The seven SEES cell lines were further characterized by short tandem repeat (STR) analysis (Supplemental Table 1), HLA-DNA typing (Supplemental Table 2), ABO typing (Supplemental Fig. 5), and cytogenetic X-chromosome inactivation status analysis (Supplemental Fig. 6). The distinct features of SEES cell lines could be observed by STR and HLA profiling. SEES-2 and SEES-5 were blood type OO, which is most suitable for cellular transplantation.

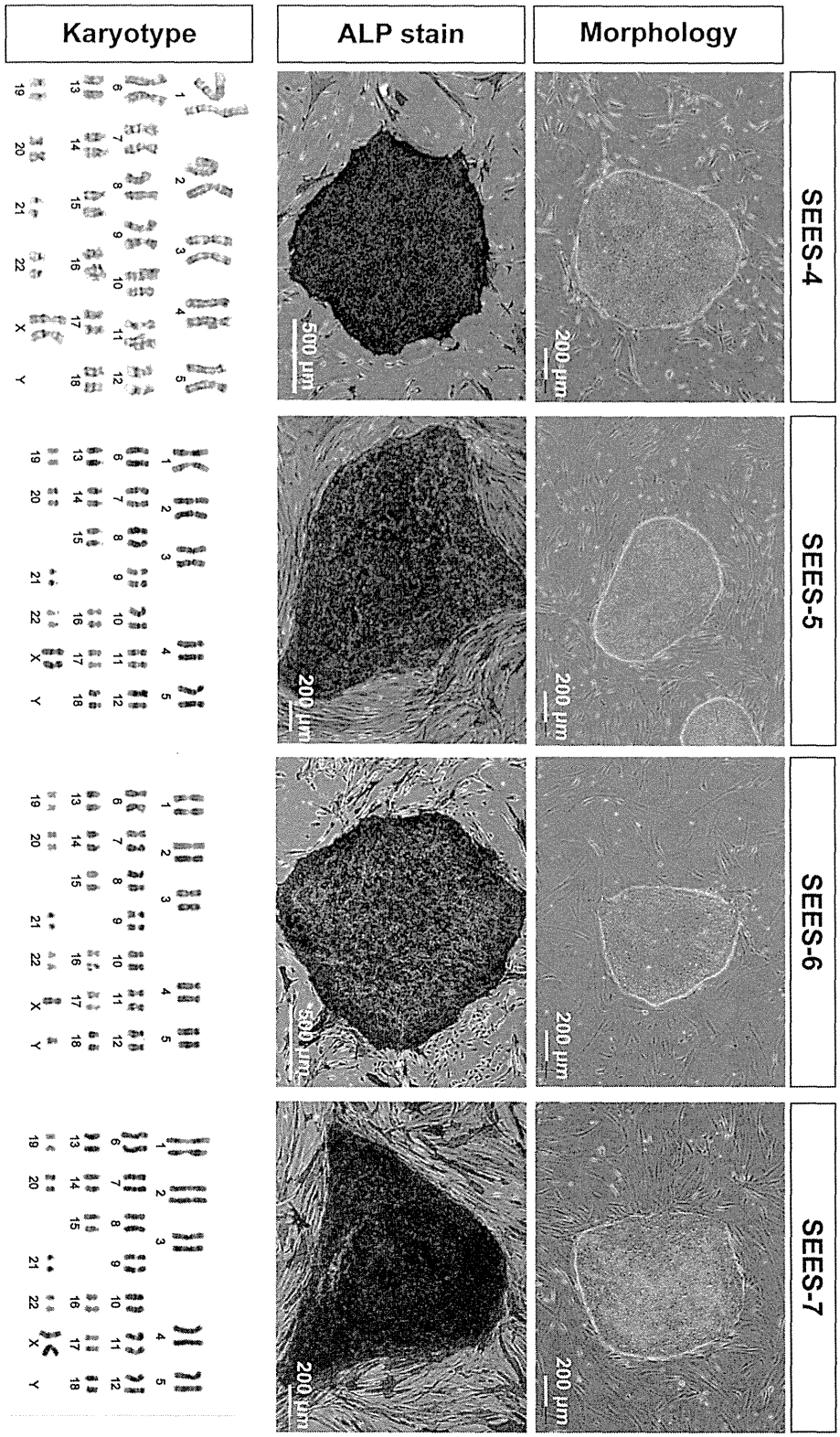

Fig. 3. Derivation of xenogeneic-free hESCs on the hMSC feeder layer Under completely xenogeneic-free conditions, four hESC lines were derived from the inactivated hMSC feeder layer using the laser ablation system. Typical ESC morphology was readily visible. Alkaline phosphatase (ALP) activity was detected in each SEES cell line. Chromosome analysis of SEES-4, SEES-5, SEES-6, and SEES-7 cells showed normal karyotypes: 46,XX, 46,XX, 46,XY, and 46,XX, respectively.

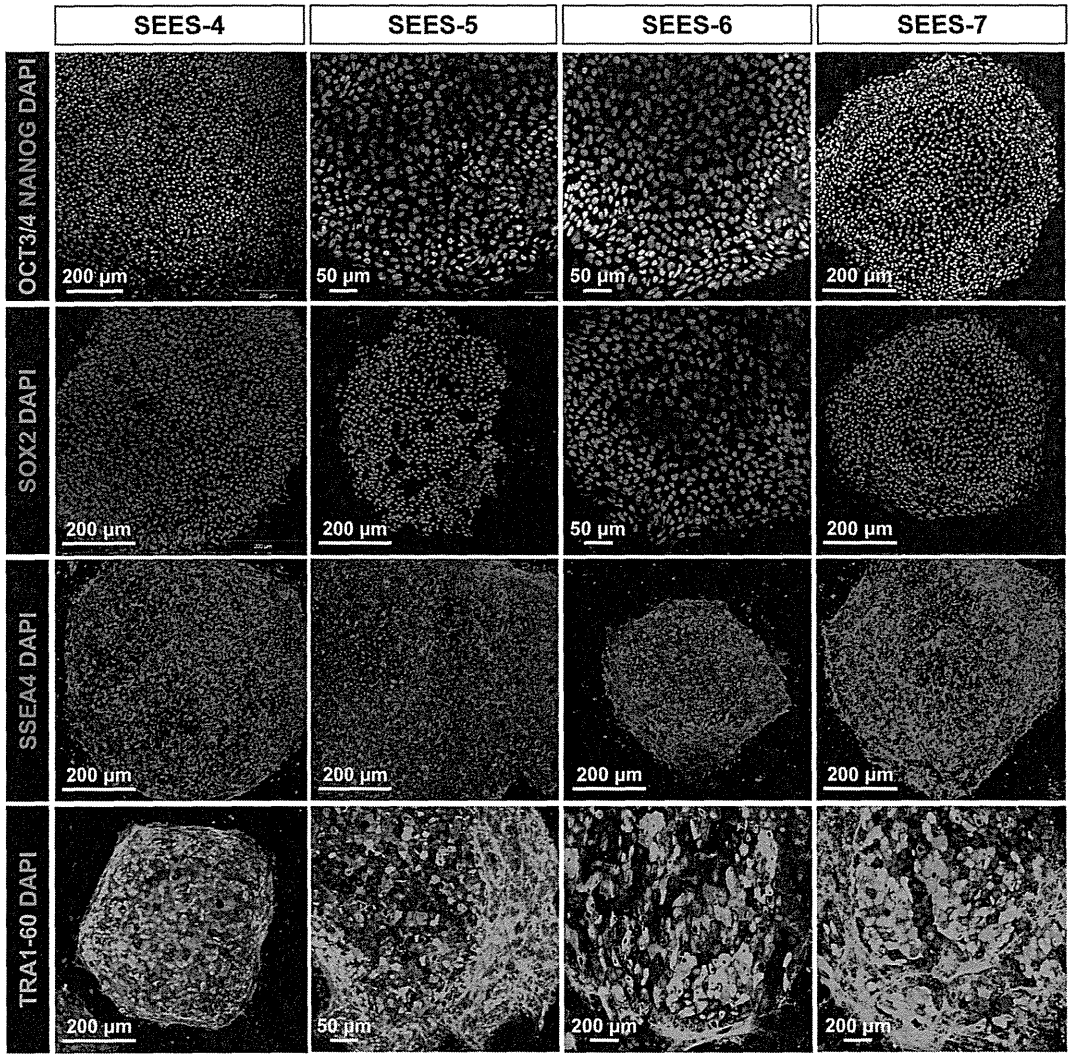

Fig. 4. Pluripotent marker expression of xenogeneic-free SEES cell lines In the undifferentiated state, xenogeneic-free SEES cell lines expressed markers characteristic of pluripotent hESCs, including OCT4, NANOG, SOX2, SSEA4, and TRA1-60. SEES-4: scale bars are 200 μm; SEES-5: scale bars are 50 μm in OCT3/4 and TRA1-60 and 200 μm in SOX2 and SSEA4; SEES-6: scale bars are 50 μm in OCT3/4 and SOX2 and 200 μm in SSEA4; SEES-7: scale bars are 200 μm.

2.5. Stable expansion of SEES cell lines under modified conventional hESC culture conditions

To overcome the shortcomings of conventional approaches, we determined which elements of conventional culture systems were necessary and sufficient for maintaining the pluripotency of hESCs. In our modified conventional hESC culture medium, human recombinant bFGF and KO-SR were replaced by pharmaceutical-grade recombinant human bFGF (trafermin) and high-dose gamma-irradiated KO-SR, respectively. SEES-2 cells were stably maintained on the qualified MEF layer in the modified medium without antibiotics (Fig. 6A). Under these conditions, the colonies expressed multiple pluripotency markers, including OCT3/4, NANOG, SOX2, SSEA4, and TRA1-60, as demonstrated by immunostaining (Fig. 6B). Interestingly, SEES-2 cells could differentiate into derivatives of all three embryonic germ layers *in vitro* and *in vivo*. The EB formation assay showed detection of TUJ1, αSMA, and AFP by immunostaining (Fig. 6C). Additionally, differentiation of the three germ layer *in vivo* was confirmed by teratoma analysis (Fig. 6D), and SEES-2 cells retained normal karyotypes following extensive passaging in culture (Fig. 6E). Under these conditions, cells were able to maintain pluripotency for over 20 passages, as confirmed by positive expression of SSEA4, TRA1-60, and TRA1-80 and absence of SSEA1 expression (Fig. 7). Taken together, our data demonstrated that the modified conventional hESC culture system, based on replacement with high-dose gamma-irradiated KO-SR and trafermin, maintained the pluripotency of hESCs. SEES-1 and SEES-3 also retaining their pluripotency, as shown by morphological analysis and immunostaining (Supplemental Fig. 7). Analysis of the expression of several genes by qRT-PCR showed that hESCs grown under conventional conditions and our modified conditions exhibited highly similar gene expression patterns (Supplemental Fig. 8). Additionally, FISH analysis of SEES cell lines showed that three out of the five SEES cell lines were X-chromosome active.

3. Discussion

Traditional methods for isolating and expanding hESCs include using MEFs as a feeder layer and supplementing medium with FBS replacement. Conventional hESC culture systems are widely used for both basic research and clinical trials under appropriate conditions for ensuring safety [15]. Currently, there are at least two options for further development of hESC cultivation systems for use in regenerative medicine; these methods seek to achieve safe

