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Supplemental Information

Platform for Induction and Maintenance of Transgene-free hiPSCs Resembling Ground State Pluripotent Stem Cells

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Inventory of Supplemental Information

Supplemental Data

Table S1. Media Formulations, related to Figure 1. Table outlining the list of ingredients for conventional culture, FRM (containing SMC4; Thiazovivin, PD0325901, CHIR99021, SB431542) and FMM.

Figure S1. Episomal induced reprogramming with FRM and FMM, related to Figure 2. (A) Day 10, SSEA4 and TRA181 flow profile of reprogramming pool. (B) Representative morphology of typical colony seen during reprogramming. Image taken at day 13 post transfection. Scale bar, 1000 µm. (C) Outline of episomal reprogrammed fibroblast cells maintained in FRM for the first 14 days, split and either maintained in FRM or switched to FMM. The reprogramming cultures were then sorted for SSEA4/TRA181/CD30 on day 21 post transfection and maintained in FRM or FMM for an additional 10 days prior to analysis. (D) Morphology and flow profile of representative cultures in FRM or FMM. White arrow points to regions of differentiated cells in a culture that consists of a mixture of undifferentiated and differentiated population. Black arrow points to sharp edges of a mostly undifferentiated population. Lower panels are representative flow profiles. FSC; Forward Side Scatter. Scale bar, 1000 µm.

Figure S2. Reprogramming of various parental lines, related to Figure 2. (A) Summary table of the starting cell lines used in this study. In addition to specific information related to each line, the percent positive SSEA4/TRA181/CD30 population at time of sort post episomal transfection is noted. (B) Illustration depicting the sort and culture of CD34 enriched umbilical cord blood cells. A volume of 0.5 ml cord blood previously maintained in a bank was used to extract 65,000 $CD34^{+}CD45^{+}Lin^{-}$ cells which were cultured in suspension for 6 days prior to episomal transfection. Scale bar, 200 µm. (C) Colony formation at day 12 post episomal transfection of FTC018. Scale bar, 1000 µm. (D) Flow cytometry profile of FTC018 on day 19

post transfection. CD30/FSC flow profile is gated off of SSEA4/TRA181 double positive quadrant.

Figure S3. Characterization during the reprogramming and maintenance process, related to Figure 2. (A) Typical colony morphology three days post single cell 96-well plate sorting. Scale bar, 400µm. (B) Representative morphology of single cell derived hiPSC-like colony 7-9 days post sort from various starting cells in 96-well plates. Scale bar, 1000 µm. (C) Immunocytochemistry for NANOG expression of hiPSC-like colonies in 96-well plates. Scale bar, 500 µm. (D) Day 16 flow profile analysis of FTC007 induced to reprogram and maintained either on Matrigel or Vitronectin coated culture plates. (E) Typical morphology of hiPSC clones 24 hours after single cell passage. Scale bar, 1000 µm. (F) Bright-field image, (G) immunofluorescence for OCT4 and NANOG and (H) flow cytomtery analysis for SSEA4 and TRA181 of FTC016-c28 maintained in FMM either continuously on Matrigel or for 5 passages on Vitronectin coated surface. Scale bar, 1000 µm for (E) and (F), 500 µm for (G).

Figure S4. Optimization of Minimal Gene Reprogramming, related to Figure 5. (A) OCT4, SOX2, NANOG and SV40LT were cloned into pCEP4 in various formats. Table represents vector systems and abbreviations. (B) Morphology of cells treated with hygromycin from day 2 to 5 post transfection with episomal construct containing hygromycin selection cassette. Note large number of floating dead cells when SV40LT was not included. Scale bar, 1000 µm. (C) Reprogramming pools were maintained for longer duration and profiled on day 16 post transfection. (D) Hygromycin kill curve analysis demonstrating cell death at 50 µM on day 5 post continuous treatment of parental line FTC007. Scale bar, 1000 µm. (E) Bright field images of cultures maintained in hygromycin selection for 5 days. In our episomal vector system (pCEP4), hygromycin-resistance gene resides in close proximity (approximately 1,500 base pair upstream) to the reprogramming genes start site. The presence of episomal DNA (integrated or non-integrated) would most likely be revealed through survival of hiPSC lines in the presence of

hygromycin. Day 21 post transfection reprogramming pool was used to demonstrate resistance to hygromycin selection as a subset of the population is anticipated to retain episomal vector during this period post transfection. Scale bar, 1000 µm. (E) Whole well image of 6 well plates containing various hiPSC populations treated with hygromycin for 5 days and stained with alkaline phosphatase. Scale bar, 2000 mm.

Figure S5. hiPSC can be cultured in multiple conditions, related to Figure 6. (A) Lentiviral derived and SMC4 maintained FTi111 displays the hallmarks of pluripotency while maintaining genomic integrity. Scale bar, 1000 µm for bright field image and 500 µm for immunofluorescence images. (B) Depiction of thaw strategy of FTi111 p43. A single vial was thawed into four culture environments as noted. Surviving cultures were passaged in respective culture with the exception of conventional culture supplemented with Thiazovivin on feeder cells, which was transitioned to conventional culture without Thiazovivin in the presence of feeder cells and passaged as clump; although direct thaw into conventional medium with feeder cells failed, we were able to recover hiPSC colonies in conventional culture by adding Thiazovivin then converting it to clump culture. (C) Morphology of recovering cells in various culture post thaw. Note that no surviving thawed single cells were identified in the conventional culture without Thiazovivin in the presence of feeder cells. Scale bar, 1000 µm. (D) Morphology of culture sets at passage 3 post thaw. Note larger colony morphology associated with conventional culture. Scale bar, 1000 µm. (E) qRT-PCR analysis for endogenous pluripotent gene expression of each culture set. Data is normalized to GAPDH and relative to H1 hESCs. Three independent experiments, SEM.

Figure S6. Gene ontology of hiPSCs in various culture conditions, related to Figure 7. (A) Table describing the derivation and maintenance of each line described in global gene expression studies. (B) A total of 300 probe sets were differentially expressed between the conventional and small molecule (FMM and SMC4) culture conditions by greater or less than

2.5-fold. Hierarchical clustering on the 300 probe sets using a complete linkage method based on Euclidean distance measurements. (C) Gene ontology biological process enrichment analysis (D.A.V.I.D.) of the 133 probe sets up-regulated 2.5-fold or greater with conventional culture (in comparison to small molecule culture). (D) Gene ontology biological process enrichment analysis (D.A.V.I.D.) of the 167 probe sets up-regulated 2.5-fold or greater with small molecule culture (in comparison to conventional culture). (E) Gene ontology biological process enrichment analysis of the 126 probe sets up-regulated 2.5-fold or greater with FMM culture (in comparison to conventional culture).

