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ARTICLE

Chemically fixed autologous feeder cell-derived niche for human induced pluripotent stem cell culture

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Binata Joddar[a,b*], Chieko Nishioka[c], Eiki Takahashi[c], Yoshihiro Ito[a]

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Conventional culture of human induced pluripotent (hiPS) cells requires feeder cell preparation that is time consuming and labour intensive. Alternatively, feeder-free culture of hiPS cells requires high cell seeding densities, specialized culture medium, and growth supplements, which raise the cost. Furthermore, recombinant systems require a long time for colony formation. Here, we employed chemically fixed autologous feeder cells for hiPS cell culture without additional time needed for colony formation. Treatment of (2.5% glutaraldehyde or formaldehyde for 10 min) autologous human dermal fibroblasts allowed hiPS cells to adhere and grow as undifferentiated colonies characterized by the expression of pluripotency markers such as alkaline phosphatase, Oct-3/4, and stage-specific embryonic antigen-4. Furthermore, hiPS cells cultured on chemically fixed feeders formed teratomas in vivo, characterized by all three germ layers. The chemically fixed autologous feeders may be used as a substitute for large scale culture of hiPS cells as a convenient in-house and a cost-effective method.

A. Introduction.

Human induced pluripotent stem (hiPS) cells can be generated from a patient's own cells, avoiding the ethical issues of human embryonic stem (hES) cells and reducing the likelihood of graft rejection[1]. It is common practice to use feeder cells when culturing iPS cells. Feeder cells supply nutrients to stem cells in culture and allow the cells to anchor and propagate, while providing an environment suitable for maintenance of pluripotency[1-3]. However, commonly used feeders for hiPS cells include SNL 76/7 cells, an immortalized cell line derived from mouse fibroblast STO cells transformed with a neomycin resistance gene[2]. Therefore, human dermal fibroblasts (HDFs) have been used as an alternative to SNL 76/7 cells for xeno-free culture of hiPS cells[2].

Feeder-free culture methods have also been developed to avoid the time-consuming and labour-intensive preparation of feeder cells for iPS cell culture. Matrigel™ is widely used for feeder-free culture of iPS cells[3, 4]. To avoid batch-to-batch variations and the risk of pathogen contamination associated with Matrigel™, extracellular matrix (ECM) proteins, including human recombinant laminin-511[5], laminin-521[6], and laminin E8[7], have been found to be most effective for iPS cell culture. The use of chemically defined peptides and synthetic acrylates has also been reported for hiPS cell culture[8], which requires specialized growth medium[9]. However, the significant increase of cost and decrease of quality control result in batch-to-batch inconsistencies[9]. In addition, a complete absence of feeder cells can be a suboptimal environment for pluripotent stem cells and often results in karyotypic abnormalities[10]. Feeder-free culture can also lead to longer doubling times for colony formation than those using feeder-based conventional methods[4,12,13]. In a previous study, we demonstrated that chemically fixed nurse cells support the growth and maintenance of hematopoietic stem cells[11] and ES cells[12]. Recently, we reported the use of chemically fixed feeders as a new culture material for mouse iPS cell culture[13]. Feeder cell fixation appears to be an attractive method to increase the therapeutic usability of iPS cells by reducing the processes involved in their propagation (**Fig. 1**) and preventing carryover of feeder cells in iPS cell colonies after passaging[13]. It should be noted that post

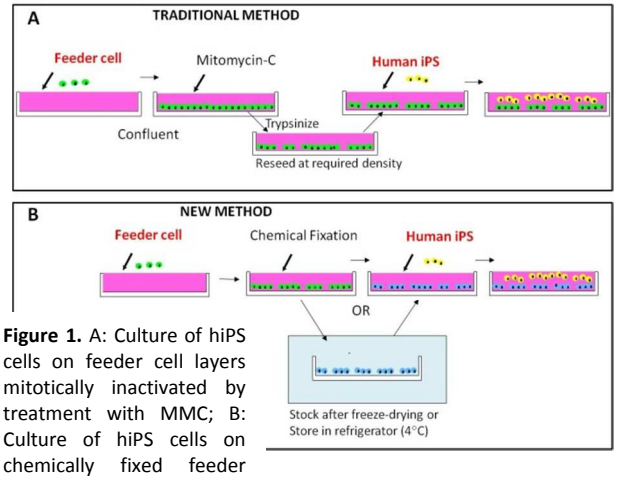


Figure 1. A: Culture of hiPS cells on feeder cell layers mitotically inactivated by treatment with MMC; B: Culture of hiPS cells on chemically fixed feeder cells.