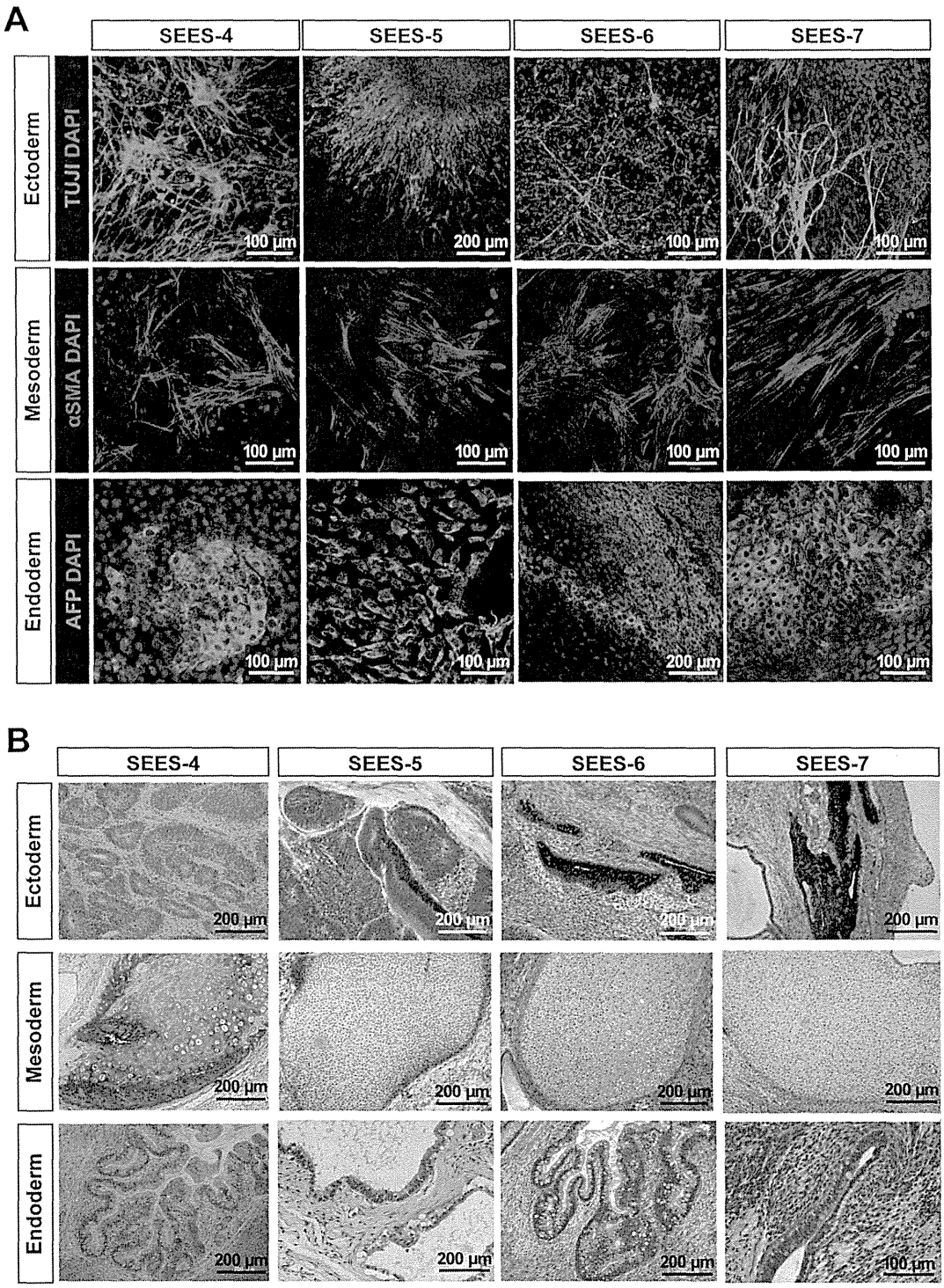

Fig. 5. Differentiation of three germ layers of xenogeneic-free SEES cell lines A) SEES cells differentiated *in vitro* via EBs expressed markers of the primary germ layers. Immunohistochemical analyses of markers of the ectoderm (TUJ1), mesoderm (αSMA), and endoderm (AFP) layers are shown. SEES-4: scale bars are 100 μm; SEES-5: scale bars are 200 μm for TUJ1 and 100 μm for αSMA and AFP; SEES-6: scale bars are 100 μm for TUJ1 and αSMA and 200 μm for AFP; SEES-7: scale bars are 100 μm. B) SEES cells differentiated *in vivo* via teratoma formation. Hematoxylin and eosin staining revealed germ layer derivatives, such as neural tissues, pigmented epithelium (ectoderm), cartilage (mesoderm), and gut epithelial tissues (endoderm). Scale bars are 200 μm.

culture conditions within a stable, conventional hESC cultivation system. Future potential uses of hESCs in clinical and industrial applications will require a reproducible, XF culture system. In this study, we evaluated the replacement of a conventional hESC culture system with high-dose, gamma-irradiated serum and pharmaceutical-grade recombinant bFGF (trafermin) in order to address the reproducibility of hESC cultivation systems. hESCs could be successfully maintained using modified conventional medium supplemented with 35 KGy-irradiated KO-SR and trafermin. Our data provided evidence supporting clinically relevant

alternative platforms of hESC culture for use in clinical and industrial applications. This study also demonstrated the development of a defined, novel, efficient culture system for the derivation of hESCs from blastocyst ICMs and described the expansion and maintenance of hESCs in completely XF conditions on human allogeneic MSC feeder layers. The XF culture system with XF hMSC feeder layers can stably expand several hiPSC lines (data not shown) and successfully allow the derivation of human iPSCs

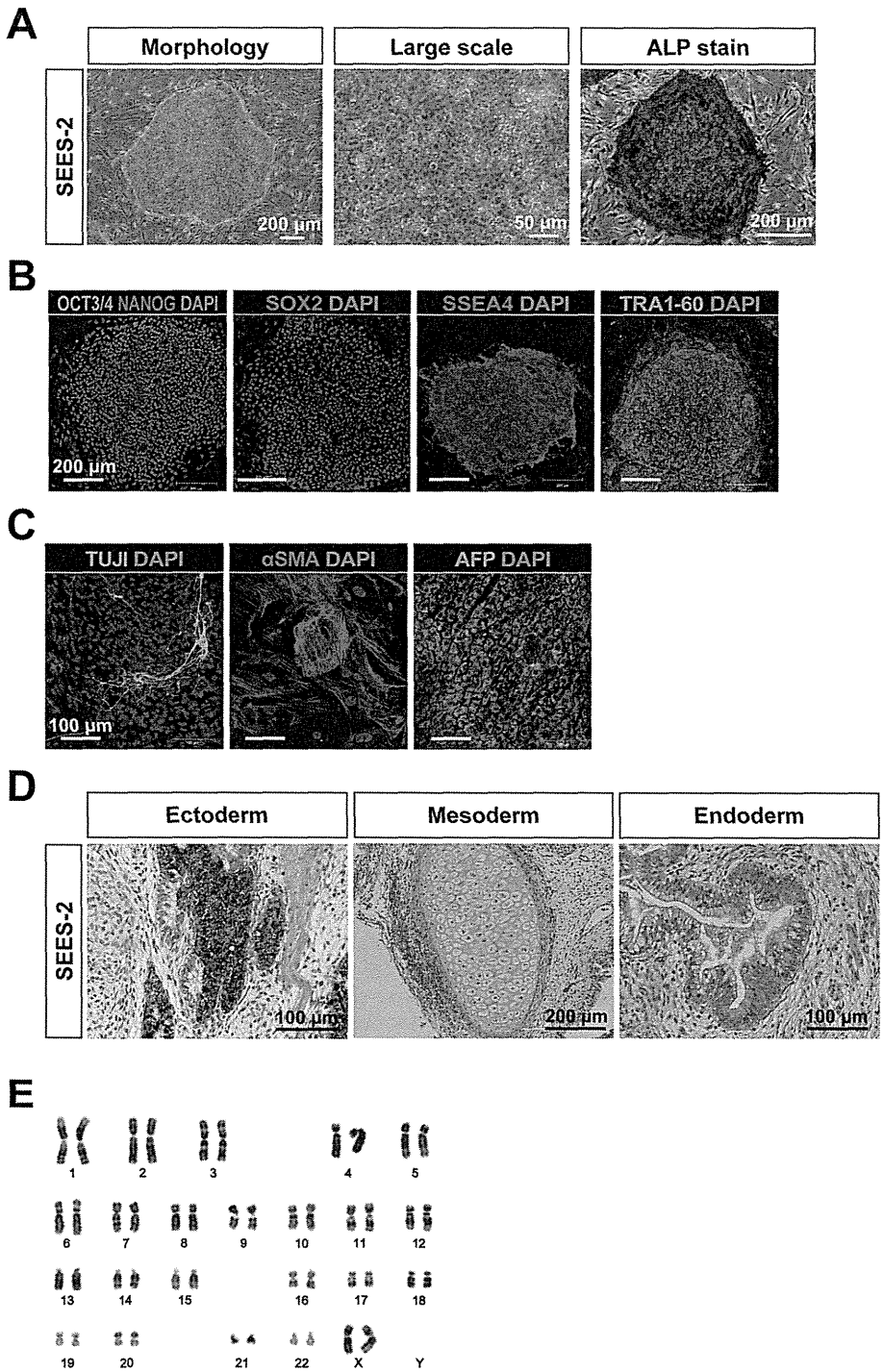

Fig. 6. Characterization of the pluripotency of SEES-2 maintained using a modified conventional hESC culture medium SEES-2 cells were stably maintained over 20 passages on the qualified MEF feeder layer in the modified medium, which contained pharmaceutical-grade recombinant human bFGF (trafermin) and high-dose (35-K) gamma-irradiated KO-SR without antibiotics. A) Typical hESC colony morphology was readily visible. ALP activity was detected. B) SEES-2 cells expressed undifferentiated hESC markers, including OCT4, NANOG, SOX2, SSEA4, and TRA1-60. SEES-2 cells could differentiate into three embryonic germ layers *in vitro* and *in vivo*. Scale bars are 200 μm. C) SEES cells that had been differentiated *in vitro* via EBs expressed markers of the primary germ layers, ectoderm (TUJ1), mesoderm (αSMA), and endoderm (AFP). Scale bars are 100 μm. D) Histological analysis of teratomas containing multidifferentiated tissues derived from SEES-2 cells. Pigmented epithelium (ectoderm), cartilage (mesoderm), and gut epithelial tissues (endoderm). E) Chromosomal analysis of SEES-2 cells cultivated through 16 passages using a modified conventional hESC culture medium showed a normal 46,XX karyotype.

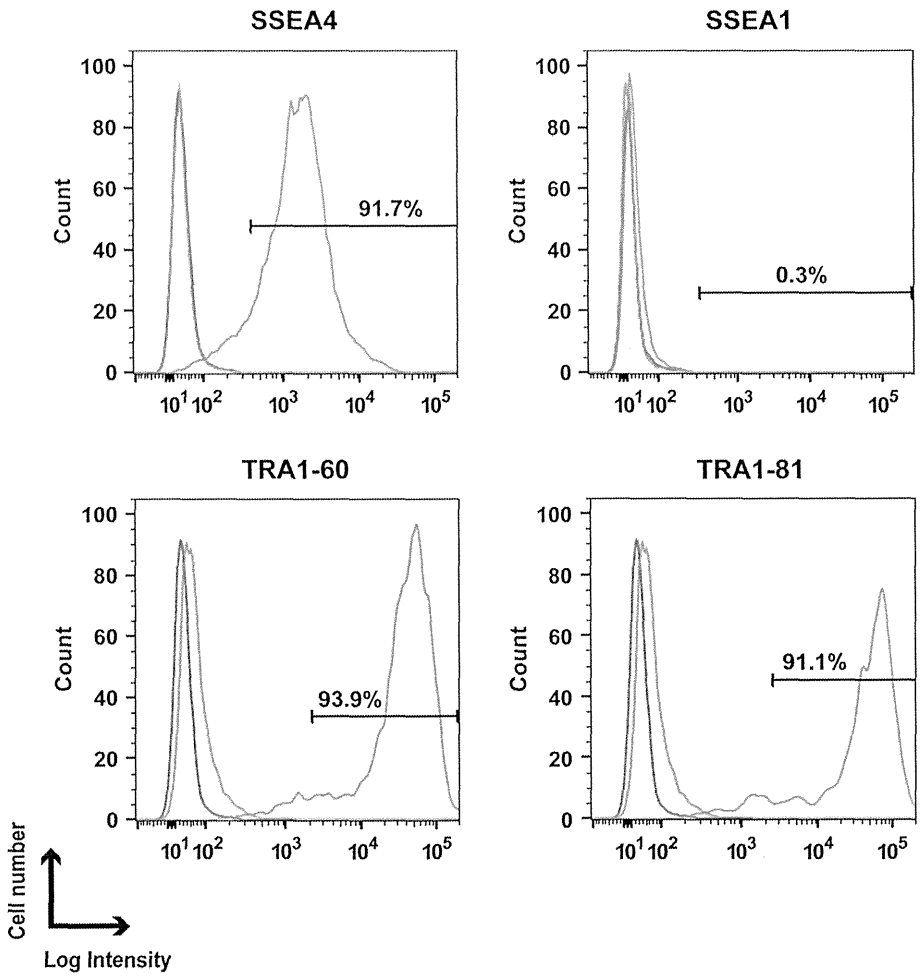

Fig. 7. Expression of pluripotency markers in SEES-2 cells was maintained using a modified conventional hESC culture medium Flow cytometric analysis of hESC-specific marker expression in SEES-2 cells. The isotype control is indicated by the blue line, and the unlabeled sample, which was used as a control, is indicated by the red line. Surface staining is shown by the yellow line for SSEA4, SSEA1, TRA-1-60, and TRA-1-81.

(Supplemental Fig. 3). These conditions are also quite applicable to iPSC derivation and cultivation as well. Importantly, the development of such an XF culture system primarily enables hESCs to culture and expand in conditions that are totally devoid of forming Neu5GC, a sialic acid glycan and xenogeneic antigen that can potentially transform cells to obtain cancer phenotypes [18,19]. In this culture system, we developed and successfully generated completely Neu5GC-free hESC lines. Our four XF hESC lines (SEES-4, SEES-5, SEES-6, and SEES-7) can be also stably cultivated under alternative xenogeneic-free conditions. Nakagawa et al. reported a novel culture system for derivation and expansion of hiPSCs with the recombinant laminin-511 E8 fragment matrices and the xenogeneic-free culture medium (StemFit™) [20]. Four XF SEES cell lines were stably grown using the StemFit™ medium under feeder-free conditions (data not shown). The XF SEES cell lines would be accustomed to being maintained in xenogeneic-free conditions with ease, as these cell lines have been derived and expanded under animal derived components-free conditions.