Conventional hESC Medium (Conv.)	Fate Reprogramming Medium (FRM)	Fate Maintenance Medium (FMM)
DMEM/F12	DMEM/F12	DMEM/F12
Knockout Serum Replacement (20%)	Knockout Serum Replacement (20%)	Knockout Serum Replacement (20%)
	N2 (1x)	
	B27 (1x)	
Glutamine (1x)	Glutamine (1x)	Glutamine (1x)
Non-Essential Amino Acids (1x)	Non-Essential Amino Acids (1x)	Non-Essential Amino Acids (1x)
β-mercaptoethanol (100μM)	β-mercaptoethanol (100μM)	β-mercaptoethanol (100μM)
bFGF (10ng/mL)	bFGF (100ng/mL)	bFGF (100ng/mL)
	LIF (10ng/mL)	LIF (10ng/mL)
	Thiazovivin (5.0 μM)	Thiazovivin (5.0 μM)
	PD0325901 (0.4 μM)	PD0325901 (0.4 μM)
	CHIR99021 (1.0 μM)	CHIR99021 (1.0 μM)
	SB431542 (2.0 μM)	
In combination with MEF feeder cells	Feeder free, in combination with Matrigel or Vitronectin	

Table S1

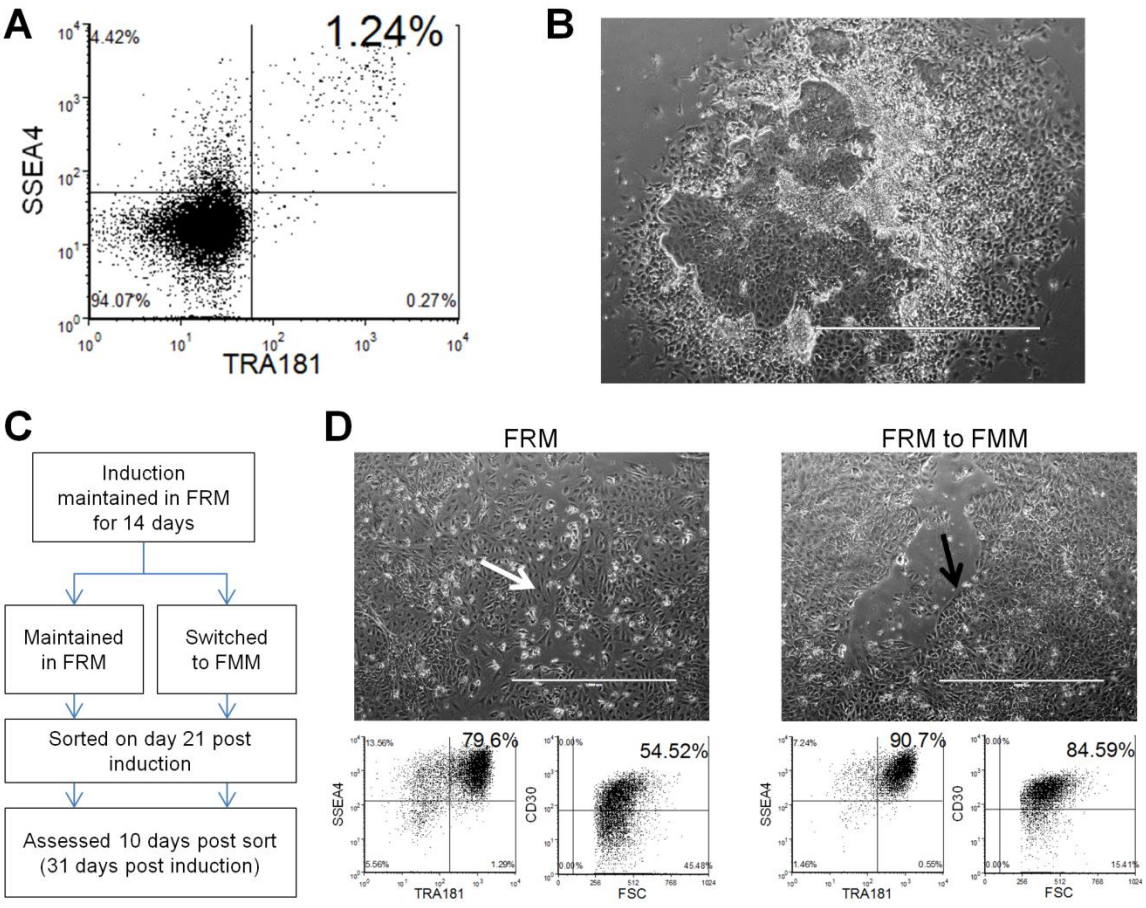
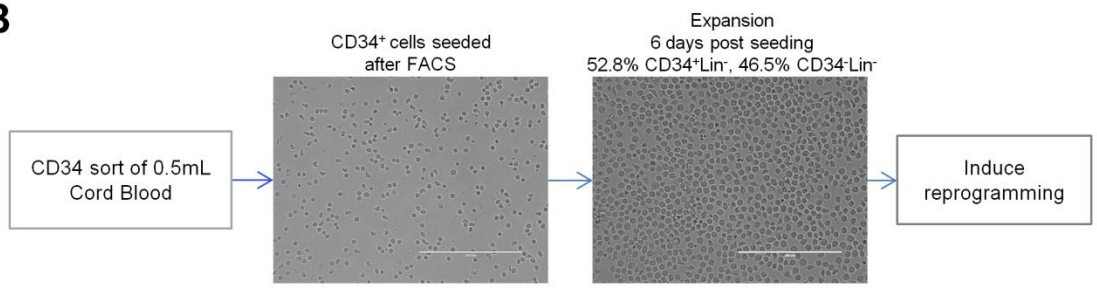

A
4.42%
1.24%
SSEA4
94.07%
0.27%
TRA181
B
C
Induction maintained in FRM for 14 days
Maintained in FRM
Switched to FMM
Sorted on day 21 post induction
Assessed 10 days post sort (31 days post induction)
D
FRM
FRM to FMM
79.6%
54.52%
90.7%
84.59%
SSEA4
CD30
TRA181
FSC

Figure S1

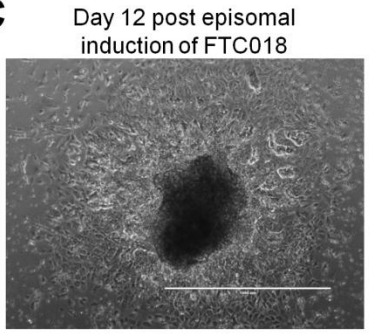
A

Nomenclature	Cell Type	Source	Ethnicity	Gender	Age	Culture Medium	Percent SSEA4/TRA181 Positive Population At Time of 96-Well Plate FACS
FTC007	Fibroblast	Neonatal Foreskin	Caucasian	Male	Neonatal	DMEM + 10%FBS	8.55% (Day 19 post induction)
FTC008	Fibroblast	Dermal Skin	Asian	Female	Adult	DMEM + 10%FBS	1.81% (Day 16 post induction)
FTC016	CD34+ Hematopoietic Cells	Cord Blood, AllCells	Pool	Pool	Neonatal	Stempro + CC110	29.3% (Day 19 post induction)
FTC017	CD34+ Hematopoietic Cells	Cord Blood, Fate derived	African American	Male	Neonatal	Stempro + CC110	41.2% (Day 21 post induction)
FTC018	CD34+ Hematopoietic Cells	Mobilized Peripheral Blood	Caucasian	Female	Adult	Stempro + CC110	10.3% (Day 19 post induction)

