Transcriptomic analysis of feeder-free culture system for maintaining naïve-state pluripotency in human pluripotent stem cells

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Background: Human pluripotent stem cells (hPSCs) such as embryonic stem cells (ESCs) and induced pluripotent stem cells (PSCs) have the capacity of self-renewal and multilineage differentiation *in vitro*. Conventional hPSCs, which are in a primed state, can produce various types of differentiated cells. However, the variability in their degree of pluripotency and differentiation propensities, which is influenced by the inductive methods and culture conditions, limit their availability. Therefore, PSCs in a naïve state are a promising source of PSCs.

Methods: We recently developed a culture system for naïve hPSCs using an inhibitor of the NOTCH signaling pathway and a histone H3 methyltransferase disruptor. This culture system requires feeder cells for stably maintaining the naïve hPSCs. We aimed to develop a culture system for hPSCs that could maintain pluripotency under feeder-free conditions.

Results: We used two inhibitors to develop an alternative feeder-free culture system to obtain naïve hPSCs. The naïve cells underwent stable cellular proliferation and were positive for naïve stem cell markers; in addition, they could differentiate into the three germ layers. These feeder-free dome-shaped induced pluripotent stem cells (FFDS-iPSCs) have characteristics similar to that of naïve-like PSCs.

Conclusions: The naive hPSCs under feeder-free conditions could ensure supply of cells for various applications in regenerative medicine and disease modeling.

Keywords: Induced pluripotent stem cells (iPS cells); naïve stem cells; regenerative medicine

Introduction

A well-designed medium containing a basic fibroblast growth factor (bFGF), maintains flat monolayer colonies of primed human induced pluripotent stem cells (hiPSCs) in feeder-independent culture conditions (1). Various differentiated cells are derived from hiPSCs for practical use. However, the variability in their degree of pluripotency and differentiation propensities, which depends on the inductive methods and culture conditions, hinders the supply of cells (2). The development of various kinds of pluripotent stem cells (PSCs), in addition to these bFGF-dependent hiPSCs, is necessary to ensure supply of various differentiated cells and to minimize variability. Leukaemia

inhibitory factor (LIF)-dependent naïve-like stem cells are derived from mouse embryonic stem cells (ESCs). These cells, characterized by dome-shaped colonies, were first developed in 2013 by adding several small molecule inhibitors in addition to growth factors, such as bFGF and LIF (3). Specific culture conditions for converting primed hiPSCs and human ESCs to LIF-dependent naïve-like PSCs are standardized (4,5). Supplementation with small molecule inhibitors for transgene-independent protocols is used to establish culture conditions for clinical applications. A combination of four or five inhibitors, LIF, and activin A (4i or 5i/L/A) (6) is used in premade commercial media. However, these protocols require feeder cells, such as mouse embryonic fibroblasts (MEFs), to maintain pluripotency. A feeder-free culture method and commercial medium is available (7); however, it requires Matrigel matrix-coated dishes. Matrigel is a biological product de-rived from mouse sarcoma; therefore, it is desirable to exclude it for culturing naïve-like PSCs for clinical applications. Both feeder cells and Matrigel contain animal-derived components and are difficult to handle. Therefore, we aimed to culture human naïve-like PSCs using recombinant vitronectin-coated dishes. In addition to avoiding the use of biological materials, this method enables easy handling.

To enhance pluripotency characteristics, we focused on small-molecule inhibitors that were associated with reprogramming, because reprogramming is closely related to the naïve state (8,9). Pharmacological inhibition of the NOTCH signaling pathway [dibenzazepine (DBZ)] and the use of the histone H3 methyltransferase disruptor of telomeric silencing 1-like inhibitor (DOT1L; iDOT1L) enhanced the reset process of hiPSCs (10). We obtained naïve-like PSCs without hypoxic conditions. The combination of DBZ, a representative γ-secretase inhibitor, and iDOT1L promoted the conversion from the primed to naïve state and the maintenance of the naïve state. Therefore, a combination of growth factors, small-molecule inhibitors, and culture conditions were assessed.

Methods

Ethics statement

Human endometrial tissues from menstrual blood and placental tissues were collected after obtaining signed informed consent. The experiments were approved by the Institutional Review Board of the National Center for Child Health and Development (NCCHD) of Japan (Permit Nos.

