Cell Freezing and Thawing

For cryopreservation, hPSCs were dissociated as follows. Semiconfluent hPSCs were treated with 4.8 m*M* EDTA/PBS for 3 min at RT and then TrypLE Select (Invitrogen, 12563011) for 1 min at 37°C. The cells were collected and pipetted for complete dispersal and then centrifuged at 200*g* for 3 min at 4°C.

For freezing, 2.0–2.2 × 10^6 hPSCs were resuspended in 0.5 ml prechilled culture medium, gently mixed with the same volume of 2× prechilled freezing medium containing 20% DMSO (final concentration: 10% DMSO), and then transferred to cryovials (NUNC, 377224). The cryovials were placed in a Mr. Frosty Freezing Container (NUNC, 5100-0001) and stored at −80°C for 24 h. The cryovials were then transferred to liquid nitrogen and stored for at least 1 week before analysis.

For thawing, the cells were warmed at 37°C in a water bath and then diluted with cold culture medium. The cells were collected by centrifugation at 200*g* for 3 min and seeded onto matrix-coated culture vessels at the indicated seeding densities.

Cell Viability Assays

The viability of hPSCs cultured with mTeSR1 medium was estimated by trypan blue exclusion or flow cytometric detection of fluorescent dye influx. To obtain accurate data of cell viability before and after cryopreservation, cell preparation for viability assays was performed by centrifugation at 500*g* for 5 min to ensure maximum recovery of more than 90% of the frozen cells. For trypan blue staining, hPSCs were incubated with a 0.2% trypan blue solution (Wako, 207-17081) for 1 min at RT, and then observed under an optical microscope in a bright field. For flow cytometric detection, hPSCs were rinsed with D-PBS by centrifugation at 500*g* for 5 min at 4°C, and then completely dissociated by treatment with 4.8 m*M* EDTA/D-PBS for 2 min at RT. The cells were rinsed with culture medium and resuspended in D-PBS. The cell suspension was incubated with 105 n*M* thiazole orange for 5 min and then 11 μ*M* propidium iodide for 2 min just prior to analysis. Considering cell damage during preparation for FACS analysis, single hPSCs were additionally treated by a dissociation process similar to that for hPSC colonies.

Cell Adhesion Assays

Freeze-thawed single hPSCs were seeded at several cell densities in 24-well plates coated with Matrigel or laminin-521. After 12 h of incubation, the cells were rinsed with prewarmed D-PBS, and the remaining live cells were detached with 0.25% trypsin/EDTA and counted using a hemocytometer.

To evaluate the effect of Y-27632 treatment, freeze-thawed single hPSCs were seeded at two cell densities and then incubated with 10 μ*M* Y-27632 (Wako, 253-00513). After 12 h, adherent live cells were counted as described above.

Karyotype Analysis

hPSCs were treated with 100 ng/ml colcemid (Life Technologies, 15212-012) for 2–3 h. After dissociation in 0.25% trypsin/EDTA, the cells were treated with a hypotonic solution and then fixed in Carnoy's solution. Cells were spread onto glass slides and stained with Giemsa. Chromosome spreads were then analyzed by randomly counting 50 cells using the Ikaros Karyotyping System (META system).

Flow Cytometric Analysis

Cells were dissociated by treatment with 4.8 m*M* EDTA/PBS for 2 min and then TrypLE Select for 1 min. To detect surface markers, the cells were rinsed twice with 10% FBS/DMEM, and 1 × 10^5 cells were incubated with primary antibodies diluted in staining buffer (0.1% BSA/D-PBS) for 30 min at 4°C. The cells were rinsed twice with staining buffer and then incubated for 30 min at 4°C with the secondary antibody diluted in staining buffer. After rinsing twice with staining buffer, the cells were resuspended in D-PBS and stained with propidium iodide just prior to analysis. To detect intracellular markers, dissociated cells were fixed with 4% formaldehyde/PBS for 15 min at 4°C. After rinsing with D-PBS, cells were incubated with saponin permeabilization buffer (SPB) (1 mg/ml saponin and 1% BSA in D-PBS) for 15 min, and then the primary antibody for 30 min at 4°C. Cells were rinsed twice with SPB, and then incubated for 30 min at 4°C with the secondary antibody diluted in SPB. After three rinses with SPB, the cells were resuspended in staining buffer for analysis by a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software. Antibodies against the following markers used were SSEA-4 (Developmental Studies Hybridoma Bank (DSHB), MC-813-70, 1 μg/ml), Tra-1-60 (Millipore, MAB4360, 1 μg/ml), GCTM2 (Millipore, MABD90, 1 μg/ml), TRA-2-54 (DSHB, 1 μg/ml), and SSEA-1 (DSHB, MC-480, 2 μg/ml) as primary antibodies and a mouse anti-Ig/FITC (Becton Dickinson, 731735, 1 μg/ml) as the secondary antibody.

Differentiation Assay

Differentiation potential was estimated by semi-quantitative polymerase chain reaction (PCR) analysis of differentiation marker genes expressed in embryoid bodies. Freeze-thawed hPSCs subcultured for two passages were detached by treatment with CTK solution (ReproCell, RCHETP002) and using a cell scraper. The cell clumps were cultured as a suspension in petri dishes with hESC medium without FGF-2. Medium changes were performed every 2 days. After 14 days of

culture, total RNA was extracted using an RNeasy Mini Kit (Qiagen, 74104), and cDNA was synthesized from 1 to 2 μg RNA using an Omniscript™ RT Kit (Qiagen, 205111) according to the manufacturer's instructions. PCR conditions were optimized to facilitate semiquantitative comparison with the log phase of amplification. The gene-specific primers are described elsewhere (Kumagai *et al.*, 2013; Miyazaki *et al.*, 2012). PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining.

