDAC4 regulates skeletogenesis and knockout mice die in the first week after birth due to excessive ossification of endochondral cartilage which interferes with breathing [24]. This effect is due to unrestricted function of MEF2 and RUNX2, two transcription factors that activate bone formation [25, 26]. RUNX2 is activated by MEF2 and both MEF2 and RUNX2 are targeted by HDAC4 [26]. HDACs 5 and 9 control, in redundant manner, cardiovascular development since single knockout mice are viable, whereas double disruption leads to lethality caused by defective cardiac development resulting from unrestricted activation of MEF2- [27], SRF-, myocardin- and Calmodulin-binding transcriptional activator 2 [28]. In addition, HDAC 4, 5, and 9 control skeletal muscle differentiation through negative regulation of MEF2, PGC1a, and NFAT in response to calcium signals [29] and motor neuron activation [30]. HDAC7 is specifically expressed in endothelial cells of the cardiovascular system [31] and HDAC7 gene deletion results in embryonic lethality due to vascular rupture and excessive hemorrhages [31]. These effects are caused by extreme activation of matrix metalloproteinase (MMP) 10 which is normaly inhibited by HDAC7 [31]. Members of the HDAC class IIb group (HDAC 6, 10) regulate cytoskeletal dynamics by controlling the acetylation of cytoskeletal proteins such as tubulin [32].

HDAC expression and activity are intimately associated with the emergence of neoplasias. In Acute Promyelocytic Leukemia (APL), fusions between Promyelocytic Leukemia (PML) and Retinoic Acid Receptor (RAR) recruit HDACs resulting in the repression of differentiation-related genes [33, 34]. In solid tumors, mutations in HATs [35] and overexpression of HDAC-associated proteins lead to relative hyperactivity of HDAC. Consequently, HDAC inhibitors are long established antitumor agents that were known before the identification of their target HDAC molecules [34, 36].

3. Inhibitors of HDACs

HDAC class I and II inhibitors (HDACi) fall into discrete structural categories such as hydroxamic acids, cyclic peptides, benzamides, benzofuranone, and sulfonamide containing molecules [37, 38]. The biological effects of HDACi result from positive or negative regulation of gene expression by induced acetylation of histones, transcription factors or other proteins. Genome-wide analyses of gene expression changes upon HDACi administration have revealed that approximately equal numbers of genes are induced and repressed [39]. The genes affected are highly dependent on the cell type and transformed cells are extremely sensitive as opposed to normal cells. Most studies have been performed

with transformed cells. The antitumor activity of HDACi results from a combination of many processes involving cell cycle arrest, apoptosis, activation of mitotic cell death, and inhibition of angiogenesis. In addition, but not unrelated to the aforementioned effects on cell functions, HDACis were reported to induce differentiation of certain cancer cell types [36]. This property gains extreme importance in light of the recently established discovery of "cancer stem cells" [40], a small population of cells that are able to reproduce the tumor and possess self-renewal and pluripotency activities.

4. HDAC Inhibitors in Stem Cell Self-Renewal and Differentiation

Due to their activity in epigenetic regulation, HDACis have been widely used in order to alter the differentiation state of stem and somatic cells as shown in Table 1.

4.1. Embryonic Stem Cell Pluripotency. Differentiation is a process of gradual loss of potency that ends up to the point where specific cell fate is acquired. Embryonic stem (ES) cells are isolated from the inner cell mass of blastocysts [1, 41, 42] and are characterized by indefinite self-renewal and pluripotency, the capability to follow all potential differentiation pathways [43, 44]. Both mouse and human ES cells express networks of pluripotency transcriptional regulators exemplified by Oct4, Sox2, and Nanog [45]. They differ in the requirements for externally provided cytokines and growth factors. For instance, mouse ES cell culture requires Leukemia Inhibiting Factor (LIF) [46], whereas human ES cell culture depends on Activin/Nodal and FGF [47]. This difference is due to the developmental stages from which these two cell types were isolated. Human ES cells are derived from later stage of embryonic development compared to the mouse and are highly similar to mouse EpiSC (epiblast stem cells) [47–49]. The differentiation of stem cells is very sensitive to epigenetic changes. Therefore, application of epigenetic regulators such as inhibitors of DNA methylation (5 Azacytidine) and HDAC inhibitors may be valuable tools for stem cell interventions [50].

In accordance with their effects on the differentiation of cancer cells, HDACis are able to promote the differentiation of ES cells. Treatment with Trichostatin A (TSA) promotes morphological and gene expression changes reminiscent of differentiation even in the presence of LIF [51, 52]. Inhibition of HDAC activity accelerated the early differentiation steps of ES cells without being sufficient for commitment to a specific lineage. Genome-wide analysis revealed two gene groups that are targeted by TSA: the first one contains genes related to pluripotency that are suppressed by TSA (Sall4, Nanog, Klf4, Oct4, and Sox2), the second is required for lineage-specific differentiation and its expression is upregulated by TSA [52].

In contrast to these studies, other studies have shown that HDACis increase self-renewal and interfere with differentiation. Low doses of TSA (10 nM) reverted mouse embryoid bodies towards the undifferentiated state [53] and employment of sodium butyrate (NaBu) was reported to support human and mouse ES cells self-renewal when administered within a narrow range of concentrations [54]. In the latter study, low doses of butyrate (and TSA) were able to substitute for FGF2 (human ES) and LIF (mouse ES). However, higher doses led to differentiation. Surprisingly, nonoverlapping transcriptional expression profile changes were observed in butyrate-treated human and mouse ES cells [54]. These findings have shown the ability of butyrate to modulate the stem cell stage pushing mouse ES forward and pulling human ES backward [54]. In agreement with these data, treatment of mouse ES with TSA was able to shift a population of epiblast-like ESC towards an ICM-like state [54, 55]. A conclusion of all these studies might be that HDACis exert an antidifferentiation effect when low doses are applied on cells that have already exited from self-renewal either as embryoid bodies [53] or epiblast-like [54, 55] cells, whereas higher doses applied on undifferentiated cells provoke differentiations [51, 52]. The same effect was observed upon HDACi treatment of two embryonic carcinoma (EC) cell lines F9 and P19. In F9 cells which belong to a less differentiated state, the expression of the pluripotency factor *Fgf4* decreased after treatment with Valproic acid (VPA) and TSA. In contrast, the same treatment of P19 cells, which are more differentiated, caused the elevation of *Fgf4* expression [56]. In agreement with this data, reactivation of pluripotency genes such as Oct4, Nanog, and Klf4 was observed in neurosphere cells treated with TSA and azacytidine, AzaC [57]. Hence, changes in the acetylation levels of stem cells result in alterations of the differentiation status in correlation with the developmental stage.

Directed differentiation of ES cells is not easy to control. Differentiation protocols generally rely either on the generation of ES cell aggregates (embryoid bodies) or on culturing on stromal cells. Effectiveness and selectivity need to be significantly improved in order for ES cell to be used as tools for cell-based therapies.

HDACi treatment was used for directed differentiation of mouse ES cells towards the cardiomyocytic lineage. TSA added on embryoid bodies between days 7 and 8 potentiated cardiac differentiation due to hyperacetylation of GATA4 [58] a master regulator of cardiogenesis. In addition, TSA induced, whereas HDAC4 overexpression inhibited, cardiomyogenesis of embryonic carcinoma P19 cells [59]. TSA was also able to facilitate the myocardial differentiation of induced pluripotent stem cells [60]. Interestingly, TSA and NaBu were reported to induce HDAC4 proteasomal degradation which in turn results in MEF2 activation and cardiac lineage commitment [61]. On the other hand, NaBu was proven effective in the induction of pancreatic and hepatic differentiation from mouse and human ES cells [62–64].

4.2. Tissue-Specific Stem Cells

4.2.1. Neural Stem Cell Differentiation. As indicated previously ablation of HDAC1 and 2 is postnatal lethal due to disorganization of brain structures [12]. However, administration of HDAC inhibitors led to the induction of neuronal and suppression of glial differentiation [65]. In addition HDAC activity is required for timing of oligodendrocyte differentiation [66].

Table 1: Functions of HDAC inhibitors in stem cell self-renewal or differentiation and somatic cell reprogramming to pluripotency.

