typically contains 10% dimethylsulfoxide (DMSO). The slow cooling method is widely used because it is applicable to various cell types including mouse embryonic stem cells. However, for cryopreservation of hPSCs, the slow cooling method results in less than 10% survivability (Wagh *et al.*, 2011). Nevertheless, for bulk storage and easy handling, slow cooling is desirable for cryopreservation of hPSCs. In this study, we aimed to optimize the slow cooling method for hPSC cryopreservation.

Cryopreservation involves a series of processes including cell detachment, freezing, storage, thawing, and reseeding. During these processes, hPSCs may suffer necrotic cell death from cell detachment to thawing because of physical damage, and apoptotic cell death at reseeding because of adhesion failure. Therefore, hPSC survivability should be verified at each step. However, previous studies have reported different timings for their analyses, various methods of measurement, and counting either colonies or individual cells (Li and Ma, 2012). In particular, counting of colonies postseeding, although it has been often performed, is inaccurate because it leads to overestimation because of an increase of colony formation caused by fragmentation of the original colonies during freeze-thaw processes. For correct evaluation of the survival efficiency, in this study, we counted individual hPSCs in a stepwise manner.

To evaluate the effect of different methods of cell detachment from culture vessels, we first compared cell survival after conventional colony dissociation or single-cell dissociation. While the viability of cells detached by conventional colony dissociation before freezing were approximately halved (42.3–59.4% survival), the majority of hPSCs cryopreserved under a colony state were already dead immediately after freeze-thawing (15.5–32.5% survival) (Fig. 1a, c, e–h). Although the conventional dissociation method maintains the colony state to prevent apoptosis caused by failure to adhere at reseeding, hPSCs had already died by necrosis before attachment. This observation suggests that it is necessary to avoid cell death mainly caused by physical damage during cell dissociation and freeze-thawing processes. Next, we performed freeze-thawing of dissociated hPSCs. The majority of dissociated hPSCs showed higher viability before cryopreservation (68.1–77.7% survival). More importantly, single hPSCs were still alive just after thawing (59.7–66.1% survival), corresponding to 80–90% viability just after dissociation (Fig. 1b, d, e–h).

Although we found that single-cell dissociation of hPSCs is advantageous for freeze-thawing, it is necessary to avoid apoptotic cell death caused by dissociation (Amit *et al.*, 2000). Because we previously showed that the seeding density significantly affects the survivability of single hPSCs (Miyazaki *et al.*, 2012), we next assessed the effect of seeding density on the viability of freeze-thawed single hPSCs. The number of hPSCs

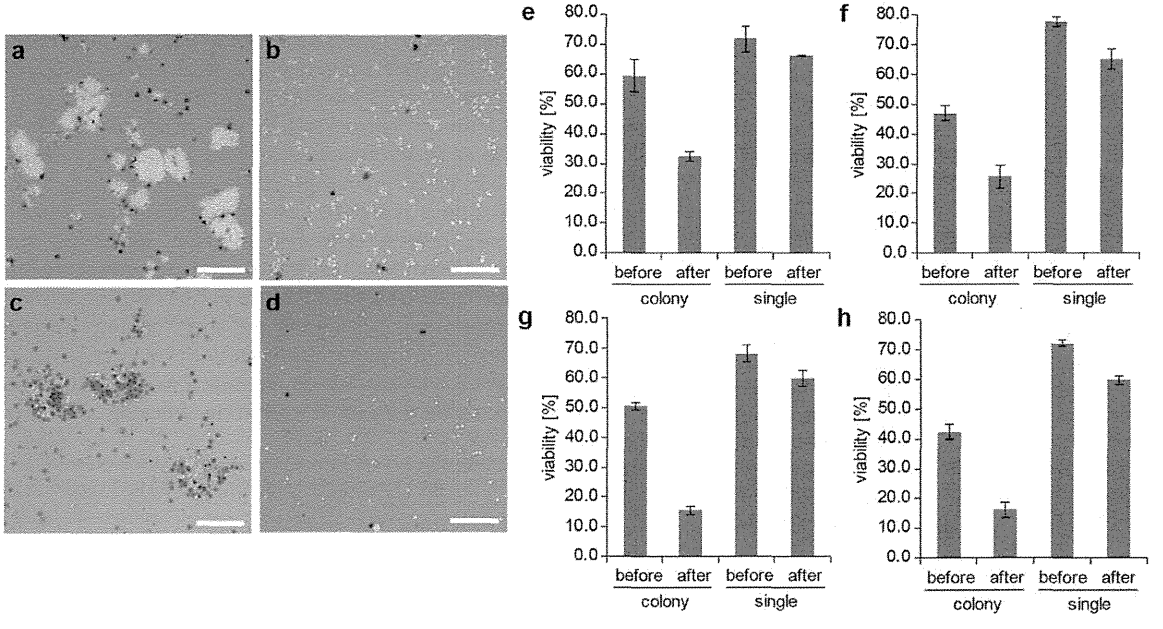

FIG. 1. hPSC survivability is influenced by the cellular state during cryopreservation. (**a–d**) Estimation of cellular state-dependent hPSC survivability by trypan blue exclusion. Representative bright field images of colony-dissociated H9 hESCs before (a) and after (b) cryopreservation and single dissociated H9 hESCs before (c) and after (d) cryopreservation. (**e–h**) Flow cytometric analysis of cellular state-dependent hPSC survivability during cryopreservation by fluorescent dye influx. e: H9 hESCs; f: 253G1 hiPSCs; g: KhES-1 hESCs; h: iPS(IMR90)-1 hiPSCs. Error bars indicate the standard deviation (SD) of three experiments. Scale bars: 200 μm.

attached to the substrate gradually decreases to the minimum at 12 h postseeding (Chen *et al.*, 2010). To evaluate survival correctly, we measured the viability of freeze-thawed single hPSCs at 12 h postseeding. On Matrigel in mTeSR1 medium, freeze-thawed single H9 hESCs seeded at 3×10^5 cells/cm^2 showed maximum survivability (36.2 ± 6.0% survival) (Fig. 2a, c), indicating the optimal condition for recovery of cryopreserved hPSCs. It is well known that administration of a Rho-associated protein kinase (ROCK) inhibitor dramatically improves the survival of single hPSCs (Watanabe *et al.*, 2007). However, ROCK inhibitor treatment did not significantly improve the survival of single hPSCs seeded at the optimal density (Fig. 2b). An effect of the ROCK inhibitor was only observed when single hPSCs were seeded at a lower cell density. These results indicate that a ROCK inhibitor is unnecessary for the optimized seeding density, whereas it should be administrated when single hPSCs are seeded at a lower seeding density. For stable survival of hPSCs, cell-cell contact is a key element (Chen *et al.*, 2010). Therefore, the seeding density of single hPSCs should be as high as possible for rapid formation of cell-cell contacts. However, seeding at very high densities leads to less efficient survival because of depletion of the adhesion surface. It is therefore important for freeze-thawed single hPSCs to be seeded at an appropriate density.

After adherence of single hPSCs, the majority of single hPSCs immediately formed cell-cell contacts as small clusters (Fig. 2c). By 3 days post-thawing, the cells had grown as colonies and reached confluency for passaging (Fig. 2d). Immediately after freeze-thawing, flow cytometric analysis showed that hPSCs remained in an undifferentiated state and maintained the undifferentiated status following subculture (Fig. 3a). After the first passage, hPSCs grew as colonies (Fig. 3b) and sustained an undifferentiated state (Fig. 3a, c) and potential for differentiation (Fig. 3c) without karyotypic abnormalities

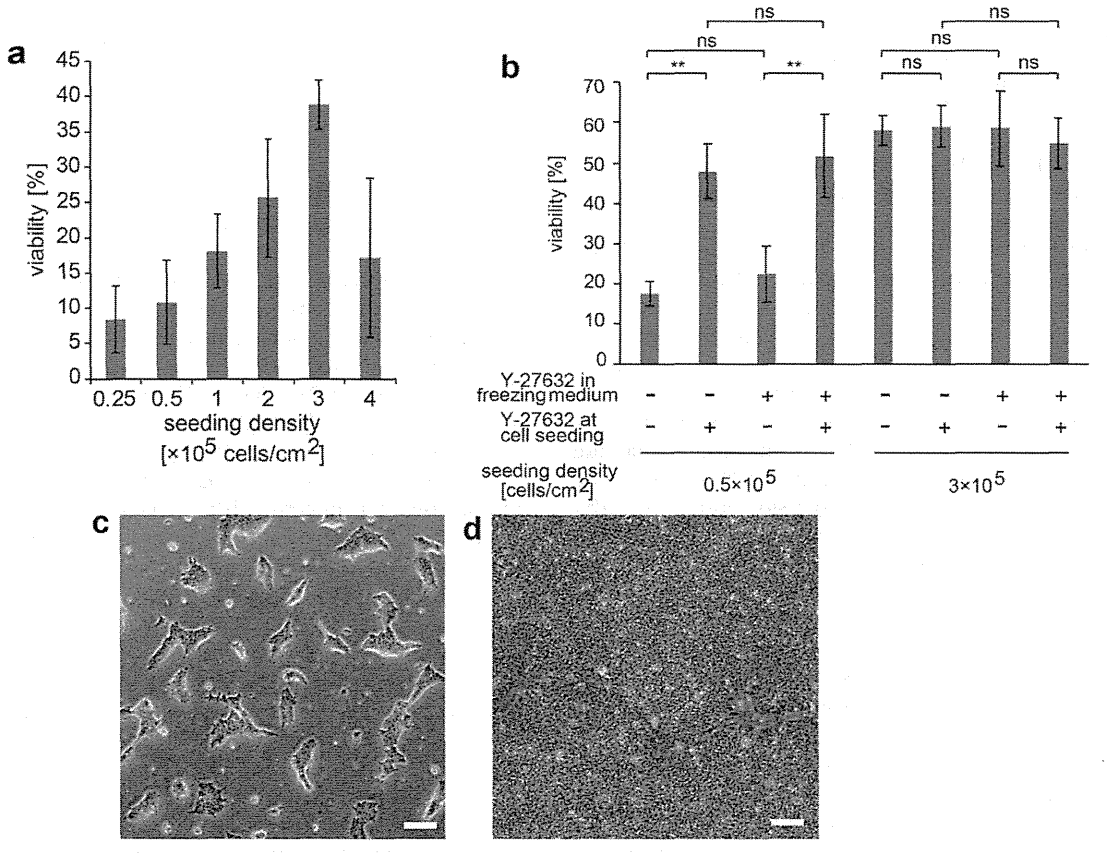

FIG. 2. Recovery of freeze-thawed single hPSCs. (**a**) Seeding density-dependent adhesion of freeze-thawed single H9 hESCs at 12 h postseeding. Cell viability is expressed as the ratio of the attached cell number to live H9 hESCs at seeding. Data represent the means ± SD of three freeze-thawing experiments of one cryopreserved batch of cells. (**b**) Effect of the ROCK inhibitor Y-27632 on adhesion of freeze-thawed single H9 hESCs at 12 h postseeding. Error bars indicate the SD. of three experiments. $^{**}P < 0.05$; ns, not significant, two-tailed Student's *t*-test. (**c, d**) Phase contrast images of freeze-thawed single H9 hESCs at 12 h (c) and 3 days (d) postseeding at 3×10^5 cells/cm^2. Scale bars: 200 μm.