Statistical Analysis

The statistical significance of differences was determined by the two-tailed Student's *t*-test. Differences with a value of $P < 0.05$ were considered significant.

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Identification of small molecules that promote human embryonic stem cell self-renewal

Hideaki Kumagai [a], Hirofumi Suemori [a], Motonari Uesugi [b,c], Norio Nakatsuji [b,d], Eihachiro Kawase [a,*]

[a] Department of Embryonic Stem Cell Research, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan
[b] Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Ushinomiya-cho, Yoshida, Sakyo-ku, Kyoto 606-8501, Japan
[c] Chemical Biology Laboratory, Institute for Chemical Research, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan
[d] Department of Development and Differentiation, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

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ABSTRACT

Human embryonic stem cells (hESCs) and induced pluripotent cells have the potential to provide an unlimited source of tissues for regenerative medicine. For this purpose, development of defined/xeno-free culture systems under feeder-free conditions is essential for the expansion of hESCs. Most defined/xeno-free media for the culture of hESCs contain basic fibroblast growth factor (bFGF). Therefore, bFGF is thought to have an almost essential role for the expansion of hESCs in an undifferentiated state. Here, we report identification of small molecules, some of which were neurotransmitter antagonists (trimipramine and ethopropazine), which promote long-term hESC self-renewal without bFGF in the medium. The hESCs maintained high expression levels of pluripotency markers, had a normal karyotype after 20 passages, and could differentiate into all three germ layers.

1. Introduction

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) have a self-renewal ability and pluripotency to differentiate into all three germ layers *in vitro* and *in vivo* [1–3]. Because of these notable properties, hESCs and hiPSCs are tools for basic biology, drug discovery research, and a cell source for regenerative medicine.

Undifferentiated hESCs and hiPSCs are usually maintained on mouse embryonic fibroblasts (MEFs) as feeders, or MEF-conditioned medium (CM) on Matrigel, which limits their clinical application owing to the potential risk of using animal components. In recent years, many commercial and non-commercial media have been reported to maintain hESCs and hiPSCs in culture under a feeder-free condition [4], but they have not been fully developed for the large-scale culture of cells because these media are expensive and often have batch-to-batch variations. One method to overcome such issues might be the addition of small molecules to the media as a replacement for growth factors and other components.

In this study, we aimed to identify small molecules to replace the role of basic fibroblast growth factor (bFGF). Most defined/xeno-free media for hESCs contain bFGF at a higher concentration. Therefore, it is thought that bFGF is one of the most important components for robust expansion of hESCs in an undifferentiated state. Moreover, small molecules are not fully defined as replacements of bFGF for the expansion of undifferentiated hESCs in culture.

Here, we adopted a high-content screening (HCS) system using green fluorescent protein (GFP) expression regulated by the OCT4 promoter to monitor changes of cell fate in media. Furthermore, we focused on not only the intensity of GFP expression under the OCT4 promoter induced by each small molecule, but also the similarities in structure and the pharmacological effects of hit compounds. We found that selected small molecules support long-term hESC self-renewal in the absence of bFGF as evidenced by various pluripotency markers, a normal karyotype and differentiation into all three germ layers.

2. Materials and methods

2.1. Construction of the hOCT4pro-EGFP reporter gene

We created an enhanced GFP (EGFP) reporter under the control of the human OCT4 promoter (hOCT4pro-EGFP) using a modified method from a previous report [5]. Briefly, the promoter region of human OCT4 was cloned from the genomic DNA of KhES-1 cells by PCR using the following primers: forward, 5′-TTCCCATGTCAAGTAAGTGGGGTGG-3′; and reverse, 5′-ACCGGTGGGGAAGGAAGGCGCCCCAAGCC-3′. The PCR product was cloned into a pBSSK(−) vector and the sequence was confirmed by DNA sequencing. The

* Corresponding author. Address: Department of Embryonic Stem Cell Research, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Fax: +81 75 751 3890.
E-mail address: kawase8@frontier.kyoto-u.ac.jp (E. Kawase).

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human OCT4 promoter was digested with HindIII and AgeI, and then inserted into pEGFP-1 (Clontech).

2.2. Maintenance and transfection of hESCs

The hESC lines (KhES-1, KhES-3, and WA09 (H9)) were routinely cultured as described previously [6–8] on mitomycin C-treated MEF feeder cells in hESC medium consisting of DMEM/F12 (D-6421; Sigma) supplemented with 20% KnockOut Serum Replacement (KSR; Invitrogen), 0.1 mM non-essential amino acids (Sigma), 2 mM L-glutamine (Sigma), 0.1 mM 2-mercaptoethanol (Sigma), and 5 ng/ml bFGF (Wako, Japan). hESC medium without bFGF was used as hESC-basal medium (BM) in this study.

For feeder-free culture, hESCs were incubated with 2 mg/ml dispase (Invitrogen) in DMEM/F12 at 37 °C for 10 min, and then detached using a cell scraper. Small clumps of hESCs were seeded onto Matrigel-coated plates in CM or hESC-BM with each compound. The plates were pre-coated with 0.2 mg/ml Matrigel (growth factor reduced; BD Biosciences) at 4 °C overnight. The medium was removed, and the plates were washed with DMEM/F-12 to remove unbound Matrigel and then warmed to room temperature before use. CM was prepared as described previously [9] with the addition of 5 ng/ml bFGF.

