Operational Design for High-density Culture of Human Induced Pluripotent Stem Cells in Suspension

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By
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Preface

This study was conducted under the supervision of Professor Masahiro Kino-oka at the Department of Biotechnology, Graduate School of Engineering, Osaka University, Japan from 2012 to 2017.

The main objective of this thesis is to improve the operational design of human induced pluripotent stem cells (hiPSCs) culture for obtaining high-density of hiPSCs through the simplification of culture operations, as well as low supplementation of growth factors to promote a cost-effective platform in suspension. The most interesting focus was the investigating the growth properties of hiPSCs to set up a boundary condition for preferential growth in single aggregate culture, revealing the temporary effect of botulinum hemagglutinin (HA) for disrupting E-cadherin mediated cell-cell connections for the establishment of an *in situ* simple method of hiPSC aggregate break-up in bioreactor for obtaining high-density, and the establishment of a culture medium refinement method by dialysis for recycling autocrine factors, as well as reducing the consumption of cytokines for the expansion of hiPSCs in suspension culture. The author believes that the findings obtained in this study will lead to the establishment of an automatic, closed, and cost-effective platform for large-scale expansion of hiPSCs in suspension culture.

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Contents

Abstract

Large numbers of human induced pluripotent stem cells (hiPSCs) are required for cell therapy applications. Suspension culture of hiPSCs is superior to obtain large numbers of cells in comparison to adherent culture since high cell number (10^8-10^9 cells), as well as better control of process parameters can be obtained in stirred suspension bioreactor. In the conventional suspension culture, hiPSCs show initial cell loss during seeding due to single cell dissociation, low growth rate due to aggregate heterogeneity, and size limitation during late-stage of culture which results in low cell density. Moreover, toxic metabolites (lactic acid, ammonium, etc.) accumulate in the culture medium which requires frequent medium change which along with remove autocrine factors from medium in conventional suspension culture. Since autocrine factors potentiate the growth of hiPSCs, removal of these components from culture medium also results in low cell density in suspension culture. By considering these limitations, this study aimed at establishing a simple and scalable method by improving the operational design of hiPSC culture for obtaining high cell density with efficient recycling of autocrine factors in suspension.

In chapter 1, size- and time-dependent growth properties of hiPSC aggregate was investigated for setting up a boundary condition in which hiPSCs can maintain high growth rate in suspension culture. hiPSCs showed size-dependent growth heterogeneity during early-stage, and time-dependent collagen type I accumulation on the aggregate periphery during late-stage of culture. Thus, considerations for initial cell number and culture time were important to maintain the balance between the secretion of autocrine factors, and the accumulation of extracellular matrix on the aggregate periphery to achieve preferable growth of hiPSCs in single aggregate culture.

In chapter 2, a simple method for *in situ* break-up of hiPSC aggregates was established by revealing the temporary effect of botulinum hemagglutinin (HA) for the disruption of E-cadherin mediated cell-cell connections in suspension culture. This method allowed high-density culture of hiPSCs through the break-up of aggregates into small sizes without initial cell loss during seeding in suspension culture. It also provided the simplification of culture operations without enzymatic treatment or washing in suspension culture.

In chapter 3, a culture medium refinement method was established by using a dialysis filter by which toxic metabolites (lactic acid, ammonium, etc.) was removed from the medium, and bFGF, TGF-β1, and other autocrine factors were recycled efficiently for the expansion of hiPSCs in suspension culture. This method allowed expansion of hiPSCs by changing the medium only once and recycling growth factors, as well as other autocrine factors that promoted a cost-effective platform in suspension culture.

In chapter 4, medium refinement system was combined with aggregate break-up by HA during late-stage of culture for obtaining high density of hiPSCs in suspension. By using this system, higher growth rate as well as higher cell density was obtained by a minimum supplementation of growth factors which provided 21-times higher yield on culture medium in comparison to the conventional suspension culture.

Overall, a simple and scalable method for high-density culture of hiPSCs was established by setting up a boundary condition for maintaining high growth rate, and breaking up the aggregates into small sizes with HA in suspension culture. This method also provided the simplification of culture operations without enzymatic treatment or washing in suspension. Moreover, the integration of medium refinement system with aggregate break-up by HA provided high-density of hiPSCs with high-yield on culture medium by efficient recycling of

autocrine factors in suspension culture. Thus, the improved operational design of hiPSC culture provided a cost-effective platform for obtaining high cell density with the simplification of culture operations which will promote simple closed-system for large-scale expansion of hiPSCs in suspension culture.

General introduction

After the first isolation of human induced pluripotent stem cells (hiPSCs) by Yamanaka group (Takahashi *et al*., 2007), application of these cells are increasing day by day as regenerative medicine for cell replacement therapies (Schulz *et al*., 2012), tissue engineering (Badylak *et al*., 2011), and drug discovery (Wernig *et al*., 2007) due to their pluripotency and self-renewal capacity. hiPSCs are reprogrammed from skin cells of similar human leukocytes antigen (HLA)-type healthy donors and stored in master cell bank (**Fig. 1**). From the master cell bank, cells are collected and expanded to make working cell bank. After expansion of hiPSCs from working cell bank, cells are differentiated into desired cell type, and transplanted into the patient as a regenerative medicine. To realize their potentiality, large-scale expansion of hiPSCs is important as practically more than 10^9–10^{10} cells are required for cell therapy applications (Lock and Tzanakakis, 2007; Jing *et al*., 2008; Serra *et al*., 2010; Kehoe *et al*., 2010; Olmer, *et al*., 2010).

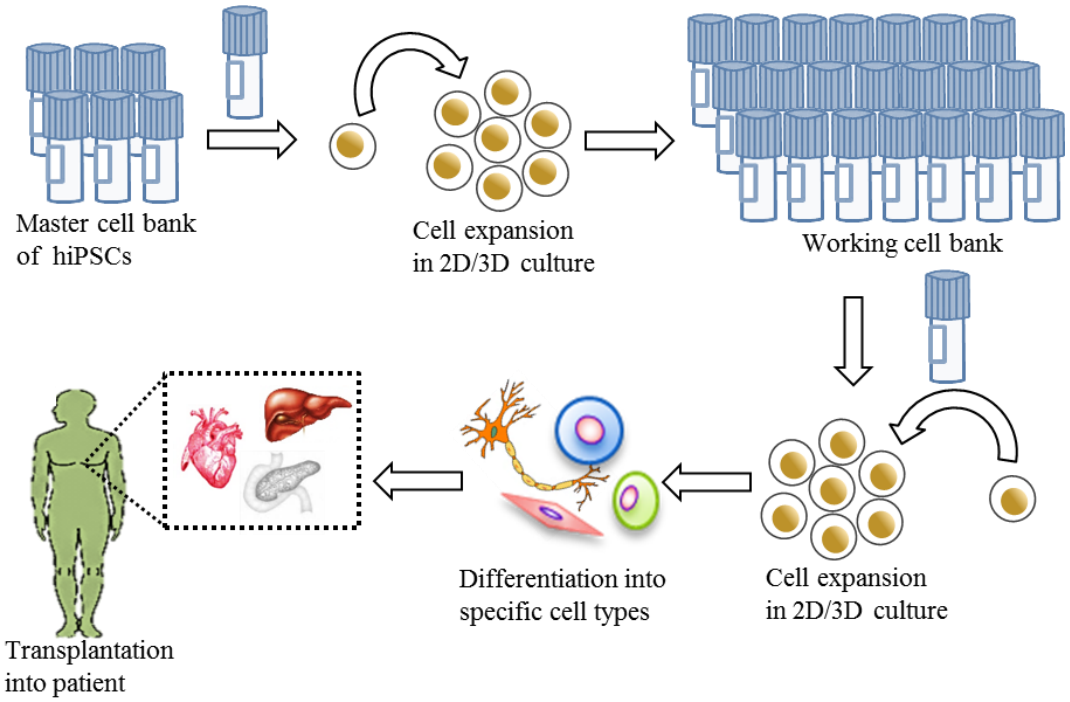


Fig. 1 Schematic diagram showing the application of hiPSCs as regenerative medicine.

Before starting large-scale expansion of hiPSC in suspension culture, some important points need to be considered, especially operational techniques (culture system, medium feeding regimen, target final cell density, etc), economic feasibility (culture medium), quality and safety of the final harvest are the most important considerations for using hiPSCs in clinical applications (Jenkins and Farid, 2015). To date, there are two main systems for hiPSCs expansion: adherent (2D) and suspension (3D) culture.

Table 1 Comparison of hiPSC culture between adherent (2D) and suspension (3D).

Characteristics	**Adherent Culture (2D)**	**Suspension culture (3D)**
Final cell density	Low	High
Medium consumption	High	Low
Growth surface area	Limited	Not limited
Required culture vessels	High numbers	Single bioreactor
Online sampling	Not possible	Possible
Labor	Intensive	Flexible
Scalability	Low	High
Cell harvesting	Difficult (need to detach from culture surface)	Easy (ready to use as aggregate)

Expansion of hiPSCs is easier in adherent culture in comparison to suspension culture, and large-scale expansion process has already been established by using automatic system, especially in Cell-STAK^{TM}, Nunc cell factory^{TM}, CompacT SelecT^{TM}, as well as by using many other systems. The major limitation in adherent culture is the growth surface area where high cell density of hiPSCs is difficult to obtain. Moreover, culture cost is high due to high consumption of medium in adherent culture (**Table 1**). On the other hand, large-scale expansion of hiPSC in suspension culture is possible as high number of cells can be obtained with low consumption of medium by using a single bioreactor (Zweigerdt *et al*., 2011; Olmer *et al*., 2012).

Although hiPSC can be cultured by many methods in suspension: aggregate, encapsulation, and microcarrier culture, aggregate culture is promising due to self-formation of aggregates from single cells and aggregates are easy to harvest from suspension culture. During initial seeding in suspension culture, single cells are inoculated into the culture vessel, and cells agglomerate to form sphere–shaped aggregates by encountering and connecting with the neighboring cells which is mediated by E-cadherin (Li *et al*., 2012; Li *et al*., 2014) (**Fig. 2**). The size of aggregates increases with the increase in culture time and some of the aggregates spontaneously fuse to form an un-uniform shape (Bauwens *et al*., 2008; Otsuji *et al*., 2014), however, some of the aggregates collapse due to high shear stress. The prolongation of culture time leads to undesired differentiation, as well as cell necrosis inside the aggregate which also cause the difficulty in maintaining proper growth in suspension culture (Sachlos *et al*., 2008). To overcome these difficulties, Singh *et al*. performed passage culture by dissociating the aggregates into single cells with Rho-associated protein kinase (Rock) inhibitor (Watanabe *et al*., 2007), but it could not prevent initial cell loss during seeding in suspension culture (Singh *et al*., 2010).

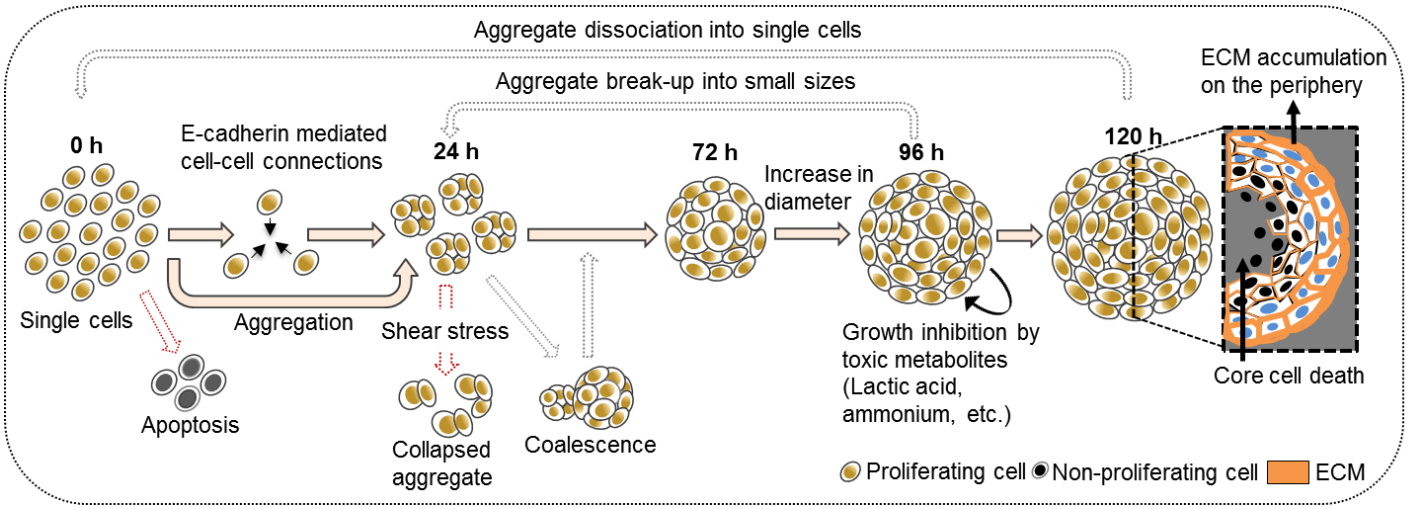
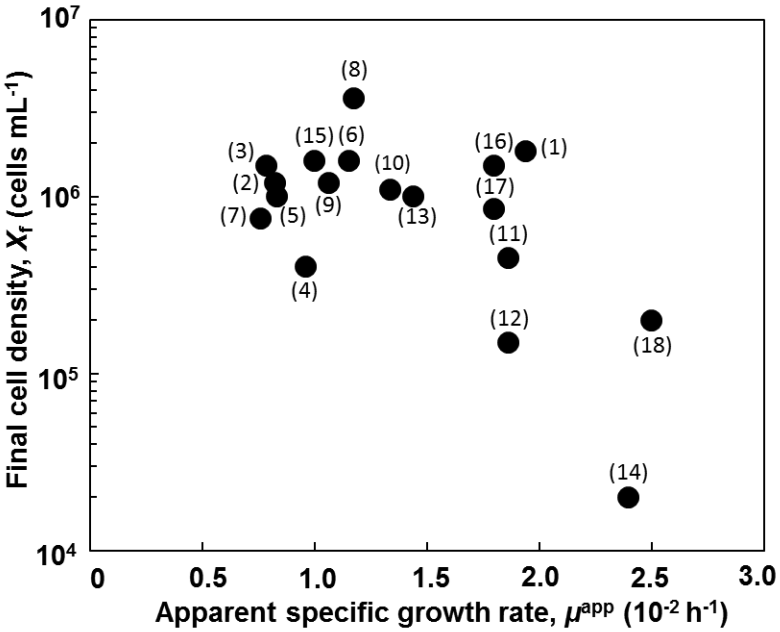


Fig. 2 Schematic illustration showing the growth properties of hiPSC aggregate in suspension culture.



Culture systems	References
Stirred suspension Bioreactor culture	Amit *et al*., 2011 (1)
Stirred suspension Bioreactor culture	Abbasalizadeh *et al*., 2012 (2)
Stirred suspension Bioreactor culture	Olmer *et al*., 2012 (3)
Stirred suspension Bioreactor culture	Matsuura *et al*., 2012 (4)
Stirred suspension Bioreactor culture	Haraguchi *et al*., 2013 (5)
Stirred suspension Bioreactor culture	Wang *et al*., 2013 (6)
Stirred suspension bioreactor culture	Miranda *et al*., 2016 (7)
Stirred suspension Bioreactor culture	Kropp *et al*, 2016 (8)
Stirred suspension Bioreactor culture	Kwok *et al*., 2017 (9)
Stirred suspension Bioreactor culture	Kino-oka lab (10)
Stirred suspension culture in tube	Elanzew *et al*., 2015 (11)
Suspension culture in plate	Olmer *et al*., 2010 (12)
Hydrogel culture system	Lei *et al*., 2014 (13)
Bag culture	Otsuji *et al*., 2014 (14)
96-well V-bottom plate	Kino-oka lab (15)
Micropatterned plate	Kino-oka lab (16)
Flow device	Kino-oka lab (17)
Adherent culture in dish	Kino-oka lab (18)

Fig. 3 Comparison of hiPSCs growth rate and final cell density in suspension culture with other culture systems.

To prevent single cell apoptosis, several techniques were applied for passaging hPSC colony as small clumps by using divalent cations (Ohnuma *et al*., 2014), or EDTA (Beers *et al*., 2012), or sodium citrate (Nie *et al*., 2014) in adherent culture. hiPSC colony can also be passaged by physically cutting into small pieces, and seeding into suspension culture (Son *et al*., 2011). Although many protocols have already been established to expand hiPSC in aggregate suspension culture by seeding single cells with Rock inhibitor in aggregate suspension culture (Watanabe et al, 2007), still there is no established method for passaging hiPSC aggregates into small clamps in suspension culture (**Fig. 3**). The highest cell density obtained in these studies is ranging from 1.0 – 2.0 × 10^6 cells mL^{-1} by frequent changing of medium (Olmer *et al*., 2012; Wu *et al*., 2014). However, accumulation of toxic metabolites increases with the increase of cell density in suspension culture which inhibits the growth of hPSCs (Chen *et al*., 2010). Although these components can be removed by perfusion culture, and hiPSCs can be expanded to a high

density (3.6×10^6 cell mL^{-1}) (Kropp *et al*., 2016), controlling aggregate size is a big challenge during the long-term suspension culture.

