STEM CELLS TRANSLATIONAL MEDICINE®

PLURIPOTENT STEM CELLS

# Derivation and Long-Term Culture of Transgene-Free Human Induced Pluripotent Stem Cells on Synthetic Substrates

LUIS GERARDO VILLA-DIAZ,[a,b] JIN KOO KIM,[a,b] JOERG LAHANN,[b,c] PAUL H. KREBSBACH[a,b]

**Key Words.** Induced pluripotent stem cells • Synthetic coating • Stem cells • Regenerative medicine • iPSC • Differentiation • Feeder-free culture

[a]Department of Biologic and Materials Sciences, [b]Biointerfaces Institute, and [c]Department of Chemical Engineering, University of Michigan, Ann Arbor, Michigan, USA

Correspondence: Paul H. Krebsbach, D.D.S., Ph.D., Department of Biologic and Materials Sciences, University of Michigan, 1011 North University Avenue, Ann Arbor, Michigan 48109, USA. Telephone: 734-936-2600; E-Mail: paulk@umich.edu

Received April 21, 2014; accepted for publication September 12, 2014; first published online in SCTM *EXPRESS* October 13, 2014.


1066-5099/2014/$20.00/0

http://dx.doi.org/10.5966/sctm.2014-0087

## ABSTRACT

**We describe a platform to derive, culture, and differentiate genomically stable, transgene-free human induced pluripotent stem cells (iPSCs) on a fully synthetic polymer substrate made of a grafted zwitterionic hydrogel: poly2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide (PMEDSAH). Three independent transgene-free iPSC lines derived in these conditions demonstrated continuous self-renewal, genomic stability, and pluripotency in vitro and in vivo after up to 9 months of continuous in vitro culture on PMEDSAH-grafted plates. Together, these data demonstrate the strength this alternative platform offers to generate and maintain human iPSCs for regenerative medicine.** STEM CELLS TRANSLATIONAL MEDICINE *2014;3:1410–1417*

## INTRODUCTION

The indefinite self-renewal and pluripotency capacity featured by human induced pluripotent stem cells (iPSCs) makes them a potential source of cells for regenerative medicine, drug discovery, disease modeling, and detailed study of self-renewal and differentiation. Since the first report demonstrating the derivation of human iPSCs [1], it was recognized that safety issues would need to be resolved for these cells to reach their full potential. The initial focus toward this goal was to avoid genomic integration of the overexpressed transgenes (*OCT4* [also known as *POU5F1*], *SOX2*, *KLF4*, and *c-MYC*) in the resulting iPSC lines to limit the inherent risk of tumor formation. Since then, several methods [2–7] have been developed to generate transgene-free human iPSCs.

To achieve appropriate safety profiles for human applications, the microenvironment in which human iPSCs are derived and cultured must also be considered. Historically, the culture conditions for human iPSCs have followed methods developed for human embryonic stem cells (ESCs), which in turn were adapted from the culture of mouse ESCs [8]. Consequently, the majority of human iPSC lines have been derived and cultured in the presence of mouse embryonic fibroblasts, human fibroblasts, or autologously derived fibroblasts. However, these commonly used culture conditions encompass biosafety risks such as xenogeneic contamination by nonhuman sialic acids [9], mycoplasma, and viral and nonviral infectious agents and may be immunologically incompatible with human use [10].

Feeder-free systems have been implemented for culturing human PSCs using extracellular matrix proteins derived from either animal sources such as Matrigel [11] or synthetically produced proteins such as human recombinant (hr) laminin [12], E-cadherin [13], and vitronectin [14]. Recently, the derivation of human iPSCs on hr vitronectin [14] and hr laminins 511 fraction E8 [15] and 521 [16] was reported, demonstrating the feasibility of generating xenogeneic-free pluripotent stem cells in feeder-free conditions. Nevertheless, the use of recombinant proteins as a substrate for the derivation and long-term and large-scale culture of human iPSCs is not cost-effective. As an alternative, synthetic coatings have been developed to support growth of human ESCs and human iPSCs while maintaining pluripotency [8]. These synthetic substrates are superior to biological substrates with respect to cost effectiveness, stability, consistency, and ease of sterilization. Thus, the use of fully synthetic substrates for derivation of human iPSCs in xenogeneic-free and feeder-free conditions could represent an ideal culture system.

Here, we describe the derivation and long-term culture of human iPSC lines using a polymer-based substrate consisting of a grafted zwitterionic hydrogel: poly2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide (PMEDSAH), which is extremely effective in sustaining the long-term feeder-free culture of human ESCs [17] and iPSCs [18]. Three independent human iPSC lines were derived and continuously cultured for 9 months on this chemically defined substrate.

Rigorous characterization of these cell lines demonstrated a fully reprogrammed character, in vitro and in vivo differentiation potential, as well as genomic stability.

## Materials and Methods

### Cell Culture

Human gingival fibroblasts (hGF) were derived with approval of the University of Michigan Institutional Review Board. The derivation procedure followed a protocol detailed previously [19]. The two hGF cell lines labeled as hGF(1) and hGF(2) and human foreskin fibroblasts (hFFs; American Type Culture Collection, Manassas, VA, http://www.atcc.org) were cultured in dedicated incubators in high humidity at 37°C and 5% $CO_2$, with 10% fetal bovine serum (vol/vol) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, http://www.invitrogen.com) and 1× antibiotic/antimycotic solution (Invitrogen). This culture medium was changed during the reprogramming cycle and expansion of human iPSC colonies to human cell conditioned medium (hCCM; GlobalStem, Gaithersburg, MD, http://www.globalstem.com) supplemented with 4 ng/ml human recombinant basic fibroblast growth factor (GlobalStem), which is herein referred to as hCCM+.