Each compound, including small molecules from the Prestwick Chemical library, was added to hESC-BM at the indicated concentrations in the presence of 0.1% DMSO. Ethopropazine, promazine, retinoic acid (RA), PD98059, and trimipramine were purchased from Sigma, methotrimeprazine from Aurora Fine Chemicals LLC, and trimeprazine from the United States Pharmacopeial Convention.

Cell lines carrying hOCT4pro-EGFP were established by transfection with the hOCT4pro-EGFP reporter plasmid. Before transfection, KhES-1 cells were seeded onto Matrigel-coated 100 mm tissue culture dishes in CM. ApaL1-linearized hOCT4pro-EGFP plasmid was transfected into KhES-1 cells using Fugene HD (Roche Diagnostics) according to the manufacturer's instructions. G418 selection (100 μg/ml) was applied at 24 h after transfection. After about 14 days of selection, the surviving colonies were picked up individually and expanded as clones.

2.3. High-content screening

For HCS, cells cultured on feeder cells were treated with a CTK solution consisting of 1 mg/ml collagenase IV (Invitrogen), 0.25% trypsin (Invitrogen), 1 mM $CaCl_2$ and 20% KSR, and then detached as small clumps [6]. The cells were seeded in hESC-BM into 96-well plates (Greiner Bio-One), and from the following day, hESC-BM containing 2 μg/ml (~5 μM) of each small molecule from the Prestwick Chemical library (in the presence of 0.1% DMSO) was changed daily. Each compound was assessed in quadruplicate. Control wells containing 0.1% DMSO in hESC-BM were included on each plate. After 6 days of culture, cells were fixed with 4% paraformaldehyde/PBS, washed with PBS, and then stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). After three washes with PBS, fluorescence images were acquired by an ArrayScan-VTI System (Cellomics) and processed with the Target Activation BioApplication (Cellomics). To normalize data, a *z*-score based on the EGFP fluorescence intensity was calculated for each medium using the negative control medium (hESC-BM containing 0.1% DMSO).

2.4. Semi-quantitative PCR

Total RNA was extracted using an RNeasy Micro Kit (Qiagen), and then 0.5–1 μg total RNA was reverse transcribed with an Omniscript RT Kit (Qiagen) according to the manufacturer's instructions. For semi-quantitative PCR analysis, PCR was performed with TaKaRa ExTaq (TaKaRa, Japan). PCRs were optimized to allow semi-quantitative comparisons within the log phase of amplification. PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. Gene-specific primers are listed in Supplementary Table S1.

2.5. Teratoma formation assay

Approximately 2×10^6 cells were injected into the testes of severe combined immunodeficiency (SCID) mice (CLEA Japan). After 8 weeks, teratomas were surgically dissected from the mice, and then fixed in 4% paraformaldehyde/PBS. Samples were embedded in paraffin, sectioned at 5 μm and processed for hematoxylin and eosin staining. Animal protocols were approved by the Institutional Board on Animal Care at Kyoto University.

2.6. Statistical analysis

Data are shown as the average ± standard deviation (SD). Statistical significance was assessed using the Student's *t*-test. The probability level accepted for significance was $P < 0.05$.

2.7. Other methods

See Supplementary Methods for remaining methods including flow cytometric analysis, immunocytochemistry, Karyotype analysis, the EdU incorporation assay, the TUNEL assay and *in vitro* differentiation assay.

3. Results

3.1. High-throughput chemical screening to identify promotion of hESC self-renewal

To carry out a screen, we first established a human OCT4-GFP reporter system in hESCs, which contained −3917 to −1 base pairs relative to the transcriptional start site [5] (Fig. 1A). OCT4 is highly expressed in hESCs and downregulated upon differentiation. We initially isolated a 3D6 hESC clone, which showed GFP expression in an undifferentiated state, and was morphologically indistinguishable from the parental KhES1 cells (Fig. 1A). Furthermore, GFP expression in the cells was lost upon differentiation by 5 days of 10 μM RA treatment, as indicated by both fluorescence microscopy (Supplementary Fig. S1A) and flow cytometric analysis (Supplementary Fig. S1B). Moreover, flow cytometric analysis showed that OCT4-GFP expression was well correlated with OCT4 expression.

Undifferentiated 3D6 hESCs were seeded onto Matrigel-coated 96-well plates at a density of 3000 cells/well in hESC-BM. After overnight incubation, a compound from the chemical library, as described in Section 2, was added to each well (2 μg/ml, ~5 μM). Medium containing compounds was changed daily for a further 5 days of incubation. Cells were analyzed for GFP expression using an Arrayscan VTI system (Cellomics).

Before performing our screening, to confirm whether the intensity of GFP fluorescence indicated the status of hESCs, we used CM, bFGF and mTeSR1 medium (StemCell Technologies) as controls for promotion of self-renewal, and RA or PD98059 as controls for induction of differentiation (Fig. 1B, and data not shown). The *z*′-factor is a parameter in statistics to assess the performance in high-throughput screening [10]. A *z*′-factor of >0.5 was routinely obtained using this assay system, thereby supporting our conclusion that the intensity of GFP fluorescence using the hOCT4pro-EGFP reporter gene system could reliably identify small molecules that maintained hESCs in an undifferentiated state.

