Analysis of essential pathways for self-renewal in common marmoset embryonic stem cells

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ABSTRACT

Common marmoset (CM) is widely recognized as a useful non-human primate for disease modeling and preclinical studies. Thus, embryonic stem cells (ESCs) derived from CM have potential as an appropriate cell source to test human regenerative medicine using human ESCs. CM ESCs have been established by us and other groups, and can be cultured in vitro. However, the growth factors and downstream pathways for self-renewal of CM ESCs are largely unknown. In this study, we found that basic fibroblast growth factor (bFGF) rather than leukemia inhibitory factor (LIF) promoted CM ESC self-renewal via the activation of phosphatidylinositol-3-kinase (PI3K)-protein kinase B (AKT) pathway on mouse embryonic fibroblast (MEF) feeders. Moreover, bFGF and transforming growth factor β (TGFβ) signaling pathways cooperatively maintained the undifferentiated state of CM ESCs under feeder-free condition. Our findings may improve the culture techniques of CM ESCs and facilitate their use as a preclinical experimental resource for human regenerative medicine.

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1. Introduction

Human regenerative medicine, including transplantation of various functional cells differentiated from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), is considered to have great potential for treating various incurable diseases, and has thus attracted much public attention. However, preclinical studies using animal disease models are required to evaluate the efficacy and safety of ESC/iPSC-derived cells prior to their clinical application. Common marmoset (CM, Callithrix jacchus) has recently been recognized as a useful non-human primate for such studies, because of its small size, high reproductive capacity, and genetic similarity to humans [1].

Understanding the molecular mechanisms governing the self-renewal of ESCs is important for the development of technologies to differentiate them into functional cells. Although both human and mouse ESCs are able to self-renew on feeder cells in vitro, their growth factor requirements for self-renewal are different. Basic fibroblast growth factor (bFGF), which activates phosphatidylinositol-3-kinase (PI3K)-protein kinase B (AKT) [2,3] and mitogen-activated protein/extracellular signal-regulated kinase (MEK)-extracellular signal-regulated kinase (ERK) pathways [2–8], and transforming growth factor β (TGFβ) leading to the activation of mothers against decapentaplegic homolog 2/3 (SMAD2/3) [2,6–11], maintain the self-renewal of human ESCs and mouse embryonic fibroblast stem cells (EpiSCs). Conversely, in mouse ESCs, leukemia inhibitory factor (LIF), which activates Janus kinase (JAK)-signal transducer and activator of transcription 3 (STAT3) and PI3K-AKT pathways, is known to play important roles in maintaining self-renewal [12–14].
ESCs derived from CM have been established by us and others [15–17]. However, the growth factors used in the culture medium are different among reports [15,17–21]. Thus, the most appropriate growth factor and its downstream pathway for maintaining the self-renewal of CM ESCs still remain to be determined.

In the present study, we characterized two CM ESC cell lines, Cj11 and CM40, and found that CM ESCs were more similar to human ESCs rather than mouse ESCs in terms of their growth factor requirement and molecular signaling pathways for self-renewal.

2. Materials and methods

2.1. CM ESC culture on mouse embryonic fibroblasts (MEFs)

CM ESC lines, CM40 and Cj11, were maintained in CM ESC medium as described before [15] with or without 1:1000 LIF (Wako, Osaka, Japan), 5 ng/ml bFGF (PeproTech, NJ, USA), 5 μM PD0325901 (MEK inhibitor, Wako) or 10 μM LY294002 (PI3K inhibitor, Santa Cruz Biotechnology, USA). CM40 cell line was established in our laboratory [15], and Cj11 cell line was obtained from WiCell Research Institute [16]. MEFs were prepared from 13.5 dpc embryos from ICR mice (Charles River, Japan) using established procedures [22].