PMEDSAH-grafted plates (GPs) were used as a synthetic substrate during the reprogramming cycle and for the long-term culture of the derived human iPSC lines. The preparation of PMEDSAH-GPs has been detailed previously [20]. Briefly, the grafting was performed on tissue culture polystyrene (TCPS) plates (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com), and polymers were obtained from Sigma-Aldrich (St. Louis, MO, http://www.sigmaaldrich.com). Graft polymerization was performed using a 0.2 M solution of methacrylate monomers in a 4:1 mixture of water and ethanol. The TCPS plates were activated using a UV-ozone cleaner (Jelight, Irvine, CA, http://www.jelight.com) for 40 minutes. Surface-activated dishes were immersed into monomer solution at 80°C for 2.5 hours. The polymer-grafted dishes were subsequently cooled to 50°C and rinsed in 1% NaCl-water solution at the same temperature overnight. The grafted plates were cleaned by ultrasonication in deionized water and dried under a stream of nitrogen gas. The cell culture on PMEDSAH was performed as previously described [20]. Briefly, PMEDSAH plates were prepared as follows: after overnight UV exposure, plates were washed two times with phosphate-buffered saline followed by addition of hCCM+. Then plates were placed and maintained until and during use in incubators in high humidity at 37°C and 5% $CO_2$. If prepared PMEDSAH-GPs were not used within a week, hCCM+ was refreshed. Matrigel-coated plates (CPs) were prepared the day of intended use following manufacturer's instructions (BD Matrigel hESC-qualified Matrix; BD Biosciences, San Diego, CA, http://www.bdbiosciences.com). Colonies of human iPSCs (hiPSCs) were passaged manually by cutting them into small clusters using either a pulled-glass pipette or the StemCell Tool (Invitrogen).

### Reprogramming Cycle

The reprogramming cycle was performed with minor modifications to the protocol provided by the CytoTune iPS reprogramming kit (Invitrogen) using Sendai virus (SeV) vectors for human (h)*OCT3/4*, h*SOX2*, h*KLF4*, and h*c-MYC*. A schematic timeline of events is given in supplemental online Figure 1. A cell-doubling time calculation was performed for all three fibroblast cell lines to determine the initial cell plating density and to achieve a density of approximately $3.3 \times 10^5$ cells per well of a six-well plate (Becton, Dickinson and Company) at the time of infection, 48 hours postplating. In total, approximately $1 \times 10^6$ cells were infected with the SV vectors at a multiplicity of infection of 3. From day 1 to day 8, parental fibroblasts were cultured with fibroblast culture medium on six-well plates; at day 8, they were treated with 0.25% trypsin-EDTA (Gibco, Grand Island, NY, http://www.invitrogen.com), inactivated with fibroblast medium, and resuspended in hCCM+. The cell suspension of each parental fibroblast line was subdivided into six parts of equal volumes and distributed into three PMEDSAH-GPs and three Matrigel-CPs. All plates were 60 mm in diameter and were pre-equilibrated with hCCM+ supplemented with Rock inhibitor (10 $\mu$M; Enzo Life Sciences, Inc., Farmingdale, NY, http://www.enzolifesciences.com). This medium was replaced every 48 hours, until day 22 when clonal expansion was performed by selecting one human iPSC colony per cell line and manually passaged into a freshly prepared 35-mm PMEDSAH-GPs or Matrigel-CPs. The resulting subcloned colonies were continuously expanded for 9 months without Rock inhibitor and characterized to assure their full reprogramming into iPSCs. The expansion of colonies on Matrigel-CP was terminated at the time of reprogramming efficiency calculation.

### Reprogramming Efficiency Calculation

The reprogramming efficiency was calculated for each cell line of human iPSCs derived on PMEDSAH-GPs and Matrigel-CPs as follows: the total number of undifferentiated colonies present in the original PMEDSAH-GPs and Matrigel-CPs plus one at day 30 post-infection was divided by the corresponding number of parental fibroblasts found in each substrate at the day of infection. A colony was considered undifferentiated if it had well defined borders and a high nucleus:cytoplasm ratio and stained positively for alkaline phosphatase.

### Evaluation of Pluripotency

Pluripotency was tested in vivo by a teratoma formation assay 6 months after derivation and continuous in vitro culture. Briefly, undifferentiated cells ($\sim 5 \times 10^6$ cells per cell line) from each derived human iPSC line on PMEDSAH-GPs were injected subcutaneously into NOD.CB17-*Prkcd*$^{SCID}$/NcrCrl mice (Charles River Laboratories, Wilmington, MA, http://www.criver.com) to induce teratomas. When tumors became palpable after 8–12 weeks, mice were euthanized, and tumors were isolated and processed for histologic analysis. A certified pathologist at the Pathology Core for Animal Research at University of Michigan Medical School analyzed and identified tissue representative from the three germ layers in hematoxylin/eosin-stained slides.

For in vitro analysis of pluripotency, embryoid body (EB) formation and directed cell-lineage differentiation from undifferentiated human iPSCs derived and cultured on PMEDSAH-GPs were performed 3, 6, and 9 months after derivation and continuous in vitro culture, using established protocols [21]. Undifferentiated human iPSCs were induced to differentiate in basal medium consisted of DMEM/F12 (Invitrogen) supplemented with 1× N2 (Invitrogen), 1× B27 (Invitrogen), 2 mM L-glutamine, 0.11 mM 2-mercaptoethanol, 1 mM nonessential amino acids, and 0.5 mg/ml bovine serum albumin (fraction V; Sigma-Aldrich). To

 

induce neuronal differentiation, 100 ng/ml human recombinant Noggin (Stemgent, Cambridge, MA, https://www.stemgent.com) was added to the basal medium, and cells were cultured in this condition for 8 days. For definitive endoderm differentiation, 100 ng/ml human recombinant activin A (Stemgent) was added to basal medium, and cells were cultured for 9 days. The induction to cardiomyocyte differentiation consisted of two sequential steps; first, cells were cultured in basal medium plus 50 ng/ml human recombinant BMP4 (Stemgent) and 50 ng/ml human recombinant activin A for 4 days and then further cultured in basal medium for an additional 10 days.

