High-density hiPSCs expansion supported by growth factors accumulation in a simple dialysis-culture platform

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Abstract

Three-dimensional aggregate-suspension culture can produce large numbers of human induced pluripotent stem cells (hiPSCs); however, use of expensive growth factors and method-induced mechanical stress potentially result in inefficient production costs and difficulties in preserving pluripotency. Here, we developed a simple, miniaturized, dual-compartment dialysis-culture device based on a conventional membrane-culture insert with deep well plates. The device allowed growth-factor accumulation and improved cell expansion up to $\sim 32 \times 10^6$ cells/mL, and reduction of excessive shear stress and agglomeration following addition of the functional polymer FP003 supported high-density expansion. The results revealed accumulation of several growth factors, including fibroblast growth factor 2 and insulin, along with endogenous NODAL, which acts as a substitute for depleted transforming growth factor- β 1 in maintaining pluripotency. Because we used the same growth-factor formulation per volume in the upper culture compartment, cost reduction increased significantly in proportional manner with cell density. We showed that growth-factor-accumulation dynamics in a low-shear-stress environment successfully improved hiPSC proliferation, pluripotency, and differentiation potential. This miniaturised dialysis-culture system demonstrated the feasibility and cost-effective mass production of hiPSCs in high-density culture.

Introduction

Human induced pluripotent stem cells (hiPSCs) are a potential source of many cell types comprising the human body. A large number of hiPSCs are essential for applications, such as regenerative therapy. Currently, suspension culture is a common standard for producing large numbers of PSCs^{1,2}; however, large-scale and cost-effective hiPSC production remains difficult because of several culture-condition requirements, including nutrition-supply limitations, waste-product removal³, and damage by mechanical stress¹. Additionally, mass production of hiPSCs requires a large amount of expensive growth factors. These problems can potentially lead to high production costs.

Dialysis-culture systems were first utilised to produce various proteins, such as monoclonal antibodies⁴⁻⁶ and tissue-type plasminogen activator⁷, from mouse hybridoma cell lines cultured at high density. Dialysis membranes permeate small molecules, such as toxic metabolites and nutrition sources, but not macromolecules, such as proteins secreted from cells. Several studies have applied this technology for PSC culture using large culture vessels at low cell density^{8,9}; however, the feasibility of high-density PSC culture has not been examined in depth due to the cost and complexity of operations in these large-scale systems. Furthermore, PSCs secrete several autocrine factors necessary to improve their proliferative efficiency¹⁰⁻¹², and these factors can be fully utilised and evaluated using this dialysis system.

Hydrodynamic conditions are important factors for controlling aggregate size and avoiding excess agglomeration, which can cause spontaneous differentiation and/or necrotic cores due to the mass-transfer limitations of oxygen, nutrition, and metabolic waste¹³. Our previous study showed the potential

of dialysis culture in low-density PSC differentiation¹⁴; however, performance of high-density suspension culture can result in mechanical stress imposed by both the rotation of the culture medium and collision between aggregates. A biopolymer comprising gellan gum is a biocompatible material used for various tissue-engineering purposes^{15,16}, with Otsuji et al.¹⁷ showing that addition of this biopolymer can potentially support suspension culture of PSCs by reducing excess agglomeration and cell damage caused by shear stress.

In this study, we assessed the feasibility of high-density expansion of hiPSCs in a simple, miniaturized, dialysis-culture system capable of evaluating the effects of various medium components, including nutrition, growth factors, and toxic metabolites, on cell proliferation, pluripotency, and differentiation capacities without the need to use a large amount of culture medium and supplemental growth factors. Because high-density culture can increase mechanical stress not only caused by shear stress but also aggregate collision¹⁸, we applied FP003 functional polymer solution containing gellan gum to create a low-mechanical-stress culture environment by increasing medium viscosity. We found that endogenous autocrine factors accumulated in this system, and that their effective use minimised the demand for their exogenous supplementation. The results demonstrate that the expansion of high-density hiPSCs was enabled by both the accumulation of endogenous autocrine factors and the low-mechanical-stress environment from using the dialysis membranes and FP003. Moreover, the increased cell density not only reduced the use of growth factors but also supported the pluripotency and differentiation potential of hiPSCs. Although this study was limited to the proliferation phase, the possibility of reducing costs by full utilisation of both endogenous and exogenous growth factors at high cell density provides new insight into large-scale organ-cell production from human stem cells and could be effective at differentiating to specific cell lineages that require more complex and expensive growth-factor-based protocols.

