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Supplemental Information

cGMP-Manufactured Human Induced Pluripotent Stem Cells Are Available for Pre-clinical and Clinical Applications

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SUPPLEMENTAL DATA

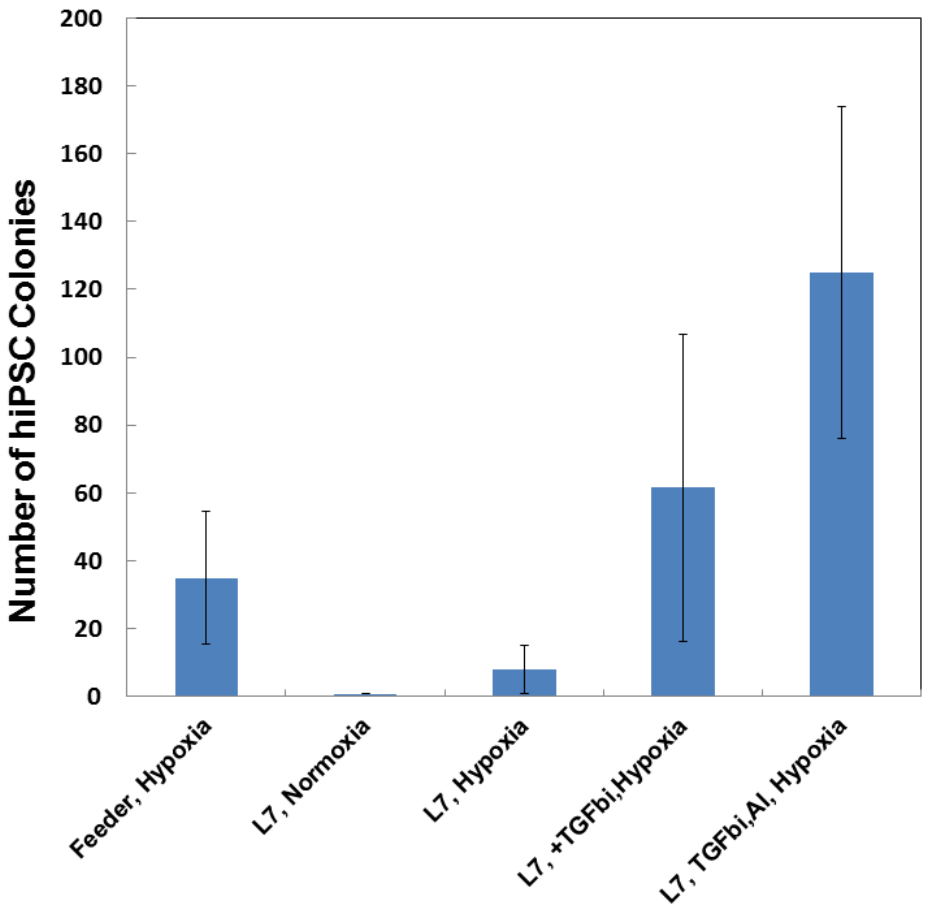

Figure S1. Improving the reprogramming efficiency under defined and cGMP conditions. The number of human iPSCs generated under defined, feeder-free and cGMP compliant condition was improved by incorporating a few enhancers in in the reprogramming process including a novel aluminum-based chemical product (i.e. 2% Alhydrogel® which is abbreviated as Al in the graph), TGF beta inhibitor (abbreviated as TGFbi), and hypoxic oxygen concentration. L7 refers to the combination of L7™ hPSC Matrix and L7™ hPSC medium. The statistical analysis is based on four technical replicates per condition (n=4).