### Quantitative Real-Time PCR

Total RNA was extracted using TRIzol (Invitrogen) and purified using RNeasy Mini Kit (Qiagen) and DNase I treatment. One milligram of total RNA was reverse transcribed into cDNA using Superscript III Reverse Transcriptase (Invitrogen). Two microliters of diluted reverse transcribed cDNA (reverse transcription [RT] reaction, 1:5 in RNase-free water) were amplified in a polymerase chain reaction (PCR) assay (30 $\mu$l) using TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com), target primers, and probes (unlabeled PCR primers and a carboxyfluorescein dye-labeled TaqMan minor groove binderprobe) (Applied Biosystems). Gene expression was determined by quantitative real-time PCR on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The relative RNA expression levels of target genes were analyzed by the comparative $\Delta\Delta C_T$ method using $\beta$-actin as an internal control. Subsequently, expression levels of investigated genes were normalized to expression levels of control samples and reported as fold changes.

### RT-PCR Analysis

Total RNA was reverse transcribed using SuperScript One-Step RT-PCR with platinum *Taq* (Invitrogen). In a single reaction (25 $\mu$l), 0.5 $\mu$g of total RNA, and 20 pmol of forward and reverse primers were used (supplemental online Table 3). The cDNA synthesis and predenaturation were carried out at 95°C for 2 minutes. The PCR amplification was performed for 35 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The final extension cycle was run at 72°C for 10 minutes. Finally, 10 $\mu$l of PCR product was loaded onto a 1.0% agarose gel and size-fractionated.

### Chromosomal Stability Studies Using High-Resolution aCGH and G-Banding Karyotype

The array-based comparative genomic hybridization (aCGH) 135K Stem Array (Ambry Genetics, Aliso Viejo, CA, http://www.ambrygen.com) was performed by Cell Line Genetics (Madison, WI, http://www.clgenetics.com) on cells continuously passaged for 9 months as follows: genomic DNA quality and concentration were determined with a NanoView spectrophotometer. Labeling reactions were prepared using the NimbleGen labeling protocol for aCGH (version 8.0; Roche, Indianapolis, IN, http://www.roche.com) with 500–600 ng of total (RNased) DNA input. The StemArray protocol consists of labeling of the DNA and hybridization. First, 500–600 ng of test sample DNA was labeled with cyanine 3-dUTP, and pooled sex-matched reference DNA was labeled with cyanine 5-dUTP by Exo-Klenow fragment. Labeled DNA was purified, and the labeling efficiency/concentration was determined by the NanoView spectrophotometer. Test and reference samples were balanced by combining 20 $\mu$g of labeled test sample and 20 $\mu$g of corresponding labeled reference sample into a single reaction tube. The labeled DNA was prepared for hybridization and placed on the 135K StemArray and hybridized at 42°C for ~72 hours. Arrays were then washed and scanned at 2 $\mu$M resolution on a NimbleGen MS 200 High Resolution Scanner. Data were extracted using Nimblescan version 2.6 image extraction software, processed, and mapped to the human genome (hg19) using FASST2 Segmentation Algorithm in Nexus Copy Number version 6.0 (Biodiscovery, Hawthorne, CA, http://www.biodiscovery.com). The aberration detection settings were: minimum probe, 5; significance threshold, 1.0× 10E-7; high gain, 1.0; gain, 0.3; loss, −0.3; big loss, −1.1. For standard G-banding karyotyping, chromosomes from at least 20 cells at the metaphase stage were prepared using standard protocols and analyzed by Cell Line Genetics.

### Western Blot Analysis

The following antibodies were used: antibody to Sendai virus protein (1:1,000; MBL International Corp., Woburn, MA, http://www.mblintl.com), Oct4-specific antibody (1:2,000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com), Nanog-specific antibody (1:1,000; Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com), $\alpha$-tubulin-specific antibody (1:2,000; Santa Cruz Biotechnology Inc.), and $\beta$-actin-specific antibody (1:1,000; Cell Signaling Technology). Whole cell lysates were prepared from cells, separated on 7.5% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membranes. The membranes were incubated with 5% milk in TBST (wt/vol) for 1 hour and then incubated with primary antibodies overnight at 4°C. Blots were incubated with peroxidase-coupled secondary antibodies (Promega, Madison, WI, http://www.promega.com) for 1 hour, and protein expression was detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, http://www.thermoscientific.com).

### Immunocytochemistry

Cells were fixed in 2% paraformaldehyde (wt/vol) for 30 minutes at room temperature and, if required, permeabilized with 0.1% Triton X-100 (vol/vol) for 10 minutes. Primary antibodies were diluted in 1% normal serum (vol/vol) and incubated overnight at 4°C and detected by respective secondary antibodies. Samples were imaged and captured using a Leica DM IRB inverted microscope (Leica, Heerbrugg, Switzerland, http://www.leica.com) with an Olympus (Center Valley, PA, http://www.olympusamerica.com) DP-30 CCD camera (Microscopy and Image Analysis Laboratory, University of Michigan, Ann Arbor, MI). The following antibodies were used: GATA4-specific antibody (R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com), HNF-3b/FoxA2-specific antibody (R&D Systems, Inc.), SOX1-specific antibody (R&D Systems, Inc.), PAX6-specific antibody (Abcam, Cambridge, U.K., http://www.abcam.com), SSEA-3-specific antibody (Millipore, Billerica, MA, http://www.millipore.com), TRA-1-60-specific antibody (Santa Cruz Biotechnology Inc.), TRA-1-81-specific antibody (Millipore), SSEA4-specific antibody (Santa Cruz Biotechnology Inc.), Oct3/4-specific antibody (Santa Cruz Biotechnology Inc.), Nanog-specific antibody (Cell Signaling Technology), and Sox2-specific antibody (Millipore).