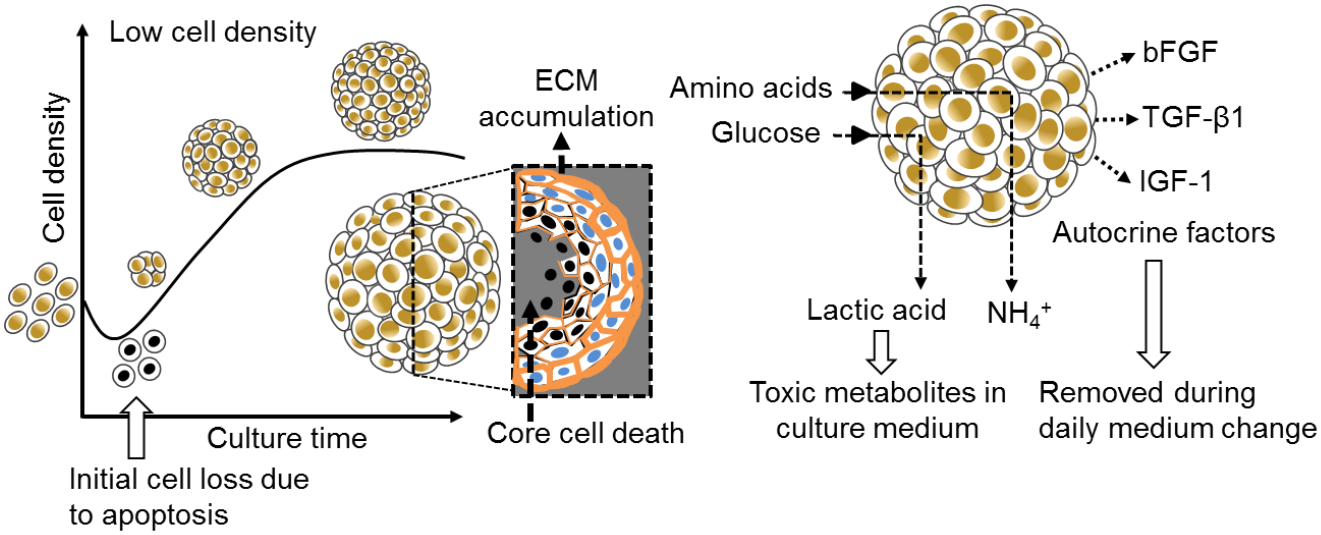


Fig. 4 Major drawbacks of hiPSC aggregate culture in suspension.

The major drawbacks in conventional suspension culture of hiPSCs are: (1) low growth rate due to aggregate heterogeneity, (2) dissociation of aggregates into single cells by enzymatic digestion which results in massive cell loss during seeding, (3) high culture cost as large volume of culture medium is required, and (4) low final cell density due to aggregate size limitation during late-stage of culture (**Fig. 4**). Aggregate size limitation as well as extracellular matrix (ECM)-shell formation hinders the diffusive transport of inductive biochemicals to and from aggregates which triggers the undesired differentiation inside the aggregate causing difficulty in maintaining proper growth of hPSCs in suspension culture. Culture cost depends on various factors, especially medium, process efficiencies, technology choices and resources. Culture cost also depends on growth rate (μ) as well as on final cell density (X_f), because prolonged culture period consumes high volume of medium in suspension culture.

By considering these limitations in conventional suspension culture, therefore, an improved operational design for hiPSC culture was proposed for obtaining high cell density with low supplementation of growth factors in stirred suspension bioreactor (**Fig. 5**). To obtain high density of hiPSCs in suspension culture, the following four chapters were considered.

Chapter 1 describes the effect of aggregate size and culture time on the growth of hiPSCs in single aggregate culture. To evaluate the growth properties of single aggregate of hiPSC, the effect of initial cell number and culture time were investigated quantitatively, and the proliferative ability of cells in the aggregates was elucidated by immunohistological analysis. A boundary condition for preferable growth of hiPSCs was proposed for obtaining high growth rate in single aggregate culture.

Chapter 2 describes the establishment of a simple and scalable method for the expansion of hiPSCs by breaking up the aggregates into small sizes with botulinum hemagglutinin (HA), and elucidation of mechanisms of aggregate break-up in suspension culture. This method allowed high-density culture of hiPSCs through the simplification of culture operations without the need of enzymatic treatment or washing in suspension culture.

Chapter 3 focuses on the establishment of a culture medium refinement system by using a dialysis filter. This method allowed the removal of toxic metabolites, as well as recycling of autocrine factors in the culture medium, and reduced the use of macromolecules (transforming growth factor-beta 1 (TGF-β1), insulin, etc.) for the expansion of hiPSCs in suspension culture. The minimum inhibitory level of lactic acid to hiPSCs, and lactic acid removal by dialysis were also investigated in this study.

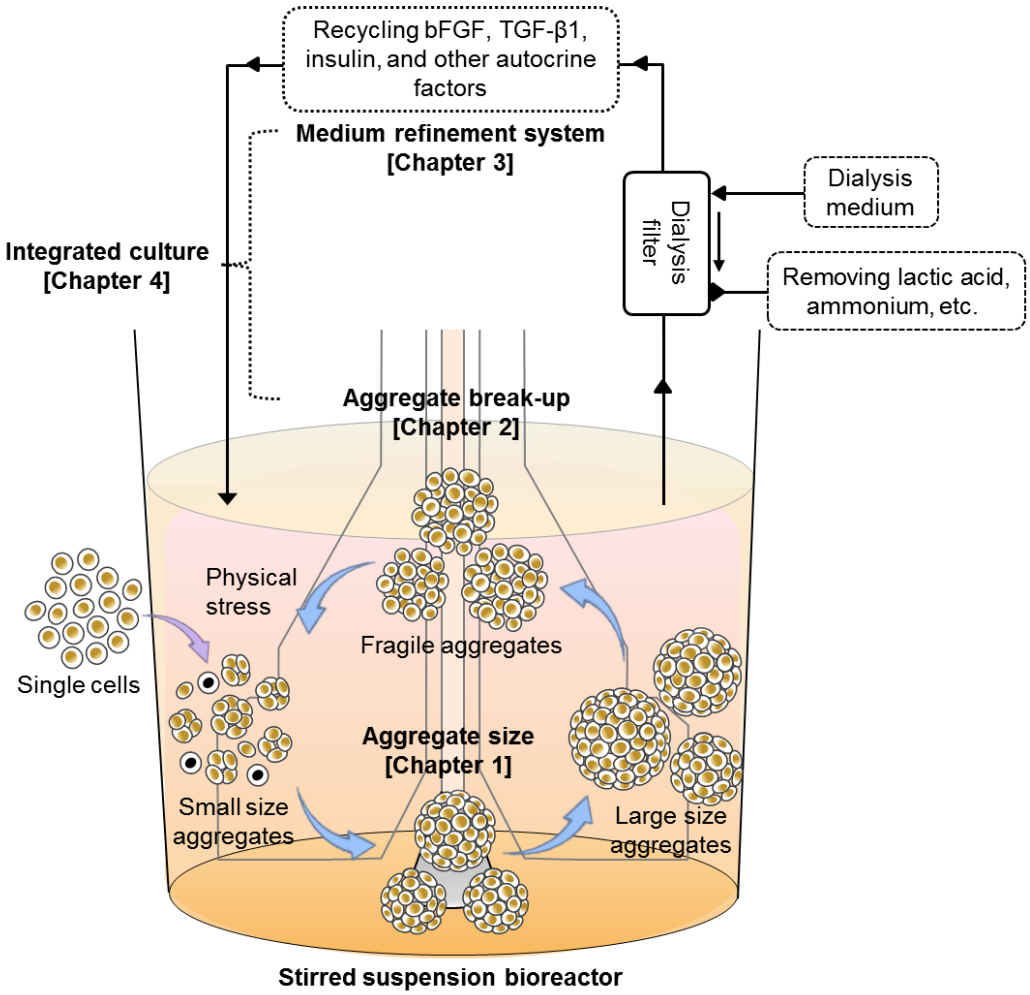


Fig. 5 Schematic diagram showing the strategy for obtaining high cell density in suspension culture by (1) analysis of growth properties of aggregate, (2) aggregate break-up to small sizes, (3) refinement of culture medium by dialysis, and (4) integration of medium refinement with aggregate break-up in suspension culture.

Chapter 4 focuses on the integration of Chapters 2 and 3 to obtain high cell density by breaking up the aggregates into small sizes, and refinement of culture medium for efficient recycling of autocrine factors, and other growth factors to reduce the supplementation of costlier macromolecules in suspension culture. The major growth-limiting factors during late-stage of culture were also investigated in this study.

Chapter 1

Size- and time-dependent growth properties of human induced pluripotent stem cells in the culture of single aggregate

1.1 Introduction

Human induced pluripotent stem cells (hiPSCs) are considered as a promising cell source for cell therapy applications owing to their self-renewal capability and differentiation potential (Takahashi *et al*., 2007; Kimbrel and Lonza, 2015). High cell numbers are generally required for clinical application, requiring robust methods for cell expansion (Kehoe *et al*., 2010; Serra *et al*., 2010). Conventional suspension culture of hiPSC aggregates has become a reliable method to obtain high numbers of cells ($10^8 - 10^9$ cells) (Olmer *et al*., 2010; Zweigerdt *et al*., 2011; Olmer *et al*., 2012), in particular after seeding single cells with ROCK inhibitor which prevents single cell apoptosis (Watanabe *et al*., 2007). During initial seeding in suspension culture, single hiPSCs are inoculated at a desired density into the culture vessel where the cells agglomerate to form sphere-shaped aggregates by encountering and connecting with neighboring cells, a process mediated by E-cadherin (Sugawara *et al*., 2010; Li *et al*., 2012).

Aggregate size increases with the increase in culture time, and coalescence occurs between aggregates to form larger ones leading to reduced proliferation in suspension culture (Otsuji *et al*., 2014; Bauwens *et al*., 2008; Abbasalizadeh *et al*., 2012). The heterogeneous size of aggregates causes low growth efficiency since micro-environmental stimuli, such as, cell–cell connections and cell–soluble factor interactions are dependent on the aggregate size (Moon *et al*., 2011). For example, oxygen concentration in the center region of large size aggregates (400 μm radius) was reported to reduce 50% in comparison to that in small sizes of aggregates (200 μm radius) of hPSCs (Kinney *et al*., 2011; Winkle *et al*., 2012; Wu *et al*., 2014). Moreover, the size

of individual aggregate has been reported to influence the growth and differentiation of hPSCs in aggregate suspension culture (Burridge *et al*., 2007; Hwang *et al*., 2009; Mohr *et al*., 2010; Moon *et al*., 2014). Therefore, determining the optimum size of aggregate is important for obtaining high proliferation rate of hiPSCs in suspension culture. Moreover, prolonged culture period leads to low inward diffusion of oxygen and nutrients from the surface to the densely agglomerated cells in the center causing necrosis inside the aggregate (Novosel *et al*., 2011; Jain *et al*., 2005). Due to aggregate size limitation, as well as ECM-shell formation during longer period of culture, the diffusive transport of inductive biochemicals to and from aggregates are affected which triggers the undesired differentiation inside the aggregate causing difficulty in maintaining proper growth of hPSCs in suspension culture (Sachlos *et al*., 2008). Consequently, detailed analysis of hiPSC aggregates is necessary to determine the boundary conditions for aggregate size and culture time that enable aggregates to retain a high proliferation rate and undifferentiated state in suspension culture.

In this study, to evaluate the growth properties of single aggregate of hiPSC, the effect of initial cell number and culture time were investigated quantitatively, and the proliferative ability of cells in the aggregates was elucidated by immunohistological analysis.

1.2 Materials and Methods

1.2.1 Cells and culture conditions

The hiPSC line, Tic, was provided by the Japanese Collection of Research Bioresources (JCRB1331, JCRB Cell Bank, Japan). Cells were routinely maintained on polystyrene substrate coated with recombinant laminin-511 E8 fragments (iMatrix™-511; Nippi Inc., Japan) in commercially available medium (mTeSR™1; STEMCELL Technologies, Canada). For

subculture, single cells were seeded with 10 μM ROCK inhibitor (Y-27632; Wako Pure Chemical Industries, Japan). Initial seeding was fixed at a viable cell density of 1.0×10^4 cells cm^{-2}. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2, and medium was exchanged daily with fresh medium. On day 4, when cells reached 80－90% confluence, cells were subcultured. Briefly, hiPSCs were treated with 5 mM ethylenediaminetetraacetic acid (EDTA)/phosphate-buffered-saline (PBS) with 10 μM ROCK inhibitor for 7 min at room temperature. Dissociation reagent (TrypLE Select™, Invitrogen, USA) with 10 μM ROCK inhibitor was then applied for another 7 min at room temperature. After dissociating the hiPSC colonies into single cells by pipetting (Miyazaki *et al*., 2012), cells were re-seeded into a new culture dish.

1.2.2 Culture of single hiPSC aggregates

For single aggregate culture, hiPSC colonies were dissociated into single cells after treating with EDTA/PBS and TrypLE Select with 10 μM Rock inhibitor as described above. After centrifugation, single hiPSCs were re-suspended in fresh medium. Viable cells were counted with a cell counter (TC20, Bio-Rad Inc., USA) by the trypan blue exclusion method. Single aggregate culture was carried out in 96-well ultra-low attachment V-bottom plates (Sumitomo Bakelite Co. Ltd., Japan), by seeding, $N_0 = 4.0 \times 10^1$ to 5.1×10^3 cells/well in 100 μL of medium/well, and cultured for 120 h (**Fig. 1.1**). Aggregates containing small number of initial cells (especially for $N_0 = 4.0 \times 10^1$, 8×10^1, 1.6×10^2, and 3.2×10^2 cells/aggregate) were cultured in multiple 96-well V-bottom plates to obtain sufficient number of cells (minimum density: 5.0×10^4 cells/mL) for counting by the automatic cell counter. Half of the spent medium was changed with fresh medium at 48 and 96 h.

1.2.3 Determining cell number, specific growth rate and aggregate size

After collecting hiPSC aggregates from the V-bottom plates at t = 24, 72, and 120 h, aggregates were washed with PBS, and dissociated into single cells using TrypLE Select with 10 μM ROCK inhibitor. After centrifugation, supernatant was discarded and cells were re-suspended in fresh medium. Numbers of viable cells in aggregates were counted using the cell counter, and cell number in a single aggregate, N (cells/aggregate) was calculated using the following equation: N = Total number of viable cells/Number of aggregates in the V-bottom plates. The values of N at t = 24, 48, 72, and 120 h denoted as N_{24}, N_{48}, N_{72}, and N_{120}, respectively.

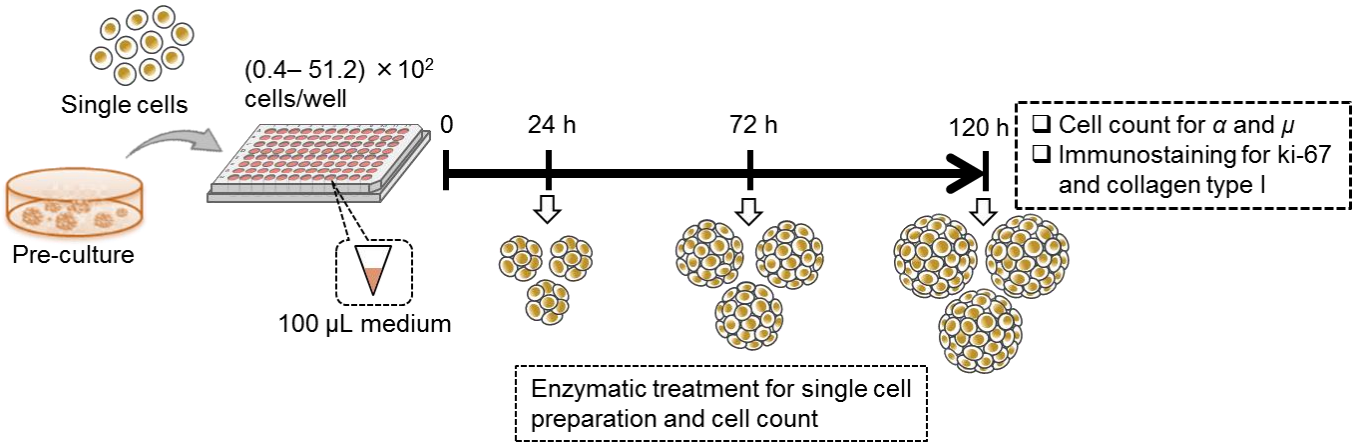


Fig. 1.1 Schematic diagram showing the experimental procedures of the present study.

Live cell ratio, α (-), was calculated using the following equation: $\alpha = N_{24}/N_0$. The apparent specific growth rate, μ^{app} (h^{-1}), was calculated using the following equation: $\mu^{app} = \ln(N_{72}/N_{24})/(\Delta t)$ at t = 24 to 72 h, and $\mu^{app} = \ln(N_{120}/N_{72})/(\Delta t)$ at t = 72 to 120 h, where Δt is the differential time of 48 h. To determine the diameter of aggregate, D (μm), bright-field images of hiPSC aggregates were captured at t = 24 h by using an image analyzer (InCell Analyzer 2000;

GE Healthcare, USA) with a 10 × objective lens, and *D* was determined by using an image processing software (Image-Pro Plus, Media Cybernetics Inc., USA).