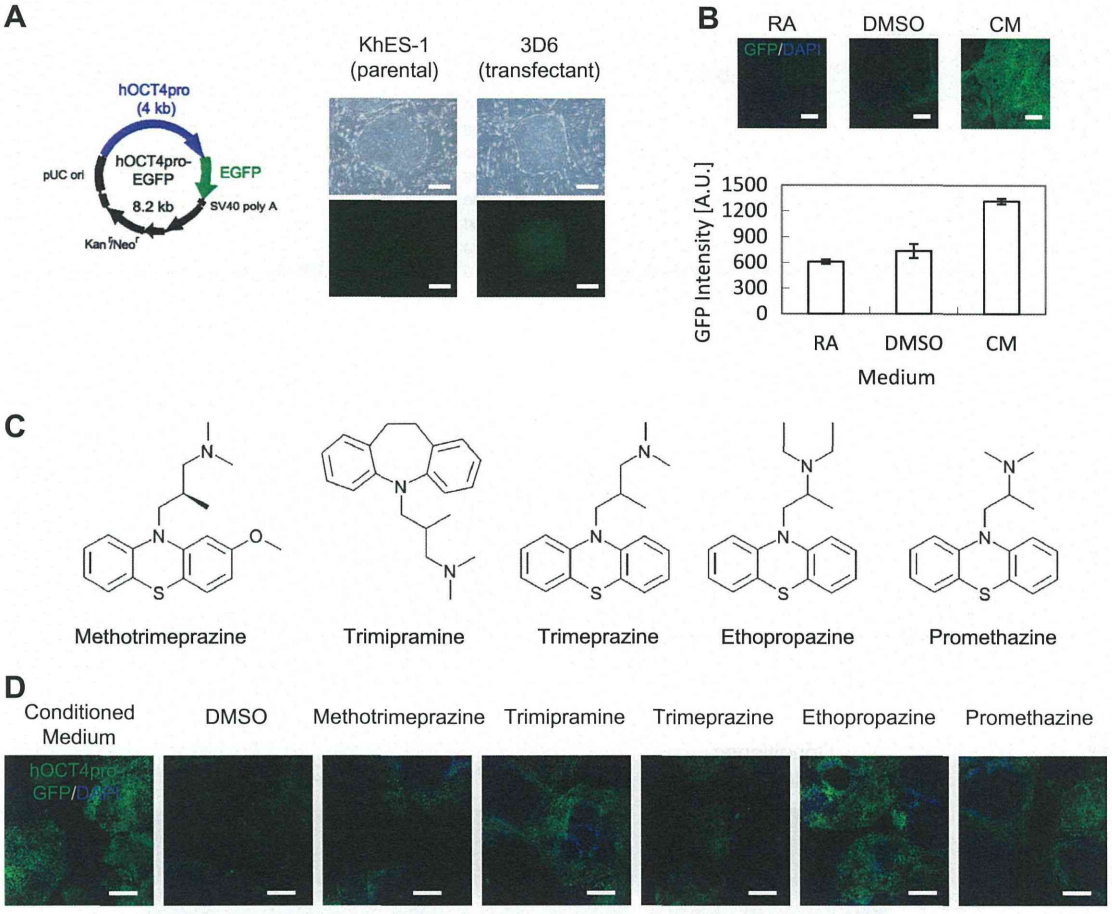

Fig. 1. High-content screening system and characterization of hit compounds. (A) (Left) Vector map of hOCT4pro-EGFP. (Right) Phase contrast and fluorescence images of parental KhES-1 and hOCT4pro-EGFP transfectant (3D6 clone) hESCs cultured on MEF feeders. Bar indicates 200 μm. (B) (upper) Representative fluorescence image of EGFP in 3D6 clones cultured in various media (CM, hESC-BM containing 0.1% DMSO, and hESC-BM containing 10 μM RA) for 5 days. Bar indicates 500 μm. (lower) Calculation of GFP intensity in upper fluorescence images. A.U. corresponds to arbitrary unit. Data are presented as the means ± SD. (C) Five of the 18 hit small molecules from the 1120-chemical library. The small molecules shared a *z*-score of higher than 2 SD, as well as structural and physiological similarities. (D) Representative fluorescence images of 3D6 hESCs cultured in hESC-BM with the five hit compounds. Expression of markers of the undifferentiated state was observed by EGFP reporter gene expression driven by the hOCT4 promoter (hOCTpro-EGFP: green) in immunocytochemistry. Cells were identified by DAPI (blue). Bar indicates 500 μm. CM, conditioned medium. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Using this assay, we screened 1120 compounds from Prestwick libraries, and 18 hit compounds (1.6% of the total chemicals screened; listed in Supplementary Table S2) that had effects on the self-renewal of hESCs were identified by exhibiting higher *OCT4-GFP expression by more than two SDs. Interestingly, we* found that five out of the 18 chemicals were neurotransmitter antagonists that may share structural and physiological similarities, namely methotrimeprazine, trimipramine, trimeprazine, ethopropazine, and promethazine (Fig. 1C and D). In this study, we used trimipramine and ethopropazine for subsequent experiments. HCS using OCT4 immunostaining further showed that almost of the cells exhibited $OCT4^{high}$ in CM as a control for the undifferentiated state (98.9%). In contrast, DMSO strongly decreased the $OCT4^{high}$ population to 7.6%, whereas trimipramine and ethopropazine were effective for recovery of the $OCT4^{high}$ population to 41.0% and 16.1%, respectively (Fig. 2A). The effect became more noticeable over several passages. Both trimipramine and ethopropazine maintained KhES-3 and WA09 hESCs in an undifferentiated state, whereas DMSO treatment resulted in continuous differentiation of the cells (Fig. 2B). Although we initially screened these chemicals using KhES-1 hESCs, the cells were differentiated even in the presence of the chemicals after several passages (data not shown). In addition, OCT4-GFP or OCT4 expression in the cells, which was increased by the addition of chemicals to the medium, was still lower than that in cells cultured in CM (Figs. 1D and 2A).