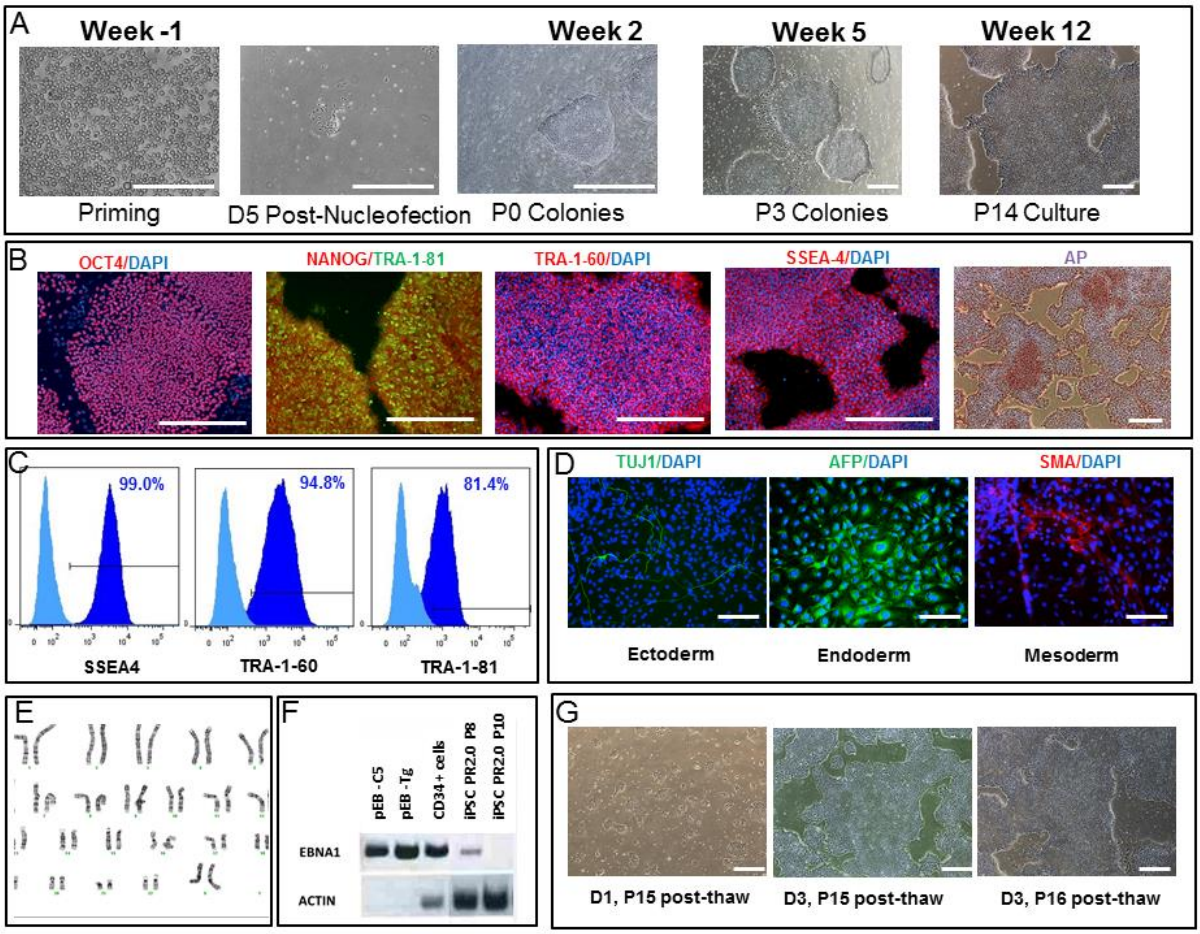

Figure S2. Generation, expansion, and characterization of human iPSCs (LiPSC-PR 2.0) - process development runs. Panel A illustrates priming of CD34+ cells isolated from cord blood unit and expanded in culture on day 3 prior to the nucleofection (Priming), iPSC colony emerged on day 5 post nucleofection (D5 Post-Nucleofection), human iPSC colonies appeared on day 10 post nucleofection (P0 colonies), iPSCs maintained in L7™ cell culture system for 3 passages (P3 Colonies), and iPSCs maintained in L7™ cell culture system for 12 passages (P12 culture). Panel B illustrates iPSCs positively stained with OCT4, NANOG, TRA-1-81, TRA-1-60, SSEA-4, and alkaline phosphatase (AP). Flow cytometry analysis (Panel C) showed that the iPSCs expressed the pluripotent stem cell surface markers including SSEA-4, TRA-1-60, and TRA-1-81(dark blue). Light blue exhibits the isotype control.. Following differentiation of iPSCs into embryoid bodies (Panel D), differentiated iPSCs expressed the markers for early ectoderm (detected beta-III-Tubulin or TUJ1), endoderm (detected Alpha-Feto Protein (AFP)), and mesoderm (detected Smooth Muscle Actin (SMA)). The iPSCs demonstrated normal karyotype after 30 passages in culture (E). Semi-qualified PCR analysis of the iPSC samples (iPSC PR2.0 at P8 and P10) exhibited plasmid clearance at P10 (F). Upon thawing iPSCs banked at passage level 14, the cells attached to the cell culture surface, grow, and maintained robust iPSC colonies over time (Panel G). Scale bar in all images in Panel A is 500 microns except the Priming image which is 250 microns. Scale bar in all images in Panel B is 500 microns. Scale bar in all images in Panel D is 125 microns. Scale bar in all images in Panel G is 500 microns.