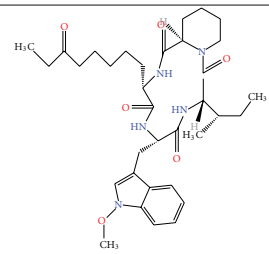
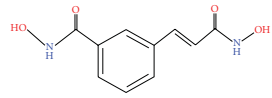
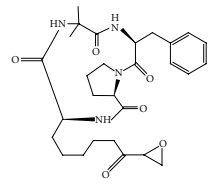
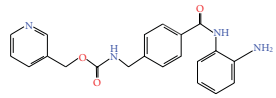
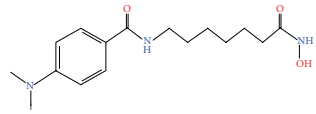
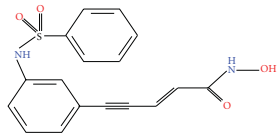
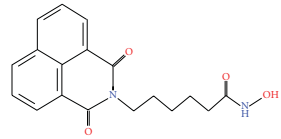
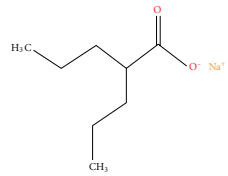
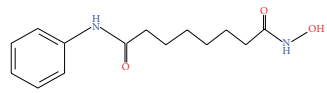
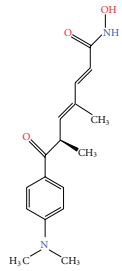
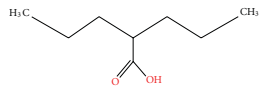
Name	Chemical structure	Self-renewal	Differentiation	Reprogramming	References
Apicidin		—	—	+	[67]
m-Carboxycinnamic acid bishydroxamide (CBHA)		—	—	+	[68]
Chlamydocin		↑ HSCs			[69]
Entinostat (MS-275)		—	↑ Neuronal ↓ Oligodentrocytic	+	[70] [67]
M344		—	↑ Neuronal ↓ Oligodendrocyte	—	[70]
Oxamflatin		—	—	+	[71]
Scriptaid		—	—	+	[72]
Sodium butyrate (NaBu)		↑ hESC ↑ mESC	↓ Adipogenic, chondrogenic neurogenic ↑ Osteogenic ↑ Ductal ↑ Pancreatic and hepatic	+	[52] [52] [73] [73] [74] [60–62] [67, 75, 76]
Suberoylanilide hydroxamic acid (SAHA)		—	↑ Neuronal ↓ Oligodendrocyte	+	[70] [71]

Table 1: Continued.

Name	Chemical structure	Self-renewal	Differentiation	Reprogramming	References
		↑ mESC			[51]
		↑ ECCs (P19)			[54]
		↑ Neurosphere cells			[55]
Trichostatin A (TSA)			↑ ESCs		[49, 50]
			↑ NSCs		[77]
			Ductal		[74]
			Cardiomyocytic		[78]
			↑ ECCs (F9)		[54]
			↑ Myocardial		[58]
				+	[67, 79–81]
Valproic acid (VPA)		↑ ECCs (P19)			[54]
			↓ Adipogenic, chondrogenic neurogenic		[73]
			↑ Osteogenic		[73]
			↑ Ductal		[74]
			↑ ECCs (F9)		[54]
			↓ Astrocyte, oligodendrocyte		[65]
			↑ Neuronal		[63, 66, 82]
				+	[81, 83]

Embryonic Stem Cells (ESCs), embryonic carcinoma Cells (ECCs), hemopoietic stem cells (HSCs), and neural stem cells (NSCs).

Specifically, VPA was reported to increase neuronal differentiation of adult neural progenitor cells and inhibit astrocyte and oligodendrocyte differentiation [65]. Moreover, VPA administration inhibited the differentiation of oligodendrocyte progenitor cells in the developing rat brain [66].

The molecular mechanism of VPA function was induction of NeuroD, a neurogenic bHLH transcription factor [65]. Derepression of NeuroD and neuronal fate activation was also caused by HDAC5 nuclear exclusion [77]. In another study, VPA promoted neuronal fate commitment via activation of the ERK pathway [70]. TSA was able to increase differentiation of neural stem cells at the expense of astrocyte production [82]. Importantly, the TSA-produced nerve cells bear normal electrophysiological properties and morphological characteristics such as the extension of long dendrites with branching points. Treatment of Adult Subventricular Zone (SVZ) precursor cells with MS-275, M344, and suberoylanilide hydroxamic acid (SAHA) increased neuronal differentiation and inhibited oligodendrocyte production via induction of NeuroD cyclinD2 and B-lymphocyte translocation gene 3 [84]. VPA was reported to promote neuronal differentiation of hippocampal neural progenitor cells by induction of proneural factors Ngn1, Mash1, and p15 and histone H4 acetylation [85]. Combination of TSA with Shh, Fgf8, and Wnt1 promotes differentiation of nonmecencephalic neural stem cells to dopaminergic neurons [69]. Interestingly, the regulatory role of histone acetylation in the nervous system is evolutionarily conserved between vertebrates and invertebrates. High levels of acetylation are required for neuronal, whereas low levels are connected to the glial differentiation of Drosophila neural stem cells [86].

4.2.2. Hemopoietic Stem Cell Self-Renewal and Differentiation. Mouse and human hemopoietic stem cells (HSC) self-renewal was potentiated by chlamydocin [87]. In another study, the application of TSA with 5-AzaC increased 12.5-fold the proliferation of HSC isolated from umbilical cord HDACi [73, 74].

Mesenchymal stem cells (MSCs) from adipose tissue and umbilical cord blood were treated with two HDAC inhibitors, VPA, and NaBu [88]. Posttreatment controlled differentiation was conducted into bone, fat, cartilage, and nervous tissue. Different results were obtained depending on the cell types which were examined. VPA and NaBu attenuated the efficiency of adipogenic, chondrogenic, and neurogenic derivation. On the other hand, osteogenic differentiation was elevated after HDACi treatment. An interesting new prospect has arisen following a publication which supports that HDAC inhibitors can be used to direct pancreatic cells to a specific lineage. It was shown that NaBu and TSA promote ductal differentiation at the expense of the acinar fate [78]. Thus, cells with exocrine function are converted to endocrine cells, capable of producing hormones such as insulin and somatostatin [89].

4.2.3. Cardiomyocytic Differentiation. Cardiac side population cells isolated from rat hearts were coaxed in cardiomyocytic differentiation by TSA treatment [90]. TSA induced the expression of transcription factors Nkx2.5, GATA4, and MEF2C that play important roles in the orchestration of the events that lead to the production of cardiomyocytes, endothelial, and smooth muscle cells [90]. In another study, TSA and azacytidine treatment promoted cardiomyocytic

differentiation of mesenchymal stem cells via induction of the same transcription factors GATA-4, NKx2.5, and MEF2c [91].

5. HDAC Inhibitors in Cell Reprogramming to Pluripotency

Reprogramming differentiated somatic cells to pluripotent stem cells has emerged as a way of producing patient-specific stem cells. These cells can be possible candidates for regenerative medicine after their differentiation to a specific cell fate.

A strategy used to reverse the differentiated state of cells was somatic cell nucleus transfer (SCNT) to enucleated eggs or oocytes [92, 93]. This proved in an emphatic way the fact that cell differentiation is not an irreversible process and that the nucleus of a differentiated cell can be reprogrammed to follow a dedifferentiation program. Additionally, it is a common belief that the more ancestral a cell is, the easier it is to be reprogrammed using the method of nuclear transfer. There are several reports showing that HDAC inhibitors can in fact be very helpful tools in the attempt to increase the efficiency of nuclear transfer experiments (Table 1).

Early reports have applied TSA to donor cells [94] or to the embryos following SCNT [68, 72] and shown that it improves both the in vivo and in vitro developmental rate. TSA was effective as cloning facilitating reagent for many species embryos, bovine ([95], mouse ([71, 96]), and porcine ([97]). TSA treatment caused chromatin rearrangements such as elevated histone acetylation and chromosome decondensation as well as increased rate of RNA synthesis [98]). Treatment of SCNT-generated mouse embryos with scriptaid improved the cloning efficiency for various inbred strains [96]. Moreover, scriptaid treatment resulted in higher levels of nascent mRNA transcription at the two-cell stage and this increase depended on the genotype of the mouse strain used. The cloned mice were both viable and fertile and there was a positive correlation between the increase in nascent mRNA synthesis and full-term development of cloned mice [96]. A different HDACi, CBHA, was reported to augment the developmental potential of cloned mouse embryos at both the pre- and postimplantation stages. Furthermore, CBHA treatment resulted in a statistically significant increase in the total ICM cell number, simultaneously reducing the ratio of apoptotic cells. In addition, it was shown that Oct4 expression was more abundant in the population of cells isolated from blastocysts of treated animals than untreated ones. Hence, those cells resembled ES cells as was confirmed by staining for pluripotency markers (Sox2, SSEA1, alkaline phosphatase) [71]. Finally two other HDAC I and IIa/b inhibitors suberoylanilide hydroxamic acid (SAHA) and oxamflatin could improve the development of cloned mice by reducing the apoptosis in blastocysts [99].

In a pioneer work, the group of Yamanaka [100] reprogrammed fetal and adult mouse fibroblasts to induced Pluripotent Stem (iPS) cells using four key transcription factors, namely Oct4, Sox2, c-Myc, and Klf4. A year later human fibroblasts were reprogrammed by the group of Takahasi et al. [101] and Park et al. [79], whereas the group of Thomson substituted the oncogenic factors Klf4 and c-Myc with Nanog and Lin28 [102]. The aforementioned iPS cells possess identical characteristics with ES cells, such as expression of pluripotency markers, ES cell morphology, self-renewal, and capability of teratoma formation [79, 101].

