Biochemical and Biophysical Research Communications xxx (2013) xxx–xxx

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 xxxx

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 bFGF. Most defined/xeno-free media for hESCs contain basic fibroblast growth factor (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 Published by Elsevier Inc.
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.

Compounds, including small molecules from the Prestwick Chemical library, were added to hESC-BM at the indicated concentrations in the presence of 0.1% DMSO. Ethopropazine, promazine, retinoic acid (RA), 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 60 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. 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 1.

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-score 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.

Please cite this article in press as: H. Kumagai et al., Identification of small molecules that promote human embryonic stem cell self-renewal, Biochem. Biophys. Res. Commun. (2013), http://dx.doi.org/10.1016/j.bbrc.2013.03.061