#146 and #518). All experiments using human cells and tissues were performed in accordance with the tenets of the Declaration of Helsinki (as revised in 2013).

The animal use protocol was approved by the Institutional Animal Care and Use Committee of the NCCHD (Permit No. A2003-002). All animal experiments were based on the three Rs (refine, reduce, and replace), and all efforts were made to minimize animal discomfort and to reduce the number of animals used.

Conventional primed hiPSC culture

All cell lines were maintained in a humidified atmosphere at 37 ℃ with 5% CO_2 in air (21% O_2, 5% CO_2). We used three types of previously established hiPSCs: cells from menstrual blood (EDOM), cells from the human skin fibroblast (HSF) derived cells, and human adipose tissue-derived stem cells (ADSCs; female, PT5006; Lonza Bioscience, Walkersville, MD, USA). EDOM-iPS cells (EDOM#6-iPSCs) were generated using a Cyto-Tune™-iPS Sendai Reprogramming Kit (Thermo Fisher Scientific, Waltham, MA, USA). The other lines (ADSC-A5 and HSF02) were established through direct delivery of mRNAs encoding reprogramming factors (Stemgent StemRNA 3rd Gen Reprogramming Kit; Reprocell, Yokohama, Japan) and cultured in StemFlex™ Medium (Thermo Fisher Scientific) on vitronectin (Thermo Fisher Scientific, for EDOM#6 and ADSC-A5) (10) or iMatrix-511 (Nippi, Tokyo, Japan, for HSF02). Cells were passaged every 5–7 days by dissociation with Accutase (Thermo Fisher Scientific), and replated onto a matrix coated plate with a ROCK inhibitor (Y-27632; FUJIFILM Wako Pure Chemical, Tokyo, Japan) at a final concentration of 10 μM. These cells were replated at a density of 2×10^3 cells/cm². The medium was replaced completely every day, except on the day after passage.

Feeder-free dome-shaped induced pluripotent stem cells (FFDS-iPSCs) culture

FFDS-iPSCs were converted from primed-state hiPSCs and cultured on vitronectin (4×10^4 cells per cm²). We used a combination of two commercial products, StemFlex™ Medium (Thermo Fisher Scientific) as the basal medium and the supplemental component RSeT™ Medium (RSeT™ 5X Supplement, STEMCELL Technologies, Vancouver, BC, Canada) to supply a combination of inhibitors, LIF, and activin A (4). We combined StemFlex™ Medium (Thermo Fisher Scientific) and RSeT™ 5X Supplement

(STEMCELL Technologies) at a ratio of 4:1; 2 µM DBZ (R&D Systems, Minneapolis, MN, USA) and 3 µM iDOT1L (Abcam, Cambridge, UK) were added to this mixed medium for maintaining naïve-state cells under feeder-free conditions. Dimethyl sulfoxide (1:1,000 dilution; Sigma-Aldrich, Missouri, USA) was included as the solvent for the two compounds. At the initiation of FFDS-iPSCs culture, single cells derived from primed hiPSCs were passaged on vitronectin-coated culture plates at a density of 4×10^4 cells/cm^2. Every 2–5 days, all differentiating FFDS-iPSCs were passaged through dissociation with Accutase and replating on vitronectin-coated plates (2×10^4 cells per cm^2) with 10 µM ROCK inhibitor for 48 h. The medium was replaced completely on alternate days. The cell growth rate and doubling time of FFDS-iPSCs (ADSCs) were calculated based on the number of replated cells, the total increase in the number of cells, and the duration of culture. Cell proliferation curves were obtained, and population doubling (PD) at the nth passage (Pn) was determined as follows: PD at Pn = PD at Pn 1 + log2 (counted cells/seeded cells).

Embroid body (EB) formation for in vitro differentiation assay

Single cells were dissociated and seeded at a density of 1×10^4 cells in 100 µL medium per well, in uncoated 96-well ultra-low attachment plates (Corning Ultra-Low Attachment Multiple Well Plate; Merck Millipore, Burlington, MA, USA) to form EBs. After 9 days, the EBs were transferred to gelatine-coated tissue culture dishes at a density of approximately three EBs per cm^2 and allowed to differentiate further for 3 weeks. The original medium contained 90% DMEM/Nutrient Mixture F-12 (DMEM/F-12), 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.1 mM nonessential amino acids (all reagents from Thermo Fisher Scientific). This medium (30 µL per well) was added at five days after seeding in 96-well plates; the medium was replaced thrice every week.