### Bisulfite Sequencing

Genomic DNA (1 mg) was treated with components of the CpGenome DNA modification kit (Chemicon, Temecula, CA, http://www.chemicon.com), according to the manufacturer's recommendations. Treated DNA was purified with QIAquick column (Qiagen, Hilden, Germany, http://www.qiagen.com). The promoter regions of human *OCT4* and *NANOG* genes were amplified by PCR. The PCR products were subcloned into pCR2.1-TOPO. Ten clones of each sample were verified by sequencing with the M13 universal primer. Primer sequences [22, 23] used for PCR amplification are shown in supplemental online Table 3.

### Statistical Analysis

Experiments were performed in triplicate, and the data are expressed as mean values ± SD and analyzed by an unpaired *t* test. Levels of statistical significance were set at $p < .05$.

## Results

### Derivation of Human Induced Pluripotent Stem Cells on PMEDSAH-GP

The parental cell lines, two derived from adult gingival tissue (hGF) and a commercial cell line from newborn foreskin (hFF), were initially cultured and infected with the reprogramming factors introduced by SeV on tissue culture plastic plates, and 5 days postinfection were subcultured, equally divided, and reseeded on PMEDSAH-GPs and Matrigel-CPs (used as control) in hCCM+ (supplemental online Fig. 1). During this period and until the first passage of colonies, the medium was supplemented with Rock inhibitor. Nine days postinfection, colonies were observed on both PMEDSAH-GPs and Matrigel-CPs. At day 16 postinfection, two colonies growing on PMEDSAH-GPs were subcloned to evaluate the expression of Nanog and alkaline phosphatase activity (supplemental online Fig. 1). At day 22 postinfection, one colony from each of the three parental lines growing on both PMEDSAH-GPs and Matrigel-CPs was selected, manually dissected, and reseeded on new substrates. Subsequently, six human iPSC lines were established, two from each parental cell line, from which one was derived and propagated on PMEDSAH-GPs and the other on Matrigel-CPs. At day 30 postinfection, the reprogramming efficiency was calculated and ranged from 0.0004% to 0.0006%. There was no statistical difference ($p > .05$) between parental fibroblasts and substrates. The number of colonies generated in each condition (178 ± 75 [average ± SD]) exceeded the required number to establish and characterize independent human iPSC lines. This suggests that PMEDSAH-GP is an effective platform to generate human iPSCs.

### Characterization of Human iPSCs Derived on PMEDSAH-GPs

The three human iPSC lines derived on PMEDSAH-GPs were subcultured on grafted plates of the same synthetic substrate for approximately 170 cell doublings over a period of 9 months and continuously characterized to demonstrate their fully reprogrammed character. Initially, Western blot analysis demonstrated that parental fibroblasts did not express Oct4. In contrast, Oct4 was highly expressed in cells undergoing reprogramming and in fully established human iPSCs, 3 months after derivation and culture (Fig. 1A). SeV protein levels were not detected in parental fibroblasts but were high 3 days postinfection and subsequently decreased to undetectable levels in human iPSCs (Fig. 1A). RNA expression of SeV was also tested by RT-PCR, showing no detectable levels in established human iPSC lines (supplemental online Fig. 2). The expression of Nanog in all iPSC lines was detected by Western blot analysis (Fig. 1B), and immunocytochemistry revealed the continuous expression of pluripotent markers: Oct4, SSEA3, SSEA4, TRA-1-60, TRA-1-81, and Sox2 (Fig. 1C). Bisulfite sequencing analysis demonstrated that promoters of *OCT4* and *NANOG* were highly methylated in parental cell lines and converted to a largely unmethylated state in established human iPSCs, 3 months after derivation and continuous culture (Fig. 1D).

The pluripotency of the human iPSC lines derived and cultured on PMEDSAH-GPs was tested up to 9 months after derivation and continuous in vitro culture, by EB formation, in vitro cell lineage differentiation, and teratoma formation. In vitro, clusters of iPSC lines formed EBs when cultured in suspension (Fig. 2A) and expressed genes representative of the three germ layers: ectoderm, endoderm, and mesoderm (data not shown). Furthermore, specific cell lineage differentiation on PMEDSAH-GPs was directed via chemically defined conditions using well-established protocols [21]. For neural differentiation, treatment with Noggin resulted in upregulation of *Pax6* RNA levels (Fig. 2B) and dual expression of Pax6 and Sox1 in treated cells (Fig. 2C). Activin A treatment induced the differentiation of human iPSCs into definitive endoderm/pancreatic cells, as indicated by high RNA levels of *SOX17* and *FOXA2* (Fig. 2B), and detection of $FoxA2^+$ cells (Fig. 2D). Cardiac muscle lineage differentiation in response to Activin A and BMP4 treatment represented in vitro mesodermal lineage development, which was confirmed by the upregulation of *T*, *TNNI3*, *NKX2.5*, and *HESX1* (Fig. 2B) and by expression of Gata4 (Fig. 2E). Teratoma analysis confirmed the pluripotency of these cell lines in vivo, as observed by identification of mature cells such as neurons, cartilage, gut glandular epithelium, and other differentiated tissues (Fig. 2F–2H).