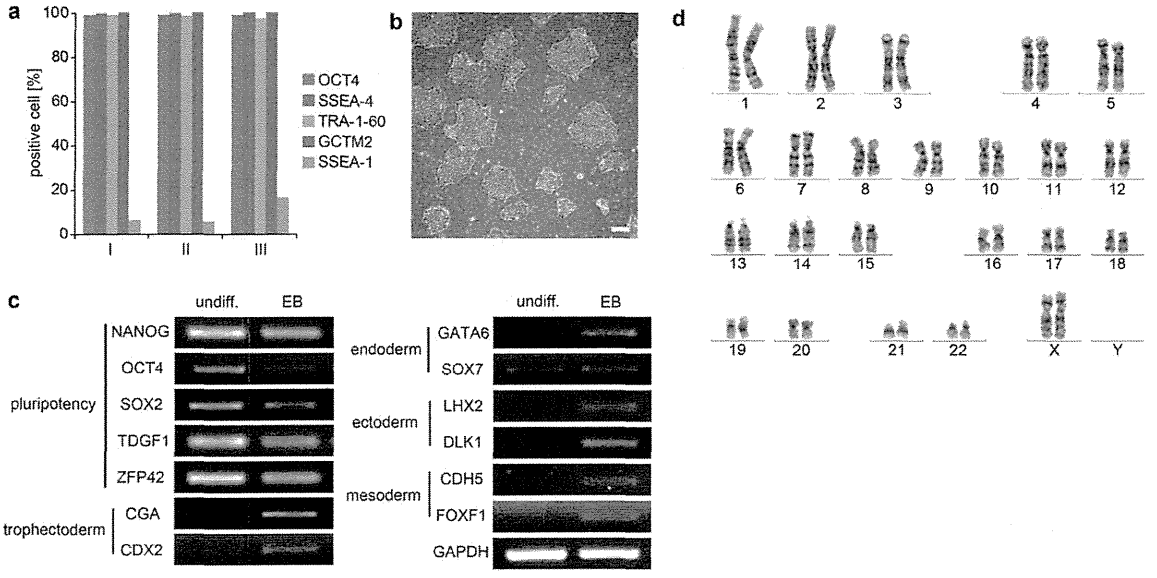

FIG. 3. Characterization of freeze-thawed hPSCs. (**a**) Flow cytometric analysis of undifferentiated markers before cryopreservation (I), at 3 days postseeding (II), and at subculture (III). (**b**) Phase contrast image of H9 hESCs at subculture. (**c**) Reverse transcription-PCR analysis of differentiation marker gene expression in embryoid bodies. Total RNA was extracted from day 14 embryoid bodies generated from cells after subculture. undiff. indicates cells at subculture. (**d**) G-banding analysis of H9 hESCs after subculture. Data for subcultured cells were obtained at two passages after freeze-thawing. Scale bars: 200 μm.

(Fig. 3d). Thus, hPSCs can be rapidly and efficiently cryopreserved by slow cooling while maintaining their pluripotent state.

Because hPSCs used for transplantation therapy would be expanded under xeno-free culture conditions, we evaluated xeno-free TeSR2 medium and recombinant human laminin-521 as the culture substrate. Laminin-521 is a laminin isoform used to improve the adhesion of dissociated hPSCs. Freeze-thawed single hPSCs in TeSR2 medium showed high survivability (86.8 ± 3.1% survival) immediately after freeze-thawing. Considering the promotion of adhesive by laminin-521, we re-evaluated the seeding density and found that freeze-thawed single hPSCs seeded at 1×10^5 cells/cm^2 showed maximal survival (59.7 ± 15.5% survival for H9 hESCs and 43.2 ± 11.2% survival for 253G1 iPSCs) (Fig. 4a and Supporting Information Fig. 1a). Moreover, on the laminin-521-coated culture surface, a higher number of single hPSCs were able to adhere and survive even at a lower seeding density (36.2 ± 6.1% survival for H9 hESCs and 32.5 ± 8.7% for 253G1 iPSCs at 0.25×10^5 cells/cm^2). After reseeding, the freeze-thawed hPSCs rapidly formed colonies on laminin-521 (Fig. 4b) and could be passaged within 3 days (Fig. 4c). The freeze-thawed hPSCs maintained their undifferentiated state (Fig. 4d, e, and Supporting Information Fig.4b), potential for differentiation (Fig. 4e), and a normal karyotype after passaging on laminin-521 in TeSR2 medium (Fig. 4f). Therefore, freeze-thawing of single hPSCs is applicable to xeno-free culture conditions, and human laminin-521 enhances the survival of single hPSCs after thawing.

In the present study, we obtained a high recovery rate following cryopreservation by optimizing the cellular state during freezing and the seeding density after thawing. These modifications achieved 80–90% survival of hPSCs post-thawing and around 60% survival following subculture on laminin-521, even in the absence of a ROCK inhibitor. hPSCs are delicate cells as compared with other types of cultured cells. Therefore, hPSCs require specific conditions not only for maintenance in culture but also for cryopreservation. Most previous approaches for effective hPSC cryopreservation have focused on development of a cryoprotectant specific for hPSCs (Ha *et al.*, 2005; T'Joen *et al.*, 2012). However, our study demonstrated that a fundamental solution for cryopreservation of hPSCs is their cellular state. It is therefore necessary to consider the cellular state for further investigation of hPSC cryopreservation. Moreover, our examination of the survivability of individual cells in a stepwise manner will contribute to estimation of the precise survival efficiency of hPSC cryopreservation.

It is still unclear why hPSC colonies are susceptible to injury after freeze-thawing. One risk is that hPSC colonies undergo unavoidable physical damage. Because cell scraping and pipetting for cell detachment is usually associated with cell rupture, freeze-thawed colonies of hPSCs may still be damaged following cell detachment. In addition, cell colonies have poor penetration of cryoprotectant. During cryopreservation, cells are

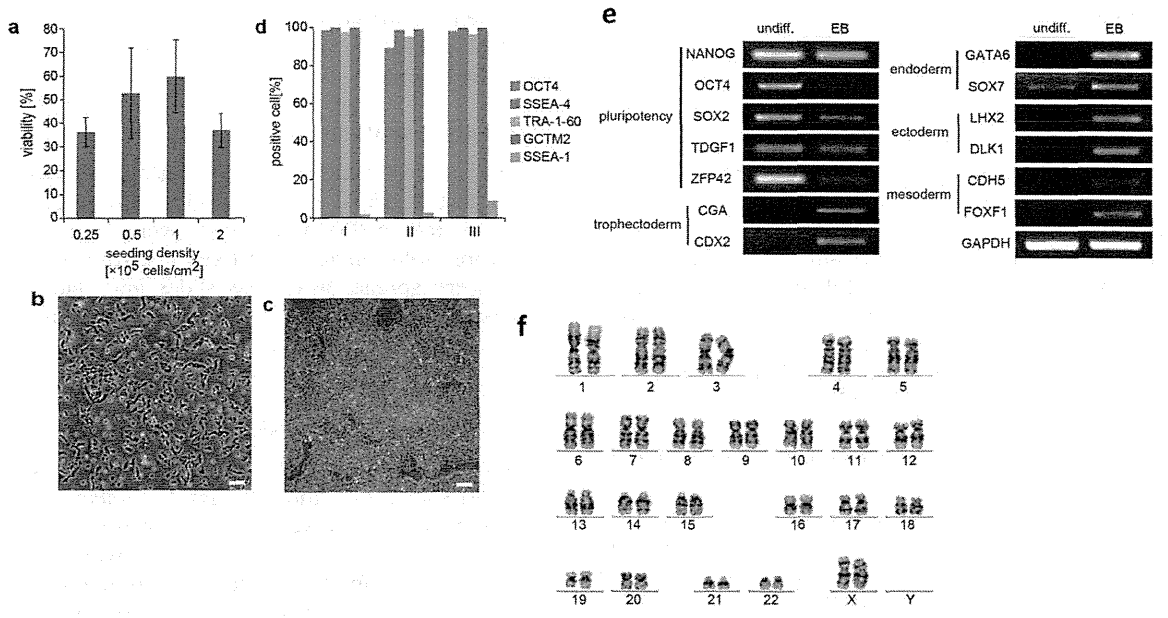

FIG. 4. Cryopreservation of single hPSCs under xeno-free culture conditions. (**a**) Seeding density-dependent adhesion of freeze-thawed single H9 hESCs on laminin-521 in TeSR2 medium at 12 h postseeding. Cell viability is expressed as the ratio of the attached cell number to live H9 hESCs at seeding. Data represent the means ± SD of three freeze-thawing experiments of one cryopreserved batch of cells. (**b**, **c**) Phase contrast images of freeze-thawed single H9 hESCs at 12 h (b) and 3 days (c) postseeding at 1×10^5 cells/cm^2. (**d**) Flow cytometric analysis of undifferentiated markers before cryopreservation (I), at 3 days postseeding (II), and at subculture (III). (**e**) Reverse transcription-PCR analysis of differentiation marker gene expression in embryoid bodies. Total RNA was extracted from day 14 embryoid bodies generated from cells after subculture. (**f**) G-banding analysis of H9 hESCs after subculture. Data for subcultured cells were obtained at two passages after freeze-thawing. Scale bars: 200 μm.

often ruptured by rapid osmotic changes caused by the cryoprotectant (Hunt, 2011). hPSC colonies are tightly connected by cell-cell interactions such as adherence junctions, and their formation generates a heterogeneous cellular state for cryoprotectant penetration. When compared with a complex cellular structure, single cells may be more suitable for cryopreservation in terms of cryoprotectant penetration. In addition, dissociation to single cells may minimize cellular damage at detachment because physical manipulation is unnecessary. Here, we used TrypLE Select as the dissociation solution, but other dissociation solutions used for single cell passaging, such as accutase and trypsin, would provide similar results.

In conclusion, our method enables highly efficient slow cooling of hPSCs. The cellular manipulation is simple and very similar to commonly used techniques for culturing most mammalian cell lines. Furthermore, our method is suitable for large-scale cryopreservation of pluripotent stem cell lines and machine-aided automation.

METHODS

Matrix Coating

Six-well flat-bottomed plates (BD Falcon, 353046) and 12-well flat-bottomed plates (BD Falcon, 353043) were coated with Matrigel (BD Biosciences, 354230) at 25 μg/cm^2 or laminin-521 (Bio Lamina, BLA-LN521-02) at 3 μg/cm^2 for 3 h at room temperature (RT) just prior to use. Matrigel and laminin-521 were diluted in DMEM/F12 or D-PBS, respectively.

