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Original Article

# The combination of dibenzazepine and a DOT1L inhibitor enables a stable maintenance of human naïve-state pluripotency in non-hypoxic conditions

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ABSTRACT

Conventional human pluripotent stem cells (hPSCs), known for being in a primed state, are pivotal for both basic research and clinical applications since such cells produce various types of differentiated cells. Recent reports on PSCs shed light on the pluripotent hierarchy of stem cells and have promoted the exploration of new stem cell states along with their culture systems. Human naïve PSCs are expected to provide further knowledge of early developmental mechanisms and improvements for differentiation programmes in the regenerative therapy of conventionally primed PSCs. However, practical challenges exist in using naïve-state PSCs such as determining the conditions for hypoxic culture condition and showing limited stable cellular proliferation. Here, we have developed new leukemia inhibitory factor dependent PSCs by applying our previous work, the combination of dibenzazepine and a DOT1L inhibitor to achieve the stable culture of naïve-state PSCs. The potential of these cells to differentiate into all three germ layers was shown both *in vitro* and *in vivo*. Such new naïve-state PSCs formed dome-shaped colonies at a faster rate than conventional, primed-state human induced PSCs and could be maintained for an extended period in the absence of hypoxic culture conditions. We also identified relatively high expression levels of naïve cell markers. Thus, non-hypoxia treated, leukemia inhibitory factor-dependent PSCs are anticipated to have characteristics similar to those of naïve-like PSCs, and to enhance the utility value of PSCs. Such naïve PSCs may allow the molecular characterization of previously undefined naïve human PSCs, and to ultimately contribute to the use of human pluripotent stem cells in regenerative medicine and disease modelling.


## 1. Introduction

Basic fibroblast growth factor (bFGF) dependent primed human induced pluripotent stem cells (hiPSCs), which have pluripotency similar to that of post-implantation epiblasts, produce various differentiated cells *in vitro*. However, the variability of their pluripotency levels and the differentiation propensities derived from inductive methods and culture conditions make supplying target cells difficult [1,2]. Thus, the development of various kinds of PSCs is desirable in order to provide various differentiated cells and to minimize variability. In this regard, leukemia inhibitory factor (LIF)-dependent PSCs, represented by mouse embryonic stem cells (ESCs), are worthy of attention. LIF-dependent mouse ESCs appear to represent a more naïve (ground state) pre-implantation state than do hiPSCs due to their association with the inner cell mass [3]. This LIF-dependent state seems to more highly resemble the biological environment of the early blastocyst stage than bFGF-dependent culture conditions for primed hiPSCs, since LIF transcripts are localized in the differentiated trophectoderm and LIF

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receptor mRNA is found in the inner cell mass of the blastocyst stage of mouse embryogenesis [4]. Therefore, specific culture conditions have been developed to covert primed hiPSCs and human ESCs to LIF-dependent naïve-like stem cells [5,6]. Supplementation with small molecule inhibitors for transgene-independent protocols has been used to easily adjust culture conditions with the goal of an eventual clinical application [6]. One such standard protocol involved a combination of four or five inhibitors, LIF, and activin A (4i or 5i/L/A) [7,8], which maintained a naïve-like state via the activity of the distal enhancer in octamer-binding transcription factor 4 (OCT4) [7]. This combination has also been utilized in pre-made commercial media to facilitate the culture of cells. However, such a method requires unconventional hypoxic culture conditions for primed hiPSCs, probably because hypoxia-related genes enhance pluripotency characteristics [9]. Since hypoxic conditions may also induce cytotoxicity to feeder cells [10], alternative methods to hypoxic conditions advantageous.

We focused on small-molecule inhibitors identified in our laboratory as being related to the reprogramming process, based on the hypothesis that reprogramming is closely related to the naïve-state [11,12], and that hypoxic conditions contribute to the reprogramming process [10,13]. In our previous study, pharmacological inhibition of the NOTCH signalling pathway (via N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester [DAPT] or dibenzazepine [DBZ]) and the histone H3 methyltransferase disruptor of telomeric silencing 1-like inhibitor (DOT1L; iDOT1L) boosted the reprogramming process of hiPSCs from somatic cells by achieving c-MYC- and Krüppel-like factor 4 (KLF4)-free iPSC production [14]. Considering the results of this technique, in which small-molecule compounds are substituted for conditions essential for the reprogramming process, the combination of DBZ, a representative γ-secretase inhibitor, and iDOT1L is expected to promote the conversion from primed to naïve state and the maintenance of the naïve state.

Here, we report a newly developed culture conditions in which two small-molecule inhibitors were added to conventional medium designed for culturing naïve-like PSCs. The new culture condition enabled us to stably maintain the naive-like state of PSCs in a LIF-dependent and non-hypoxic manner. We subsequently evaluated LIF-dependent PSC lines for pluripotential characteristics.

## 2. Materials and methods

### 2.1. Ethics statement

Human endometrial tissues from menstrual blood and placental tissues were collected after receiving signed informed consent, with ethical approval from the Institutional Review Board of the National Center for Child Health and Development (NCCHD) of Japan (permit numbers EDOM #146, PL #55) [15]. All experiments conducted in this study using human cells and tissues were performed in accordance with the tenets of the Declaration of Helsinki.

The animal use protocol was approved by the Institutional Animal Care and Use Committee of the NCCHD (Permit Number: 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.

