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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 are expected as an appropriate cell source to test human regenerative medicine using human ESCs. CM ESCs has been established by us and other groups, and known to 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 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.

Keywords

Embryonic stem cells

Common marmoset

bFGF

TGFβ

Self-renewal

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, *Callithrixjacchus*) 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 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 epiblast 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, CA, 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 embryoid 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 Supplementary Table 1. Nuclei were counterstained with DAPI. Images were obtained under a fluorescence microscope (Axiovert 135M; Carl Zeiss, Germany, or BZ-9000; Keyence) and then analyzed by Axiovert software (Carl Zeiss) or BZ-Analyzer software (Keyence).

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 (Tomy 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 (Supplementary 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 (Figs. 2A and 2B). 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±1.59×10^6 cells vs. bFGF+LY294002, 2.36±1.25×10^6 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, $NaHCO_3$, 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 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 (Figs. 3A and 3B). And the addition of TGFβ resulted in an increase of phosphorylated SMAD2/3 (Figs. 3A and 3B), suggesting that SMAD2/3 was activated downstream of TGFβ. Moreover, the addition of LIF resulted in an increase of phosphorylated STAT3, suggesting that STAT3 was activated downstream of LIF (Supplementary Figs. S4B and S4D). These results suggested that bFGF-PI3K-AKT, bFGF-MEK-ERK, TGFβ-SMAD2/3 and LIF-JAK-STAT3 pathways known to regulate self-renewal of human or mouse ESCs were activated in CM ESCs under feeder-free condition. It should be noted that ERK was not activated by 5 ng/ml of bFGF that was used for the culture on feeder cells as described in Figs. 2A and 2B (Supplementary Fig. S4B), but it was remarkably activated by 100 ng/ml of bFGF generally used for feeder-free culture of human ESCs (Figs. 3A and 3B) [30].

Next, to determine the growth factors maintaining the undifferentiated state of CM ESCs under feeder-free condition, CM ESCs were cultured in the feeder-free system with various combinations of growth factors, followed by analysis of their undifferentiated state morphologically and immunocytochemically. Most of the colonies cultured in Essential 6 medium with bFGF, bFGF+TGFβ, bFGF+LIF, or bFGF+TGFβ+LIF showed a well-packed appearance, and a majority of the cells expressed NANOG (Fig. 3C). In contrast, most of the

colonies cultured in Essential 6 medium with TGFβ, LIF, or TGFβ+LIF showed an unpacked appearance, and a majority of the cells did not express NANOG (Fig. 3C). Moreover, $NANOG^+$ and well-packed colonies were found at the highest proportion (98.00±0.88%) when the cells were cultured in the presence of TGFβ+bFGF (Fig. 3D). In addition, almost all of the colonies were positive for OCT3/4, SOX2, SSEA-4, TRA1-60 and TRA1-81 (Supplementary Fig. S3), and these colony forming cells kept the capability of differentiating three lineages (Supplementary Fig. S5). 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 human 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. S1 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 (Figs. 3C and 3D).

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-triggered acquisition of pluripotency (STAP) cells have given us realistic expectations for human regenerative medicine [22,34-38]. And the need for the development of methods to test new therapeutic approach using such cells is increasing. CM is a useful experimental animal that can suit such needs, and therefore, the characterization of CM ESCs is important. In this study, we investigated essential signaling pathways for the self-renewal of CM ESCs under feeder-dependent and feeder-free culture conditions.

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 RT-PCR under different conditions, particularly PCR primers used in our previous report were human LIFR sequence-originated because there were no available marmoset genomic sequence data.

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 (Figs. 1B and 1C). 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 (Figs. 3A and 3B). 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 cells 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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Figure legends

Fig.1 bFGF promotes self-renewal of CM ESCs in the presence of feeder support.

(A) RT-PCR analysis showing the expression of FGFR1, FGFR2, FGFR3, FGFR4, LIFR, and gp130 genes in CM ESCs (CM40). (B) No effect of bFGF on the proportion of OCT3/4$^+$ cells. CM ESCs (CM40; 1.4×10^5) were seeded on mitomycin C (MMC)-treated MEFs and cultured with LIF (open square), bFGF (open circle), or without growth factors (control; open triangle). The percentage of OCT3/4$^+$ cells was determined by FCM. (C) Enhancement of undifferentiated CM ESC growth by bFGF. CM ESCs (CM40; 1.4×10^5) were seeded on mitomycin C (MMC)-treated MEFs and cultured with LIF (open square), bFGF (open circle), or without growth factors (control; open triangle). The number of cells was then counted by trypan blue exclusion. The number of OCT3/4$^+$ cells was determined by multiplying the number of cells by the percentage of OCT3/4$^+$ cells and the passage ratio together. Data are shown as the mean±SD (n=4). $^{**}P<0.01$ (bFGF vs. control) and $^{\dagger\dagger}P<0.01$ (bFGF vs. LIF). Note that all of PCR in (A) was performed with 30 cycles, and no bands were detected for FGFR expression in MEFs in (A), however, they were faintly done when PCR was performed with 40 cycles.