1.2.4 Fluorescent staining

hiPSC aggregates were washed with PBS (Sigma-Aldrich, USA) and embedded in optical cutting temperature compound (OCT, Tissue-Tek, Japan). Thin sections (20 μm) were prepared using a cryostat microtome (Leica, Germany), and fixed with 4% paraformaldehyde (Wako Pure Chemical Industries) for 10 min at room temperature. After washing with PBS, the specimens were permeabilized with PBS containing 0.5% Triton X-100 (Wako Pure Chemical Industries) for 5 min, washed twice with PBS, and blocked in Block Ace (DS Pharma Co. Ltd., Japan) at 4°C overnight. The sections were then probed with primary antibodies against ki-67 (Abcam, USA), and collagen type I (Abcam) at 4°C overnight. Sections were then washed twice with Tris-buffered saline (TBS: Dako, Japan), and immersed in PBS containing 10% Block Ace and Alexa Fluor 488-conjugated secondary antibody (Life technologies, USA) for 60 min at room temperature. Cell nuclei were stained with 4´, 6-diamidino-2-phenylindole (DAPI, Life Technologies, USA) for 20 min. The specimens were washed with PBS, and observed with a confocal laser scanning microscope (FV1000, Olympus, Japan).

1.2.5 Preparation of conditioned medium

To investigate the effect of autocrine factors on small size aggregates, used medium was collected from aggregate culture ($(2.7 \pm 0.2) \times 10^2$ cells/aggregate) in the V-bottom plates after 72 h, and concentrated using a dialysis system with a molecular weight cut off (MWCO) of 5 kDa (Vivaspin 20, Vivaproducts, USA). This method diluted the low molecular weight toxic

components from the collected medium (lactic acid, ammonium), and retained the high molecular weight components (cytokines) and autocrine factors. The resulting medium was then mixed with an equal volume of fresh mTeSR™1 medium to prepare the conditioned medium (CM). Aggregates containing $(2.8 \pm 0.5) \times 10^1$ and $(8.8 \pm 0.7) \times 10^1$ cells/aggregate were cultured for 72 h with fresh medium and CM in 96-well V-bottom plates. Aggregates were harvested at t = 24 and 72 h, and dissociated into single cells using TrypLE Select. After counting the cells with a cell counter, the apparent specific growth rates were determined.

1.2.6 Statistical analysis

Data presented in this study were obtained from three independent experiments performed in 96-well ultra-low attachment V-bottom plates and are expressed as means ± standard deviation (SD). Statistical comparisons were evaluated using the Student's t-test and values of $p < 0.01$ and $p < 0.05$ were considered significant.

1.3 Results

To investigate the effect of aggregate size on hiPSC growth, aggregates containing different initial cell numbers were cultured for t = 72 h, and evaluated quantitatively. With increased N_0, α also increased, and the maximum $\alpha = 0.7 \pm 0.1$ was observed for $N_0 = 6.4 \times 10^2$ cells/aggregate, whereas, an N_0 higher than this, resulted in decreased α (**Fig. 1.2A**). However, D increased with the increase of N_{24} (**Fig. 1.2B**).

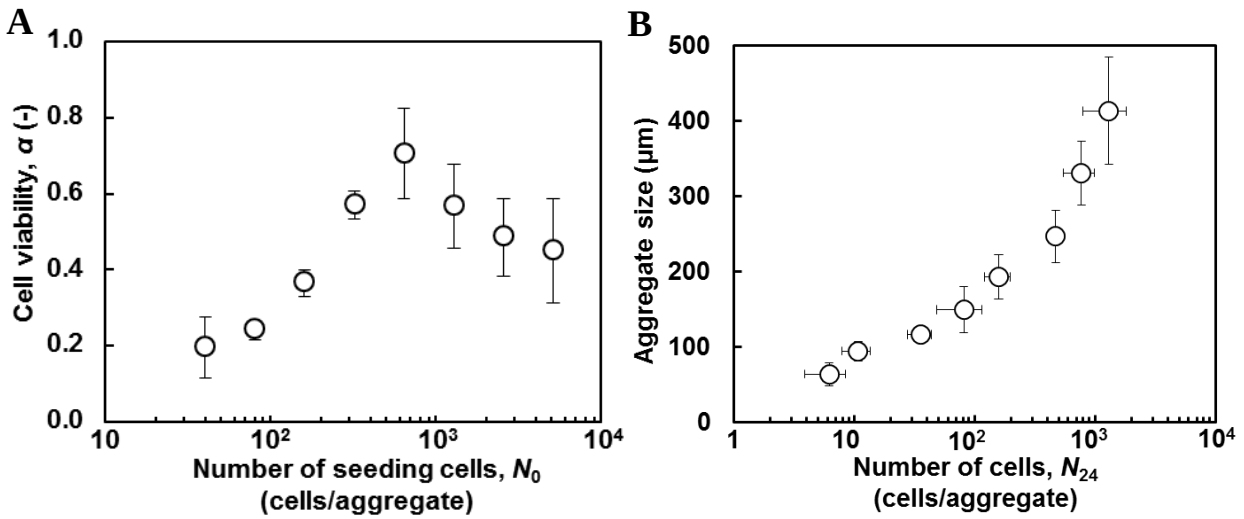


Fig. 1.2 Live cell ratio (**A**), and aggregate size (**B**) after 24 h of seeding.

Aggregates containing $N_{24} = (8.8 \pm 0.8) \times 10^1$ to $(2.7 \pm 0.2) \times 10^2$ cells showed higher cell expansion ratio after culturing for 120 h (**Fig. 1.3A**). Initial aggregate size played a major role in hiPSC growth, whereby μ^{app} increased with increased N_{24}, and maximum μ^{app} was observed in medium size aggregates ($(8.8 \pm 0.8) \times 10^1$ to $(6.8 \pm 1.1) \times 10^2$ cells/aggregate) during early-stage of culture (**Fig. 1.3B**). Small ($(1.1 \pm 0.4) \times 10^1$ to $(2.8 \pm 0.5) \times 10^1$ cells/aggregate), and large ($(1.1 \pm 0.2) \times 10^3$ to $(3.5 \pm 1.1) \times 10^3$ cells/aggregate) size aggregates showed a lower μ^{app} in comparison to medium size aggregates during t = 24 to 72 h. However, small size aggregates containing $N_{24} = (2.8 \pm 0.5) \times 10^1$ cells/aggregate showed higher μ^{app} in CM ($(3.8 \pm 0.3) \times 10^{-2}$ h$^-$

^{1}) in comparison to fresh medium ((3.1± 0.2) × 10^{-2} h^{-1}) after culturing for 72 h which was similar to the μ^{app} of medium size aggregates containing N_{24} = (8.8 ± 0.7) × 10^{1} cells/aggregate (**Fig. 1.4**). To evaluate the effect of culture time on hiPSC growth, aggregates containing different N_0 were cultured for 120 h, and μ^{app} was determined at t = 24 to 72 h, and t = 72 to 120 h of culture. Decreased μ^{app} at t = 72 to 120 h was observed in comparison with t = 24 to 72 h for the whole range of N_{72} and N_{24}, respectively, indicating growth inhibition during late-stage of culture (**Fig. 1.3B, C**). The highest μ^{app} was observed in the aggregates containing (1.0 ± 0.2) × 10^{2} to (1.4 ± 0.5) × 10^{3} cells/aggregate during late-stage of culture. To investigate the effect of aggregate size on spatial distribution of proliferating hiPSCs inside the aggregates, histological analysis of total nuclei, and cell proliferation-

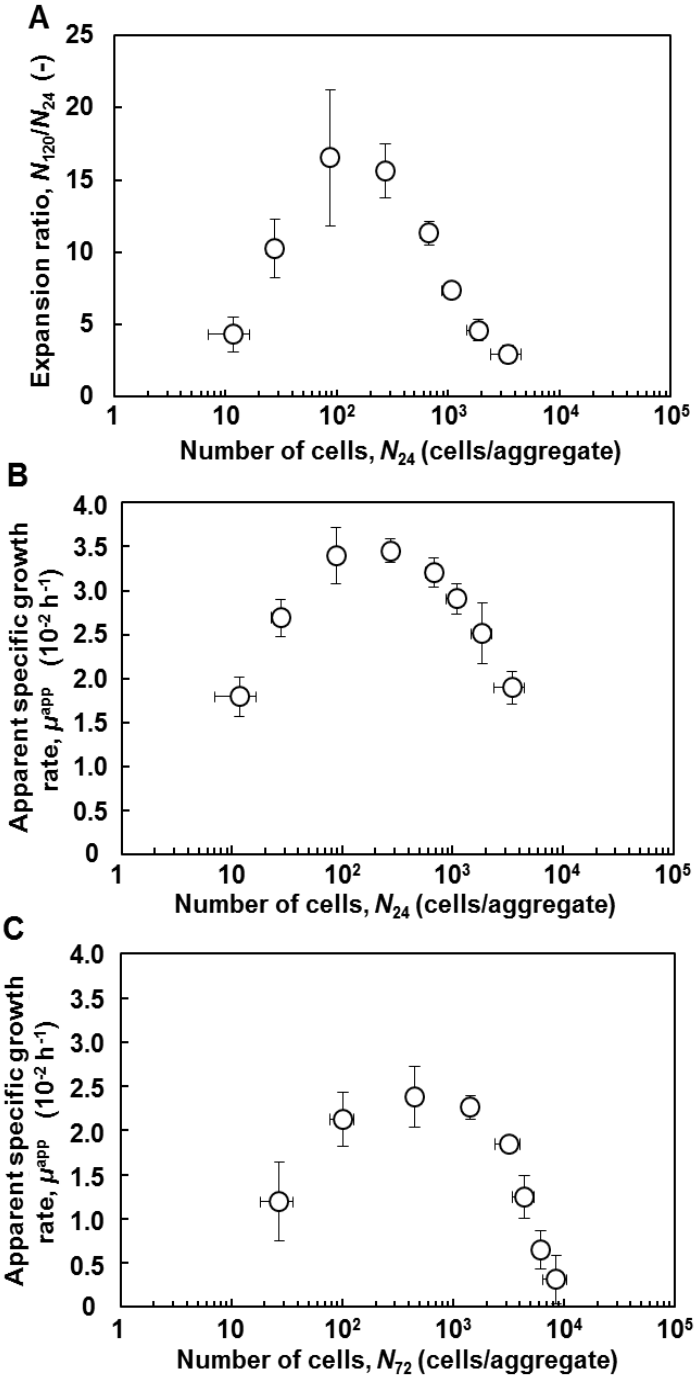


Fig. 1.3 Growth profiles of single hiPSC aggregate. (**A**) Expansion ratio of hiPSC after 120 h culture. (**B**) Growth rate of hiPSC aggregates containing different initial cell numbers from t = 24 to 72 h, and (**C**) from t = 72 to 120 h. Data presented as practical number of cells/aggregate.

associated marker, ki-67, was performed with different cell numbers (N_{48}) at t = 48 h. Small size aggregates containing N_{48} = (2.0 ± 0.2) × 10^1 cells showed weak expression of ki-67 (**Fig. 1.5A–C**), whereas medium size aggregates containing N_{48} = (2.8 ± 0.2) × 10^2 cells showed homogeneous expression of ki-67 across the whole aggregate regions (**Fig. 1.5D–F**). Conversely, large size aggregates containing N_{48} = (1.3 ± 0.2) × 10^3 cells showed lower expression of ki-67 at the center region in comparison with the periphery (**Fig. 1.5G–I**).

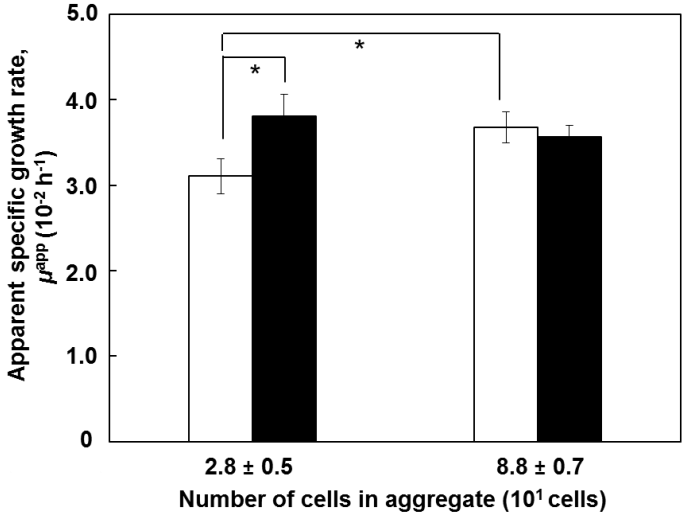


Fig. 1.4 Effect of conditioned medium on small size aggregate growth. Growth rate of hiPSC aggregates containing (2.8 ± 0.5) × 10^1 and (8.8 ± 0.7) × 10^1 cells/aggregate after culturing for 72 h with fresh medium (open bar) and conditioned medium (close bar). * $p < 0.05$.

Expression of collagen type I, an ECM member found in hiPSC aggregates, was determined in specimens collected at t = 24, 72, and 120 h for medium size aggregates containing (6.8 ± 1.1) × 10^2 cells/aggregate (**Fig. 1.6**). Collagen type I was distributed homogeneously throughout the aggregate at t = 24, and 72 h, with accumulation at the peripheral region at t = 120 h, indicating formation of a shell-like structure during late-stage of culture. Moreover, the frequency of DAPI-positive hiPSCs in the center region of the aggregate at t = 120 h was lower than that at t = 24, and 72 h, suggesting reduced growth over time at the center region. To assess ECM accumulation, similar size aggregates at N_{24} = (1.8 ± 0.4) × 10^3 and N_{120} = (1.4 ± 0.4) × 10^3

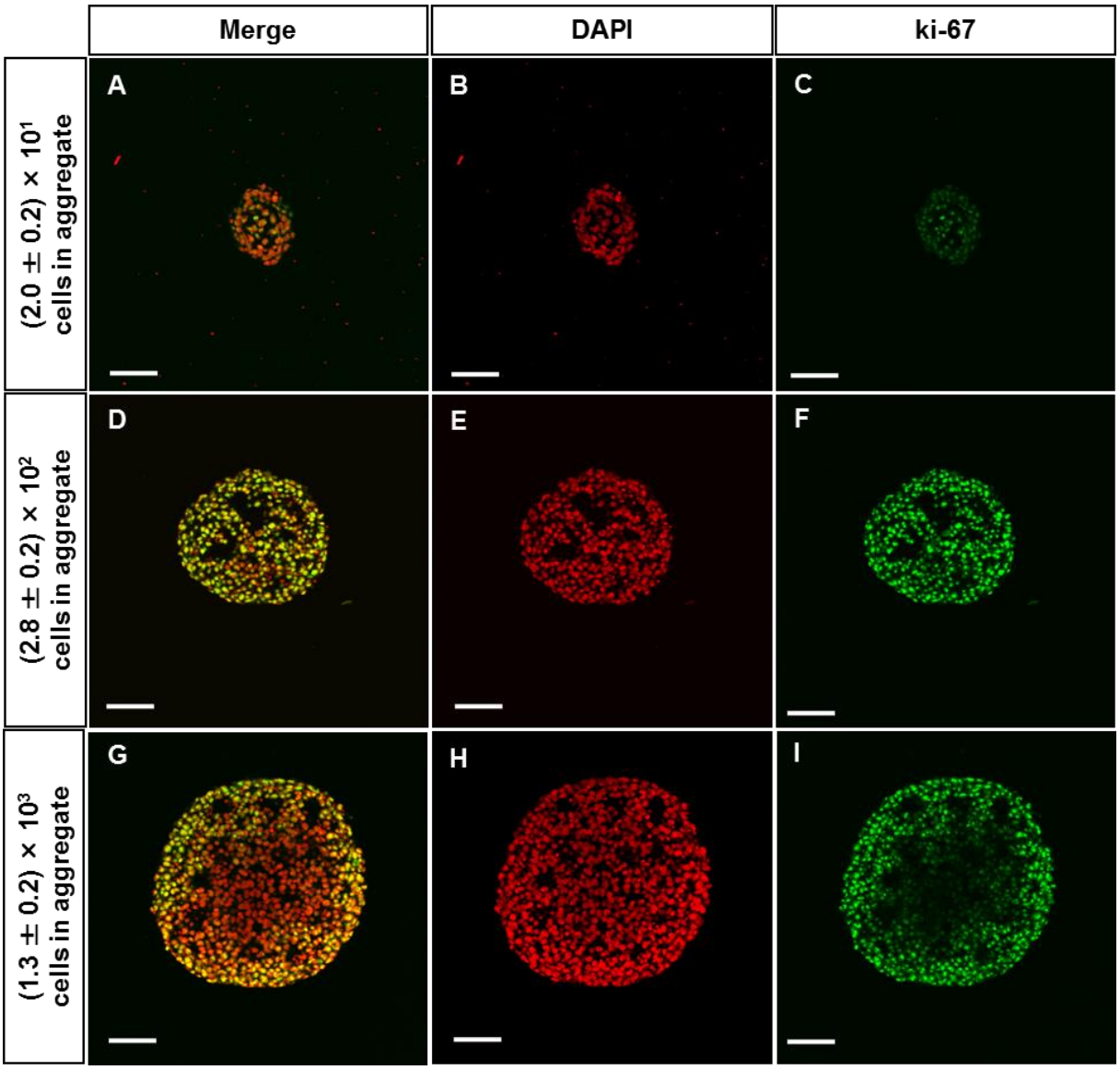


Fig. 1.5 Florescent images of hiPSC aggregates after 48 h of culture, showing for nuclei (red) and ki-67 (green). (A-C) aggregates containing (2.0 ± 0.2) × 10^1 cells, (D-F) aggregates containing (2.8 ± 0.2) × 10^2 cells, and (G-I) aggregates containing (1.3 ± 0.2) × 10^3 cells. Scale bars: 100 µm.

cells/aggregate were investigated for collagen type I expression after culturing for 24, and 120 h, respectively (**Fig. 1.7**). A higher amount of collagen type I accumulated in the peripheral region of N_{120} = (1.4 ± 0.4) × 10^3 cells/aggregate in comparison to N_{24} = (1.8 ± 0.4) × 10^3 cells/aggregate.