3.2. Effect of trimipramine and ethopropazine on long-term culture of hESCs

We found that two hESC lines (KhES-3 and WA09) cultured on Matrigel in hESC-BM containing trimipramine or ethopropazine proliferated for at least 20 passages without bFGF in the medium. Under these culture conditions, the cells showed compact colonies (Fig. 3A), which were similar to the undifferentiated colonies cultured in CM generally used as gold standard. We karyotyped 50 randomly selected cells by G-banding of both cell lines after 20 passages. Cells cultured with trimipramine or ethopropazine had normal karyotypes (Fig. S2A). Furthermore, RT-PCR analysis revealed that hESCs maintained high expression levels of pluripotency markers including OCT4 and NANOG, while the expression of differentiation marker genes was suppressed (Fig. 3B). Immunofluorescence analysis showed that the cells maintained the expression of pluripotency markers OCT4, NANOG and SOX2 (Fig. 3C). Flow cytometric analysis further indicated that the majority of hESCs cultured with these chemicals expressed pluripotency markers SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and OCT4 with only

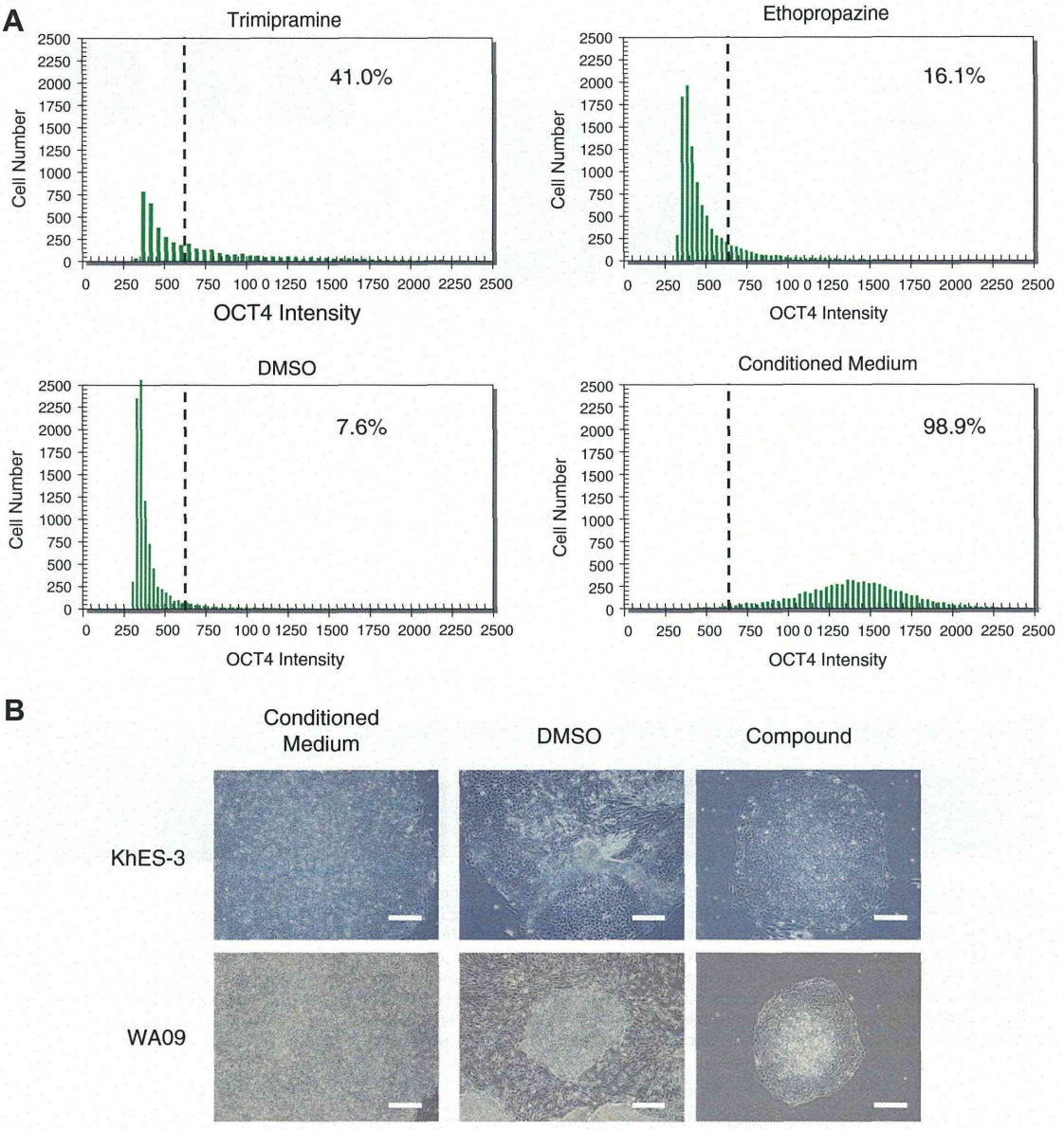

Fig. 2. Small molecules support short-term culture of hESCs without bFGF in the medium. (A) Histograms of the intensity of OCT4 immunostaining in hESCs treated with CM, DMSO, or small chemicals. (B) Morphology of KhES-3 and WA09 hESCs cultured in hESC-BM with trimipramine or ethopropazine after several passages, respectively. DMSO caused differentiation of hESCs, whereas CM and small molecule-containing media maintained the undifferentiated state of hESCs.