In order to improve the efficiency of reprogramming, several strategies were developed [83] using small molecules such as DNA methyltransferase inhibitors (5 AzaC, [75]), histone methylotransferase inhibitors (BIX, [76]), and HDAC inhibitors (Table 1). Important steps have been made towards the direction of replacing the oncogenic factors with chemical compounds. In particular, Valproic acid (VPA) was used to substitute for c-myc [80]. VPA and the pluripotency factors Oct4, Sox2, and Klf4 were able to reprogram primary human fibroblasts. The presence of VPA increased the number of iPS colonies by 50-fold. Produced iPS cells resemble ES cells in pluripotency and gene expression profiles [80]. In another study, Klf4 was fully dispensable [67]. The combination of Oct4, Sox2, and VPA was sufficient to reprogram somatic cells with a similar efficiency compared to three-factor reprogramming (Oct4, Sox2, and Klf4). These iPS cells exhibited several desired characteristics, such as increased levels of pluripotency markers and alkaline phosphatase activity. In addition, they seemed morphologically similar to human ES cells and were karyotypically normal. Finally, the two factor-induced human iPS cells were able to form teratomas derived from all three lineages. It is possible that VPA treatment sets somatic cells in a transition state before their complete dedifferentiation [67]. These results offer great possibilities in attaining full reprogramming with chemical reagents, a procedure both safe and practical to be used in human therapies.

In a recent study [75], human fetal fibroblasts were reprogrammed to pluripotency using human ES cell extracts with the addition of 5-azacytidine, TSA, and retinoic acid. This proves that the epigenetic state of cells has a great impact on the efficiency of reprogramming by this method. During the process, upregulation of pluripotency markers (Oct4, Sox2) and morphological changes were observed. In parallel, markers of differentiation (LAMIN A/C) were downregulated, showing a positive correlation between dedifferentiation, and increase in acetylation status of cells.

Another HDAC inhibitor NaBu used at low doses improved the generation of iPS cells by 50-fold by using retroviral or "piggyback" vectors for reprogramming human fibroblasts even in the absence of Klf4 and c-myc [81]. In another study, butyrate was reported to potentiate iPS cell generation from mouse embryonic fibroblasts in the presence of c-myc [103]. This difference might be due to differences in the endogenous c-myc levels between the human and mouse cells.

In addition to the typical iPS cells, reversion of differentiation was assisted by the addition of HDACi in other cell types. Dedifferentiation of primordial germ cells (PGC) into pluripotent embryonic germ (EG) cells was achieved using TSA to replace FGF-2 [104]. A high-throughput screen has revealed the ability of four HDAC inhibitors (NaBu, TSA, MS-275 and Apicidin) to reprogram oligodendrocyte progenitors (OPC) into multipotent neural stem-like cells that

can generate both neurons and glia [105]. Finally, an intriguing new possibility emerged from a recent publication using the nematode *C. elegans* as model [106]. The researchers employed two common HDAC inhibitors (VPA and TSA) to mimic the removal of histone chaperone LIN-53 and managed to reprogram germ cells into specific neuron types. It would be interesting to examine the effect of HDAC inhibition in efforts of direct reprogramming from one type to the other in the more complex context of mammalian cells.

6. Conclusions and Perspectives

Stem cell methodologies have revolutionized modern therapeutic strategies that aim to replace damaged cells or tissues. Controlling the pluripotent stem cell fate [95] is dependent on important transcription, signaling, and epigenetic factors. Among other epigenetic regulators, Histone deacetylases have important roles in cell physiology, differentiation, developmental decisions, and tumor formation [10]. Compared to HDAC genes deletions, HDAC inhibitors elicit cell restricted, albeit pleiotropic effects. A vast collection of natural and synthetic HDAC inhibitors has shown very potent effects in embryonic stem cell differentiation pathways. They may promote either self-renewal [54, 55] or differentiation [51, 52] depending on the stem cell status and the dose employed. These effects might result from reorganization of the embryonic stem cell chromatin that is remarkably dynamic and decondensed [107]. Therefore, HDACi can reverse the repressive or activating epigenetic traits that characterize genes involved in the regulation of self-renewal or differentiation.

Most importantly, HDACis have shown considerable activity in directing the neuronal, cardiomyocytic, and hepatic lineages differentiations. In most cases where the molecular mechanism was examined, it involved the induction of differentiation-regulating transcription factors. Moreover, HDACis were used in somatic cell reprogramming processes. Treatment of donor cells before transfer or embryos following transfer resulted in facilitation of embryo cloning and improvement of embryo developmental potential. These effects were due to enhanced histone acetylation, chromatin decompaction, increase of RNA synthesis, and inhibition of apoptosis. Due to the ethical issues raised by embryo cloning, these techniques are not yet applicable to humans. Therefore, the recent achievement of iPS generation has offered great expectations in custom-specific stem cells for human health. In that field, there is increasing effort in omitting retroviral vectors, oncogenes, and—if possible—all kinds of exogenous genetic material. Substituting transcription or signaling factors with simple small molecule reagents can render the therapies both safer and simpler. For that purpose, HDAC inhibitors have shown activity to enhance reprogramming and substitute for the presence of transcription factors, importantly the oncogenes c-myc and Klf4 [67]. However, the exact molecular mechanism whereby VPA, TSA, and other HDACi function needs to be elucidated. Future researches are expected to elucidate the mechanism of HDACi action in order to design novel reagents with increased effectiveness and specificity. On the other hand, genome-wide analyses have shown that acetylation is a modification as frequent as phosphorylation. Considering that nonhistone proteins are also targets for acetylation, it is expected that analysis of the "acetylome" [108, 109] changes in the course of stem cell differentiation will shed light on the functions and applications of HDAC inhibitors. In addition to mRNA profiling, analysis of miRNA expression changes that follow HDACi may reveal mechanisms whereby these reagents have so specific effects on different cell differentiation backgrounds. HDACis are able to potentiate both stem cell differentiation and somatic cell reprogramming to pluripotency. This may suggest that common mechanisms are involved in opposite changes of the differentiation status. Elucidation of these mechanisms is expected to open new opportunities in the interface between chemistry and stem cell biology. Combining HDAC inhibitors with other small molecule effectors and miRNAs [110] can provide valuable tools to overcome challenges due to genetic interventions and improve stem cell applications for tissue regeneration therapies.

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Review Article

Analysis of Embryoid Bodies Derived from Human Induced Pluripotent Stem Cells as a Means to Assess Pluripotency

Steven D. Sheridan,[1] Vasudha Surampudi,[2] and Raj R. Rao[2]

[1] *Center for Human Genetic Research, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA*
[2] *Department of Chemical and Life Science Engineering, School of Engineering, Virginia Commonwealth University, Richmond, VA 23284, USA*

Correspondence should be addressed to Raj R. Rao, rrrao@vcu.edu

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Human induced pluripotent stem cells (hiPSCs) have core properties of unlimited self-renewal and differentiation potential and have emerged as exciting cell sources for applications in regenerative medicine, drug discovery, understanding of development, and disease etiology. Key among numerous criteria to assess pluripotency includes the *in vivo* teratoma assay that has been widely proposed as a standard functional assay to demonstrate the pluripotency of hiPSCs. Yet, the lack of reliability across methodologies, lack of definitive clinical significance, and associated expenses bring into question use of the teratoma assay as the "gold standard" for determining pluripotency. We propose use of the *in vitro* embryoid body (EB) assay as an important alternative to the teratoma assay. This paper summarizes the methodologies for creating EBs from hiPSCs and the subsequent analyses to assess pluripotency and proposes its use as a cost-effective, controlled, and reproducible approach that can easily be adopted to determine pluripotency of generated hiPSCs.

1. Introduction

Human induced pluripotent stem cells (hiPSCs) are developing as exciting cell sources for applications in regenerative medicine [1] and drug discovery, primarily based on their extensive similarities to their human embryonic stem cell counterparts and shared properties of self-renewal and multilineage differentiation capabilities. The strategy of inducing pluripotency by activating the pluripotent network has proven to be successful in the reprogramming of somatic cells back to an embryonic-like state [2, 3]. However, there is a critical need to assess the pluripotent capabilities of the hiPSCs on a line-by-line basis once reprogramming has occurred to demonstrate this differentiation potential.

Multiple criteria have been proposed to evaluate the pluripotent state of generated hiPSCs [4–6]. Key among these criteria includes routine morphological analysis of cells for the presence of high nuclear-cytoplasmic ratio, cell surface and gene expression of pluripotent markers, demonstration of differentiation capabilities into derivatives from the three developmental germ layers (ectoderm, endoderm, and mesoderm), and specialized functional outcomes to demonstrate developmental potency. The functional assays developed thus far include *in vitro* differentiation, teratoma formation, chimera development, germline transmission, and tetraploid complementation [7]. The most stringent test to screen for pluripotency is the ability to demonstrate germline competency after chimera development, a test that can easily be conducted for mouse iPSCs. For hiPSCs, where testing for germline transmission and tetraploid complementation is not possible, teratoma formation of hiPSCs injected into immunocompromised mice and subsequent analysis of tissue formation has been widely used as an important methodology to investigate the developmental ability of the generated hiPSCs.