Teratoma formation for the in vivo differentiation assay

Approximately $1–5\times10^6$ cells were injected into the subcutaneous tissue and kidney capsule of nude mice (BALB/cAJcl-nu/nu; CLEA Japan, Tokyo, Japan); tumor masses were collected after 2 to 3 months (11). The harvested tumours were fixed with 4% paraformaldehyde, embedded in paraffin, cut into 5 µm sections, and stained with haematoxylin and eosin. Various parts of the tumours were subjected to histological analysis; the three germ layers were identified based on representative histological features (11).

Immunofluorescence staining

Cells were cultured in a glass-bottom dish (AGC Techno Glass, Shizuoka, Japan) and fixed with 4% paraformaldehyde for 10 min at 4 ℃ before being permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 min at room temperature (RT). The samples were blocked with 5% normal goat serum in Dulbecco's phosphate-buffered saline (DPBS; Thermo Fisher Scientific) for 30 min at RT. They were incubated with primary antibodies at 4 ℃ overnight. After washing with DPBS, the samples were incubated with secondary antibodies conjugated to Alexa 488 or 546 (Thermo Fisher Scientific) for 30 min at RT. After washing with DPBS, the samples were placed in mounting medium with 4',6-diamidino-2-phenylindole (DAPI). The following primary antibodies were used: OCT4 (C-10; Santa Cruz Biotechnology, Dallas, TX, USA), NANOG (ReproCell), Tra1-60 (MAB4360; Sigma-Aldrich), SSEA4 (MAB1435; R&D Systems), SSEA1 (MAB2155; R&D Systems), KLF4 (ab216875; Abcam), PRDM14 (ab187881; Abcam), anti-βIII Tubulin (TUJ1, Promega, Madison, Wisconsin, USA), α-smooth muscle actin (α-SMA; A2547; Sigma-Aldrich), and SOX17 (MAB1924; R&D Systems). Images were acquired using an LSM510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). All antibodies except for the anti-TUJ1 antibody (1:300) were used at a 1:150 dilution in 5% normal goat serum.

Quantitative reverse transcription polymerase chain reaction (PCR)

Total RNA from the cell pellet was extracted using ISOGEN II (Nippon Gene, Tokyo, Japan). cDNA was prepared using Superscript® IV Reverse Transcriptase (Thermo Fisher Scientific), according to the manufacturer's instructions. Gene expression was analysed using Qiagen RT2 Profiler PCR Arrays (Qiagen, Hilden, Germany), which were commercially produced for simultaneously and reliably assessing the expression of genes in various pathways. Total RNA (500 ng) was used with a PCR array kit; PCR was performed using the SYBR Green method (RT2 SYBR® Green qPCR Mastermixes; Qiagen) in a 7300 Real-Time PCR System (Thermo Fisher Scientific), following the manufacturer's instructions. Threshold cycle

values were converted to relative values. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control; the expression level in each sample was normalized to that in a primed ADSC iPS cell line. Representative mRNA levels of pluripotency-related genes in 12 cell lines, including six FFDS-iPSCs lines and six primed hiPSC lines, were assessed with RT² Profiler™ PCR Array Human Induced Pluripotent Stem Cells (#PAHS-092Z; Qiagen).

Karyotypic analysis

Chromosomal G-band analyses of primed hiPSCs and FFDS-iPSCs were performed at Chromosome Science Labo. Ltd. (Sapporo, Hokkaido, Japan). At least 20 metaphase spreads were examined for each cell line.

Statistical analysis

Quantitative data are reported as the mean ± standard error of the mean (SEM) from at least three independent experiments. ExAtlas (https://lgsun.irp.nia.nih.gov/exatlas/) software for gene expression meta-analysis was used to construct heatmaps and calculate P values. A P value <0.05 was considered statistically significant by Student's *t*-test.