B



C



D

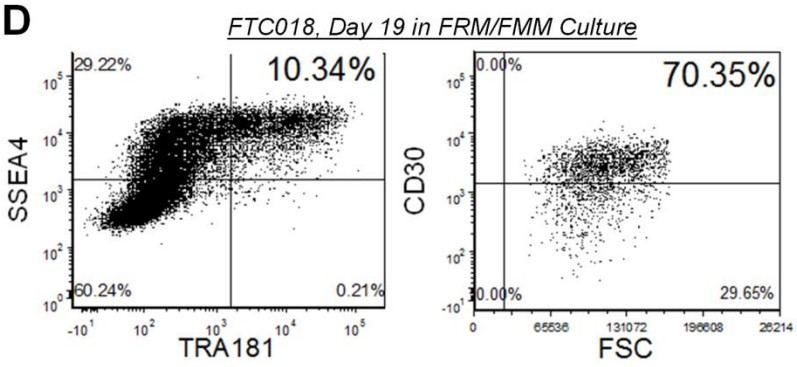


Figure S2

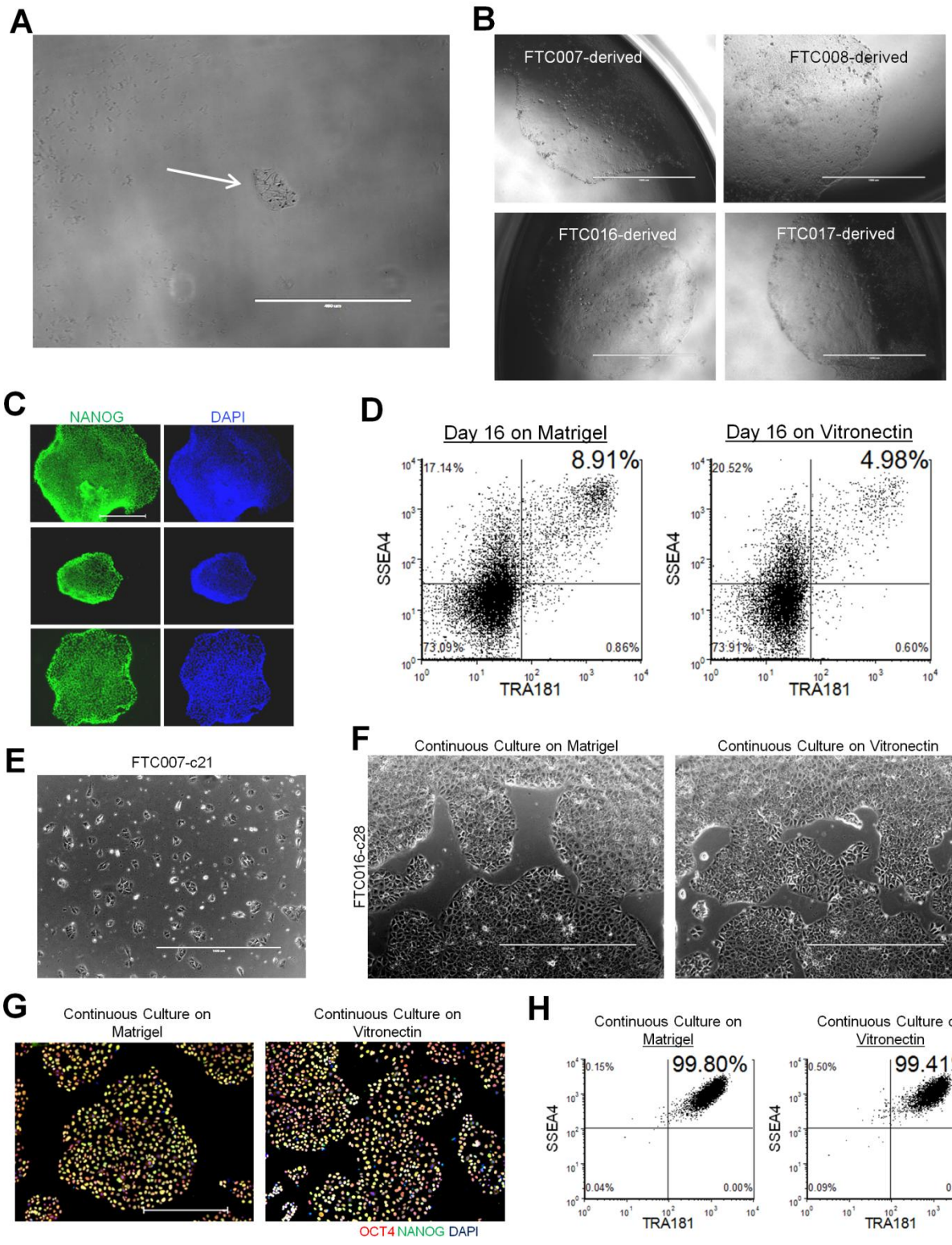

A
B
FTC007-derived
FTC008-derived
FTC016-derived
FTC017-derived
C
NANOG
DAPI
D
Day 16 on Matrigel
Day 16 on Vitronectin
8.91%
4.98%
17.14%
20.52%
73.09%
73.91%
0.86%
0.60%
SSEA4
TRA181
E
FTC007-c21
F
Continuous Culture on Matrigel
Continuous Culture on Vitronectin
FTC016-c28
G
Continuous Culture on Matrigel
Continuous Culture on Vitronectin
OCT4 NANOG DAPI
H
Continuous Culture on Matrigel
Continuous Culture on Vitronectin
99.80%
99.41%
0.15%
0.50%
0.04%
0.09%
0.00%
0.00%
SSEA4
TRA181