2. The Teratoma Assay for Pluripotency Assessment

Teratomas are solid, defined tumors, often germ-line derived, composed of the highly organized differentiated cells and tissues containing representatives of the three developmental germ layers that can also be generated artificially by transplanting pluripotent stem cells (hESCs or hiPSCs) into immunodeficient mice [8]. The histopathology of teratomas is remarkable in that they can serve as an important tool to observe early morphogenesis into organized tissues. The number of cells transplanted into the immunodeficient animal model in order to generate the fully differentiated teratoma *in vivo* can range anywhere between a hundred to a million cells or more [9]. As part of this methodology, the generated hiPSCs are usually transplanted at the following sites: intramuscular, subcutaneous, under the testis capsule, or under the kidney capsule in an immune deficient mouse. After a period of at least three weeks, the mature teratomas are excised out of the animal to be assessed for the presence of the cells derived from the three germ layers. The teratoma assay has been proposed to be the most stringent means to assess pluripotency of hiPSCs [10] but is not as rigid as the tetraploid complementation and germline transmission that can be conducted with mouse iPSCs [11]. In addition, the high costs associated with the assay, use of dozens or hundreds of animals, lack of a definitive clinical or biological relevance of the ability to form teratomas to the specific cell types subsequently derived from the hiPSCs, and the nonsystematic way this assay has been employed bring into question the use of the teratoma assay as the "gold standard" [4, 6, 8, 11].

Recent reports have highlighted the need to standardize the protocol to generate and examine the teratomas induced in the immunodeficient mouse models, if they are to be used as the gold standard for assessing pluripotency in generated hiPSCs [8, 10]. Studies have shown that some of the hiPSC lines have not been successful in forming all three germ layers in the teratomas but have been successful in deriving certain cell types that may have clinical significance [12]. Other researchers have also found that partially reprogrammed hiPSCs can form teratomas even if they do not meet other criteria for pluripotency, leading many to question the overall significance of the teratoma assay [6]. Generation of systematic protocols for teratoma generation and analysis that can be adopted across different labs also remains a challenge [8].

3. Embryoid Bodies as a Means to Assess Pluripotency of hiPSCs

An important alternative method to the teratoma assay is an *in vitro* approach involving the generation of embryoid bodies (EBs) from hiPSCs. EBs are three-dimensional aggregates of cells that are an amalgam of the three developmental germ layers [7]. In this approach, the undifferentiated hiPSCs are placed in suspension, which promotes stochastic differentiation into cells of all three germ layers. Formation of EBs is a routine approach used in the differentiation of the hiPSCs into different cell lineages [13]. One of the major advantages of this approach is that it is performed *in vitro* with standard tissue culture methods and materials, thus avoiding the regulatory issues and extensive expenses associated with maintaining immune-deficient mice. The EBs can be easily grown in a suspension culture in a petri dish in the laboratory and can be scaled up without much difficulty once the appropriate conditions for scale-up are established [14]. Unlike the teratoma assay in which hiPSCs that have passed all other pluripotency tests yet fail to form teratomas for unknown reasons [12], hiPSCs readily form EBs by a number of methods providing the ability to demonstrate trilineage differentiation and analysis in a more controlled, reproducible manner. The numerous approaches that have been developed to generate EBs along with the established analyses used for their pluripotent assessment are summarized in the following sections.

4. Methodologies for the Formation of hiPSC Embryoid Bodies

There have been several methods developed to create embryoid bodies for a variety of purposes, from the generation of specific tissue types to stochastic *in vitro* germ layer differentiation, to illustrate potency of candidate pluripotent stem cell lines [13, 15–17] (summarized in Table 1). For specific tissue lineages, EBs have been shown to be beneficial in the initiation of differentiation and to enhance the differentiation towards certain lineages [18] such as hematopoietic [15, 19], neural [20, 21], and cardiac tissues [22–25]. Methods for developing EBs differ in their ability to form aggregates of uniform size and the maintenance of their long-term viability. Typically, it is advantageous to control the uniform size of EBs for the reproducible differentiation of specific tissue types; however, the ability to form EBs of varied size for extended culture periods facilitates the formation of diverse tissues representing the three germ layers as a means to demonstrate differentiation potential. Both types of techniques can be used for the assessment of pluripotent stem line (hESC, hiPSC) quality.

4.1. Heterogeneous Methods of Embryoid Body Formation. Methods for the creation of stochastic EBs are the most straightforward and useful for the generation of varied germ layer representatives for subsequent demonstration of pluripotency [16]. Liquid suspension culture (LSC, [15]) is a common method for creating EBs and depends on the ability to grow cellular aggregates without attachment to the tissue culture vessel (Figure 1(a)). Typical tissue culture vessels for this purpose range from nontissue culture-treated petridishes to specially treated ultra-low attachment surfaces available through several vendors. These low-attachment tissue culture vessels are typically coated to provide a neutral, hydrophilic surface to prevent protein adsorption and subsequent cell attachment [26]. Hydrogels, such as naturally occurring agarose, or chemically defined, synthetic materials such as polyhydroxyethylmethacrylate (pHEMA) have been used to coat vessel surfaces to prevent the attachment of cells

Table 1: Common techniques to form human embryoid bodies.

	Controls EB size	Need for single cell suspension	Large-scale bioreactor production	Need for special equipment	References
Heterogeneous methods					
Liquid suspension culture	No	No	No	No	[15, 16]
Stirred flask culture	No	No	Yes	Yes	[27]
Rotary cell culture systems (RCCSS)	No	No	Yes	Yes	[28, 29]
Homogeneous methods					
Hanging drop culture	Yes	Yes	No	No	[13, 30, 31]
Low adhesion U-bottom multiwell plates	Yes	No	No	No	[14]
Indented solid microsurfaces (Aggrewell™)	Yes	Yes	No	Yes	[32]
Other methods					
Hydrogels (e.g., methylcellulose, agarose, alginate)	No	Yes	No	No	[15, 33, 34]

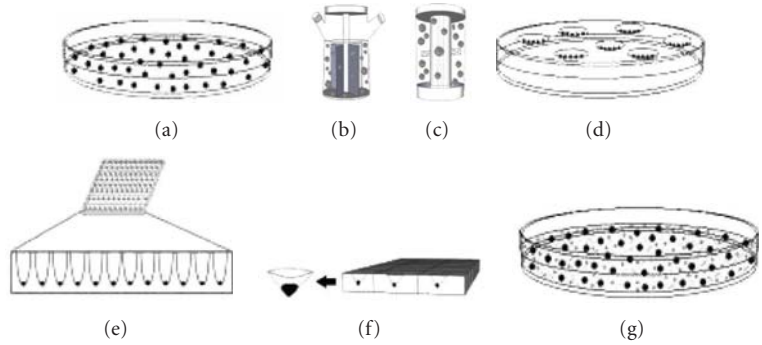

Figure 1: Schematic representation of methods to form embryoid bodies from pluripotent stem cells includes (a) liquid suspension culture, (b) stirred flask culture, (c) rotary cell culture systems, (d) hanging drop culture, (e) low adhesion U-bottom multiwall plates, (f) indented solid microsurfaces, and (g) hydrogel culture systems.

for the purpose of creating suspension cellular aggregates [26]. Though these surface treatments prevent the cell attachment to the tissue culture vessel resulting in aggregation, initial seeding densities need to be optimized in order to facilitate the cell-cell interactions required to form appropriately sized EBs. Seeding densities that are too low result in poor aggregation thus resulting in poorly established EB cultures. Thus, typical seeding densities are kept quite high during the initial aggregation (minimum of 10^6 cells per 2 mL media in a 50 mm plate [16] or 6-well plate well). In the case of pluripotent hESC and hiPSC cultures, it is often desirable to facilitate the initial aggregation step by transferring isolated sections of colonies to low attachment vessels, since these cultures tend to be negatively effected by being in single cell suspension resulting in poor viability by apoptosis [35]. Once formed, suspension EBs cultures are typically cultured in the low attachment vessel for an extended period of time, typically from 28 to 35 days with fresh medium exchange every 2-3 days, though longer culture times may be desirable. Medium conditions have been demonstrated to have an important influence on the viability and germ layer differentiation of the suspended EBs. For instance, studies have shown that culture of EBs in a physiological glucose concentration (5.5 mM as opposed to 25 mM high glucose formulations typical of hESC and hiPSC expansion medium) in the presence of basic fibroblast growth factor (bFGF) prolonged the viability and increased the complexity of tissues in the EBs showing that representatives of all three germ layers in cultures could be maintained up to 105 days [36].