Fig. 2 bFGF-PI3K-AKT pathway supports self-renewal of CM ESCs.

(A) Western blot analysis showing the activation of AKT by bFGF in CM ESCs. CM40 cells were starved of bFGF and KSR overnight, and then stimulated with 5 ng/ml of bFGF for the indicated durations. AKT, ERK1/2 and α-Tubulin are shown as loading controls. The relative band intensities of p-AKT/AKT and p-ERK/ERK are shown in (B). Band intensities were measured by ImageJ software. Data are shown as the mean±SD. The Student's t-test was used to test inter-group differences. $^{**}P<0.01$. (C) Inhibition of self-renewal by LY294002. CM ESCs

(CM40; 1.4×10^5) were seeded on MMC-treated MEFs and cultured in medium containing bFGF (open circle), control medium (open triangle), bFGF+LY294002 (closed circle) or LY294002 (closed triangle). The percentage of $OCT3/4^+$ cells was then determined by FCM at the indicated day as shown in (C). The number of live cells was counted by trypan blue exclusion. Growth curves were generated by multiplying the number of live cells by the percentage of $OCT3/4^+$ cells and passage ratio together as shown in (D). Data are shown as the mean±SD. bFGF, n=4; control, n=4; bFGF+LY294002, n=3; LY294002, n=3; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$, bFGF vs. control; $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.005$, bFGF+LY294002 or LY294002 vs. control.

Fig. 3　bFGF and TGFβ maintain the undifferentiated state of CM ESCs under feeder-free conditions.

(A) Western blot showing the activation of AKT, ERK1/2 and SMAD2/3 by 100 ng/ml of bFGF or 2 ng/ml of TGFβ for 30 min in CM ESCs. CM40 were starved of growth factors overnight and then pre-treated with LY294002 or PD0325901for 1 h before stimulation with bFGF. AKT, ERK1/2, SMAD2/3, and α-Tubulin are shown as loading controls. The relative band intensities of p-AKT/AKT, p-ERK/ERK and p-SMAD2/3/SMAD2/3 are shown in (B). Band intensities were measured by ImageJ software. Data are shown as the mean±SD. One-way ANOVA followed by the Tukey's post-hoc test was used to test inter-group differences. $^{**}P<0.01$ and $^{***}P<0.005$. (C) Immunocytochemical analyses of NANOG expression in CM ESCs (CM40) cultured with various growth factors for 4 days. Merged images of NANOG (red) and nuclei (DAPI; blue) are shown. Scale bars represent 200 μm. NANOG expression in cells cultured without any growth factors is shown as a control. (D) Proportion of $NANOG^+$ colonies. The percentage of $NANOG^+$ colonies cultured with various growth factors was analyzed by immunocytochemistry. For

statistical analyses, 300 colonies were examined in each experiment (n=3). Data are shown as the mean±SD. One-way ANOVA followed by the Tukey's post-hoc test was used to test inter-group differences. $^{***}P<0.005$. The difference between bFGF- and bFGF+TGFβ-treated cells was statistically analyzed using the Student's t-test. $^{\dagger\dagger}P<0.01$.

Table 1. The characters of mouse EpiSCs and mouse, human and CM ESCs.

		Mouse ESCs	Mouse EpiSCs	human ESCs	CM ESCs
Morphology of colony		small, dome	large, flat	large, flat	large, flat
Growth factor dependency	LIF	+	-	-	-
	bFGF	-	+	+	+
	TGFβ/Activin	-	+	+	+
Marker expression	NANOG	+	+	+	+
	OCT3/4	+	+	+	+
	T (brachyury)	-	+	+	+
	CER1	-	+	+	+
	EOMES	-	+	+	+
	FOXA2	-	+	+	+
	GATA6	-	+	+	+
	SOX17	-	+	+	+
Tolerance to single cell dissociation		+	-	-	-
Contribution in chimera		+	-	N/D	N/D

N/D = not determined.