Figure S3

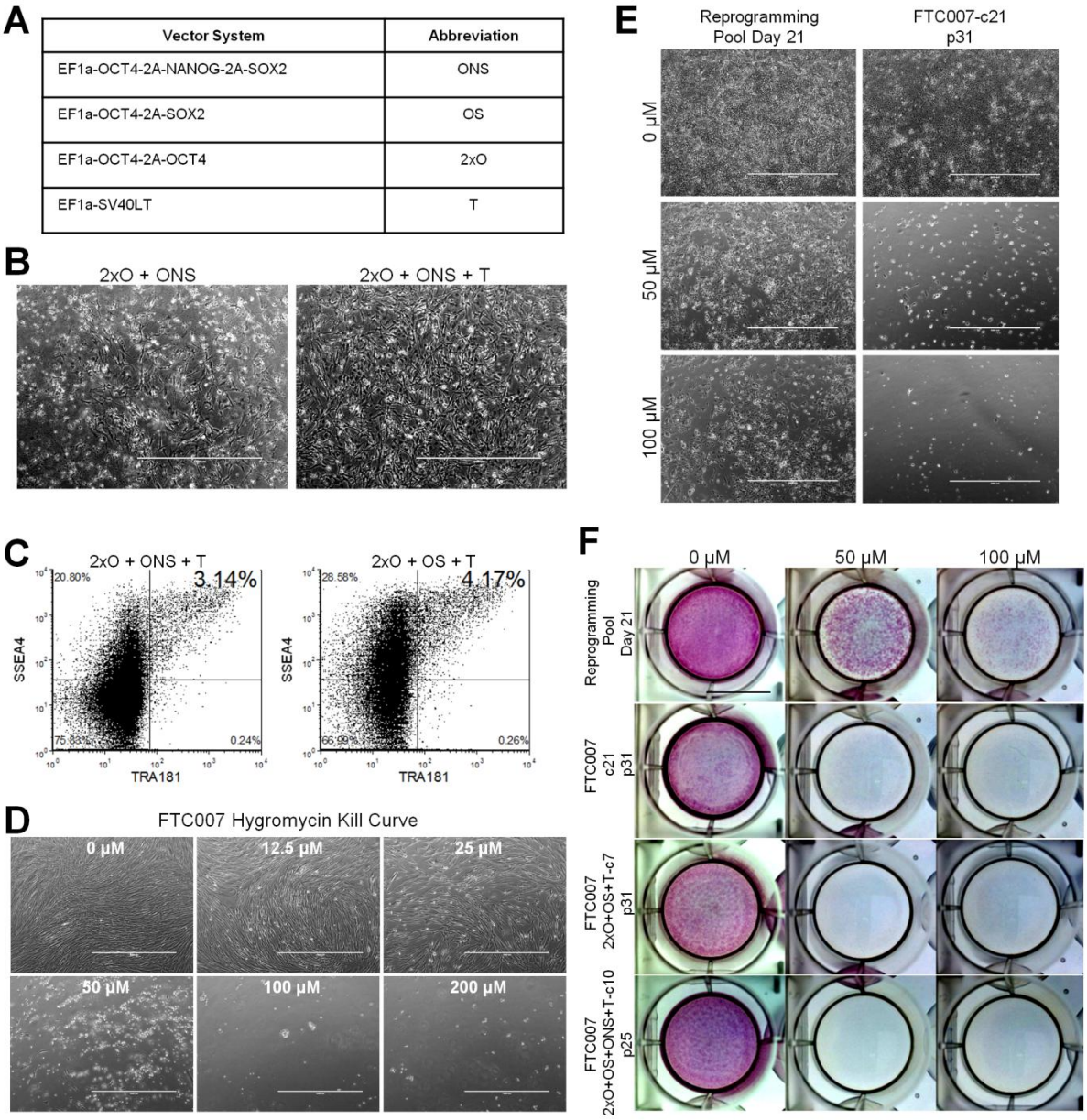

A
Vector System
Abbreviation
EF1a-OCT4-2A-NANOG-2A-SOX2
ONS
EF1a-OCT4-2A-SOX2
OS
EF1a-OCT4-2A-OCT4
2xO
EF1a-SV40LT
T
B
2xO + ONS
2xO + ONS + T
C
2xO + ONS + T
3.14%
20.80%
75.83%
0.24%
2xO + OS + T
4.17%
28.58%
66.99%
0.26%
SSEA4
TRA181
D
FTC007 Hygromycin Kill Curve
0 µM
12.5 µM
25 µM
50 µM
100 µM
200 µM
E
Reprogramming Pool Day 21
FTC007-c21 p31
0 µM
50 µM
100 µM
F
0 µM
50 µM
100 µM
Reprogramming Pool Day 21
FTC007 c21 p31
FTC007 2xO+OS+T-c7 p31
FTC007 2xO+OS+ONS+T-c10 p25

Figure S4

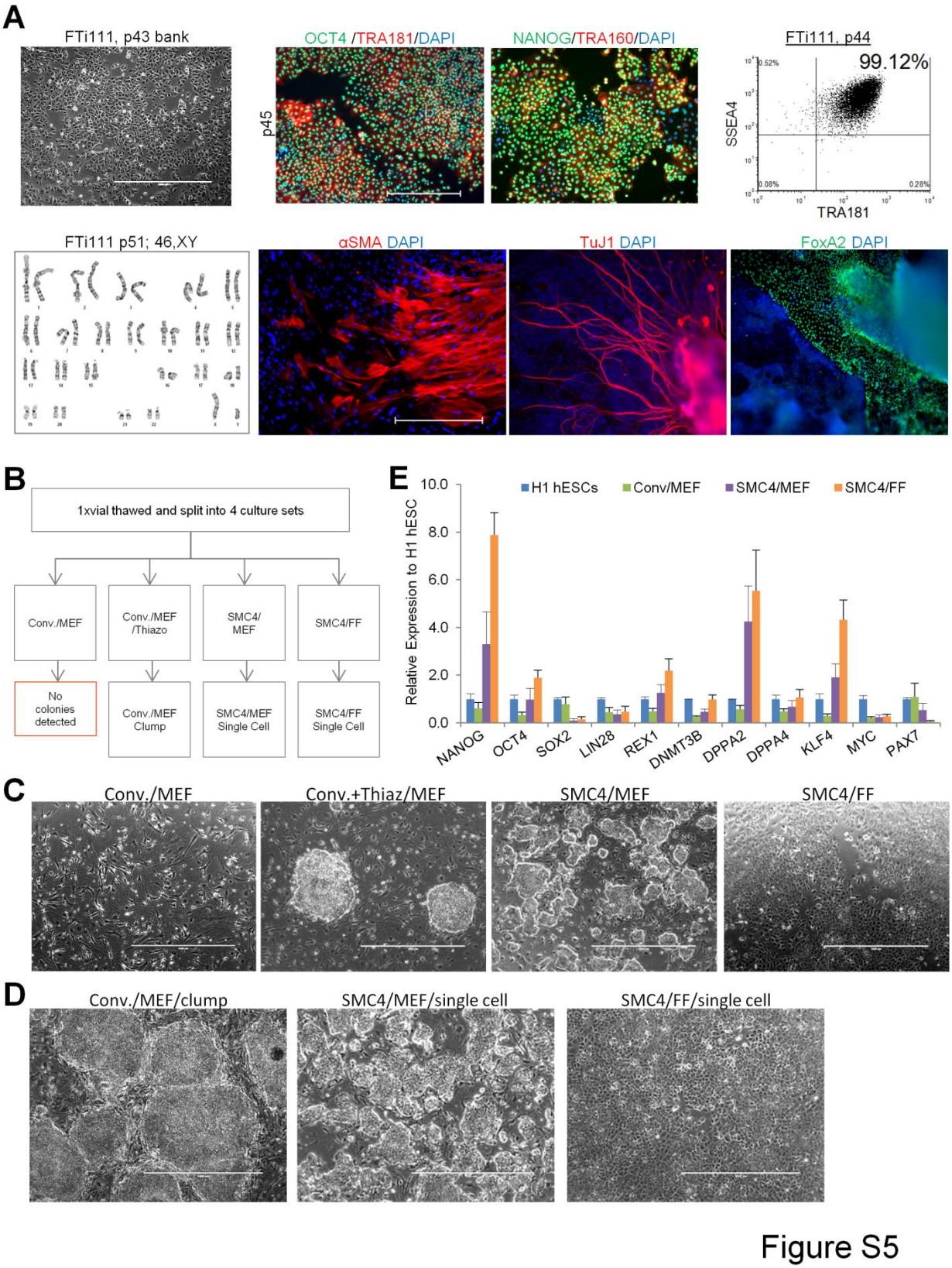
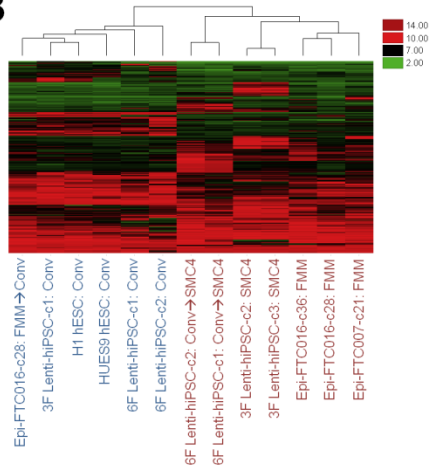