Results

Stable cultivation of FFDS-iPSCs

Three independent primed hiPSC lines (ADSC-iPSCs, EDOM-iPSCs and HSF-iPSCs) were used (10). We developed a culture condition for the maintenance of pluripotency of naïve-like hiPSCs under feeder-free conditions. The hiPSCs culture condition contains a NOTCH inhibitor, DBZ, and a histone methyltransferase DOT1L inhibitor (iDOT1L), which we have previously reported in maintaining the naïve state using feeder cells (10). We applied these conditions to culture feeder-free naïve human pluripotent stem cells (hPSCs). However, we were unable to maintain the conversion of naïve hiPSCs from primed hPSCs using the RSeT™ medium supplemented with DBZ and iDOT1L in initial experiments (data not shown). Furthermore, applying another culture condition using the StemFlex™ medium as the basal medium supplemented with LIF and 6 inhibitors, MEK inhibitor, GSK-3b inhibitor, BRAF inhibitor, SRC inhibitor, DBZ, and iDOT1L was unstable in the naïve state (data not

shown). We eventually optimized a specific combination of the human PSC culture products supplemented with DBZ and iDOT1L. All investigated PSC lines were maintained as dome-shaped colonies characteristic of naïve-like stem cells over ten passages. Therefore, the culture of the ADSC-iPS cell line was further evaluated (*Figure 1A,1B*). A cellular proliferation assay showed that FFDS ADSC-iPSCs (FFDS-ADSCs) had a PD time similar to that of primed ADSC-hiPSCs (primed-ADSCs) (FFDS-ADSCs: 1.05±0.27 *vs.* primed-ADSCs: 0.91±0.22 days, P=0.16; *Figure 1C*).

Pluripotency markers and karyotype evaluation of feeder-free iPSCs

To confirm the pluripotency of FFDS-iPSCs, qRT-PCR analysis and immunofluorescence staining for representative pluripotency markers were performed. The transcript levels of the pluripotency markers (NANOG, POU5F1, SOX2 and TERT) in the FFDS-iPSCs were equal to that in primed-hiPSCs (*Figure 2A*). In addition, immunofluorescence staining indicated NANOG expression in the nuclei of FFDS-ADSCs (*Figure 2B*); SSEA4 and Tra1-60 were detected in the cytoplasm. In addition, we detected representative naïve pluripotency-related proteins, SSEA1, in the cytoplasm and KLF4 and PRDM14, in the nuclei (*Figure 2B*). Culture systems used for producing naïve-like stem cells indicated a tendency towards inducing aneuploidy (6,12,13). Therefore, metaphase chromosome analyses with G-banding were performed on the FFDS-iPSCs at passage 12 and displayed normal karyotypes in FFDS-iPSCs (*Figure 2C*). The pluripotency of the established FFDS-iPSCs was evaluated based on EB formation during *in vitro* differentiation and teratoma formation *in vivo*. EBs differentiated from FFDS-iPSCs expressed markers associated with the three germ layers: TUJ1 (ectoderm), alpha-smooth muscle cell (αSMA) (mesoderm), and SOX17 (endoderm; *Figure 2D*). In the *in vivo* analysis, representative ectoderm, mesoderm, and endoderm derivatives were detected in all transplant experiments with FFDS-iPSCs (*Figure 2E*).

