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Activin A Is Essential for Feeder-Free Culture of Human Induced Pluripotent Stem Cells

Minoru Tomizawa,[1*] Fuminobu Shinozaki,[2] Takao Sugiyama,[3] Shigenori Yamamoto,[4] Makoto Sueishi,[3] and Takanobu Yoshida[5]

[1]*Department of Gastroenterology, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan*

[2]*Department of Radiology, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan*

[3]*Department of Rheumatology, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan*

[4]*Department of Pediatrics, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan*

[5]*Department of Internal Medicine, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan*

ABSTRACT

Feeder-free culture of human induced pluripotent stem (hiPS) cells is necessary for their clinical application to avoid adverse effects of foreign proteins. hiPS cells were cultured with combinations of activin (A), CHIR99021 (C), basic fibroblast growth factor (F), and leukemia inhibitory factor (L) under feeder-free conditions. Culture was terminated after 12 passages or when the cell morphology changed from pluripotency. Pluripotency was analyzed by alkaline phosphatase (ALP) staining and immunostaining with antibodies to Oct3/4, Nanog, SSEA4, and TRA-1-60. SB431542 (SB), an activin inhibitor, was added to the culture, and the morphology of the cells was observed. hiPS cells cultured with A, AC, and ACL after 12 passages were positive for ALP staining. Oct3/4 was positive in hiPS cells cultured with A, AC, and ACL. hiPS cells were positive for Nanog when cultured with A and AC; however, Nanog signal was weaker in cells cultured with ACL. SSEA4 was positive in hiPS cells cultured with A and AC but almost negative in those cultured with ACL. hiPS cells were positive for TRA-1-60 when cultured with A, AC, and ACL. hiPS cells lose their undifferentiated morphology at six passages when cultured with A + SB, five passages with AC + SB, and nine passages with ACL. We conclude that feeder-free culture of hiPS cells requires A or AC to maintain pluripotency. J. Cell. Biochem. 114: 584–588, 2013.

KEY WORDS: PASSAGE; ALKALINE PHOSPHATASE STAINING; CHIR99012; LEUKEMIA INHIBITORY FACTOR; BASIC FIBROBLAST GROWTH FACTOR; SB431542

Human induced pluripotent stem (hiPS) cells are a promising cell source for regenerative medicine. Mouse fibroblasts are used as feeder cells for hiPS cells. hiPS cells are desirably free of proteins from other species [Meng et al., 2012] but not suitable for transplantation when cultured on mouse feeder cells because they are exposed to mouse proteins. Feeder-free culture conditions need to be developed. The mechanisms associated with the maintenance of pluripotency of iPS cells under feeder-free culture are not fully understood.

Activin A, a member of the transforming growth factor-β superfamily, mimics Nodal, binds activin receptors, and activates Smad2 by phosphorylation [Sulzbacher et al., 2009]. Once activated, Smad2 associates with Smad4, translocates to the nucleus, and regulates gene expression in concert with other transcription factors. Human ES (hES) cells rely on activin A [James et al., 2005]. Activin A regulates Nanog expression [Shin et al., 2011] and is therefore involved in pluripotency because Nanog regulates the

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*Correspondence to: Dr. Minoru Tomizawa, MD, PhD, Department of Gastroenterology, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan.
E-mail: nihminor-cib@umin.ac.jp

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ground-state pluripotency of hES cells [Miyanari and Torres-Padilla, 2012]. Basic fibroblast growth factor (bFGF) sustains self-renewal of hES cells [Xu et al., 2005].

CHIR99021, a glycogen synthase kinase-3β inhibitor, can produce hiPS cells from human embryonic keratinocytes with Oct3/4 and Klf4 [Li et al., 2009]. Mouse iPS cells are generated with Oct3/4 and CHIR99021 in the medium [Li et al., 2011]. Leukemia inhibitory factor (LIF) is necessary for mouse embryonic stem (mES) cells to maintain pluripotency [Hirai et al., 2011]. mES cells start differentiating upon withdrawal of LIF [Tomizawa et al., 2008].

Culture conditions, to maintain pluripotency under feeder-free conditions, are not clear for hiPS cells because there is a difference in self-renewal between mES and hES cells [Xu et al., 2005]. Activin and bFGF cooperate to maintain pluripotency of hiPS cells [Vallier et al., 2009b]. CHIR99021 and LIF improve the efficiency of generating hiPS cells with episomal vectors under feeder-free conditions [Yu et al., 2011]. Withdrawal of CHIR99021 and LIF induce differentiation of hiPS cells under feeder-free conditions [Hirano et al., 2012]. These reports suggest that cocktails of growth factors and small molecules may be solutions for feeder-free culture of hiPS cells.