A
FTi111, p43 bank
OCT4 /TRA181/DAPI
NANOG/TRA160/DAPI
p45
FTi111, p44
99.12%
SSEA4
TRA181
FTi111 p51; 46,XY
αSMA DAPI
TuJ1 DAPI
FoxA2 DAPI
B
1xvial thawed and split into 4 culture sets
Conv./MEF
Conv./MEF /Thiazo
SMC4/ MEF
SMC4/FF
No colonies detected
Conv./MEF Clump
SMC4/MEF Single Cell
SMC4/FF Single Cell
E
H1 hESCs
Conv/MEF
SMC4/MEF
SMC4/FF
Relative Expression to H1 hESC
10.0
8.0
6.0
4.0
2.0
0.0
NANOG
OCT4
SOX2
LIN28
REX1
DNMT3B
DPPA2
DPPA4
KLF4
MYC
PAX7
C
Conv./MEF
Conv.+Thiaz/MEF
SMC4/MEF
SMC4/FF
D
Conv./MEF/clump
SMC4/MEF/single cell
SMC4/FF/single cell

Figure S5

A

Cell Line	Description		Cell Line	Description
FTC016-c28 & -c36	Cord blood parental, episomal-generated and maintained in FRM/FMM		6F Lenti-hiPSC-c1, -c2 & -c3	Fibroblast parental, OSKMNL lentiviral hiPSC clone generated and maintained in conventional hESC culture
FTC007-C21	Fibroblast parental, episomal-generated and maintained in FRM/FMM		3F Lenti-hiPSC-c1 & -c4	Fibroblast parental, OKS lentiviral hiPSC clone generated and maintained in conventional hESC culture
3F Lenti-hiPSC-c2 & -c3	Fibroblast parental, OKS lentiviral hiPSC clone generated and maintained in SMC4			

B



C

133 Probe sets upregulated in conventional hESC culture group vs. FMM/SMC4 culture group			
GO ID	**GO Term**	**%**	**p-value**
GO:0007389	pattern specification process	9.7	3.50E-05
GO:0048598	embryonic morphogenesis	8.6	5.90E-04
GO:0001775	cell activation	7.5	2.30E-03
GO:0046649	lymphocyte activation	6.5	2.50E-03
GO:0042110	T cell activation	5.4	3.00E-03
GO:0002521	leukocyte differentiation	5.4	3.40E-03
GO:0030217	T cell differentiation	4.3	3.60E-03
GO:0030097	hemopoiesis	6.5	5.10E-03
GO:0045321	leukocyte activation	6.5	5.70E-03
GO:0007610	behavior	8.6	6.50E-03
GO:0048534	hemopoietic or lymphoid organ development	6.5	7.60E-03
GO:0048568	embryonic organ development	5.4	8.90E-03
GO:0002520	immune system development	6.5	9.80E-03

D

167 Probe sets upregulated in small molecule culture group vs. conventional culture group			
GO ID	**GO Term**	**%**	**p-value**
GO:0042127	regulation of cell proliferation	12.4	5.20E-04
GO:0051205	protein insertion into membrane	2.5	1.50E-03
GO:0009725	response to hormone stimulus	7.4	2.60E-03
GO:0051668	localization within membrane	2.5	4.20E-03
GO:0009719	response to endogenous stimulus	7.4	4.80E-03
GO:0008284	positive regulation of cell proliferation	7.4	5.50E-03
GO:0008406	gonad development	4.1	6.10E-03
GO:0009636	response to toxin	3.3	7.30E-03
GO:0014070	response to organic cyclic substance	4.1	8.00E-03
GO:0043066	negative regulation of apoptosis	6.6	8.20E-03
GO:0048545	response to steroid hormone stimulus	5	8.30E-03
GO:0008585	female gonad development	3.3	8.30E-03
GO:0043069	negative regulation of programmed cell death	6.6	8.80E-03
GO:0060548	negative regulation of cell death	6.6	9.00E-03
GO:0048608	reproductive structure development	4.1	9.20E-03
GO:0045137	development of primary sexual characteristics	4.1	9.40E-03
GO:0046660	female sex differentiation	3.3	1.00E-02
GO:0046545	development of primary female sexual characteristics	3.3	1.00E-02

E

126 Probe sets upregulated in FMM samples group vs. conventional culture group			
GO ID	**GO Term**	**%**	**p-value**
GO:0007267	cell-cell signaling	16.5	8.20E-07
GO:0040008	regulation of growth	10.3	1.10E-04
GO:0042981	regulation of apoptosis	15.5	1.20E-04
GO:0043067	regulation of programmed cell death	15.5	1.30E-04
GO:0010941	regulation of cell death	15.5	1.30E-04
GO:0010817	regulation of hormone levels	7.2	1.90E-04
GO:0045926	negative regulation of growth	6.2	3.60E-04
GO:0031100	organ regeneration	4.1	3.90E-04
GO:0001558	regulation of cell growth	7.2	7.10E-04
GO:0043066	negative regulation of apoptosis	9.3	7.40E-04
GO:0043069	negative regulation of programmed cell death	9.3	8.20E-04
GO:0060548	negative regulation of cell death	9.3	8.30E-04
GO:0016358	dendrite development	4.1	9.40E-04
GO:0006916	anti-apoptosis	7.2	9.70E-04
GO:0045596	negative regulation of cell differentiation	7.2	1.20E-03
GO:0030308	negative regulation of cell growth	5.2	1.70E-03
GO:0050768	negative regulation of neurogenesis	4.1	2.00E-03
GO:0045792	negative regulation of cell size	5.2	2.20E-03
GO:0010721	negative regulation of cell development	4.1	2.40E-03
GO:0042445	hormone metabolic process	5.2	2.80E-03
GO:0032535	regulation of cellular component size	7.2	3.90E-03
GO:0034754	cellular hormone metabolic process	4.1	4.30E-03
GO:0051270	regulation of cell motion	6.2	4.30E-03
GO:0048754	branching morphogenesis of a tube	4.1	5.60E-03
GO:0008361	regulation of cell size	6.2	5.70E-03
GO:0001569	patterning of blood vessels	3.1	5.90E-03
GO:0031099	regeneration	4.1	6.60E-03
GO:0035295	tube development	6.2	7.50E-03
GO:0009991	response to extracellular stimulus	6.2	7.50E-03
GO:0001763	morphogenesis of a branching structure	4.1	8.00E-03
GO:0006917	induction of apoptosis	7.2	8.60E-03
GO:0012502	induction of programmed cell death	7.2	8.70E-03

Figure S6

Supplemental Experimental Procedures

hiPSC Maintenance in Conventional Culture System

Conventionally cultured hiPSCs were routinely maintained on mitomycin C treated MEF (Millipore) feeder cells and cultured with conventional medium (referred to as conventional medium in the text) containing DMEM/F12 (Mediatech), 20% v/v knockout serum replacement (Life Technologies), 1% v/v non-essential amino acids (Mediatech), 2 mM L-glutamine (Mediatech), 100 μM β-mercaptoethanol (Life Technologies) and 10 ng/mL bFGF (Life Technologies). Upon confluency, conventionally cultured hiPSCs were enzymatically dissociated using 1 mg/mL collagenase IV (Life Technologies) for 7 min at 37°C followed by mechanical dissociation into small pieces (termed as clump passaging), collected and dilute passaged 1:3–1:4 onto freshly seeded feeder cells every 5–7 days with daily addition of conventional medium. In case of excessive spontaneous differentiation, undifferentiated colonies were manually picked and cut into small pieces using the tip of Insulin Syringe (Becton Dickinson) and transferred to freshly seeded feeder cells. Cell cultures were maintained in a humidified incubator set at 37°C and 5% CO_2.