Naïve characterization of FFDS-iPSCs at transcription level

To examine the differences in gene expression between FFDS-iPSCs and primed-iPSCs, we used RT-PCR array kits in which the major transcription factors associated with pluripotency and various differentiation-related genes were
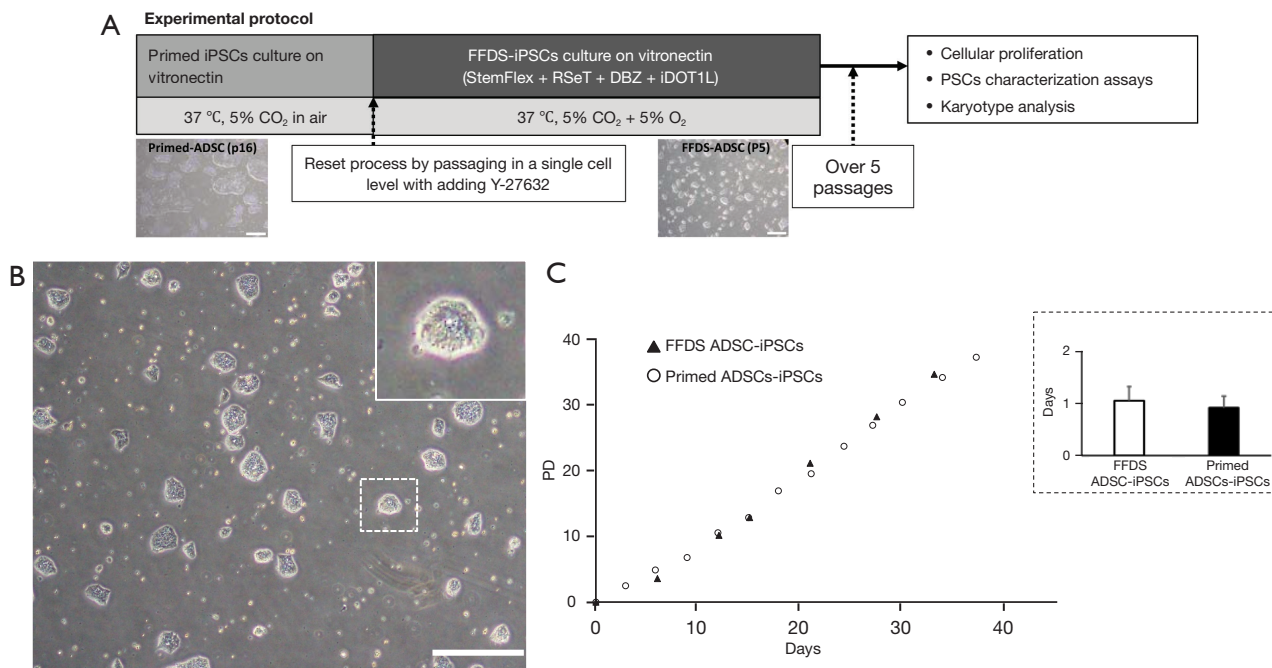
Figure 1 Cellular characteristics of feeder-free dome-shaped pluripotent stem cells. (A) Summary of the FFDS-iPSCs culture process. FFDS-iPSCs were passaged every 3–5 days by dissociation with Accutase. All scale bars denote 500 µm. (B) Morphological changes in FFDS-iPSCs colonies were observed. Dome-shaped colonies were maintained throughout the observation period for over 10 passages. Scale bar =500 µm. (C) The growth rate of FFDS-ADSCs (white circles) was equal to that of primed ADSCs (black triangles), and they had identical doubling times (days). The left white bar indicates FFDS-ADSCs, and the right black column indicates primed ADSCs. iPSC, induced pluripotent stem cell; FFDS, feeder-free dome-shaped; PSC, pluripotent stem cell; DBZ, dibenzazepine; ADSC, adipose tissue-derived stem cell; PD, population doubling.

included. A total of 84 genes were used for cluster analysis, and ExAtlas (https://lgsun.irp.nia.nih.gov/exatlas/) gene expression meta-analysis software was used for constructing heatmaps. In total, 12 samples, six primed-iPSC and six FFDS-iPSC samples, were used. Hierarchical clustering classified the 12 samples into two groups based on culture conditions (*Figure 3A*). Based on earlier studies that systematically assessed the characteristics of naïve-like PSCs (4,7,14-16), the well-known naïve pluripotency-related markers, such as DPPA2, DPPA3, FGF4, GATA2, GDF3, KLF4, NODAL, SOX15, TBX3, and UTF1, tended to be highly expressed in FFDS-iPSCs (*Figure 3B*).

Discussion

Since the first report of human naïve PSCs (3-5), the insights into the hierarchy of human PSCs have evolved. Regarding application for differential works, especially early human developmental, culturing PSCs under naive conditions are becoming indispensable models (17). The latest advancements in generating human embryo-like 3D structures including the blastocyst-like structure, known as blastoid, require naïve state PSCs (18-21). However, there is a lack of standardized culture protocols for human naïve PSCs (22). Further studies for improving the culture conditions of human naïve PSCs are necessary, because they could be aided modelling early human development (17).