### 2.2. Conventional primed hiPSC culture

All cell lines, including naïve-like cell lines, were maintained in a humidified atmosphere of 37 °C, 5% $CO_2$ in air. We used three types of previously established hiPSCs: cells from menstrual blood (EDOM), cells from placental artery endothelium (PL), and human adipose tissue-derived stem cells (ADSCs; Female, PT5006; Lonza Bioscience, Walkersville, MD). EDOM-iPS cells (EDOM#6-iPSCs) were generated by CytoTune™-iPS Sendai Reprogramming Kit (ThermoFisher Scientific, Waltham, MA). PL-iPS cells were established by retroviral infection of four reprogramming factors (OCT4, SRY box transcription factor 2 [SOX2], c-MYC, and KLF4) [15]. Cells were maintained on tissue culture plates precoated with 0.1% gelatine and irradiated mouse embryonic fibroblasts (MEFs; $30 \times 10^3$ per $cm^2$) in medium consisting of 80% KnockOut™ Dulbecco's modified Eagle's medium (DMEM), 20% KnockOut™ Serum Replacement, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM nonessential amino acids (all reagents from Thermo Fisher Scientific); 0.2 mM 2-mercaptoethanol (Sigma, St. Louis, MO), and 10 ng/mL recombinant human basic fibroblast growth factor (bFGF; FUJIFILM Wako Pure Chemical, Tokyo, Japan). The other line (ADSC) was established by direct delivery of mRNAs encoding reprogramming factors [16] (Stemgent StemRNA 3rd Gen Reprogramming Kit; Reprocell, Yokohama, Japan) and cultured in StemFlex™ Medium (Thermo Fisher Scientific) on vitronectin (Thermo Fisher Scientific).

Cells were passaged every 5–7 days. For EDOM- and PL-iPSCs lines, after detaching the MEFs with CTK solution (Reprocell), colonies were dissociated manually with a cell scraper (Sumitomo Bakelite, Tokyo, Japan). For ADSC-iPSCs, cells were dissociated by Accutase (Thermo Fisher Scientific) with a ROCK inhibitor (Y-27632; FUJIFILM Wako Pure Chemical) at a final concentration of 10 μM. EDOM-iPSCs and PL-iPSCs were replated at about a ratio of approximately 1:5 at 80% confluency, and ADSC-iPSCs were replated at a density of $2 \times 10^3$ cells/$cm^2$. All medium was changed daily except for the day after passage.

### 2.3. Non-hypoxic LIF-dependent PSCs culture

Non-hypoxic LIF-dependent (NHL)-PSCs were converted from primed-state hiPSCs and cultured on irradiated MEFs ($40 \times 10^3$ per $cm^2$). We used a commercial product, ReproNaïve™ (ReproCELL) according to Theunissen et al. [8]. as base medium. ReproNaive includes LIF in spite of bFGF and is used under low-oxygen culture conditions according to the manufacturer's instructions. To prepare naïve-state maintaining medium under non-hypoxic condition, 2 μM DBZ (R&D Systems, Minneapolis, MN) and 3 μM iDOT1L (Abcam, Cambridge, UK) were added to this medium. This customized medium also contained dimethyl sulfoxide (1:1000 dilution; Sigma–Aldrich) as a solvent for these two compounds. At the initiation of NHL-PSCs culture, single cells derived from primed hiPSCs were prepared for passaging on MEF-coated culture plates at a density of $4 \times 10^4$ cells per $cm^2$. All converting NHL-PSCs from primed-state conditions were passaged every 2–5 days by dissociation with CTK or Accutase, and replated onto MEFs layers ($2 \times 10^4$ cells per $cm^2$) with 10 μM ROCK inhibitor for 48 h. All medium was changed daily except for the day after passaging.

The cell growth rate and doubling time of NHL-PSCs (EDOM) were simply calculated from the number of replated cells, the total number of increased cells, and the duration of culture. However, since primed hiPSCs (EDOM) were cultured without cell counting, the increased number of cells was considered equal at each replating at 80% confluency. Cell proliferation curves were obtained and population doubling (PD) at the nth passage (Pn) was described as follows: PD at Pn = PD at Pn-1 + log 2 (counted cells/seeded cells).

### 2.4. Embryo body formation for in vitro differentiation assay

Single cells were dissociated and seeded at $1 \times 10^4$ cells per well in uncoated 96-well ultra-low attachment plates (Corning®

Costar® Ultra-Low Attachment Multiple Well Plate size 96 well; Merck Millipore, Burlington, MA) to form embryo bodies (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% foetal 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 was added at 30 μL per well five days after seeding for cultures in 96-well plates and changed three times weekly for cultures in tissue culture dishes.

### 2.5. Teratoma formation for in vivo differentiation assay

For teratoma formation according to our previous reports [17], approximately from $1 \times 10^6$ to $5 \times 10^6$ cells were injected into the subcutaneous tissue and kidney capsule of nude mice (BALB/cAJcl-nu/nu; CLEA Japan Inc., Tokyo, Japan) and tumor masses collected after 2–3 months. Harvested tumours were fixed with 4% paraformaldehyde, embedded in paraffin, serially sectioned into 5 μm sections and stained with haematoxylin and eosin. Various parts of the tumours were subjected to histological analysis and classified into the three germ layers by representative histological features [17]. Ectoderm derivatives were classified into neural tissue, including neural rosettes, neural tubes and neuropils, pigmented cells, and squamous epithelium, including squamous cells and nests. Endoderm derivatives were classified into endodermal tubes. Mesoderm derivatives were classified into cartilage cells, bone tissue, blood vessels, smooth muscle cells and fat cells.