We describe the search for suitable combinations of factors for feeder-free culture during the passage of hiPS cells with activin A, bFGF, CHIR99021, or LIF.

MATERIALS AND METHODS

CELL CULTURE

hiPS cells (201B7) (RIKEN Cell Bank, Tsukuba, Japan) were cultured on 6-well plates (Asahi Techno Glass, Tokyo, Japan) coated with Matrigel™ (Becton Dickinson, Franklin Lakes, NJ) in ReproFF (Reprocell, Yokohama, Japan) for feeder-free culture in 5% carbon dioxide at 37°C in a humidified chamber, and harvested with Accutase® (Innovative Cell Technologies, Inc., San Diego, CA) for experiments. We added 10 ng/ml of activin A (A) (R&D Systems, Inc., Minneapolis, MN), 2 μM of CHIR99021 (GSK-3 beta inhibitor) (C) (Wako Pure Chemicals, Osaka, Japan), 1,000 U/ml of human LIF (L) (Sigma Aldrich, St. Louis, MO) at, or 5 ng/ml of bFGF (Wako Pure Chemicals) was added to Dulbecco's minimum essential medium-F12 (Sigma Aldrich) supplemented with 20% Knockout Serum Replacement (Life Technologies, Grand Island, NY), 10% minimum essential amino acids (Life Technologies), 2 mM L-glutamine (Life Technologies), and 1 mM 2-mercaptoethanol (Sigma Aldrich) (iPSm[−]). Culture of hiPS cells was completed when they differentiated, disappeared, or reached 12 passages. SB431542 (SB), an activin inhibitor, was purchased from Wako Pure Chemicals, and 10 μM of it was added.

ALKALINE PHOSPHATASE STAINING

Alkaline phosphatase (ALP) staining was performed for cells cultured on 6-well plate (Asahi Techno Glass) coated with Matrigel™ and Leukocyte Alkaline Phosphatase (Sigma Aldrich) following the manufacture's instruction.

IMMUNOSTAINING

Cells cultured on 4-well chamber slides (Becton Dickinson) coated with Matrigel™ were fixed in 4% paraformaldehyde (Sigma Aldrich) and incubated with hydrogen oxide in 100% methanol for 30 min at 4°C. Specimens were incubated with 2% fetal bovine serum in phosphate-buffered saline (PBS) (wash buffer) for 30 min at 4°C. Anti-Oct3/4 (Becton Dickinson) and anti-Nanog (Repro Cell) antibody specimens were incubated in 0.1% sodium citrate (Wako Pure Chemicals) and 0.1% Triton X-100 (Wako Pure Chemicals) in distilled water. Cells in 1:500 diluted anti-Oct3/4, anti-Nanog, anti-SSEA-4 (Millipore, Billerica, MA), and anti-TRA-1-60 (Millipore) were incubated in the wash buffer overnight at 4°C. After washing thrice with PBS, 500-times diluted horseradish peroxidase labeled anti-mouse (GE Healthcare, Pittsburgh, PA) or rabbit antibody (GE Healthcare) was incubated in the wash buffer for 3 h at 4°C. Diaminobenzidine (DAKO, Glostrup, Denmark) was applied, and nuclei were stained with hematoxylin (Muto Pure Chemicals, Tokyo, Japan) for 15 s. Specimens were observed and photographed with AX80 (Olympus, Tokyo, Japan).

STATISTICAL ANALYSIS

Statistical analysis was performed by one-factor analysis of variance with JMP 8.0 (SAS Institute, Cary, NC). $P < 0.05$ was considered statistically significant.