### Genomic Stability of Human iPSCs Derived and Cultured on PMEDSAH-GPs

The genetic stability of human iPSCs derived on PMEDSAH-GPs was analyzed 9 months after derivation and continuous in vitro culture. Standard G-band analysis demonstrated normal male chromosomal karyotypes for all three human iPSC lines (Fig. 3A), whereas aCGH analysis detected minimal gains and losses in specific chromosomal regions in all cell lines (Fig. 3B; supplemental online Table 1). It was observed that some of the aberrations were recurrent in two of the three human iPSC lines and only in one occasion (chromosome 9q34.3) in all cell lines (supplemental online Table 2).

## Discussion

Three independent human iPSC lines derived in xenogeneic and integration-free conditions demonstrated continuous self-renewal, genomic stability, and pluripotency in vitro and in vivo after up to 9 months of continuous in vitro culture on PMEDSAH-GPs. These results demonstrate that PMEDSAH-GPs support the derivation and long-term self-renewal of genomically stable human iPSCs, as well as differentiation of into derivatives of the three germ lineages in chemically defined conditions. The fully reprogrammed character of the cell lines was demonstrated by continuous expression of

 

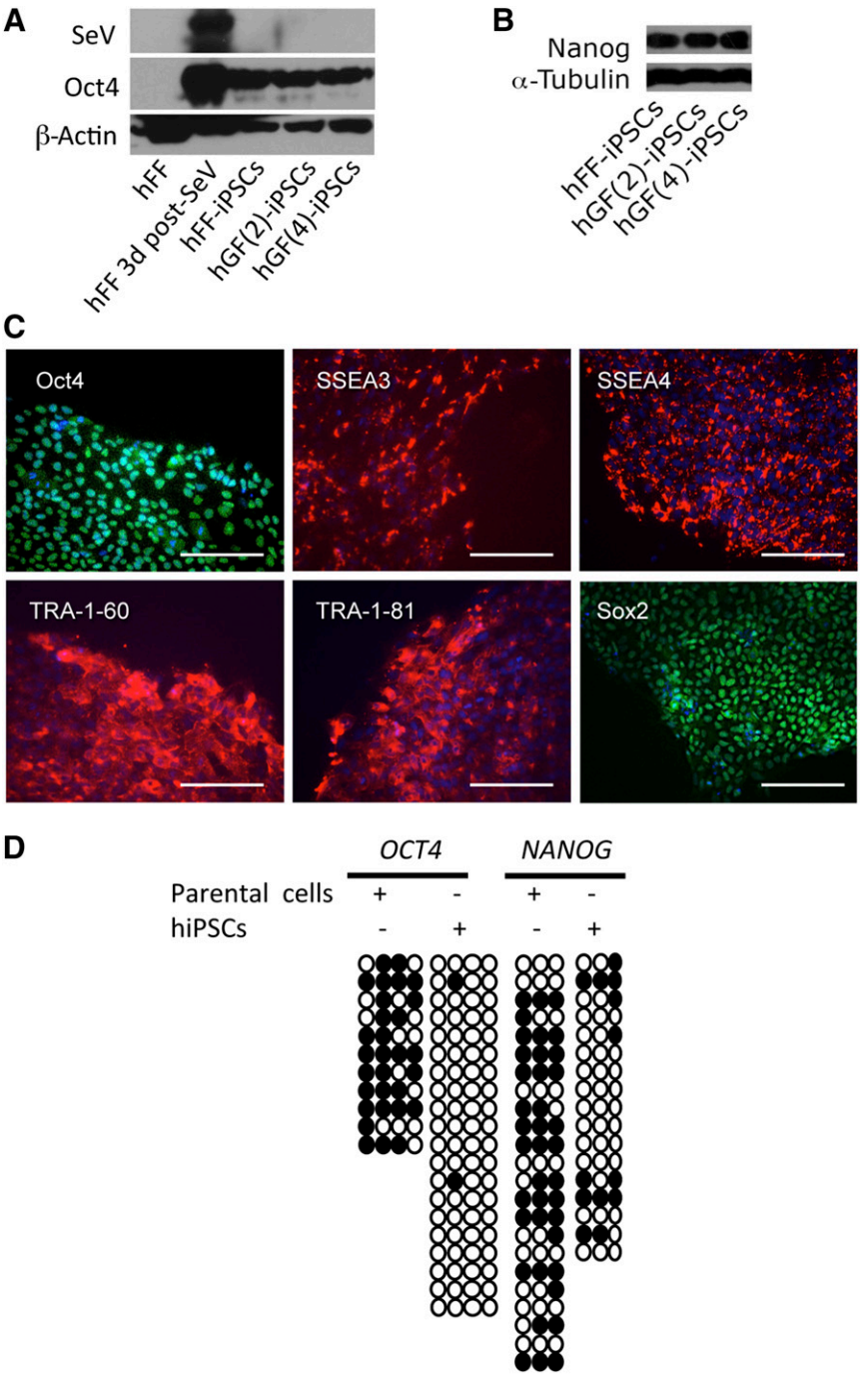


**Figure 1.** Expression of pluripotent-related markers and methylation status of human iPSCs derived on poly2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide (PMEDSAH)-grafted plates (GPs). **(A):** Western blot analysis demonstrating the expression of SeV and Oct4 in the three independent human iPSC lines derived and cultured for 3 months on PMEDSAH-GPs and compared with parental fibroblasts before and 3 days after infection. $\beta$-Actin was used as loading control. **(B):** Western blot confirming the expression of Nanog in the three established cell lines 3 months after derivation and continuous in vitro culture. $\alpha$-Tubulin was used as loading control. **(C):** Representative epifluorescent micrographs of human iPSC colonies 9 months after derivation and continuous in vitro culture, showing expression of Oct4, SSEA3, SSEA4, TRA-1-60, TRA-1-81, and Sox2. Scale bars = 100 $\mu$m. **(D):** Representative comparative bisulfite genomic sequencing analysis of *OCT4* and *NANOG* promoters in parental cells and human iPSCs 3 months after derivation and continuous in vitro culture. Open and closed circles indicate unmethylated and methylated CpGs, respectively. Abbreviations: d, day(s); hFF, human foreskin fibroblast; hGF, human gingival fibroblast; hiPSC, human induced pluripotent stem cell; iPSC, induced pluripotent stem cell; SeV, Sendai virus.