### 2.6. Immunofluorescence analysis of stem cell and differentiation markers

Cells were cultured in a glass-bottom dish (AGC TECHNO GLASS, Shizuoka, Japan) and fixed with 4% paraformaldehyde for 10 min at 4 °C before being permeabilized with 0.1% Triton X-100 (Sigma) for 10 min at room temperature (RT). After blocking with 5% normal goat serum in Gibco™ Dulbecco's phosphate-buffered saline (DPBS; Thermo Fisher Scientific) for 30 min at RT, samples were incubated with primary antibodies at 4 °C overnight. After washing with DPBS, samples were incubated with secondary antibodies conjugated to Alexa 488 or 546 (Thermo Fisher Scientific) for 30 min at RT. After washing with DPBS, mounting medium with DAPI was used.

Primary antibodies specific for the following proteins were used in this study: OCT4 (C-10; Santa Cruz Biotechnology, Dallas, TX), NANOG (ReproCell), Tra 1-60 (MAB4360; Sigma–Aldrich), SSEA4 (MAB1435, R&D Systems), KLF4 (ab216875; Abcam), PRDM14 (ab187881; Abcam), anti-βIII Tubulin (TUJ1, Promega, Madison, Wisconsin), α-smooth muscle cell actin (α-SMA; A2547; Sigma), 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.

### 2.7. Copy DNA preparation and gene expression analysis

Quantitative reverse transcriptase PCR (qRT–PCR) was performed. After total RNA from the cell pellet was extracted with ISOGEN II (Nippon Gene, Tokyo, Japan), cDNA was prepared with Superscript® IV Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturer's instructions. Gene expression was analysed using Qiagen $RT^2$ Profiler PCR Arrays (Qiagen, Hilden, Germany), which were commercially produced for the simultaneous reliable assessment of gene expression in various pathways. Total RNA (500 ng) was used with a PCR array kit, and PCR was performed based on a 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 normalized to those of housekeeping genes, including actin-beta (ACTB), beta-2-microglobin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1) and ribosomal protein large P0 (RPLP0), and translated to relative values. GAPDH was used as an internal control, and the expression level in each sample was normalized to that in a primed EDOM cell line.

Representative mRNA expression levels of pluripotency-related genes in 12 cell lines, including six NHL-PSC lines and six primed hiPSC lines, were assessed with $RT^2$ Profiler™ PCR Array Human Induced Pluripotent Stem Cells (#PAHS-092Z; Qiagen) and $RT^2$ Profiler™ PCR Array Human Embryonic Stem Cells (#PAHS-081Y; Qiagen) arrays.

In addition, the gene expression levels in EBs derived from three NHL-PSC lines and three primed hiPSC lines were compared with an $RT^2$ Profiler™ PCR Array Human Induced Pluripotent Stem Cells (#PAHS-092Z; Qiagen) array.

### 2.8. Karyotypic analysis

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

### 2.9. Statistical analysis

Quantitative data are reported as means ± 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.

## 3. Result

### 3.1. Stable cultivation of non-hypoxia, LIF-dependent pluripotent stem cells

Three independent primed hiPSC lines (EDOM-iPSCs, PL-iPSCs and ADSC-iPSCs) were used in this research. The differential properties of the primed cell lines were confirmed *in vitro* and *in vivo* before naïve converting experiments (Supplementary Figs. S1a and b). In order to establish a convenient culture system for human naïve stem cells, we evaluate the cellular states of PSCs under physiological conditions of oxygen. In our preliminary experiments, we failed to convert and maintain the pluripotency of hiPSCs using a commercial product (ReproNaive™) based on previously reported work [8] by Theunissen et al. in non-hypoxic culture conditions (37 °C, 5% $CO_2$ in air; data not shown). Considering any additional factors required to sustain naïve conditions, we previously reported that a combination of Notch signalling and histone methyltransferase inhibition induced pluripotency at a high efficiency even without KLF4 and c-MYC reprogramming factors [14]. For conversion to a LIF-dependent condition, primed hiPSC lines were adapted to ReproNaive medium (naïve-based medium) on MEFs by treating with a NOTCH inhibitor, DBZ, and a histone methyltransferase DOT1L inhibitor (iDOT1L) under non-hypoxic culture conditions (Supplementary Fig. S2). All examined

PSC lines were maintained as dome-shaped colonies over twenty PDs (Supplementary Fig. S3a). We selected EDOM-iPSCs in order to primed hiPSCs (Supplementary Fig. S2). Dome-shaped colonies formed after the initiation of non-hypoxia, LIF-dependent culture conditions; following further passaging, most colonies displayed a stable small dome shape (Fig. 1a). NHL-EDOM cells were maintained as dome-shaped colonies for over 100 days in culture (Fig. 1b). A cellular proliferation assay showed that NHL-EDOM cells had a much shorter population doubling time than primed hiPSCs (NHL-EDOM: 23.4 ± 8.1 vs. Primed-EDOM: 50.8 ± 13.3 days, $P < 0.01$; Fig. 1b).

To confirm the pluripotency of NHL-EDOM, qRT-PCR analysis and immunofluorescence staining of representative pluripotency markers were performed. Transcript levels of pluripotency markers (POU5F1, NANOG, SOX2 and TERT) in the NHL-EDOM were similarly expressed compare to primed EDOM-iPSCs (Fig. 1c). In addition, immunofluorescence staining showed that OCT4 and NANOG were positively expressed in the nuclei of NHL-EDOM cells (Fig. 1d).

### 3.2. Chromosomal stability in NHL-PSCs culture

Since some culture systems used in previous studies to produce naïve-like stem cells, showed a tendency for aneuploidy [8,18,19], metaphase chromosome analyses by G-banding were performed on hiPSCs and NHL-PSCs. In this study, we examined the two primed hiPSC lines (EDOM and ADSC) in chromosomal types (chromosomal abnormalities existed in PL-iPSCs even before the initiation of NHL culture conditions). Widespread karyotypic abnormalities were not observed, and sufficient the maintenance of a diploid karyotype was detected in NHL-PSCs derived from these hiPSCs (Fig. 1e, Supplementary Fig. 3b). These results indicated that NHL-PSCs culture conditions could maintain chromosome stability.