chemical fixation the feeder cells are non-viable and cannot release active growth factors or chemokines into the cell culture[14]. Therefore, fixed feeder cells can be refrigerated for long-term storage prior to use[13]. However, this technique has not been extended to cultures of hiPS cells, because they are much more susceptible to slight changes in culture conditions. As an alternative to animal feeders, HDFs can be used as feeder cells to culture hiPS cells[10]. In this study, we developed an optimized chemical fixation protocol using Glutaraldehyde (GA) and Formaldehyde (FA) to prepare a niche matrix from autologous feeder cells for hiPS cell culture (**Figure 1**). The behavior of hiPS cells cultured on these feeders was monitored in terms of their colony size and expression of pluripotency markers. The simple method developed in this study may have implications in research and clinical applications of hiPS cells.

B. Results

I. Optimization of the chemical fixative concentration for autologous feeders in hiPS cell culture

Various concentrations of GA (2.5–10%), were assessed to optimally crosslink autologous feeder cells for hiPS cell culture. After 3 days of culture, the average colony width of hiPS cells cultured on HDF-GA (2.5%) was ~500 μm, which was comparable with that of hiPS cell colonies cultured on mitomycin-C (MMC)-HDFs (control). However, increasing the concentration of GA used for chemical fixation of autologous feeders resulted in a significant reduction in the size of hiPS cell colonies (**Fig. 2**).

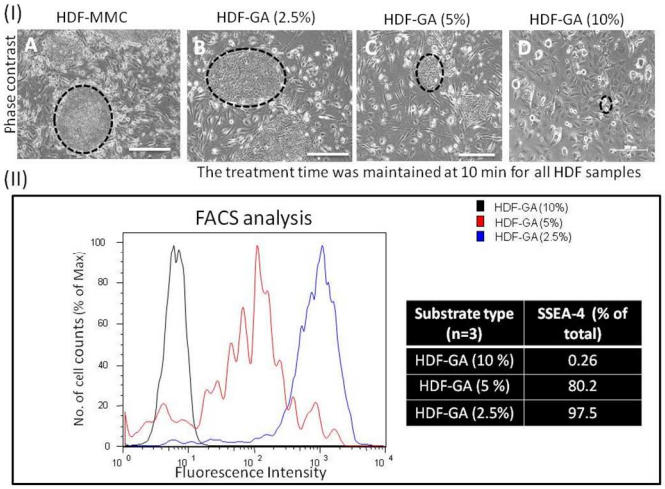


Figure 2. I. Morphology of hiPS cells cultured on MMC-treated HDFs (A) and chemically fixed HDFs (B–D). The size of the hiPS colonies was reduced gradually as the GA concentration was increased from 2.5 to 10% (B–D). Scale bar, 200 μm.

II. Flow cytometric analysis of the percentage of SSEA-4-expressing hiPS cells cultured on chemically fixed HDFs. The percentage of SSEA-4-expressing hiPS cell colonies was gradually increased as the GA concentration was reduced from 10 to 2.5%.

The average diameters of hiPS cell colonies on 5% and 10% GA-treated feeders were reduced to ~100 μm and ~20 μm, respectively (**Fig. 2** I, A–D). Furthermore, hiPS cells cultured on 5% GA-treated feeders exhibited a significantly lower level of the pluripotency marker stage-specific embryonic antigen-4 (SSEA-4) compared with that in hiPS cells cultured on 2.5% GA-treated feeders (**Fig. 2** II).Very few SSEA-4-expressing hiPS cells were detected when cultured on 10% GA-treated feeders (**Fig. 2** II). These results imply that increasing the concentration of the chemical fixative above 2.5% adversely affects the feeder substrate properties, thereby reducing hiPS cell adhesion and pluripotency. Therefore, in subsequent experiments, fixation of autologous HDF feeders for 10 min with 2.5% GA or FA was used for hiPS cell cultures.

II. Chemically fixed feeders maintain the adhesive phenotype of hiPS cells

Human iPS cells adhered and were maintained as colonies when cultured on fixed feeder cells (**Fig. 3**A–D), even after eight passages. The morphology of the hiPS cell colonies was similar on HDF-GA or FA and MMC-HDFs (control) (**Fig. 3**B–D). Furthermore, the distinct adhesive phenotype of hiPS cells was similar when cultured on GA- or FA-treated feeders, which is a characteristic of undifferentiated hiPS cells as reported by others[15]. Average diameters of colonies varied from 200 to 500 μm on all feeder cell-based substrates (**Fig. 3**A–D). Human iPS cells cultured on laminin-5 formed much smaller clusters (up to 100 μm in diameter) even when seeded at a higher cell density compared with that on feeder cells (**Fig. 3**E). Furthermore, these colonies appeared to be differentiated around the edges (**Fig. 3**E).