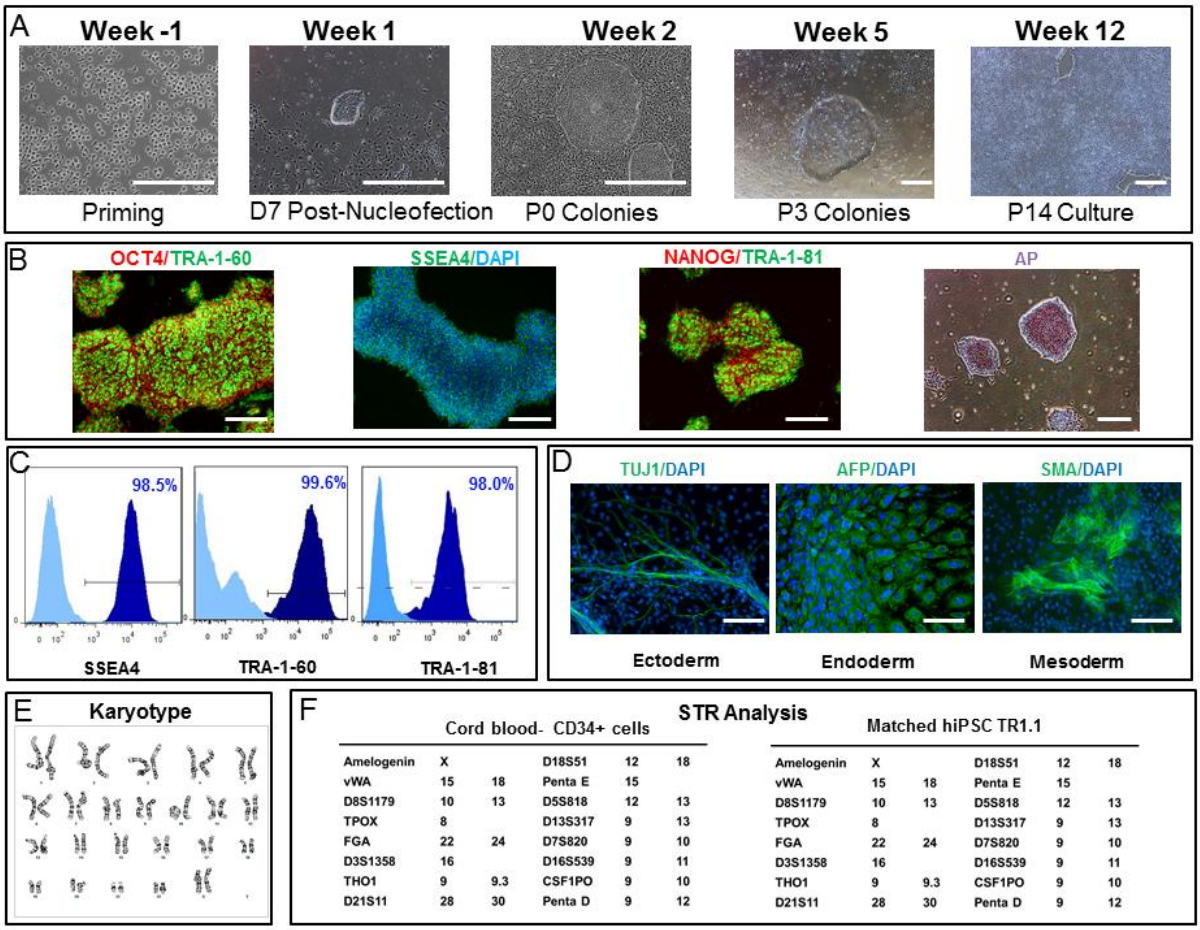

Figure S3. Generation, expansion, and characterization of human iPSCs (LiPSC-TR1.1) - training runs. Panel A illustrates priming of CD34+ Cells isolated from cord blood unit and expanded in culture on day 3 prior to the nucleofection (Priming), iPSC colony emerged on day 7 post nucleofection (D7 Post-Nucleofection), iPSC colonies appeared on day 14 post nucleofection (P0 colonies), iPSCs maintained in L7™ cell culture system for 3 passages (P3 Colonies), and iPSCs maintained in L7™ cell culture system for 14 passages (P14 culture). Panel B illustrates iPSCs positively stained with OCT4, NANOG, TRA-1-60, TRA-1-81, and alkaline phosphatase (AP). Flow cytometry analysis (Panel C) showed that the iPSCs expressed the pluripotent stem cell surface markers including SSEA-4, TRA-1-60, and TRA-1-81(dark blue). Light blue exhibits the isotype control. Following differentiation of iPSCs into embryoid bodies (Panel D), differentiated iPSCs readily expressed the markers for early ectoderm (detected TUJ1), endoderm (detected Alpha-Feto Protein (AFP)), and mesoderm (detected Smooth Muscle Actin (SMA)). The iPSCs demonstrated normal karyotype after 16 passages in culture (E). STR analysis showed that the human iPSCs matched the starting CD34+ donor sample (F). Scale bar in all images in Panel A is 500 microns except the Priming image which is 250 microns. Scale bar in all images in Panel B is 250 microns except the alkaline phosphatase image which is 500 microns. Scale bar in all images in Panel D is 125 microns.

Table S1. Assays used to characterize human iPSCs manufactured under cGMP condition, related to Section - Quality Control (QC) Testing and Release Assays.

Assay Release			
Pluripotency Markers	Identity & Purity	SSEA-4 >70%, Tra-1-60 >70%, Tra-1-81 >70%, Oct3/4 >70%; Purity: CD34 <5%	Release assay
Karyotype Analysis	Safety	46, XX or 46, XY	Release assay
Mycoplasma Testing	Safety	Negative	Release assay
Sterility Testing	Safety	Negative	Release assay
Endotoxin Testing	Safety	Standard QC release (<0.5 EU/ml)	Release assay
Vector Clearance	Safety	No trace of episomal plasmid DNA detected	Release assay
STR Genotyping	Purity & Identity	STR Profile of starting population and iPSC line are identical	Release assay
Cell Count & Viability	Viability	% viability >50; minimum cell number/vial	Release Assay
Viral Panel Testing	Safety	Standard MCB Release Panel	Release Assay
Characterization Assays			
EB Formation	Identity & Potency	Detection of at least one marker per germ layer	FIO*
Gene Array Analysis	Identity	Clustering with established hPSCs	FIO*
Colony morphology	Identity & Purity	Characteristic morphology of culture/colonies; lack of spontaneously differentiated cells	FIO*
Post-thaw Plating	Thawing efficiency and Viability	20+ colonies / vial (after 7 days or 50% confluency)	FIO*

* For Information Only (FIO)

Table S2. R2 values for gene array data for human iPSCs developed using GMP compliant process, related to Figure 4E and Section - Gene Expression Profiling of iPSC Lines.