Though static suspension cultures of EBs are the most widely used due to their simplicity and the minimal cost of low-attachment treated standard vessels (dishes and multiwell plates), recent advances in bioreactor suspension culture have demonstrated usefulness of these techniques to optimize, standardize, and scale up the formation of EBs [17, 28, 29]. Unlike static suspension cultures, these systems require specialized culture equipment such as stirred flasks or the more recent Rotating Cell Culture Systems (RCCSs), developed by NASA, either the Slow Turning Lateral Vessel (STLV) or the larger capacity High Aspect Rotating Vessel (HARV) [37–39]. Typical stirred flask methodologies involve seeding hESC or hiPSC suspensions in a specialized flask utilizing a magnetic stirring bar to continually rotate the culture in order to facilitate aggregation of the cells into sustained cellular aggregates and provide better gas exchange than static systems [27] (Figure 1(b)). This method has been used to derive EBs and is amendable to scale up for larger yields [27]. Unlike these stirred flask suspension systems which allow for

the EBs to be agitated over culture time allowing for greater gas exchange, less hypoxia, and less agglomeration than the static suspension system [27], the RCCS systems greatly reduce shear forces, present in the stirred flask systems, which can greatly damage the EBs [40, 41]. Often termed "microgravity" cell culture, RCCS systems allow for the continuous horizontal rotation resulting in very low shear stress on the cells, active membrane-based gas diffusion to prevent hypoxia and to equally distribute both oxygen and expiration waste gas, and can partially control EB size by regulating rotation speed and initial seeding densities (Figure 1(c)). Using these RCCS systems, the STLV in particular with its high membrane surface area to medium volume compared to the larger HARV, it has been demonstrated that EBs cultured in this fashion demonstrated higher viability, more complex tissue differentiation, and, in a specific example of neural induction, enhancement of neural progenitor differentiation [42].

4.2. Homogenous Methods of Embryoid Body Formation. As opposed to the heterogeneous methods of EB formation that result in representatives of the three germ layers in a stochastic culture, it is frequently the case that more controlled EB formation methods for increased reproducibility and size control can facilitate the differentiation towards specific tissue types. In particular, control of EB size has been demonstrated to influence viability, proliferation, and differentiation potential [26] to cardiomyocytes [22, 25, 30, 43], endothelial tissue [43], as well as instruct hematopoiesis [19].

Several methods have been developed in order to form EBs of defined size. These methods share in their methodology ways to segregate a defined number of cells in order to allow them to aggregate before being collected for further culture. The hanging drop method utilizes 20–25 μL drops containing a defined number of cells (typically 1000–10000) in single cell suspension [13, 30, 31]. The drops are placed onto the underside of a flipped 100 mm tissue culture plate lid, typically a maximum of 96 drops if using a multichannel pipette to place the drops (Figure 1(d)). Once placed, the lid is carefully flipped allowing the drops to remain attached to the lid and the lid is placed over a buffer-filled plate to prevent evaporation of the hanging drops. This technique is limited in the upper size limit of the initial cell aggregate due to the limited volume of the hanging drop required to allow fluid tension to adhere the drop to the lid (20 to 25 μL). Initial formation of the cellular aggregates typically takes 1 to 2 days, after which the EBs are individually collected manually and placed in low-adhesion plates for further culture and maturation. A variation on the hanging drop using round bottom ultra-low attachment-treated multiwall plates has also been developed which allows for the derivation of larger EBs than the hanging drop method, in that larger amounts of cells can be placed into each well (i.e., 100–200 μL of cell suspension per well in 96-well round-bottom plates) (Figure 1(e)) [14]. More recently, using a silicon wafer-based microfabrication technology containing hundreds or thousands of micrometer sized wells per cm^2 adhered to the well bottoms of a standard multiwall plate, studies have demonstrated the ability to form large numbers of uniform and synchronized human EBs of defined size [32] in a commercially available format (AggreWell^{TM}, Stem Cell Technologies) (Figure 1(f)). Though these techniques allow for the controlled aggregation of hESCs and hiPSCs, both require the formation of single cell suspensions exposing the cells to low viability and poor EB formation and, in the case of the plate based systems, centrifugation in order to force settle the cells into the bottom of the wells. It is common, therefore, that protective agents, such as the addition of Rho-associated kinase (ROCK) inhibitor, Y-27632, are required for these methods [35].

4.3. Other Embryoid Body Formation Methodologies. The EB formation methods discussed previously represent the most commonly used for the purpose of assessing hESC and hiPSC pluripotency *in vitro*. Other more specific approaches exist for niche applications such as encapsulation techniques utilizing hydrogels such as methylcellulose [15, 33] or hyaluronic acid (HA) [34]. These techniques allow for the entrapment of single cells in suspension and subsequent growth of cellular aggregates (Figure 1(g)). These techniques have been mostly utilized for more specific goals than pluripotency assessment such as when cell clusters derived from single-cell clones are desirable. These techniques often suffer from very low yields [15] due to the intrinsic instability of prolonged single cell culture of the pluripotent stem cells as well as complicating the isolation of EBs from the hydrogel for subsequent downstream analyses.

5. Analysis of Germ Layer Formation in hiPSC-Derived Embryoid Bodies

In order to assess the pluripotency of hiPSCs by the *in vitro* method of deriving EBs, it is imperative to have definitive downstream assays that demonstrate the ability to form representatives of the three developmental germ layers, thus demonstrating their increased differentiation potency upon reprogramming. There are several methods to show this that range from less stringent (showing expression of germ layer-specific genes) to the more stringent and definitive demonstration of tissue-similar structures (histology and immunohistochemistry) that resemble early embryonic development along with the concomitant expression of markers. This section will briefly review the typical requirements for such analyses.

5.1. Expression of Germ Layer-Specific Genes. Perhaps the least stringent test of pluripotency in EBs is the demonstration of germ layer-specific gene expression by biochemical means. The reason for the lower stringency is that this only represents the ability to detect such gene expression without knowledge of higher-order structural or temporal expression within organized structures which can be discerned by more extensive histological and immunohistochemical examination (see the following). However, it is clear that the input cells, often fibroblasts in the case of their derivation toward hiPSCs, do not express these genes and can be used as an indicator of acquired tissue specific expression [61] (i.e., neural-specific genes being expressed). Gene expression analysis

Table 2: Typical markers for the analysis of human embryoid bodies.

	Marker name	Alternate name	Marker type	Suitable marker for gene expression	Suitable marker for IHC	Reference
Pluripotency						
	Oct4 (POU5F1)	Octamer-binding transcription factor 4	Transcription factor	Yes	Yes	[44]
	Nanog	n/a	Transcription factor	Yes	Yes	[45]
	REX-1	Zinc finger protein 42 homolog (*ZFP*42)	Transcription factor	Yes	Yes	[2]
	SOX-2	SRY (sex determining region Y)-box 2	Transcription factor	Yes	Yes	[46]
	SSEA-3	Stage-specific embryonic antigen 3 (SSEA-3)	Surface antigen	No	Yes	[47]
	SSEA-4	Stage-specific embryonic antigen 3 (SSEA-4)	Surface antigen	No	Yes	[47]
	Tra-I-60	n/a	Surface antigen	No	Yes	[48]
	Tra-I-81	n/a	Surface antigen	No	Yes	[48]
Ectoderm						
	GFAP	Glial fibrillary acidic protein	Glial intermediate filament	Yes	Yes	[49]
	Nestin	n/a	Intermediate filament	Yes	Yes	[49]
	Pax-6	Paired box gene 6	Transcription factor	Yes	Yes	[50]
	Sox-1	Sex determining region Y-box 1	Transcription factor	Yes	Yes	[51]
Endoderm						
	AFP	Alpha-fetoprotein	Plasma Protein	Yes	No	[52]
	Amylase	Alpha-amylase	Metabolic Enzyme	Yes	Yes	[53]
	FOXA2	Hepatocyte nuclear factor 3-beta (HNF-3B)	Transcription Factor	Yes	Yes	[54]
	PDX1	Pancreatic and duodenal homeobox 1, insulin promoter factor 1	Transcription factor	Yes	Yes	[55]
	GATA4	GATA binding protein 4	Transcription factor	Yes	Yes	[56]
Mesoderm						
	Brachyury	n/a	Transcription factor	Yes	Yes	[57]
	CD34	Cluster of differentiation molecule 34	Surface antigen	No	Yes	[58]
	FLT1	Vascular endothelial growth factor receptor 1	Surface receptor	Yes	Yes	[59]
	RUNX1	AML1, Runt-related transcription factor 1	Transcription factor	Yes	Yes	[60]

is also often used to demonstrate the lack of pluripotency genes upon EB differentiation, thus demonstrating the completeness of differentiation. This is of particular concern with hiPSCs, in order to demonstrate that the factors used to reprogram in the case of integrating retroviruses are in fact silenced [62]. There are several pluripotent and germ layer-specific markers used for the purpose of ascertaining differentiation within EBs (Table 2).