We aimed to develop a more convenient and stable cultivation system for human naïve PSCs in a feeder-free culture, to further facilitate human stem cell-based embryology. We evaluated representative culture conditions for naïve-like PSCs, when selecting growth factors and inhibitors (4,6). We used a most popular commercial product, containing a combination of four or five inhibitors, LIF, and Activin A for performing the reset process. Several applications require maintaining naïve state PSCs without feeders; therefore, we added inhibitors of the NOTCH signaling pathway (DBZ) and histone H3 methyltransferase
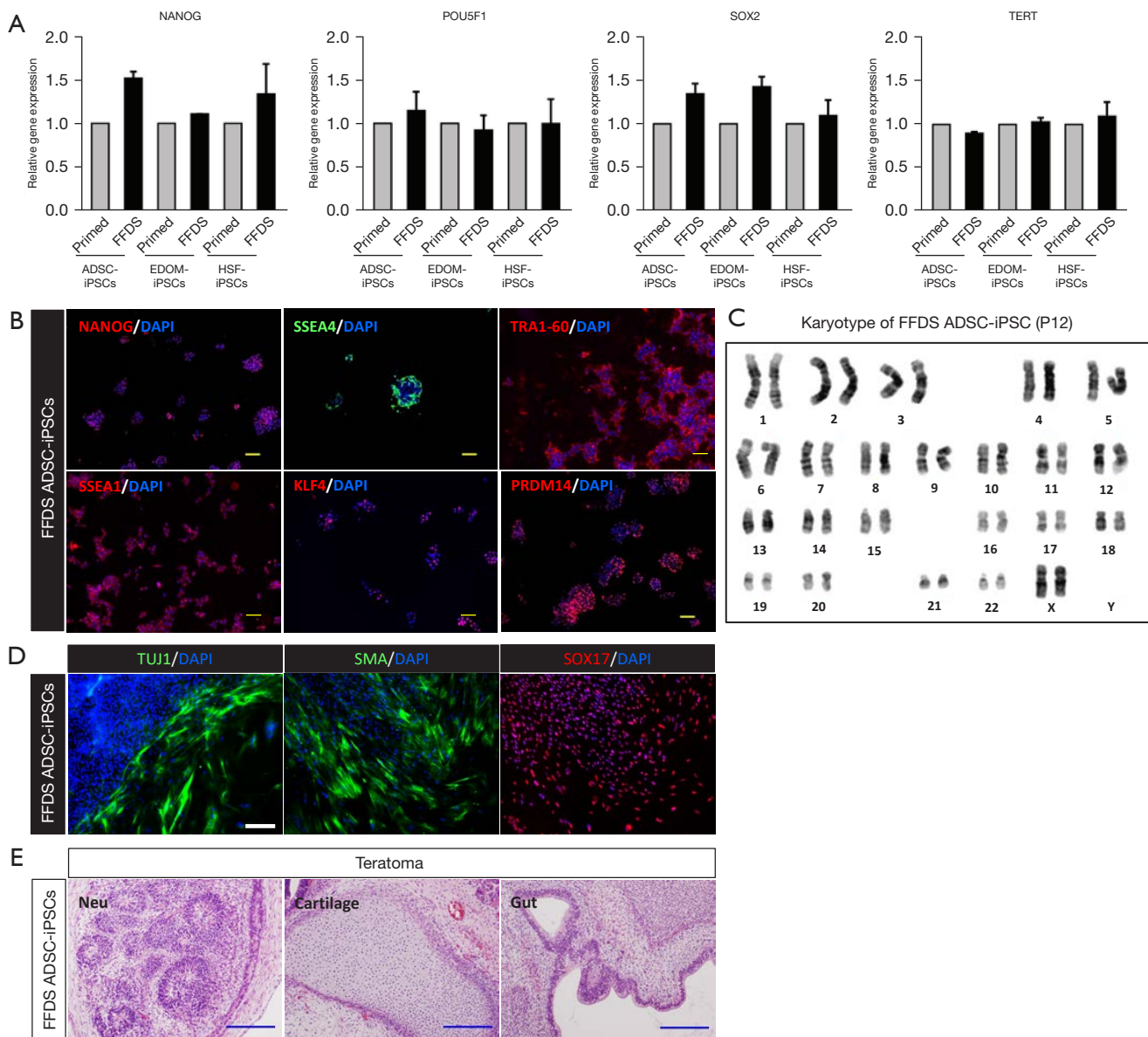
Figure 2 Pluripotency markers and karyotype evaluation of feeder-free dome-shaped pluripotent stem cells. (A) The expression levels of representative pluripotency markers were compared in three independent human iPS cell lines (ADSC-iPSC, EDOM-iPSC, HSF-iPSC). Relative expression of *NANOG*, *POU5F1*, *SOX2*, and *TERT* in FFDS-iPSCs (black columns) compared following normalization to the levels in primed iPSCs (grey columns). Data are reported as the mean ± SE. Statistically significant differences were identified between primed PSCs and FFDS-iPSCs using Student's *t*-test (n=6). (B) Expression of NANOG, SSEA1, SSEA4, TRA1-60, KLF4, and PRDM14 in undifferentiated FFDS-iPSCs from ADSC-iPSCs (FFDS ADSC-iPSCs). DAPI (blue), SSEA4 (green), and NANOG, SSEA1, TRA1-60, KLF4, and PRDM14 (red). Scale bar =100 µm. (C) Normal female karyotype of the FFDS ADSC-iPSCs at 12 passages was detected through G-banding. (D) FFDS ADSC-iPSCs differentiated *in vitro* EBs expressed markers of the three germ layers. Immunohistochemical analyses of markers of the ectoderm (TUJ1, green), mesoderm (SMA, green), and