low-level of expression of the differentiation marker SSEA-1 (Fig. 3D). Thus, trimipramine and ethopropazine may become substitutes for bFGF to maintain hESCs in an undifferentiated state. Furthermore, we found that trimipramine and ethopropazine treatments resulted in a similar proliferation rate of both hESC lines compared with those cultured in CM (Fig. 3E). We could not detect significant differences between trimipramine or ethopropazine in hESC-BM and CM in terms of cell cycling using a 5-ethyl-2′-deoxyuridine (EdU) uptake assay (S-phase) (Supplementary Fig. S2B). In addition, the chemicals did not induce apoptosis of hESCs using the TUNEL assay, although they showed insignificantly increased apoptosis of KhES-3 hESCs compared with those cultured in CM (Supplementary Fig. S2C). Together, we concluded that hESCs cultured with trimipramine or ethopropazine showed similar behavior compared with those cultured in CM.

Finally, we confirmed the pluripotency of hESCs that had been expanded in the presence of trimipramine or ethopropazine by examining their ability to form teratomas comprised of all three germ layers including neuroepithelium (ectoderm), intestinal epithelium (endoderm) and cartilage (mesoderm) (Fig. 4A). To assess the pluripotency of hESCs *in vitro*, the cells were cultured in a differentiation medium described in the Supplementary Methods. We found that hESCs could differentiate into ectoderm (β-III tubulin), mesoderm (α-smooth muscle actin (SMA)) and endoderm (α-fetoprotein (AFP)) as determined by immunohistochemical analyses (Fig. 4B).

4. Discussion

Small molecules are different from proteins in terms of molecular size, and can penetrate multi-layer tissues easily. Therefore, small molecules are expected to be more efficient for maintenance of the undifferentiated state of hESCs and hiPSCs to subsequently