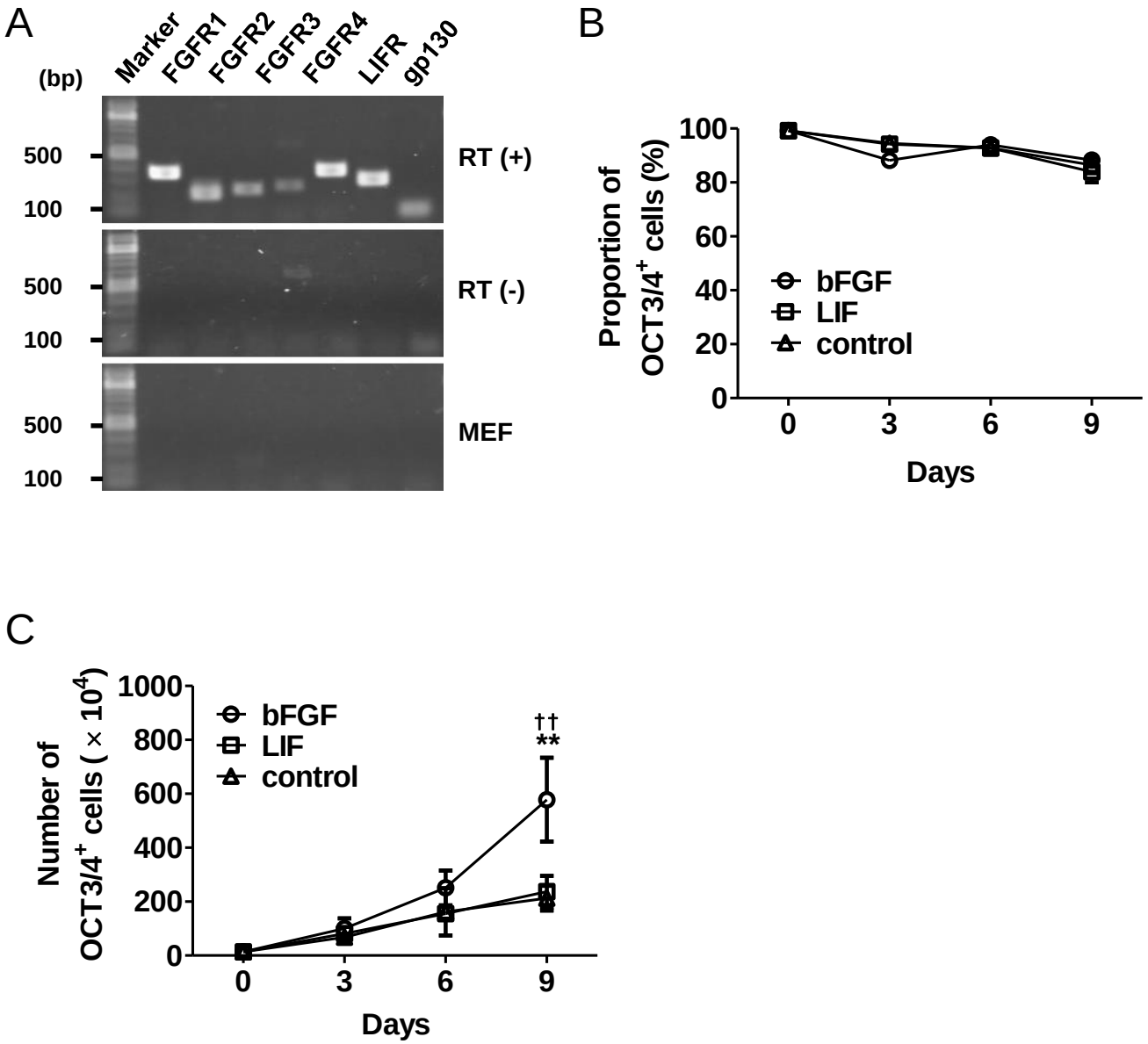
A
Marker
FGFR1
FGFR2
FGFR3
FGFR4
LIFR
gp130
(bp)
500
100
RT (+)
RT (-)
MEF
B
Proportion of OCT3/4+ cells (%)
100
80
60
40
20
0
bFGF
LIF
control
0
3
6
9
Days
C
Number of OCT3/4+ cells (× 10^4)
1000
800
600
400
200
0
bFGF
LIF
control
††
**
0
3
6
9
Days