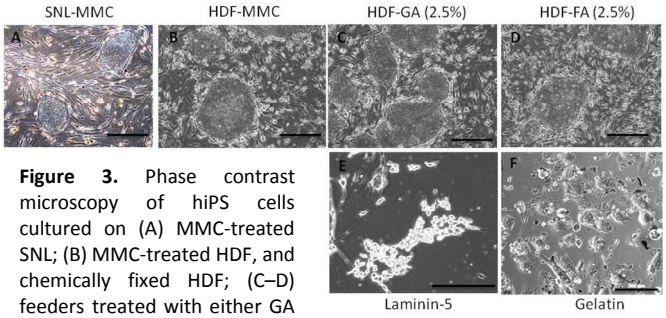


Figure 3. Phase contrast microscopy of hiPS cells cultured on (A) MMC-treated SNL; (B) MMC-treated HDF, and chemically fixed HDF; (C–D) feeders treated with either GA or FA. Very few hiPS cell colonies were detected in feeder-free culture on; (E) laminin-5; and (F) gelatin. Scale bar, 200 μm.

In contrast, very few hiPS cells attached to gelatin and they failed to form colonies (**Fig. 3**F).

III. The colony doubling time of hiPS cells is unaltered when cultured on chemically fixed feeders

Colony doubling times of hiPS cells grown on either GA- or FA-treated feeders were similar to those of hiPS cells grown

on MMC-treated SNL or HDF feeders[2] and Matrigel™[3], but lower than those of cells cultured on laminin-5 (**Fig. 4** I). These results indicated that the hiPS cells readily adapted to culture on chemically fixed feeders.

The average number of colonies per passage was also significantly higher for hiPS cells cultured on feeder cells compared with that for hiPS cells cultured without feeders (**Fig. 4** II). Collectively, these results imply that feeder-based hiPS cell culture generates a higher number of hiPS cell colonies compared with that in feeder-free culture. Human iPS cells cultured on gelatin did not grow beyond the first passage.

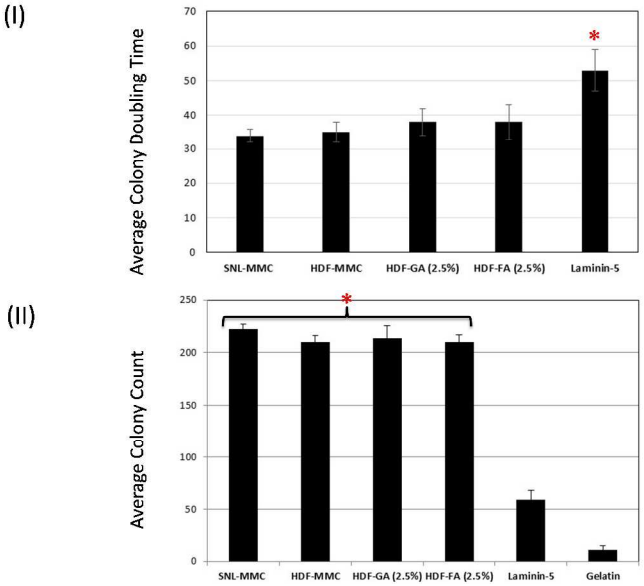


Figure 4. I. Average colony doubling time of hiPS cells on various substrates. Data were averaged from five passages in all samples. Samples cultured on Laminin-5 required significantly longer times to double in culture ($p>0.05$ *). II. Average number of hiPS cell colonies (50–200 μm) per passage after 3 days of culture on various substrates. Cultures grown on feeder cells had significantly more colonies than those in cultures without feeders ($p>0.05$ *).

IV. Human iPS cells cultured on chemically fixed feeders maintain their pluripotency

Positive alkaline phosphatase (ALP) staining confirmed by colorimetric staining indicated maintenance of the undifferentiated state of hiPS cell colonies for at least eight passages on feeder cells (**Fig. 5** I, A–D), even after fixation (**Fig. 5** I, C, D). On laminin-5, we observed very few ALP-positive colonies (**Fig. 5** I, E). No ALP-positive colonies were detected in hiPS cell cultures on gelatin (**Fig. 5** I, F). Furthermore, the gross morphology and relative number of ALP-stained colonies were similar on live (MMC-HDF) and chemically fixed (GA- or HD) feeder cells (**Fig. 5** I, G, H).