RESULTS

Before the present study, screening was necessary to reveal reagents that could maintain pluripotency. There was a possibility that combinations of reagents could maintain pluripotency while single use of reagents could not. Single or combinations of reagents were, thus, analyzed. hiPS cells were passaged with combinations of A, C, L, or F, and the cells reached 12 passages when cultured with A, A + C (AC), and A + C + L (ACL) (Fig. 1). The passage number did not reach 12 with the other combinations of reagents. Average passage number was 9.25 ± 2.76, and 4.00 ± 1.41 in media with or without A, respectively ($P = 0.0003$); the hiPS cells were maintained in an undifferentiated state longer with combinations involving A than those without it. Figure 2 shows that hiPS cells cultured with A, AC, and ACL at 12 passages show an undifferentiated morphology. ALP staining was performed to confirm that iPS cells maintained pluripotency (Fig. 3). hiPS cells in A, AC, and ACL were positive for ALP staining at 12 passages. Morphology and ALP staining seemed different among cells incubated with A, AC, and ACL. Pluripotency of hiPS cells were further investigated immunohistochemically (Fig. 4). Oct3/4 was positive in hiPS cells cultured with A, AC, and ACL. hiPS cells were positive for Nanog with A and AC. hiPS cells cultured with AC displayed significantly stronger immunostaining with Oct3/4 and Nanog. SSEA4 was positive in hiPS cells cultured with A and AC, whereas SSEA4 was almost negative in those cultured with ACL. hiPS cells were positive for TRA-1-60 when cultured with A, AC, and ACL. Data suggested that A was a key reagent to maintain pluripotency of hiPS cells under a feeder-free condition. SB431542 was added to A, AC, and ACL to inhibit the activity of A. iPS cells lost undifferentiated morphology at six passages when cultured with A + SB, five passages with AC + SB, and nine passages with ACL + SB (Fig. 5). On the other hand, iPS cells cultured without SB reached 12 passages.

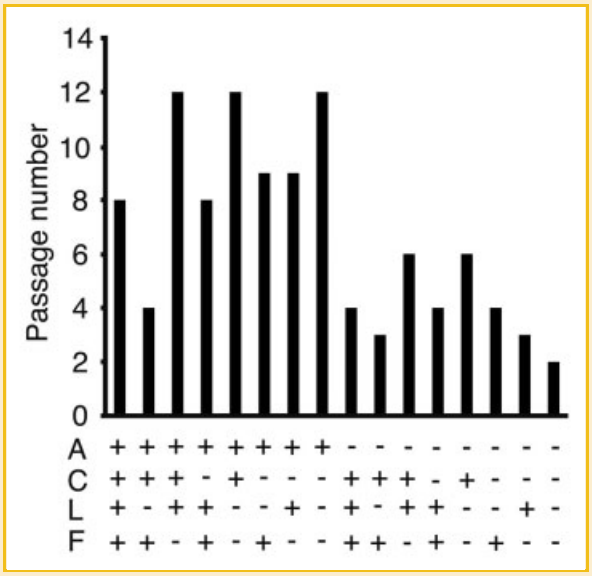

Fig. 1. Passage numbers of human induced pluripotent stem cells maintaining undifferentiated morphology. Human induced pluripotent stem cells were cultured with activin A, CHIR99021, leukemia inhibitory factor (LIF), or basic fibroblast growth factor (FGF). Endpoint of passage was defined as disappearance or morphological changes suggesting differentiation. Passage numbers of cells cultured with activin A were significantly higher than those cultured without it (see text). A: activin A (10 ng/ml); C: CHIR99021 (2 μM); L: LIF (1000 U/ml); F: basic FGF (5 ng/ml).

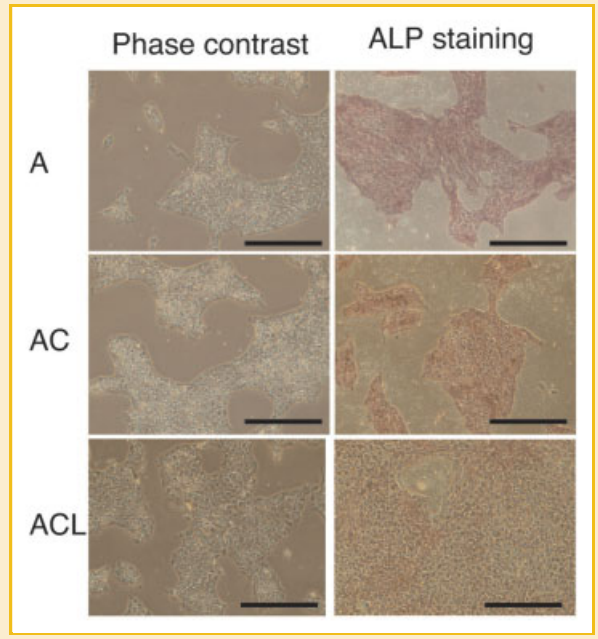

Fig. 3. Alkaline phosphatase staining of human induced pluripotent stem cells after 12 passages. Alkaline phosphatase (ALP) staining was performed for human induced pluripotent stem cells when they reached passage 12. All cells were positive for ALP staining. Original magnification: 40×, A: activin A (10 ng/ml), AC: activin A (10 ng/ml) plus CHIR99021 (2 μM), ACL: activin A (10 ng/ml) plus CHIR99021 (2 μM) plus leukemia inhibitory factor (1,000 U/ml). Original magnification: 40×, scale bar: 250 μm.