Results

Mass-transfer of the system under cell-free conditions

We applied the dialysis-culture system to continuously support medium refinement through the dialysis membrane (Fig. 1A). The permeability evaluation device was able to maintain the equilibrium of glucose concentration after 6 h of culture and attain equilibrium of the lactic acid concentration in 8 h.

We confirmed the ability of the device to retain high-molecular-weight growth factors using a permeability test with fluorescein isothiocyanate (FITC), which evaluates the capability of size-selective accumulation of macromolecules, such as growth factors. During 24 h, the only macromolecule capable of passing through the 12-kDa molecular-weight cut-off (MWCO) dialysis membrane was a small amount of 4-kDa FITC, whereas 10-kDa FITC and 20-kDa FITC remained in the upper culture compartment with no detection in the lower dialysate compartment (Fig. 1D–F).

To achieve a mild dynamic culture environment, we added FP003 solution to the medium in the upper culture compartment (Fig. 1G), findings that FP003 addition did not significantly alter the mass-transfer

capability between the two compartments (data not shown).

The effects of FP003 on shear-stress-induced cell injury

To evaluate possible cell injury caused by the dynamic culture conditions, the hiPSC aggregates were cultured for 6 h under rotational culture conditions in the presence and absence of FP003. The results showed that addition of FP003 significantly reduced lactate dehydrogenase (LDH) leakage caused by membrane rupture of damaged cells (Fig. 1F). This showed that FP003-mediated decreases in mechanical stress improved pluripotency (Supplementary Fig. 1).

Improved cell proliferation and aggregate morphology

After expansion in different culture configurations (Fig. 2A), the hiPSC aggregates showed different morphologies and proliferation levels. The aggregate population expanded in dialysis culture showed decent morphology and improved growth relative to a population expanded under conventional suspension culture with regular medium replacement (Fig. 2B). To evaluate the effect of cell density, we performed expansion of the three groups with different inoculum cell densities (1×10^6 , 2×10^6 , and 4×10^6 cells/mL), which were expected to produce different final cell densities at day 4 (ranging from lower density to higher density based on high-density mammalian cell culture criteria). As a control for evaluating the simple dialysis culture, we added a conventional suspension culture with and without manual daily medium replacement. We found that the higher initial cell-seeding density tended to produce smaller aggregates at the end of the expansion period among the same culture configurations (Fig. 2C). Histological morphology showed that the aggregates grew at a tolerable size for expansion, as indicated by the absence of necrotic areas at the centre of the aggregate and often caused by the mass transfer limitation of oxygen, nutrients, and waste products (Fig. 2D). This level of aggregate growth in the dialysis-culture group resulted in a significant increase in cell proliferation within a high-density cell number. By contrast, the proliferation rate of all initial cell-seeding density populations at the end of the expansion period was limited to $\sim 5 \times 10^6$ cells/mL, with significant cell-number depletion occurring during the first and second days of culture without medium replacement (Fig. 2E).

Continuous nutrient supply and toxic metabolite removal

Based on glucose measurements, the dialysis systems demonstrated better performance in maintaining glucose levels as compared with the other two groups. According to the lower dialysate compartment, glucose concentrations in the dialysis systems were maintained in a density dependent manner at between ~ 2.2 g/L and 3.0 g/L, which was nearly the original concentration of the culture medium (Fig. 3A). These results showed that in terms of glucose delivery, the dialysis systems were capable of supporting feeding requirements for high-density culture.

The lactate concentration in the dialysis-culture system was successfully maintained at a lower range than critical, which can lead to cell damage and reduce pluripotency (Fig. 3B)^{19,20}. Additionally, we

confirmed this by higher lactate accumulation in groups without dialysis and with and without 24-h medium replacement, in which lactate concentrations rose above the critical limit.

Growth factor accumulation in culture medium

Without the necessity for complete medium replacement in the dialysis-culture system, we observed accumulation of exogenous growth factors, such as fibroblast growth factor 2 (FGF2), during the expansion period. By contrast, conventional daily replacement of the medium completely removed the remaining FGF2 (Fig. 4A). A similar phenomenon was observed with insulin accumulation, which accumulated in the dialysis culture and promoted continuous hiPSC proliferation relative to the other culture systems (Fig. 4B). However, we found that transforming growth factor- β 1 accumulation decreased over time in the dialysis culture along with an increase in cell number (Fig. 4C).