The expression level of Oct-3/4 mRNA was significantly higher in hiPS cells cultured on feeder cells compared with

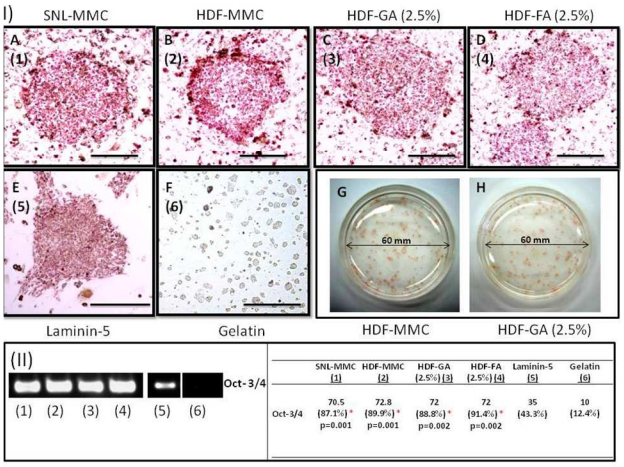
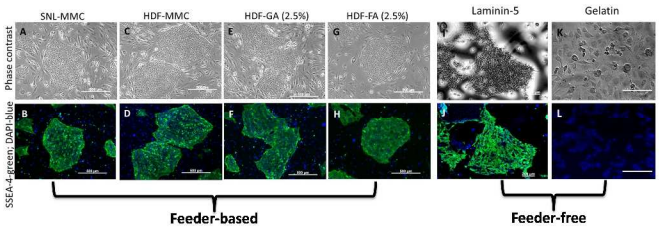


Figure 5. I. ALP stained hiPS cells cultured on MMC-treated SNL (A), MMC-treated HDF (B; control), and chemically fixed HDF (C, D) feeders. ALP staining (pinkish-red) of hiPS cells cultured on HDF-GA or HDF-FA was performed after eight passages. In feeder-free culture, ALP stain intensity was greatly reduced in hiPS cells cultured on laminin-5 (E) and absent in those cultured on gelatin (F). Scale bars, 200 μm. Gross morphologies were very similar for ALP-stained hiPS cells cultured on MMC-HDFs (G) or chemically fixed HDFs (H).

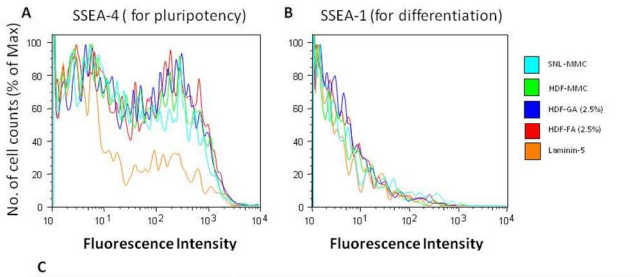
II. RT-PCR analysis and quantitative grey value measurements of the expression levels of Oct-3/4 in hiPS cells cultured on various substrates. (1)-MMC-SNL; (2)-MMC-HDF; (3)-HDF-GA (2.5%); (4)-HDF-FA (2.5%); (5)-Laminin-5; (6)-Gelatin. Values in parenthesis are intensities normalized to GAPDH and expressed as a percentage. Values marked with an * are significantly greater from Laminin-5. p-values are indicated in parenthesis alongside.



that in hiPS cells cultured without feeders (**Fig. 5** II). This trend confirmed the undifferentiated state of hiPS cells on feeder cells. On laminin-5 and gelatin, we found significantly low levels of Oct-3/4 expression (**Fig. 5** II).

Immunostaining revealed high expression of SSEA-4 in hiPS cells cultured on feeder cells (**Fig. 6**A–H) compared with that in cells cultured on gelatin (**Fig. 6**K, L). No hiPS cell colonies cultured on laminin-5 showed SSEA-4 expression (**Fig. 6**I, J; indicated with white arrows). Negative controls omitting the primary antibody against SSEA-4 did not exhibit any fluorescence (data not shown).

Figure 6. Phase contrast imaging and SSEA-4 expression in hiPS cells cultured on (A, B) MMC-treated SNL, (C, D) MMC-treated HDF, or chemically fixed HDF feeders treated with either GA (E, F) or FA (G, H).