### 3.3. Differentiation into three germ layers

To assess the pluripotency of the established NHL-PSCs, *in vitro* differentiation assays were performed to evaluate embryoid body (EB) formation *in vitro* and teratoma formation *in vivo*.

EBs differentiated from NHL-EDOM expressed markers associated with the three germ layers: TUJ1 (ectoderm), αSMA (mesoderm), and SOX17 (endoderm; Fig. 2a). OLIG2 and PAX6 (Ectoderm), MESP1 and PECAM1 (Mesoderm), HNF4A and GATA4 (Endoderm) differentiation markers in transcription levels were detected in EBs from the NHL-EDOM compared to EBs from primed EDOM-iPSCs, although there were statistically signified (Fig. 2b).

In the *in vivo* analysis, representative ectoderm, mesoderm and endoderm derivatives were detected in all transplant experiments of NHL-EDOM (Fig. 2c). Additionally, in *in vitro* and *in vivo* assays of other NHL-ADSC and NHL-PL, all three germ layers were detected (Supplementary Figs. 3c and d).

### 3.4. Expression level changes and hierarchical cluster analysis of pluripotency-related genes

To examine the differences in gene expression between NHL-PSCs and primed hiPSCs, we used RT-PCR array kits in which the major transcription factors associated with pluripotency and various differentiation-related genes were included. A total of 132 genes were used for cluster analysis, and ExAtlas (https://lgsun.irp.nia.nih.gov/exatlas/) gene expression meta-analysis software was used to construct heatmaps. Cells in samples were allowed to differentiate with regard to passage numbers of primed hiPSCs provided for the NHL-PSCs culture process. In total, 12 samples, including six primed hiPSC and six NHL-PSC samples, were used in this analysis. Hierarchical clustering indicated that the 12 samples could be divided into two groups based on culture conditions (Fig. 3a). No approximate gene expression similarity between the six NHL-PSCs was detected based on primed hiPSC cell lines. In accordance with studies that systematically assessed the characteristics of naïve-like PSCs [6,8,20–22], lists of previously reported naïve pluripotency-related markers were extracted from 132 genes. Primed pluripotency-related genes were also extracted [6,20]. This method did not allow comprehensive analyses of markers to be performed (Fig. 3b). However, the majority of naïve pluripotency-related markers, including DPPA2, DPPA3, DPPA5, FGF4, GATA6, GDF3, KLF4, LEFTY2, NODAL and NR0B1, tended to be highly expressed. The expression of three genes, UTF1, *PRDM14* and *TDGF1*, was similar to that in NHL-PSCs (Fig. 3b). Interestingly, major primed pluripotency-related genes (*ZIC2*, *DNMT3B* and *THY1*) were significantly downregulated in NHL-PSCs (Fig. 3c). Immunofluorescence staining also revealed that PRDM14 and SSEA1, as naïve markers, localized in NHL-PSCs (Fig. 3d). Furthermore, qRT–PCR analysis on EBs from three primed hiPSC and three NHL-PSC samples, were performed. Hierarchical clustering indicated that the six samples could be clearly divided into two groups based on culture conditions (Supplementary Fig. 4a). The expression of four genes-*BMP2*, *CD34*, *FOXA2* and *SOX17*-were significantly upregulated in EBs of NHL-PSCs (Supplementary Fig. 4b).

## 4. Discussion

Since bFGF-dependent primed hiPSCs of different cell lines show marked differences in performance [2,23], we sought to establish a new type of LIF-dependent PSC. During the development of a new culture method, we examined representative culture conditions for naïve-like PSCs derived from previous studies [7,8]. This is because the reset process, during which primed-like PSCs are converted to naïve-like PSCs, might allow further developmental potential, as observed for mouse ESCs [24].

To perform the reset process and solve problems specific to primed hiPSCs, a combination of four or five inhibitors, plus LIF and Activin A, was used [6–8]. However, several laborious conditions are still required to maintain naïve-state hPSCs in the laboratory. Current naïve-state culture needs cells to be grown under hypoxic conditions [5–8]. Naïve PSCs show limited cellular proliferation and so stop proliferating at ~20 passages without continuously expressing exogenous factors [25]. To provide practical advantages over current naïve-state PSCs, the stable culture of naïve-state PSCs under non-hypoxic conditions, even without the genomic integration of any exogenous factors, might benefit further applications. Currently, pre-made commercial media developed based on previous report, have enabled the initial maintenance of naïve-like PSCs; however, this still requires the use of a non-physiological concentration of oxygen. In fact, we failed to maintain naïve-state PSCs using such media under non-hypoxic conditions (data not shown). Therefore, current conditions need improvement to achieve practical and convenient conditions for culturing naïve-state PSCs.

With regard to additional factors to improve cellular pluripotency, we previously reported that two inhibitors of the NOTCH signalling pathway and histone H3 methyltransferase, namely, DAPT or DBZ, and iDOT1L, respectively, strongly enhanced the efficiency of the reprogramming process [14]. In this study, we found that two reprogramming-enhancing chemicals of DBZ and iDOT1L applied to naïve-state PSCs stably enhanced the naïve state under non-hypoxic conditions. In short, conventional naïve medium with DBZ and iDOT1L enabled primed PSCs to convert to non-hypoxic LIF-dependent-PSCs without any exogenous factors in cells. Interestingly, NHL-PSCs can be maintained for >100 passages. Karyotypic analysis showed a normal karyotype after 11 passages.