Additionally, endogenous autocrine activity successfully accumulated endogenous autocrine factors produced by the cells. This accumulation was confirmed by the existence of NODAL in the culture medium. NODAL is a member of the TGF- β family and is natively secreted by PSCs²¹. Together with other TGF- β family members, such as TGF- β 1 and activin A, a sufficient amount of NODAL is important to regulate pluripotency^{21,22}. Because NODAL acts within the same pathway, it might represent a substitute for TGF- β 1 in maintaining pluripotency, suggesting that NODAL accumulation can contribute to the total cost reduction of the dialysis system by limiting the need for TGF- β 1. According to the medium formulation in this study (Table 1), we did not exogenously supplement NODAL, which was confirmed by its absence in the culture medium at day 0. These results showed that at the end of the expansion, NODAL concentration increased at higher inoculation densities in culture medium from dialysis culture based on its accumulation and relative to levels in other culture systems both with and without medium replacement (Fig. 4D).

Table 1. The medium formulation used in this study

Culture medium component	Molecular Weight	Concentration
<i>Basal Medium Component</i>		
DMEM/F-12 (Life technologies, USA)	< 1 kDa	2 ml (Culture compartment)
Carbohydrate		
Amino Acids		
Inorganic salt		15 ml (Dialysate compartment)
Vitamins		
Buffer		
Antioxydant		
Other small molecule component		
<i>Supplemented growth factors</i>		
FGF2 (Life technologies, USA)	18 kDa	100 ng/mL
Insulin (Life technologies, USA)	5.74 kDa	19.4 μ g/mL
Transferrin (Life technologies, USA)	80 kDa	10.7 μ g/mL
TGF β -1 (Life technologies, USA)	25 kDa	2 ng/mL
FP003 (Nissan Chemical, Japan)	500 kDa	2%

High-density culture improves pluripotency/differentiation

Characterisation of hiPSCs after expansion showed that the aggregates growing in the dialysis culture exhibited better pluripotency. Although early identification of the pluripotent state by alkaline phosphatase staining showed similar results (Fig. 5A), fluorescence-associated cell sorting (FACS) analysis showed higher stage-specific embryonic antigen 4 (SSEA-4)-positive cells in the higher-inoculum-density groups of dialysis culture (Fig. 5B). In general, most pluripotency markers, such as SRY-box transcription factor 2 (SOX2), octamer-binding transcription factor 4 (OCT4), and NANOG, were upregulated in dialysis culture as compared with conventional suspension culture with medium replacement. Among the selected genes, expression levels of *NANOG* were significantly higher along with increasing cell density and mainly in the highest inoculation density of dialysis culture (Fig. 5C). Immunostaining confirmed the significant differences in these markers at the protein level between the highest inoculation density in the dialysis culture and the density used for expansion during regular medium replacement without dialysis (Fig. 5D).

Additionally, the hiPSC aggregates that previously grew in dialysis culture showed higher differentiation capability into a specific cell type, which reconstituted three germ layers with different morphological properties mainly in the higher-inoculum cell density (Fig. 6A). This population exhibited better gene-expression levels of lineage-specific markers for ectoderm (*orthodenticle homeobox 2 (OTX2)* and *NESTIN*) (Fig. 6B), mesoderm [*T-box transcription factor T* (Brachyury) and *runt-related transcription factor 1 (RUNX1)*] (Fig. 6C), and endoderm (*SOX17*, *C-X-C chemokine receptor 4 (CXCR4)*, and *GATA-binding protein 4 (GATA4)*] (Fig. 6D). Morphologically, the spherical hiPSC aggregates were altered into organoid-like embryoid bodies, followed by distinguished morphological shifting observed after the random differentiation assay. This population of embryoid bodies showed less cavitated structure in higher-inoculation-density dialysis culture during the previous expansion. The cavitation phenomenon is often related to cell damage during aggregate expansion, which potentially decreases pluripotency^{23,24}.

Discussion

One of the main problems in clinical application of PSCs is the high cost associated with their production, with recombinant growth factors representing the most expensive aspect of medium formulation for PSC culture. To save growth-factor usage, strategies have been employed to retain the accumulated growth factors by using dialysis-based culture systems. However, most of these large culture systems are not well optimised to push their potential to enable low-cost expansion by increasing cell density (i.e., $\geq 1 \times 10^7$ cells/mL)²⁵. High-density culture might also increase additional risks, such as high mechanical stress due to aggregate collision¹⁸ and excess agglomeration¹⁷. To address this problem, we designed a miniaturised dialysis-culture system for first-line evaluation of high-density culture. Our findings showed that this system was able to support the production of hiPSCs in high-density volumetric yield up to $\sim 32 \times 10^6$ cells/mL and with decent pluripotency preservation and differentiation capability. Additionally, adequate hiPSCs were acquired by reductions in mechanical stress and excess agglomeration via the

addition of FP003 biopolymer solution. The increase in final cell density might allow a reduced requirement for expensive growth factors^{8,9}. The dialysis culture mimicked *in vivo* conditions, where the production of autocrine factors is utilised in compact, three-dimensional tissue receiving a continuous supply of nutrition from the blood. Mimicking this phenomenon in an *in vitro* system allows acquisition of healthy and high-quality PSCs in a cost-effective manner through maintenance of their autonomous homeostasis in high-density cell culture. This culture system offers new insight into utilisation of exogenous and endogenous growth-factor accumulation in a cost effective dialysis-culture system (Supplementary Fig. 2).