2.2. CM ESC culture under feeder-free conditions

CM40 and Cj11 ESC lines were cultured on Matrigel (BD Biosciences, CA, USA)-coated dishes in Essential 8 medium (Life Technologies, NY, USA) or Essential 6 medium (Life Technologies) with or without 1:1000 LIF (Wako), 100 ng/ml bFGF (PeproTech), 2 ng/ml TGFβ (PeproTech), 5 μM PD0325901 (MEK inhibitor, Wako), 10 μM LY294002 (Santa Cruz Biotechnology).

2.3. CM ESC differentiation

Undifferentiated ESCs were detached from the feeder cells by treatment with 0.25% trypsin (NacalaiTesque, Kyoto, Japan) for 1 min. The collected colonies were processed for embryo body (EB) formation assay in CM ESC medium on low cell-binding 12-well plates (Nalge Nunc International Kk, Japan) for 4 or 8 days. Detailed protocols to differentiate CM ESCs into three germ layers are described in “Supplementary Materials and Methods”.

2.4. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS) (NacalaiTesque), permeabilized with 0.3% Triton X-100/PBS, blocked with staining buffer (2% fetal bovine serum (FBS)/PBS), the primary antibodies used are shown in Supplemental Table 1. Nuclei were counterstained with DAPI. Images were obtained using a LAS3000 (Fujifilm, Japan). Band intensities were measured by ImageJ software (NIH).

2.5. Flow cytometry (FCM)

CM ESCs were fixed in 4% PFA/PBS, permeabilized with 0.3% Triton X-100/PBS, blocked with staining buffer (2% FBS/PBS), and then incubated with an anti-OCT3/4 antibody (Santa Cruz Biotechnology, sc-5279 or sc-8628). The cells were detected on a FACSVerse flow cytometer (Becton Dickinson, USA), followed by data analysis using FlowJo software (Tommy Digital Biology, Japan).

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using an RNeasy Mini Kit (Qiagen, USA), and cDNA was synthesized using Superscript III reverse transcriptase (Life Technologies). Then PCR was carried out using the synthesized cDNA as templates and gene-specific primers (see Supplemental Table 2). The primers were designed based on different exons to span the intervening intron and avoid amplification of contaminating genomic DNA.

2.7. Western blotting

Cells were incubated on ice with RIPA buffer containing protease inhibitors (Complete Mini, EDTA-free; Roche, Basel, Switzerland) and a phosphatase inhibitor cocktail (NacalaiTesque). The cell lysates were then resolved by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting. The primary antibodies used are shown in Supplementary Table 3. The signals were detected using a LAS3000 (Fujifilm, Japan). Band intensities were measured by ImageJ software (NIH).

2.8. Statistical analysis

Unless otherwise noted, inter-group differences were analyzed using analysis of variance (ANOVA) followed by the Tukey's post-hoc test with GraphPad Prism 5 (GraphPad Software, CA, USA).

3. Results

3.1. bFGF promotes self-renewal of CM ESCs on feeder cells

bFGF and LIF have been reported to be essential for the maintenance of human and mouse ESCs, respectively [3,12–14,23–27], and either or both of these growth factors were considered to be required for the maintenance of CM ESCs. To determine the optimal condition for culturing CM ESCs, we first examined the expression of receptors for bFGF (FGFR1, FGFR2, FGFR3, and FGFR4) and LIF (LIFR and gp130). RT-PCR analysis demonstrated that all of these receptors were expressed in the CM ESCs (Fig. 1A), suggesting that both growth factors play important roles in the biology of CM ESCs.