5.2. Histological Analysis of Embryoid Bodies. Though gene expression analysis can be used to identify the presence of germ layer-specific markers and the concomitant lose of pluripotent markers, a more definitive assessment of tissue differentiation is based on the histological evaluation of sectioned EBs followed by assessment of tissue organization, cellular morphology, and localized protein expression [16, 28, 29, 36, 63]. Typically, EBs are first pelleted and embedded (i.e., low-melting point agarose) in order to concentrate the EBs for subsequent paraffin embedding and sectioning for mounting on microscope slides [63]. These slide-mounted sections are then stained with hematoxylin-eosin (H&E) in order to enhance contrast between the tissues and cells (Figure 2). Using these techniques, various tissues can be commonly recognized such as neural rosettes (ectoderm-Figures 2(a), 2(d), 2(g), and 2(j)), connective tissue (mesoderm-Figures 2(b), 2(e), 2(h), and 2(k)), and putative endoderm (Figures 2(c), 2(f), 2(i), and 2(l)). Though this technique is commonly used for both EBs and teratomas to determine the presence of germ layer representatives once their histomorphologies are identified, the assessment can

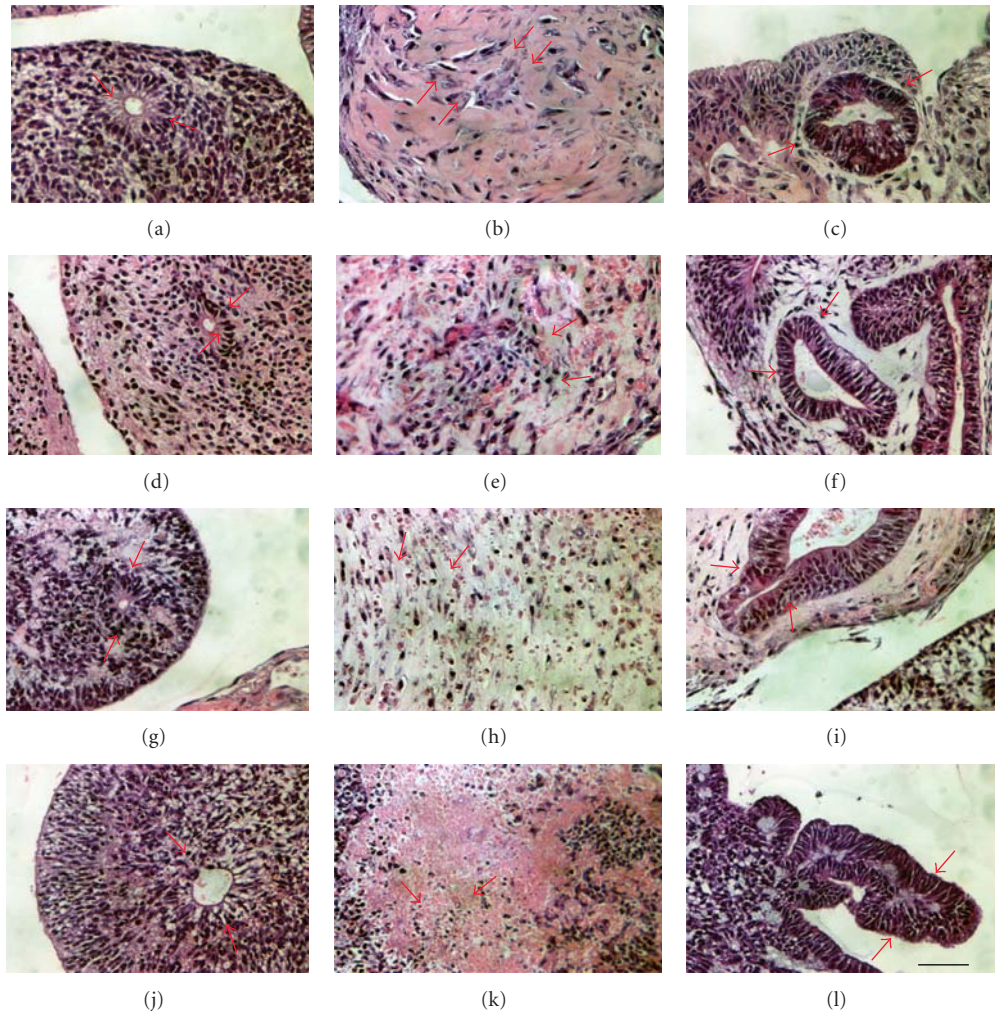

Figure 2: Histological evidence of germ layer differentiation in embryoid bodies generated from human pluripotent stem cells cultured by different methods to illustrate equivalence of culture techniques [63]. Shown are images of hematoxylin and eosin-stained histologic sections of EBs from hESCs propagated directly on mouse embryonic fibroblast (MEF) feeder layer (top row, a–c) or hESCs propagated in indirect coculture with MEFs (second row, d–f), hiPSCs propagated on MEFs (third row, g–i), or hiPSCs propagated in indirect coculture with MEFs (bottom row, j–l). Equivalent trilineage potential is demonstrated by presence of ectodermal (neuroepithelial) (a, d, g, and j); mesodermal (fibrous connective) (b, e, h, and k), and endodermal (intestinal) (c, f, i, and l) differentiation in these EBs. Arrows point to the corresponding tissue in each figure. Magnification is 400x total (10x ocular, 40x objective). Each scale bar represents 50 μm in length.

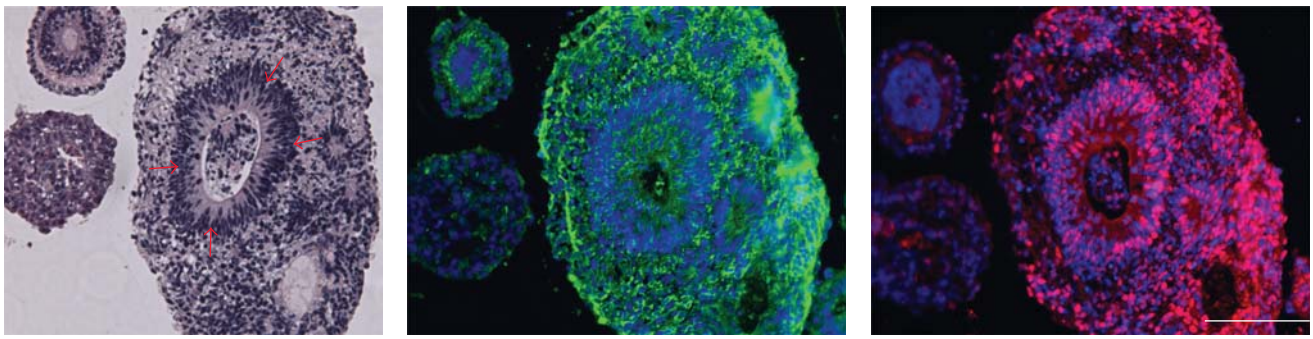

Figure 3: Immunohistochemical analysis of embryoid body sections confirming neuroepithelial tissue in adjacent sections, hematoxylin, and eosin-stained neural rosettes in hiPSCs-derived EBs (a), antinestin immunofluorescent staining (b), green-nestin, blue-nuclei stain, and anti-Sox2 immunofluorescent staining (c), red-Sox2, blue-nuclei stain. Magnification is 400x total (10x ocular, 40x objective). Each scale bar represents 50 μm in length.

be subjective and may require further immunohistochemical analysis in order to confirm the interpretation using specific antibodies (Table 2 and Figure 3) in sections adjacent to H&E stained sections. This is particularly true of putative endoderm, which is less prevalent and typically less mature in embryoid bodies than the more straightforward identification of neural and connective tissues.

6. Conclusions

Although hiPSCs reprogrammed from human somatic cells have been well documented as a source of pluripotent stem cells with numerous shared similarities with hESCs, the increasing establishment of many new hiPSC lines requires the use of multiple assays and extensive resources to demonstrate their pluripotency on a line-by-line basis. Key among these is assays to demonstrate the potency towards formation of the three developmental germ layers and subsequent derivation of specific differentiated cell types in order to demonstrate their therapeutic potential. Recently, there has been enormous debate in the international stem cell community on the feasibility and use of the *in vivo* teratoma assay to demonstrate the pluripotency of derived hiPSCs [4, 6, 8–11]. Our paper proposes the embryoid body (EB) assay as a useful *in vitro*, cost-effective alternate to demonstrate the differentiation potential of derived hiPSCs. Methods for the generation of EBs and subsequent biochemical and histological analysis have gone through steady and tremendous improvements which permits its use across different laboratories. Further refinement and automation of the proposed methodologies provides opportunities for applications of the EB assay as a gold standard for assessing pluripotency of generated hiPSCs.

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Review Article

Prospect of Induced Pluripotent Stem Cell Genetic Repair to Cure Genetic Diseases

Jeanne Adiwinata Pawitan

Department of Histology, Faculty of Medicine, Universitas Indonesia, Jakarta 10430, Indonesia

Correspondence should be addressed to Jeanne Adiwinata Pawitan, jeanneadiwip@fk.ui.ac.id

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In genetic diseases, where the cells are already damaged, the damaged cells can be replaced by new normal cells, which can be differentiated from iPSC. To avoid immune rejection, iPSC from the patient's own cell can be developed. However, iPSC from the patients's cell harbors the same genetic aberration. Therefore, before differentiating the iPSCs into required cells, genetic repair should be done. This review discusses the various technologies to repair the genetic aberration in patient-derived iPSC, or to prevent the genetic aberration to cause further damage in the iPSC-derived cells, such as Zn finger and TALE nuclease genetic editing, RNA interference technology, exon skipping, and gene transfer method. In addition, the challenges in using the iPSC and the strategies to manage the hurdles are addressed.