Figure 1

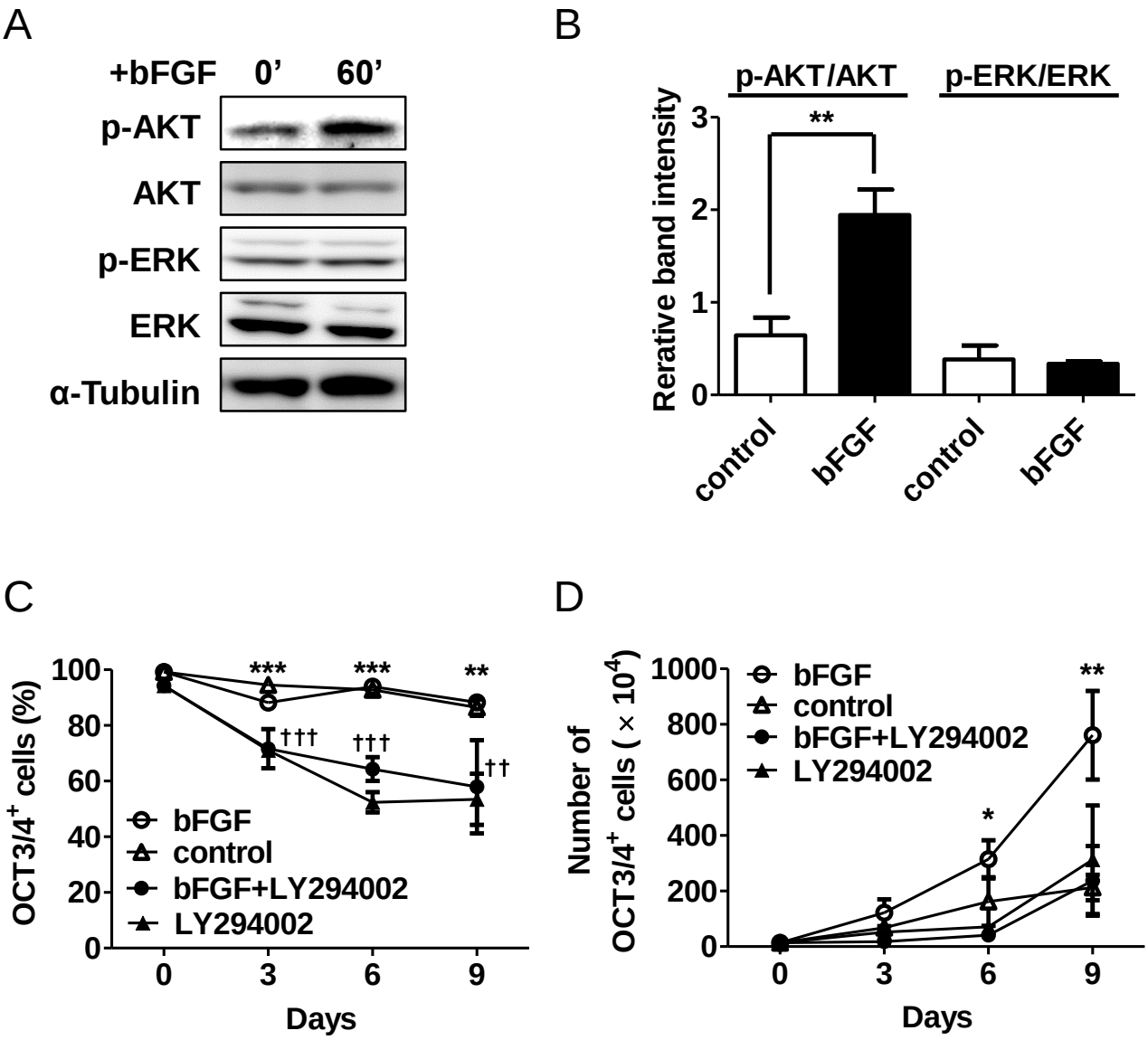
A
+bFGF
0’
60’
p-AKT
AKT
p-ERK
ERK
α-Tubulin
B
p-AKT/AKT
p-ERK/ERK
Rerative band intensity
**
control
bFGF
control
bFGF
C
Proportion of OCT3/4+ cells (%)
***
***
**
†††
†††
††
bFGF
control
bFGF+LY294002
LY294002
Days
D
Number of OCT3/4+ cells (× 10^4)
bFGF
control
bFGF+LY294002
LY294002
*
**
Days