R^2	LiPSC-PR1.0	LiPSC-PR2.0	LiPSC-PR3.0	LiPSC-TR1.1	LiPSC-TR1.2	LiPSC-ER1.1	LiPSC-ER1.2	LiPSC-ER1.3	H7 P37	NL9 p11	LiPSC-GR1.1	LiPSC-GR1.2
LiPSC-PR1.0	1.000	0.978	0.982	0.973	0.982	0.949	0.974	0.977	0.979	0.977	0.949	0.956
LiPSC-PR2.0	0.978	1.000	0.985	0.978	0.985	0.974	0.983	0.980	0.983	0.977	0.949	0.948
LiPSC-PR3.0	0.982	0.985	1.000	0.972	0.984	0.974	0.990	0.977	0.983	0.982	0.953	0.954
LiPSC-TR1.1	0.973	0.978	0.972	1.000	0.990	0.961	0.970	0.979	0.974	0.970	0.929	0.939
LiPSC-TR1.2	0.982	0.985	0.984	0.990	1.000	0.966	0.982	0.980	0.980	0.980	0.942	0.948
LiPSC-ER1.1	0.949	0.974	0.974	0.961	0.966	1.000	0.982	0.964	0.975	0.956	0.931	0.928
LiPSC-ER1.2	0.974	0.983	0.990	0.970	0.982	0.982	1.000	0.976	0.983	0.979	0.952	0.949
LiPSC-ER1.3	0.977	0.980	0.977	0.979	0.980	0.964	0.976	1.000	0.980	0.965	0.941	0.950
H7 P37	0.979	0.983	0.983	0.974	0.980	0.975	0.983	0.980	1.000	0.973	0.951	0.954
NL9 P11	0.977	0.977	0.982	0.970	0.980	0.956	0.979	0.965	0.973	1.000	0.952	0.950
LiPSC-GR1.1	0.949	0.949	0.953	0.929	0.942	0.931	0.952	0.941	0.951	0.952	1.000	0.966
LiPSC-GR1.2	0.956	0.948	0.954	0.939	0.948	0.928	0.949	0.950	0.954	0.950	0.966	1.000

Table S3. List of MCB viral test performed to test the final human iPSCs bank for absence of adventitious viruses using *in vitro* and *in vivo* assays, related to Supplemental Experimental Procedure - MCB Viral Testing

Assay	Type of Sample	No. of Sample	No. of Cells per Sample	Volume (mL)	Storage
TEM for Virus & Retrovirus	Cryopreserved	1	1×10^7 cells/ml	1 mL	Vapor Phase < -135^0C
Test for Inn-apparent Viruses	Cell Lysate	1 x 32 ml 1 X 8 ml 1 x 3 ml 2 x 2 ml	1×10^7 cells/ml	47 ml	≤ -70^0C or Ship Immediately
In vitro for Presence of Viral Contaminants	Cell Lysate	2 x 10 ml	1×10^7 cells/ml	20 ml	
Bovine Virus	Cell Lysate	1 x 12 ml	1×10^7 cells/ml	12 ml	
Porcine Virus	Cell Lysate	1 x 4 ml	1×10^7 cells/ml	4 ml	
PERT Assay	Supernatant.	3 x 0.5 ml	N/A	1.5 ml	
Adeno-Associated Virus	Cell Pellet	2	1×10^7 cells/ml	2 ml	
PCR (HIV, EBV, ETC) Human Panel 11	Cell Pellet	2 x 11 Tests	2×10^7 cells/ml	22 ml	

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

hPSC Culture

Human embryonic stem cell (hESC) lines (i.e. WA07, WA09 and WA14) were used to establish the defined, feeder-free cell culture system. These cells were obtained from WiCell and initially cultivated on mitomycin-C treated mouse embryonic fibroblasts (MEFs) in 80% Knockout-Dulbecco's Modified Eagle's Medium/F12 (Life Technologies, 12660-012), 20% Knockout Serum Replacement (Life Technologies, 10828-028), 2mM L-glutamine (Life Technologies, 25030-081), 55μM β-mercaptoetanol (Life Technologies, 21985-023), 1% non-essential amino acids (Life Technologies, 11140-050), and 8 ng/ml human basic fibroblast growth factor (Life Technologies, PHG0026). The hESC lines were adapted and continuously maintained in feeder-independent conditions using L7 hPSC Medium on defined L7™ hPSC Matrix (Lonza, FP-5020). The L7™ hPSC medium included L7™ hPSC basal medium (Lonza, CTM-5058) and L7™ hPSC medium supplement (Lonza, CTM-5059). The cells were serially subcultured using L7™ hPSC Passaging Solution (Lonza, FP-5013 or CTM-5070) per protocol suggested by the manufacturer and described elsewhere (Ying et al. 2014). All hPSC lines were cultivated in a humidified 37°C cell incubator equilibrated with 5% CO_2, unless noted otherwise.