1. Introduction

Since the first generation of induced pluripotent stem cell (iPSC) from mouse adult fibroblast using four inducing factors by Takahashi and Yamanaka in 2006 [1], followed by the generation of human iPSC [2], various vectors to introduce various inducing factors have been published, and various combinations of the inducing factors, in the form of transcription factors or microRNA, were used. Further, there are chemical compounds, for example, butyrate that may enhance the inducing capacity of the transcription factors [3], so that Oct 4 alone is enough to induce somatic cells into iPSC [4].

Further, various patient-derived iPSCs were developed that may be used to reveal the pathogenesis of various genetic diseases. These genetic abnormality-harboring iPSCs may be repaired, and the genetically repaired iPSC may be differentiated into normal required cells [5]. In the future, these patient-derived normal cells may be used to a patient-tailored therapy to replace the damaged cells due to the disease.

To date, iPSCs for various genetic diseases have been developed, such as for certain type of Parkinson's disease [5], spinal muscular atrophy [6], lentigines, electrocardiographic abnormalities, ocular hypertelorism, pulmonary valve stenosis, abnormal genitalia, retardation of growth, and deafness (LEOPARD) syndrome [7], long Q-T syndrome [8], Timothy syndrome [9], Hurler syndrome [10], epidermolysis bullosa [11], and thalassemia [12].

The iPSC resembles embryonic stem cell in the differentiation capacity into various kinds of cells and in inducing teratoma in laboratory animal [1]. However, various researches have shown that iPSC is not identical to embryonic stem cell. Moreover, various aberrations, which may arise during induction or subsequent propagation, pose challenges in the use of iPSC for the cure of genetic diseases.

Therefore, this review discusses the prospect of iPSCs to cure genetic disease, in term of the efficient methods for genetic repair that may be used to repair genetic disease-harboring iPSCs, and the challenges that should be resolved when iPSCs are to be used to cure genetic diseases.

2. Methods for Genetic Repair

To date, there are several efficient methods for genetic repair of genetic diseases, that is, zinc finger and transcription activator-like effector (TALE) nuclease method, RNA

interference (RNAi), exon skipping technology, and gene transfer. However, when the cells are already damaged, they should be replaced by new normal cells, which can be differentiated from iPSC. Those methods may be used to repair the genetic disease-harboring cells that may be done either in the somatic cells before induction to pluripotency [13], or somatic cell derived iPSC [5].

2.1. Zinc Finger Nuclease Method. The zinc finger nuclease method is one of the efficient genetic editing methods. A Zn finger nuclease consists of a Zn finger domain and FokI endonuclease. The Zn finger domain contains Zn finger motifs that recognize and bind to a specific DNA sequence. The FokI endonuclease works as a dimer to cause a double-strand break (DSB) in the DNA. Therefore, Zn finger nucleases should work in pairs. One of the Zn finger motifs recognizes and binds to the sequence up stream and the other to the sequence down stream to the site to be cleaved by the endonuclease (Figure 1). Principally, a certain Zn finger nuclease can be engineered to recognize any specific sequence and to cause a DSB at any specific site. The DSB is then repaired by homologous recombination, which is facilitated by the presence of exogenous donor DNA homologous to the sequence to be repaired, or by error-prone nonhomologous end joining [14, 15]. To deliver the Zn finger nucleases into a cell, an expression vector containing the Zn finger nucleases can be engineered. The results of this genetic editing may be either mutation repair or insertion of a certain DNA sequence, when a certain exogenous donor DNA is used, or error prone repair when no donor DNA is used, or deletion when two pairs of Zn finger nucleases are used and causing 2 DSB [15]. Therefore, this method may be used to correct a mutation, or to insert or delete a certain DNA sequence (Figure 2).

A study used exogenous donor DNA that was packed in a double-stranded plasmid, or in the form of a single-strand oligodeoxynucleotide. This method was successfully used to repair a point mutation A53T (G209) in α-synuclein gene in a Parkinson's disease patient-derived iPSC. Further, the repaired iPSC was successfully differentiated into functional dopaminergic neurons [5].

A drawback of this method is off target DSB due to homodimerization, which may cause undesired mutation or cytotoxicity. Therefore, genome-wide putative-off target mutation assay should be performed, to ascertain that there is no off-target mutation in the genetically repaired iPSC [5]. Recently, to reduce off-target DSB, engineered FokI nuclease that cannot form a homodimer was developed. This method showed that the obligate FokI heterodimer greatly reduces the off target DSB [16, 17].

2.2. TALE Nuclease Method. Transcription activator-like effectors from a plant pathogen, the *Xanthomonas sp.*, are sequence-specific DNA-binding proteins. As Zn fingers, TALEs can be engineered to bind to any specific sequence, and linked to a FokI nuclease to work in pair and cleave the sequence [18, 19]. This method was tested in iPS and showed that TALE nuclease mediated site-specific genetic

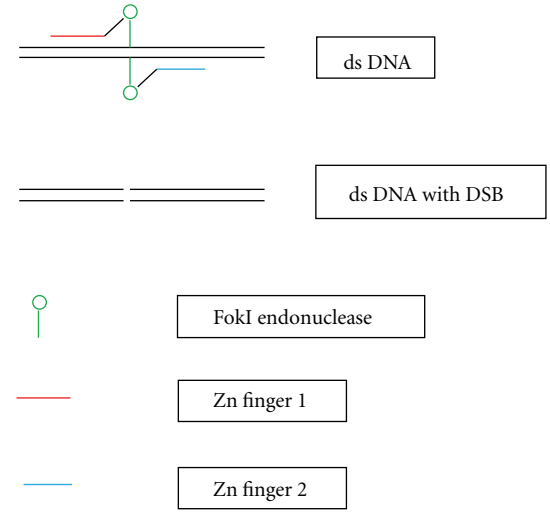

Figure 1: Generation of a double strand break by zinc finger nucleases, ds: double strand, DSB: double strand break, Zn: zinc.

modification with similar precision and efficiency as Zn finger nuclease [20], but with lower degree of off target activity and cytotoxicity [21].

2.3. RNA Interference (RNAi) Technology. RNA interference involves micro- (mi-)RNA and small interfering (si)RNA, which, upon base-pairing to their target sequence in a certain mRNA, cause degradation or prevent translation of the mRNA [22]. This method may be useful to suppress the expression of a toxic mutant allele that causes the symptoms of a certain genetic disease. However, this method does not repair the underlying genetic aberration. Therefore, to suppress the expression of the mutant allele in a genetically abnormal iPSC, a method to continuously deliver the interfering RNA is needed.

Various expression systems for either miRNA or siRNA have been developed using various vectors and promoters [23–27]. The expression system for siRNA involves the formation of short hairpin (sh)RNA before the formation of a double-strand functional siRNA, while that for miRNA involves the formation of primary miRNA transcripts, followed by the formation of pre-miRNA, and finally a functional mature miRNA [23, 24].

However, the use of strong promoter results in high level expression of miRNA or shRNA that may lead to cytotoxicity [28–30]. Cytotoxicity of siRNA expression system may be due to competition of the artificial with the natural RNA interference system and lead to disruption of the natural system [31], or off-target silencing [32], possibly due to miRNA-like binding of siRNA at the 3′ UTR region [33]. In addition, shRNA or the viral vector may induce cellular interferon response that leads to universal silencing [34, 35].

Comparison between miRNA and siRNA expression system showed that siRNA was more potent [36], but miRNA expression system was safer [31, 37, 38]. Cytotoxicity and off target effect of siRNA can be reduced by reducing the siRNA concentration [33]. Therefore, using less potent promoter

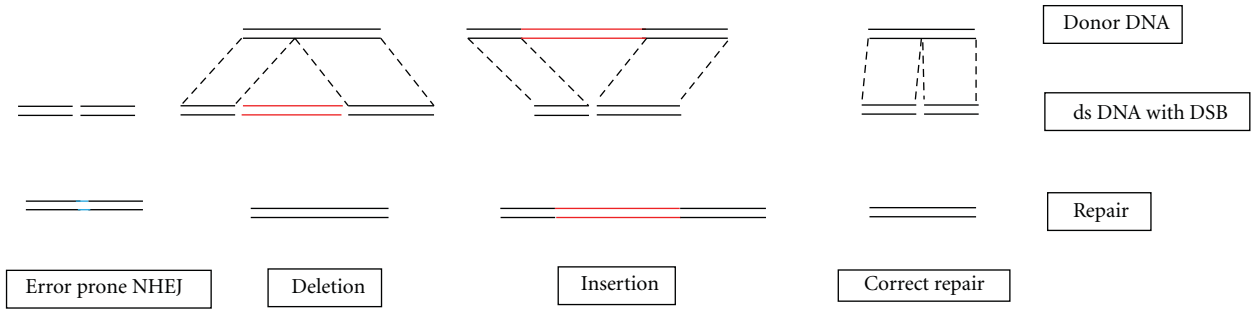

Figure 2: Possibilities of genetic repair using zinc finger nucleases, ds: double-strand, DSB: double-strand break, NHEJ: non-homologous end joining.

in siRNA expression system may resolve the problem. Alternatively, engineering a single nucleotide bulge in the siRNA expression system may overcome the problem [39].

This RNA interference technology was proven useful to suppress the expression of a mutant allele in Alzheimer's disease in cell culture [40], spinocerebellar ataxia [23], Huntington's disease [41], and amyotrophic lateral sclerosis, in animal models [42, 43].