Figure 2

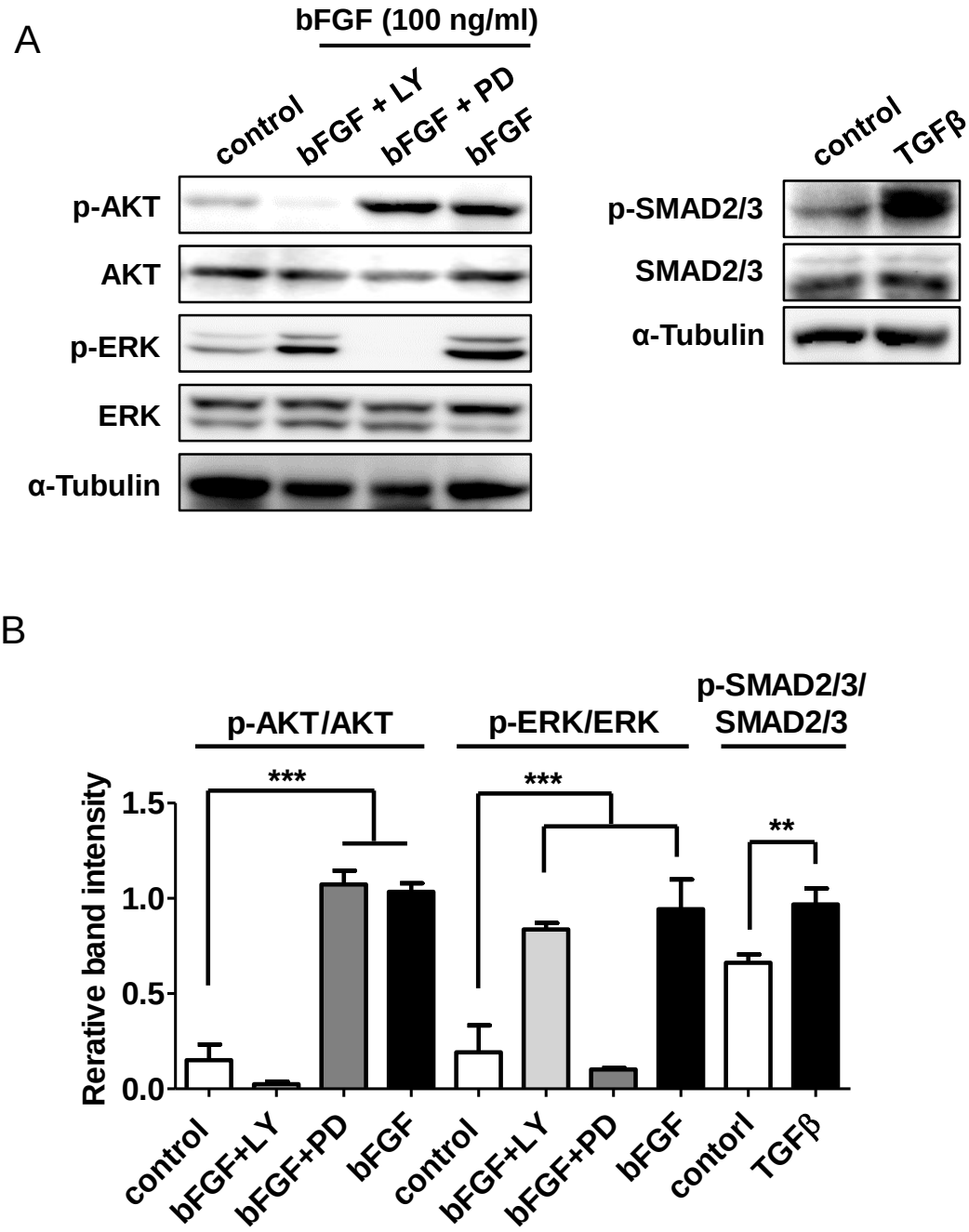
A
bFGF (100 ng/ml)
control
bFGF + LY
bFGF + PD
bFGF
p-AKT
AKT
p-ERK
ERK
α-Tubulin
control
TGFβ
p-SMAD2/3
SMAD2/3
α-Tubulin
B
p-AKT/AKT
p-ERK/ERK
p-SMAD2/3/
SMAD2/3
Rerative band intensity
1.5
1.0
0.5
0.0
***
***
**
control
bFGF+LY
bFGF+PD
bFGF
control
bFGF+LY
bFGF+PD
bFGF
contorl
TGFβ