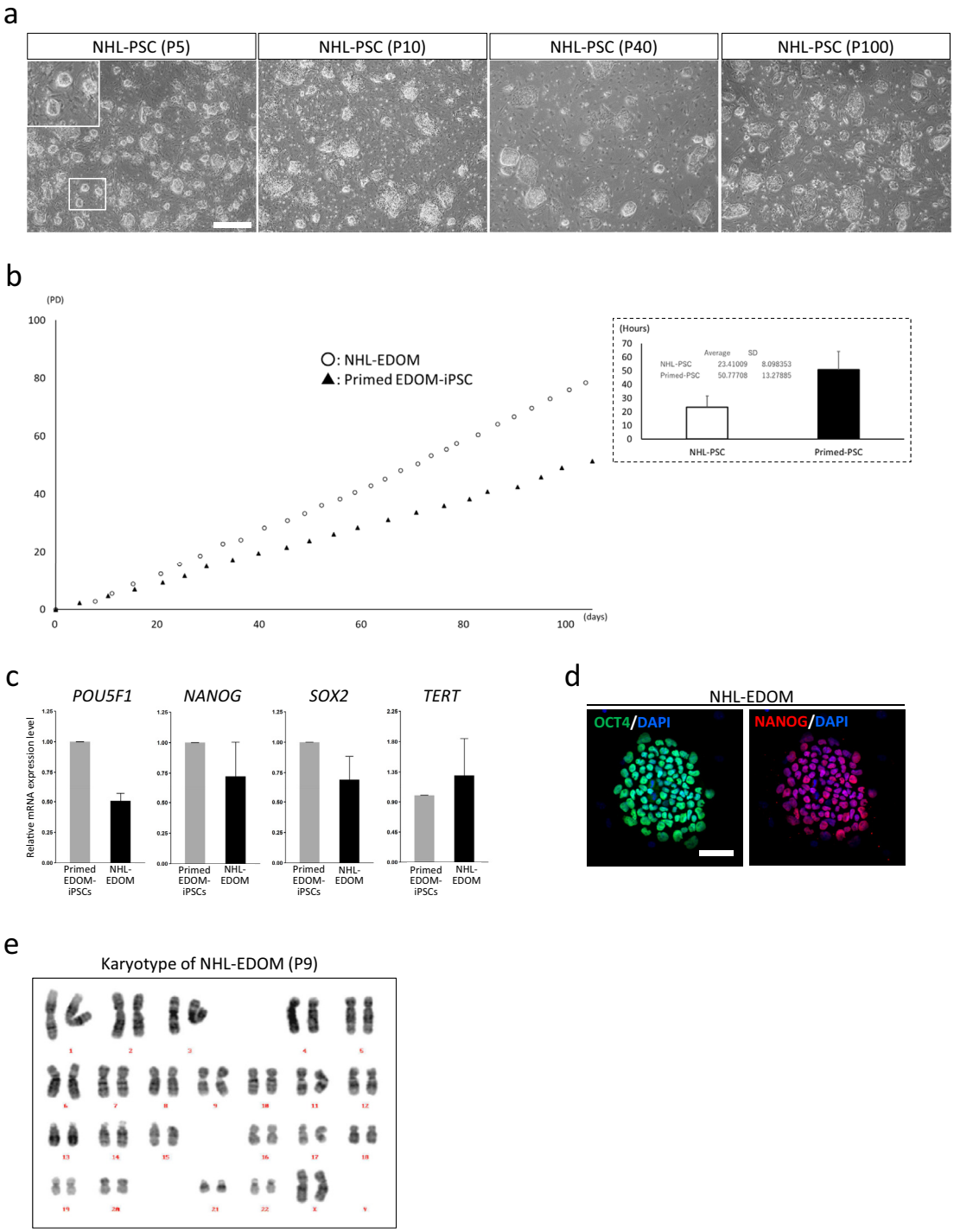


**Fig. 1. Cellular characteristics of non-hypoxia, LIF-dependent pluripotent stem cells.** (a) Morphological changes in non-hypoxia leukemia inhibitory factor-dependent pluripotent stem cell (NHL-PSC) colonies could be observed throughout the long culture period. Dome-shaped colonies were maintained throughout the observation period for over 100 passages. Scale bar = 500 μm. "P" indicates the passage number. (b) The growth rate of non-hypoxia leukemia inhibitory factor-dependent human endometrial cells (NHL-EDOM; white circles) cells was higher than that of primed EDOM-iPS cells (black triangles), and a much shorter doubling time (hours) was observed (NHL-PSC: 23.4 ± 8.1 vs. Primed-PSC: 50.8 ± 13.3, $P < 0.01$). The left white bar indicates NHL-EDOM cells, and the right black column indicates primed EDOM-iPS cells. (c) The expression levels of representative pluripotent markers were compared. Relative expression of POU5F1, NANOG, SOX2, and TERT in NHL-EDOM (black columns) cells compared with normalization to primed EDOM-iPSCs (grey columns) expression levels. Data are reported as mean ± SE. Statistically significant differences were not identified between primed EDOM-iPSCs vs. NHL-EDOM using Student's *t*-test (n = 3). (d) Expression of OCT4 and NANOG in undifferentiated NHL-EDOM cells. DAPI (blue), OCT4 (green), and NANOG (Red). Scale bar = 100 μm. (e) Normal female karyotype of the NHL-EDOM cells at 9 passages was detected by G-banding.