2.4. Exon Skipping Technology. Exon skipping technology causes deletion of selected exon(s) by targeting a sequence in the adjacent intron using an antisense oligonucleotide. This method can be used in genetic aberration where there is a mutation that causes a frameshift or a stop in the mRNA, and deletion of one/several frame-shifted exon(s) leads to a shorter, but still functional protein, for example, dystrophin in Duchene muscular dystrophy (DMD) [44].

However, the use of exogenous antisense oligonucleotide to cause exon skipping in iPSC needs continuous supply of the antisense oligonucleotide. Therefore, to repair a genetic aberration in iPSC, an expression vector needs to be engineered.

A study on DMD mouse model used an expression vector to deliver the antisense oligonucleotide by a single-dose injection into skeletal muscle. The expression vector was engineered using AAV-2-based vector combined to a modified U7 small nuclear (sn)RNA, which was linked to the antisense sequence to both flanking intron of the exon(s) to be deleted. The U7 snRNA guides the antisense sequence to the proper subcellular site and facilitates splicing and exon skipping. The study showed a sustained production of functional dystrophin and correction of the muscular dystrophy [44]. Another study used the same method but administered the expression vector into the hippocampus and showed normalized synaptic plasticity [45].

2.5. Gene Transfer Method. Gene transfer method may be useful in genetic diseases where there is genetic aberration that causes the absence of expression of a certain gene, such as in tyrosinemia type 1 due to fumarylacetoacetate hydrolase (FAH) deficiency [46], β thalassemia [12], and Fanconi anemia [13]. A study has developed an iPSC from an FAH deficient mouse, corrected the genetic aberration by transduction of FAH cDNA using lentiviral vector, and successfully generated healthy mice from the corrected iPSC [46].

3. Challenges in Using iPSCs

As iPSC resembles embryonic stem cell in teratoma-inducing capacity, and the detection of various genetic and epigenetic aberrations, caution should be paid to solve these problems. Moreover, delivery route and the high cost in this patient-tailored therapy may pose other problems. Further, there is still a question of which cell type should be differentiated and transplanted, whether the differentiated iPSCs can integrate and cooperate with other cells in the target site, and whether the differentiated iPSC is not rejected by the immune system. Finally, safety issues concerning the use of genetically repaired iPSC need to be considered.

3.1. Teratoma-Inducing Capacity. Theoretically, iPSC can be differentiated into any required cells. A study on human embryonic stem cells used multiple passages under differentiation-inducing condition to eliminate residual tumor-forming cells and proved the absence of pluripotent cells using Oct3/4 marker, and by grafting the differentiated cells in rats [47]. However, human iPSC line-derived dopaminergic neuron progenitor transplantation in a rat model showed the presence of Nestin-positive tumor-like cells at the site of transplantation [48].

Therefore, to prevent tumor formation in patients receiving transplantation of differentiated iPSC, it should be ascertained that the differentiated cells are free from residual pluripotent iPSC, and methods should be developed to purify the differentiated iPSC and to check the absence of tumorigenic potential in the desired cells.

3.2. Genetic and Epigenetic Aberrations. A recent study showed that there were variations in the copy number of certain genes in the form of duplications and deletions, in human iPSC [5, 49–51]. Another study showed that there were point mutations in certain somatic protein-coding genes [52].

Apart from teratoma-inducing capacity of iPSC, copy number variation may be present in the form of amplification of oncogenes or deletion of tumor suppressor genes that may lead to tumor formation. Moreover, point mutation may lead to either up regulation or down-regulation of certain important genes other than tumor-related genes. Therefore, caution is warranted before iPSC-derived cells are used in therapy. Substantial genetic abnormalities may be observed by chromosomal analysis, but subtle changes need more careful examination.

An epigenetic study showed aberrant DNA methylation of certain single bases in human iPSC [53]. Moreover, comparison of human iPSC developed from various types of cells representative of ectoderm, mesoderm, and endoderm revealed retention of transcription memory of the original cells due to incomplete promoter DNA methylation [54]. A study on DNA methylation and transcription profiles of 20 different human embryonic stem cell and 12 iPSC lines showed large variations [55]. Analysis of methylation and transcription profiles [54], and transcription and expression profiles enable the prediction of a cell line efficiency to be differentiated into a certain required cell type [56].

Therefore, before attempts to induce iPSC from a patient, determination of the iPSC-derived cells that are required to replace damaged cells may be useful in choosing the cell source for iPSC induction. Using a cell from the same germ layer as the desired differentiated cell is an advantage.

3.3. Delivery Route. Delivery route depends on the cells, tissue, or organ to be repaired, which may not be the same for all diseases. For some diseases, the target site may be difficult to reach, for example, Alzheimer, Parkinson's, and other neurological disease, where the target site is inside the brain. Attempts to deliver cells into the brain have been done and showed variable results [57, 58]. Therefore, the simplest way is intravenous delivery. However, it is still a question whether the cells home to the intended target site.

A study on intravenous injection of either bone marrow mesenchymal stem cells or epidermal neural crest stem cells in an animal model showed that both types of cells homed into inflamed corpus callosum. The result of the study suggests that intravenous delivery may work on neurodegenerative diseases where inflammation is present on the target site [59].

Therefore, for each genetic disease, studies are highly needed to identify the most effective, efficient, and safe route of delivery.

3.4. High Cost in Developing a Patient-Tailored Therapy. The possibility to repair genetic defect in patient-derived iPSC may lead to individual patient-tailored therapy for genetic diseases. This approach has an advantage compared to allogenic cell therapy, as no immunosuppressive regiment is required, though a study showed that autologous iPSC may elicit immune response [60]. However, the presence of genetic abnormality in various iPSCs necessitates careful screening to ensure the safety of iPSC-derived differentiated cells. To date, technologies to check subtle abnormalities, such as copy number variation and genome wide mutation analysis, are available, though they need a high cost. Therefore, for each genetic disease, development of efficient, effective, and economical method to screen and check the safety of the desired cells is highly needed.

3.5. Type of Cell to Be Transplanted and Integration into the Target Site. Requirement of cell type depends on the damaged cells due to the genetic disease. The option is whether to use fully or partly differentiated iPSC. Transplantation of partly differentiated iPSC is intended to resume the differentiation *in vivo* into the mature desired cells.

A study showed that *in vitro* differentiated murine iPSC-derived neurons functionally integrated into the brain and alleviated the symptoms in Parkinson's rat model [61]. Another study on embryonic stem-cell-derived neural stem cells that were transplanted in the putamen of a Parkinson's disease animal model showed that the transplanted cells differentiated *in vivo* into functional dopaminergic neurons [62].

Therefore, studies are required for each genetic disease to determine which cell type and degree of differentiation give the best result in term of cell function, integration, and cooperation with surrounding cells, which finally alleviate the symptoms.

3.6. Immune Rejection Problem. Using patient's own cells to provide iPSC-derived cells is believed to handle immune rejection problems. However, a recent study showed that even the patient's own iPSC may induce immune rejection [60]. Moreover, when viral vectors are used to engineer the expression vector for the various methods for genetic repairs or to repress the symptoms, immune rejection may be developed towards the viral vectors. A study showed that viral vectors induced adaptive immune response *in vivo* [63], which leads to inflammatory responses [64]. In addition, innate immune rejection may be developed towards the RNA in case exon skipping or RNA interference method is used. Several studies showed interferon production due to RNA-expressing vectors *in vitro* [34, 35], which can be overcome by reducing the length of RNA to below 21-mers [35].

Although in iPSC genetic repair, the expression vector is transduced into the iPSC, and the presence in blood or tissue may be minimal to be able to induce immune response, studies are highly needed to find a method to cope with immune rejection problems and to ascertain that the corrected iPSC is really safe and will not be rejected.

3.7. Safety Issues in Using Genetically Repaired iPSC. Most of the methods of genetic repair, which may be used to repair patient-derived iPSC, use viral vectors as expression vectors, such as lentiviral-based vector in gene transfer technology [13], TALE nuclease genetic editing [18], RNAi technology [24, 25], or AAV-based vector in RNAi technology [29] and exon skipping technology [44, 45].

Viral vectors especially lentiviral-based vectors are known to cause side effects that range from immortalization to clonal dominance *in vitro*, and oncogenesis *in vivo*, due to

integration of the vector into host genome. The integration or insertional mutagenesis activates the expression of a protooncogene or cancer promoting genes near the integration site [65].

Therefore, it is very important to address the clinical safety of the vectors. This purpose can be achieved by deletion of promoter element in the viral long terminal repeat (LTR), which is termed self-inactivating (SIN) LTR, which may significantly decrease cellular transformation *in vitro*. Another approach to reduce oncogenesis is by insertion of an insulator element into the LTR [65]. Thus, vector design and safety assessment of the vector before constructing an expression vector for the purpose of genetically repairing iPSC is of high importance.

4. Conclusion

Induced pluripotent stem cells are very promising as the source of the required cells to replace the damaged cells in various genetic diseases. However, further studies are needed to resolve the various challenges.

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