DISCUSSION

hES cells cultured with 5 ng/ml activin A on Matrigel™-coated flasks for 15 passages maintain pluripotency [Xiao et al., 2006]. hiPS cells maintain pluripotency with activin A. Our results suggest that activin A supports pluripotency of hiPS cells for longer passages, and these results are consistent with those of previous reports. Activin A maintains pluripotency of hES cells by regulating the expression of Nanog [James et al., 2005; Miyanari and Torres-Padilla, 2012]. SB431542 suppresses the expression of Nanog in hES cells and promotes differentiation to neuroectoderm [Vallier et al., 2009a]. In our experiments, hiPS cells lost morphology of pluripotency with SB431542 (Fig. 5). It is speculated that SB431542 inhibits the activity of activin A and suppresses the expression of Nanog because the gene is essential for pluripotency [Miyanari and Torres-Padilla, 2012].

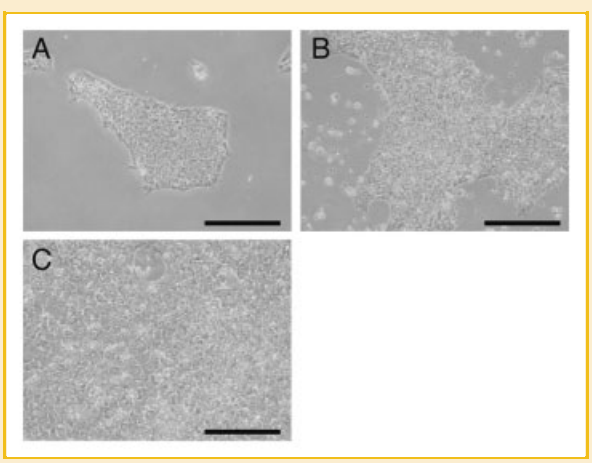

Fig. 2. Morphology of human induced pluripotent stem cells after passages. Human induced pluripotent stem cells maintained the morphology of an undifferentiated state after 12 passages with activin A (10 ng/ml) (**A**), activin A plus CHIR99021 (2 μM) (**B**), or activin A plus CHIR99021 plus leukemia inhibitory factor (1,000 U/ml) (**C**), respectively. Original magnification: 100×; scale bar: 100 μm.

Wnt signals are essential for self-renewal of mES cells [ten Berge et al., 2011]. CHIR99021 enables self-renewal of mES cells under feeder-free conditions with β-catenin as a downstream effecter [Ye et al., 2012]. CHIR99021 alone induces non-neural differentiation although it enhances survival [Ying et al., 2008]. hiPS cells consistently differentiated at six passages with CHIR99021 alone (Fig. 1). Tsai et al. [2010] report that hES cells can be cultured with 4 ng/ml bFGF and 5 ng/ml activin A for seven passages. In our study, hiPS cells lost pluripotency at nine passages with 5 ng/ml bFGF and 10 ng/ml activin A. The previous report and our data suggested that hiPS cells lose pluripotency when cultured with a combination of bFGF and activin A [Tsai et al., 2010]. bFGF maintains pluripotency of hiPS cell with feeder cells [Takahashi et al., 2007]. bFGF is, in contrast, used for hiPS cells to differentiate to endoderm under feeder free condition [Inamura et al., 2010]. hiPS cells might lose pluripotency with bFGF without feeder cells. The reason for these is not clear.

Pluripotent stem cells are divided into naïve and primed type [Hirai et al., 2012]. Naïve pluripotent stem cells depend on LIF and