Reprogramming of Somatic Cells

To initiate reprogramming, ectopic expression of reprogramming factors were induced by lentiviral transduction or episomal vector transfection. Lentiviral transfection was followed as previously described (Valamehr et al., 2012). Briefly, the starting cells were plated at $1x10^5$ cells per well of a 6-well plate on Matrigel (BD Biosciences) coated surface. Unless specified, all Matrigel coatings consists of adding Matrigel solution (one aliquot of Matrigel resuspended in 25 mL DMEM/F12) to tissue culture surfaces and allowing for 2-4 hrs incubation at 37°C. Supernatant from 293T cells generating lentivirus expressing transgene *OCT4/SOX2/KLF4* was added to the starting cells at a dilution of 1:2 (one part lentiviral supernatant : one part fibroblast

medium), supplemented with 4 µg/mL polybrene (Millipore), and transferred to 37°C and 5% CO_2 for 12-16 hrs. Fibroblast medium: DMEM (Mediatech), 10% FBS (Life Technologies), 1x glutamax (Life Technologies), 1x non-essential amino acids (Mediatech). After incubation with lentivirus, the cells were washed three times with PBS and fed with fibroblast medium. 48hrs post transfection, the culture medium was switched to 50/50 medium containing one part FRM (or SMC4) and one part fibroblast medium. The medium was completely switched to FRM (or SMC4) once the culture was passaged into a larger vessel, usually between days 4 to 6 post infection. Passaging consists of dissociation with Accutase (Millipore) onto Matrigel coated surface. Cultures were maintained in FRM (or SMC4) until the next application.

For episomal vector reprogramming, transfection of fibroblast or cord blood cells using gene set OCT4/SOX2/NANOG/KLF4/LIN28/MYC/SV40LT (A14703, Life Technologies) was conducted using NEON Transfection System (Life Technologies). Approximately, 4 µg of vector set was transfected into $5x10^5$ fibroblast cells or $2.5x10^5$ cord blood cells using settings 1650v/10ms/3pulses in appropriate buffers as described by product manual. The transfected cells were plated directly into a 10 cm dish (fibroblast) or a well of 6-well plate (cord blood) coated with Matrigel and containing either fibroblast culture medium or cord blood culture medium (depending on the cell type) supplemented with 10 ng/mL bFGF and 5 µg/mL fibronectin (BD Biosciences). Cord blood culture medium: SFMII + CC110 (Stem Cell Technologies). Twenty-four hours post transfection, FRM was added to the culture in equal volume. For fibroblast cultures, forty-eight hours post transfection 50 µg/mL hygromycin (Mediatech) was added to the culture. The culture medium was switched to entirely FRM on day 5 with hygromycin removed on day 7 post transfection. All reprogramming cultures were switched to FMM on day 14 post transfection. For cord blood cultures, twenty-four hours post transfection, FRM was added in equal volume and continuously added every few days until day 14 post transfection where the culture was aspirated and replaced with entirely FMM. In both cases, cluster of adherent rounded cells were seen around days 5 to 7 post transfection. Once

in FMM all reprogramming cultures were maintained and single cell passaged using Accutase on either Matrigel or Vitronectin coated surface. The single cell dissociated cells were expanded onto Matrigel coated plates with FMM and maintained until flow cytometry sorting. In Vitronectin (Life Technologies) surface coating studies, all aspects were kept the same except for the substitution of Matrigel for Vitronectin. For reduced factor episomal reprogramming, pCEP4 (Life Technologies) vector backbone was constructed to contain *OCT4-P2A-OCT4*, *OCT4-P2A-SOX2* or *OCT4-P2A-NANOG-T2A-SOX2* under the regulation of EF1α promoter. The transfection of reduced factor episomal vectors followed the same protocol as described above with the exception of few modifications. EBNA was co-transfected as either EBNA mRNA (20 µg) or vector cassette (2 µg) (Howden et al., 2006). Hygromycin selection was maintained for 10 days and FMM was introduced on day 16.

Flow Cytometry Analysis and Sorting

Single cell dissociated (described above) reprogramming pools were resuspended in chilled staining buffer containing Hanks' Balanced Salt Solution (MediaTech), 4% fetal bovine serum (Invitrogen), 1x penicillin/streptomycin (Mediatech) and 10 mM Hepes (Mediatech); made fresh for optimal performance. Conjugated primary antibodies, including SSEA4-FITC, TRA181-Alexa Fluor-647 and CD30-PE (BD Biosciences), were added to the cell solution and incubated on ice for 15 min. All antibodies were used at 7-10 µL in 100 µL staining buffer per million cells. The solution was washed once in staining buffer, spun down at 225 g for 4min and resuspended in staining buffer containing 10 µM Thiazovivn and maintained on ice for flow cytometry sorting. Flow cytometry sorting was performed on FACS Aria II (BD Biosciences) using gating strategy described in the Results section. The sorted cells were directly ejected into 96-well plates using the 100 µM nozzle, at concentrations of 3 and 9 events per well. Sorting 3 cells per well was our preferred concentration as we noticed that events sorted did not necessarily correlate to actual number of cells seen in each well post sort and that 3 cells per well gave us a preferred

number of wells containing individual colonies. Each well was prefilled with 200 µL FMM supplemented with 5 µg/mL fibronectin and 1x penicillin/streptomycin (Mediatech) and previously coated overnight with 5x Matrigel. 5x Matrigel precoating includes adding one aliquot of Matrigel into 5 mL of DMEM/F12, then incubated overnight at 4°C to allow for proper resuspension and finally added to 96-well plates at 50 µL per well followed by overnight incubation at 37°C. The 5x Matrigel is aspirated immediately before the addition of media to each well. Upon completion of the sort, 96-well plates were centrifuged for 1-2 min at 225 g prior to incubation. The plates were left undisturbed for seven days. On the seventh day, 150 µL of medium was removed from each well and replaced with 100 µL FMM. Wells were refed with an additional 100 µL FMM on day 10 post sort. Colony formation was detected as early as day 2 and most colonies were expanded between days 7-10 post sort. In the first passage, wells were washed with PBS and dissociated with 30 µL Accutase for approximately 10 min at 37°C. The need for extended Accutase treatment reflects the compactness of colonies that have sat idle in culture for prolonged duration. After cells are seen to be dissociating, 200 µL of FMM is added to each well and pipetted several times to break up the colony. The dissociated colony is transferred to another well of a 96-well plate previously coated with 5x Matrigel and then centrifuged for 2 min at 225 g prior to incubation. This 1:1 passage is conducted to spread out the early colony prior to expansion. Subsequent passages were done routinely with Accutase treatment for 3-5 min and expansion of 1:4-1:8 upon 75-90% confluency into larger wells previously coated with 1x Matrigel in FMM. Flow cytometry analysis was performed on Guava EasyCyte 8 HT (Millipore) and analyzed using FCS Express 4 (De Novo Software).