In culture, ESCs are generally known to spontaneously differentiate. However, the addition of appropriate growth factors inhibits such spontaneous differentiation. To evaluate the effects of bFGF and LIF on the proliferation and differentiation of CM ESCs in vitro, we passaged CM ESCs at a ratio of 1:3 every three days for three passages, and then counted the numbers of undifferentiated OCT3/4+ cells. We found that the proportion of OCT3/4+ cells was unchanged regardless of the addition of bFGF or LIF (Fig. 1B). However, the numbers of OCT3/4+ cells were significantly increased by the addition of bFGF, but not LIF, compared with those of controls cultured without bFGF and LIF (Fig. 1C). Similar results were obtained when the cells were cultured for more than ten passages (Supplemental Fig. S1 and data not shown). The above experiments were performed using CM40 cell line, and similar results were obtained with Cj11 cell line (Supplementary Fig. S2). These results strongly suggest that bFGF promotes the proliferation of CM ESCs rather than maintaining the undifferentiated state of CM ESCs.

3.2. bFGF-PI3K-AKT pathway supports self-renewal of CM ESCs on feeder cells

bFGF and its downstream PI3K-AKT and MEK-ERK pathways are important for the self-renewal of human ESCs [2,3,5,6]. We therefore examined whether these pathways were activated by bFGF for CM ESC self-renewal on feeder cells. CM ESCs were cultured overnight in medium lacking knockout serum replacement (KSR) and any growth factors. Then, we added bFGF (5 ng/ml), and examined the activation of AKT and ERK1/2 in the cells by Western blotting.
The results showed that the band intensity of phosphorylated AKT was significantly increased after the treatment with bFGF, while that of phosphorylated ERK1/2 was not changed (Fig. 2A and B). These data suggested that PI3K-AKT, but not MEK-ERK, pathway was activated by bFGF in CM ESCs under feeder-dependent culture condition.

Next, to examine whether bFGF-PI3K-AKT pathway plays any roles in the maintenance of self-renewal of CM ESCs, the cells were cultured in medium containing bFGF in the presence or absence of the PI3K inhibitor, LY294002. We found that the proportion of OCT3/4+ cells was maintained at approximately 90% for at least three passages when the cells were cultured without LY294002, whereas it was gradually decreased when the cells were cultured with LY294002 (day0, 96.75 ± 2.83% vs. day9, 57.97 ± 16.76%, Fig. 2C). In addition, OCT3/4 cell proliferation was inhibited in the presence of LY294002 (day9, bFGF, 7.61 ± 1.59 × 10⁶ cells vs. bFGF+LY294002, 2.36 ± 1.25 × 10⁶ cells, Fig. 2D). Additionally, even when bFGF was not added, the proportion of OCT3/4+ cells was significantly reduced by the treatment with LY294002 (Fig. 2C), indicating that PI3K-AKT pathway is activated by unknown factors from MEFs and play roles for self-renewal of CM ESCs. Overall, these results strongly suggest that bFGF-PI3K-AKT pathway is essential for the self-renewal of CM ESCs under feeder-dependent culture condition.

To examine the expression of OCT3/4, we used an antibody against amino acids 1–134 of human OCT3/4 (monoclonal OCT3/4 antibody, sc-5279) that was known to be useful for detecting the expression of CM OCT3/4 [28]. And recent study reported that another antibody raised against amino acids 1–19 of human OCT3/4 (polyclonal OCT3/4 antibody, sc-8628) was more useful to detect ESC-specific OCT3/4 [29]. Thus we performed immunocytochemistry and FCM analysis using sc-8628, and obtained the similar results (Supplementary Fig. S3).

3.3. bFGF and TGFβ signaling cooperate to maintain the undifferentiated state of CM ESCs under feeder-free conditions

All of the experiments described above were performed with feeder support. Thus, the various secreted factors including cytokines and adhesion molecules might have affected the results. To examine the dependency of CM ESCs on feeder cells, CM ESCs were cultured on a high or low density of feeder cells, and then the undifferentiated state was examined by immunocytochemistry using an anti-NANOG antibody (Supplementary Fig. S1). We found that CM ESCs on low-density feeder cells lost their expression of NANOG after four passages, whereas those on high-density feeder cells maintained NANOG expression even after ten passages (Supplementary Fig. S1). Therefore, it is conceivable that the self-renewal of CM ESCs is maintained by unknown factors derived from feeder cells.