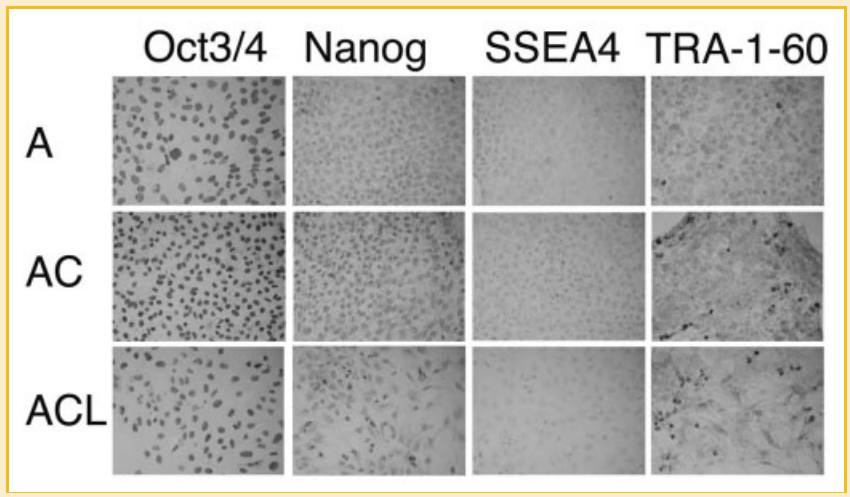

Fig. 4. Immunostaining of human induced pluripotent stem cells with markers of undifferentiated state. Immunostaining was performed for human induced pluripotent stem (hiPS) cells after 12 passages with antibodies to Oct3/4, Nanog, SSEA4, or TRA-1-60. Oct3/4 and Nanog were positive for all cells. hiPS cells cultured with activin A (10 ng/ml) were marginally weaker for SSEA4 than those cultured with activin A plus CHIR99021 (2 μM), and activin A plus CHIR99021 plus leukemia inhibitory factor (1,000 IU/ml). Original magnification: 200×. A: activin A (10 ng/ml), AC: activin A (10 ng/ml) plus CHIR99021 (2 μM), ACL: activin A (10 ng/ml) plus CHIR99021 (2 μM) plus LIF (1,000 U/ml).

BMP for self-renewal while primed ones on bFGF and activin A [Tesar et al., 2007]. Mouse ES cells are naïve while human ES cells and hiPS cells are primed [Hirai et al., 2012]. hiPS cells were primed and depended on activin A rather than LIF. The differentiation state might be different among hiPS cells incubated with A, AC, and ACL. That might be the reason why morphology and ALP staining were different among hiPS cells cultured in three conditions. Mouse ES cells show stronger expression of Oct34 and Nanog when incubated with CHIR999021 under serum free condition suggesting that the small molecule promotes self-renewal [Ye et al., 2012]. It was speculated that hiPS cells incubated with AC had the strongest potential of self-renewal.

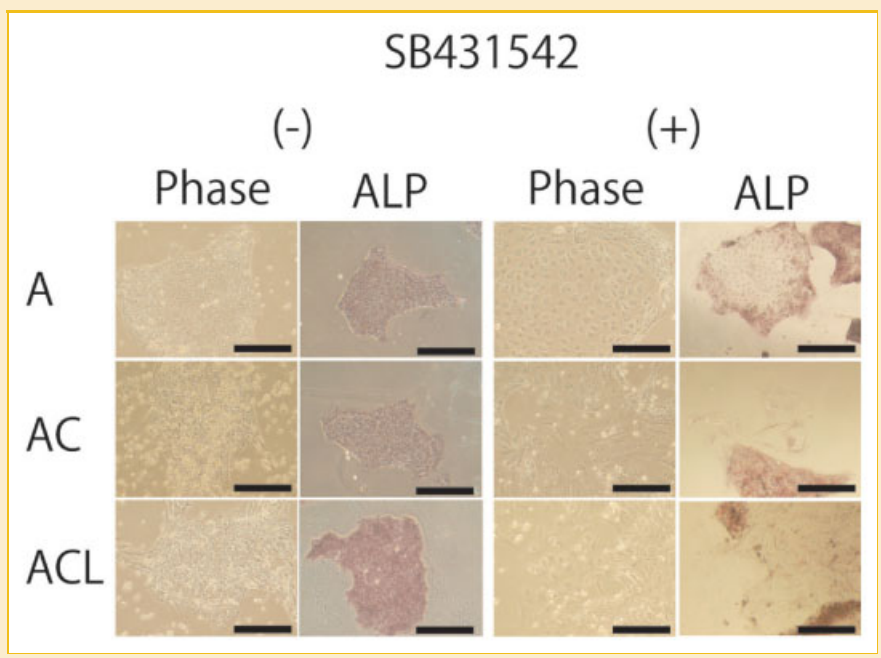

Fig. 5. Passage of human induced pluripotent stem cells with activin A and SB431542. Human induced pluripotent stem (hiPS) cells were passaged with activin A (10 ng/ml) (A), activin A plus CHIR99021 (2 μM) (AC), or activin A plus CHIR99021 plus leukemia inhibitory factor (ACL) (1,000 IU/ml), with or without SB431542 (SB). hiPS cells differentiated at six passages with A + SB, five passages with AC + SB, and nine passages with ACL + SB. Passage number of hiPS cells without SB reached 12. Phase: phase contrast without alkaline phosphatase staining, ALP: phase contrast with alkaline phosphatase staining, original magnification: 100×; scale bar: 100 μm.

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