Maintenance and Passaging of hPSCs

hESC lines, H9 and KhES-1, and hiPSC lines, iPS(IMR90) clone 1, and 253G1 were maintained on mitomycin C-treated mouse embryonic fibroblasts as described previously (Suemori *et al.*, 2006). hPSCs were transferred onto Matrigel-coated culture vessels in mTeSR1 medium (StemCell Technologies, ST-05850) or laminin-521-coated culture vessels in TeSR2 medium (StemCell Technologies, ST-05860) following the manufacturer's instructions.

Subculture was performed by colony dissociation as follows. Semi-confluent hPSCs were incubated with 2 mg/ml dispase in DMEM/F12 at 37°C for 3 min, and then rinsed twice with DMEM/F12. After addition of culture medium, weakly adherent colonies were detached using a cell scraper. The cells were collected and centrifuged at 200*g* for 3 min at 4°C. Small colonies were passaged onto fresh matrix-coated culture vessels at a ratio of 1:4. The cells were cultured at 37°C with 3% CO_2 in a humidified incubator. Medium changes were performed daily.

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.

ACKNOWLEDGMENTS

The authors thank Ms. Mari Hamao and Ms. Ritsuko Goto for technical assistance and the members of our laboratory for helpful discussions.

LITERATURE CITED

Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, Itskovitz-Eldor J, Thomson JA. 2000. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol 227:271–278.

Chen G, Hou Z, Gulbranson DR, Thomson JA. 2010. Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells. Cell Stem Cell 7:240–248.

Ha SY, Jee BC, Suh CS, Kim HS, Oh SK, Kim SH, Moon SY. 2005. Cryopreservation of human embryonic stem cells without the use of a programmable freezer. Hum Reprod 20:1779–1785.

Heng BC, Kuleshova LL, Bested SM, Liu H, Cao T. 2005. The cryopreservation of human embryonic stem cells. Biotechnol Appl Biochem 41:97–104.

Hunt CJ. 2011. Cryopreservation of human stem cells for clinical application: A review. Transfus Med Hemother 38:107–123.

Kumagai H, Suemori H, Uesugi M, Nakatsuji N, Kawase E. 2013. Identification of small molecules that promote human embryonic stem cell self-renewal. Biochem Biophys Res Commun 434:710–716.

Li T, Mai Q, Gao J, Zhou C. 2010. Cryopreservation of human embryonic stem cells with a new bulk vitrification method. Biol Reprod 82:848–853.

Li Y, Ma T. 2012. Bioprocessing of cryopreservation for large-scale banking of human pluripotent stem cells. Biores Open Access 1:205–214.

Miyazaki T, Futaki S, Suemori H, Taniguchi Y, Yamada M, Kawasaki M, Hayashi M, Kumagai H, Nakatsuji N, Sekiguchi K, Kawase E. 2012. Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells. Nat Commun 3: 1236.

Reubinoff BE, Pera MF, Vajta G, Trounson AO. 2001. Effective cryopreservation of human embryonic stem cells by the open pulled straw vitrification method. Hum Reprod 16:2187–2194.

Serra M, Brito C, Correia C, Alves PM. 2012. Process engineering of human pluripotent stem cells for clinical application. Trends Biotechnol 30:350–359.

Suemori H, Yasuchika K, Hasegawa K, Fujioka T, Tsuneyoshi N, Nakatsuji N. 2006. Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. Biochem Biophys Res Commun 345:926–932.

T'Joen V, De Grande L, Declercq H, Cornelissen M. 2012. An efficient, economical slow-freezing method for large-scale human embryonic stem cell banking. Stem Cells Dev 21:721–728.

Wagh V, Meganathan K, Jagtap S, Gaspar JA, Winkler J, Spitkovsky D, Hescheler J, Sachinidis A. 2011. Effects of cryopreservation on the transcriptome of human embryonic stem cells after thawing and culturing. Stem Cell Rev 7:506–517.

Watanabe K, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, Takahashi JB, Nishikawa S, Muguruma K, Sasai Y. 2007. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat Biotechnol 25:681–686.

Biochemical and Biophysical Research Communications 434 (2013) 710–716

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

ARTICLE INFO

Article history:
Received 17 March 2013
Available online 27 March 2013

Keywords:
Human embryonic stem cell
High-content screening
Small molecule
Basic fibroblast growth factor

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).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.03.061

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.

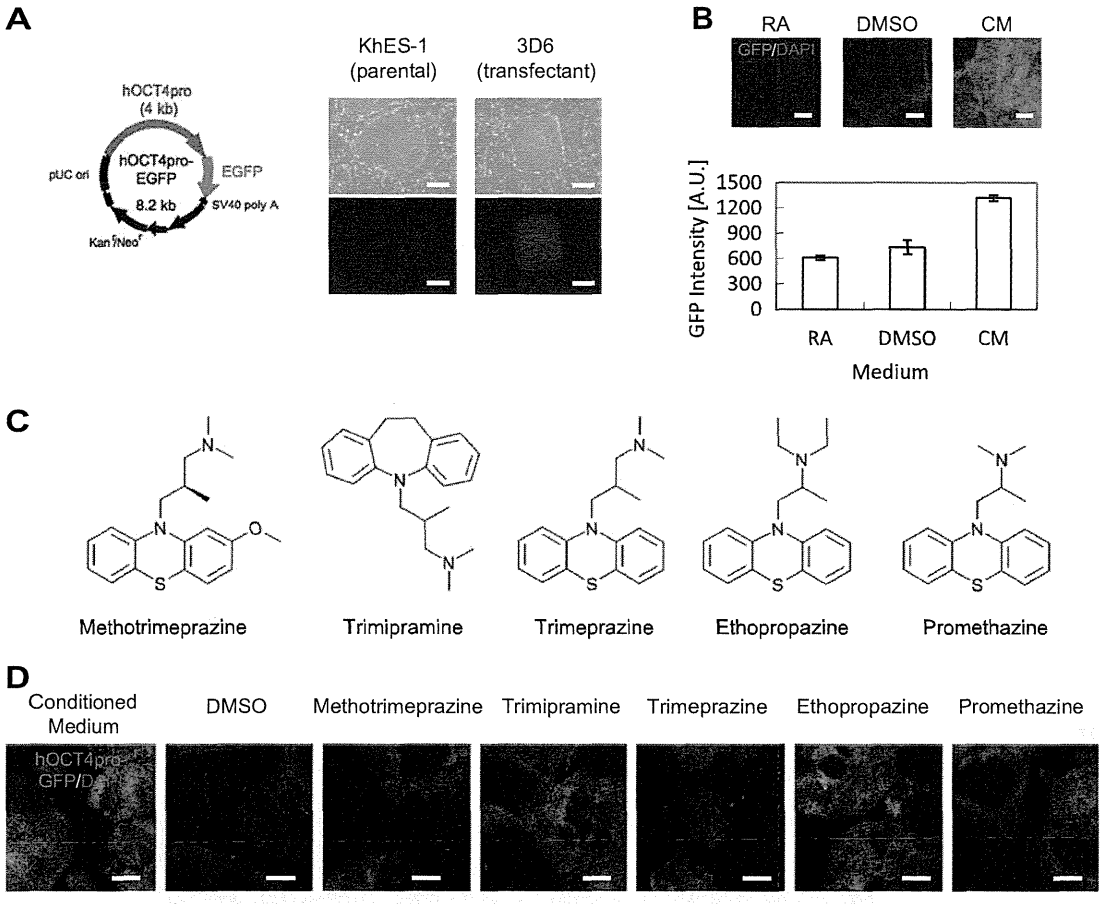

Fig. 1. High-content screening system and characterization of hit compounds. (A) (Left) Vector map of hOCT4pro-EGFP. (Right) Phase contrast and fluorescence images of parental KhES-1 and hOCT4pro-EGFP transfectant (3D6 clone) hESCs cultured on MEF feeders. Bar indicates 200 μm. (B) (upper) Representative 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

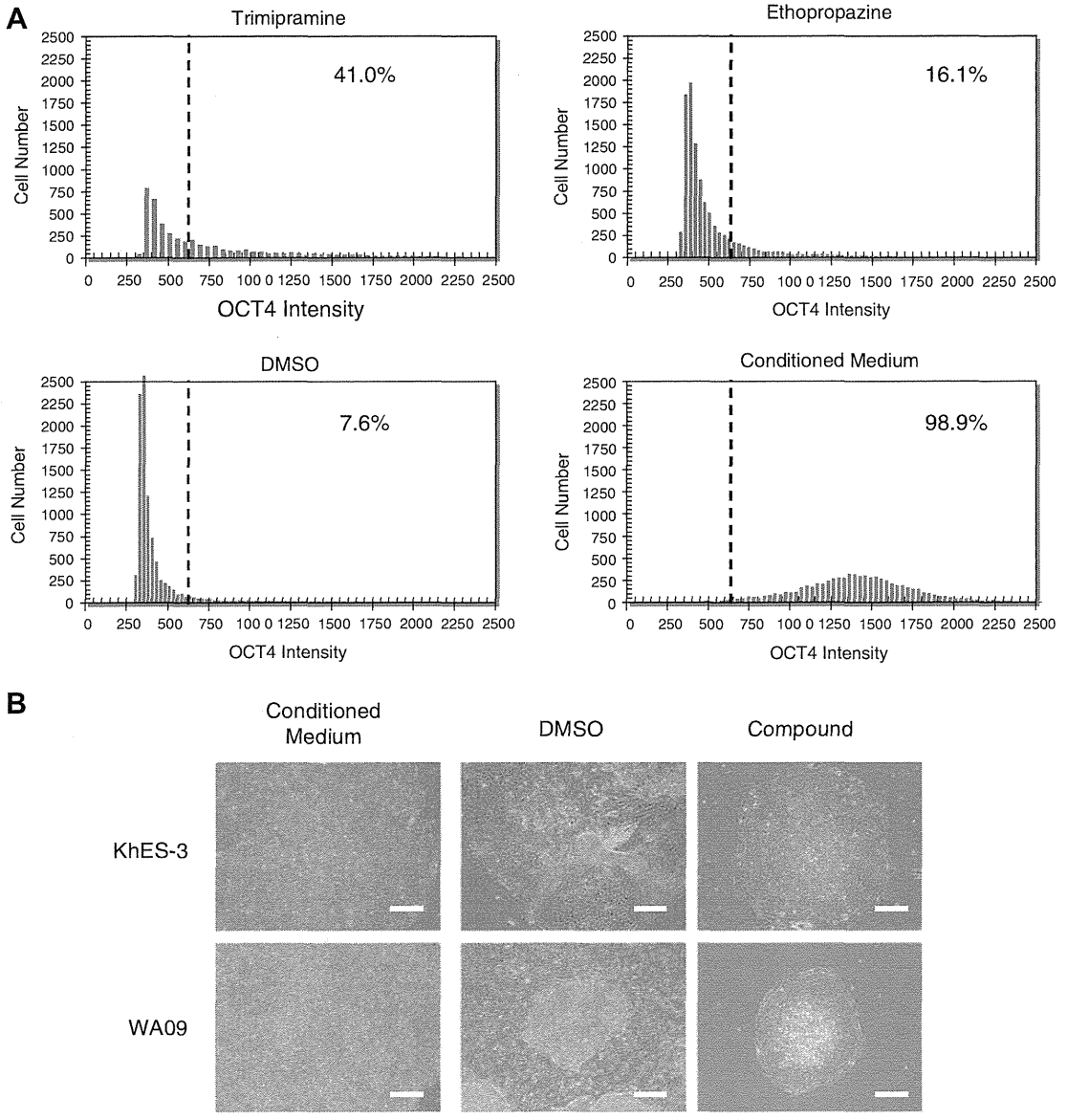

Fig. 2. Small molecules support short-term culture of hESCs without bFGF in the medium. (A) Histograms of the intensity of OCT4 immunostaining in hESCs treated with CM, DMSO, or small chemicals. (B) Morphology of KhES-3 and WA09 hESCs cultured in hESC-BM with trimipramine or ethopropazine after several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