Chen et al. showed that Essential 8 medium (Dulbecco’s modified Eagle’s medium/F12 supplemented with l-ascorbic acid-2-phosphate magnesium, insulin, transferrin, sodium selenium, NaHCO₃, bFGF, and TGFβ) supports the self-renewal of human ESCs and iPSCs under feeder-free conditions [30]. To clarify the essential growth factors required for maintaining the undifferentiated state of CM ESCs, CM ESCs were cultured under feeder-free condition. We found that CM ESCs could be cultured on Matrigel in Essential 8 medium without feeder support, although they could not be maintained for more than three passages (data not shown). Next, we cultured CM ESCs on Matrigel in Essential 6 medium lacking bFGF and TGFβ overnight, and then the activation of signaling pathwa...
pathways known to maintain mouse and human ESCs (bFGF-PI3K-AKT, bFGF-MEK-ERK, TGFβ-SMAD2/3, and LIF-JAK-STAT3 pathways) were analyzed by Western blotting after the addition of bFGF, TGFβ, or LIF to the medium. We found that phosphorylation of AKT and ERK was increased by the addition of bFGF, while it was decreased by the treatment with LY294002 or PD0325901, suggesting that both of AKT and ERK were activated downstream of bFGF under feeder-free condition. And the addition of TGFβ resulted in an increase of phosphorylated SMAD2/3 (Fig. 3A and B). Moreover, the addition of LIF resulted in an increase of phosphorylated STAT3, suggesting that STAT3 was activated downstream of LIF (Supplementary Figs. S4B and D). This observation indicates that the addition of both TGFβ and bFGF is the most appropriate growth factor combination for maintenance of the undifferentiated state of CM ESCs under feeder-free condition, which is similar to a characteristic of mouse ESCs [2,6,9–11].

3.4. CM ESCs show phenotypes similar to those of human ESCs and mouse EpiSCs

Human ESCs and mouse EpiSCs share a number of similar phenotypes as shown in Table 1 [7,8]. CM ESCs formed flattened colonies and expressed NANOG as well as markers for both mouse EpiSCs and human ESCs, such as T, CER1, EOMES, FOXA2, GATA6, and SOX17 (Supplementary Figs. S5 and S6A) [7]. Moreover, bFGF and TGFβ signalings play crucial roles in maintaining the undifferentiated state of human ESCs and mouse EpiSCs [2,7,8,11,30], and the same roles of these signaling pathways were also found in CM ESCs (Fig. 3C and D).

Previous reports have shown that apoptosis of human ESCs and mouse EpiSCs is induced by culturing after complete dissociation [31,32]. Watanabe et al. showed that dissociation-induced apoptosis of human ESCs is suppressed by treatment with the Rho-associated kinase (ROCK) inhibitor Y27632 [33]. To examine whether dissociation-induced apoptosis of human ESCs and mouse EpiSCs was similarly found in CM ESCs, colonies of CM ESCs were dissociated into single cells by trypsinization, and then the cells were plated on Matrigel-coated dishes with or without Y27632. Compared with untreated controls, we found that Y27632-treated CM ESCs produced...
significantly more colonies, suggesting that dissociation-induced apoptosis of CM ESCs occurred and was suppressed by Y27632 (Supplementary Figs. S6B and S6C). Thus, we concluded that CM ESCs are similar to human ESCs and mouse EpiSCs.

### 4. Discussion

Recent advances in the field of basic research for pluripotent stem cells such as the generation of ESCs, iPSCs and stimulus-trig-
LIF has been widely used to establish and maintain non-human primate ESCs [15, 17, 18, 39–42], although some researchers claim that LIF cannot maintain the self-renewal capacity of these cells [16, 41–43]. We found that LIF did not affect the capacity for self-renewal of CM ESCs (Figs. 1 and 3), although it activated the JAK-STAT3 pathway (Supplementary Fig. S3). More extensive studies are needed to further explore the roles of the LIF-JAK-STAT3 pathway in CM ESCs. In our previous report, the expression of LIFR was not found in undifferentiated CM ESCs [15], but it was found in this study after repetitive experiments (Fig. 1A). This discrepancy was considered to be caused by the detection threshold of this study after repetitive experiments (Fig. 1A). This discrepancy was considered to be caused by the detection threshold of this study after repetitive experiments (Fig. 1A).