We observed successful accumulation of several exogenous growth factors in the dialysis-culture medium to support further proliferation. We used the simple medium formulation developed by Chen et al.²⁶ and based on the minimum components required for PSC culture, including Dulbecco's modified Eagle medium (DMEM)/F12 basal medium and supplementation with insulin, transferrin, TGF- β 1, and FGF2 (Table 1)²⁷. Similar to that used for other PSC cultures, this medium includes a large amount of FGF2²⁸ and insulin²⁹, whereas TGF- β 1 is often supplemented in small amounts to support the pluripotency³⁰. A previous study reported that some autocrine factors, such as FGF2³¹⁻³³, TGF- β 1³⁴⁻³⁶, and insulin-like growth factors³⁷⁻³⁹, are secreted by stem cells to support their homeostasis, which is reinforced by the addition of exogenous FGF2 and insulin to the culture medium. However, the remaining excess growth factors, such as insulin and FGF2, are usually removed when the medium is replaced. Therefore, utilisation of accumulated insulin and FGF2 by dialysis culture might further reduce costs. Additionally, TGF- β 1 is significantly depleted over time in dialysis culture, making it insufficient to support high-density hiPSCs culture. Although recent studies show that PSCs can still proliferate and maintain their pluripotency in the absence of TGF- β 1 and FGF2^{40,41}, PSCs cultured in their absence are less dependent on glycolytic pathways, which reduces their proliferation⁴⁰. By contrast, in the present study, we showed that gene expression associated with pluripotency remained enhanced, suggesting that support might come from other autonomous regulatory pathways.

The endogenous growth factors secreted by hiPSCs affect pluripotency maintenance and proliferation during high-density expansion. Previous reports show that better cellular metabolism and yield can be achieved by increasing inoculation density⁴². Increases in cell density might also play an important role in maintaining pluripotency through improved accumulation of endogenous growth factors and their autocrine derivatives. To confirm their advantageous accumulation, we chose NODAL as a selected candidate factor to represent the accumulation of important hiPSC autocrine factors. NODAL and TGF- β 1 levels are mediated by a similar mechanism via SMAD2/3 signalling to maintain PSC pluripotency by enhancing NANOG upregulation to maintain balanced differentiation into an ectoderm or endoderm lineage (Fig. 7)^{22,43-47}. In response to these mechanisms, we found that NODAL levels increased in a density dependent manner and consistently correlated with *NANOG* gene-expression patterns. This indicated that NODAL might reconstitute the role of TGF- β 1 in maintaining pluripotency and retaining differentiation potential into three human embryonic germ layers cell type.

As expected, medium refinement by the dialysis-culture system successfully supported further hiPSC proliferation. To compensate for the accumulation of toxic metabolic products resulting from cellular metabolism, small molecules, such as lactate, need to be frequently removed from the culture medium when performing high-density culture. Continuous lactate removal using dialysis fed-batch support successfully maintained lactate concentrations in the upper culture compartment below the critical concentration, thereby eliminating the growth-limiting conditions caused by lactate accumulation while supplying glucose. Moreover, we observed an exponential growth curve associated with the maximum cell density and its relationship with lactate concentration, suggesting that there remains potential for further increases in cell density for this system. Interestingly, a significant amount of glucose remained, even without use of the dialysis culture and in the absence of medium replacement, from the first day of culture, although the cell number was extremely depleted relative to the early days of the culture. This suggested that lactate accumulation rather than glucose starvation was the primary limiting factor for cell proliferation in suspension culture. This phenomenon occurs, because hiPSCs exhibit higher anaerobic respiration and metabolism relative to their differentiated cell types. Consequently, lactate secretion was much higher than glucose consumption due to the dependency of PSCs on glycolysis for their energy demands⁴⁸.