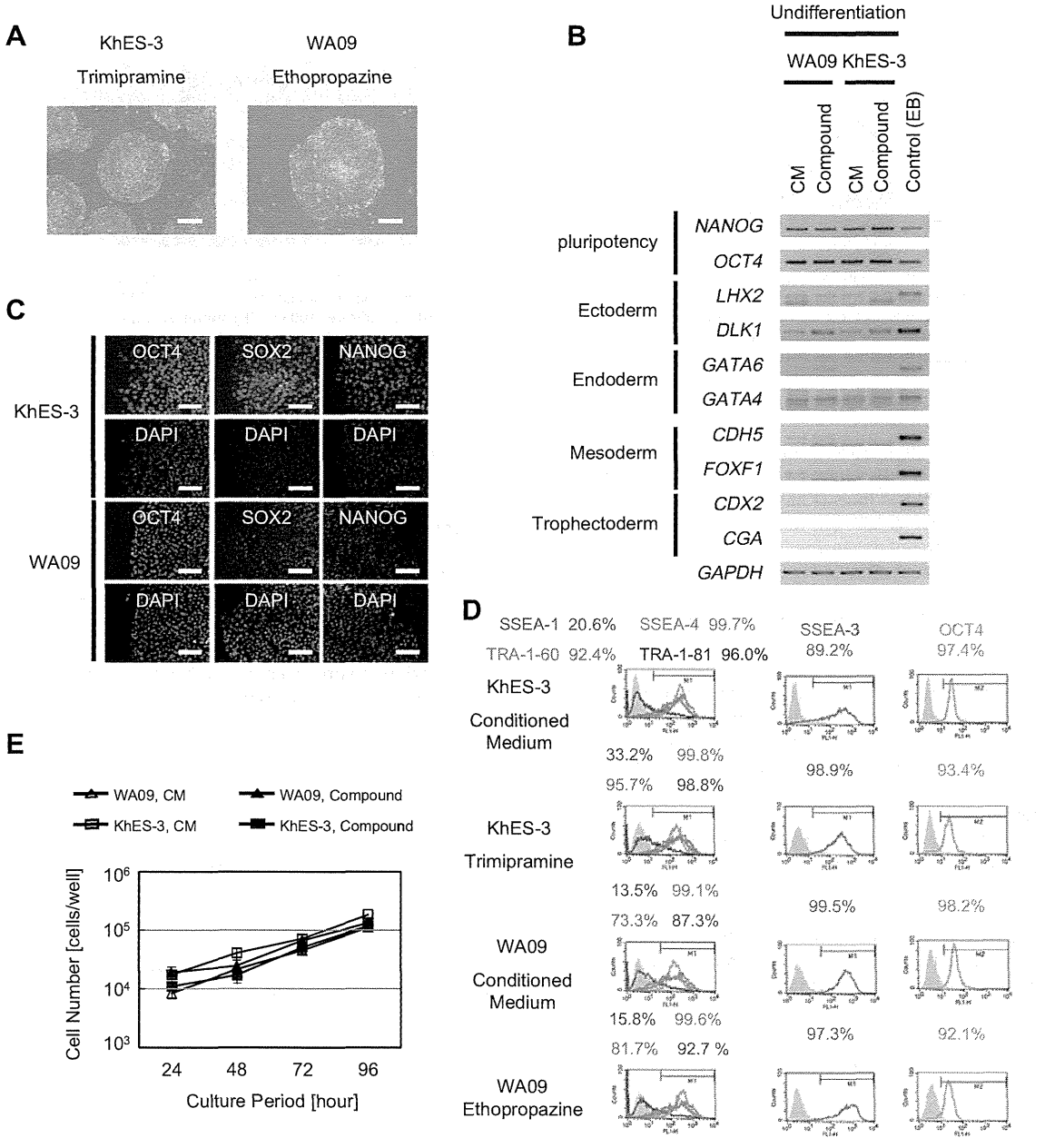

Fig. 3. Small molecules support long-term culture of undifferentiated hESCs without bFGF in the medium. (A) Phase contrast micrographs of KhES-3 and WA09 hESCs cultured in hESC-BM with trimipramine or ethopropazine after 25 passages. Bar indicates 200 μm. (B) Expression of pluripotency marker genes (*OCT4* 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

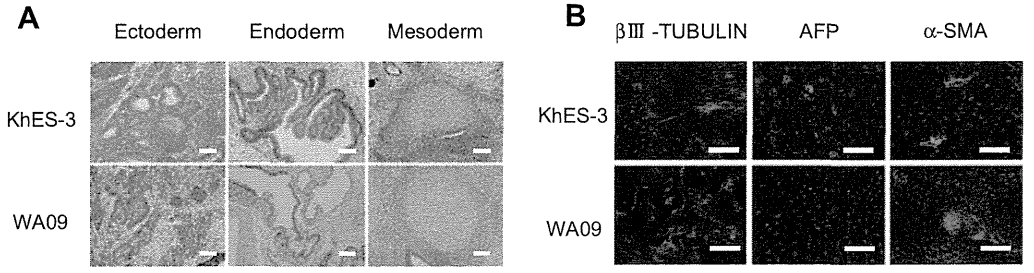

Fig. 4. Small molecules sustain the pluripotency of KhES-3 and WA09 hESCs without bFGF in the medium. (A) Characterization of teratomas derived from KhES-3 and WA09 hESCs cultured with trimipramine or ethopropazine. Hematoxylin and eosin staining of paraffin-embedded teratoma sections identified hESC 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.

Acknowledgments

We thank Ms. Mari Hamao and Ms. Ritsuko Goto for their valuable technical assistance and the members of the laboratories of N. Nakatsuji for insightful discussions. We also thank Mr. Yoshishiro Koyama for his valuable technical assistance with the HCS analysis. This work was supported by the New Energy and Industrial Technology Development Organization (NEDO) of Japan, and partially supported by a Grant-in-Aid for Scientific Research (to E.K.) and the "Funding Program for Next Generation World-Leading Researchers" (to M.U.). H.K. was supported, in part, as a predoctoral fellow for the Global COE Program "Center for Frontier Medicine" by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan. iCeMS is supported by the World Premier International Research Center Initiative (WPI), MEXT, Japan.

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.

References

[1] J.A. Thomson, J. Itskovitz-Eldor, S.S. Shapiro, M.A. Waknitz, J.J. Swiergiel, V.S. Marshall, J.M. Jones, Embryonic stem cell lines derived from human blastocysts, Science 282 (1998) 1145–1147.

[2] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, S. Yamanaka, Induction of pluripotent stem cells from adult human fibroblasts by defined factors, Cell 131 (2007) 861–872.

[3] J. Yu, M.A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J.L. Frane, S. Tian, J. Nie, G.A. Jonsdottir, V. Ruotti, R. Stewart, Slukvin II, J.A. Thomson, Induced pluripotent stem cell lines derived from human somatic cells, Science 318 (2007) 1917–1920.

[4] V. Akopian, P.W. Andrews, S. Beil, N. Benvenisty, J. Brehm, M. Christie, A. Ford, V. Fox, P.J. Gokhale, L. Healy, F. Holm, O. Hovatta, B.B. Knowles, T.E. Ludwig, R.D. McKay, T. Miyazaki, N. Nakatsuji, S.K. Oh, M.F. Pera, J. Rossant, G.N. Stacey, H. Suemori, Comparison of defined culture systems for feeder cell free propagation of human embryonic stem cells, In Vitro Cell Dev. Biol. Anim. 46 (2010) 247–258.

[5] L. Gerrard, D. Zhao, A.J. Clark, W. Cui, Stably transfected human embryonic stem cell clones express OCT4-specific green fluorescent protein and maintain self-renewal and pluripotency, Stem Cells 23 (2005) 124–133.

[6] H. Suemori, K. Yasuchika, K. Hasegawa, T. Fujioka, N. Tsuneyoshi, N. Nakatsuji, Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage, Biochem. Biophys. Res. Commun. 345 (2006) 926–932.

[7] T. Miyazaki, S. Futaki, K. Hasegawa, M. Kawasaki, N. Sanzen, M. Hayashi, E. Kawase, K. Sekiguchi, N. Nakatsuji, H. Suemori, Recombinant human laminin isoforms can support the undifferentiated growth of human embryonic stem cells, Biochem. Biophys. Res. Commun. 375 (2008) 27–32.