Although the mechanism of action of these two inhibitors was not elucidated, the pluripotency of the new NHL-PSCs was clearly verified by their *in vitro* and *in vivo* differentiation into all three germ layers. This pluripotency was maintained for a relatively long culture period. Although the EDOM-iPSC line was mainly used, similar effects were found in the other two lines, ADSC-iPSCs and PL-iPSCs. Our findings are important initial steps to overcoming the current inconvenience of culturing native-state PSCs; however, the

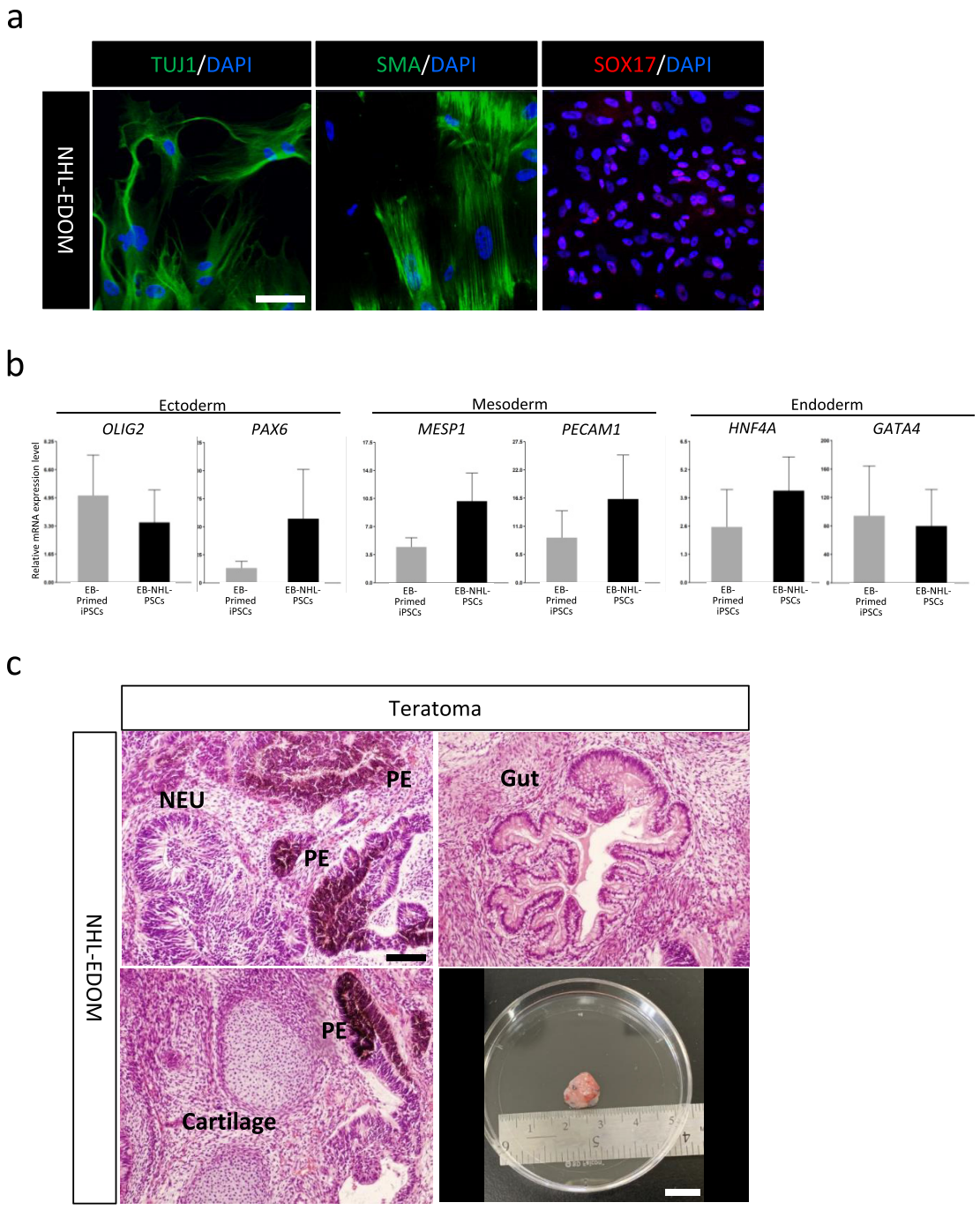


**Fig. 2. Differentiation into the three germ layers of NHL-PSCs.** (a) NHL-EDOM cells differentiated *in vitro* via EBs expressed markers of the three germ layers. Immunohistochemical analyses of markers of the ectoderm (TUJ1), mesoderm (SMA), and endoderm (SOX17) layers are shown. Cells were double stained with the cell nucleus marker, DAPI. Scale bar is 100 μm. (b) The expression levels of representative differentiation markers were compared. The average expression levels and standard deviations of each gene in three primed human-induced pluripotent stem cell (hiPSC) lines (grey columns) and three non-hypoxia leukemia inhibitory factor-dependent pluripotent stem cell (NHL-PSC) lines (black columns) are indicated. Data are reported as mean ± SE. Statistically significant differences were not identified between primed iPSCs vs. NHL-PSCs using Student's *t*-test (n = 3). (c) Non-hypoxia leukemia inhibitory factor-dependent human endometrial (NHL-EDOM) cells differentiated *in vivo* via teratoma formation. Hematoxylin and eosin staining revealed germ layer derivatives, such as neural tissues (NEU), pigmented epithelium (PE; ectoderm), cartilage (Cartilage; mesoderm), and gut epithelial tissues (Gut; endoderm). Scale bars are 200 μm.

molecular mechanisms involved in enhancing the naïve-state of NHL-PSCs under non-hypoxic conditions need to be resolved.