Generation of human induced pluripotent stem cells

Cryopreserved human umbilical Cord Blood (hUCB) $CD34^+$ cells (Lonza, 2C-101) were thawed and expanded in a priming medium comprised of a basal medium [including IMDM (Iscove's Modified Dulbecco's Medium; Life Technologies, 12440-053), Ham's F12 (Life Technologies, 31765-035), Chemically Defined Lipid Concentrate (Life Technologies, 11905-031), Bovine Serum Albumin Fraction V (Life Technologies, 15260-037), and Insulin,-Transferrin-Selenium-Ethanolamine (ITS-X) (Life Technologies, 51500-056)] supplemented with 100 ng/mL

recombinant human (rh)SCF (PeproTech, AF-300-07), 100 ng/ml rhFlt3-ligand (PeproTech, AF-300-19), 20 ng/ml rhThrombopoietin (PeproTech, 300-18) and 10 ng/ml IL-3 (PeproTech, 200-03). The $CD34^+$ cells were seeded in 12-well plates (Corning, 3513) at a density of 4-6 $\times 10^5$ cells per well. Confluent cells (approximately day 3 post-thaw) were passaged the day prior to Nucleofection. 1×10^6 hUCB $CD34^+$ cells were reprogramed using the episomal plasmids encoding Oct4, Sox2, Klf4, c-Myc and Lin28 and pEB-Tg (Chen et al. 2011; Dowey et al. 2012). These plasmids were introduced into the cells using the 4D-Nucleofector™ System and P3 solution Kit (Lonza, V4XP-3012). After nucleofection, the cells were plated in the priming medium in a 37°C humidified incubator containing 5% CO_2 and 3% O_2. In some experimental conditions, thirty micrograms of Alhydrogel® adjuvant 2% (InvivoGen, vac-alu-250) were immediately supplemented into the expansion medium to enhance the reprogramming efficiency. Two days post-plating, the cells were transferred into 6-well plates pre-coated with L7™ hPSC Matrix (Lonza, FP-5020) in the L7™ hPSC medium supplemented with 1μM TGFβ inhibitor (Stemgent, 04-0014). Cells were placed in a 37°C humidified incubator containing 5% CO_2 and 3% O_2. The cell culture medium was changed every other day during the course of reprogramming step until hiPSC colonies appeared and isolated for further expansion (using L7 cell culture system) and characterization.

Xeno-components: The reagents and medium used for the isolation of CD34+ cells, priming and derivation and expansion of human iPSC contain Xeno products. The xeno-containing materials used in the isolation of the CD34+ cells are unique to the isolation process but already available as CE-marked medical devices in the EU, FDA approved in the US, and / or already being used for the clinical applications. The materials used during the priming, reprogramming and expansion of human iPSCs have xeno products, for instance Bovine Serum Albumin. While the

main strategy was to remove the xeno components, those difficult to replace or remove at the time of process optimization were only materials already used in the cell therapy applications. Recently, we have developed an entirely xeno-free process (except for the CD34+ cells isolation) that supports robust reprogramming and expansion of human iPSCs and will be used in the cGMP manufacturing of the future hiPSC lines. Moreover, a non-animal origin (NAO) version of the cell culture medium has been developed and will be used the future reprogramming / expansion procedures per clients' requests.

L7™ Cell Culture System: L7™ Cell Culture System include L7™ hPSC Passaging Solution (Lonza, FP-5013 or CTM-5070), L7™ hPSC Meidum, and L7™ hPSC Matrix. The L7™ hPSC Passaging Solution, which is commercially available, has been disclosed in this manuscript (i.e. it is a non-enzymatic passaging solution based on sodium citrate for serial subculture of hiPSCs) and comprehensively discussed in our previous publication (Nie et al. 2014). The L7™ hPSC Medium is a proprietary medium and its development and features will be discussed in detail in a separate manuscript (Manuscript in Preparation). The L7™ hPSC Medium is comprised of L7™ hPSC basal medium (Lonza, CTM-5058) and L7™ hPSC medium supplement (Lonza, CTM-5059). The basal medium is a xeno-free basal medium specifically designed for maintenance and expansion of human pluripotent stem cells by screening hundreds of basal medium supplements. The L7™ hPSC medium supplements contains xeno components commonly used in clinical manufacturing applications. Moreover, a xeno-free version of the medium, which is currently restricted to research use, is commercially available. A cell therapy (CT) manufactured version of the medium can be available for interested end users as a custom service. The CT version of the medium was used in the current work. L7™ hPSC Matrix is a proprietary and commercially available product.

Reprogramming Plasmids: The reprogramming and hiPSC generation process was carried out by using a single transfection of two plasmids: pEB-C5 and pEB-Tg. The pEB-C5 is a polycistronic episomal reprogramming plasmid containing five genes encoding the transcription factors Oct4, Sox2, Klf4, c-Myc, and Lin28. pEB-Tg is an episomal plasmid expressing SV40 Large T antigen that was demonstrated to increase reprogramming efficiency (Chou et al. 2011). The plasmid was manufactured by an approved vendor (GENEWIZ LLC, NJ). Detailed information regarding the pEB-C5 and pEB-Tg plasmids can be found at http://www.addgene.org/28213/ and http://www.addgene.org/28220/, respectively. The approved vendors were mainly production oriented suppliers and evaluated using a written vendor questionnaire along with specific tests shown on the certificate of analysis (COA). Regarding the plasmids, in particular, a detailed certificate of analysis (COA) including information regarding the DNA yield, DNA concentration, DNA quality, DNA formulation, sterility, endotoxin, type of host, PCR confirmation, and QC confirmation by restriction digestion was established and used in ordering and material review / approval process.