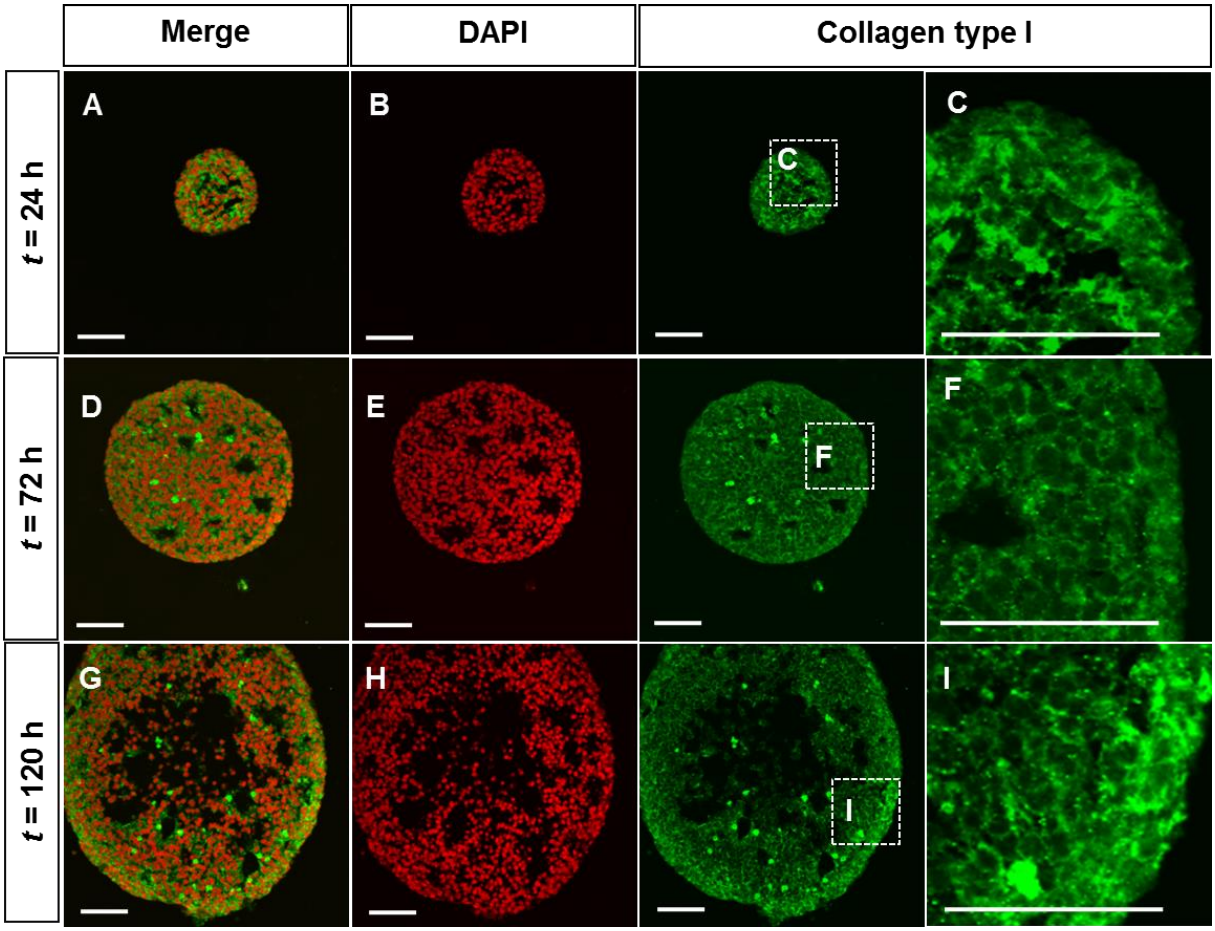


Fig. 1.6 Florescent images of hiPSC aggregate containing (6.8 ± 1.1) × 10^2 cells for nuclei (red), and collagen type I (green). (A-C) aggregates cultured for 24 h, (D-F) aggregates cultured for 72 h, and (G-I) aggregates cultured for 120 h. Panels C, F, and I show magnified views of the boxed areas. Scale bars: 100 μm.

Furthermore, hiPSC aggregates containing N_{120} = (1.4 ± 0.4) × 10^3 cells/aggregate showed lower expression of ki-67 in the center region in comparison to N_{24} = (1.8 ± 0.4) × 10^3 cells/aggregate (**Fig. 1.8**). To confirm the undifferentiated state of hiPSCs, flow cytometry was performed, and similar frequency of Octamer-binding transcription factor (OCT) 3/4 was observed during t = 24 h (98.3% ± 1.8%), 72 h (99.6% ± 0.3%), and 120 h (99.7% ± 0.3%) of culture.

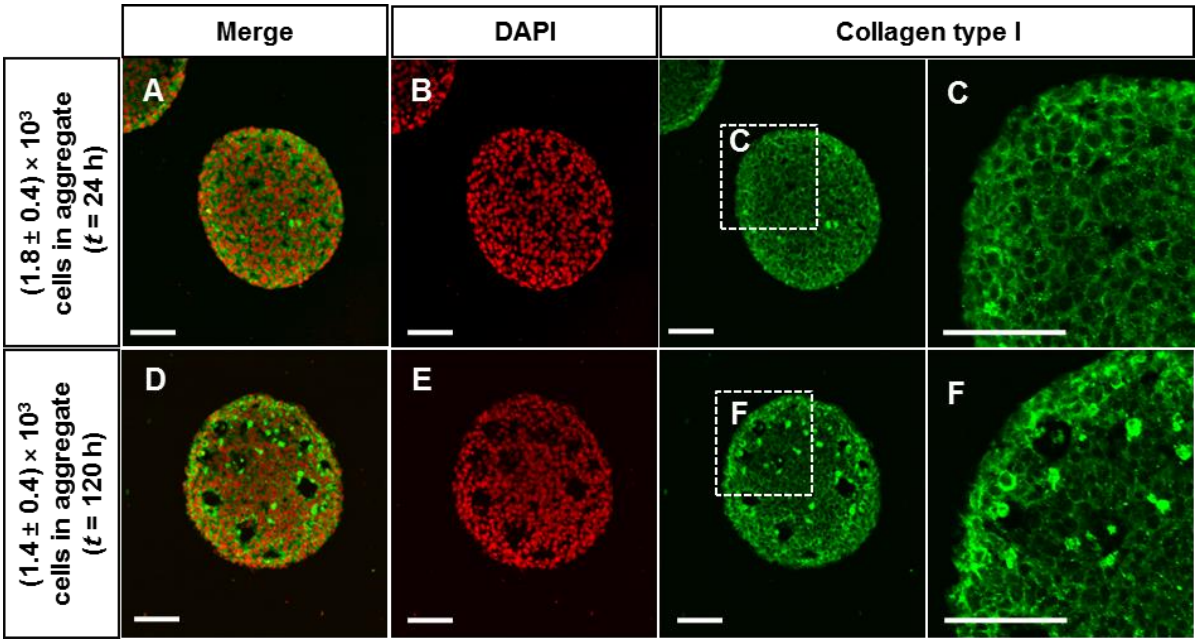


Fig. 1.7 Florescent images of hiPSC aggregates for nuclei (red), and collagen type I (green). (A-C) show (1.8 ± 0.4) × 10^3 cells/aggregate cultured for 24 h, and (D-F) show (1.4 ± 0.4) × 10^3 cells/aggregate cultured for 120 h. Panels C and F are the magnified views of boxed areas. Scale bars: 100 μm.

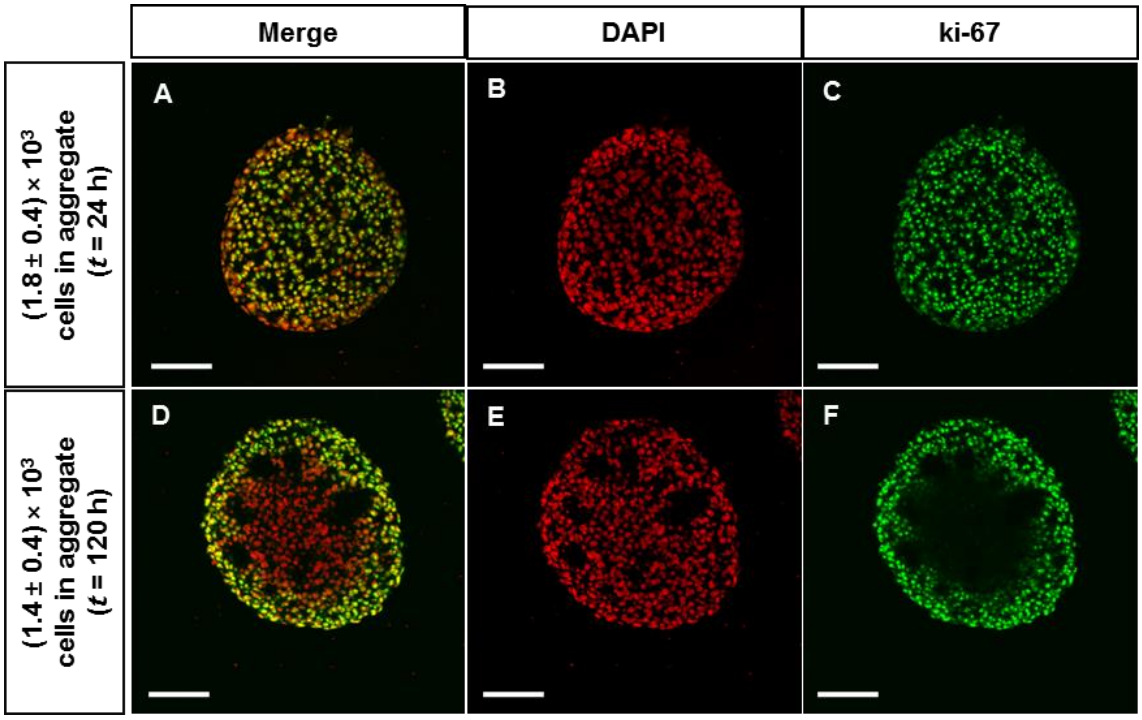


Fig. 1.8 Florescent images of hiPSC aggregates for nuclei (red), and ki-67 (green). (A-C) show (1.8 ± 0.4) × 10^3 cells/aggregate cultured for 24 h, and (D-F) show (1.4 ± 0.4) × 10^3 cells/aggregate cultured for 120 h. Scale bars: 100 μm.

1.4 Discussion

Elucidation of the aggregate growth properties is important for hiPSC expansion in suspension culture. This study quantitatively evaluated the growth properties of single hiPSC aggregates with respect to initial cell number and culture time. Factors that reduce the growth rate of hiPSC aggregates during early- and late-stage of culture were also investigated (**Fig. 1.9**).

Aggregate size played an important role because hiPSC growth rate decreased in both small and large size aggregates during t = 24 to 72 h of culture (**Fig. 1.3B**). In the small size aggregates, lower α was observed in comparison to medium size aggregates (**Fig. 1.2A**). E-cadherin is co-expressed with other undifferentiation markers and plays a central role in cell survival (Li *et al*., 2012). Ohgushi *et al*. reported high cell loss after dissociating hESCs into single cells due to disruption of cell-cell connections mediated by E-cadherin (Ohgushi *et al*., 2010). In addition, Xu *et al*. suggested that E-cadherin played a central role in the survival and self-renewal of hESCs since cell death occurred due to irreparable disruption of E-cadherin following enzymatic dissociation (Xu *et al*., 2010). Together with these suggestions, low cell–cell connection mediated by E-cadherin in small size aggregates was considered to lead low level of cell survival rate. Moreover, small size aggregates showed a lower μ^{app} in comparison with the medium size aggregates at t = 24 to 72 h (**Fig. 1.3B**). Because small size aggregates contained fewer cells in comparison with the medium size aggregates, it was assumed that small size aggregates had low secretion of growth–promoting factors in comparison to medium size aggregates. When small size aggregates were cultured with CM, a similar μ^{app} was observed in comparison with the medium size aggregates (**Fig. 1.4**).

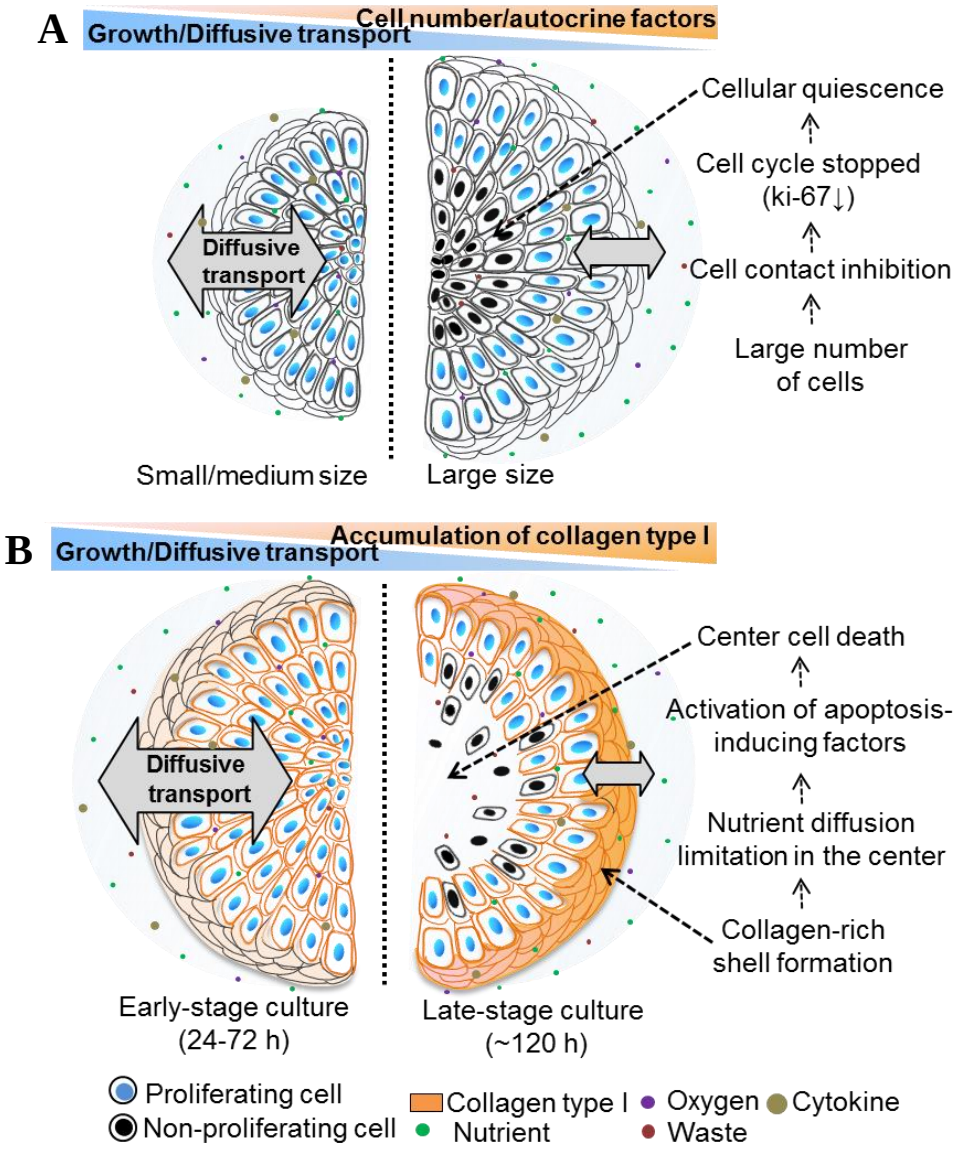


Fig. 1.9 Schematic illustration of the present study focusing on the growth of a single hiPSC aggregate considering the effect of (**A**) aggregate size and (**B**) culture time.

CM is known to contain a high concentration of autocrine factors. In fact, hPSCs secrete many essential components via cell–cell interactions especially basic fibroblast growth factor (bFGF), TGF-β1, and insulin as autocrine factors which act to maintain culture homeostasis, and thus stimulate cell growth (Bendall *et al*., 2007; Greber *et al*., 2007; Montes *et al*., 2009). It is

therefore hypothesized that the CM containing autocrine factors was responsible for the enhanced growth of the small size aggregates.

In the large size aggregates, μ^{app} was decreased with increased N_{24} at t = 24 to 72 h (**Fig. 1.3B**), and expression of ki-67 was suppressed in the center region (**Fig. 1.5**). It was assumed that diffusion of oxygen and nutrients from the periphery to the center were limited, resulting in reduced cell growth and enhanced cell necrosis in the center region (Novosel *et al*., 2011; Jain *et al*., 2005). In the present study, the maximum D of large size aggregates was more than 400 μm (**Fig. 1.2B**) which was assumed to cause low diffusive transport of oxygen and nutrients into the densely agglomerated cells in the center region of aggregates. It has already been reported that oxygen concentration in the center region of larger embryoid bodies (EBs) (400 μm) is 50% lower in comparison to that in medium size EBs (200 μm) (Kinney *et al*., 2011; Winkle *et al*., 2012; Wu *et al*., 2014) which triggers apoptosis of the core cells due to low oxygen diffusion (Carmeliet *et al*., 1998). Moreover, the morphology and cell–cell connections are reported to alter at high cell number, which in turn stimulates the Hippo pathway signaling, triggering the phosphorylation of Yap in the nucleus, and reducing cell proliferation (Ota *et al*., 2008; Wada *et al*., 2011). Together with this suggestion, low expression of ki-67 in the center region of large size aggregate was considered to occur due to cell contact inhibition of proliferation because the cells entered to a quiescent state during early-stage of culture (**Fig. 1.9A**). However, the higher expansion ratios observed in the medium size aggregates containing $N_{24} = (8.8 \pm 0.8) \times 10^1$ to $(2.7 \pm 0.2) \times 10^2$ cells after culturing for 120 h, indicated the feasible range for initial cell seeding number for higher proliferation of hiPSC in aggregate culture (**Fig. 1.3A**).