In summary, two reprogramming-enhancing chemicals, DBZ and iDOT1L, in current naïve medium enabled us to stably convert and maintain naïve-state PSCs from a primed state under non-hypoxic conditions. The newly acquired PSCs were found to have naïve human pluripotent characteristics and showed stable proliferation for over 100 passages. Naïve PSCs without exogenous genome integrations under non-hypoxic conditions may allow the molecular dissection of previously undefined naïve PSCs in humans, and also contribute to expanding the use of human pluripotent stem cells in regenerative medicine and disease modelling.

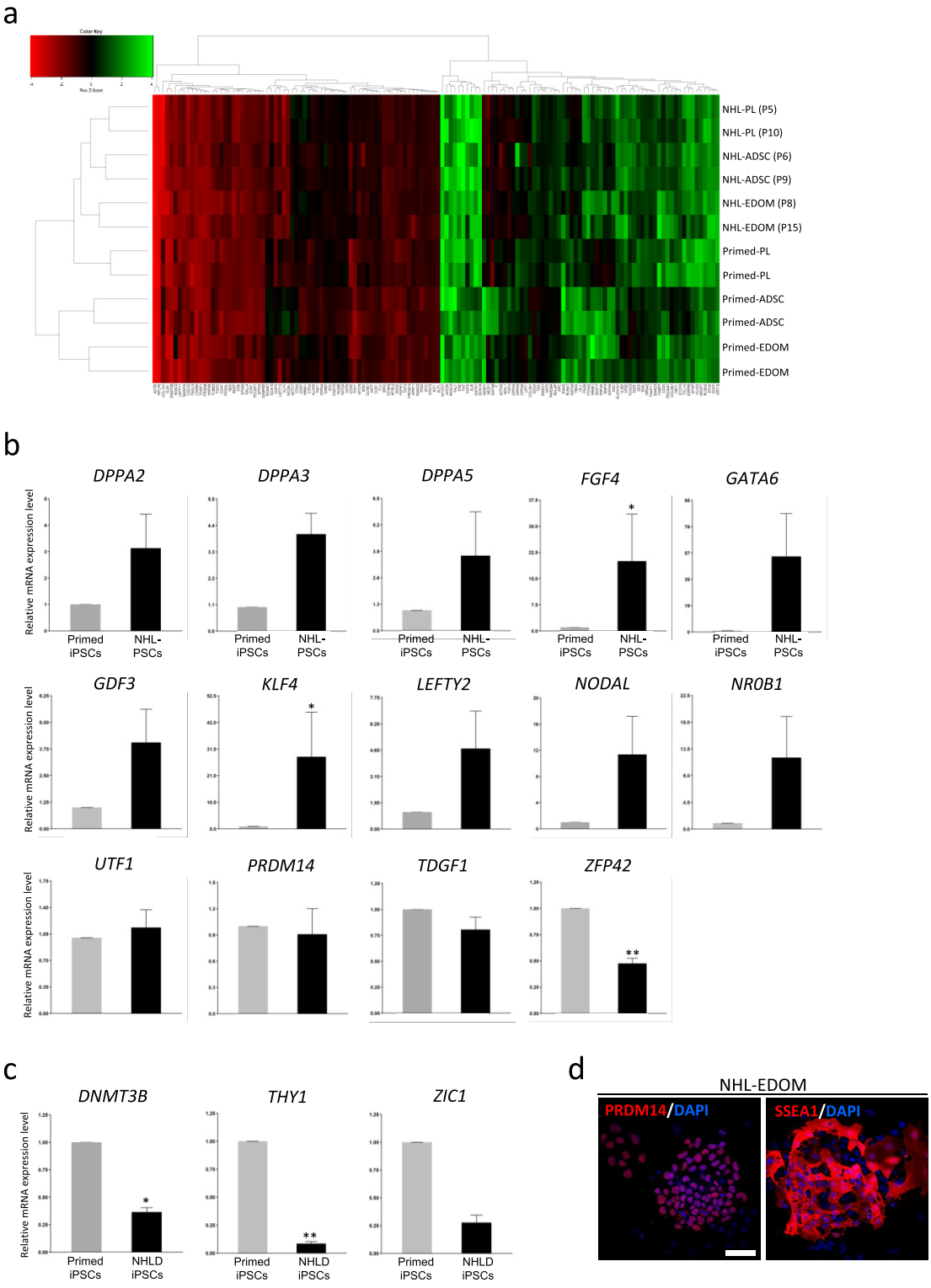


**Fig. 3. Naïve characterization of NHL-PSCs in transcription level.** (a) The heat map shows the expression level of each gene with six primed hiPSC lines and six non-hypoxia leukemia inhibitory factor-dependent pluripotent stem cell (NHL-PSC) lines. Clustering analysis is based on 132 gene expression patterns. GAPDH was used as an internal control and the expression level in each sample was normalized to the expression level in the primed human endometrium (EDOM) cell line. The passage number of NHL-PSCs is indicated as "P", and the total passage number is indicated. (b) (c) The expression levels of representative naïve and primed cell markers were compared. The average expression levels and standard deviations of each gene in six primed-iPSC lines (grey columns) and six NHL-PSC lines (black columns) are indicated. The data are reported as mean ± SE. Statistically significant differences were identified between primed iPSCs vs. NHL-PSCs using Student's *t*-test (n = 3). $*P < 0.05$, $**P < 0.01$. (d) NHL-EDOM cells expressed the human primed cell markers, PRDM14 and SSEA1. Scale bar is 100 μm.

## Author contribution statement

WI performed experiments, sample collection and data analysis, wrote the main manuscript text, and prepared all figures and tables. TK performed experiments and data analysis. HA was involved in data acquisition, manuscript drafting, and critical manuscript revision for important intellectual content. WI, HA and AU made substantial contributions to the conception and design of the study. TA and JI provided final approval for the submission of this manuscript version.