[8] T. Miyazaki, S. Futaki, H. Suemori, Y. Taniguchi, M. Yamada, M. Kawasaki, M. Hayashi, H. Kumagai, N. Nakatsuji, K. Sekiguchi, E. Kawase, Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells, Nat. Commun. 3 (2012) 1236.
[9] C. Xu, M.S. Inokuma, J. Denham, K. Golds, P. Kundu, J.D. Gold, M.K. Carpenter, Feeder-free growth of undifferentiated human embryonic stem cells, Nat. Biotechnol. 19 (2001) 971–974.
[10] J.H. Zhang, T.D. Chung, K.R. Oldenburg, A simple statistical parameter for use in evaluation and validation of high throughput screening assays, J. Biomol. Screen 4 (1999) 67–73.
[11] K. Watanabe, M. Ueno, D. Kamiya, A. Nishiyama, M. Matsumura, T. Wataya, J.B. Takahashi, S. Nishikawa, K. Muguruma, Y. Sasai, A ROCK inhibitor permits survival of dissociated human embryonic stem cells, Nat. Biotechnol. 25 (2007) 681–686.
[12] Y. Shi, J.T. Do, C. Desponts, H.S. Hahm, H.R. Scholer, S. Ding, A combined chemical and genetic approach for the generation of induced pluripotent stem cells, Cell Stem Cell 2 (2008) 525–528.
[13] T. Lin, R. Ambasudhan, X. Yuan, W. Li, S. Hilcove, R. Abujarour, X. Lin, H.S. Hahm, E. Hao, A. Hayek, S. Ding, A chemical platform for improved induction of human iPSCs, Nat. Methods 6 (2009) 805–808.
[14] D. Huangfu, R. Maehr, W. Guo, A. Eijkelenboom, M. Snitow, A.E. Chen, D.A. Melton, Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds, Nat. Biotechnol. 26 (2008) 795–797.
[15] C.A. Lyssiotis, R.K. Foreman, J. Staerk, M. Garcia, D. Mathur, S. Markoulaki, J. Hanna, L.L. Lairson, B.D. Charette, L.C. Bouchez, M. Bollong, C. Kunick, A. Brinker, C.Y. Cho, P.G. Schultz, R. Jaenisch, Reprogramming of murine fibroblasts to induced pluripotent stem cells with chemical complementation of Klf4, Proc. Natl. Acad. Sci. USA 106 (2009) 8912–8917.
[16] Z. Li, T.M. Rana, A kinase inhibitor screen identifies small-molecule enhancers of reprogramming and iPS cell generation, Nat. Commun. 3 (2012) 1085.
[17] S. Chen, M. Borowiak, J.L. Fox, R. Maehr, K. Osafune, L. Davidow, K. Lam, L.F. Peng, S.L. Schreiber, L.L. Rubin, D. Melton, A small molecule that directs differentiation of human ESCs into the pancreatic lineage, Nat. Chem. Biol. 5 (2009) 258–265.
[18] S.C. Desbordes, D.G. Placantonakis, A. Ciro, N.D. Socci, G. Lee, H. Djaballah, L. Studer, High-throughput screening assay for the identification of compounds regulating self-renewal and differentiation in human embryonic stem cells, Cell Stem Cell 2 (2008) 602–612.
[19] R. Damoiseaux, S.P. Sherman, J.A. Alva, C. Peterson, A.D. Pyle, Integrated chemical genomics reveals modifiers of survival in human embryonic stem cells, Stem cells 27 (2009) 533–542.
[20] I. Barbaric, M. Jones, D.J. Harley, P.J. Gokhale, P.W. Andrews, High-content screening for chemical modulators of embryonal carcinoma cell differentiation and survival, J. Biomol. Screen 16 (2011) 603–617.
[21] A.I. Lukaszewicz, M.K. McMillan, M. Kahn, Small molecules and stem cells. Potency and lineage commitment: the new quest for the fountain of youth, J. Med. Chem. 53 (2010) 3439–3453.
[22] K. Dorey, E. Amaya, FGF signalling: diverse roles during early vertebrate embryogenesis, Development 137 (2010) 3731–3742.
[23] H. Tsutsui, B. Valamehr, A. Hindoyan, R. Qiao, X. Ding, S. Guo, O.N. Witte, X. Liu, C.M. Ho, H. Wu, An optimized small molecule inhibitor cocktail supports long-term maintenance of human embryonic stem cells, Nat. Commun. 2 (2011) 167.
[24] X. Feng, J. Zhang, K. Smuga-Otto, S. Tian, J. Yu, R. Stewart, J.A. Thomson, Protein kinase C mediated extraembryonic endoderm differentiation of human embryonic stem cells, Stem cells 30 (2012) 461–470.
[25] A.M. Singh, D. Reynolds, T. Cliff, S. Ohtsuka, A.L. Mattheyses, Y. Sun, L. Menendez, M. Kulik, S. Dalton, Signaling network crosstalk in human pluripotent cells: a Smad2/3-regulated switch that controls the balance between self-renewal and differentiation, Cell Stem Cell 10 (2012) 312–326.

nature COMMUNICATIONS

ARTICLE

Received 30 Jul 2012 | Accepted 29 Oct 2012 | Published 4 Dec 2012

DOI: 10.1038/ncomms2231

OPEN

Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells

Takamichi Miyazaki[1], Sugiko Futaki[2], Hirofumi Suemori[1], Yukimasa Taniguchi[2], Masashi Yamada[2], Miwa Kawasaki[2], Maria Hayashi[2], Hideaki Kumagai[1], Norio Nakatsuji[3,4], Kiyotoshi Sekiguchi[2] & Eihachiro Kawase[1]

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) have the potential to provide an infinite source of tissues for regenerative medicine. Although defined xeno-free media have been developed, culture conditions for reliable propagation of hESCs still require considerable improvement. Here we show that recombinant E8 fragments of laminin isoforms (LM-E8s), which are the minimum fragments conferring integrin-binding activity, promote greater adhesion of hESCs and hiPSCs than do Matrigel and intact laminin isoforms. Furthermore, LM-E8s sustain long-term self-renewal of hESCs and hiPSCs in defined xeno-free media with dissociated cell passaging. We successfully maintained three hESC and two hiPSC lines on LM-E8s in three defined media for 10 passages. hESCs maintained high level expression of pluripotency markers, had a normal karyotype after 30 passages and could differentiate into all three germ layers. This culture system allows robust proliferation of hESCs and hiPSCs for therapeutic applications.

[1] Department of Embryonic Stem Cell Research, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. [2] Laboratory of Extracellular Matrix Biochemistry, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan. [3] Department of Development and Differentiation, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. [4] Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Ushinomiya-cho, Yoshida, Sakyo-ku, Kyoto 606-8501, Japan. Correspondence should be addressed to E.K. (email: kawase8@frontier.kyoto-u.ac.jp) for general correspondence or K.S. (email: sekiguch@protein.osaka-u.ac.jp) for recombinant laminin products.

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) have great potential for a wide range of applications in regenerative medicine, and as tools for human disease modelling and drug discovery. However, defined xeno-free and feeder-free systems have not been fully established for the realization of these applications. Several types of defined medium have been developed, which consist of known components and avoid the use of animal products[1–5]. However, the widespread use of Matrigel as a culture substrate is potentially problematic[6]. Matrigel is not an optimal substrate because it is derived from mouse Engelbreth–Holm–Swarm tumours and contains many unknown components. Additionally, hESCs usually propagate as colonies in culture, and their passaging involves either enzymatic dissociation by gently pipetting or manual microdissection of the cells[7]. hESC colonies grow as a monolayer and sustain an undifferentiated state, whereas the formation of large aggregates during passaging accelerates their spontaneous differentiation. Thus, it is somewhat difficult to precisely control appropriate dissociation of hESCs while passaging. As a result, conventional colony-based protocols often have considerable variations in culture outcomes. One method to overcome such difficulties might be complete dissociation of hESCs by an optimized and standardized protocol. However, complete dissociation of hESC clumps into single cells causes extensive cell death, and the survival is generally $<1\%$ (refs 8,9). Furthermore, complete cell dissociation is thought to be associated with an increased risk of karyotypic abnormalities[10,11]. In this study, we aimed to identify an optimal substrate with the greatest adhesive property for the culture of completely dissociated hESCs and hiPSCs, and we further examined whether it could promote robust propagation of these cells while retaining a normal karyotype.

Although there are reports of the replacement of Matrigel with defined substrates, such as small peptides, to allow sustained hESC self-renewal in defined or xeno-free media[3–5,12–18], considerable improvements and refinements of these substrates are still needed. Laminin, the main component of Matrigel, is a heterotrimeric glycoprotein composed of three covalently linked chains that are termed α, β and γ[19].Thus far, 15 laminin isoforms formed by various combinations of each chain (α1–α5, β1–β3 and γ1–γ3) have been identified. We have previously shown that human recombinant laminin isoforms (laminin-511, -332 and -111) support hESC proliferation in an undifferentiated state, although laminin-111 shows weak cell adhesive properties[20]. Laminin-511, the first and only alternative substrate with a greater adhesive property for hESCs than that provided by Matrigel, also permits sustained self-renewal of hESCs in xeno-free culture conditions[12]. Typically, procedures for the isolation of native pure laminins from tissues are arduous or highly impractical. Recently, some recombinant laminins have been produced using mammalian expression systems[21–25]. However, full-length laminins are large heterotrimeric proteins (Fig. 1a), and mass production of these recombinant proteins is still very difficult. Therefore, we examined whether recombinant laminin fragments could support adhesion and undifferentiated proliferation of hESCs and hiPSCs. These fragments are obviously smaller molecules and have a much higher yield compared with that of the full-length protein.

Laminin isoforms bind to multiple receptors on the cell surface, including integrins, syndecans and dystroglycans, of which integrins serve as the major adhesion receptors of hESCs[26]. Laminin-511, -332 and -111 share the characteristic of specific high affinity for α6β1 integrin[27]. Using function-blocking antibodies, α6β1 integrin has been identified as the major cell adhesion receptor for both Matrigel and laminin-511 (refs 12,28). Furthermore, α6β1 integrin is abundantly expressed by both hESCs[20] and hiPSCs (Supplementary Fig. S1). Thus, we examined whether the integrin-binding site in laminin is solely necessary for the maintenance of hESCs and hiPSCs.

Laminin E8 fragments (LM-E8s) are truncated proteins composed of the C-terminal regions of the α, β and γ chains. These truncated proteins contain the active integrin-binding site comprising the laminin globular 1-3 domains of the α chain[21] and the glutamate residue in the C-terminal tail of the γ chain[29], but lack other activities such as the heparin/heparan sulphate-binding activity, which are associated with intact laminins[21]. Therefore, LM-E8s serve as a functionally minimal form that retains the full capability for binding to α6β1 integrin[30]. When LM-E8s are separated into an α chain and a disulphide-bonded dimer of β and γ chains, they lose their cell adhesive activity[31], underscoring the requirement for all three chains for their integrin-binding activity. Thus, the integrin-binding site of laminin appears to be a composite epitope composed of multiple polypeptides[32], thereby making it difficult to recapitulate the integrin-binding site with synthetic peptides modelled on individual laminin subunits.

In this study, we produced two recombinant LM-E8s, LM511-E8 from laminin-511 and LM332-E8 from laminin-332, both of which have high cell adhesive properties (Fig. 1a), and examined their capabilities as adhesion substrates for hESCs and hiPSCs. Both fragments display stronger adhesion affinities for hESCs and hiPSCs than those of other substrates. Furthermore, LM-E8s enable robust propagation of hESCs and hiPSCs in an undifferentiated state in cultures with defined and xeno-free media using dissociated cell passaging.

Results

LM-E8s maximize hESC and hiPSC adhesion. Extracellular matrix (ECM) proteins vary considerably in their affinities for receptors, and in protein size and charge. Consequently, different proteins vary in their optimal use as a cell culture support matrix. To determine whether LM511-E8 and LM332-E8 were suitable for hESC and hiPSC adhesion, we first compared the adhesive properties of completely dissociated H9 hESCs and iPS(IMR90)-1 cells in mTeSR1 medium on matrices containing LM511-E8 or LM332-E8 with those on other matrices. Cell adhesion on all matrices showed dose-dependent increases, but with different maxima: 15 $\mu g\,cm^{-2}$ for Matrigel; 3–6 $\mu g\,cm^{-2}$ for intact laminin-511 and -332; and 1.5 $\mu g\,cm^{-2}$ for LM511-E8 and LM332-E8 (Fig. 1b and Supplementary Figs S2a and S3a). In agreement with a previous report[12], adhesion to intact laminin-511 or -332 was higher than that to Matrigel. However, we found that LM511-E8 and LM332-E8 had significantly higher adhesive properties than those of intact laminin-511 and -332. We therefore used LM511-E8 and LM332-E8 at a coating concentration of 1.5 $\mu g\,cm^{-2}$ in subsequent experiments.