Culture time was also considered as an important factor for hiPSC growth because μ^{app} at t = 72 to 120 h was lower than that at t = 24 to 72 h for the whole range of N_{72} and N_{24}, respectively (**Fig. 1.3C**). The highest μ^{app} in the aggregates containing $(1.0 \pm 0.2) \times 10^2$ to $(1.4 \pm 0.5) \times 10^3$ cells indicated the time-dependent boundary for high proliferation of cells during late-stage of culture. Immunofluorescent staining revealed that a higher amount of collagen type I was accumulated at the aggregate periphery at t = 120 h in comparison with t = 24 and 72 h of culture (**Fig. 1.6**). Collagen precursors are predominantly biosynthesized in the rough endoplasmic reticulum of the cell, and secreted via a transmembrane protein transport system to the extracellular space where they accumulate, and form a network with other ECM components (Ishikawa *et al*., 2016). However, accumulation of collagen type I was time-dependent as a higher amount of collagen type I was observed at the peripheral region of the medium size aggregates in comparison with the large size aggregates after 120 and 24 h culture, respectively (**Fig. 1.7**). Due to the formation of a collagen-rich, shell-like structure on the periphery of hiPSCs aggregate, the diffusive transport of soluble factors, especially cytokines including bFGF, TGF-β1, as well as the diffusion of oxygen and toxic metabolites to and from the aggregate was suggested to hinder during late-stage of culture (**Fig. 1.9B**). hESC aggregates were also reported to form a collagen-rich, shell-like structure on their surface hindering the diffusive transport of inductive biochemicals during late-stage of culture (Sachlos *et al*., 2008). Moreover, reduction in the diffusive transport of nutrients was suggested to lead to the activation of apoptosis-inducing factors of the core cells (Joza *et al*., 2001), which likely resulted in low expression of ki-67 in the center region of the hiPSC aggregates during late-stage of culture in the present study (**Fig. 1.8**). Shortening the culture time (~72 h) to avoid ECM shell formation on the aggregate periphery is therefore important for obtaining high growth rate during hiPSC culture. Thus, we propose that

medium size (100 μm－200 μm) aggregates should be maintained for better supply of nutrients and oxygen, and that the culture time should be shortened with frequent subculture to reduce ECM accumulation at the aggregates periphery for obtaining high number of hiPSCs in conventional suspension culture.

In conclusion, hiPSCs showed heterogeneous growth properties depending on aggregate size and culture time in the culture of single aggregate. Growth of small size aggregates was autocrine factor-dependent, whereas large size aggregates had low center cell proliferations due to cellular quiescence. Culture time was also important for influencing heterogeneous growth during prolonged culture period by uneven distribution of factors including oxygen, nutrients and toxic metabolites both inside and outside of the hiPSC aggregates, due to formation of an ECM-shell at the periphery. Therefore, understanding the growth properties of hiPSC aggregates to maintain balance between secretion of autocrine factors and accumulation of ECM on the aggregate periphery is essential to ensure optimal growth in suspension culture. Furthermore, consideration of the boundary conditions for aggregate size and culture time is needed to prevent aggregate heterogeneity, and to obtain high growth rate during large-scale expansion of hiPSCs in suspension culture.

1.5 Summary

This study quantitatively evaluated the effects of initial cell number and culture time on the growth of hiPSCs in the single aggregate culture. Small size aggregates showed a lower growth rate in comparison to medium size aggregates during early-stage of culture (24–72 h). However, when small size aggregates were cultured in conditioned medium, their growth rate increased significantly. On the other hand, large size aggregates showed a lower growth rate and lower expression level of proliferation marker (ki-67) in the center region of aggregate in comparison to medium size aggregate during early-stage of culture. Medium size aggregates showed the highest growth rate during early-stage of culture. Furthermore, hiPSCs proliferation was dependent on culture time because the growth rate decreased significantly during late-stage of culture (72–120 h) at which point collagen type I accumulated on the periphery of aggregate, suggesting blockage of diffusive transport of nutrients, oxygen and metabolites into and out of the aggregates. Consideration of initial cell number and culture time are important to maintain balance between autocrine factors secretion and extracellular matrix accumulation on the aggregate periphery to achieve optimal growth of hiPSCs in single aggregate culture.

Chapter 2

Botulinum hemagglutinin-mediated *in situ* break-up of human iPS cell aggregates for high-density suspension culture

2.1 Introduction

hPSCs comprising of hESCs and hiPSCs are potential source of specialized cells for cell therapy application owing to their self-renewal (Takahashi *et al*., 2007) and differentiation capability to any types of mature cells of human body (Chambers *et al*., 2009; Zhang *et al*., 2009). hiPSCs are superior to hESCs for clinical applications because hiPSCs circumvents the complication of ethical issues and criticism associated with their use in research. To realize their potential, bio-process developments for the robust expansion of hiPSCs are important, since high numbers of hPSCs-derived targeted cells are required for cell therapy application (Lock and Tzanakakis, 2007; Jing *et al*., 2008). The large-scale expansion of hiPSCs in adherent culture is difficult; therefore, suspension culture is garnering increasing attention because high cell density and better control of process parameters can be obtained in stirred suspension bioreactor culture (Olmer *et al*., 2012; Haraguchi *et al*., 2015).

Since large size of hiPSC aggregates show low growth rate, aggregates are dissociated into single cells for maintaining high growth rate in conventional suspension culture by the following basic steps: enzyme treatment and pipetting to dissociate the aggregates, centrifugation and washing for enzyme removal, and re-seeding into the culture vessel (Olmer *et al*., 2010; Haraguchi *et al*., 2015). After seeding single cells in suspension culture, cells encounter the neighboring cells and establish cell–cell connections, mediated by E–cadherin, and form spherical aggregates (Sugawara *et al*., 2010; Li *et al*., 2012). However, some cells fail to

encounter the neighboring cells and undergo apoptosis (Watanabe *et al*., 2007; Ohgushi *et al*., 2010). As a result, massive cell loss occurs during hiPSC aggregate dissociation with enzymatic digestion (Singh *et al*., 2010; Haraguchi *et al*., 2015). Moreover, aggregate size increases with the increase in culture time (Bauwens *et al*., 2008; Otsuji *et al*., 2014), and large size aggregates show undesirable differentiation and cell necrosis within the aggregate, as collagen–rich shell forms on the outer surface of the aggregate, hindering the diffusive transport of oxygen and nutrients (Sachlos *et al*., 2008; Kinney *et al*., 2011; Winkle *et al*., 2012; Wu *et al*., 2014). In the previous chapter, we have also observed decrease of hiPSC proliferation as a result of aggregate size limitation, and accumulation of collagen type I on the aggregate periphery reducing the growth rate of hiPSCs during late–stage of culture. These heterogeneous phenomena of aggregate growth lead to low cell density in conventional suspension culture (Amit *et al*., 2010; Abbasalizadeh *et al*., 2012; Olmer *et al*., 2012). Therefore, routine break–up of aggregates into smaller ones is important for maintaining high growth rates to obtain a high–density of hiPSCs during long–term suspension culture.

In this study, we have developed a simple and scalable method for the expansion of hiPSCs by aggregate break–up into small ones using botulinum hemagglutinin (HA), and have illustrated the mechanism of aggregate break–up in suspension culture. This method allowed high–density culture of hiPSCs without the need for enzymatic treatment or centrifugation by simplifying culture operations in suspension.

2.2 Materials and Methods

2.2.1 Cells and Culture Conditions

The hiPSC line, Tic, provided by the Japanese Collection of Research Bioresources (JCRB1331, JCRB Cell Bank, Japan), was routinely maintained on polystyrene substrate coated with recombinant laminin-511 E8 fragments (iMatrix™-511; Nippi Inc., Japan) in commercially available medium (mTeSR™1; STEMCELL Technologies, Canada). For subculture, single cells were seeded with 10 μM ROCK inhibitor (Y-27632; Wako Pure Chemical Industries, Japan). The initial seeding was fixed at a viable cell density of 1.0×10^4 cells cm^{-2}. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2, and the medium was exchanged daily with fresh medium. On day 4, when cells reached confluence, cells were subcultured. Briefly, hiPSCs were treated with 5 mM EDTA/PBS with 10 μM ROCK inhibitor for 7 min at room temperature. Dissociation reagent (TrypLE Select™, Invitrogen, USA) with 10 μM ROCK inhibitor was then added for another 7 min at room temperature, and hiPSC colonies were dissociated into single cells by pipetting. After centrifugation, the supernatant was discarded, and cells were re-suspended in fresh medium. Viable cells were counted with a cell counter (TC20; Bio-Rad Inc., USA) by the trypan blue exclusion method, and cells were re-seeded in a new culture dish.

2.2.2 HA Addition Conditions for Aggregate Break-up

HA complexes (His-HA, His-BHA1-FLAG: His-BHA2: Strep-BHA3) were kindly provided by Professor Yukako Fujinaga (Kanazawa University, Japan). The procedure used for reconstituting the functional HA complex was similar to that described elsewhere (Sugawara *et al*., 2014). To determine HA addition time, hiPSC aggregates were cultured in 30 mL bioreactor (BWV-S03A; Able Co., Japan) at an initial seeding density of 1.0×10^5 cells mL^{-1} with 10 μM

ROCK inhibitor at an agitation rate of 55 rpm, and incubated at 37º C with 5% CO_2 (**Fig. 2.1**). At 96 h, hiPSC aggregates (5.0×10^5 cells mL^{-1}) were dispensed in a 24-well plate (Corning Inc., USA) containing 10, 20, and 30 nM HA in the culture medium, and observed at 0, 6, 9, 12, and 24 h. In each condition, aggregates were broken up by pipetting, and images were captured using a confocal microscope (Olympus, Japan) before and after pipetting. To investigate the mechanisms of aggregate break-up by HA, aggregates were immunostained for E-cadherin and HA at each sampling point.

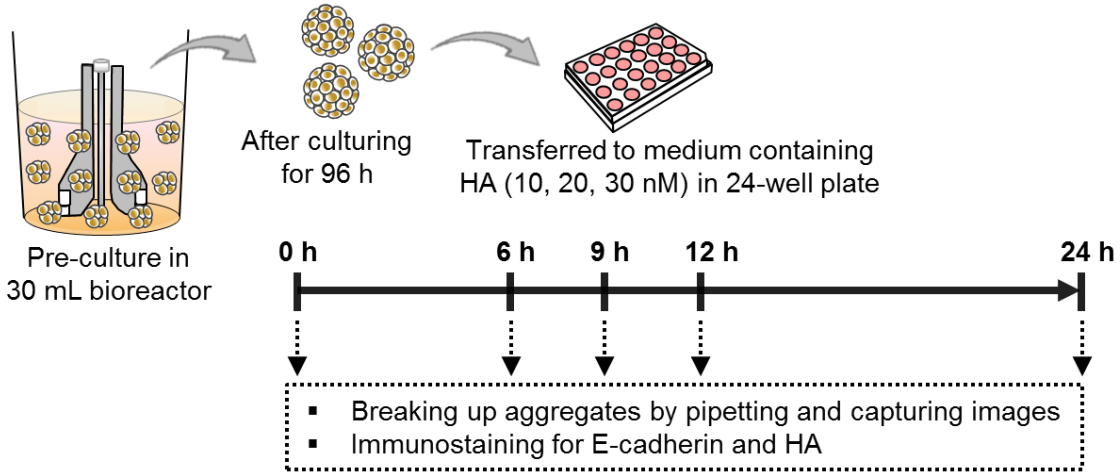


Fig. 2.1 Schematic diagram showing the experimental procedures for aggregate break-up with HA.

2.2.3 Suspension Cultures of hiPSCs

To investigate the effect HA on hiPSC aggregate break-up, cells were detached from the culture substrate by following the method described before. After dissociating the hiPSC colony into single cells, 1.0×10^5 cells mL^{-1} were seeded with 10 μM ROCK inhibitor in 30 mL bioreactor. At 87 h, HA (20 nM) was added to the culture medium and kept for 9 h (**Fig. 2.2**). Then, aggregates were broken into small sizes by pipetting, and re-seeded in 30 mL bioreactor at a seeding density of 1.0×10^5 cells mL^{-1} with 10 μM ROCK inhibitor, with or without washing to remove HA from the medium, and cultured for an additional 96 h. In the control culture,

aggregates were treated with dissociation reagent (ACCUMAX; STEMCELL Technologies, Canada) with 10 μM ROCK inhibitor for 10 min. Single cells were re-seeded in 30 mL bioreactor at 1.0×10^5 cells mL^{-1} with 10 μM ROCK inhibitor, and cultured for an additional 96 h. In both cases, medium was exchanged with fresh one at every 24 h.

For high–density culture of hiPSC aggregates, single cells with 10 μM ROCK inhibitor were seeded in 30 mL bioreactor (1.0×10^5 cells mL^{-1}) to prepare aggregates. At 87 h, HA (20 nM) was added to the culture medium and kept for 9 h. Next, aggregates were broken into small sizes by pipetting, and all the cells were re-seeded in 30 mL bioreactor with 10 μM ROCK inhibitor and cultured for an additional 96 h. In the control culture, aggregates were not treated with HA, or not broken into small sizes, and cultured for an additional 96 h. The culture medium was exchanged with fresh medium at frequent intervals to reduce the toxic effect of metabolites during late–stage of culture.

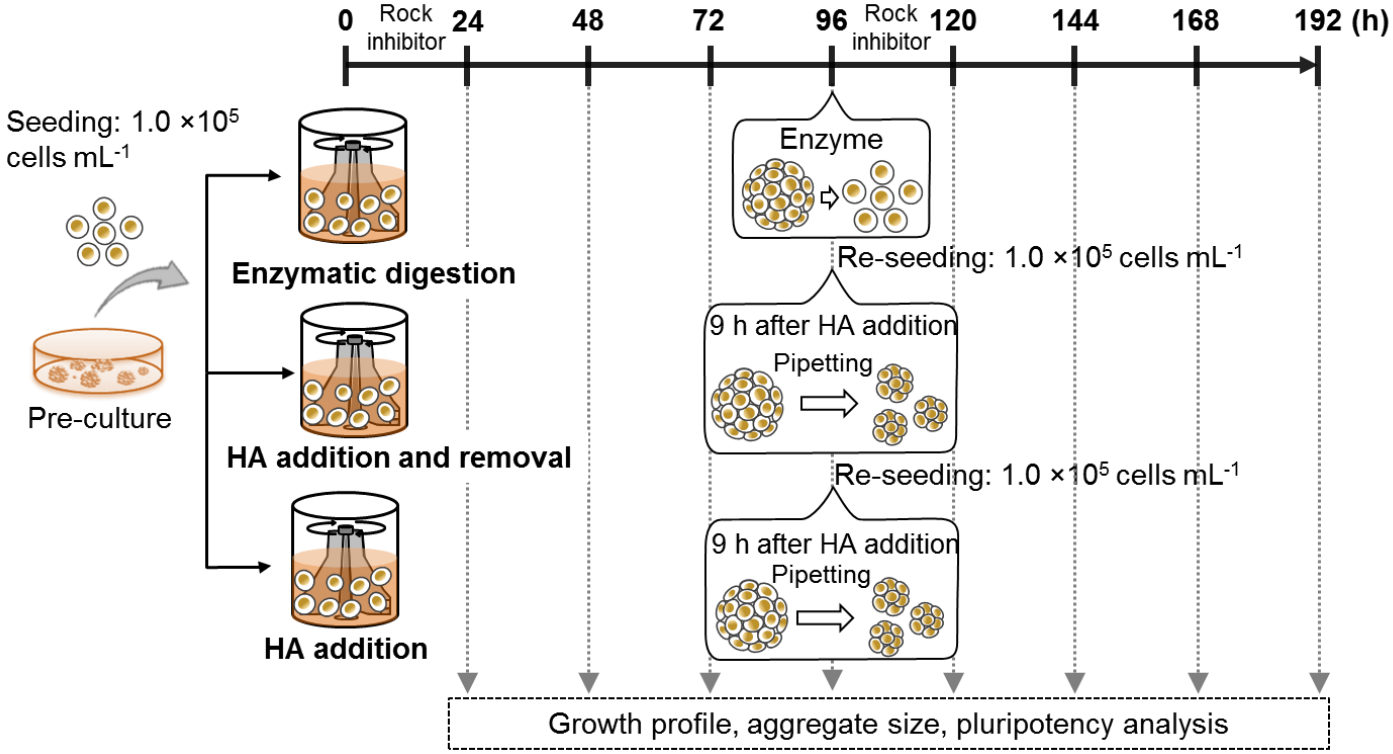


Fig. 2.2 Schematic diagram showing the experimental procedures for hiPSCs aggregate passage in suspension culture.