Pluripotent hESCs are isolated from preimplantation embryos [1] and general characteristics of hESCs include flat morphology, dependence on FGF2 signaling, differentiation into three germ layers *in vitro* and *in vivo*, and pluripotent markers expression [21]. Numerous hESC lines have been derived [3]. It is indicated that many of hESC lines differ in the manner in which they were derived and maintained in culture, and such differences may have significant effects on the characteristics of the cell lines [22,23]. In female hESCs, culture conditions may have significant effects on the X chromosome inactivation status and contribute to the cellular characteristics [24]. Three of five female SEES cell lines showed none of XIST expression in this study. However, it has demonstrated that there are not notable differences in gross hESCs characters including pluripotent markers and differentiation *in vitro* and *in vivo*, despite the significant differences in XIST expression status among SEES 1, 2, 4, 5 and 7. Notably, SEES cell lines are characterized not only the biological properties of human ESCs, also the genomic signatures including ABO blood typing, STR genotyping and HLA isotyping. The distinct properties of the SEES cell lines offer a scalable cell resource for clinical application.

The culture medium we described in this study consisted of a basal culture medium with well-known growth factors that define the maintenance of pluripotency. Since these ingredients are well known and are added to a simple culture medium formulation, the cells derived/expanded in such a system have the potential for reduced contamination, better kinetics of growth, good ability to differentiate into all the three germ layer derivative lineages, maintain a normal karyotype, and, most importantly, will not contribute to tumorigenicity. This defined culture system serves as a better and safer alternative to derive/culture/expand hESCs/iPSCs for larger cell therapy purposes.

4. Materials and methods

4.1. Derivation of hESC lines on inactivated mouse embryonic fibroblast (MEF) feeder cultures

All derivations and cultures of hESC lines in this study were performed in full compliance with the Guidelines on the Derivation and Distribution of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science and Technology, Japan (Notification No. 156 of 2009), after approval of the Institutional Review Board regarding hESC research at the National Center for Child Health and Development (NCCHD; "Sei-iku" in Japanese title of the affiliation), Japan. Surplus frozen human embryos, donated by consenting couples, were thawed using a Cryotop Safety Thawing Kit (Kitazato BioPharma, Shizuoka, Japan; #VT602) according to the manufacturer's instructions and cultured in BlastAssist System medium (MediCult, Jyllinge, Denmark; #12150010) until they reached the blastocyst stage. The derivation of three hESC lines, i.e., SEES-1, SEES-2, and SEES-3, was performed using modified HUES derivation methods, as described previously [4,5,25]. Briefly, the inner cell mass (ICM) was isolated by immunosurgery by using rabbit antiserum (Rockland Immunochemicals, PA, USA; #109-4139) and guinea pig serum complement (Sigma–Aldrich, MO, USA; #S-1639) and then seeded onto a feeder layer of freshly plated gamma-irradiated MEFs, isolated from ICR embryos at 12.5 gestations and passaged two times before gamma irradiation (30 Gy), in hESC conventional derivation media. The hESC conventional derivation media consisted of Knockout Dulbecco's modified Eagle's medium (KO-DMEM; Life Technologies, CA, USA; #10829-018) supplemented with 20% Knockout Serum Replacement (KO-SR; #10828-028), 2 mM GlutaMAX-I (#35050-079), 0.1 mM nonessential amino acids (NEAAs; #11140-076), 50 U/mL penicillin/50 μg/mL streptomycin (Pen-Strep; #15070-063), 0.055 mM beta-mercaptoethanol (#21985-023), and recombinant human full-length bFGF (#PHG0261) at 10 ng/mL (all reagents were from Life Technologies). Seven to 14 days after ICMs were plated, expanded ICMs were dissected mechanically into small clumps using a finely drawn glass Pasteur pipette and transferred onto a new MEF feeder layer as previously described [4,25]. Secondary colonies were similarly dispersed and plated onto new feeder layers of MEFs until passages 2–4. Cells were then further expanded manually using a Stem Cell Cutting Tool (Vitrolife, Kungsbacka, Sweden; #14601) and Dispase II (Eidia, Ibaraki, Japan; #GD81070).

4.2. hMSCs as feeder layers

4.2.1. Production and culture of hMSCs under XF conditions

To derive and expand hMSC feeder layers under XF conditions, we eliminated the use of media with all animal-derived components during the derivation, propagation, and passaging process in the preparation of new hMSC feeders from human subjects. Parental written informed consent was obtained from all families, and the study was approved by the Institutional Review Board (IRB #88) of the NCCHD. hMSCs were isolated from human dermal tissue samples collected from juvenile donors undergoing surgical procedures for polydactyly in the Division of Orthopedics of the NCCHD. Human dermal tissues were first washed in Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium (#14190-250) containing penicillin/streptomycin and then minced into small pieces by using a sterile scalpel in a laminar flow cabinet. The minced tissue was centrifuged and filtered in sequential steps to separate the tissue debris. The isolated cells were then expanded in StemPro MSC SFM XenoFree (MSC-XF; #A10675-01) supplemented with StemPro LipoMax Defined XenoFree Lipid Supplement (#A10850-01) on culture dishes coated with a recombinant humanized matrix (CELLstart CTS; #A10142-01). The isolated cells were expanded in MSC-XF medium with CELLstart using animal-component free TrypLE Select (#12563-011; all reagents from Life Technologies). After approximately 20 days, a confluent monolayer of primary cells was established (passage 0).

4.2.2. Proliferation assay

To assess the proliferative capacity of the isolated cells, primary cultures from two donors were analyzed through 22 serial passages using MSC-XF medium with CELLstart. Cells were counted using a cell viability analyzer (Vi-CELL Cell Viability Analyzer; Beckman Coulter, CA, USA), and cells were subcultured at 10^5 cells/100-mm dish every 4 days for approximately 15 days. At each passage, the population doubling (PD) rate was calculated based on the total cell number using the following formula: $[\log_{10}(N_h) - \log_{10}(N_1)]/\log_{10}(2)$, where N_1 was the number of cells plated and N_h was the number of cells harvested [26]. Growth curves were generated in triplicate using two independent cell lines.

4.2.3. Flow cytometric analysis and in vitro multilineage differentiation assay

Flow cytometric analysis was performed as described previously [27] in order to characterize the cells. Cells were incubated with primary antibodies or isotype-matched control antibodies, followed by immunofluorescence secondary antibody staining and analysis using an EPICS ALTRA analyzer (Beckman Coulter). The following cell surface epitopes were detected with anti-human fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated antibodies: CD29 (Beckman Coulter; #6604105), CD44 (Beckman Coulter; #IM1219), CD90 (BD Pharmingen, CA, USA; #555596), CD117 (Beckman Coulter; #IM1360), and CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany; #130-080-801). The potential of the isolated cells to differentiate into osteogenic and adipogenic lineages was examined according to the manufacturer's instructions. Adipogenesis and osteogenesis were induced by culturing cells in medium from an hMSC Adipogenic BulletKit (Lonza, PA, USA; #PT-3004) or an hMSC Osteogenic BulletKit (Lonza; #PT-3002), respectively. After 8 weeks under differentiation conditions, cells were processed for lineage-specific staining. Oil red staining was used for the detection of accumulated oil droplets in the cytoplasm of cells maintained with adipogenic differentiation media. Alkaline phosphatase activity was used to determine the extent of osteogenesis in cells grown in osteogenic differentiation medium.

4.2.4. Preparation of hMSCs for the feeder layer

At passages 5–20, XF cells were detached from the culture plate with TrypLE Select, washed with MSC-XF medium, and counted. The cells were resuspended in culture medium at a concentration of 1×10^7 cells per tube and then gamma-irradiated with 30 Gy. The irradiated cells were washed in medium and frozen at a concentration of 2×10^6 cells per vial in freezing medium (STEM-CELL-BANKER, ZENOAQ, Fukushima, Japan; #CB043).

4.3. XF hESCs

4.3.1. Derivation and expansion of new hESC lines under XF conditions

The procedure for derivation of XF hESCs was approved by the Institutional Review Board at the NCCHD, and embryos were collected from donors undergoing fertility treatment after obtaining informed consent. Frozen embryos were thawed and cultured to the blastocyst stage under the same methods as used for conventional hESC derivation. Blastocysts without immunosurgery were plated on inactivated XF hMSC feeder layers (Yub-1896 cells)

in XF hESC culture medium composed of 15% Knockout SR Xeno-Free CTS (KO-SR XF; Life Technologies; #12618-013), 85% KO-DMED, 2 mM GlutaMAX-I, 0.1 mM NEAAs, penicillin/streptomycin, 50 μg/mL L-ascorbic acid 2-phosphate (Sigma–Aldrich; #A4544), 10 ng/mL heregulin-1β, recombinant human NRG-beta 1/HRG-beta 1 EGF domain (R&D Systems, MN, USA; #396-HB-050/CF), 200 ng/mL LONG R^3-IGF1, recombinant human insulin-like growth factor-1 (Sigma–Aldrich; #85580C), and recombinant human full-length bFGF (Life Technologies; #PHG0261) at 20 ng/mL. Cells were initially maintained in a drawer-type incubator (IVF CUBE, ASTEC, Fukuoka, Japan; #AR-3100) at 37 °C under the appropriate humidified gas mixture (usually 3%–5% O_2/5% CO_2/90%–92% N_2). Within 7 days after whole blastocysts were plated on the feeder layers, ICMs were isolated by laser-mediated ablation of trophectoderm (TE) cells by using a XYClone laser system (Hamilton Thorne Biosciences, MA, USA) with 80% pulse strength and a pulse length of 300 μs [5]. After 2 weeks, the ICM outgrowth began to resemble morphologically distinct hESCs, and cells could be handpicked and transferred to fresh plates. After the first splitting, new colonies were disaggregated with a recombinant trypsin (Roche Applied Science, Basel, Switzerland; #06369880103) and transferred to fresh mitotically inactivated XF hMSC feeder plates every 5–7 days. Undifferentiated cells, as judged by morphology, were chosen for each further passage. A total of four XF hESC lines were derived and stably maintained (SEES-4, SEES-5, SEES-6, and SEES-7). All four SEES cell lines were maintained in a standard tissue culture incubator at 37 °C under the appropriate humidified gas mixture (3%–5% O_2/5% CO_2/90%–92% N_2) in multigas incubator (Sanyo, Osaka, Japan; #MCO-18M) in a separate culture room to avoid any contamination. SEES cells were cryopreserved using a conventional slow-rate cooling/thawing method with STEM-CELLBANKER. Validation of the cryopreservation by subsequent thawing of individual tubes resulted in efficient recovery of viable, undifferentiated hESCs.

4.3.2. Immunohistochemical analyses of stem cell and differentiated markers

Immunohistochemistry was performed as previously described [28,29]. The primary antibodies used for hESCs were specific for Nanog (1:300; ReproCELL, Kanagawa, Japan; #RCAB0003P), Oct3/4 (1:300; Santa Cruz Biotechnology, CA, USA; #sc-5279), Sox2 (1:300; Merck Millipore, Darmstadt, Germany; #AB5603), TRA1-60 (1:300; Merck Millipore; #MAB4360), and SSEA4 (1:300; Merck Millipore; #MAB4304). To assess the differentiation of the three germ layers, the primary antibodies used for embryoid bodies (EBs) were anti-α-fetoprotein (AFP; 1:200; R&D Systems; MAB1368), mouse (ascites) anti-β-tubulin III (TUJ1; 1:1000; Promega; #G712A), and anti-α-smooth muscle actin (αSMA; 1:400; Sigma; #A2547). Cells were fixed in 4% paraformaldehyde and incubated with primary antibodies overnight at 4 °C. Cells were then probed with Alexa Fluor 546 Goat Anti-Mouse IgG- or Alexa Fluor 488 Goat Anti-Rabbit IgG-conjugated secondary antibodies (1:300 each; Life Technologies), counterstained with 1 μg/mL DAPI, for 1 h in the dark at room temperature. After incubation, cells were mounted in Vectashield mounting medium containing 4′, 6-diamidino-2-phenylindole (Vector Laboratories, CA, USA). The labeled cells were visualized using a laser-scanning confocal microscope (LSM 510 META; Carl Zeiss, Oberkochen, Germany). Alkaline phosphatase (ALP) was detected with a Vector Red kit (Vector Laboratories; #SK-5100) according to manufacturer's instructions.