To confirm that LM511-E8 and LM332-E8 were recognized by α6β1 integrin on the cell surface, we used integrin function-blocking antibodies to investigate whether α6 and/or β1 integrins were required for the adhesion of H9 hESCs to LM-E8-coated substrata in mTeSR1 medium (Fig. 1c; Statistical results of multiple comparisons are shown in Supplementary Tables S1a and S1b). Cell attachment to both LM-E8s was significantly reduced by the addition of antibodies against α6 and/or β1 integrins. We further confirmed that an inactive form of LM511-E8 (LM511(EQ)), in which the glutamic acid residue (E) at the third position from the C-terminus in the γ chain was replaced by a glutamine residue (Q)[29], showed dramatic abrogation of cell adhesion (Fig. 1c). Taken together, these data showed that α6β1 integrin mediated the adhesion of completely dissociated hESCs to LM511-E8 and LM332-E8.

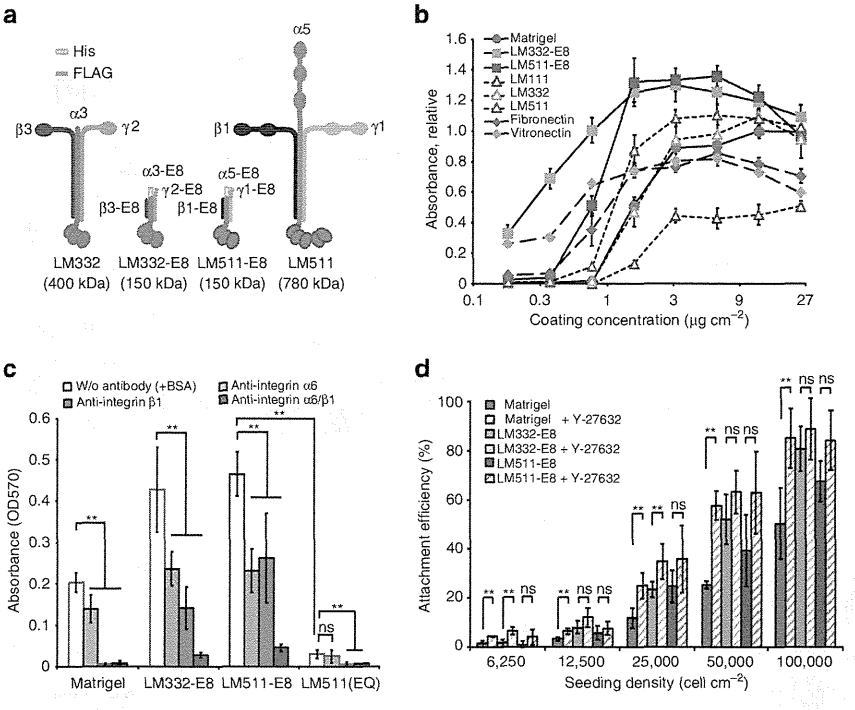

Figure 1 | Validation of LM-E8s for H9 hESC attachment. (**a**) Schematic representations of the two LM-E8s and the original intact laminin isoforms. A 6xHis-tag and FLAG-tag were attached to the N-termini of the α and γ chains, respectively, of LM-E8s to facilitate the purification of recombinant LM-E8s. The molecular weights of individual proteins are shown in parentheses. (**b**) Dose-response adhesion curves of H9 hESCs on various ECMs. LM-E8s at 1.5 μg cm^{-2} showed higher efficiency for supporting the adhesion of dissociated cells than did other matrices. The means of absorbance (OD570) represent the relative number of adherent cells normalized against the values at the maximum effect on Matrigel, which was arbitrarily set as 1. Error bars show the s.e.m. of five independent assays, except for vitronectin, which shows the average of duplicate assays. (**c**) Inhibition of integrins on LM-E8s. Completely dissociated H9 hESCs were incubated with an integrin-specific blocking antibody for 30 min in mTeSR1 medium. LM511(EQ): an inactive form of LM511-E8. Error bars indicate the s.d. of five individual assays. $^{**}P<0.05$; ns, non-significant; two-way ANOVA or Tukey's test. (**d**) Seeding density-dependent adhesion of H9 hESCs on LM-E8s. LM-E8s showed a higher cell adhesive activity for completely dissociated H9 hESCs than did Matrigel. Completely dissociated cells were incubated on LM-E8s in mTeSR1 medium for 6 h with or without Y-27632. Data represent the means ± s.d. of three experiments. $^{**}P<0.05$; ns, non-significant, two-tailed Student's *t*-test.

Both LM332-E8 and LM511-E8 showed higher adhesion for completely dissociated hESCs or hiPSCs than did Matrigel at various cell densities (Fig. 1d and Supplementary Fig. S3b). Furthermore, using the optimal cell density (more than 50,000 cells per cm^2), we observed attachment of 40–70% of cells to LM-E8s, which raised the possibility of passaging cultures by completely dissociating hESCs and hiPSCs.

It has been previously shown that the Rho-associated protein kinase (ROCK) inhibitor Y-27632 increases the adhesion of dissociated hESCs[33]. However, the effect of Y-27632 on hESC and hiPSC cultures in LM-E8-coated plates was unclear (Fig. 1d and Supplementary Fig. S3b). Y-27632 pretreatment before complete dissociation did not significantly improve the attachment of dissociated H9 hESCs on LM-E8s (Supplementary Fig. S2b). We therefore concluded that the addition of Y-27632 to the medium was unnecessary for cultures using LM-E8s, unlike attachment onto Matrigel, fibronectin, vitronectin and Synthemax that has a synthetic culture surface consisting of short peptides derived from vitronectin (Supplementary Fig. S2c). To focus on the effectiveness of LM-E8s, we did not use Y-27632 in subsequent experiments. In addition, we selected 50,000 cells per cm^2 as the seeding density for passaging hESCs and hiPSCs.

LM-E8s support vigorous proliferation in defined media. We next examined whether LM-E8s supported undifferentiated growth of completely dissociated hESCs and hiPSCs in mTeSR1 medium. H9 hESC colonies dissociated by a conventional method showed superior attachment to LM-E8s (Fig. 2a). Likewise, completely dissociated H9 hESCs showed strong adherence on LM-E8s. Cells quickly adhered and spread on LM-E8-coated substrates, with extending lamellipodium-like protrusions (Fig. 2b and Supplementary Movies 1 and 2). H9 hESCs adhered to LM-E8s promptly recovered cell–cell contacts, formed highly dense colonies (Fig. 2c), and showed steady proliferation similar to that of conventional colony cultures (Fig. 2d). Similarly, completely dissociated iPS(IMR90)-1 cells also exhibited steady proliferation on LM-E8 fragments (Supplementary Fig. S3c). In contrast, completely dissociated H9 hESCs displayed weak adhesion on Matrigel or considerably poor adhesion on fibronectin and vitronectin, although H9 hESC colonies normally adhere to these substrates (Fig. 2a). Most dissociated cells on Matrigel, fibronectin and vitronectin remained as single cells and rapidly underwent cell death (Fig. 2b and Supplementary Movies 3–5). In agreement with the cell adhesion and survivability on LM-E8s, molecular analysis revealed that LM-E8s stimulated the signalling pathways downstream of integrins in hESCs and iPSCs. AKT, FAK and ERK1/2, signalling molecules closely associated with cell survival and migration[34], were activated to a greater extent in cells cultured on LM-E8s than in cells cultured on fibronectin or vitronectin (Fig. 2e). Y-27632 treatment abrogated MLC2 phosphorylation regardless of culture substrates, indicating that