Addition of the FP003 biopolymer successfully created a low-shear-stress culture environment and prevented hiPSC agglomeration. PSCs exhibit a high tendency to aggregate when cultured in suspension⁴⁹; therefore, we utilized dynamic conditions to control aggregate size and prevent excess agglomeration^{2,49-52}. The lack of agitation can result in large aggregates with necrotic areas often caused by unequal exposure to nutrition and secreted toxic metabolites due to mass-transfer limitations and failure to reach some aggregated cells⁵³. However, the excessive mechanical stress resulting from this condition can also potentially induce unwanted spontaneous differentiation and affect cellular viability^{51,54}. To prevent the negative effect of this agitation, we added FP003 to the culture medium. Otsuji et al.¹⁷ revealed the potential of FP003 for preventing agglomeration in static hiPSC suspension culture. Interestingly, addition of FP003 did not significantly affect the transfer of micromolecules, such as glucose and lactic acid, in our dialysis-culture system. Additionally, comparison with rotary suspension culture indicated that FP003 significantly decreased cellular injury, possibly due to increased culture-medium viscoelasticity via modification of rheological properties. In suspension culture, low-acyl gellan gum in FP003 forms a microfiber-network structure that keeps cells floating in a well-dispersed manner and blocks agglomeration between aggregates (Fig. 1B). As a result, the high-density expansion resulted in a uniform aggregate population with decent growth in size. Moreover, the 4-day culture grew to a tolerable size that allowed the transfer of oxygen, nutrition, and waste products throughout the aggregates. This condition was also confirmed by the absence of necrotic areas inside the aggregates.

This smaller-scale dialysis-culture platform would be useful for evaluating the feasibility of dialysis operations in PSC culture using minimal resources. The complexity of currently available large-scale culture systems makes technical operations during PSC culture challenging to optimise. Additionally, medium refinement could overcome the requirement for a high metabolic rate demanded by PSCs in the

forms of nutrition transfer, waste-product removal, and growth-factor supplementation. This culture system allows various culture conditions simultaneously, enabling optimization of parameters, such as cell metabolism, growth-factor use, device permeation associated with cell growth, pluripotency, and differentiation capacity. Moreover, mathematical simulations describing the metabolic kinetics associated with both exogenous and endogenous growth factors represent useful references when designing larger-scale dialysis-culture systems. However, larger-scale systems, such as slow-turning lateral vessels⁸ or stirred-suspension bioreactors with dialysis support⁹, might still have several problems related to oxygenation and mechanical stress that are broadly design dependent. Although this study was limited to the proliferation phase, the potential reductions in production costs associated with utilisation of both endogenous and exogenous growth factors might contribute to future optimisation of larger-scale and cost-effective hiPSC-production methods. Furthermore, such methods could increase PSC proliferation and differentiation, which require optimal growth-factor and autocrine utilisation.

In summary, we described a dialysis-culture system that promotes efficient hiPSC expansion at a high density while maintaining their pluripotency and differentiation capacities by facilitating growth-factor accumulation together with important autocrine factors under a low-hydrodynamic-stress culture environment. This study provides novel insight into the feasibility of minimum growth-factor usage, which might significantly promote cost reductions in dialysis-based PSC production.

Methods

Dialysis device for high-density suspension culture

The dialysis-culture system included upper and lower dialysate compartments (Fig. 1A). A 40-µm mesh bottom-cell strainer (PluriSelect, Leipzig, Germany) was used as the upper culture compartment, with this insert modified by cutting and removing the bottom mesh layer. To selectively permeate nutrition or waste products, we affixed a 12-kDa MWCO Spectra/Por 4 dialysis membrane (Spectrum Chemical, New Brunswick, NJ, USA) to the bottom side of the strainer using alkyl-α-cyanoacrylate-based surgical-grade tissue adhesive (Aron Alpha A; Daiichi Sankyo, Japan). The upper compartment was then placed in 6-well plates (Corning, Corning, NY, USA) as dialysate-compartment inserts. As a control condition, the cell strainer was directly affixed to the bottom surface of six-well untreated plates (Iwaki, Tokyo, Japan) using Aron Alpha A tissue adhesive (Daiichi Sankyo). All devices were sterilised using an ethylene oxide gas steriliser before use.

Permeability test in cell-free conditions

To evaluate the effect of FP003 on device performance, glucose and lactic acid measurements were performed during a 12-h penetration test under cell-free conditions. Low-glucose DMEM (2 mL; Sigma-Aldrich, St. Louis, MO, USA) containing 0.2% FP003 and 0.8 g/L lactic acid (Sigma-Aldrich) was added to the upper culture compartment, and 15 mL of high-glucose DMEM (Sigma-Aldrich) was placed in the lower dialysate compartment. The dialysis-culture system was then placed in a 120-rpm rotary shaker. We

collected 50- μ m samples of the medium every 2 h and measured changes in glucose and lactic acid concentration using a YSI 2950 multipurpose bioanalyzer (YSI, Yellow Springs, OH, USA).