Figure 3

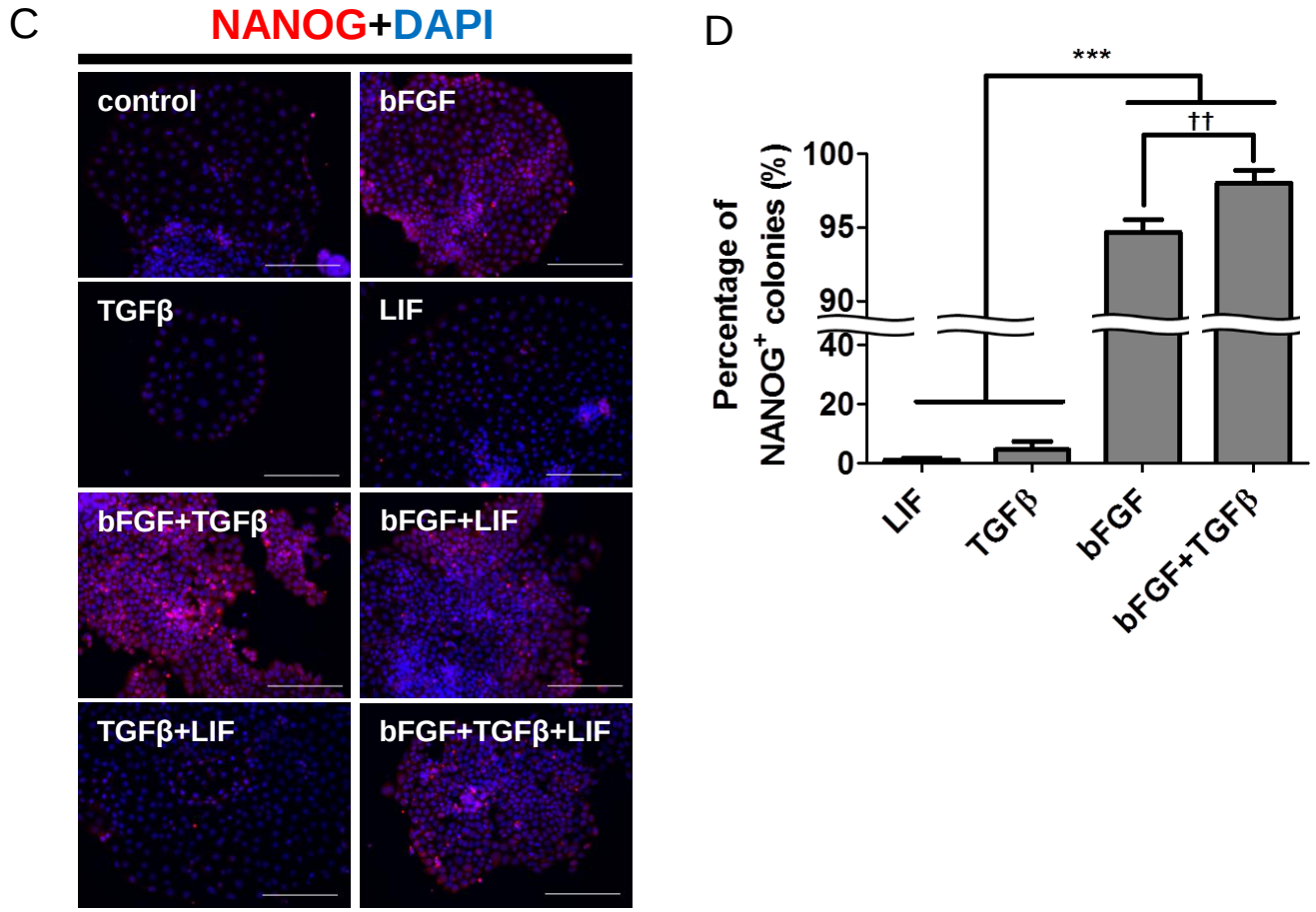
C
NANOG+DAPI
control
bFGF
TGFβ
LIF
bFGF+TGFβ
bFGF+LIF
TGFβ+LIF
bFGF+TGFβ+LIF
D
***
††
Percentage of NANOG+ colonies (%)
100
95
90
40
20
0
LIF
TGFβ
bFGF
bFGF+TGFβ

Figure 3-2