protein markers related to human pluripotent stem cells, the demethylated status of both *Nanog* and *Oct4* promoters in these cells, and the capability to differentiate both in vivo and in vitro into representative cells and tissues of all three germ layers.

The human iPSC lines derived in these conditions were transgene-free because nonintegrating Sendai virus constructs encoding *OCT4*, *SOX2*, *KLF4*, and *c-MYC* were used to induce the reprogramming. The Sendai viruses are nonsegmented

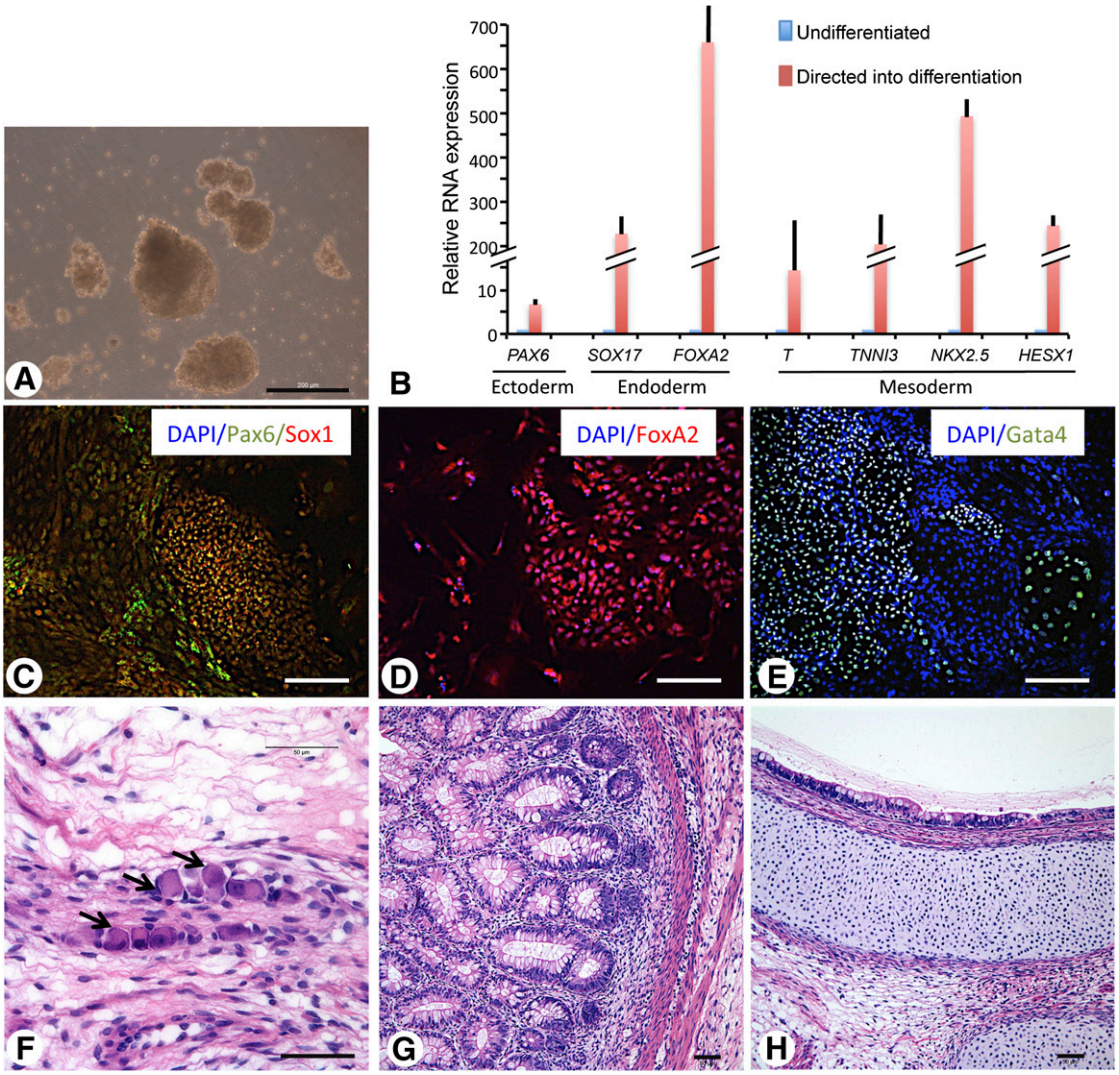


**Figure 2.** Evaluation of pluripotency of human induced pluripotent stem cells (iPSCs) derived and cultured on poly2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide (PMEDSAH)-grafted plates (GPs). The pluripotency of the three human iPSCs derived and cultured on PMEDSAH was tested by embryoid body (EB) formation, directed in vitro cell lineage differentiation, and teratoma induction. **(A):** Representative micrograph of EBs from human foreskin fibroblast induced pluripotent stem cells 9 months after derivation and continuous in vitro culture. **(B):** Graph showing relative RNA transcription levels of genes expressed in cells after directed in vitro differentiation of human iPSCs on PMEDSAH-GPs. **(C–E):** Representative micrographs of directed in vitro cell lineage differentiation on PMEDSAH-GPs of human iPSCs 9 months after derivation and continuous in vitro culture. Neural differentiation (ectoderm) was achieved after treatment with Noggin **(B, C)**. Definitive endoderm/pancreatic differentiation was induced by activin A treatment **(B, D)**. Mesoderm lineage was obtained after treatment with activin A and BMP4 to induce cardiac muscle differentiation **(B, E)**. Teratoma formation was performed 6 months after derivation and continuous in vitro culture of human iPSCs. **(F–H):** Representative micrographs of neurons **(F)**, gut glandular epithelium **(G)**, and cartilage **(H)** identified in teratomas. Scale bars = 200 $\mu$m **(A)**, 100 $\mu$m **(C–E)**, and 50 $\mu$m **(F–H)**. Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

negative-strand RNA viruses that do not have a DNA phase and thus do not integrate into the genome of infected cells [24]. In addition, Sendai virus constructs were also selected to induce reprogramming because of their highly effective infection rates and simplicity of use compared with other methods that require multiple transfections to achieve strong gene expression, continuous protein translation, or further manipulations to excise integrated constructs. Indeed, our data demonstrated the effectiveness of these vectors to induce reprogramming on PMEDSAH-GPs. Our results demonstrate that in fully