4.3.3. Flow cytometry analysis of pluripotency markers

SEES cells cultured in appropriate conditions were stained for 30 min at 4 °C with primary antibodies and immunofluorescence secondary antibodies. Cells were analyzed with a Cytomics FC 500 Cytometer (Beckman Coulter), and data were analyzed with FC 500 CXP Software ver. 2.0 (Beckman Coulter). Antibodies against human SSEA-1 (R&D Systems; #FAB2155C), SSEA4 (R&D Systems; #FAB1435F), TRA1-60 (Merck Millipore; #MAB4360), and TRA1-81 (Merck Millipore; #MAB4381) were used as primary antibodies in hESCs. PE-conjugated anti-mouse IgG antibodies (BD Pharmingen; #555578) and PE-conjugated anti-mouse IgM antibodies (BD Pharmingen; #553472) were used as secondary antibodies. X-Mean, the sum of intensity divided by the total cell number, was automatically calculated and was utilized for our evaluation.

4.3.4. Differentiation assays in vitro and in vivo

For induction of differentiation *in vitro*, the cells were dissociated using either StemPro Accutase (Life Technologies; #A11105-01) or TrypLE Select for 5 min at 37 °C, plated into 96-well plates (low attachment surface; Lipidure; NOF Corp., Tokyo, Japan), and cultured in differentiation medium containing KO-DMEM supplemented with 20% fetal bovine serum (FBS), 2 mM GlutaMAX-I, 0.1 mM NEAAs, and penicillin/streptomycin, to generate EBs. After 7 days in suspension, EBs were transferred onto poly-L-ornithine-coated chamber slides and cultured for an additional 10–14 days. The cultures were fixed with 4% paraformaldehyde for 20 min before immunohistochemical analysis.

In vivo pluripotency was assessed by teratoma formation in severe combined immunodeficient nude mice (BALB/cAJcl-nu/nu) purchased from CLEA Japan. A 60-mm plate of undifferentiated hESCs was washed with DPBS, and the cells were harvested with a cell scraper. The cell suspension was collected into a 15-mL conical tube and centrifuged at 1000 rpm for 4 min. The cell pellet was resuspended in hESC culture medium and Matrigel (BD Biosciences, NJ, USA; #356234) to a final total volume of 400 μL. Approximately 2–5 × 10^6 cells in 200 μL were injected subcutaneously into the dorsolateral area on both sides. Mice were sacrificed after 8–10 weeks. Tumors were then excised surgically, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Hematoxylin and eosin-stained paraffin-embedded sections were histologically examined for the presence of differentiated human tissue derived from all three embryonic germ layers.

The animal use protocol was approved by the Institutional Animal Care and Use Committee of the National Research Institute for Child Health and Development (NRICHD, Permit Number: A2003-002). All experiments with mice were subject to the 3 R consideration (refine, reduce, and replace), and all efforts were made to minimize animal suffering and to reduce the number of animals used.

4.3.5. Sialic acid analysis

Sialic acid was released from cell homogenate samples by acid hydrolysis with 0.05 M HCl at 80 °C for 3 h. Sialic acid samples were ultrafiltered using Amicon Ultra-0.5 mL filters (cut-off, 10 kDa; Millipore; #UFC503008), and filtrates were dried in a vacuum concentrator. Sialic acid was derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB; Sialic Acid Fluorescence Labeling Kit; Takara Bio, Shiga, Japan) and analyzed by reverse-phase fluorometric HPLC1 using a PALPAK Type R column (Takara Bio). The excitation and emission wavelengths were 310 and 448 nm, respectively. The DMB-derivatized sialic acid was identified by comparing retention times with those of known standards (Glyko Sialic acid reference panel; ProZyme, CA, USA; #GKRP-2503) that were similarly treated. This method can evaluate sialic acids at a minimum concentration of 0.01 nmol/mg protein.

4.3.6. Karyotype analysis

Chromosomal G-band analyses were performed at the Nihon Gene Research Laboratories, Sendai, Japan. The chromosomes were

classified according to the International System for Human Cytogenetic Nomenclature. At least 20 metaphase chromosomes were analyzed per cell line.

4.4. Generation of human iPSCs

Human iPSCs were generated from XF Yub-1896 cells by transduction with of a lentiviral vector carrying three reprogramming factors (OCT3/4, SOX2 and KLF4) under XF culture conditions. The vector was a generous gift of Konrad Hochedlinger (Harvard University). XF iPSCs were successfully maintained on inactivated XF hMSC feeder layers in XF hESC culture medium over 20 passages. We confirmed the XF iPSCs expressed pluripotent markers including OCT3/4, NANOG, SSEA4, and TRA1-60.

4.5. Modified conventional hESC cultivation

4.5.1. Production of MEF feeder stock

Pregnant ICR mice were obtained from a breeding colony under specific pathogen-free (SPF) conditions in a barrier room with extensive health monitoring at CLEA Japan (Atsugi facility, Kanagawa, Japan). MEFs were generated from ICR embryos at 12.5 gestations as previously described [5,25] and expanded using MEF feeder sock medium composed of 90% KO-DMEM, 30 KGy gamma-irradiated FBS (HyClone; Thermo Fisher Scientific, MA, USA), and 2 mM GlutaMAX-I without any antibiotics. At passage 2, cells were mitotically inactivated by gamma irradiation (30 Gy) and frozen at a concentration of 4×10^6 cells per vial. To minimize the risk of introducing murine viruses and other pathogens, MEF feeder stock was tested in GMP/GLP studies by Vitology Limited (Glasgow, UK). The specifications and results for the testing of lot MEF-0001 are presented in Supplemental Table 3.

4.5.2. hESC expansion using gamma-irradiated KO-SR and pharmaceutical-grade recombinant human bFGF without antibiotics

To develop a safer culture system for clinical application of hESCs, we replaced KO-SR and recombinant human full-length bFGF with pharmaceutical recombinant human bFGF and high-dose gamma–irradiated KO-SR. Frozen KO-SR products ware gamma irradiated at 35 KGy (KOGA ISOTOPE, Ltd., Shiga, Japan). Pharmaceutical-grade recombinant human bFGF (generic name: trafermin), supplied by Kaken Pharmaceutical (Tokyo, Japan) as Fiblast Spray, which is prepared using the powdered form of trafermin, was applied for hESC culture. hESC lines were cultivated in a modified conventional culture medium composed of 20% gamma-irradiated KO-SR, 80% KO-DMEM, 2 mM GlutaMAX-I, 0.1 mM NEAAs, and 50 ng/mL trafermin without any antibiotics on plates coated with 0.1% type I collagen (Nippon Ham, Ibaraki, Japan; #307-31611) and a mitotically inactivated layer of MEFs.

Competing financial interests

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.reth.2014.12.004.

References

[1] Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145–7.
[2] Schuldt BM, Guhr A, Lenz M, Kobold S, MacArthur BD, Schuppert A, et al. Power-laws and the use of pluripotent stem cell lines. PLoS One 2013;8:e52068.
[3] Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, et al. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol 2000;227:271–8.
[4] Cowan CA, Klimanskaya I, McMahon J, Atienza J, Witmyer J, Zucker JP, et al. Derivation of embryonic stem-cell lines from human blastocysts. N Engl J Med 2004;350:1353–6.
[5] Chen AE, Egli D, Niakan K, Deng J, Akutsu H, Yamaki M, et al. Optimal timing of inner cell mass isolation increases the efficiency of human embryonic stem cell derivation and allows generation of sibling cell lines. Cell Stem Cell 2009;4:103–6.
[6] Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, et al. Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 2001;19:971–4.
[7] Richards M, Fong CY, Chan WK, Wong PC, Bongso A. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat Biotechnol 2002;20:933–6.
[8] Martin MJ, Muotri A, Gage F, Varki A. Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat Med 2005;11:228–32.
[9] Pham T, Gregg CJ, Karp F, Chow R, Padler-Karavani V, Cao H, et al. Evidence for a novel human-specific xeno-auto-antibody response against vascular endothelium. Blood 2009;114:5225–35.
[10] Varki A. Colloquium paper: uniquely human evolution of sialic acid genetics and biology. Proc Natl Acad Sci U S A 2010;107(Suppl. 2):8939–46.
[11] Ludwig TE, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, Frane JL, et al. Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol 2006;24:185–7.
[12] Lu J, Hou R, Booth CJ, Yang SH, Snyder M. Defined culture conditions of human embryonic stem cells. Proc Natl Acad Sci U S A 2006;103:5688–93.
[13] Yao S, Chen S, Clark J, Hao E, Beattie GM, Hayek A, et al. Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. Proc Natl Acad Sci U S A 2006;103:6907–12.
[14] Hasegawa K, Pomeroy JE, Pera MF. Current technology for the derivation of pluripotent stem cell lines from human embryos. Cell Stem Cell 2010;6:521–31.
[15] Schwartz SD, Hubschman JP, Heilwell G, Franco-Cardenas V, Pan CK, Ostrick RM, et al. Embryonic stem cell trials for macular degeneration: a preliminary report. Lancet 2012;379:713–20.
[16] Fraga AM, Souza de Araújo É, Stabellini R, Vergani N, Pereira LV. A survey of parameters involved in the establishment of new lines of human embryonic stem cells. Stem Cell Rev 2011;7:775–81.
[17] Wang L, Schulz TC, Sherrer ES, Dauphin DS, Shin S, Nelson AM, et al. Self-renewal of human embryonic stem cells requires insulin-like growth factor-1 receptor and ERBB2 receptor signaling. Blood 2007;110:4111–9.
[18] Hedlund M, Padler-Karavani V, Varki NM, Varki A. Evidence for a human-specific mechanism for diet and antibody-mediated inflammation in carcinoma progression. Proc Natl Acad Sci U S A 2008;105:18936–41.
[19] Pearce OM, Läubli H, Verhagen A, Secrest P, Zhang J, Varki NM, et al. Inverse hormesis of cancer growth mediated by narrow ranges of tumor-directed antibodies. Proc Natl Acad Sci U S A 2014;111:5998–6003.

[20] Nakagawa M, Taniguchi Y, Senda S, Takizawa N, Ichisaka T, Asano K, et al. A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells. Sci Rep 2014;4:3594.
[21] Hanna JH, Saha K, Jaenisch R. Pluripotency and cellular reprogramming: facts, hypotheses, unresolved issues. Cell 2010;143:508–25.
[22] Hoffman LM, Carpenter MK. Characterization and culture of human embryonic stem cells. Nat Biotechnol 2005;23:699–708.
[23] Hoffman LM, Hall L, Batten JL, Young H, Pardasani D, Baetge EE, et al. X-inactivation status varies in human embryonic stem cell lines. Stem Cells 2005;23:1468–78.
[24] Nguyen HT, Geens M, Spits C. Genetic and epigenetic instability in human pluripotent stem cells. Hum Reprod Update 2013;19:187–205.
[25] Akutsu H, Cowan CA, Melton D. Human embryonic stem cells. Methods Enzymol 2006;418:78–92.
[26] Escobedo-Lucea C, Bellver C, Gandia C, Sanz-Garcia A, Esteban FJ, Mirabet V, et al. A xenogeneic-free protocol for isolation and expansion of human adipose stem cells for clinical uses. PLoS One 2013;8:e67870.
[27] Cui CH, Miyoshi S, Tsuji H, Makino H, Kanzaki S, Kami D, et al. Dystrophin conferral using human endothelium expressing HLA-E in the non-immunosuppressive murine model of Duchenne muscular dystrophy. Hum Mol Genet 2011;20:235–44.
[28] Nagata S, Toyoda M, Yamaguchi S, Hirano K, Makino H, Nishino K, et al. Efficient reprogramming of human and mouse primary extra-embryonic cells to pluripotent stem cells. Genes Cells 2009;14:1395–404.
[29] Makino H, Toyoda M, Matsumoto K, Saito H, Nishino K, Fukawatase Y, et al. Mesenchymal to embryonic incomplete transition of human cells by chimeric OCT4/3 (POU5F1) with physiological co-activator EWS. Exp Cell Res 2009;315:2727–40.

References in supplemental figures

[1] Hosoi E. Biological and clinical aspects of ABO blood group system. J Med Invest 2008;55:174–82 (For Supplemental Figure S1).
[2] Ota M, Fukushima H, Kulski JK, Inoko H. Single nucleotide polymorphism detection by polymerase chain reaction-restriction fragment length polymorphism. Nat Protoc 2007;2:2857–64 (For Supplemental Figure S2).
[3] Muro T, Fujihara J, Imamura S, Nakamura H, Kimura-Kataoka K, Toga T, et al. Determination of ABO genotypes by real-time PCR using allele-specific primers. Leg Med Tokyo 2012;14:47–50 (For Supplemental Figure S3).

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MINI REVIEW

Generation of pluripotent stem cells without the use of genetic material

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Induced pluripotent stem cells (iPSCs) provide a platform to obtain patient-specific cells for use as a cell source in regenerative medicine. Although iPSCs do not have the ethical concerns of embryonic stem cells, iPSCs have not been widely used in clinical applications, as they are generated by gene transduction. Recently, iPSCs have been generated without the use of genetic material. For example, protein-induced PSCs and chemically induced PSCs have been generated by the use of small and large (protein) molecules. Several epigenetic characteristics are important for cell differentiation; therefore, several small-molecule inhibitors of epigenetic-modifying enzymes, such as DNA methyltransferases, histone deacetylases, histone methyltransferases, and histone demethylases, are potential candidates for the reprogramming of somatic cells into iPSCs. In this review, we discuss what types of small chemical or large (protein) molecules could be used to replace the viral transduction of genes and/or genetic reprogramming to obtain human iPSCs.