C

Substrate type (n=5)	SSEA-4 (% of total)	SSEA-1 (% of total)
SNL-MMC	81	1.13
HDF-MMC	83.7	1.47
HDF-GA (2.5%)	77.7	1.21
HDF-FA (2.5%)	75.8	2.49
Laminin-5	63.2	0.56
Gelatin	0.01	0.00

FACS analysis

Figure 7. Percentages of SSEA-4 (pluripotency marker)- and SSEA-1 (differentiation marker)-expressing hiPS cells cultured on various substrates, and analysed by flow cytometry after eight passages on all substrates except for cells cultured on gelatin.

Flow cytometric analysis revealed similar levels of SSEA-4 expression in hiPS cells cultured on chemically fixed HDFs or on controls. However, when cultured on laminin-5, there was a decrease in percentage of SSEA-4-expressing cells, and no SSEA-4 expression was found in cells cultured on gelatin (**Fig. 7**A, B). A very low percentage of SSEA-1 expression was detected in hiPS cells cultured on all substrates, indicating that the cells analysed were not differentiated (**Fig. 7**A, B).

We further detected the presence of Collagen IV retained in chemically fixed feeders which is essential for stem cell ac pluripotency (**Supplementary F (Fig. 5–7, Supplementary** C two types of chemically fixed F A and FA) can be used as H HDF feeders to culture hiPS su oubling time, which normally ce labour-intensive protocols to re pr

The pluripotency of hiPS cells cultured on chemically fixed HDFs was confirmed by assessment of in vivo teratoma formation (**Fig. 8**B–J) in comparison with the normal histology of the mouse testis (**Fig. 8**A). Haematoxylin and Eosin (H&E) staining of teratomas demonstrated that all three germ layers were generated from hiPS cells, including the ectoderm (indicated by epidermal tissues), mesoderm (indicated by muscles and adipose), and endoderm (indicated by epithelial tissues). These results suggested that the hiPS cells cultured on chemically fixed HDFs

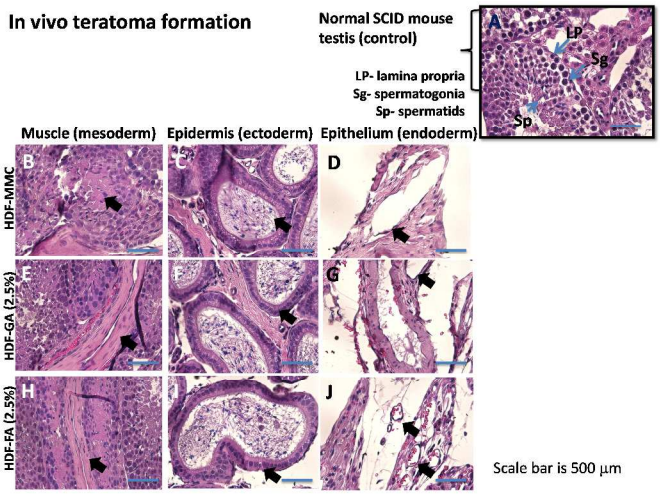


Figure 8. In vivo teratoma formation of hiPS cells cultured on chemically fixed HDFs. After seven passages on GA- or FA-HDFs, hiPS cells were collected and injected subcutaneously into the testis of a 7-week-old SCID mouse to induce formation of teratomas. At 4 weeks after injection, the teratomas were fixed, sectioned, and stained with H&E. Scale bars, 500 μm.

maintained their pluripotency to differentiate into tissues of all three germ layers.
Please see Online Supplement

C. Discussion

In a recent study[13] we showed that stem cells thrived on chemically preserved non-viable feeder cells which supported mouse iPS colony formation and pluripotency. Chemical fixation used to physically preserve the feeder cell protein structures killed these cells, yet maintained the intactness of their physical structure. This resulted in a cell culture surface that retained virtually all of the proteins which are essential for stem cell adhesion and culture atop them. Most importantly the results of this previous (mouse iPS)[12] and our current (human iPS) study demonstrate that the right contact environment is a priority for stem cell culture. It is well known that substrate topography can be engineered to control cell adhesion and functions[17]. Given the fact that chemical fixation generates a micro-or a nano-scaled rough protein-rich surface which is favourable for stem cell culture, using micro- or nano-engineering approaches to mimic this surface topography with ECM proteins might be a commercially alternative strategy for large scale culture of human iPS cells.

When feeder cells are treated with GA or FA, the amino-containing protein molecules are crosslinked at their intrinsic locations (**Supplementary Figure S1**), retaining the essential protein structures of the feeder cells. ECM molecules secreted by feeder cells are required for interactions with hiPS cells to maintain their pluripotency[18]. The chemically preserved ECM and cell adhesion molecules, which also localize to stem cell niches in vivo[19, 20], allow hiPS cells to grow on these niche-like matrices in vitro.