2.2.4 Kinetic Analysis of Growth Properties of hiPSC Aggregates

To determine the apparent specific growth rate, μ^{app} (h^{-1}), hiPSC aggregates were collected from the bioreactor (3 samples each time) at every 24 h, and dissociated into single cells by using TrypLE Select. After centrifugation, the supernatant was discarded and cells were re-suspended in fresh medium. Live cells were counted using the cell counter. The initial live cell ratio, α (-), was determined by $\alpha = X_{24}/X_0$, where X_0 is the cell density at seeding, and X_{24} is the cell density at t = 24 h. The μ^{app} was calculated by the following equation: $\mu^{app} = \ln(X_f/X_i)/(t_f - t_i)$, where X_f is the final cell density at t_f = 87 or 192 h, and X_i is the initial cell density at t_i = 24 or 120 h. Expansion fold was calculated by X_f/X_i, where X_f is the final cell density at t_f = 87 or 192 h, and X_i is the initial cell density at t_i = 0 or 96 h. The yield on culture medium, Y (cells mL^{-1}), was calculated by the following equation: $Y = N_t/V_m$, where N_t is the total number of cells (cells), and V_m is the total volume of medium consumption (mL). To determine the aggregate size, images were captured every 24 h using an image analyzer with a 4× objective lens (IN Cell Analyzer 2000; GE Healthcare, USA), and aggregate diameter was measured by using Image-Pro Plus software (Version 6.0; Media Cybernetics, USA).

2.2.5 Analysis of Medium Components

To analyze the components of culture medium associated with cell cultivation, medium was collected at every time during medium change, and analyzed for glucose and lactic acid by using a biochemical analyzer (Bioprofile 400; Nova Biomedical, USA).

2.2.6 Flow Cytometry

To perform flow cytometry, cells were collected at t = 192 h for checking the expression of the pluripotency markers OCT 3/4 and SSEA 4. Briefly, single cells were prepared, treated with the Cytofix/Cytoperm™ permeabilization kit (BD Biosciences, USA), and incubated with primary antibody (mouse monoclonal OCT 3/4, Santa Cruz Biotechnology, USA), and secondary antibody (Allophycocyanin (APC) anti-mouse IgG, BD Biosciences, USA). Direct staining of stage-specific embryonic antigen (SSEA) 4 was performed by using human/mouse SSEA 4 fluorescein (R&D Systems, USA). Flow cytometry was performed with a flow cytometer (Sysmex, USA), and data were analyzed using commercially available software (FlowLogic, Inivai Technologies, Australia).

2.2.7 Fluorescence Staining

The procedure for staining was similar to that described previously (Kim *et al*., 2014). Briefly, hiPSC aggregates were transferred to a 24-well plate containing HA in the culture medium and incubated for 0, 6, 9, 12, and 24 h (**Fig. 2.1**). Then embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Fine Chemical Co., Ltd., Japan), and sectioned into 20–μm–thick slices using a cryostat microtome (Leica, Germany). The sections were washed with PBS and fixed with 4% paraformaldehyde (Wako Pure Chemical Industries) for 10 min at room temperature. The specimens were washed with PBS, and permeabilized with PBS containing 0.5% Triton X-100 (Wako Pure Chemical Industries) for 5 min; washed twice with PBS containing 0.1% Tween 20 (Sigma-Aldrich); and blocked in Block Ace for 90 min at room temperature. The sections were washed with Tris-buffered saline (TBS; Dako) and then probed with anti-E-cadherin antibody (24E10; Cell Signaling Technology, USA) and anti-Flag M2

antibody (Sigma-Aldrich) to detect HA, and incubated overnight at 4°C. Next, they were washed twice with TBS and immuno-labeled with Alexa Fluor 488-conjugated goat anti-rabbit or Alexa Fluor 594-conjugated goat anti-mouse IgG for 60 min at room temperature. The cell nuclei were stained with 4´, 6-diamidino-2-phenylindole (DAPI; Life Technologies) for 20 min at room temperature. Images were captured using a confocal laser scanning microscope (FV 1000; Olympus) with a 60× objective lens.

2.2.8 Statistical Analysis

Data presented in this study were obtained from three independent experiments and expressed as mean ± standard deviation (SD). Statistical comparisons were evaluated using the Student's t-test and values of $p < 0.01$ and $p < 0.05$ were considered to be significant.

2.3 Results

2.3.1 hiPSC Aggregates Break-up into Small Sizes by HA

To break-up hiPSC aggregates into smaller ones, the aggregates were cultured in medium containing 10, 20, and 30 nM HA for 0, 6, 9, 12, and 24 h (**Fig. 2.3**). At 10 nM HA, hiPSCs aggregates could not be broken into small sizes, whereas, at 30 nM HA, aggregates were broken into small sizes earlier than at 20 nM HA, and the activity of HA persisted longer. At 20 nM HA, aggregates could be broken into small sizes only for a short period (at 9h). Moreover, hiPSC aggregates showed time-dependent break-up after HA addition in suspension culture. The aggregates could not be broken up shortly after HA addition (0–6 h) or even, long after it (12–24 h). The aggregates were easily broken up into smaller ones by pipetting 9 h after HA addition.

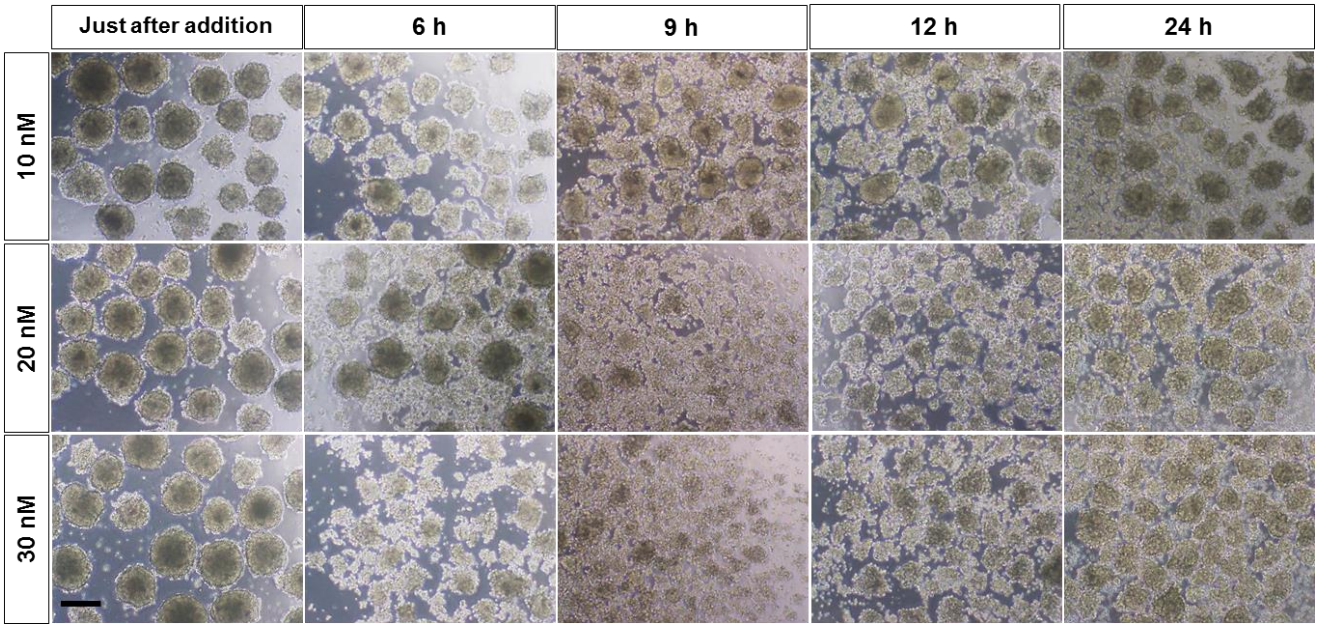


Fig. 2.3 Fluorescent images of hiPSC aggregates for determining HA concentration, and timing for breaking up the aggregates into small sizes by pipetting in suspension culture. Scale bar: 200 µm.

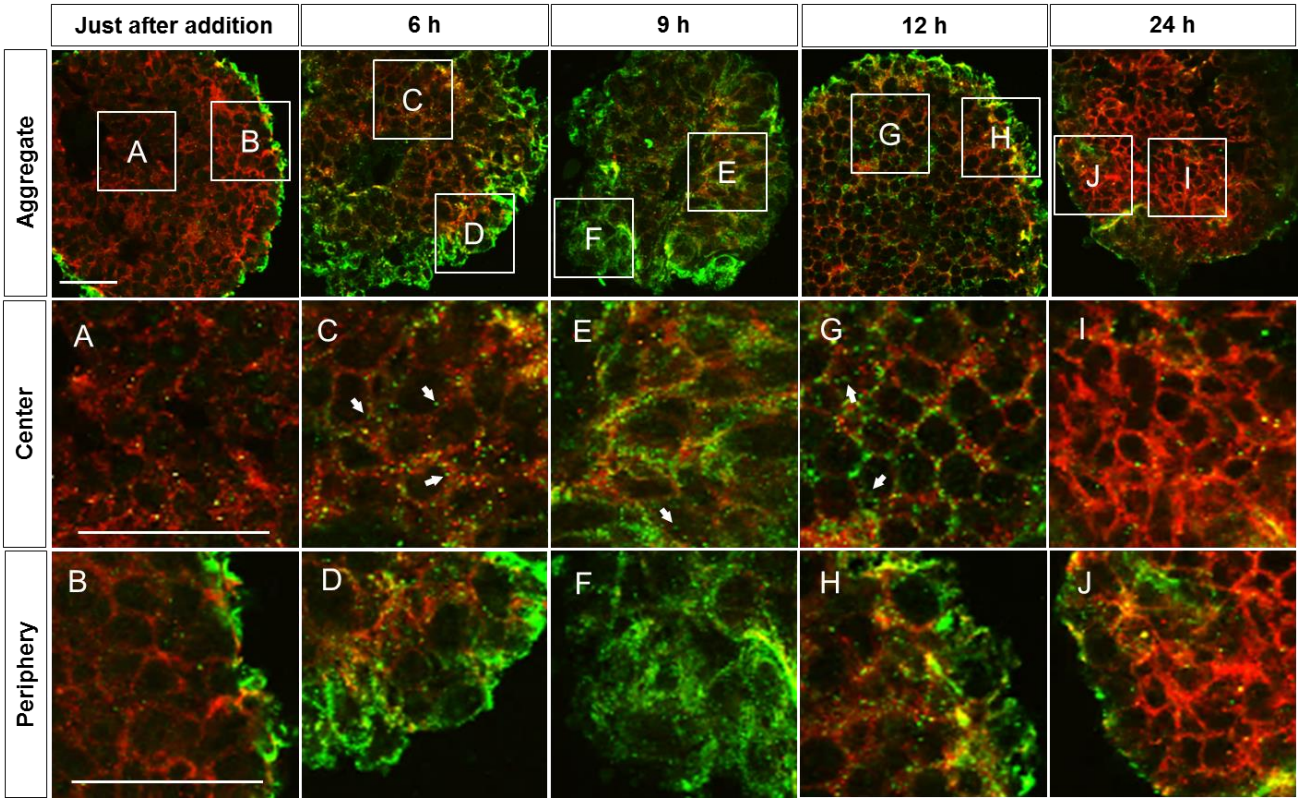


Fig. 2.4 Immunostaining of hiPSC aggregates for E-cadherin (red) and HA (green) in suspension culture. Panels (A-J) are magnified views of merge images of aggregate centre and peripheral cells at (A, B) just after addition, (C, D) 6 h, (E, F) 9 h, (G, H) 12 h, and (I, J) 24 h of HA addition. Arrow heads indicate the localization of HA in the cytoplasm and intercellular barrier of cells. Scale bars: 50 µm.

To investigate HA localization and E-cadherin expression in the intercellular barrier, hiPSC aggregates were stained with anti-Flag M2 antibody for visualizing FLAG-tagged HA1 and HA2, and anti-E-cadherin antibody, respectively (**Fig. 2.4**). HA localization was observed in the peripheral cells of the aggregate, and E-cadherin expression was observed throughout the aggregate immediately upon adding HA to the culture medium (**Fig. 2.4A and B**). Six hours after HA addition, a higher localization of HA was observed in the peripheral cells than in the center cells of the aggregate, whereas lower expression of E-cadherin was observed in the peripheral cells compared to the center cells (**Fig. 2.4C and D**). However, HA localization was observed only in the intercellular barrier of the center cells of the aggregate (arrow head) 6 h after HA addition, indicating the specific binding of HA to E-cadherin. Nine hours after HA addition, low E-cadherin expression and high HA localization were observed throughout the aggregate (**Fig. 2.4E and F**). Furthermore, 12 h after HA addition, E-cadherin expression increased and HA localization decreased in the intercellular barrier of center cells compared to the peripheral cells of aggregate (**Fig. 2.4G and H**). HA localization was observed in the cell cytoplasm (arrow head) 12 h after HA addition. Twenty four hours after HA addition, high expression of E-cadherin and low localization of HA were observed throughout the aggregate (**Fig. 2.4I and J**).

2.3.2 Effect of aggregate break-up by HA on hiPSCs growth

To investigate the effect of aggregate break-up by HA, hiPSCs aggregates were prepared by seeding single hiPSCs in 30 mL bioreactor. At 96 h, aggregates were broken with HA or enzymatic digestion. hiPSCs showed a higher live cell ratio, as well as higher cell density with both HA addition (1.3 ± 0.3, and (1.3 ± 0.2) × 10^6 cells mL^{-1}, respectively), and HA addition and removal (1.3 ± 0.2, and (1.3 ± 0.3) × 10^6 cells mL^{-1}, respectively) than with enzymatic digestion (0.7 ± 0.1, and (5.5 ± 1.3) × 10^5 cells mL^{-1}, respectively) in suspension culture (**Fig. 2.5A**). The size of aggregates increased with the increase of culture time and reached to an average of 253.4 ± 65.3 μm before passaging at 87 h (**Fig. 2.5B**). The size of aggregates after break–up with HA was on an average 88.2 ± 25.3 μm at 120 h, and it increased with the increase in culture time with both HA treatment and enzymatic digestion.

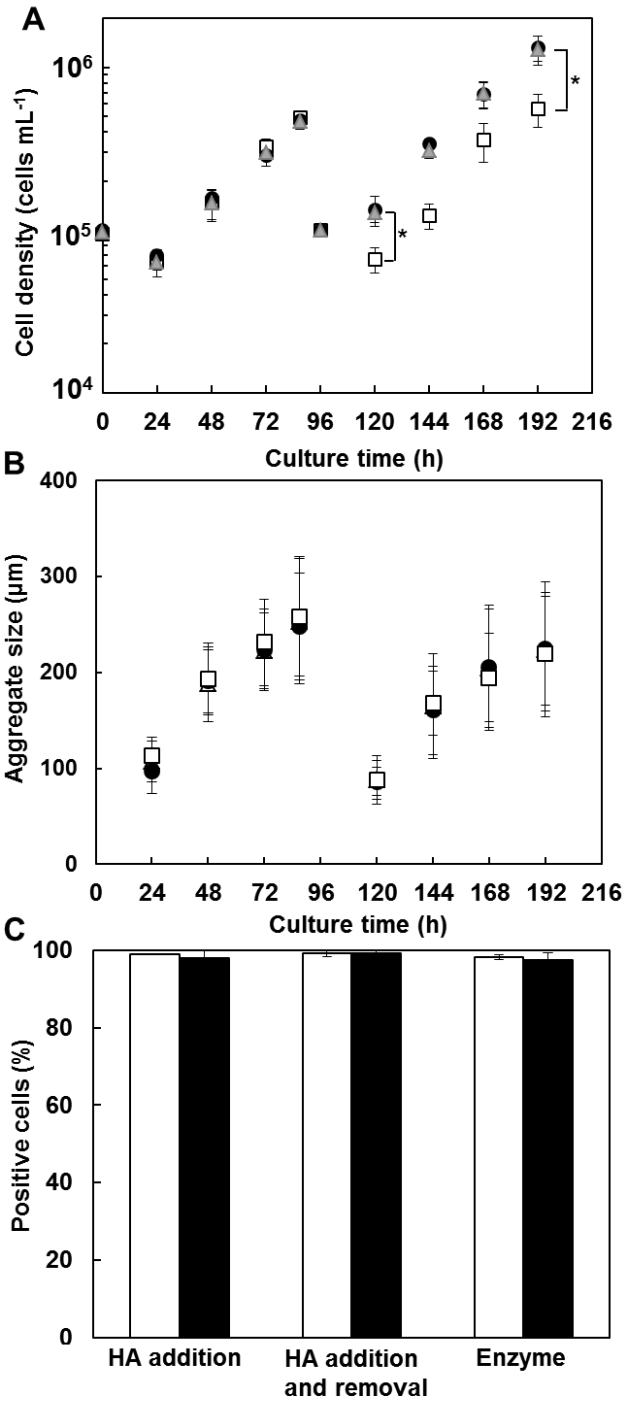


Fig. 2.5 Effect of hiPSC aggregates break-up with HA and enzymatic digestion. (**A**) Growth profile and (**B**) size of hiPSC aggregate broken up with HA addition (triangle), HA addition and removal (circle), and enzymatic digestion (square). (**C**) Flow cytometry analysis for OCT 3/4 (open bar) and SSEA 4 (close bar). *$p < 0.05$.

Table 2.1. Comparison of growth properties of hiPSC aggregate dissociated with enzyme and aggregate broken up with HA in suspension culture (at t = 96–192 h).

	Aggregate dissociation with enzyme	Aggregate break-up with HA
Live cell ratio, α (-)	0.7 ± 0.1	1.3 ± 0.3*
Apparent specific growth rate, μ^{app} (10^{-2} h^{-1})	3.1 ± 0.2	3.2 ± 0.1
Final cell density, X_f (10^5 cells mL^{-1})	5.5 ± 1.3	13.2 ± 2.3**
Expansion fold (-)	5.5 ± 1.3	13.2 ± 2.3**

* $p < 0.05$, ** $p < 0.01$

The μ^{app} was similar in both HA ((3.2 ± 0.1) × 10^{-2} h^{-1}) and enzymatic digestion ((3.1 ± 0.2) × 10^{-2} h^{-1}) (**Table 2.1**). However, higher expansion fold was obtained in the culture with aggregate break-up with HA (13.2 ± 2.3) than with enzymatic digestion (5.5 ± 1.3). hiPSCs showed similar expression of OCT 3/4 (98.9% ± 0.2%, 99.1% ± 0.8%, and 98.2% ± 0.6%), and SSEA 4 (98.0% ± 2.1%, 99.2% ± 1.2%, and 97.6% ± 1.7%) in HA addition, HA addition and removal, and enzymatic digestion, respectively (**Fig. 2.5C**).