Real-Time RT-PCR and Fluidigm Analysis

Total RNA was isolated using Pico Pure RNA Isolation Kit (Life Technologies). Complimentary DNA (cDNA) was reverse transcribed from 100 ng of isolated total RNA using the iScript cDNA Synthesis Kit (Bio-Rad). The cDNA was then used for pre-amplification of specific target genes

and two reference control genes using the TaqMan PreAmp Master Mix Kit (Life Technologies) and a 0.2x concentration of pooled TaqMan assays. Specific target amplification (STA) from cDNA was performed using 14 cycles of amplification with the standard cycling conditions stated in the manufacturer's protocol. The pre-amplified cDNA reactions (n=48) were diluted 1:5 (in sterile water) and used as template for the real-time quantitative PCR reactions. 48.48 Dynamic arrays (Fluidigm) were loaded using a NanoFlex IFC Controller MX (Fluidigm) with TaqMan assays loaded in duplicate and real-time reactions were performed using a BioMark Real-Time PCR System (Fluidigm). Results were analyzed using BioMark Real-Time PCR Analysis software (Fluidigm). Samples with cycle thresholds (Cts) above 32 were excluded from the calculations. In case of hESC control analysis, assay replicates were used to determine SEM. Average Cts were calculated using the mean of two reference genes (GAPDH and HPRT1) against the median of six control MEF cell lines (OSK hiPSCs on MEF and H1 ESCs). Relative gene expression results are displayed in Excel (Microsoft) in heat map format.

FAM-labeled TaqMan probes

Assay ID	Catalog Number (Life Technologies)	Gene Symbol	RefSeq
Hs00232764_m1	4331182	*FOXA2*	NM_021784.4;NM_153675.2
Hs00173490_m1	4331182	*AFP*	NM_001134.1
Hs00171403_m1	4331182	*GATA4*	NM_002052.3
Hs00751752_s1	4331182	*SOX17*	NM_022454.3
Hs00610080_m1	4331182	*T*	NM_003181.2
Hs00607978_s1	4331182	*CXCR4*	NM_003467.2;NM_001008540.1
Hs00415443_m1	4331182	*NODAL*	NM_018055.4
Hs02330075_g1	4331182	*MYOD1*	NM_002478.4
Hs00240871_m1	4331182	*PAX6*	NM_001127612.1
Hs00801390_s1	4331182	*TUBB3*	NM_001197181.1;NM_006086.3
Hs00374280_m1	4331182	*STAT3*	NM_139276.2;NM_213662.1;NM_003150.3
Hs04260366_g1	4331182	*NANOG*	NM_024865.2
Hs00602736_s1	4331182	*SOX2*	NM_003106.3
Hs00399279_m1	4331182	*ZFP42*	NM_174900.3
Hs01003405_m1	4331182	*DNMT3B*	NM_001207055.1;NM_001207056.1;NM_006892.3;NM_175848.1;NM_175850.2;NM_175849.1
Hs00702808_s1	4331182	*LIN28A*	NM_024674.4
Hs99999003_m1	4331182	*MYC*	NM_002467.4
Hs01081364_m1	4331182	*DNMT3L*	NM_013369.2;NM_175867.1
Hs00360439_g1	4331182	*KLF2*	NM_016270.2
Hs00222238_m1	4331182	*OTX2*	NM_172337.1;NM_021728.2
Hs00242962_m1	4331182	*PAX7*	NM_001135254.1;NM_002584.2;NM_013945.2
Hs00414521_g1	4331182	*DPPA2*	NM_138815.3
Hs00216968_m1	4331182	*DPPA4*	NM_018189.3
Hs99999905_m1	4331182	*GAPDH*	NM_002046.4
Hs01003267_m1	4331182	*HPRT1*	NM_000194.2
Custom-made TaqMan Gene Expression Assays			
Gene	Forward Primer	Reverse Primer	
OCT4	GGGTTTTTGGGATTAAGTTCTTCA	GCCCCCACCCTTTGTGTT	
KLF4	AGCCTAAATGATGGTGCTTGGT	TTGAAAACTTTGGCTTCCTTGTT	

Testing Presence of Transgenes

Genomic DNA was isolated using QIAamp® DNA Mini Kit and Proteinase K digestion (Qiagen). 100 ng of the genomic DNA was amplified using transgene-specific primer sets (Table 2 below) (Yu et al., 2007) using Taq PCR Master Mix Kit (Qiagen). The PCR reactions were run for 35 cycles as follows: 94°C for 30 sec (denaturation), 60-64°C for 30 sec (annealing) and 72°C for 1 min (extension). Genomic DNA from fibroblasts and hiPSCs generated using lentiviral methods were used as negative controls. DNA of the episomal constructs was used as positive control.

Transgene specific primer sets

Amplified region	Forward	Reverse
Oct4-Oct4 region of episomal transgene	CAGGCCCGAAAGAGAAAGCG	GGAGGGCCTTGGAAGCTTAG
Oct4-NANOG region of episomal transgene	TATACACAGGCCGATGTGGG	TTGACCGGGACCTTGTCTTC
OCT4-SOX2 region of episomal transgene	GTGGTCCGAGTGTGGTTCTG	GTTCTCCTGGGCCATCTTGC
Lin28-SV40pA episomal transgene	AAGCGCAGATCAAAAGGAGA	CCCCCTGAACCTGAAACATA
WPRE lentiviral element	TGCTTCCCGTATGGCTTTC	AAAGGGAGATCCGACTCGTCTG
EBNA1	ATCGTCAAAGCTGCACACAG	CCCAGGAGTCCCAGTAGTCA
Human *GAPDH*	GTGGACCTGACCTGCCGTCT	GGAGGAGTGGGTGTCGCTGT

Immunocytochemistry Analysis

Cells were fixed using 4% v/v paraformaldehyde (Alfa Aesar), washed three times with PBS containing 0.2% v/v Tween (PBST) (Fisher Scientific) and permeablized using 0.15% v/v TritonX-100 (Sigma-Aldrich) in PBS for 1 hr at 25°C. After permeabilization, cells were blocked with 1% v/v BSA (Sigma) in PBST (PBSTB) (Fisher Scientific) for 30 min at 25°C. After gentle removal of PBSTB, cells were incubated with primary antibody in PBSTB overnight at 4°C. Primary antibodies used in this study include OCT4 (Santa Cruz), NANOG (Santa Cruz), TRA160 (Millipore), TRA181 (Millipore), SSEA4 (Millipore), β-III Tubulin (TUJ1, R&D Systems), α-Smooth Muscle Actin (Sigma), FoxA2 (R&D Systems), Sox17 (R&D Systems), NESTIN (Abcam) and Alpha-1-Fetoprotein (Dako). After the overnight incubation, cells were washed three times with PBST and stained with secondary antibody (Alexa Fluor 488 or 555; Invitrogen) diluted 1:250 in PBSTB for 1 hr at 37°C. The cells were washed three times in PBST and stained with Hoechst dye (Invitrogen). For H3K27me3 staining analysis, hiPSCs were grown 72 to 96 hrs on cover slips and fixed with 4% parafomaldehyde (Electron Microscopy Science, EMS) in PBS for 15 min at 25°C. Cell permeabilization was performed with 0.1% Triton X-100 in PBS for 1 hour at 25°C, and then cells were incubated with blocking solution (1% BSA in PBS) for 30 min at 25°C. After blocking, cover slips were incubated with 1:1600 dilution of anti-trimethyl-histone H3 (Lys27) antibody (Millipore 07-449, H3K27me3) in blocking solution, overnight at 4°C. Secondary antibodies were Alexa Fluor 555 Goat-anti-Rabbit IgG (Life Technologies, A21429). The nuclei were counterstained with DAPI and viewed with an Axio Observer Inverted Microscope (Carl Zeiss). Images were captured with the AxioVS40 v4.8.1.0 (Carl Zeiss Imaging Solutions Gmbh).