Compared with either traditional feeder-based[1-3] culture or feeder-free systems[4,12,13,19-26] for iPS cells, our chemically fixed autologous feeder substrate poses itself as a convenient substitute. It consists of essential cell membrane proteins, is non-xenogenic, requires less than an hour for preparation, and is cost effective because it does not require a specialized medium such as mTeSR™ or growth factors such as TGF-β1 for long term culture of hiPS cells.

The doubling time for colony formation of hiPS cells cultured on chemically fixed substrates was also unaltered compared with that of hiPS cultured on mitotically inactivated feeders[2], on Matrigel™[4,13,23] and shorter compared with that of hiPS cultured in other feeder-free systems[21]. Although Nagaoka et al.[4] have reported shorter colony doubling times (~26 h) for hiPS cells grown without feeders, the recombinant protein surface supports the adherence of only 30% of the cells using equal volumes of mouse embryonic fibroblast-conditioned medium and mTeSR™1. This result implies that their initial cell seeding density was much higher than ours. Here, more than 80% of the hiPS cells adhered and remained viable when cultured on chemically fixed feeders. Similarly, Nakagawa et al.[22] reported shorter colony doubling times (~28 h) of hiPS cells grown under feeder-free conditions, compared with those of cells grown on feeders, by addition of a ROCK inhibitor during seeding on various substrates. Yamasaki et al.[23] have reported the shortest colony doubling times (~17 h) of hiPS cells grown on ECM substrates by addition of transforming growth factor (TGF)-β1.

In our study the hiPS cells failed to grow on gelatin although gelatin-coated surface is one of the simplest matrices for cell culture. Others have been successful in using gelatin for human ES and iPS culture, but in the presence of soluble fibronectin and ROCK inhibitor which was added to these cultures [24].

In the absence of a ROCK inhibitor in our study, the hiPS cells attached to laminin-5 but the edges of the colonies appeared differentiated. The cell seeding density of hiPS cells cultured on laminin-5 (1.6×10^5 cells/ cm^2) was much higher compared to that of chemically fixed feeders (5.0×10^3 cells/ cm^2). Although other studies have reported lower cell seeding densities[19,22], a ROCK inhibitor was deemed necessary to maintain the viability of cultured hiPS cells in feeder-free culture systems such as laminin-5[19,22].

D. Methods

I. Preparation of MMC-treated SNL and HDF feeders

All cells were procured from the RIKEN Bioresource Center at Tsukuba unless indicated otherwise. HDFs were kindly provided by our collaborators at RIKEN CLST. MMC-treated SNL and HDF feeder cells were prepared by incubating confluent cells in a 100-mm dish with DMEM/F12 (Invitrogen, Carlsbad, CA, USA) containing 10 µg/ml MMC (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 2.25 h. The medium was then changed to complete medium for hiPS cells (formulation described below). MMC-treated feeder cells were trypsinized, reseeded into 60-mm cell culture dishes at 6.0×10^5 cells/dish, which corresponded to 80% confluence, and incubated overnight at 37°C before use.

II. Preparation of GA- and FA-treated HDF feeders

Chemically fixed HDFs were not pre-inactivated by MMC treatment. HDFs were trypsinized and reseeded into 60-mm cell culture dishes at 6.0×10^5 cells/dish and incubated for 2 days in a CO_2 incubator at 37°C. Prior to chemical fixation, the HDFs were washed with PBS and then treated with a solution of 2.5% GA (Sigma-Aldrich) or 2.5% FA (Sigma-Aldrich) at room temperature for 10 min. The cells were then washed up to 12 times with PBS (30 min per wash at 30 rpm in a shaker). Completion of the wash cycle was achieved when the pH of the wash buffer was ~7. To optimize the concentration of GA, the cells were also treated with 5% and 10% GA. Chemically fixed HDFs were refrigerated at 4°C in PBS until use.

III. Preparation of feeder-free cultures on gelatin and laminin-5

To prepare gelatinized surfaces, cell culture dishes were treated with 0.1% (w/v) gelatin (Millipore, Billerica, MA, USA) for 2 h at 37°C and then washed before subsequent use.