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Pluripotent stem cells (PSCs) can be derived from terminally differentiated tissues by altering the epigenetic status of cells. These PSCs have the potential to differentiate into any cell type derived from the three germ layers.[1–5] Cell type determination is heavily dependent on epigenetic process. The generation of iPCSs from differentiated cells is partly regulated by epigenetics. PSCs provide an unlimited cell source with the potential for use in studying diseases, drug screening, and regenerative medicine. Human PSCs provide a promising platform for obtaining patient-specific cells for various therapeutic and research applications. In general, induced pluripotent stem cells (iPSCs) are generated via genetic manipulation or by nuclear transfer to generate PSCs from somatic cells.[6] However, nuclear transfer-generated PSCs raised ethical concerns and are technically difficult to prepare. The genetic manipulation of PSCs limits their clinical uses. Although embryonic stem cells (ESCs) do not need to be genetically manipulated, there are strong ethical concerns regarding human ESCs (hESCs), limiting their use in clinical applications.

iPSCs were first generated in 2006–2007 by the transduction of four transcription genes, Oct3/4, Sox2, c-Myc, and Klf-4[7–10] or Oct 4, Sox2, Nanog, and Lin28.[11] Following these studies, several researchers succeeded in generating iPSCs using fewer pluripotent genes. Of note, researchers generated iPSCs without transducing c-Myc, a potent oncogene.[12–14] Currently, mouse iPSCs (miPSCs) can be generated by reprogramming a single gene, such as *Oct4*,[15,16] or with the aid of small or large molecules in place of gene transduction.[17–19] However, the low reprogramming efficiency of human iPSCs (hiPSCs) is a major drawback. The use of virus-mediated delivery of reprogramming factors, which leads to the permanent integration of oncogenes and potentially harmful genomic alterations,[20] is a serious concern. The use of genome-integrating viruses could cause insertional mutagenesis and unpredictable genetic dysfunction.[9,21]

Therefore, several reprogramming technologies that do not use viral integration have been developed for iPSC production.[22,23] These approaches include the use of non-integrating viruses,[24–26] transposon-based systems,[27] and the delivery of reprogramming factors on plasmids.[22,28–30] Adenovirus, lentivirus, Sendai virus, miRNA, and plasmid transfection methods have been reported to generate miPSCs[28,31] and

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hiPSCs[24,30,32–35] to minimize chromosomal disruption.[18] Yu *et al.* generated hiPSCs by transfecting non-integrating episomal vectors.[30] In addition, the piggyBac transposon[18,27,31] and Cre-recombinase excisable viruses[36] have been used to generate hiPSCs. The transgenes can be excised by inducible gene expression once reprogramming is established.[25,27,36] However, there is evidence that there can be problems with residual DNA and chromosomal disruptions, resulting in harmful genetic alterations.[18]

The repeated transfection of modified mRNA encoding the reprogramming factors is also efficient for generating iPSCs.[22,37] Although these strategies eliminate the threat of random viral integration into the host cell genome, these approaches are technically challenging and less efficient than viral transduction. Therefore, it is important to identify new conditions and small or large molecules that can promote reprogramming and ultimately replace all of the reprogramming transcription factors (TFs).[14,38–41] For clinical applications, using small or large molecules to generate PSCs are preferable to genetic manipulations. Recently, several novel methods have been reported for generation of iPSCs without the use of genetic material; these methods include protein-induced PSCs (piPSCs) that are reprogrammed from somatic cells using cell-penetrating TF proteins,[18,19] and chemically iPSCs (CiPSCs) that are reprogrammed from somatic cells using small molecules.[14,17,20,38–49]

Human CiPSCs are a promising tool in the clinical application of PSCs. CiPSCs can be reprogrammed to become iPSCs from somatic cells, without genetic manipulation, through the addition of small-molecule chemicals in the culture medium. In this review, we will discuss the generation of iPSCs without the use of genetic material and instead using small or large (protein) molecules. We will discuss types of small and/or large (protein) molecules that can replace specific viral transduction of pluripotent TFs to obtain piPSCs and CiPSCs from somatic cells.

PSCs REPROGRAMMING WITH PROTEINS (piPSCs)

In the reprogramming of somatic cells into iPSCs, one of the methods that avoids exogenous genetic introduction to the target cells is delivering the reprogramming proteins directly into cells rather than transducing the cells with TF genes. Previous researchers have reported that the proteins can be delivered into mammalian cells *in vitro* and *in vivo* by conjugating the proteins with a short peptide to guide their transduction, such as HIV transactivator of transcription (tat) or polyarginine.[30,50–52] Various solubilization and refolding techniques have been developed so that recombinant proteins expressed in *E. coli* and contained in inclusion bodies can be re-folded into bioactive proteins. This allows for easy, large-scale production of therapeutic proteins.[30,53] Currently, recombinant forms of Oct4, Sox2, Klf4, and c-Myc are commercially available. Table 1 summarizes the reprogramming of mouse and human somatic cells into piPSCs with the aid of proteins by transduction without TFs.

The challenge in delivering proteins into cells is the proteins' limited capacity to penetrate the cell membranes. Proteins that are capable of crossing the cell membrane barrier generally contain high proportions of basic amino acids, such as lysine and arginine.[54,55] and the HIV tat protein has a short, basic segment of 48–60 amino-acid residues that is known to cross the cell membrane and to activate HIV-specific genes.[18,54] In 2009, Zhou *et al.* attached a transfection domain of a polyarginine protein to the C terminus of the four reprogramming factors Oct4, Sox2, Klf4, and c-Myc to generate recombinant proteins that are able to permeate the plasma membranes of mouse embryonic fibroblasts (MEFs).[19] The mouse piPSCs that were generated were stably expanded for 30 passages, and the morphology of the piPSCs was similar to ESCs, as they formed small, compact, domed colonies.[19] The piPSCs expressed typical pluripotency markers, including ALP, SSEA1, Nanog, Oct4, and Sox2, as assayed by immunostaining. The expression of endogenous pluripotency genes was verified by RT-PCR. The piPSCs generated embryoid bodies in suspension and differentiated into cells characteristic of the three germ layers: (a) endoderm cells expressing AFP, FoxA2, GATA4, Sox17, albumin (hepatic marker), and Pdx1 (pancreatic marker); (b) mesoderm cells expressing Brachyury and mature beating cardiomyocytes expressing the CT3 and MHC markers; and (c) ectoderm cells expressing Pax5 and Sox1 (neural markers) and βIII-tubulin and MAP2ab (mature neuronal markers).[19] Although these findings are promising, the extremely low efficiency (0.006%) and poor reproducibility of the generation of piPSCs hinders its use as a general method for generating iPSCs.

The same year, Kim *et al.* reported the generation of piPSCs from human newborn fibroblasts (HNFs) using four recombinant cell-penetrating reprogramming proteins (Oct4, Sox2, Klf4, and c-Myc) fused with a cell-penetrating peptide (CPP, polyarginine with nine repeating arginine residues).[18] The authors generated HEK293 cell lines that expressed each of the four human reprogramming proteins fused to CPP. HNFs were treated with cell extracts from the HEK293 cell lines. After repeated treatment with the cell extracts containing the reprogramming proteins, the HNFs ultimately became human piPSCs.[18] The human piPSCs showed similar characteristics to hESCs (H9) in cell morphology and pluripotent marker expression and were cultured for more than 35 passages without loss of pluripotency, which suggests that the appropriate epigenetic reprogramming events occurred in these cells. The human piPSCs were successively differentiated into cells derived from the three germ layers both *in vitro* (embryonic formation) and *in vivo* (teratoma formation).[18] Interestingly, Kim *et al.* could not generate mouse piPSCs when they applied the same method to mouse cells.[18] This discrepancy might be due to a low concentration of reprogramming proteins, as they used whole-protein extracts from HEK293 cells as the source of the reprogramming proteins. Furthermore, Kim *et al.* did not add

Table 1 Reprogramming of mouse and human somatic cells into iPSCs with the aid of proteins by transduction without transcription factors (TFs)

TFs required	*Somatic cells*	*PSCs*	*Small molecules*	*Efficiency (%)*	*Reference*
Mouse					
No factor	MEFs (NIH3T3)	miMPCs	mESC (D3) extract with streptolysin O treatment	NA	60
No factor	MEFs	miPSCs	VPA, four recombinant proteins (OSKM) with polyarginine tag	0.006%	19
No factor	MEFs	miPSCs	VPA, three recombinant proteins (OSK) with polyarginine tag	0.002%	19
No factor	Mouse skin fibroblasts	miMPCs	Fish egg extract	NA	57
No factor	Mouse cardiac fibroblasts	miPSCs	mES (C57) cell line extract	0.001%	59
No factor	Mouse dermal fibroblasts	miPSCs	mES (C57) cell line extract	NA	56
Human					
No factor	Human new born fibroblasts	hiPSCs	HEK293 Cell extract containing Recombinant proteins with polyarginine tag	0.001%	18
No factor	Human NPCs derived from cord blood	hiPSCs	TSA, RG-108, reprogramming proteins (Oct4, Klf4, Sox2)	0.001%	90
No factor	Human newborn foreskin fibroblast BJ (CRL-2522)	hiPSCs (hiMPCs)	Fibromodulin	0.03%	58

small molecules to the culture medium,[18] whereas Zhou *et al.* used valproic acid (VPA) for the reprogramming to generate mouse piPSCs.[19] VPA is known to enhance the reprogramming efficiency of cells to generate miPSCs.[42]

piPSC GENERATION USING CELL EXTRACTS

Jin *et al.* also generated mouse piPSCs using cell extracts instead of viral transduction. They found that cell extracts from only specific ESC lines could promote the reprogramming of mouse dermal fibroblasts into piPSCs.[56] Therefore, the authors performed protein microarrays to characterize the proteomes of different ESC lines that can and cannot reprogram the mouse dermal fibroblast. Protein extract from the ESC line that could promote reprogramming showed high levels of proteins that regulate protein synthesis and metabolism, compared with the other ESC lines that could not promote reprogramming into piPSCs; this result suggests that there are threshold concentrations of specific synthetic and metabolic proteins that the cells must exceed to initiate reprogramming.

Several researchers have reported that, in addition to ESC extracts, other cell extracts such as fish oocyte extracts[57] or a single extracellular matrix proteoglycan of fibromodulin[58] can promote the reprogramming of human fibroblast cells into human piPSCs or induced multipotent stem cells. In most cases, the specific proteins from the extracts that drive reprogramming are unknown.[57,59,60] Furthermore, it has not yet been verified whether human piPSCs can be reprogrammed with high efficiency and high reproducibility from somatic cells incubated with extracts of hESCs or other animal cells.

COMPARISON OF hiPSCs DERIVED FROM GENETIC MATERIAL AND PROTEINS

Rhee *et al.* compared multiple hiPSC lines derived from retrovirus-based, lentivirus-based, and protein-based reprogramming to hESCs based on their capacity to differentiate into neuronal stem cells (NSCs) and dopaminergic neurons.[61] Optimized co-culturing and fine selection methods led to the efficient generation of NSCs and dopaminergic neurons from hESCs and all hiPSC lines tested, including piPSCs. NSCs and dopaminergic neurons derived from hiPSCs using lentiviral transduction showed residual expression of exogenous reprogramming genes, whereas hiPSCs derived from retroviral vectors and piPSCs did not express the exogenous reprogramming genes.[61] In addition, the virus-based iPSCs exhibited early cellular senescence and limited expansion because of apoptosis during passaging, whereas hESCs and hiPSCs exhibited unlimited expansion without cellular senescence. NSCs derived from piPSCs were grafted into rats with striatal lesions, a model of Parkinson's disease. NSC engraftment resulted in striking behavioral recovery associated with a high proportion of TH^+ (ie, dopamine-secreting) neurons, and the transplantation of NSCs rescued motor deficits. However, tumors formed when a large number of NSCs was grafted into the rats.[61] The researchers also found that fully differentiated dopamine neurons are too vulnerable to survive transplantation; therefore, almost no functional recovery and no TH^+