To test the ability of the device to accumulate macromolecules in the upper culture compartment and differences in permeation of differently sized molecules, 2 μ g/mL FITC (Sigma-Aldrich) at different molecular weights (4, 10, and 20 kDa) was added to the upper culture compartment. This experiment was performed using DMEM basal medium in both compartments, similar to the penetration test for micromolecules. Medium samples were collected after 12 h, and FITC concentration was measured using a Wallac Arvo SX 1420 multilabel counter (PerkinElmer, Waltham, MA, USA).

Monolayer hiPSC culture

The TkDN-4M hiPSC line was provided by the Stem Cell Bank, Centre for Stem Cell Biology and Regenerative Medicine, University of Tokyo (Tokyo, Japan)⁵⁵. Cells were cultured and maintained in vitronectin-coated tissue-culture dishes using complete supplemented Essential 8 (E8) culture medium (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer instructions.

LDH assay

To evaluate cell injury caused by hydrodynamic stress, we determined LDH leakage from cells. hiPSC aggregates were formed by inoculation of a 2×10^6 single-cell suspension/well in 6-well plates for 24-h culture in complete medium supplemented with 0.2% free fatty acid-bovine serum albumin (BSA) in a 90-rpm rotary shaker. The formed aggregate was transferred to 12-well plates with 2 mL complete medium with or without 0.2% FP003 and cultured with shaking at 120 rpm. The cells were harvested by sedimentation in 10-mL tubes, and the supernatant was isolated for LDH analysis using an LDH cytotoxic assay kit (Wako Pure Chemical, Osaka, Japan) according to manufacturer instructions.

High-density hiPSC suspension culture

To increase simplicity and reduce costs, the culture medium was divided into two main components. Basal medium included DMEM/F12 (Life Technologies, Carlsbad, CA, USA) supplemented with complex micromolecule nutrition and macromolecule growth factors (Table 1). hiPSC aggregates were formed by inoculation of a 2×10^6 single-cell suspension/well in 6-well plates for 24-h culture in complete medium supplemented with 0.2% free fatty acid-bovine serum albumin in a 90-rpm rotary shaker. On the day of culture, the dialysis-culture insert was placed in 6-well plates, and the dialysis membrane was activated by pre-wetting the dialysis layer using 2 mL of sterile H₂O 15 min to 30 min before starting the culture. After removal of the H₂O, 15 mL of DMEM/F12 basal medium was added to the lower dialysate compartments. The hiPSC aggregates were harvested and transferred to the upper culture compartment using 2 mL of DMEM/F12 basal medium along with 0.2% BSA and 0.2% FP003, which were added according to manufacturer instructions to the upper culture compartments on the first day of culture (Fig. 4B). The hiPSCs expansion was performed on a 120-rpm rotary shaker for 4 days with daily replacement of basal medium in the lower dialysate compartment and supplementation with 2% growth factors in groups not undergoing medium replacement in the upper culture compartment. To simplify the

operation, growth-factor supplementation consisting 100 ng/mL FGF2, 19.4 µg/mL Insulin, 10.7 µg/mL Transferrin, and 2 ng/mL TGFβ-1 was added only in upper culture compartment everyday, and the culture medium was not replaced to preserve the growth factors accumulation. (Fig. 1). As a control group, suspension culture using the same conditions was performed without dialysis support and with or without medium replacement every 24 h.

Morphological analysis

Each of the aggregate groups was moved to 12-well plates, and macroscopic and microscopic images were obtained by light microscope (Olympus, Japan), and aggregate diameter was analysed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell counting

After morphological analysis by light microscopy (Olympus, Tokyo, Japan), the aggregates were collected, centrifuged at 1000 rpm for 3 min, and the supernatant was removed from the tube. To obtain single cells, 1 mL of TrypLE dissociation reagent (Thermo Fisher Scientific) was added to the aggregates and incubated for 10 min to 15 min at 37 °C, followed by homogenisation by gentle pipetting. The cells were then diluted 100-fold in phosphate-buffered saline (PBS) and counted using a haemocytometer (Tatai-type; Japan).

Measurement of glucose and lactate concentrations

Glucose and lactate concentrations during the 4-day culture in different high-density configurations was measured by collecting 50-µL samples every 24 h and assessment using a YSI 2950 multipurpose bioanalyzer (YSI).

Haematoxylin-eosin staining of cross-sectioned aggregates

Aggregates were collected and fixed with 4% paraformaldehyde (Wako Pure Chemical) in PBS for 1 h at room temperature, washed in PBS, and cultured in 30% PBS-sucrose solution (Wako Pure Chemical) at 4 °C overnight. The sucrose solution was removed, and the aggregates were placed in a cryomold after embedding with Tissue-Tek OCT compound (Sakura, Alphen aan den Rijn, The Netherlands) at - 20 °C until hardened. Thin sections (10 µm) were obtained using a cryostat and mounted onto glass slides for haematoxylin-eosin staining.