Embryoid body (EB) differentiation

Confluent cultures of human pluripotent stem cell colonies were dissociated using L7™ hPSC Passaging Solution. Cell aggregates were suspended in EB formation medium consisting of DMEM/F12 (Life Technologies, 11330-032) containing 10μM Rock Inhibitor Y27632 (Millipore, SCM075) and allowed to settle by gravity in a conical tube. After removing the supernatant, cells were suspended in fresh EB medium. Cell aggregates were then seeded using a split ratio of 1:1 on Ultra Low Attachment (Corning, YO-01835-24) plates and returned to the incubator for 12 to 24 hours. Once large cell aggregates formed, they were collected into a conical tube and allowed to settle by gravity. The medium was then removed and replaced with

differentiation medium (80% DMEM High Glucose (Life Technologies, 11965-092), 20% defined fetal bovine serum (Hyclone, SH30070.03), 1X non-essential amino acids (Life Technologies, 11140-050), 2 mM L-glutamine (Cellgro/Mediatech, 25-005-CI) and 55 μM β-Mercaptoethanol (Life Technologies, 21985-023)). The cell aggregates were placed on Ultra Low Attachment plates using a split ratio of 1:1 in 0.4 ml differentiation medium/cm^2. The culture medium was then changed every second day for six days. On the seventh day, EBs were seeded on gelatin-coated plates (EmbryoMax® ES Cell Qualified Gelatin Solution (Millipore, ES006-B)) at 10 EBs/cm^2. The EBs were allowed to attach undisturbed for 2 days. The differentiation medium was changed after the second day and every other day afterward with 0.4 ml/cm^2 differentiation medium. The cultures were prepared for immunocytochemistry on day 14. Differentiated hPSCs were fixed with 4% PFA and permeabilized with 0.1% Triton X-100 PBS solution as described above. After rinsing the fixed cells with PBS-T solution, the cells were incubated with DPBS containing 10% goat serum (Life Technologies, 10000C) for 2 hours at room temperature. Primary antibodies detecting alpha-1 Fetoprotein (Abcam, ab3980; 1:200 or R&D systems, MAB1369, 1:100), beta III tubulin (Millipore, MAB1637; 1:400) and Smooth Muscle Actin (DAKO, M0851; 1:500) were added to blocked cultures and incubated overnight at 2-8°C. The cells were rinsed twice with PBS-T, and the secondary antibody, Alexa Fluor 488-conjugated goat anti-mouse IgG(H+L) (Life Technologies, A11001; 1:1000) or Alexa Fluor 494-conjugated goat anti-mouse IgG(H+L) (Life Technologies, A-11032; 1:1000) were added and incubated on the cells for at least 2 hours at room temperature. The cultures were then rinsed three times (5 minutes each) in 1X DPBS prior to counterstaining with DAPI. The cells were maintained in 50% glycerol for analysis.

Flow cytometry

Flow cytometry of hPSCs was performed when they reached approximately 70 to 80% confluency in hPSC medium. The hPSC cultures were then dissociated into a single-cell suspension using a solution of 0.05% Trypsin/EDTA (CellGro, 25-052-CI) containing 2% chick serum (Sigma-Aldrich, C5405). The cells were fixed and permeabilized for intracellular staining with the Cytofix/Cytoperm Kit (Becton Dickinson, 554714) following the manufacturer's recommended protocol. Permeabilized cells were incubated with PE-conjugated anti-OCT3/4 (R&D Systems, IC1759P) or respective PE-conjugated anti-IgG isotype control.

Extracellular antigens were detected on unfixed cells stained with PE-conjugated antigen-specific antibodies and respective isotypes using the manufacturer's recommended concentration: anti-TRA-1-60 (Becton Dickinson, 560193), anti-TRA-1-81 (Becton Dickinson, 560161), anti-IgG3 isotype (Becton Dickinson, 556659); anti-SSEA4 (Becton Dickinson, 560128) and anti-IgM isotype (Becton Dickinson, 555584). The samples were then processed through a FACSCanto™ II flow cytometer (Becton Dickinson). Data were acquired using BD FACS Diva software and analyzed with Flowjo 7.6 software.

Immunocytochemistry

Human pluripotent stem cells were cultured in the hPSC cell culture medium. hiPSC colonies present in the cultures on days 3 through 5 were prepared for immunocytochemical analysis as follows. The culture medium was aspirated, and washed twice with 1X Dulbecco's Phosphate Buffered Saline (Lonza Biosciences, 17-513F). The cells were then fixed in 1X DPBS containing 4% PFA (Electron Microscopy Sciences, 15710) for 20 minutes, then rinsed twice with PBS-T (0.2% Tween-20 in 1X DPBS) for 5 minutes (Sigma-Aldrich, P9416) followed by a 2 hour incubation with 10% donkey serum in PBS-T at room temperature. The hPSCs were then

treated with primary antibodies detecting extracellular antigens SSEA4 (Millipore, MAB4304; 1:100), TRA-1-60 (Millipore, MAB4360; 1:100) and TRA-1-81 (StemGent, 09-0011; 1:100) overnight at 2-8°C prior to being permeabilized for 20 minutes in 1X DPBS containing 0.1% Triton X-100 (Sigma-Aldrich, T9284). A second blocking step with 10% donkey serum solution was performed before incubating the cells with intracellular primary antibodies overnight at 2-8°C. Primary antibodies raised against pluripotency-associated antigens OCT4 (Abcam, ab19857; 1:350) and NANOG (R&D Systems, AF1997; 6.7μg/ml) were used in combination with the secondary antibodies Cy3-conjugated Donkey anti-rabbit IgG (Jackson ImmunoResearch, 711-165-152; 1:200) and Cy3-conjugated donkey anti-Goat IgG (H+L) (Jackson ImmunoResearch, 805-165-180; 1:200), respectively. Primary antibodies specific for SSEA4 and TRA-1-60/TRA-1-81 were used in combination with secondary antibodies, Alexa Fluor 488-cojugated donkey anti-mouse IgG (H+L) (Jackson Immunoresearch, 715-545-150; 1:200) and Alexa Fluor 488-cojugated donkey anti-mouse IgM (H+L) (Jackson Immunoresearch, 715-545-140; 1:200), respectively. All cells were incubated with secondary antibodies for 2 hours and then counterstained with 300 nM DAPI (Life Technologies, D3571) in 1X DPBS at room temperature for 15 -30 minutes. Cells were rinsed after permeabilization and between the incubation of the primary and secondary antibodies. 50% Glycerol was immediately added to the wells after the final wash with PBS-T. All fluorescence detection was visualized using an EVOS FL all-in-one microscope equipped with software version 17625.