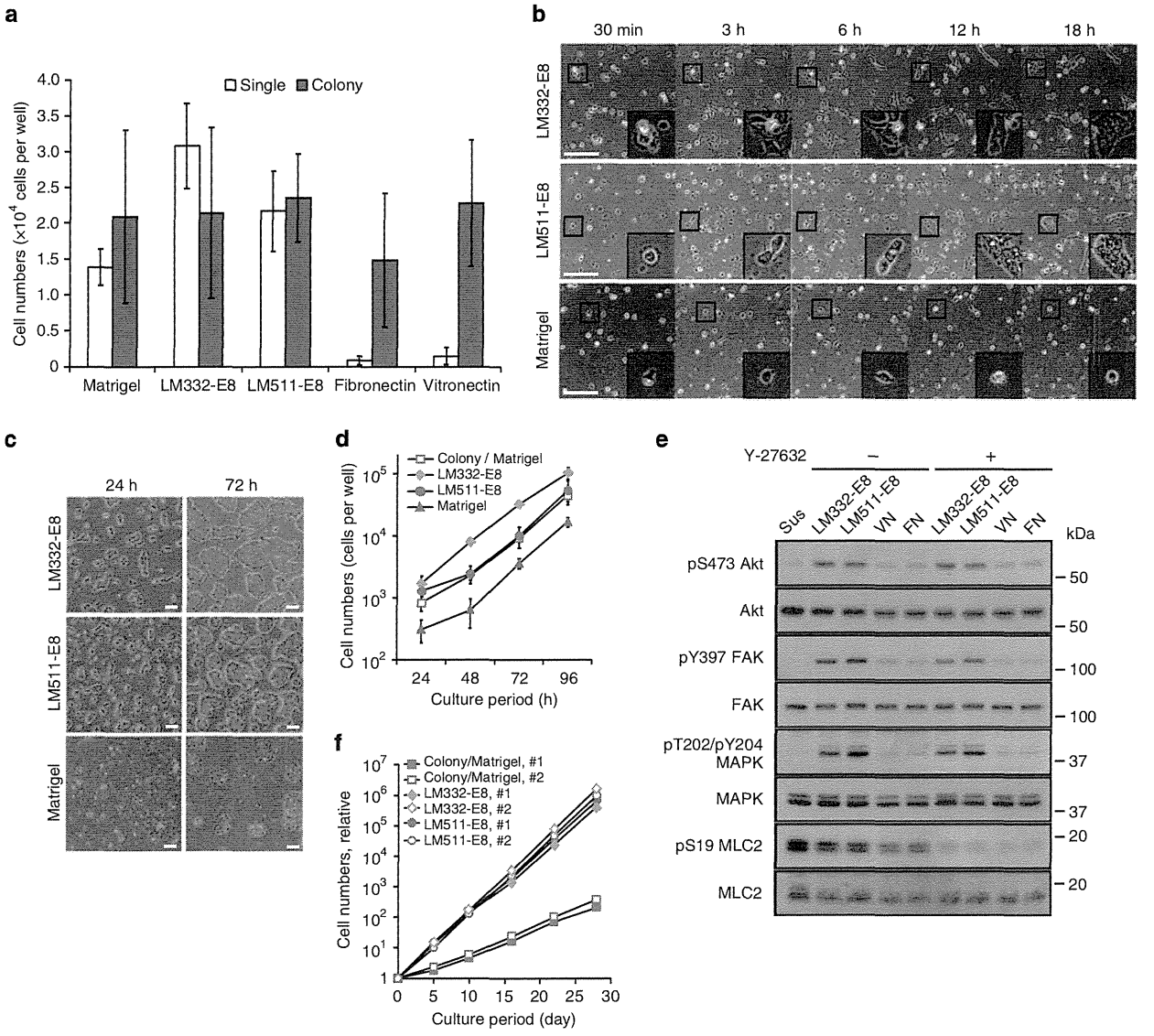

Figure 2 | LM-E8s support undifferentiated proliferation in defined medium. (**a**) Distinct cell survival of H9 hESCs depending on ECMs. Data represent the numbers of live cells at 24 h after seeding 3.75×10^4 cells as single cells (single) or colonies (colony). (**b**) Serial phase-contrast images of H9 hESCs on LM-E8s or Matrigel in mTeSR1 medium at initial attachment. Images at the bottom right show an enlarged view of enclosed boxes. Note that dissociated H9 hESCs promptly extended and reached neighbouring cells on LM-E8s, but failed cellular extension on Matrigel. See also Supplementary Movies 1–5. (**c**) Phase-contrast images of H9 hESCs in mTeSR1 medium on LM-E8s or Matrigel during the growth phase. (**d**) Short-term growth curves of H9 hESCs on LM-E8s in mTeSR1 medium. Dissociated H9 hESCs on LM-E8s proliferated and had a similar growth rate as that in conventional colony culture during logarithmic growth. Data represent the means ± s.d. of three individual experiments. (**e**) Detection of phosphorylated proteins associated with cell adhesion by western blot analysis. Cell lysates were prepared from H9 hESCs incubated for 1 h on each substrate with or without Y-27632. Sus, dissociated H9 hESCs incubated for 1 h in suspension; FN, fibronectin; VN, vitronectin. (**f**) Long-term growth curves of H9 hESCs on LM-E8s in mTeSR1 medium. The numbers of seeded cells at the initial passage were converted to 1, and the counted cell numbers at each passage are shown as cumulative cell numbers. Note that dissociation culture on LM-E8s resulted in a more than 200-fold increase in cell numbers relative to conventional colony culture over 1 month. Scale bars, 200 μm.

the signalling pathways promoting cell survival on LM-E8s were not the same as those targeted by Y-27632. Once H9 hESCs adhered on each culture substrate, the cells similarly proliferated at an invariable rate that was regardless of the culture substrate, cluster size and Y-27632 treatment (Fig. 2d and Supplementary Fig. S2d). Therefore, the expansion efficiency was simply dependent on the initial adhesion efficiency and passaging ratio. LM-E8s allowed a higher passaging ratio during subculture, which was approximately 1:10 compared with 1:4 for conventional colony culture. Indeed, LM-E8s yielded more than 200-fold of the number of cells compared with that obtained by conventional culture over a 1-month period (Fig. 2f). Thus, LM-E8s enabled rapid expansion of completely dissociated hESCs and hiPSCs by supporting robust adhesion and rapid cell expansion.

Using our culture protocol with LM-E8s and two chemically defined media (mTeSR1 and StemPro), three hESC lines (H9, HES3 and KhES-1) and two hiPSC lines (iPS(IMR90)-1 and 253G1) showed robust proliferation for at least 10 serial passages. Flow cytometric analysis further indicated that the majority of dissociated hESCs and hiPSCs cultured on LM-E8s

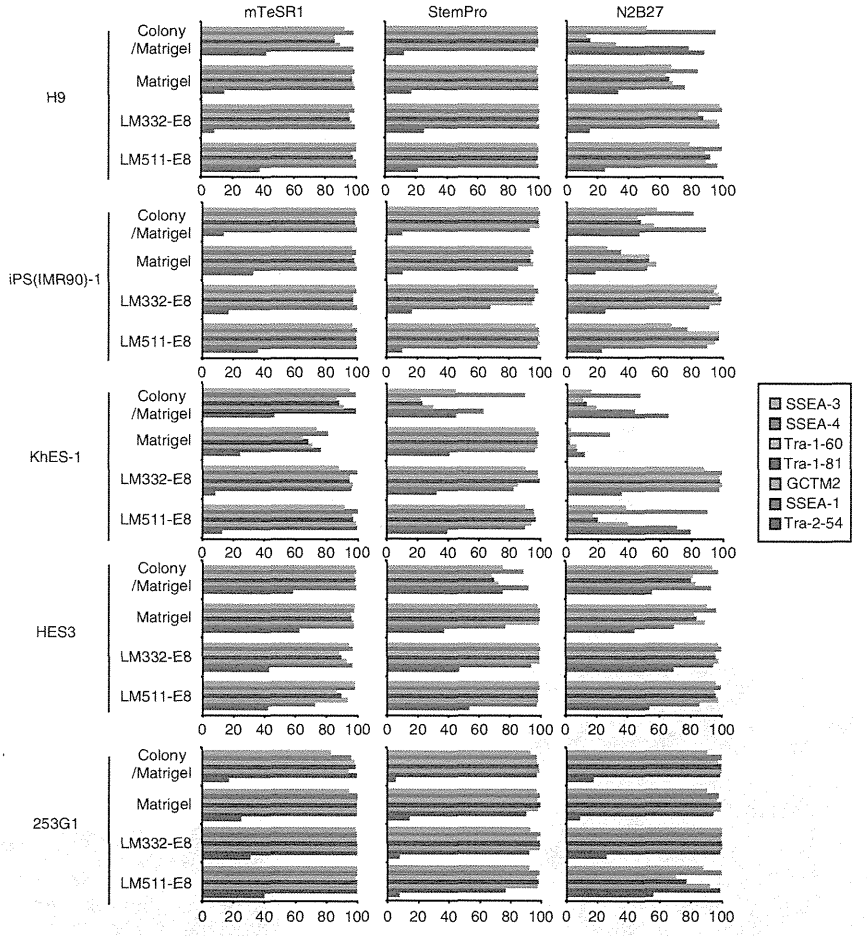

Figure 3 | Flow cytometric analysis of hESC or hiPSC lines. Three hESC lines (H9, HES3 and KhES-1) and two hiPSC line (iPS(IMR90)-1 and 253G1) were cultured on LM-E8s after complete dissociation. These cells maintained higher expression levels of the undifferentiated markers, compared with that on Matrigel with either colony or complete dissociation culture. Undifferentiated markers: SSEA-3, SSEA-4, Tra-1-60, Tra-1-81, GCTM2 and Tra-2-54. Differentiation marker: SSEA-1. Data were collected from cells cultured for 10 passages. The percentages of positive cells are indicated on the graph.

expressed pluripotency markers SSEA-3, SSEA-4, Tra-1-60, Tra-1-81, GCTM2 and Tra-2-54, with only low level of expression of the differentiation marker SSEA-1 (Fig. 3). It has been reported that N2B27 medium is less robust for hESC maintenance, compared with two other media[2], but almost all hESCs and hiPSCs, except for KhES-1, cultured on LM-E8s showed undifferentiated growth in N2B27 medium. We karyotyped 50 randomly selected cells after G-banding of each cell line for all experiments at passage 10. Cells propagated on LM-E8s after complete dissociation had normal karyotypes (Table 1 and Supplementary Fig. S4a, b). Using multiplex fluorescence *in situ* hybridization (M-FISH) analysis, we showed that completely dissociated H9 hESCs and iPS(IMR90)-1 cells had a normal karyotype after 30 and 20 passages on LM-E8s, respectively (Supplementary Fig. S4c–f).

To assess the pluripotency of hESCs that had been cultured on LM-E8s in mTeSR1 medium for 10 passages, the cells were treated with the growth factors described in the Methods. We found that hESCs could differentiate into ectoderm (βIII tubulin), mesoderm (α-smooth muscle actin) or endoderm (α-fetoprotein), as determined by immunohistochemical analyses (Fig. 4a). Furthermore, reverse transcriptase PCR (RT–PCR) analysis showed that, when hESCs were placed in suspension culture, they formed embryoid bodies with increased expression of gene markers for all three germ layer lineages after 10 days of incubation (Fig. 4b). Similar results were obtained for iPS (IMR90)-1 cells cultured on LM-E8s (Supplementary Fig. S5). We further confirmed that hESCs cultured on LM-E8s in mTeSR1 medium for 25 passages formed teratomas consisting of all three germ layers, including neuroepithelium (ectoderm), intestinal epithelium (endoderm) and cartilage (mesoderm) (Fig. 4c).

Thus, our new culture method using completely dissociated cells on LM-E8s supported long-term maintenance and efficient cell expansion of hESCs and hiPSCs in an undifferentiated state.

LM-E8s support vigorous proliferation in xeno-free medium. The final goal of this study was to characterize LM-E8s as substrates that support hESCs in a sustainable undifferentiated state under a xeno-free culture condition. We cultured completely dissociated H9 hESCs and 253G1 iPSCs on LM511-E8 or LM332-E8 in TeSR2 medium that is chemically defined and animal protein free. Dissociated H9 hESCs tightly adhered on LM-E8s in TeSR2 medium (Fig. 5a). Similarly to the results described for defined media, we found that H9 hESCs and 253G1 iPSCs proliferated robustly for more than 30 and 10 passages, respectively. Flow cytometric analysis also confirmed that the cells retained their undifferentiated state (Fig. 5b and Supplementary Fig. S6a). RT–PCR analysis of H9 hESCs and 253G1 iPSCs

Table 1 | Summary of karyotypic analysis.

Cell line	Medium	Colony culture	Matrigel	LM332-E8	LM511-E8
H9	mTeSR1	Normal*	Normal*	Normal	Normal*
iPS (IMR90)-1	mTeSR1	Normal*	Normal*	Normal*	Normal*
HES3	mTeSR1	Normal*	Normal*	Normal*	Normal
KhES-1	mTeSR1	46, xx, add(13)[1]	46, xx, add(13)[13]	Normal*	Normal*
253G1	mTeSR1	Normal	Normal	Normal	Normal
H9	StemPro	Normal*	Normal*	Normal*	Normal*
iPS (IMR90)-1	StemPro	Normal	Normal	Normal	Normal*
HES3	StemPro	Normal*	Normal	Normal*	Normal*
KhES-1	StemPro	Normal	Normal*	Normal*	Normal*
253G1	StemPro	Normal	Normal	Normal	Normal
H9	N2B27	Normal	Normal	Normal	Normal
iPS (IMR90)-1	N2B27	Normal*	Normal*	Normal*	Normal*
HES3	N2B27	Normal*	Normal	Normal*	Normal
KhES-1	N2B27	Normal	Normal	Normal	Normal
253G1	N2B27	Normal	Normal	Normal	Normal

The table lists the abnormalities found in 50 randomly selected G-banded cells from tenth passage cultures.
*Indicates that a single defect of one chromosome was observed, suggesting that it may have been due to technical/procedural issues.