Laminin-5 (laminin-332) (Reprocell, Kyoto, Japan) is a recombinant human coating material for hES/hiPS cell culture. Human iPS cells can be maintained stably in an undifferentiated state in long-term culture on laminin-5 using ReproFF[25]. To prepare laminin-5-coated surfaces, a 35-mm dish was coated with 1–2 µg laminin-5 following the manufacturer's protocol[25]. Prior to coating, 1 ml PBS (−Ca++, −Mg++) was added to the dish. Then, laminin-5 was added directly to the PBS, followed by manual rotation for uniform coating of the dish. The coated dishes were stored at 4°C overnight prior to use.

IV. Culture of hiPS cells

Human iPS cells (RBRC-HPS0063 201B7 established by Shinya Yamanaka; Riken Cell Bank) were cultured in complete hiPS cell culture medium or ReproFF (Reprocell) following protocols established at the Center for iPS Cell Research and Application (CiRA; Kyoto University). After preparation of MMC-SNL and MMC-HDF feeders (**Fig. 1**) for the first passage, 1×10^6 hiPS cells were thawed, seeded onto a 60-mm culture dish, and then cultured for 3–4 days in a CO_2 incubator at 37°C. At confluency, the hiPS cells were detached with CTK solution (0.25% trypsin-EDTA, 0.1% collagenase type IV, 20% Knockout serum replacement (KSR), and 1 mM $CaCl_2$) for 5 min at 37°C to obtain smaller colonies for passaging. From the second passage onwards, the hiPS cells were passaged onto various substrates including feeders (1×10^5 cells/60-mm dish or 5.0×10^3 cells/cm^2) or feeder-free culture substrates (2×10^6 cells/35-mm dish or 1.6×10^5 cells/cm^2).

Based on the manufacturer's recommendation for laminin-5[25], the cell seeding density of hiPS cells on laminin-5 and gelatin was higher compared with that of hiPS cells cultured on feeder cells.

V. Human iPS cell culture medium and supplements

DMEM/F12 (Invitrogen) was supplemented with 20% KSR (Invitrogen), 2 mM L-glutamine (Invitrogen), 1×10^{-4} M non-essential amino acids (Invitrogen), 1×10^{-4} M 2-mercaptoethanol, 50 U/ml penicillin, and 50 mg/ml streptomycin. Prior to use, 0.2 ml of 10 μg/ml basic fibroblast growth factor (bFGF) was added to 500 ml of the medium. This complete hiPS cell medium was aliquoted, stored at 4°C for up to a week, and used to culture hiPS cells on all substrates unless indicated otherwise.

To culture hiPS cells on laminin-5-coated dishes, ReproFF medium supplemented with 5 ng/ml bFGF was used according to the manufacturer's recommendations.

VI. Measurement of hiPS cell proliferation

Cell Proliferation was estimated using techniques described previously[26]. Human iPS colonies after being cultured for 16 hours on all substrates were detached with CTK solution and dispersed as single cells by trypsinization with 0.25% trypsin/1×10^{-3} M EDTA (Sigma). The number of cells was then measured using Trypan Blue (Sigma) uptake and TC10 automated cell counter (Bio-Rad). In this manner, the numbers of cells at harvest per dish (60 mm diameter) were counted to calculate the doubling time of cells cultured on the various substrates[21]. The hiPS cell doubling time was calculated as follows:

$$doubling\ time\ (hours) = h \times \ln(2) / \ln(\frac{c2}{c1})$$

h = hours in culture (16 hours), c2 = cell number per vessel at harvest, and c1 = cell number initially seeded per vessel (100000 cells).

Doubling times were averaged from 5 passages for each sample and reported.

VII. Human iPS cell colony counts

The number of colonies (>50 μm) was estimated from phase contrast images at 3 days post-cell seeding[11,30]. After incubation at 37°C in a humidified atmosphere with 5% CO_2, the colonies were washed with PBS and then stained for ALP activity (as described below). The stained colonies were washed with PBS twice and counted.

VIII. ALP staining

Human iPS cell colonies were fixed with 4% FA at room temperature for 10 min and then washed with PBS. ALP staining was done with a Vector Red Alkaline phosphatase Substrate Kit I (Vector Laboratories, Burlingame, CA, USA).

IX. RT-PCR analysis of Oct-3/4 expression in hiPS cells

Cells cultured for up to 3 days were collected by trypsinization. Total RNA was then extracted using an RNeasy Protect Mini Kit (Qiagen, Hilden, Germany). For RT-PCR analysis, cDNA was prepared from 5 μg total RNA using an oligo-dT primer and the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). cDNA was amplified using primers specific for Oct-3/4 (468 bp, NM_013633, forward, AGCTGCTGAAGCAGAAGAGG; reverse, CCTGGGAAAGGTGTCCCTGTA). RT-PCR products were visualized by electrophoresis in a 1.8% ethidium bromide-stained agarose gels. The bands were viewed under UV light and analysed by densitometric analysis using an Image Master W DS gel analyser (Amersham Biosciences Europe, Freiburg, Germany). Band intensities were further analysed quantitatively by Image J software (NIH). Oct-3/4 expression was normalized to β-tubulin mRNA levels.