reprogrammed cells, RNA expression and protein levels of Sendai virus become undetectable after the establishment of fully reprogrammed cells (Fig. 1; supplemental online Fig. 2). We have also derived human iPSCs using Cre/LoxP constructs on PMEDSAH-GPs (data not shown), which suggests that other nonintegrating methods could be used in combination with this synthetic substrate to derive transgene-free human iPSC lines.

It has been established that during long-term culture of pluripotent stem cells, chromosomal changes may occur, and these aberrations may confer growth advantages and disrupt differentiation capacity [25]. In addition, it has been reported that *SOX2*, *c-MYC*, and *KLF4* may be duplicated in retrovirus-generated iPSCs because of genomic integration [26]. Therefore, we evaluated the genomic stability of the three human iPSC lines derived and propagated continuously on PMEDSAH-GPs after 9 months (Fig. 3). The standard G-banding assay demonstrated a normal karyotype

 

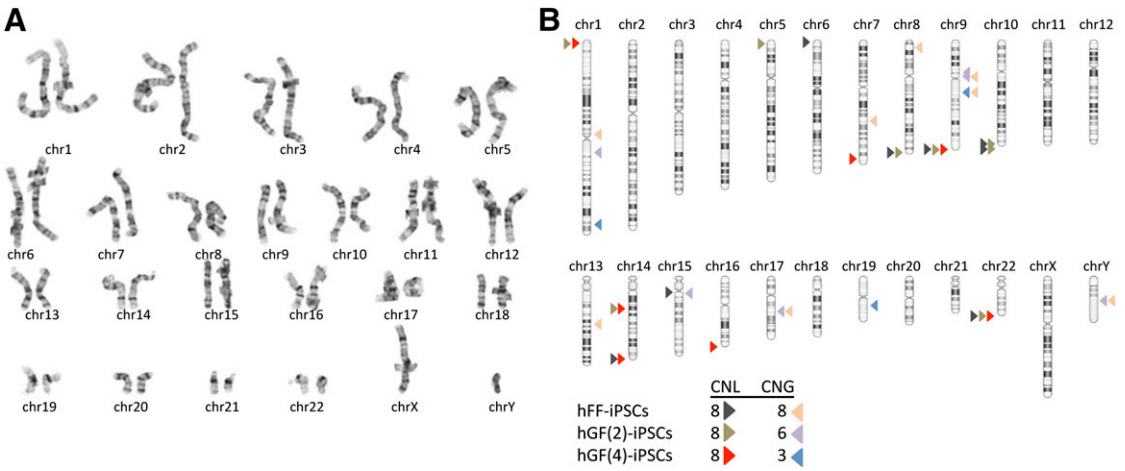


**Figure 3.** Genetic stability of the human iPSCs derived on poly2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide (PMEDSAH)-grafted plates (GPs). The genomic stability of the three human iPSC lines derived on PMEDSAH-GPs was tested 9 months after derivation and continuous in vitro culture. **(A):** Representative standard G-banding metaphase karyotyping of one of the three human iPSCs derived on PMEDSAH-GPs showing normal male karyotype. **(B):** Ideogram summarizing chromosome losses and gains (left and right, respectively) of the three human iPSCs as detected by high-resolution array-based comparative genomic hybridization. No mutations are localized in chromosome loci where genes related to stem cells, cancer, or culture adaptation are localized. Abbreviations: chr, chromosome; CNG, copy number gain; CNL, copy number loss; hFF, human foreskin fibroblast; hGF, human gingival fibroblast; iPSC, induced pluripotent stem cell.

for all three cell lines, confirming previous results with hESC culture on PMEDSAH-GPs [17]. The aCGH analysis demonstrated minimum modifications in the three cell lines. However, these mutations do not match chromosomal loci where key stem cell and cancer-related genes are contained [26] or where genes related to culture adaption, such as *BCL2L1*, are localized [27]. Because the aCGH analysis was performed only in parental cells and after 9 months of continuous culture of the derived human iPSC lines, we could not determine the time at which these mutations occurred, or whether other mutations were corrected [27]. Thus, these results indicate the genomic stability of human iPSCs derived and cultured long term on PMEDSAH-GPs.