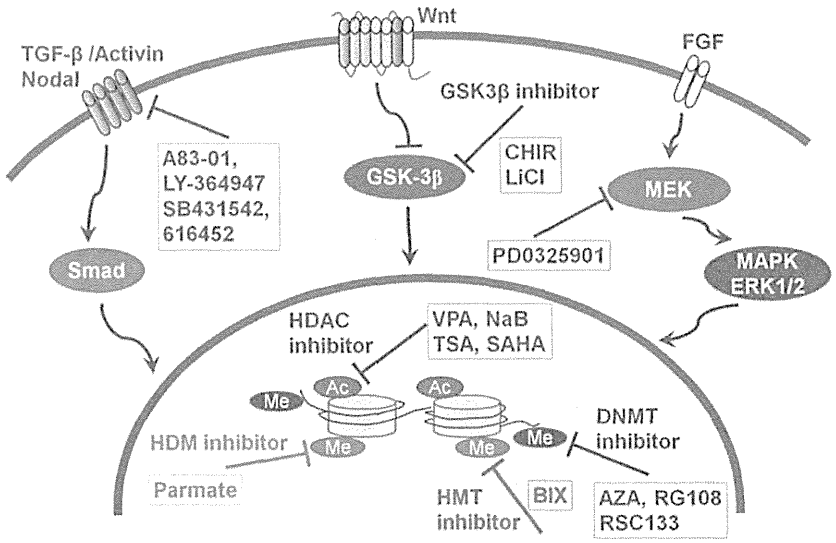

Figure 1 The signaling pathways underlying the chemical manipulation of stem cell fate and reprogramming. Epigenetic modulators can modify the chromatin structure to make it more permissive to changes in the epigenome during reprogramming. Small molecules that target signaling pathways can regulate the expression of pluripotent transcription factor genes, leading to the reprogramming of somatic cells into iPSCs. Several small-molecule inhibitors of epigenetic modification enzymes, such as DNA methyltransferases (DNMTs), histone deacetylases (HADC), histone methyltransferases (HMT), and histone demethylases (HDM) that act as major players in forming the epigenetic landscape, have been selected as small chemical molecules for the reprogramming of somatic cells into iPSCs. The abbreviations of the small molecules are shown in Table 2.

neurons were generated in their study.[61] This study elegantly described the superiority of hiPSCs generated by virus-free methods, although optimization is still required to remove residual undifferentiated cells and to avoid tumor generation. Such work will pave the way for standardized quality control before moving hiPSCs into clinical trials[62] and will support the use of human piPSCs in clinical applications, such as personalized cell therapy for specific diseases.

The success of human piPSCs opens an avenue to generate safe production protocols for hiPSCs, as human piPSCs can eliminate the potential risks from the use of viral vectors and DNA transfection. However, the extremely low efficiency of piPSC generation (approximately 0.001%) is an issue blocking the use of human piPSCs in clinical applications.

SMALL MOLECULES THAT PROMOTE iPSC PRODUCTION INVOLVING FEW TFs

Small molecules that target specific signaling pathways and/or functions are valuable chemical tools in the reprogramming of somatic cells into iPSCs. These small molecules have the potential to play extensively important roles in both evaluating the basic biology of stem cells and promoting the development of clinical approaches toward regenerative medicine. Such approaches could open the door to cell replacement therapies, which use functional homogenous cells produced under chemically defined conditions *in vitro* and the development of small-molecule drugs to stimulate the endogenous patient cells to repair themselves and regenerate.[20] Here, we review recent progress made in using small molecules to either sustain pluripotency or induce the reprogramming of somatic cells into iPSCs.

Previous studies have demonstrated that the expression of four TFs can reprogram several somatic cell types into iPSCs. Furthermore, iPSCs can be generated by viral integration of *Oct4/Sox2/Klf4* without expressing the oncogene *c-Myc*, which contributes to iPSC tumorigenicity, as determined by experiments that generated chimeras and their progeny mice.[63] For therapeutic usage, it is important to minimize the number of genes transduced into somatic cells to generate iPSCs.[64] However, decreasing the number of TFs leads to decreased iPSC generation efficiency. Therefore, several small molecules have been developed to enhance the generation efficiency of iPSCs.

Epigenetic changes are important for reprogramming somatic cells into PSCs. Therefore, several small-molecule inhibitors against epigenetic-modifying enzymes that are major players in building the epigenetic landscape,[47] such as DNA methyltransferases (DNMTs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases, were selected for somatic cell reprogramming into iPSCs. Epigenetic modulators can modify chromatin structure and make it more permissive to transcriptional machinery during somatic cell reprogramming. Some small molecules are summarized in Figure 1, which target transcription factors that are downstream in the signaling pathways. These small molecules can regulate gene expression level of pluripotent TFs, leading to somatic cell reprogramming into iPSCs, and it will be further discussed later in this review.

Small molecules with molecular weights less than 500–800 Da can freely permeate across the cell membrane and can be used to reprogram somatic cells into iPSCs.[65] It is systematically approached to identify small molecules to replace the reprogramming TFs or to promote reprogramming efficiency. One high-throughput method used to discover small molecules is as follows. Each well on the plate is seeded with somatic cells that are transduced with Oct4 promoter-driven enhanced green fluorescence protein and subsequently transcription factors, as endogenous *Oct4* expression is the first indication that iPSCs are being generated. Next, a panel of small molecules are screened, which can either replace Sox2, Oct4, and/or promote the efficiency of reprogramming. Colony numbers expressing GFP were evaluated as read out of this experiment. The colony generation efficiency is estimated from cells treated with and without small molecules.

Table 2 shows the small molecules that can replace TFs, which are required for reprogramming and increase reprogramming efficiency in generating iPSCs. These small molecules are inhibitors of epigenetic marks and singling pathways.

SMALL MOLECULES THAT CAN REPLACE *Oct4*

Oct4 is the master regulatory pluripotency gene and may serve as a pluripotency determinant in reprogramming.[15,66–68] BIX01294, a G9a HMTase inhibitor, was reported to induce miPSCs in place of *Oct4* (Table 2).[69] Furthermore, Forskolin (FSK, cAMP agonist), D4476 (casein kinase 1 inhibitor), and 2-methyl-5-hydroxytryptamine (5-HT3 agonist) can replace *Oct4* in mouse cells with the aid of other small molecules.[17] RG108, a DNMT inhibitor, can replace *Oct4* during mouse skeletal muscle cell reprogramming into miPSCs where skeletal muscle cells endogenously express *Sox2*, *Klf4*, and *c-Myc*.[70] Figure 2 shows the chemical scheme of small molecules that may functionally replace *Oct4* and *Klf4* during somatic cell reprogramming into iPSCs.

The compounds 616452 (E-616452) and SB431542 are transforming growth factor (TGF)-β inhibitors that could replace *Sox2* during mouse and human iPSC generation (Figure 1).[15,69–71] Compound 616452 can replace *Sox2* in the MEF reprogramming. However, 616452 does not actually act by inducing *Sox2* expression in the target cells; rather, it enables reprogramming through the induction of *Nanog* transcription.[69] Another TGF-β inhibitor, LY-364947, can replace *Sox2* in miPSC generation (Figure 1).[69,72] Furthermore, the glycogen synthase kinase-3 (GSK-3) inhibitor CHIR99021 (CHIR), which activates the Wnt signaling pathway, was reported to induce the reprogramming of both mouse and human somatic cells without the use of a *Sox2* transgene.[71] The protein arginine methyltransferase inhibitor AMI-5 enables *Oct4*-induced reprogramming of MEFs in combination with the TGF-β inhibitor A83-01 (A-83-01) and can replace *Sox2* during mouse somatic cell reprogramming.[73] BayK8644 (BayK), an L-channel calcium agonist, was also reported to be able to replace *Sox2* in combination with BIX01294 during MEF reprogramming into miPSCs.[63] Shh, purmorphamine, and oxysterol, the activator of Sonic hedgehog signaling, have been reported to upregulate *Bmi1*, *Sox2*, and *N-Myc* expression in mouse fibroblasts.[74] Therefore, these molecules can replace *Sox2* in mouse fibroblast reprogramming into NSCs that are naturally expressing *Sox2*.[74]

Staerk *et al.* applied a cell-based, high-throughput chemical screening method to identify small molecules that can replace *Sox2* during mouse somatic cell reprogramming.[72] From their *Nanog* reporter-based screening, they discovered that the Pan-Src family kinase inhibitors ipyrazine, dasatinib, and PP1 could replace *Sox2* in MEF reprogramming into miPSCs.[72] Figure 2 also shows the chemical scheme of small molecules that may functionally replace *Sox2* during somatic cell reprogramming into iPSCs.

SMALL MOLECULES THAT CAN REPLACE *Klf4* and *c-Myc*

Kenpaullone has been reported as a substitute for *Klf4* in mouse cells, although the underlying mechanism is unknown.[75] Furthermore, several small molecules can increase the iPSC generation efficiency, indicating that the small molecules can replace *Klf4* and *c-Myc* during somatic cell reprogramming into iPSCs. For example, VPA (an HDAC inhibitor) improves reprogramming efficiency by more than 100-fold and enables efficient iPSC induction without the introduction of *c-Myc*.[76]

SMALL MOLECULES THAT PROMOTE THE EFFICIENCY OF iPSC GENERATION

Figure 3 shows the chemical scheme of small molecules that promote somatic cell reprogramming into iPSCs. 5-Azacytidine, which is a DNMT inhibitor is reported to facilitate the transition of partially reprogrammed MEFs into fully reprogrammed iPSCs from 0.41% in partially reprogrammed MEFs to 77.8% with 5-azacytidine treatment after five passages.[77]

The MEK inhibitor PD0325901 can inhibit the mitogen-activated protein kinase/extracellular signal-regulated kinase signaling pathway to promote late somatic cell reprogramming into mouse and human iPSCs (Figure 1).[20,67,71] PD0325901 enhances the efficiency and completion of the iPSC reprogramming process. PD0325901 inhibits the growth of non-iPSC colonies and promotes the growth of reprogrammed iPSCs, leading to larger and more homogeneous iPSC colonies.[67]

Tranylcypromine (brand name: Parnate), an H3K4 demethylation inhibitor, can also promote the reprogramming efficiency of mouse somatic cells into miPSCs (approximately 20-fold).[15] Tranylcypromine is also known to activate endogenous *Oct4* expression in mouse embryonic carcinoma cells.[78]

Sodium butyrate, a natural small fatty acid and HDAC inhibitor, increases the efficiency of mouse and human iPSC

Table 2 Small molecules facilitating somatic cell reprogramming and their function

Small molecules	*C*[a]	*Host animal*	*Function*	*TFs to be replaced or function*	*Reference*
5-aza-CR, AZA	0.5 mM	Mouse	DMNT inhibitor	Promotion of reprogrammming	77
RG108	0.04–500 μM	Mouse	DMNT inhibitor	Sox2 (with BIX) or Oct4	63
RSC133	10 μM	Human	DMNT inhibitor, Histone deacetylase inhibitor	Promotion of reprogramming	82
Sodium butyrate	0.5–1 mM	Mouse, human	HDAC inhibitor	Promotion of reprogramming	79
SAHA	5 μM	Mouse	HDAC inhibitor	Promotion of reprogramming	42
TSA	20 nM	Mouse	HDAC inhibitor	Promotion of reprogramming	42
VPA	0.5–2 mM	Mouse, human	HDAC inhibitor	Promotion of reprogramming	15
Tranylcypromine (Parnate)	5–10 μM	Mouse	H3K4 demethylation inhibitor (epigenetic modulator)	Promotion of reprogramming	71
BIX	0.5–2 μM	Mouse	G9a HMTase inhibitor	Oct4	67
CHIR	3–10 μM	Mouse, human	GSK-3β inhibitor that activate Wnt signalling pathway	Sox2	15
Kenpaullone	5 μM	Mouse	GSK-3/CDKs inhibitor	Klf4	75
Compound B6	1 μM	Mouse	AKt-mediated inhibitor of GSK3-β	Promotion of reprogramming	84
LiCl	5–10 mM	Mouse and human	GSK-3β inhibitor, LSD1 inhibitor	Promotion of reprogramming	70
616452 (E-616452, RepSox)	1 μM	Mouse, human	TGF-β inhibitor (ALKinhibitor II)	Sox2	69
A83-01	0.5 μM	Mouse, human	TGF-β inhibitor	Promotion of reprogramming	43
LY-364947	1 μM	Mouse	TGF-β inhibitor	Sox2	72
SB431542	2 μM	Mouse, human	TGF-β inhibitor	Promotion of reprogramming	71
PD0325901 (PD)	0.5–1 μM	Mouse, human	MEK inhibitor	Promotion of reprogramming	67
AMI-5	5 μM	Mouse	Protein arginine methyltransferase inhibitor	Sox2, Klf4 (with A-83-01)	73
N-oxaloylglycine	1 μM	Human	Prolyl-4-hydroxylase inhibitor	Promotion of reprogramming	43
Compound B4 (TGFb-RI)	1 μM	Mouse	ALK4 inhibitor	Promotion of reprogramming	84
Dasatinib	0.5 μM	Mouse	Src family tyrosine kinase inhibitor	Sox2	72
iPY razine (iPY)	10 μM	Mouse	Src family tyrosine kinase inhibitor	Sox2	72
PP1	10 μM	Mouse	Src family tyrosine kinase inhibitor	Sox2	72
Rapamycin	0.3 nM	Mouse	mTOR inhibitor	Promotion of reprogramming	83
Compound B8	1–2 μM	Mouse	IP3K inhibitor	Promotion of reprogramming	84
Compound B10	1–2 μM	Mouse	P38 kinase inhibitor	Promotion of reprogramming	84
D4476	5 μM	Mouse	CK1 inhibitor	Oct 4 with FSK and 2-Me-5HT	17
BayK	2 μM	Mouse	a L-channel calcium agonist	Sox2	63
FSK	10–50 μM	Mouse	cAMP agonist	Oct4 with 2-Me-5HT), and D4476	17
Prostaglandin E2	5 μM	Mouse	cAMP agonist	Promotion of reprogramming	17
Rolipram	10 μM	Mouse	cAMP agonist	Promotion of reprogramming	17
2-Me-5HT	5 μM	Mouse	5-HT3 agonist	Oct4 with FSK and D4476	17
5-(4-Chloro-phenyl)-3-phenyl-pent-2-enoic acid (PS48)	5 μM	human	PDK1 activator	Promotion of reprogramming	43
8-Br-cAMP	0.1–0.5 mM	Human	cAMP-dependent protein kinase activator	Promotion of reprogramming	65
Fructose 2,6-bisphosphate	10 mM	Human	Phosphofructokinase 1 activator	Promotion of reprogramming	43
Quercetin	1 μm	Human	Hypoxia-inducible factor pathway activator	Promotion of reprogramming	43