endoderm (SOX17, red) layers. Cells were double stained with the cell nucleus marker DAPI (blue). Scale bar is 200 µm. (E) FFDS-ADSCs differentiated *in vivo* via teratoma formation. Hematoxylin and eosin staining revealed germ layer derivatives, such as neural tissues (Neu; ectoderm), cartilage (mesoderm), and gut epithelial tissues (Gut; endoderm). Scale bars: 200 µm. FFDS, feeder-free dome-shaped; ADSC, adipose tissue-derived stem cell; iPSC, induced pluripotent stem cell; EDOM, menstrual blood; HSF, human skin fibroblast; DAPI, 4',6-diamidino-2-phenylindole; SMA, smooth muscle actin; iPS, induced pluripotent stem; PSC, pluripotent stem cell; SE, standard error; EB, embryoid body.
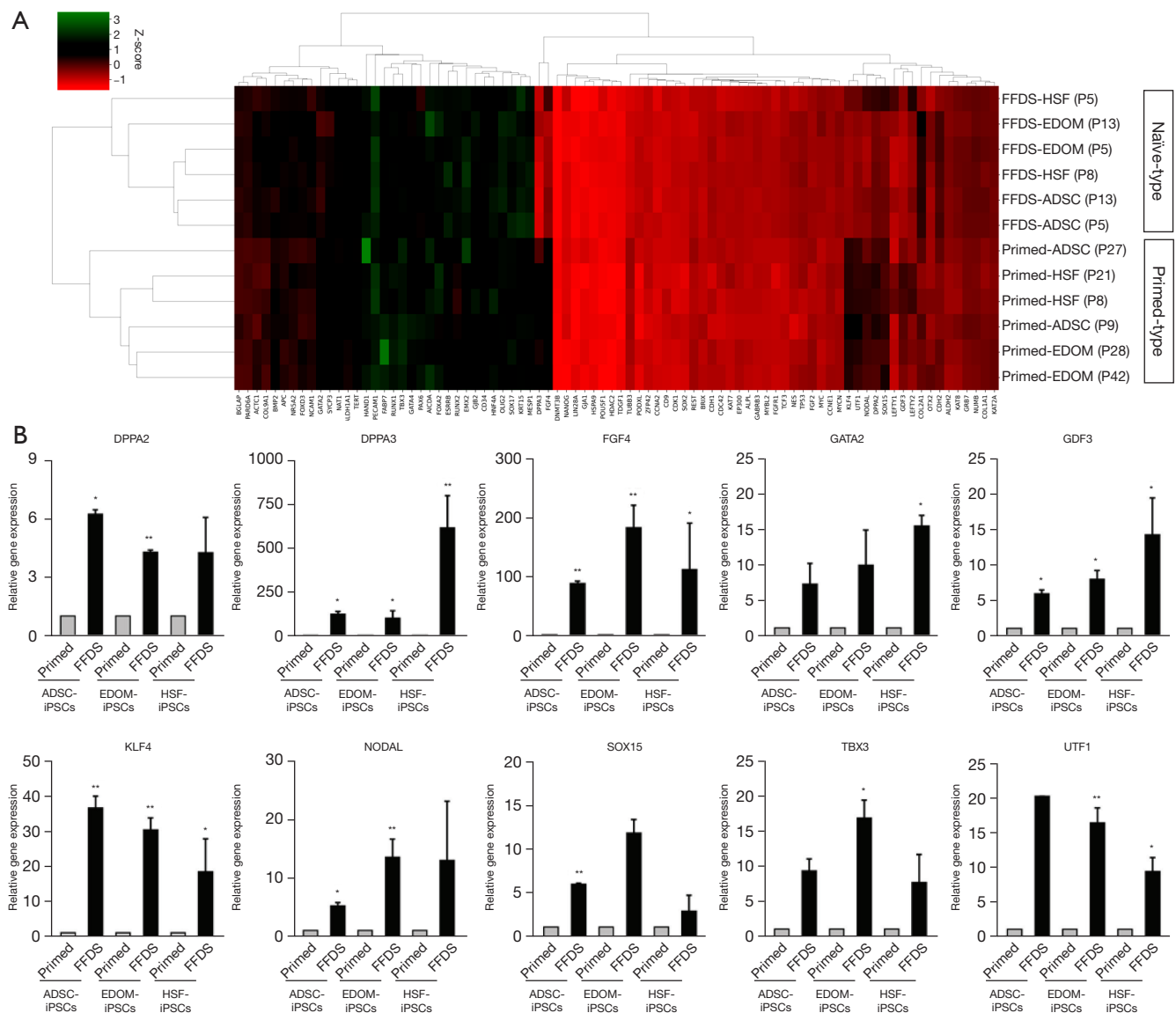
Figure 3 Naïve characterization of FFDS ADSC-iPSCs at transcription level. (A) The heat map shows the expression level of each gene in six primed hiPSC lines and six FFDS-iPSC lines. Clustering analysis was based on 84 gene expression patterns. GAPDH was used as an internal control. The passage number of iPSCs is indicated as "P". (B) The expression levels of representative naïve pluripotency-related markers were compared. The average expression levels and standard deviations of each gene in primed-iPSC lines (grey columns) and FFDS-iPSC lines (black columns) are indicated. The data are reported as mean ± SE. Statistically significant differences were identified between primed iPSCs and FFDS-iPSCs using Student's *t*-test (n=3). *, P<0.05; **, P<0.01. FFDS, feeder-free dome-shaped; HSF, human skin fibroblast; iPSC, induced pluripotent stem cell; EDOM, menstrual blood; ADSC, adipose tissue-derived stem cell; hiPSC, human induced pluripotent stem cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SE, standard error.