During the preparation of this work, it was reported that a new chemically defined and xeno-free medium supports the derivation of human iPSCs on vitronectin [14]. When this medium was used in combination with PMEDSAH-GPs, only pre-iPSC colonies were formed, and cells were not induced to be fully reprogrammed (data not shown). This observation indicates that further optimization of chemically defined media is required to achieve a complete chemically defined and xeno-free platform for the derivation and culture of human iPSCs on PMEDSAH-GPs, and work is in progress to achieve this goal.

## Conclusion

This study demonstrated a feeder-free platform to derive, expand, and differentiate genomically stable and transgene-free human iPSCs. This platform has the potential to be in compliance with good tissue practice and good manufacturing practice. Compared with currently used methods, the conditions presented here represent an alternative to the goal of generation and long-term propagation of human iPSCs with potential for clinical application.

## Acknowledgments

We thank John Erby Wilkinson, D.A.C.V.P., for his pathology expertise in the teratoma tissue interpretation. This work was supported by NIH Grant DE016530.

## Author Contributions

L.G.V.-D.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; J.K.K.: collection and/or assembly of data, data analysis and interpretation; J.L.: provision of study material, data analysis and interpretation, final approval of manuscript; P.H.K.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

## Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

## References

1 Takahashi K, Tanabe K, Ohnuki M et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007; 131:861–872.

2 Yu J, Hu K, Smuga-Otto K et al. Human induced pluripotent stem cells free of vector and transgene sequences. Science 2009;324: 797–801.

3 Woltjen K, Michael IP, Mohseni P et al. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature 2009; 458:766–770.

4 Jia F, Wilson KD, Sun N et al. A nonviral minicircle vector for deriving human iPS cells. Nat Methods 2010;7:197–199.

5 Fusaki N, Ban H, Nishiyama A et al. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc Jpn Acad Ser B Phys Biol Sci 2009;85:348–362.

6 Warren L, Manos PD, Ahfeldt T et al. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell 2010;7: 618–630.

7 Kim D, Kim CH, Moon JI et al. Generation of human induced pluripotent stem cells by direct

delivery of reprogramming proteins. Cell Stem Cell 2009;4:472–476.

**8** Villa-Diaz LG, Ross AM, Lahann J et al. Concise review: The evolution of human pluripotent stem cell culture: From feeder cells to synthetic coatings. STEM CELLS 2013;31:1–7.

**9** Martin MJ, Muotri A, Gage F et al. Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat Med 2005;11:228–232.

**10** Stacey GN, Cobo F, Nieto A et al. The development of 'feeder' cells for the preparation of clinical grade hES cell lines: Challenges and solutions. J Biotechnol 2006;125:583–588.

**11** Xu C, Inokuma MS, Denham J et al. Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 2001;19: 971–974.

**12** Miyazaki T, Futaki S, Hasegawa K et al. Recombinant human laminin isoforms can support the undifferentiated growth of human embryonic stem cells. Biochem Biophys Res Commun 2008;375:27–32.

**13** Nagaoka M, Si-Tayeb K, Akaike T et al. Culture of human pluripotent stem cells using completely defined conditions on a recombinant E-cadherin substratum. BMC Dev Biol 2010;10:60.

**14** Chen G, Gulbranson DR, Hou Z et al. Chemically defined conditions for human iPSC derivation and culture. Nat Methods 2011;8: 424–429.

**15** Nakagawa M, Taniguchi Y, Senda S et al. A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells. Sci Rep 2014;4:3594.

**16** Lu HF, Chai C, Lim TC et al. A defined xeno-free and feeder-free culture system for the derivation, expansion and direct differentiation of transgene-free patient-specific induced pluripotent stem cells. Biomaterials 2014;35: 2816–2826.

**17** Villa-Diaz LG, Nandivada H, Ding J et al. Synthetic polymer coatings for long-term growth of human embryonic stem cells. Nat Biotechnol 2010;28:581–583.

**18** Villa-Diaz LG, Brown SE, Liu Y et al. Derivation of mesenchymal stem cells from human induced pluripotent stem cells cultured on synthetic substrates. STEM CELLS 2012;30: 1174–1181.

**19** Raya A, Rodríguez-Pizà I, Guenechea G et al. Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. Nature 2009;460:53–59.

**20** Nandivada H, Villa-Diaz LG, O'Shea KS et al. Fabrication of synthetic polymer coatings and their use in feeder-free culture of human embryonic stem cells. Nat Protoc 2011;6: 1037–1043.

**21** Yao S, Chen S, Clark J et al. Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. Proc Natl Acad Sci USA 2006;103:6907–6912.

**22** Maherali N, Ahfeldt T, Rigamonti A et al. A high-efficiency system for the generation and study of human induced pluripotent stem cells. Cell Stem Cell 2008;3:340–345.

**23** Deb-Rinker P, Ly D, Jezierski A et al. Sequential DNA methylation of the Nanog and Oct-4 upstream regions in human NT2 cells during neuronal differentiation. J Biol Chem 2005; 280:6257–6260.

**24** Li HO, Zhu YF, Asakawa M et al. A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression. J Virol 2000;74:6564–6569.

**25** Baker DEC, Harrison NJ, Maltby E et al. Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. Nat Biotechnol 2007;25:207–215.

**26** Elliott AM, Elliott KA, Kammesheidt A. High resolution array-CGH characterization of human stem cells using a stem cell focused microarray. Mol Biotechnol 2010;46:234–242.

**27** Amps K, Andrews PW, Anyfantis G et al. Screening ethnically diverse human embryonic stem cells identifies a chromosome 20 minimal amplicon conferring growth advantage. Nat Biotechnol 2011;29:1132–1144.

See www.StemCellsTM.com for supporting information available online.

 