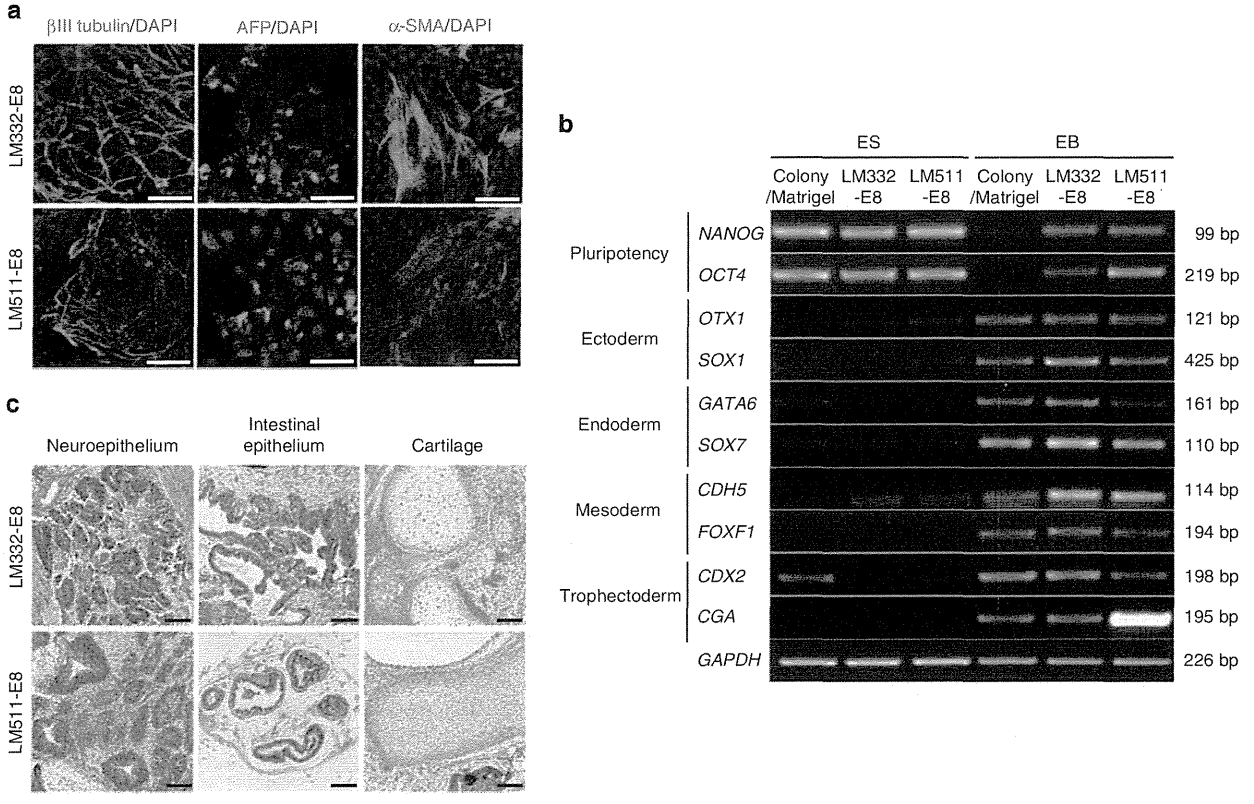

Figure 4 | LM-E8s sustain pluripotency in defined medium. (**a**) Immunostaining for markers of the three germ layers in differentiating H9 hESCs cultured on LM-E8s in mTeSR1 medium after 10 passages: ectoderm (βIII tubulin), endoderm (alpha-fetoprotein (AFP)), and mesoderm (α-smooth muscle actin (SMA)). (**b**) RT-PCR analysis of marker genes for the differentiation of embryoid bodies. H9 hESCs were cultured in mTeSR1 medium for 10 passages on LM-E8s after complete dissociation, or on Matrigel after colony dissociation. Cells were then allowed to form embryoid bodies for 10 days. (**c**) Teratomas consisting of the three germ layers developed following the transfer of H9 hESCs cultured on LM-E8s after 25 passages in mTeSR1 medium. Cells were injected into the testes of SCID mice. After 8 weeks, the fate of the cells was analysed. Haematoxylin and eosin staining showed differentiation into various tissues including neuroepithelium (ectoderm), intestinal epithelium (endoderm) and cartilage (mesoderm). Scale bars, 200 μm.

cultured on LM-E8s after complete dissociation showed that the expression of pluripotency markers, including *NANOG* and *OCT4*, remained high, whereas the expression of differentiation lineage markers was suppressed (Fig. 5c and Supplementary Fig. S6b). H9 hESCs and 253G1 iPSCs had a normal karyotype after 35 and 10 serial passages, as indicated by G-band analysis (Supplementary Fig. S7a–d), and M-FISH analysis corroborated these findings (Supplementary Fig. S7e,f). In addition, we

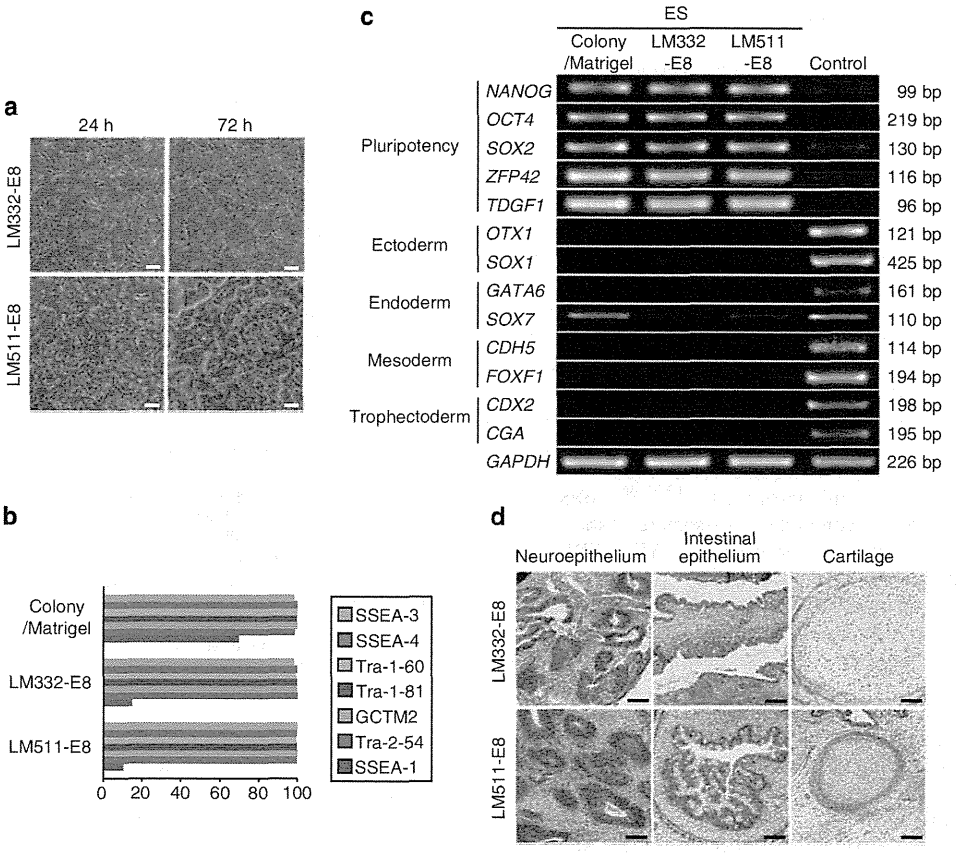

Figure 5 | LM-E8s sustain the pluripotency in xeno-free TeSR2 medium. (**a**) Phase-contrast images of H9 hESCs. Dissociated H9 hESCs in TeSR2 medium reformed clusters (24 h) and actively proliferated (72 h). (**b**) Flow cytometric profiles of H9 hESCs cultured on LM-E8s for 34 passages after complete dissociation, or on Matrigel for 30 passages after colony dissociation. H9 hESCs maintained expression of markers of the undifferentiated state. Numbers indicate percentages of cells that were positive for surface markers. (**c**) RT-PCR analysis of the expression of pluripotency marker genes in H9 hESCs cultured in TeSR2 medium. H9 hESCs cultured for 30 passages on LM-E8s after complete dissociation maintained high-level expression of pluripotency marker genes, while differentiation lineage marker genes were suppressed. Embryoid bodies derived from H9 hESCs cultured as colonies in mTeSR1 medium were used as the control to assess differentiation. (**d**) Teratomas consisting of the three germ layers developed from H9 hESCs cultured on LM-E8s for 34 passages in TeSR2 medium after complete dissociation. Cells were injected into the testes of SCID mice. After 8 weeks, the fate of the cells was analysed. Haematoxylin and eosin staining showed differentiation into various tissues including neuroepithelium (ectoderm), intestinal epithelium (endoderm) and cartilage (mesoderm). Scale bars, 200 μm.

confirmed the pluripotency of hESCs cultured on LM-E8s by their ability to form teratomas comprising all three germ layers (Fig. 5d).

Discussion

Stem cell–ECM interactions are important for maintaining stem cell adhesion, survival and self-renewal both *in vivo* and *in vitro*. The development of an ECM that sustains hESC and hiPSC cultures still requires substantial effort, particularly to avoid the use of feeder cells. In this study, we have demonstrated that LM-E8s support stronger adhesion of hESCs and hiPSCs than do other substrates, including Matrigel and intact laminin-511. We showed that LM-E8s enabled rapid and steady expansion of these cells. Furthermore, the cells cultured on LM-E8s for more than 30 passages in defined xeno-free media maintained an undifferentiated state and retained a normal karyotype and pluripotency. The adhesion of these cells to LM-E8s is primarily α6β1 integrin-dependent, because a function-blocking antibody against α6β1 integrin strongly inhibits the cell adhesion. Furthermore, LM511(EQ), a mutant LM-E8 that cannot bind to α6β1 integrin owing to a single amino-acid substitution[29], does not support the adhesion of hESCs. Thus, we believe that the interaction of LM-E8s with α6β1 integrin is the principal route for binding to hESCs and promotion of proliferation in an undifferentiated state.

Although laminin-511 was shown to be a potential substrate for feeder-free culture of hESCs, intact laminin-511 is a large heterotrimeric protein of 780 kDa (Fig. 1a) and requires three independent transfections of expression vectors encoding the α, β and γ chains, each of which is larger than 10 kb, for recombinant protein production. It is therefore an arduous protocol to produce recombinant laminin-511 in sufficient quantities for the clinical application of hESCs. Here, we have shown that LM-E8s, the minimum structure harbouring the full integrin-binding activity of laminins, are superb substrates for the long-term culture of hESCs, with a substantial advantage over intact laminin-511 and laminin-332. LM-E8s are much smaller and easier to produce recombinantly than intact laminins. Indeed, the smaller size of LM-E8s significantly improves recombinant protein expression, allowing LM-E8s to yield more than five times more recombinant protein than intact laminin-511 (see Methods). In addition, LM-E8s can be easily purified using 6xHis- and FLAG-tags attached to the α and γ chains, respectively (Fig. 1a). This method reduces the time required