Table (Continued)

Small molecules	*C*[a]	*Host animal*	*Function*	*TFs to be replaced or function*	*Reference*
DZNep	0.05–0.1 μM	Mouse	Epigenetic modulators	Promotion of reprogramming	17
DNP	1 μM	Human	Oxidative phosphorylation uncoupler	Promotion of reprogramming	43
TTNPB	1 μM	Mouse	Retinoic acid receptor ligand	Promotion of reprogramming	17
Oxysterol	0.5–1 μM	Mouse	Sonic hedgehog signaling	Sox2, Klf4, and C-Myc	74
Purmorphamine	0.5–1 μM	Mouse	Sonic hedgehog signaling	Sox2, Klf4, and C-Myc	74
Shh	500 ng/ml	Mouse	Sonic hedgehog signaling	Sox2, Klf4, and C-Myc	74

Abbreviations: BayK, Bay K8644; BIX, BIX-01294; CHIR, CHIR99021; CK1, Casein kinase 1; DNMT, DNA methyltransferase; DNP, 2,4-dinitrophenol; DZNep, 3-deazaneplanocin; FSK, forkolin; HDAC, histon deacetylase; G9a HMTase, G9a histone methyltransferase; IP3K, inositol triphosphate 3-kinase; PDK1, 3′-phosphoinositide-dependent kinase 1; SAHA, suberoylanilide hydroxamic acid; TF, transcription factor; TSA, trichostain A; VPA, valproic acid; 2-Me-5HT, 2-methyl-5-hydroxytryptamine; 5-aza-CR, AZA, 5-azacytidine; 8-Br-cAMP, 8-Bromoadenosine 3′′5′′-cyclic monophosphate.
[a]C; Concentration used typical reprogramming experiments.

cell generation over a range of concentrations, such as 0.5–1 mM.[79,80] The effect of sodium butyrate on reprogramming seems to be mediated by *c-Myc* and works during an early stage of reprogramming. In addition, sodium butyrate enhances the percentage of fully reprogrammed iPSCs by reducing the number of incomplete or partially reprogrammed cells.[79] Genome-wide gene expression analysis suggests that the upregulation of several pluripotent genes in sodium butyrate-treated MEFs during reprogramming is not through the suppression of the p53-p21 pathway, even though reprogramming efficiency is also known to be increased by the suppression of the p53-p21 pathway.[80]

The anti-psychotic drug lithium chloride (LiCl) can accelerate the generation of mouse and human iPSCs.[70] The effect of LiCl on promoting reprogramming is partially dependent on its role as a GSK-3β inhibitor. However, LiCl also upregulates *Nanog*, which has not been observed after the treatment of somatic cells with other GSK-3β inhibitors. Furthermore, LiCl exerts its effects by promoting epigenetic modifications via the downregulation of LSD1, an H3K4-specific HDAC gene.[70]

PS48, an activator of 3-phosphoinositide-dependent protein kinase 1, can also accelerate the reprogramming efficiency into hiPSCs.[40] PS48 activates the phosphatidylinositol 3-kinase/Akt pathway and significantly induces the expression of glycolytic genes, which results in the facilitation of metabolic reprogramming from the mitochondrial oxidation process used mainly by adult somatic cells into the glycolysis process used in PSCs.[40,43] Owing to its lower oxidative stress, glycolytic metabolism is favorable over mitochondrial respiration in PSCs during cell proliferation and cell-cycle transition. The hypoxic condition and its effector gene, hypoxia-inducible factor 1α, which is extensively linked to promoting glycolytic metabolism, can also enhance the reprogramming efficiencies of both mouse and human cells.[40,81]

Several other small molecules, such as A83-01 (TGF-β inhibitor),[43,70] 3-deazaneplanocin (epigenetic modulator),[17] 2,4-dinitrophenol (DNP, oxidative phosphorylation uncoupler),[43] RSC133 (DNMT inhibitor),[82] rapamycin (mTOR inhibitor),[83] compound B6 (AKT-mediated inhibitor of GSK3-β),[84] compound B8 (inositol triphosphate 3-kinase inhibitor),[84] compound B10 (P38 kinase inhibitor),[84] and vitamin C,[70] can facilitate the reprogramming efficiency of somatic cells into mouse and/or human iPSCs (Table 2).

SMALL MOLECULES CAN REPLACE SEVERAL TFS DURING MOUSE iPSC REPROGRAMMING

From the available knowledge of small molecules that can substitute for the essential TFs *Oct4*, *Sox2*, *Klf4*, and *c-Myc*, and promote reprogramming efficiency, several researchers tried to generate iPSCs by reducing the numbers of the TFs transduced into mammalian somatic cells. Figure 4 shows the schematic representation of some of the processes developed for miPSC generation from MEFs by reducing the numbers of TFs with and without small molecules. Table 3 summarizes the research for mouse somatic cell reprogramming into miPSCs by transduction with and without TFs and with the aid of small molecules.

NSCs and keratinocytes are known to endogenously express *Sox2*, *Klf4*, and/or *c-Myc* to some extent. Some examples of the gene expression levels of *Oct4*, *Sox2*, and *Nanog* in human fibroblasts, keratinocytes, $CD133^+$ cells from cord blood, and NSCs are described in Figure 5.[13,85,86] Several researchers tried to use endogenously expressing *Sox2*, *Klf4*, and/or *c-Myc* and to generate iPSCs with fewer numbers of pluripotent TFs. Kim *et al.* succeeded in preparing miPSCs from mouse NSCs using two exogenous TFs (*Oct4* and *Klf4* or *Oct4* and *c-Myc*).[87] They did not use small molecules to enhance reprogramming efficiency. Shi *et al.* also generated iPSCs from mouse NSCs using the G9a HMTase inhibitor BIX-01294 (BIX) in conjunction with the transduction of *Oct4* and *Klf4*, but without the use of oncogenic *c-Myc*.[67] BIX induced *Oct4* overexpression and facilitated the depression of *Oct4*, which promoted the iPSC reprogramming efficiency.[20,67]

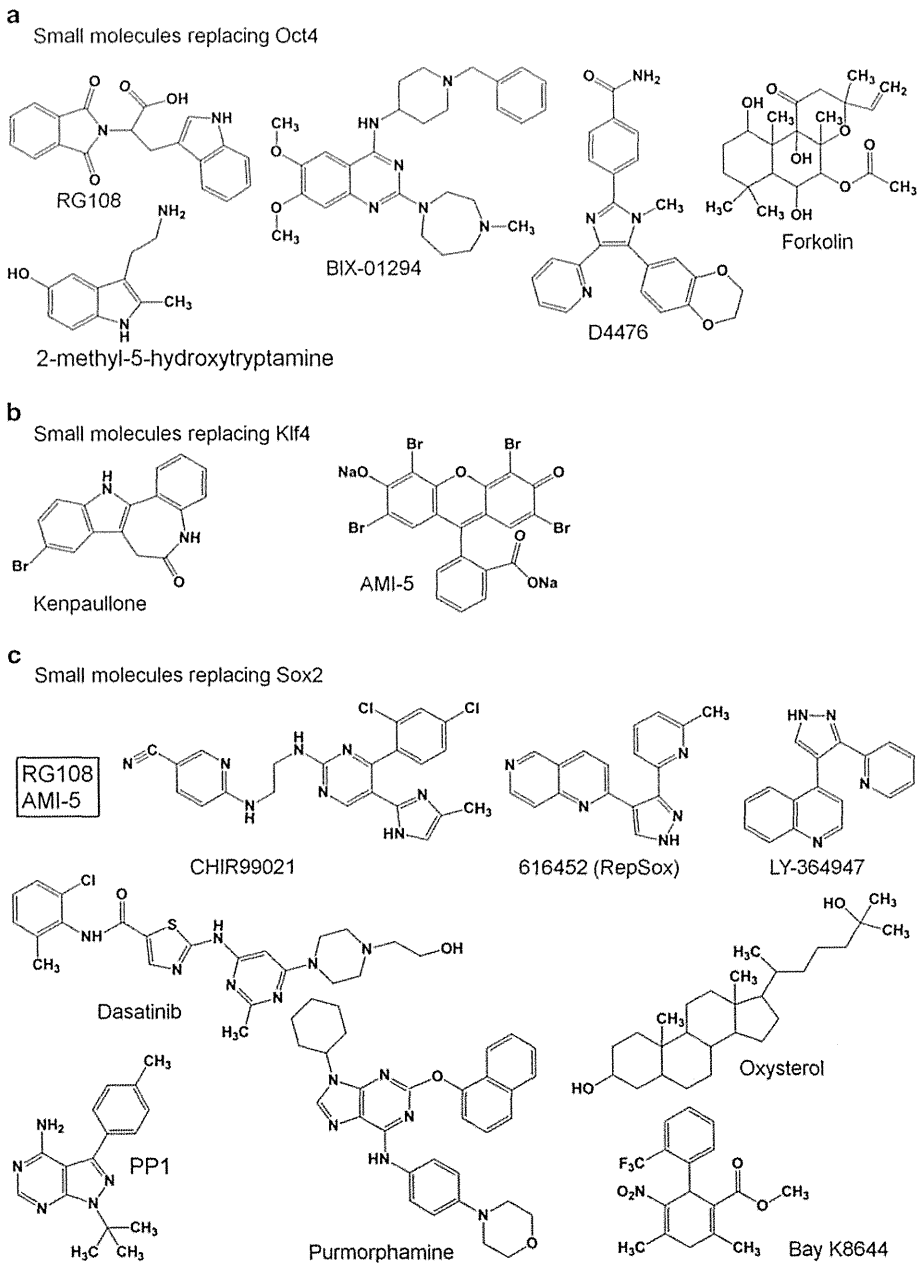

Figure 2 Representative schematic structures of small molecules that can replace *Oct4*, *Klf4*, and *Sox2* transduction in reprogramming to generate iPSCs. (**a**) Small molecules replacing *Oct4*; (**b**) small molecules replacing *Klf4*; (**c**) small molecules replacing *Sox2*.