2.3.3 High-density Culture of hiPSC by Aggregate Break-up

To obtain high cell density in a single vessel, hiPSCs were cultured for 192 h with or without aggregate break-up by HA in suspension culture. A higher cell density of (4.5 ± 0.2) × 10^6 cells mL^{-1} was obtained with aggregate break–up by HA than that without aggregate break–up ((1.7 ± 0.1) × 10^6 cells mL^{-1}) at 192 h (**Fig. 2.6A**). Aggregate size increased gradually and reached an average of 216.9 ± 47.0 μm before breaking up at 87 h (**Fig. 2.6B**). When aggregates were broken up with HA, small size of aggregates (98.9 ± 31.7 μm) was observed at 120 h, and

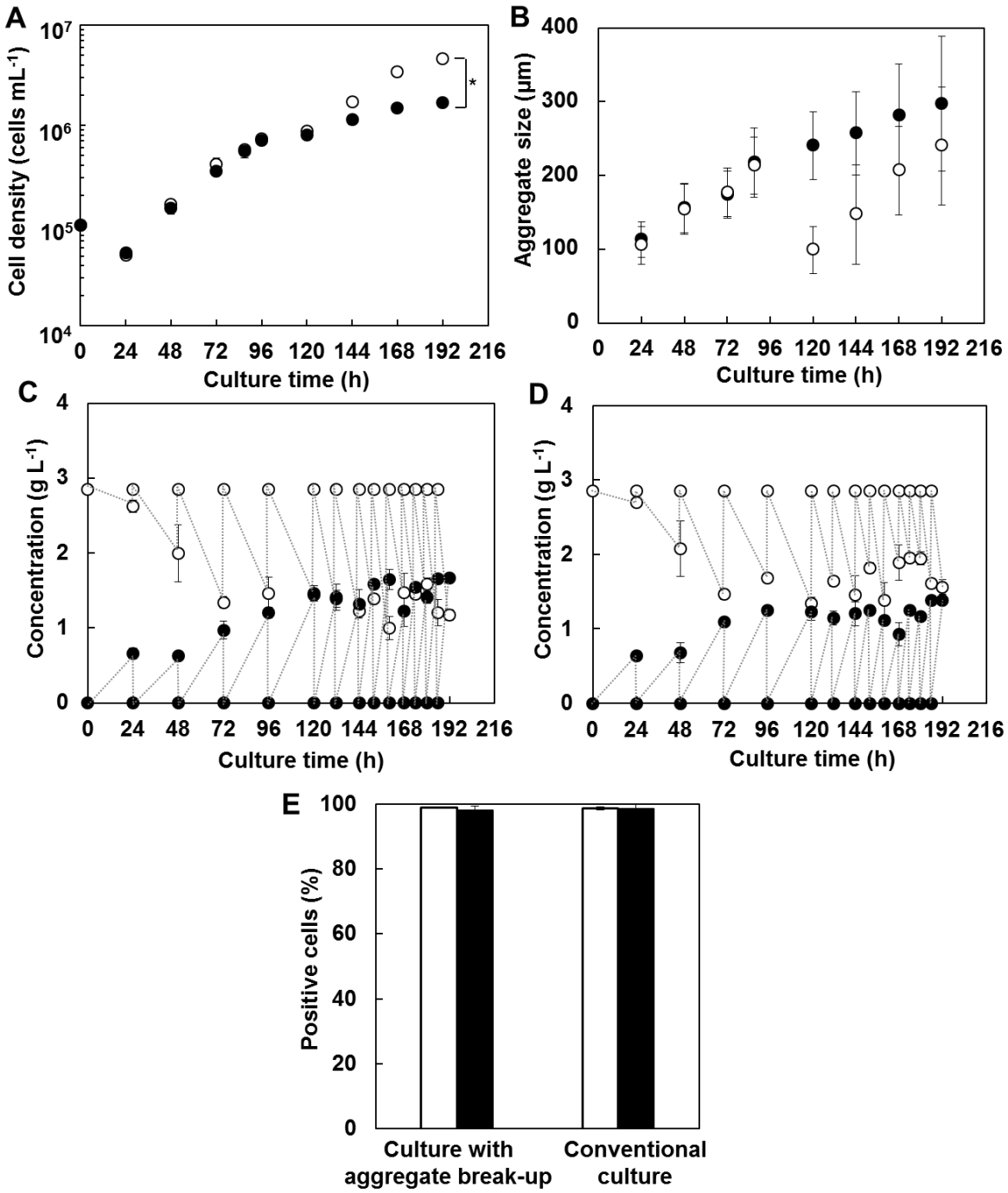


Fig. 2.6 High-density culture of hiPSC with aggregate break-up. (**A**) Growth profile, and (**B**) size of hiPSC aggregate with aggregate break-up by HA (open circle), and without aggregate break-up (close circle). Glucose consumption (open circle), and lactic acid production (close circle) in the (**C)** culture of aggregate break-up by HA, and **(D**) conventional culture without aggregate break-up. (**E**) Flow cytometry analysis for OCT 3/4 (open bar), and SSEA 4 (close bar) in the culture of aggregate break-up by HA, and in the conventional culture without aggregate break-up. *$p < 0.01$.

Table 2.2. Comparison of growth properties of hiPSC with or without aggregate break-up by HA in suspension culture.

	Without aggregate break-up		With aggregate break-up	
	Early-stage (0–87 h)	Late-stage (96–192 h)	Early-stage (0–87 h)	Late-stage (96–192 h)
Apparent specific growth rate, μ^{app} (10^{-2} h^{-1})	3.5 ± 0.1	1.1 ± 0.1	3.6 ± 0.2	2.4 ± 0.2*
Final cell density, X_f (10^5 cells mL^{-1})	5.4 ± 0.7	16.6 ± 1.2	5.6 ± 0.6	45.3 ± 2.3*
Expansion fold (-)	5.4 ± 0.7	2.4 ± 0.1	5.6 ± 0.6	6.3 ± 0.3*

*$p < 0.01$

the size increased gradually thereafter. However, aggregate size did not increase significantly in the control culture without aggregate break–up during late-stage of culture (120–192 h). Higher consumption of glucose and production of lactic acid were observed in the suspension culture of hiPSCs with aggregate break–up by HA than in the control culture without aggregate break–up (**Fig. 2.6C and D**). hiPSCs cultured with the aggregate break–up with HA showed higher μ^{app} ((2.4 ± 0.2) × 10^{-2} h^{-1}) than that of the control culture without aggregate break-up ((1.1 ± 0.1) × 10^{-2} h^{-1}) during late-stage of culture (**Table 2.2**). Higher fold of expansion (6.3 ± 0.3) was obtained in the culture with aggregate break–up compared to the control culture without aggregate break–up (2.4 ± 0.1). hiPSCs showed similar expression of OCT 3/4 (98.8% ± 0.1%, and 98.6% ± 0.5%) and SSEA 4 (98.1% ± 1.1%, and 98.5% ± 1.3%) in the culture with HA and the control culture without HA, respectively (**Fig. 2.6E**).

2.4 Discussion

The spontaneous formation of aggregates after seeding single cells in culture vessel provides an advantage for large-scale expansion of hiPSCs in suspension culture. However, maintaining high growth rate of aggregates during long-term culture is difficult because aggregate size increases with an increase in culture time, and large size aggregates show heterogeneous growth owing to the occurrence of cell necrosis in the center region of the aggregate. Therefore, controlling aggregate size is required to obtain high number of cells in suspension culture. In this study, we established a simple method for breaking up aggregates into small sizes by using HA and investigated the mechanisms underlying the disruption of E-cadherin–mediated cell–cell connections by HA to obtain high-density of hiPSCs for simplifying the culture operations in suspension as shown in **Fig. 2.7**.

2.4.1 Temporal activity of HA facilitated the disruption of E-cadherin-mediated cell-cell connections in hiPSCs aggregate

After adding HA in the culture medium, compact aggregates turned into easy to mold which was further broken into small sizes by pipetting (**Fig. 2.3**). However, during short (0–6 h) and long (12–24 h) time after HA addition; aggregates could not be broken into small sizes. Aggregates could be broken into small sizes only 9 h after HA addition. Generally, cell-cell connections in hiPSC aggregates are mediated by E-cadherin (Chen *et al*., 2010; Li *et al*., 2012). While hiPSC aggregates were exposed to HA, cell-cell connections were loosened by directly disrupting the E-cadherin located in the intercellular barrier of hiPSCs aggregate (**Fig. 2.4**). HA was reported to disrupt the E-cadherin mediated cell-cell connections in different types of cells in adherent culture (Sugawara *et al*., 2010; Lee *et al*., 2014; Kim *et al*., 2017). However, HA bound

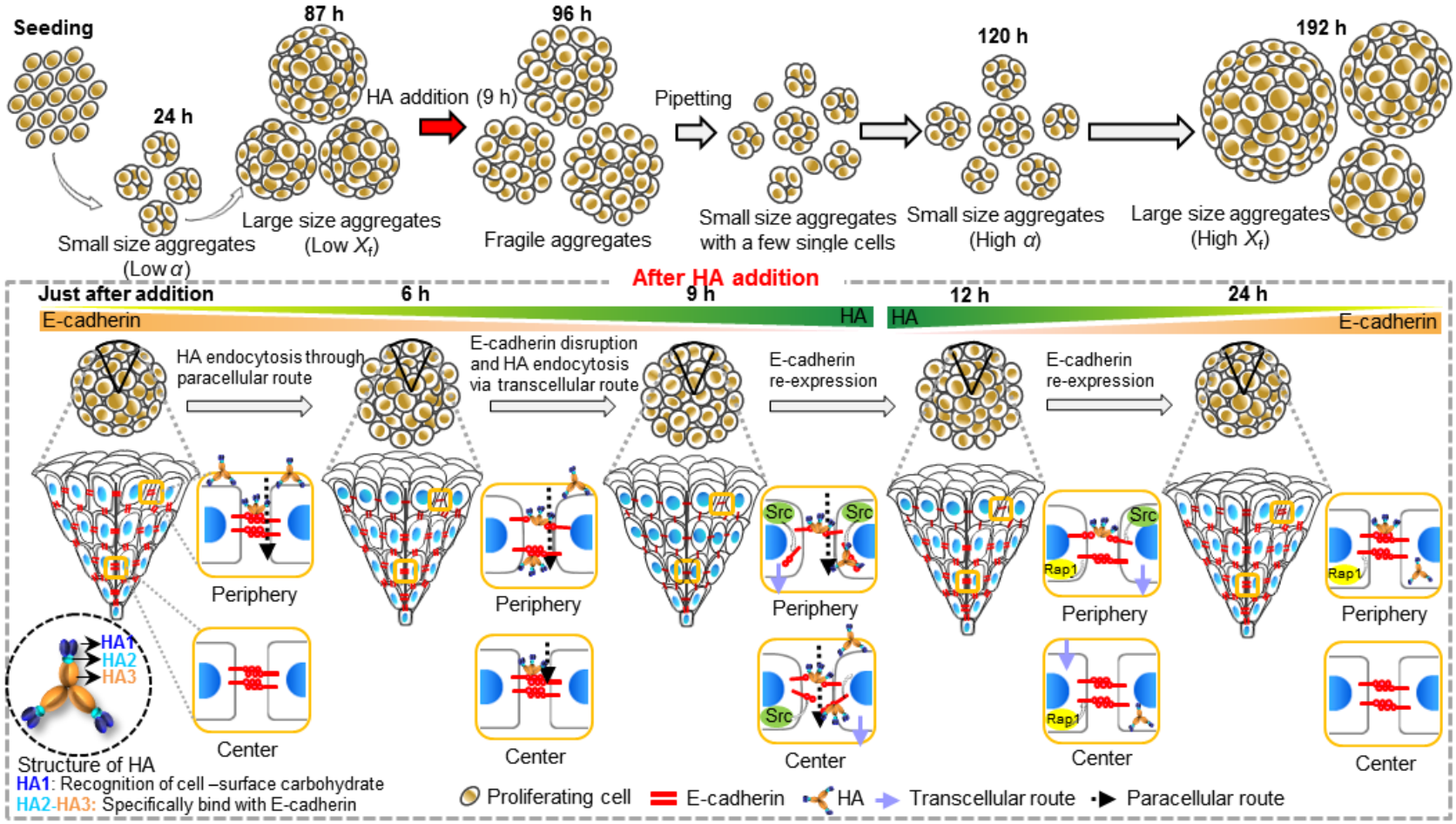


Fig. 2.7 Schematic illustration of the present study focusing on HA-mediated E-cadherin disruption for obtaining high density of hiPSCs in suspension culture.

to the E-cadherin of aggregate peripheral cells just after adding in the culture medium as shown in **Fig. 2.7**. After 6 h of addition, HA disrupted the E-cadherin mediated cell-cell connections of the peripheral cells and penetrated into the aggregate. The center cells of aggregate maintained high frequency of cell-cell connections mediated by E-cadherin after 6 h of addition since the time was not enough to reach the HA at the center region of aggregate. Furthermore, the localization of HA was observed only in the intercellular barrier of center cells of aggregate after 6 h of HA addition (**Fig. 2.4C, arrow head**), indicating the specific binding of HA to E-cadherin, as well as, primary way of HA transportation into the aggregate via paracellular route. Lee *et al* reported three ways of HA transportation into the epithelial cells in adherent culture: transcellular route, E-cadherin and carbohydrate-mediated basolateral route, and paracellular route (Lee *et al*., 2014). However, most of the E-cadherin in the intercellular barrier of hiPSC aggregate was disrupted after 9 h of HA addition, causing weak cell-cell connections which promoted breaking up the aggregates into small sizes by pipetting. The weak cell-cell connection was reported previously to stimulate the Src kinase activity which in turn activates the endocytic machinery for E-cadherin internalization into the cells (Balzac *et al*., 2005). Together with this suggestion, the least expression of E-cadherin after 9 h of HA addition was considered due to the internalization of E-cadherin into the cells. However, after 12 h of HA addition, aggregates could not be broken into small sizes by pipetting as E-cadherin re-expressed in the intercellular barrier of center cells of aggregate to promote strong cell-cell connections. The re-expression of E-cadherin in the center cells of aggregate was suggested due to the stimulation by Rap1 activation which promoted the recycling of internalized E-cadherin from the cell (Balzac *et al*., 2005; Kim *et al*., 2017). However, the activity of HA retained after 12 h of addition on the peripheral cells of aggregate since lower expression of E-cadherin was observed

in comparison to the center cells. The localization of HA in the cytoplasm after 12 h of addition indicated the secondary way of HA transportation into the aggregate via trans-cellular route (**Fig. 2.4G, arrow head**). After 24 h, the localization of HA decreased significantly and E-cadherin re-expressed throughout the aggregate suggesting the absorption of HA into hiPSC aggregate since binding of HA complex to cells facilitates the absorption into the cells (Balzac *et al*., 2005). Thus, it was clarified that primary transportation of HA into the aggregate was mediated by paracellular route; whereas, secondary transportation was mediated by transcellular route after HA addition in suspension culture. The temporal activity of HA was also confirmed for breaking up aggregates into small sizes in suspension culture.

2.4.2 hiPSC aggregates break-up with HA had no effect on growth rate rather it enhanced live cell ratio and simplified culture operations in comparison to enzymatic digestion

hiPSC aggregates break-up by HA resulted in higher initial live cell ratio and higher cell density in comparison to aggregates dissociation with conventional enzymatic digestion (**Fig. 2.5**). Since aggregates were broken into small sizes, high cell-cell connections and ECM were assumed to maintain within the aggregates which provided a higher live cell ratio in comparison to aggregate dissociation with enzymatic digestion. In the enzymatic digestion, aggregates were dissociated into single cells which resulted in massive cell loss during initial seeding in suspension culture. Singh *et al*. also reported high percentage of apoptotic cells during initial seeding after dissociating hPSC aggregate into single cells with enzymatic digestion (Singh *et al*., 2010). Generally, E-cadherin is co-expressed with other undifferentiation markers and plays a central role in cell survival and proliferation (Li *et al*., 2012). Ohgushi *et al*. and Xu *et al*. reported high cell loss after dissociating hESCs into single cells due to E-cadherin disruption

following enzymatic dissociation (Ohgushi *et al*., 2010; Xu *et al*., 2010). Taken together, low cell-cell connections due to irreparable loss of E-cadherin was considered to lead to low live cell ratio in the aggregate dissociation with enzymatic digestion. On the other hand, washing of cells is mandatory in the conventional aggregate dissociation with enzymatic digestion to remove enzyme which along with remove ECM from cell suspension. Removal of ECM triggers apoptosis as ECM support growth and proliferation of hPSCs (Suh and Han, 2011; Chang *et al*., 2015). As a result, low expansion of cells (~5 fold) was obtained in the aggregate dissociation with enzymatic digestion in comparison to the aggregate break-up with HA (~13 fold) (**Table 1**). Haraguchi *et al*. and Singh *et al*. reported 2-fold expansion (Singh *et al*., 2010, Haraguchi *et al*., 2015), whereas, Zweigerdt *et al*. reported 3-6 fold expansion (Zweigerdt *et al*., 2011) of hPSCs during aggregate dissociation with enzymatic digestion in suspension culture. Since HA had no negative effect on hiPSC proliferation (**Fig. 2.5**), as well as in maintaining pluripotency (**Fig. 2.5C**), and inactivated spontaneously after addition (24 h); removal of HA from medium by washing was not needed which promoted simplification of culture operations in suspension. Therefore, a simple method for hiPSC aggregate break-up was established which can be applied for large-scale expansion of hiPSCs without washing or centrifugation during passage in suspension culture.