Differentiation Analysis (EB and Directed)

hiPSC were differentiated as EBs in differentiation medium containing DMEM/F12 (Mediatech), 20% fetal bovine serum (Invitrogen), 1% non-essential amino acids (Mediatech), 2 mM L-glutamine (Mediatech) and 100 μM β-mercaptoethanol. Briefly, for EB formation hiPSCs were seeded in FMM and switched to conventional the following day to prime the cells. After 3 to 4 days in conventional medium, cultures were single cell dissociated with Accutase (Millipore) and resuspended in differentiation medium including 10 μM Y27632 to a final concentration of 100,000 cells/mL. Note that ROCK inhibitor Y27632 instead of Thiazovivn is used for EB formation. Cells were seeded at 100 μL/well in V-bottom 96-well non-tissue culture plate (Nunc) and centrifuged at 950 g for 5 min. The following day compact “ball-like clumps” were transfer to ultra-low binding 6-well plate (Corning) using P1000 at approximately 30–40 EBs/well in differentiation medium. After 7 days, EBs were transferred at 1:1 to Matrigel coated 6-well plate and fed with differentiation medium every three days. After 3 weeks in culture, cells were fixed and stained. For directed monolayer differentiation, hiPSCs were seeded on Matrigel coated wells in FMM to deliver 50% and 90% confluency the following day. Both densities were induced to differentiate. For neural induction (Lee et al., 2007), FMM media was replaced with hESC media supplemented with 10 μM SB431542 and 100 nM LDN-193189 (both SMAD inhibitors, Biovision). Following 2 days, differentiation media with supplemented with 3 μM CHIR99021 (Biovision) in addition to the dual SMAD inhibitors. Cells were fixed two days later and stained for Nestin (Abcam). For mesoderm differentiation, media was replaced with RPMI (Mediatech) supplemented with 1x B27 media additive (Life Technologies), 3 μM CHIR99021, 4 ng/ml bFGF and 10 ng/ml BMP4. Media changed every other day and cells fixed on the 4th day and stained for αSMA (Sigma). Endoderm differentiation was performed using the Human Pluripotent Stem Cell Functional Identification Kit (R&D Systems). hiPSCs were incubated with endoderm differentiation media for 3 days, fixed and stained for SOX17 (R&D Systems).

Gene Expression Analysis

RNA was extracted using the PicoPure RNA Isolation kit (Life Technologies) using the manufacturers recommended protocol. Total RNA was quantified using the Nanodrop 2000 Spectrophotometer (Thermo Scientific). In brief, biotinylated aRNA was prepared from roughly 100 ng of total RNA using the standard protocol for MessageAmp II aRNA Amplification Kit (Applied Biosystems/Ambion, Austin, TX) utilizing the optional Second Round Amplification and then transcribed into biotin labeled aRNA using MessageAmp II Biotin Enhanced Kit (Applied Biosystems/Ambion, Austin, TX) using the standard protocol. Biotin labeled aRNA was purified and fragmented according to Affymetrix recommendations. 20 µg of fragmented aRNA were used to hybridize to the Human Genome U133-plus-2.0 chips (Affymetrix Inc. Santa Clara, CA) for 16 hours at 45°C. The arrays were washed and stained in the Affymetrix Fluidics Station 450 and scanned using the Affymetrix GeneChip Scanner 3000 7G. Raw expression data files are available on Gene Expression Omnibus (GSE50868). The image data were analyzed using Affymetrix Expression Console software using default analysis settings. Arrays were normalized by log scale robust multi-array analysis (RMA, Affymetrix) and visualized in Spotfire for Genomics 4.5 (Tibco Spotfire, Palo Alto, CA). Biological pathway enrichment analysis of the differentially expressed probes was performed against the Gene Ontology (GO) database (Singular Enrichment to GO Biological Process and p-value < 0.01) using Database for Annotation, Visualization and Integrated Discovery (DAVID v6.7) (Huang da et al., 2009a, b). Hierarchical clustering was performed to compare the gene expression profiles between samples based on Log2 expression levels using a complete linkage clustering method with Euclidean distance measurements (Spotfire for Genomics 4.5). Probe sets for clustering were selected by either an overall differential in expression levels (> or < 2.5-fold) or presence on targeted gene lists defining a ground or metastable state. For X Chromosome gene expression comparison, RMA normalized Affymetrix gene chip probe set intensities were converted to linear expression values by taking the 2^[RMA log2 intensity]. Linear expression ratios were

calculated as the naïve expression set divided by the primed expression set. The expression ratios for all 1688 probe sets mapped to the X chromosome were visualized in Spotfire 4.5 with the probe sets greater or less than 2 fold enrichment ratio highlighted.

Microarray (aCGH+SNP) Analysis

High resolution array comparative genomic hybridization + single nucleotide polymorphism (Agilent SurePrint G3 Human Genome CGH+SNP Microarray Kit, 4x180K; NCBI Build 37/hg19) and subsequent copy number variation and loss of heterozygosity analysis was conducted by WiCell Cytogenetics (Madison,WI). Relative copy number and regions of homozygosity was determined by comparative differential hybridization of labeled genomic DNA to the 180,000 oligonucleotide whole genome tiling array.

Alkaline Phosphatase Staining

Cells were fixed in 4% v/v paraformaldehyde (Alfa Aesar), washed three times with PBS and stained with Alkaline Phosphatase Staining Kit (Millipore). Briefly, two parts Fast Red Violet, one part Naphtol AS-BI Phosphaste and one part water were mixed, added to the fixed cells and incubated at 25°C for 15 min followed by a PBS wash.

Karyotype Analysis

Cytogenetic analysis was performed on G-banded metaphase cells by WiCell Research Institute (Madison, WI). Each karyotype analysis includes a minimum count of 20 spreads with analyses expanded to 40 spread counts when nonclonal aberrations are identified in the first 20.

Teratoma Formation

Single cell dissociated hiPSCs, at concentrations of 0.5 and 3 million cells per 200 µL solution (100 µL FMM and 100 µL Matrigel) were injected subcutaneously into NOD/SCID/γ^{null} mice. After 5-6 weeks (3 million cells injection) and 7-8 weeks (0.5 million cells injection), teratomas

were harvested in PBS, fixed overnight at room temperature in 4% paraformaldehyde and maintained thereafter in 70% ethanol at room temperature for processing. Samples were submitted to UCSD Histology Core Facility for sectioning and hematoxylin and eosin staining. Sections were examined, interpreted and photographed using a Nikon Eclipse TS100 microscope equipped with a Nikon DS-Fi1 camera.

Statistical Analysis

At least three independent experiments were performed. Values are reported as mean $\pm$ SEM. For hESC controls, assay triplicates were used to determine mean and SEM. Statistical analysis was done with ANOVA with $p < 0.05$ considered significant.

Ethics Statement

Fate Therapeutics has established and maintains a program for activities involving all animals in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" and section IV.C.6 of the "Public Health Service Policy on Humane Care and Use of Laboratory Animals".

Supplemental References

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