We also found that the self-renewal of CM ESCs cultured on feeder cells was remarkably promoted by bFGF, which is similar to the characteristic of human ESCs (Fig. 1). However, even in the absence of bFGF, most CM ESCs could be maintained in an undifferentiated state by culture on feeder cells, although they showed slower growth compared to those cultured in bFGF containing medium (Fig. 1B and C). This observation indicates that growth factors secreted from feeder cells such as activin, noggin and bFGF, maintain growth compared to those cultured in bFGF containing medium (Fig. 1B and C). This observation indicates that growth factors secreted from feeder cells such as activin, noggin and bFGF, maintain this state by culture on feeder cells, although they showed slower growth compared to those cultured in bFGF containing medium (Fig. 1B and C). This observation indicates that growth factors secreted from feeder cells such as activin, noggin and bFGF, maintain growth compared to those cultured in bFGF containing medium (Fig. 1B and C). This observation indicates that growth factors secreted from feeder cells such as activin, noggin and bFGF, maintain the undifferentiated state of CM ESCs [44, 45]. Indeed, CM ESC colonies cultured on low-density feeder cells differentiated within four passages (Supplementary Fig. S1).

Previous studies have demonstrated the critical roles of PI3K-AKT and MEK-ERK pathways in the self-renewal of human ESCs [2–6]. Our results showed that AKT, but not ERK1/2, was activated by the addition of bFGF (5 ng/ml), while ERK1/2 was continuously activated even in the absence of bFGF on feeder support (Fig. 2A). Moreover, inhibition of either MEK-ERK or PI3K-AKT pathways resulted in reduced self-renewal of CM ESCs (Fig. 2 and Supplementary Fig. S7). Therefore, activation of the PI3K-AKT pathway downstream of bFGF as well as the MEK-ERK pathway by unknown mechanisms is required for self-renewal of CM ESCs on feeder support. On the other hand, both AKT and ERK1/2 were activated by the addition of bFGF (100 ng/ml) under feeder-free condition (Fig. 3A and B). And treatment with LY294002 resulted in the elevated expression of endoderm and mesoderm markers, and treatment with PD0325901 caused the reduced expression of these markers, indicating that modulation of these pathways affects the differentiation process in CM ESCs (Supplementary Fig. S8). We are now extensively investigating the effect of these inhibitors on the differentiation process of CM ESCs induced by the treatment with specific cytokines and EB formation assay.

Several studies have demonstrated differences in the mechanisms of ESC self-renewal between mice and humans. Mouse ESCs require LIF for their self-renewal, whereas human ESCs require bFGF and TGFβ. Mouse EpiSCs originating from post-implantation embryos depend on bFGF and TGFβ, and show characteristics similar to those of human ESCs originating from the inner cell mass of blastocysts as shown in Table 1 [7, 8, 10]. Mouse EpiSCs are therefore considered to be the counterpart of human ESCs. In this study, we demonstrated that CM ESCs were very similar to human ESCs and mouse EpiSCs in terms of their morphology, gene expression, growth factor dependency for self-renewal, and vulnerability to single cell dissociation.

Our findings strongly suggest that CM ESCs are phenotypically similar to human ESCs. Therefore, CM ESCs may facilitate the development of valuable preclinical experimental systems to test new therapeutic modalities for incurable human diseases, particularly in the field of regenerative medicine.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [http://dx.doi.org/10.1016/j.fob.2014.02.007](http://dx.doi.org/10.1016/j.fob.2014.02.007).

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