(iDOT1L), to promote pluripotency (8,10). However, we could not maintain dome-shaped colonies in this system. Therefore, we evaluated the supplementation of two growth factors, bFGF and LIF. We combined two commercial products, a base medium for primed hiPSCs and a supplement for naïve-like PSCs. We obtained FFDS-iPSCs, which could be maintained for over 10 passages. FFDS-iPSCs could not be maintained without DBZ and iDOT1L;

therefore, these two inhibitors were necessary for stably maintaining the naïve state under feeder-free conditions.

The pluripotency of the native-state iPSCs, FFDS-iPSCs, was verified in both *in vitro* and *in vivo* differentiation into the three germ layers; this pluripotency was maintained for a certain culture period. A normal karyotype was retained after 12 passages. The expression of the naïve markers was consistently higher in all three cell lines of FFDS-iPSCs, compared to that in the primed iPSCs. Characterization of the FFDS-iPSCs showed that culturing of human naïve PSCs under refined conditions with feeder-free could facilitate applications using naïve hPSCs.

Conclusions

Combining two growth factors, bFGF and LIF, and further supplementing the medium with DBZ and iDOT1L could stably convert and maintain dome-shaped PSCs from primed hiPSCs under feeder-free conditions in vitronectin-coated plates. These FFDS-iPSC had naïve-like characteristics.

Acknowledgments

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://sci.amegroups. com/article/view/10.21037/sci-2022-043/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Human endometrial tissues from menstrual blood and placental tissues were collected after obtaining signed informed consent. The experiments were approved by the Institutional Review Board of the National Center for Child Health and Development (NCCHD) of Japan (Permit Nos. #146 and #518). All experiments using human cells and tissues were performed in accordance with the tenets of the Declaration of Helsinki (as revised in 2013). The animal use protocol was approved by the Institutional Animal Care and Use Committee of the NCCHD (Permit No. A2003-002). All animal experiments were based on the three Rs (refine, reduce, and replace), and all efforts were made to minimize animal discomfort and to reduce the number of animals used.

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