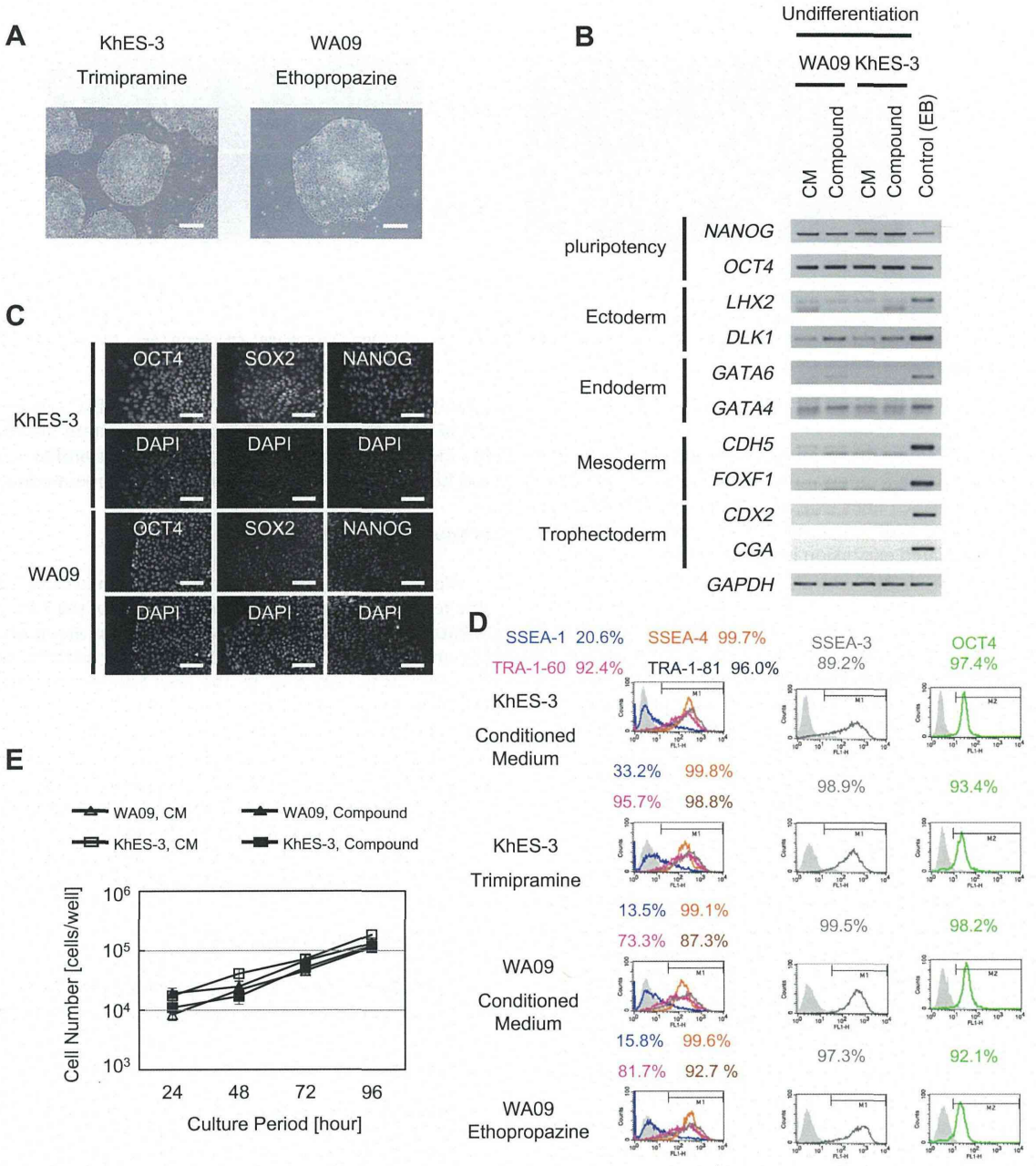

Fig. 3. Small molecules support long-term culture of undifferentiated hESCs without bFGF in the medium. (A) Phase contrast micrographs of KhES-3 and WA09 hESCs cultured in hESC-BM with trimipramine or ethopropazine after 25 passages. Bar indicates 200 μm. (B) Expression of pluripotency marker genes (*OCT4* and *NANOG*) and differentiation marker genes (ectoderm: *LHX2* and *DLK1*; endoderm: *GATA6* and *GATA4*; mesoderm; *CDH5* and *FOXF1*; trophectoderm: *CDX2* and *CGA*) was analyzed by RT-PCR. (C) Immunocytochemical detection of pluripotency marker genes (OCT4, SOX2, and NANOG) in KhES-3 and WA09 hESCs cultured in hESC-BM with trimipramine or ethopropazine after 40 passages. Bar indicates 100 μm. (D) Flow cytometric analysis of markers of the undifferentiated state (OCT4, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81) and a differentiation marker (SSEA-1) after 40 passages of culture in CM or hESC-BM with trimipramine or ethopropazine. (E) Growth rates of KhES-3 and WA09 hESCs cultured in CM or hESC-BM with trimipramine or ethopropazine during 4 days of culture after seeding. Cell numbers were counted every 24 h. The data represent means ± SD.

induce specific differentiation. For example, Y-27632 has been identified as effective for maintaining the survival of dissociated hiPSCs [11]. Several small chemicals have also been identified to promote the generation of iPSCs from somatic cells [12–16]. Moreover, (−)-indolactam V has been found to promote pancreatic differentiation of human pluripotent cells [17]. Here, we show that some neurotransmitter antagonists support the expansion of undifferentiated hESCs without bFGF in the medium.

Although a previous study using a HCS system identified small molecules that regulate undifferentiated proliferation of hESCs, the small molecules used in the study cannot support long-term culture [18]. In this study, we first addressed following points to develop a more reliable HCS system for hESCs. Previous HCS systems evaluate the undifferentiated state of hESCs by the expression of OCT4 or SSEA-3 using immunohistochemistry [18–20]. Instead, we used a reporter gene system consisting of GFP regulated by the hOCT4 promoter, which enables evaluation of OCT4 expression in cells directly without immunostaining procedures. Furthermore, we assessed not only the score of the effect of each small molecule, but also their similarities in terms of structure and pharmacological effects for hit compounds previously proposed by Lukaszewicz et al. [21]. This strategy may enable more reliable HCS for the

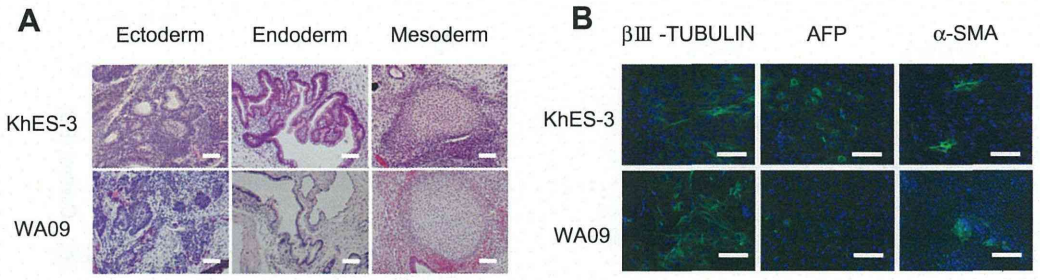

Fig. 4. Small molecules sustain the pluripotency of KhES-3 and WA09 hESCs without bFGF in the medium. (A) Characterization of teratomas derived from KhES-3 and WA09 hESCs cultured with trimipramine or ethopropazine. Hematoxylin and eosin staining of paraffin-embedded teratoma sections identified hESC differentiation into various tissues including neural pigment (ectoderm), a gut-like tube (endoderm), and cartilage (mesoderm). Bar indicates 100 μm. (B) Immunostaining for markers of the three germ layers in differentiating KhES-3 and WA09 hESCs cultured with hit compounds after 40 passages: ectoderm (βIII-tubulin), endoderm (AFP), and mesoderm (α-SMA).

identification of small molecules that drive hESC self-renewal as a replacement for bFGF.

In this study, we show that trimipramine and ethopropazine permit long-term hESC self-renewal without bFGF in the medium. We examined three hESCs lines (KhES-1, KhES-3, and WA09) and found that KhES-3 and WA09, but not KhES-1, hESCs successfully maintain undifferentiated expansion in the presence of these small molecules. Indeed, as indicated in Figs. 1D and 2A, OCT4 expression increased in the presence of the small molecules compared with that of DMSO, but OCT4 expression was still lower than that in CM. Therefore, the small molecules were not fully effective for the maintenance of undifferentiated proliferation of some hESC lines. We examined higher concentrations of the small molecules, but they showed cytotoxicity at such concentrations (data not shown). Taken together, further improvement of the small molecules to reduce their cytotoxicity while increasing the self-renewal of many hESCs lines needs to be achieved for complete replacement of bFGF in media.