Measurement of growth-factor concentrations

A 100-µL solution containing 1 g/L FGF2 primary antibody (R&D Biosystems, Minneapolis, MN, USA) was immobilised in each well of 96-well enzyme-linked immunosorbent assay (ELISA) plates and incubated at 37 °C for 3 h. The plates were then washed with 200 µL of PBS-Tween-20 (PBST), followed the addition of 100 µL of blocking buffer and incubation for 1 h at 25°C. Samples (100 µL) were collected, and standard solution was used as a replacement and incubated for 3 h at 37 °C. The plates were washed with PBST, and 100 µL/well of 1-g/L FGF2 diluted secondary antibody (R&D Biosystems) was added and incubated for 2 h at 37 °C. The plates were then washed with PBST, and 50 µL of streptavidin-horseradish

peroxidase solution (R&D Biosystems) was added and incubated at room temperature for between 30 min and 1 h. To obtain a colour reaction, the plates were washed with PBST, and 100 µL of a colour solution comprising 2.5 mg *O*-phenylene diammonium chloride and 0.5 µL/mL H₂O₂ citrate buffer were added to each well, followed by incubation for 30 min at room temperature. The colour reaction was stopped by adding 50 µL/well H₂SO₄ (4N). Fluorescence intensity was measured using a Wallace Arvo SX 1420 multilabel counter (PerkinElmer). The same method are applied for Insulin and TGFβ-1. The antibodies used in this analysis are listed in Supplementary table 1.

NODAL detection was performed using a human NODAL ELISA kit (LSBio, Seattle, WA, USA) according to manufacturer instructions. Fluorescence intensity was measured using a Wallac Arvo SX 1420 multilabel counter (PerkinElmer).

Random differentiation assay

The random differentiation assay was performed by transferring 50 to 100 hiPSC aggregates to a 60-mm tissue-culture dish (Iwaki) with 5 mL of DMEM/F12 medium containing 10% foetal calf serum for 7 days in a static suspension culture. The differentiated embryoid bodies were observed under a light microscope (Olympus), and gene expression of tri-lineage markers was measured using a StepOnePlus quantitative reverse transcription polymerase chain reaction (qRT-PCR) kit (Thermo Fisher Scientific).

qRT-PCR

mRNA was isolated using Trizol reagent (Life Technologies) and reverse transcribed using ReverTra Ace master mix (Toyobo, Osaka, Japan), followed by qPCR analysis using Thunderbird SYBR qPCR mix (Toyobo) according to manufacturer instructions. Gene amplification was performed using a StepOnePlus kit (Thermo Fisher Scientific) following the reagent manufacturers instruction. The primer sequences used in this analysis are listed in Supplementary Table 2.

Alkaline phosphatase staining

To detect intracellular alkaline phosphatase, aggregates were harvested and stained with an AP staining kit II (Stemgent, Cambridge, WA, USA), with several modifications for aggregate staining using gentle rotational agitation. After washing with 1 × PBST, the aggregates were incubated with 2 mL fixed solution in 6-well plates at room temperature for 30 min and then washed with 1 × PBST and incubated with 3 mL AP substrate solution in the dark for 90 min, followed by washing with 1 × PBST. The stained aggregates were observed under a light microscope (Olympus).

FACS analysis

hiPSC aggregates were harvested and dissociated by 30-min incubation in Accutase solution at 37 °C. The cell suspension was then filtered through a 40-µm cell strainer (Corning) and counted using a haemocytometer (Tatai, Japan). Cells (1 × 10⁶) were fixed with 4% paraformaldehyde for 3 h and stained with the Alexa Fluor-conjugated SSEA-4 antibody (Biolegend, San Diego, CA, USA). FACS was performed using a Coulter Epics flow cytometer (Beckman Coulter, Brea, CA, USA).

Immunostaining of sectioned aggregates

Aggregates were collected and fixed with 4% paraformaldehyde overnight at 4 °C. To preserve the morphology during cross-sectioning, the aggregates were incubated with an 8% sucrose solution overnight following sample fixation in a cryomold using Tissue-Tek OCT compound (Sakura) and freezing for at least 6 h at – 80 °C. A 20-μm cross-section was obtained using a Leica CM1850 cryostat (Leica Biosystems, Wetzlar, Germany) and fixed on poly-L-lysine-coated slides (Sigma-Aldrich). The sections were then stained using a pluripotency marker kit (R&D Biosystems) according to manufacturer instructions.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (v.8.3.0; GraphPad Software, San Diego, CA, USA). Statistical significance was determined by one-way analysis of variance with Tukey's multiple comparison test. A $p < 0.05$ was considered significant.