X. Immunofluorescence staining of hiPS cells

Cells from 5-8 passages which had been in culture for at least 35-40 hours were fixed with Mild form 20 N (8% FA, pH 7.0–7.5; Wako Pure Chemical Industries, Osaka, Japan) for 30 min and then permeabilized with 0.2% Triton X-100/PBS for 2 min. After blocking with 1% bovine serum albumin for 30 min at room temperature, the samples were incubated with a mouse monoclonal antibody against SSEA-4 (Cell Signalling Technology, Beverly, MA, USA) followed a FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Please see Online Supplement for Collagen IV staining.

XI. Flow cytometric analysis of hiPS

To determine the percentages of SSEA-4 (pluripotency marker)- and SSEA-1 (differentiation marker)-expressing hiPS cells from 5-8 passages which had been in culture for at least 35-40 hours cultured on various substrates were washed and dissociated with TrypLE Express (12604039; Invitrogen) to obtain single cell suspensions. Dissociated single cells were washed again and passed through a 70-µm cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA). The cells were centrifuged for 2 min and re-suspended in DMEM/F-12 supplemented with 20% (v/v) fetal bovine serum. Then, the cells were prepared for flow cytometric analysis using a BD Stemflow™ Human and Mouse Pluripotent Stem Cell Analysis Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. First, the cells were fixed with BD Cytofixation buffer (4% paraformaldehyde), followed by permeabilization with 1× BD Perm/Wash buffer. The cells were then stained with Alexa Fluor 647-conjugated mouse anti-SSEA-4 (Clone MC813) and PE-conjugated mouse anti-SSEA-1 (MC480) antibodies on a rocking platform for 30 min on ice. A FACSCanto™ instrument (Becton Dickinson) was calibrated before each analysis with Rainbow Calibration Particles (Becton Dickinson). The negative gate was set using isotype controls, Alexa Fluor 647 Mouse IgG_3 (Clone J606), κ Isotype Control, and PE Mouse IgM (Clone G155-228), κ Isotype Control. Before analysis of the stained cells, BD CompBead Plus positive and negative beads were analysed to facilitate application setup. Nonviable cells were excluded by staining with 0.1% (v/v) propidium iodide (Sigma-Aldrich).

XII. In vivo teratoma formation

All experimental and surgical procedures involving animals were approved by RIKEN's Institutional Animal Care and Use Committee. A total of 24 mice was used for in-vivo experiments. A cell suspension of 5×10^5 cells/20 µl was injected subcutaneously into one side of the testis of a 7-week-old SCID mouse (n=6 per sample, × 3 samples; 18 mice total). Control mice received 1×PBS only (n=6). At 4 weeks after injection, the teratoma formed was fixed with 4% paraformaldehyde using a perfusion fixation method and then embedded in paraffin[13]. Sections (4 µm thick) were stained with H&E. Images were obtained under a Nikon microscope.

XIII. Statistical analysis

Three samples were prepared for each condition and each experiment was repeated at least three times unless otherwise mentioned. Data are expressed as the mean ± standard deviation. One-way analysis of variance followed by Bonferroni's post-hoc tests were performed in all statistical analyses. p-values of less than 0.05 were considered significant.

E. Conclusions

Considering that the hiPS cells cultured on chemically fixed feeders exhibit both in-vitro pluripotency by forming colonies that are positive for ALP stain, Oct-3/4, SSEA-4; and in-vivo pluripotency by differentiating into cell types that are characteristic of the ectoderm, endoderm, or mesoderm when implanted in vivo, it is concluded that the present method is very simple and useful for hiPS cell culture because no additional supplements are required.

F. Acknowledgements

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Notes and references

[a]*Nano Medical Engineering Laboratory, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan*

[b]**Currently at: Department of Mechanical Engineering & Biomedical Engineering Program at The University of Texas at El Paso, A-104 Engineering Building, 500 W. University Ave. El Paso, TX 79968-0521, USA.*

[c]*Support Unit for Animal Experiment, Research Resources Center, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan*

**Corresponding author: bjoddar@utep.edu*

Tel.: +1 (915) 747-8456; Fax: +1 (915) 747-5019.

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