The FGF pathway is activated by FGF ligands binding to FGF receptors, which in turn may trigger activation of various downstream signaling pathways including MAP kinase, PI3K kinase/AKT and PLC/PKC pathways [22]. However, the molecular mechanism by which bFGF promotes undifferentiated expansion of hESCs is still unclear. A MAP kinase kinase (MEK) inhibitor, PD0325901, is reported to be effective for hESC maintenance [23]. Activation of the PLC/PKC pathway may induce hESC differentiation [24]. PIK3/AKT signaling activity has been reported to participate in undifferentiated proliferation by suppression of MAP and canonical Wnt signaling pathways [25]. Therefore, the PIK3/AKT pathway may be a potential target of bFGF signaling. However, it remains unclear whether such small molecules related to PIK3/AKT pathways are ideal replacements for bFGF. Thus, small molecules related to growth factors and their signaling pathways have not been fully identified for the replacement of bFGF to expand undifferentiated hESCs in culture.

In this study, we showed that trimipramine and ethopropazine permit long-term hESC self-renewal without bFGF in the medium. Further studies will clarify the molecular mechanisms of trimipramine and ethopropazine, which enable long-term expansion of hESCs, including the interactions between these chemicals and the FGF or PIK3/AKT pathways.

For feeder-free culture systems, CM is usually used as the current gold standard. In this study, we showed that trimipramine and ethopropazine treatments resulted in similar growth curves and cell cycles as those of hESCs cultured in CM. Thus, the small molecules enabled similar performance compared with that of CM for the expansion of undifferentiated hESCs.

In conclusion, we established a HCS platform to identify small chemicals that promote hESC self-renewal. Using the screening system, we identified trimipramine and ethopropazine as novel small molecules that promote long-term hESC self-renewal without bFGF in the medium. This screening system may help to identify more small molecules to achieve robust proliferation of hESCs and hiPSCs in low-cost and growth factor-free medium.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.061.

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REVIEW

Modeling human neurological disorders with induced pluripotent stem cells

Yoichi Imaizumi*,† and Hideyuki Okano*

*Department of Physiology, Keio University School of Medicine, Tokyo, Japan

†Next Generation Systems CFU, Eisai Co. Ltd., Ibaraki, Japan

Abstract

Human induced pluripotent stem (iPS) cells obtained by reprogramming technology are a source of great hope, not only in terms of applications in regenerative medicine, such as cell transplantation therapy, but also for modeling human diseases and new drug development. In particular, the production of iPS cells from the somatic cells of patients with intractable diseases and their subsequent differentiation into cells at affected sites (e.g., neurons, cardiomyocytes, hepatocytes, and myocytes) has permitted the *in vitro* construction of disease models that contain patient-specific genetic information. For example, disease-specific iPS cells have been established from patients with neuropsychiatric disorders, including schizophrenia and autism, as well as from those with neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease. A multi-omics analysis of neural cells originating from patient-derived iPS cells may thus enable investigators to elucidate the pathogenic mechanisms of neurological diseases that have heretofore been unknown. In addition, large-scale screening of chemical libraries with disease-specific iPS cells is currently underway and is expected to lead to new drug discovery. Accordingly, this review outlines the progress made via the use of patient-derived iPS cells toward the modeling of neurological disorders, the testing of existing drugs, and the discovery of new drugs.

Keywords: human disease model, induced pluripotent stem cells, neurological disorders, Parkinson's disease.

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The advent of an aging society is accompanied not only by increases in cancer and heart disease but also by increases in chronic and age-related diseases. Analyses of the pathological mechanisms of various chronic diseases and the development of new therapies for their management are currently underway, based in large part on the impressive research advances of recent years. However, numerous disorders remain with no established means of treating the underlying cause. The fact that complete human disease models are unavailable for these conditions is cited as a major problem in terms of developing new drugs for their control. While animal disease models and human disease-mimetic cell lines have been developed, construction of models that can accurately and thoroughly reproduce human pathology remains difficult. Furthermore, there is ample room for debate as to whether animal and cell line disease models can correctly reflect the phenomena that actually occur in human patients, because of species-specific differences and differences in cell line specificity. Moreover, no disease models exist for many of the rarer conditions.

In recent years, however, Professor Yamanaka of Kyoto University (Kyoto, Japan) launched a method for the preparation of induced pluripotent stem (iPS) cells that have almost the same pluripotency as embryonic stem (ES) cells. This was done by introducing four reprogramming genes, *Oct4, Sox2, Klf4*, and *c-Myc*, into differentiated somatic cells (Takahashi and Yamanaka 2006; Takahashi *et al.* 2007). Yamanaka's method made it possible (with some exceptions) to establish iPS cells from the somatic cells of any individual, regardless of race, genetic background, or state of health (i.e., whether afflicted with a disease). Moreover, the development of *in vitro* differentiation protocols for ES cells toward each

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Address correspondence and reprint requests to Hideyuki Okano, Department of Physiology, Keio University, School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: hidokano@a2.keio.jp

Abbreviations used: AD, Alzheimer's disease; APP, amyloid precursor protein; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazine; ERK1/2, extracellular signal-regulated kinase 1/2; ES cell, embryonic stem cell; FD, familial dysautonomia; GSH, reduced glutathione; iPS cell, induced pluripotent stem cell; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; TALEN, transcription activator-like effector nuclease; ZFN, zinc-finger nuclease.