Declarations

Competing interests

The authors declare no competing interest in this research

Author contributions

Y.S., I.H., and F.G.T. designed the experimental project. F.G.T. and Q.Y.L. performed experiments and analyzed the data. M.H. and M.M. provide the FP003. F.G.T., I.H., Y.S., M.I. and Y.K. design the dialysis device. F.G.T., Q.Y.L., I.H., M.N., Y.S. wrote the manuscript.

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Figures

Figure 1

Main concept, mechanism and evaluations of simple dialysis culture with FP003 addition. (A). The schematic illustration of high-density hiPSCs expansion in a miniaturized dual compartment dialysis system. The transfer of small molecule such as (B). glucose and (C). lactic acid also measured during 12 hours. The cell-free penetration assays of various large molecule : (D). 20 kDa FITC, (E). 10 kDa FITC, (F). 4 kDa FITC. (F). The mechanism of the FP003 addition in the culture medium to prevent excess

agglomeration and mechanical stress. (H). The mechanical stress-induced cellular injury was reduced during 6 hours culture with the addition of FP003 (blue) compared to the normal culture medium (red). Mean + or - standard deviation are indicated in each graph (n=3). Statistical significance : ****p<0.0001; **p<0.01; *p<0.05.

Figure 2

Experimental procedure, aggregates morphology, and proliferation. (A) In this study, the aggregates formed by culturing the single cell in 6 well plates in 24 hours before moved into each culture system. (B). The different aggregates morphology among different culture configuration and inoculum density. (C). The aggregates growth was evaluated by comparing the diameter between aggregates population expanded in D/MR(-) and C/MR(+) (N=500). The diameter of aggregates population in C/MR(-) was excluded due to the limiting number of aggregates caused by lactate toxicity induced-cell death at the end of the expansion. (D). The cross sectioned-histological analysis of resulted hiPSCs aggregates. (E). The fold expansion and proliferation curve of hiPSCs during expansion. Mean standard deviation are indicated in each graph (n=3). *Inoculum density **High-density mammalian cell culture referring to Griffiths et al. (1990).

Figure 3

The concentration of glucose and lactate in the culture medium which partially reflects the culture environment condition in different configuration. (A) The glucose concentration and (B). The lactate concentration in culture medium. *the glucose concentration in culture medium **critical lactate concentration for PSCs culture mentioned by Ouyang et al. (2007) and *** critical lactate concentration for PSCs culture which mentioned by Horiguchi et al. (2018)

Figure 4

The concentration of the growth factors in the culture compartment during 4 days of hiPSCs expansion: (A). FGF2, (B). Insulin, and (C). TGFβ-1. (D). The Nodal concentration was measured at the end of expansion period. Mean standard deviation are indicated in each graph (n=3). Statistical significance : ****p<0.0001; ***p<0.001

Figure 5

The characterization of the pluripotent state is evaluated after 4 days of expansion by different pluripotency markers. (A). The aggregates staining by basic pluripotent marker alkaline phosphatase. (B). SSEA4 protein surface marker by FACS and (C). Gene expression level of the three main transcription factors regulating pluripotency (OCT4, SOX2, and Nanog). The C/MR(-) was excluded from the analysis due to the insufficient cell yield. . Mean standard deviation are indicated in each graph (n=3). Statistical significance: ***p<0.001; **p<0.01; *p<0.05; (D). The crosssectioned immunostaining of OCT4, SOX2, and Nanog of the highest density group of D/MR(-) and C/MR(+).

Figure 6

The differentiation potential demonstrated by performed random differentiation assays of the embryoid bodies. (A). The differentiated embryoid body morphology derived from the hiPSCs expansion. These embryoid bodies showing the different gene expression levels of the trilineage marker of the germ layers: (B) ectoderm (OTX2, Nestin), (C). mesoderm (Brachyury, RUNX1), and (D). endoderm (SOX 17, CXCR4, and GATA4). The non-dialysis culture group without medium replacement was excluded from the analysis due to the insufficient yield. Mean standard deviation are indicated in each graph (n=3). Statistical significance: ***p<0.001; **p<0.01; *p<0.05

Figure 7

The mechanism of The Nanog regulation by the TGFB family (such as TGFB-1 and Nodal) through SMAD2/3 which affecting the balance of the pluripotency by controlling ectoderm and endoderm lineage development.

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