The immunocytochemistry and staining procedures of human pluripotent stem cells differentiated into neural lineage were as described previously (Zeng et al. 2003). Briefly, cells were fixed with 4% paraformaldehyde for 20 minutes, blocked in blocking buffer (10% goat serum, 1% BSA, 0.1% Triton X-100) for one hour followed by incubation with the primary

antibody at 4°C overnight in 8% goat serum, 1% BSA, 0.1% Triton X-100. Appropriately coupled secondary antibodies, Alexa350-, Alexa488-, Alexa546-, Alexa594- or Alexa633 (Molecular Probes, and Jackson ImmunoResearch Lab Inc., CA) were used for single or double labeling. All secondary antibodies were tested for cross reactivity and non-specific immunoreactivity.

Whole genome expression analysis

Total RNA was isolated using the RNeasy Mini kit according to the manufacturer's instructions (Qiagen, CA) and hybridized to Illumina Human HT-12 BeadChip (Illumina, Inc., CA, performed by Microarray core facility at the Burnham Institute for Medical Research). All the data processing and analysis was performed using the algorithms included with the Illumina BeadStudio software. The background method was used for normalization. The maximum expression value of gene for probe set was used as the expression value of the gene. For the processed data, the dendrogram was represented by global array clustering of genes across all the experimental samples using the complete linkage method and measuring the Euclidian distance. Expression of sample correlations was a measure of Pearson's coefficient, implemented in R System.

Assay Qualification, Characterizations, and in process control

Flow cytometry assay for pluripotent stem cells: The flow cytometry assay for evaluation of human pluripotent stem cells was qualified according to the current Good Manufacturing Practices, the International Conference on Harmonization Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) validation guidelines (O'Hara et al. 2011). The qualification study was conducted using stage-specific embryonic antigen-4 (SSEA4), TRA-1-60, and TRA-1-81. These three markers are known pluripotent stem cell associated surface

antigens widely accepted for evaluation of the identity and purity of hiPSCs in the field of pluripotent stem cells. In addition, an intracellular marker, OCT4, a transcription factor thought to play a key role in maintaining the self-renewal and pluripotency of the embryonic stem cells (Shi and Jin 2010), was also included in the qualification study. The release criteria for pluripotency markers were established based on the positive expression of four different markers (SSEA4, TRA-1-60, TRA-1-81, and OCT3/4) according to published data as well as data generated during the process development phase. Since cord blood derived CD34 positive cells were utilized as a starting material for reprogramming and generation of the final product hiPSCs, negative expression of surface marker CD34 was also included in the qualification study. Precision (intra-assay, inter-assay, and intermediate), accuracy, specificity and sensitivity of this flow cytometry assay was determined during the qualification study. The qualified flow cytometry assay with established release criteria was later used to evaluate the purity and identify of human iPSCs.

Quantitative PCR for evaluation of residual plasmid clearance: Since human induced pluripotent stem cells (iPSCs) were generated using pEB-C5 (i.e an EBNA1/OriP episomal plasmid expressing *Oct4, Sox2, Klf4, c-Myc and Lin28*) and pEB-Tg (i.e. an EBNA1/OriP plasmid for transient expression of SV40 T antigen), a quantitative PCR (qPCR) assay ("Residual qPCR") was developed to quantitatively detect residual EBNA/OriP sequences originating from either pEB-C5 or pEB-Tg. Both pEB-C5 and pEB-Tg are non-integrating plasmids that are supposed to become clear following serially passaging of hiPSCs (Chou et al. 2011; Dowey et al. 2012). The residual qPCR was carried out using the following primer sequences:

(1) Forward primer sequence: AGTGTAGGAATGAAACATTCTGAATATCTTTAACAA

(2) Reverse primer sequence: CCCATAGCCATAAATTCGTGTGAGA

(3) Reporter sequence: TCCAGTCTTTACGGCTTGTCCC

Considering the goal of assay to determine the clinical safety of the hiPSC clones generated by episomal plasmids, the residual qPCR assay was qualified according to the International Conference on Harmonization Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) validation guidelines. Accuracy, specificity, limit of detection (LOD), and limit of quantification (LOQ) were determined during the PCR qualification studies. This qualification study was executed based on 9 total assays conducted by 3 analysts on 3 separate days using a validated qPCR machine. Appropriate control positive, control negative, and reference materials were used in the qualification study.