## Author disclosure statement

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## Conflict of interest

The authors declare no potential conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2020.08.001.

## References

[1] Mawaribuchi S, Aiki Y, Ikeda N, Ito Y. mRNA and miRNA expression profiles in an ectoderm-biased substate of human pluripotent stem cells. Sci Rep 2019;9:11910.
[2] Kim K, Doi A, Wen B, Ng K, Zhao R, Cahan P, et al. Epigenetic memory in induced pluripotent stem cells. Nature 2010;467:285–90.
[3] Ghimire S, Van der Jeught M, Neupane J, Roost MS, Anckaert J, Popovic M, et al. Comparative analysis of naive, primed and ground state pluripotency in mouse embryonic stem cells originating from the same genetic background. Sci Rep 2018;8:5884.
[4] Nichols J, Davidson D, Taga T, Yoshida K, Chambers I, Smith A. Complementary tissue-specific expression of LIF and LIF-receptor mRNAs in early mouse embryogenesis. Mech Dev 1996;57:123–31.
[5] Theunissen TW, Friedli M, He Y, Planet E, O'Neil RC, Markoulaki S, et al. Molecular criteria for defining the naive human pluripotent state. Cell Stem Cell 2016;19:502–15.
[6] Han X, Chen H, Huang D, Chen H, Fei L, Cheng C, et al. Mapping human pluripotent stem cell differentiation pathways using high throughput single-cell RNA-sequencing. Genome Biol 2018;19:47.
[7] Takashima Y, Guo G, Loos R, Nichols J, Ficz G, Krueger F, et al. Resetting transcription factor control circuitry toward ground-state pluripotency in human. Cell 2014;158:1254–69.
[8] Theunissen TW, Powell BE, Wang H, Mitalipova M, Faddah DA, Reddy J, et al. Systematic identification of culture conditions for induction and maintenance of naive human pluripotency. Cell Stem Cell 2014;15:471–87.
[9] Hasegawa Y, Tang D, Takahashi N, Hayashizaki Y, Forrest AR, FANTOM consortium, et al. CCL2 enhances pluripotency of human induced pluripotent stem cells by activating hypoxia related genes. Sci Rep 2014;4:5228.
[10] Yoshida Y, Takahashi K, Okita K, Ichisaka T, Yamanaka S. Hypoxia enhances the generation of induced pluripotent stem cells. Cell Stem Cell 2009;5:237–41.
[11] Guo G, Yang J, Nichols J, Hall JS, Eyres I, Mansfield W, et al. Klf4 reverts developmentally programmed restriction of ground state pluripotency. Development 2009;136:1063–9.
[12] Stuart HT, van Oosten AL, Radzisheuskaya A, Martello G, Miller A, Dietmann S, et al. NANOG amplifies STAT3 activation and they synergistically induce the naive pluripotent program. Curr Biol 2014;24:340–6.
[13] Huang X, Trinh T, Aljoufi A, Broxmeyer HE. Hypoxia signaling pathway in stem cell regulation: good and evil. Curr Stem Cell Rep 2018;4:149–57.
[14] Ichida JK, Tcw J, Williams LA, Carter AC, Shi Y, Moura MT, et al. Notch inhibition allows oncogene-independent generation of iPS cells. Nat Chem Biol 2014;10:632–9.
[15] Nishino K, Toyoda M, Yamazaki-Inoue M, Fukawatase Y, Chikazawa E, Sakaguchi H, et al. DNA methylation dynamics in human induced pluripotent stem cells over time. PLoS Genet 2011;7:e1002085.
[16] Warren L, Manos PD, Ahfeldt T, Loh YH, Li H, Lau F, et al. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell 2010;7:618–30.
[17] Akutsu H, Nasu M, Morinaga S, Motoyama T, Homma N, Machida M, et al. In vivo maturation of human embryonic stem cell-derived teratoma over time. Regen Ther 2016;5:31–9.
[18] Pastor WA, Chen D, Liu W, Kim R, Sahakyan A, Lukianchikov A, et al. Naive human pluripotent cells feature a methylation landscape devoid of blastocyst or germline memory. Cell Stem Cell 2016;18:323–9.
[19] Sahakyan A, Kim R, Chronis C, Sabri S, Bonora G, Theunissen TW, et al. Human naive pluripotent stem cells model X chromosome dampening and X inactivation. Cell Stem Cell 2017;20:87–101.
[20] Szczerbinska I, Gonzales KAU, Cukuroglu E, Ramli MNB, Lee BPG, Tan CP, et al. A chemically defined feeder-free system for the establishment and maintenance of the human naive pluripotent state. Stem Cell Reports 2019;13:612–26.
[21] Nichols J, Smith A. Naive and primed pluripotent states. Cell Stem Cell 2009;4:487–92.
[22] Du P, Pirouz M, Choi J, Huebner AJ, Clement K, Meissner A, et al. An intermediate pluripotent state controlled by MicroRNAs is required for the naive-to-primed stem cell transition. Cell Stem Cell 2018;22:851–864 e855.
[23] Osafune K, Caron L, Borowiak M, Martinez RJ, Fitz-Gerald CS, Sato Y, et al. Marked differences in differentiation propensity among human embryonic stem cell lines. Nat Biotechnol 2008;26:313–5.
[24] Mathieu J, Detraux D, Kuppers D, Wang Y, Cavanaugh C, Sidhu S, et al. Folliculin regulates mTORC1/2 and WNT pathways in early human pluripotency. Nat Commun 2019;10:632.
[25] Hanna J, Cheng AW, Saha K, Kim J, Lengner CJ, Soldner F, et al. Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. Proc Natl Acad Sci U S A 2010;107:9222–7.