Silva *et al.* also prepared miPSCs from mouse brain-derived NSCs using *Oct4* and *Klf4* transduction.[88] The cells rapidly adopted an undifferentiated morphology after a single round of transduction. However, these cells were partially reprogrammed and did not stably express endogenous *Oct4* or *Nanog*, and they were unable to colonize chimeras.[88] Therefore, the authors cultured the partially reprogrammed cells in medium containing a dual inhibitor of mitogen-activated protein kinase signaling and GSK-3, 2i medium, along with leukemia inhibitory factor, which is a self-renewal cytokine for mouse PSCs.[88] The cells cultured in 2i medium with leukemia inhibitory factor induced the stable upregulation of Oct4 and Nanog, transgene silencing, and competence for somatic and germline chimerism, which demonstrated that the cells were completely reprogrammed into miPSCs.[88] 2i medium is a powerful tool for generating miPSCs and

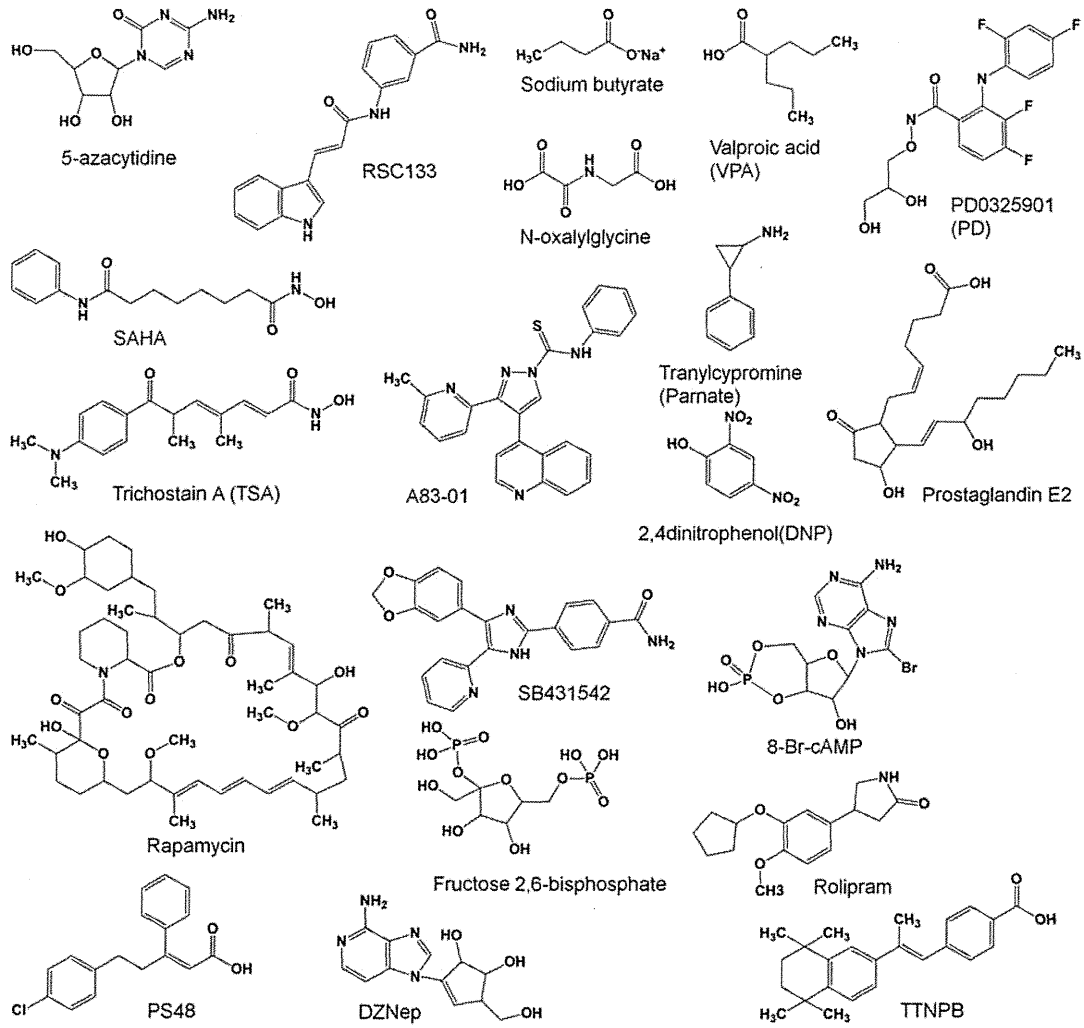

Figure 3 Representative schematic structures of small molecules that promote reprogramming to generate iPSCs.

seems to aid in the completion of mouse somatic cell reprogramming into miPSCs, although the effect of 2i medium on the generation of hiPSCs is currently unclear.

Other groups have also examined whether they could generate miPSCs from other somatic cells that do not endogenously express pluripotent TFs, such as MEFs, using small molecules and transducing fewer TFs. Huangfu *et al.* reprogrammed MEFs into miPSCs using VPA and transducing three factors without introducing the oncogene *c-Myc*. VPA, an HDAC inhibitor, improved the reprogramming efficiency by more than 100-fold under their conditions.[42]

Shi *et al.* screened several drugs to identify small molecules that can generate iPSCs from MEFs transduced with *Oct4* and *Klf4* and thus compensate for the lack of *Sox2* overexpression.[63] They found that a combination of BIX and Bayk8644 (BayK), an L-channel calcium agonist, was effective at compensating for the lack of *Sox2* expression.[63] This combination of small molecules (BIX and BayK) enabled the reprogramming of *Oct4*/*Klf4*-transduced MEFs, which do not endogenously express the TFs (ie, *Oct4*, *Sox2*, and *Klf4*) essential for reprogramming into iPSCs.[7,8] RG108, a DNMT inhibitor, can act as a direct epigenetic modifier to shift epigenetic landscapes for cell reprogramming. In combination with BIX, RG108 can reprogram cells transduced with *Oct4* and *Klf4* into miPSCs with high efficiency.[20,63]

VPA and CHIR99021 can improve the efficiency of miPSC colony generation after the transduction of three factors into MEFs, and Sox2 can be replaced by the TGF-β inhibitor 616452.[15] Therefore, Li *et al.* also generated miPSCs using only two TFs, *Oct4* and *Klf4*, in combination with VPA, CHIR99021, and 616452.[15] The authors further discovered that miPSC colonies were generated using only one TF, *Oct4*, in combination with VC6 (VPA, CHIR99021, and 616452)

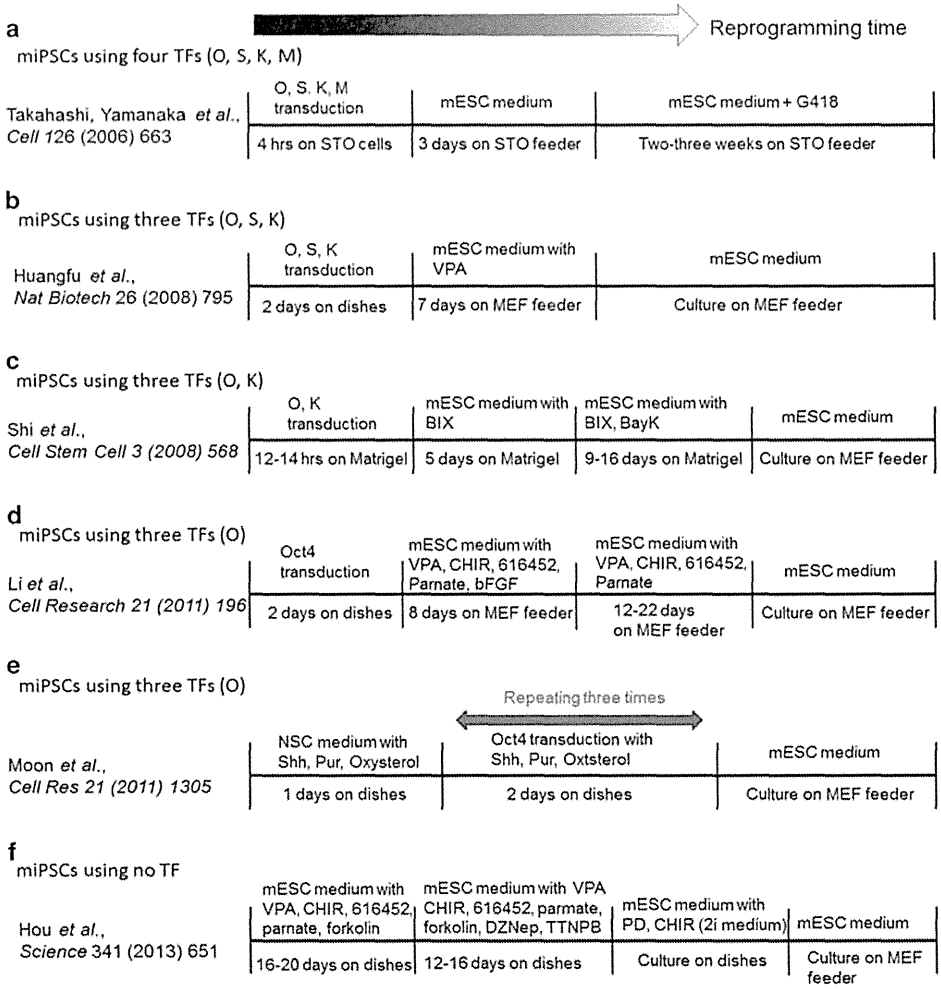

Figure 4 Schematic representation of the process of miPSC generation from MEFs by reducing the number of TFs with and without small molecules. (**a**) miPSCs using four TFs (Oct4 [O], Sox2 [S], Klf4 [K], and c-Myc [M]);[7] (**b**) miPSCs using three TFs (O, S, K);[42] (**c**) miPSCs using two TFs (O, K);[63] (**d**, **e**) miPSCs using one TF (O);[15,74] and (**f**) miPSCs using no TFs.[17]

treatment when MEFs and adult mouse fibroblasts were cultured for 30 days. However, the efficiency was only 0.0005% (one colony in 2×10^5 cells).[15] They confirmed that miPSCs could not be generated in the absence of VPA, CHIR 99021, or 616452 in their experiments.[15] Because tranylcypromine significantly promoted miPSC generation by approximately 20-fold, Li *et al.* succeeded in generating miPSCs by transduction of *Oct4* alone with the addition of VPA, CHIR99021, 616452, and tranylcypromine (VC6T) in the culture medium.[15] It is thought that the small-molecule combination including VC6T facilitated miPSC generation by lowering several major barriers to the reprogramming process. VPA (an HDAC inhibitor) and tranylcypromine (an H3K4 demethylation inhibitor) are epigenetic modulators that have been reported to facilitate iPSC generation.[42,89] From the effects of VPA and tranylcypromine, H3K4 demethylation and HDAC may be two major epigenetic barriers in the generation of miPSCs, suppressing the pluripotency transcriptional network. GSK3-β inhibition by CHIR99021 or TGF-β signaling inhibition by 616452 could efficiently replace *Sox2* for reprogramming.[15] GSK3-β and TGF-β signaling should also be another two critical barriers that suppress the reprogramming process. Therefore, overcoming these four major reprogramming barriers, ie, two epigenetic barriers and two signaling barriers, may allow researchers to generate miPSCs by *Oct4* induction alone.[15]

miPSC GENERATION USING ONE TF

Yuan *et al.* generated miPSCs from MEFs using only one factor, *Oct4*. They treated MEFs with a combination of small molecules, including AMI-5, a protein arginine methyltransferase inhibitor, and A83-01, a TGF-β inhibitor, to generate miPSCs.[73] miPSCs were able to generate live-born pups through tetraploid complementation assays, indicating

Table 3 Reprogramming of mouse somatic cells into miPSCs by transduction with and without transcription factors (TFs) with the aid of small molecules

TFs required[a]	*Somatic cells*	*PSCs*	*Small molecules*	*Efficiency (%)*	*Reference*
Four factors					
OSKM	MEFs	miPSCs	None	0.02%	7
OSKM	Mouse hepatocyte and gastric epithelial cells	miPSCs	None	0.01%	10
OSKM	Mouse liver and stomach cells	miPSCs	None	0.01%	10
OSKM	MEFs	miPSCs	Compound B6, B8, and/or B10	0.14–0.35%	84
Three factors					
KSM	Mouse NPCs	miPSCs	None	0%	67
KSM	Mouse NPCs	miPSCs	BIX	0.004%	67
OSK	MEFs	miPSCs	None	0.001–0.0026%	12
OSK	MEFs	miPSCs	VPA	0.089%	42
OSK	MEFs	miPSCs	CHIR	0.18%	71
Two factors					
OK	Mouse NPCs	miPSCs	None	0.004–0.006%	87
OK	Mouse NPCs	miPSCs	BIX	0.034%	67
OK	Mouse NPCs	miPSCs	PD + CHIR (2i)	0.0125%	88
OK	MEFs	miPSCs	None	0.003%	69
OK	MEFs	miPSCs	BIX/BayK	0.022%	63
OK	MEFs	miPSCs	CHIR	0.006%	71
OK	MEFs	miPSCs	616452	0.05%	69
OK	MEFs	miPSCs	VPA, CHIR, 616452	0.01–0.04%	15
One factor					
O	Mouse NPCs	miPSCs	None	0.004%	16
O	MEFs	miPSCs	None	0%	15
O	MEFs	miPSCs	VPA, CHIR, 616452, Parnate	0.05–0.08%	15
O	MEFs	miPSCs	AMI-5, A-83-01	0.02%	73
O	MEFs	miPSCs	Shh, purmorphamine, or oxysterol	0.11%	74
No factor					
No factor	Mouse skeletal myoblasts	miPSCs	RG108	0.11%	68
No factor	MEFs	miPSCs	VPA, CHIR, 616452, Parnate, Forkolin, DZNep	0.04%	17
No factor	MEFs	miPSCs	VPA, CHIR, 616452, Parnate, Forkolin, DZNep, TTNPB	0.2%	17

[a]O, S, K, and M indicate Oct4, Sox2, Klf-4, and c-Myc, respectively.

that the miPSCs achieved full reprogramming. Their study suggests that the regulation of protein arginine methylation by inhibition with A83-01 is also involved in the reprogramming process into miPSCs.[73] The efficiency of miPSC generation was reported to be 0.02% in their study.[73]

Moon *et al.* also generated miPSCs from mouse fibroblasts using only one factor, *Oct4*, with the aid of the small molecules of Shh, purmorphamine, and oxysterol.[74] These small molecules can activate sonic hedgehog signaling, which compensates for Bmi1 gene expression. Bmi1 expression in