Characterization assays: Evaluation of human iPSC colony morphology, plating efficiency of iPSCs post-thaw, and EB formation were classified as FIO assays due to the challenges associated with qualification of these assays, in particular the subjective interpretation of the results. EB formation was used to demonstrate the identity and potency of hiPSCs by investigating spontaneous differentiation into three germ layers (i.e. ectoderm, mesoderm, and endoderm) and evaluating the results through immunofluorescence at the protein level or qPCR analysis at the transcript level. Post-thaw plating efficiency was evaluated based on alkaline phosphatase (AP) staining. AP, a hydrolase enzyme responsible for dephosphorylating molecules such as nucleotides, proteins, and alkaloids under alkaline conditions, has been widely used for evaluation of undifferentiated pluripotent stem cells including both embryonic stem cells and iPSCs (Pease et al. 1990; Takahashi et al. 2007; Chin et al. 2010; Goh et al. 2013). Upon staining, the undifferentiated cells appear red or purple, whereas the differentiated cells appear

colorless. However, considering the inconsistencies observed in the quality of AP reagents offered by different suppliers and subsequent intensity of AP staining, it was difficult to set specifications and cut off values for the cells positively stained with AP marker, which in turn resulted in inability to qualify this assay.

Karyotype and Short Tandem Repeat (STR): Karyotype and STR analyses were performed by a qualified service provider (Cell Line Genetics, Inc., Madison, Wisconsin) using standard methods.

Human G-banding karyotyping was performed in accordance with FDA Good Laboratory Practice by Cell Line Genetics, which was audited by Lonza Walkersville, with clinically certified cytogeneticists experienced with identifying chromosomal abnormalities from pluripotent stem cells. For each cell line, 20 chromosomes were analyzed from live or fixed cells in metaphase. The analysis was performed using G-banding and Leishman stain, and the Cells were analyzed according to the Clinical Cytogenetics Standards and Guidelines published by the American College of Medical Genetics (Meisner and Johnson 2008).

The STR assay utilized PCR and capillary electrophoresis on a PowerPlex 16 mutiplex STR platform (Promega) to determine a match of ≥80% of the 16 loci evaluated. Data analysis was performed with SoftGenetics Genemarker software. Each assay was evaluated for off ladder peaks, considered artifacts, and cross contamination prior reporting.

MCB viral testing: According to FDA regulations, release of allogeneic MCBs for clinical use requires extensive testing for the presence of viral contaminates. The scope of the MCB viral testing for human iPSCs was adjusted based on the cellular characteristics of pluripotent stem cells and comprised of both *in vitro* and *in vivo* assays (Table S3). Following preparation of

samples per standard procedures recommended by the contract lab (BioReliance Corporation, Rockville, Maryland), samples were submitted to the BioReleiance in appropriate condition and format. BioReliance is fully accredited for GLP, and all studies conducted by BioReliance are performed in compliance with the requirements of the UK and German GLP Regulations, the US FDA Good Laboratory Practice Regulations (21 CFR 58), the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice (http://www.bioreliance.com/us/about-us).

In Process Assay: One unique aspect of current iPSC manufacturing process was to incorporate appropriate in process control assays to monitor safety, identity, and robustness of the human iPSCs. This was particularly important considering the length of manufacturing process (about 3-4 months) and the need to expand high quality human iPSCs under stringent GMP conditions. For instance, a flow cytometry assay was implemented following the isolation of CD34+ cells but before proceeding with the priming step. This step ensured appropriate population of actively proliferating CD34+ cells (i.e. a minimum of 40% CD34+ cells) were included in the expansion phase (priming step) prior to the reprogramming (Nucleofection) step. Moreover, the selection of best human iPSC colonies for expansion was based on the quality of iPSCs observed during the expansion phase as well as level of residual plasmid present in the samples taken from each human iPSC clone. A scoring system was established to evaluate the quality of iPSC cultures after isolation and throughout the course of serial subculturing of the cells based on the attachment of iPSC colonies the day after passaging, evaluation of the confluency and amount of spontaneous differentiation at each passage and every day, and elapsed time (days) per passage. Following selection and establishing the iPSC clones, in process samples were submitted at the end of each passage to detect the level of residual plasmids (i.e. residual EBNA/OriP sequences) using qPCR analysis and by recording the Ct value. The Ct values and the scores achieved for

each clone were used to evaluate the best clone(s) to use in further manufacturing, scale up and banking process.

Neural Differentiation

Generation of NSC from PSC was as previously described (Swistowski et al. 2009). In brief, NSC were derived from iPSC lines and were cultured in Neurobasal® Medium supplemented with 1% nonessential amino acids, 1% GlutaMAX^{TM}, 1 x B-27®, and 10 ng/ml bFGF (all from Life Tech., NJ), and passaged using Accutase.

Gene Targeting in Safe Harbors by ZFN/TALENs

Gene targeting by TALEN or ZFN in safe harbor site AAVS1 on Chr.19 was as previously described (Pei 2015). Specifically, 4-6 ug of a pair of TALEN RNA targeting AAVS1 site were used for co-nucleofection with 10ug donor vector AAVS1-GFP using Amaxa Human Stem Cell Nucleofection Kit (Lonza, NJ). After nucleofection, cells were plated and selected by Puromycin (Life Tech., NJ). Drug-resistant colonies were re-plated, and colonies growing from single cells that were uniformly green were selected manually under a fluorescent microscope. These selected clones were screened by PCR and sequencing to identify targets with correct donor vector integrations.

Statistics

The quantitative data are present as mean ± SEM. Comparison of the reprogramming methods and calculation of statistical significance was conducted using one-way ANOVA (analysis of variance).

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