2.4.3 Overcoming size-dependency on aggregate growth resulted in high cell density and high growth rate in suspension culture

The apparent specific growth rate of hiPSC decreased significantly during late-stage (96–192 h) of control culture without aggregate break-up due to aggregate size limitation (**Table 2.2**). Large size of aggregates have been previously reported to show undesired differentiation as well

as cell necrosis in the center region of aggregate as collagen-rich shell forms on the outer surface of aggregate which hinders the diffusive transport of oxygen and nutrients during late-stage of culture (Sachlos *et al*., 2008; Kinney *et al*., 2011; Winkle *et al*., 2012; Wu *et al*., 2014). In the previous chapter, lower growth rate was observed both in large size aggregate as well as with the increase of culture time due to collagen type I formation on the aggregate periphery during late-stage of culture. In this study, while hiPSC aggregates were broken into small sizes with HA in the late-stage of culture, high cell density ($(4.5 \pm 0.2) \times 10^6$ cells mL^{-1}) was obtained which was three times higher than the control culture without aggregate break-up (**Fig. 2.6**). Moreover, higher consumption of glucose and production of lactic acid was observed in the culture with aggregate break-up by HA in comparison to without aggregates break-up. Several recent studies have also been reported to obtain high-density of hPSCs (2.0×10^6 cells mL^{-1}) and high growth rate in the conventional aggregate suspension culture (Amit *et al*., 2010; Abbasalizadeh *et al*., 2012; Olmer *et al*., 2012;), which are significantly lower in comparison to the current study. Although hiPSCs can be expanded to a high-density (3.6×10^6 cells mL^{-1}) by continuous perfusion (Kropp *et al*., 2016), maintaining such density in aggregate suspension culture is still challenging due to aggregate size limitation. In the current study, we have succeeded to overcome the aggregate size limitation through the break-up of hiPSC aggregates into small sizes with HA to obtain high cell density with high expression of pluripotency markers (**Fig. 2.6E**) in suspension culture. Moreover, higher expansion fold (~6 folds) was obtained with the break-up of aggregates by HA which was higher than those previously reported for hPSCs expansion (2–4 folds) during long-term suspension culture (Abbasalizadeh *et al*., 2012; Chen *et al*., 2012; Olmer *et al*., 2012; Hunt *et al*., 2014). This successful performance suggests HA treatment as a promising technique to prevent initial cell loss during seeding, simplifying passage operations

without centrifugation or washing, and for obtaining high cell density with high yield on medium in suspension culture.

In conclusion, a simple and scalable method for *in situ* break-up of hiPSC aggregates was established by revealing the temporal activity of HA for disrupting E-cadherin mediated cell-cell connections by minimizing the initial cells loss during seeding in suspension culture. This method also allowed high density culture of hiPSCs without the need of enzymatic treatment or centrifugation in suspension culture. Thus, this promising technique of aggregate break-up with HA will lead to the establishment of a simple closed-system for robust expansion of hiPSCs in suspension culture.

2.5 Summary

In this study, a simple method for break-up of hiPSC aggregates into small sizes was established by using botulinum hemagglutinin (HA) which specifically bound with E-cadherin, and disrupted cell-cell connections in hiPSC aggregate. The primary transportation of HA into the aggregate was mediated by paracellular route, whereas, the secondary transportation of HA was mediated by transcellular route after HA addition in the aggregate suspension culture. Since HA showed temporary activity for aggregate break-up the, removal of HA from culture medium was not needed during suspension culture. hiPSC aggregates broken with HA showed higher number of live cells, higher final cell density, and higher yield on culture medium in comparison to the aggregate dissociation with enzymatic digestion in suspension culture. Moreover, a maximum density of $(4.5 \pm 0.2) \times 10^6$ cells mL^{-1} was obtained by breaking up the aggregates into small sizes that was 3 times higher than the conventional culture without aggregate break-up. Therefore, the temporal activity of HA for disrupting E-cadherin mediated cell-cell connections paved the way to establish a *in situ* simple method of hiPSC aggregate break-up in bioreactor without initial cell loss which led to a high cell density in suspension culture.

Chapter 3

Culture medium refinement by dialysis for the expansion of human induced pluripotent stem cells in suspension

3.1 Introduction

Human induced pluripotent stem cells (hiPSCs) (Takahashi *et al*., 2007) hold great promise in the field of regenerative medicine for cell-based therapies (Schulz *et al*., 2012), tissue engineering (Badylak *et al*., 2011), and drug discovery because of their pluripotency and self-renewal capacity (Wernig *et al*., 2007). To realize their potential, the developments of bioprocesses for the expansion of hiPSCs are important, as a large number of cells are required for clinical application (Lock and Tzanakakis, 2007; Jing *et al*., 2008; Kehoe *et al*., 2010; Serra *et al*., 2010; Olmer *et al*., 2010). Compared to adherent culture, suspension culture of hiPSC aggregate is promising to obtain such numbers of cells, as a higher cell density and better control of process parameters can be achieved in a stirred-suspension bioreactor (Zweigerdt *et al*., 2011; Olmer *et al*., 2012; Haraguchi *et al*., 2015).

Production of lactic acid is higher in hPSC culture than it is in other cell types (Varum *et al*., 2011), and lactic acid has potential toxic effects on the proliferation of hPSC (Ouyang *et al*., 2007; Chen *et al*., 2010). As a result, frequent changing of culture medium is required to remove toxic metabolites (Fernandes-Platzgummer *et al*., 2014), although the consumption of glucose is not high in hPSC culture compared to that in other types of cells (Terstegge *et al*., 2007). Moreover, suspension culture of hiPSCs in a bioreactor requires a large volume of culture medium, since spent medium is replaced daily with fresh medium to reduce the accumulation of lactic acid, ammonium (NH_4^+), and other toxic metabolites, which increase with an increase in

cell density (Abbasalizadeh *et al*., 2012). These toxic metabolites reduce the growth of hiPSCs in suspension culture. On the other hand, hPSCs secrete various autocrine factors in the culture medium that maintain culture homeostasis and potentiate the growth of cells. hESCs have been reported to spontaneously secrete TGF-β1 and IGF-II in response to bFGF in feeder-free adherent cultures (Bendall *et al*., 2007; Montes *et al*., 2009). These autocrine factors are removed from culture medium along with toxic metabolites during daily medium changes in conventional suspension culture. The cost of culturing hiPSC increases with increasing medium consumption, as the culture medium contains many costly macromolecules including bFGF, TGF-β1, and insulin (Tian *et al*., 2004; Ludwig *et al*., 2006).

In this study, a method to refine culture medium by dialysis was established. This method allowed the removal of toxic metabolites, as well as recycling of autocrine factors in the culture medium and reduced the use of macromolecules (TGF-β1, insulin, etc.) for the expansion of hiPSCs in suspension culture. The minimum inhibitory level of lactic acid for hiPSCs and lactic acid removal by dialysis were also investigated.

3.2 Materials and methods

3.2.1 Culture of hiPSCs

hiPSCs were obtained from Japanese Collection of Research Bioresources (Tic, JCRB number: 1331, Japan). For subculture, single cells (1.0×10^4 cells cm^{-2}) were seeded with 10 μM ROCK inhibitor (Tocris Bioscience, UK) in a 55 cm^2 culture dish (Corning Inc., USA) coated with laminin fragment (iMatrix™-511, Nippi Inc., Japan), with hiPSC expansion medium, mTeSR™1 (STEMCELL Technologies, Canada), 2 mm in depth. Cells were incubated at 37°C

in a humidified atmosphere with 5% CO_2, and medium was exchanged daily with fresh one. On day 4, when cells reached 80－90% confluence, cells were subcultured. For routine passage, hiPSCs were treated with 5 mM EDTA/PBS for 7 min at room temperature with 10 μM ROCK inhibitor. After that, dissociation reagent (TrypLE Select^{TM}, Invitrogen, USA) with10 μM ROCK inhibitor was applied for another 7 min at room temperature. After dissociating the hiPSC colony into single cells, 1.0×10^4 cells cm^{-2} were seeded with 10 μM ROCK inhibitor into a new culture dish.

3.2.2 Effect of lactic acid

The effect of lactic acid on hiPSC growth was studied in two-dimensional (2D) adherent culture. Media containing various initial concentrations of lactic acid (0, 0.5, 1.0, 1.5, 2.0 and 2.5 g L^{-1}) were used to culture hiPSCs in 6-well plates (Corning Inc., USA) coated with laminin at a seeding density of 1.0×10^4 cells cm^{-2}. The initial pH of the medium was adjusted with sodium hydroxide (NaOH) to 7.4. To determine the adhesion ratio, α and the apparent specific growth rate, μ^{app}, live cells were harvested, and dissociated into single cells by using TrypLE select. Then live cells were counted by using a cell counter (TC20, Bio-Rad Inc., USA) with trypan blue exclusion method. The adhesion ratio was determined by the equation, $\alpha = X_{24}/X_0$, where X_0 and X_{24} are cell densities at seeding and t = 24 h, respectively. The apparent specific growth rate, μ^{app} (h^{-1}), was calculated by the following equation: $\mu^{app} = \ln(X_f/X_i)/(t_f - t_i)$, where X_f is the final cell density at t_f = 72 h, and X_i is the initial cell density at t_i = 24 h. Flow cytometry was performed to detect the frequency of pluripotency markers of hiPSC, OCT 3/4, and TRA-1-60 at t = 72 h.

3.2.3 Medium refinement system

The medium refinement system was designed with a cross flow dialysis membrane filter by which used suspension culture medium was refreshed with DMEM/F-12 (Sigma-Aldrich, USA) as the dialysate. The cross flow dialysis membrane filter contained 300 hollow fibers, and had a 200 μm internal diameter, a MWCO of 10 kDa, and a surface area of 150 cm^2 (AKH-5S, Toyobo, Japan) (**Fig. 3.1**). Aseptic connections were maintained using silicone tubing. The used medium was collected from the aggregate suspension culture through a circular filter inside the bioreactor, which was prepared with hollow fibers (surface area, 25 cm^2; internal diameter, 1.3 mm; MFC-K1, Toyobo). The flow rate of used medium was 2.6 mL min^{-1}, whereas, the cross flow rate of fresh basal medium (DMEM/F12), which is considered to be equivalent to the basal component in expansion medium (Ludwig *et al*., 2006), was 6.6 mL min^{-1}. To analyze metabolites and cytokines, medium was sampled before and after dialysis. The concentrations of glucose, lactic acid and ammonium were analyzed using a biochemical analyzer (Bioprofile 400, Nova Biomedical). The concentrations of cytokines, specifically bFGF and TGF-β1, in the culture medium were determined by an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, UK).

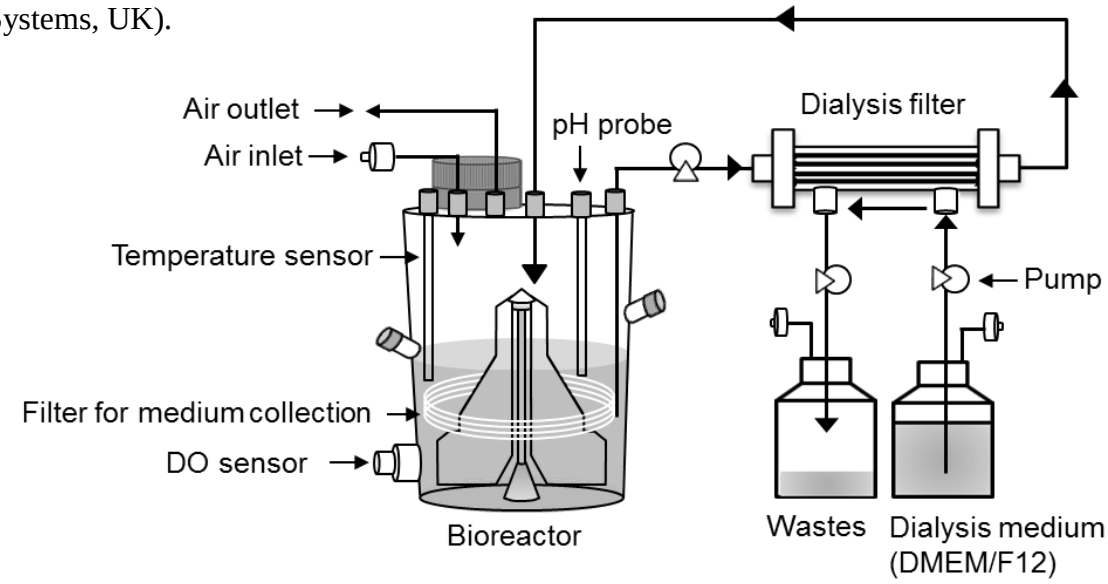


Fig. 3.1 Schematic diagram of medium refinement system for hiPSC culture in a stirred suspension bioreactor.

3.2.4 Adherent culture of hiPSCs with refined medium

Human iPSCs were cultured in a 6-well plate with fresh medium, refined medium with or without bFGF (100 μg L^{-1}, DS Pharma, Japan), and unrefined medium for 72 h. To determine the values of α and μ^{app}, live cells were counted at t = 24 and 72 h. Flow cytometry was used to detect OCT 3/4 and TRA-1-60 expression at t = 72 h.

3.2.5 Suspension culture with the medium refinement system

Suspension culture of hiPSC aggregates was performed by seeding single cells at a density of 2.0×10^5 cells mL^{-1} in a stirred suspension bioreactor (working volume: 100 mL, BWV-S10A, Able Co., Japan) with 10 μM ROCK inhibitor (**Fig. 3.1**). In the expansion medium, dissolved oxygen (DO, 3.2 mg L^{-1}) was regulated by air and N_2 gas. Moreover, pH was regulated by CO_2 and the agitation rate was maintained at 40 rpm. In the control culture, medium was exchanged with fresh medium every 24 h during the first 48 h and every 12 h thereafter. During culture with the medium refining system, spent medium was exchanged with fresh medium only at t = 24 h to remove the ROCK inhibitor. At t = 48 h, hiPSC aggregates, along with the used medium, were transferred to another 100 mL bioreactor which was equipped with the medium refinement system as the cell separation filter inside the bioreactor interrupted the formation of aggregates during the initial seeding of single cells at t = 24 h. Further culture was performed by dialyzing the used medium with the basal medium (600 mL at each time) supplemented with bFGF (100 μg L^{-1}) every 12 h, and TGF-β1 (1 μg L^{-1}, Peprotech, USA) every 24 h. Heparin (100 μg L^{-1}, STEMCELL Technologies, Canada) was added to both the control culture and medium refinement culture to stabilize bFGF. This experiment was repeated three times separately with three independent bioreactors. To determine specific growth rate, μ (h^{-1}), hiPSC aggregates were

collected from bioreactor (3 samples in each time) at t = 24, 48, 72, and 96 h, and dissociated into single cells by using TrypLE select. Then live cells were counted by using a cell counter with trypan blue exclusion method. The apparent specific growth rate, μ^{app} (h^{-1}), was calculated by the following equation: $\mu^{app} = \ln(X_f/X_i)/(t_f - t_i)$, where X_f is the final cell density at t_f = 96 h, and X_i is the initial cell density at t_i = 24 h. Culture medium was sampled every 12 h for analyzing metabolites (glucose and lactic acid). At t = 96 h, flow cytometry was performed for checking the expression of OCT 3/4 and TRA-1-60. To determine the amounts of remaining growth factors in the culture medium, used medium was collected at t = 24 and 96 h, and analyzed by ELISA.

3.2.6 Flow cytometry

Cells were treated with the Cytofix/Cytoperm™ permeabilization kit (BD Biosciences), and incubated with primary antibody, mouse monoclonal OCT 3/4 (Santa Cruz Biotechnology), and secondary antibody, APC anti-mouse IgG (BD Biosciences). Direct staining of TRA-1-60 was performed using FITC-conjugated mouse anti-human TRA-1-60 (BD Pharmingen, USA). Flow cytometry was performed with Japan-made sorter, analyzer (JSAN, Bay Bioscience, Japan), and data were analyzed by commercially available software (FlowLogic, Inivai Technologies, Australia).

3.2.7 Stabilization of bFGF

For bFGF stabilization, fresh medium was kept at 4°C and 37°C in a 30 mL bioreactor (BWV-S03A, Able Co., Japan) with or without heparin solution (100 μg L^{-1}) for 24 h, and then analyzed by ELISA (Furue *et al*., 2008; Chen *et al*., 2012). To investigate the consumption of

bFGF, hiPSC aggregates (5.0×10^5 cells mL^{-1}) were cultured in a 30 mL bioreactor with fresh medium supplemented with heparin. After 24 h of culture, the used medium was collected and analyzed by ELISA.

3.2.8 Statistical analysis

Data presented in this study were obtained from three independent experiments and expressed as the mean ± standard deviation (SD). Comparisons were evaluated statistically using Student's *t*-test, and values of $p < 0.05$ and $p < 0.01$ were considered significant.

3.3 Results

3.3.1 Effect of lactic acid on hiPSC growth and removal of lactic acid by dialysis

To investigate the effects of lactic acid on hiPSC growth, various concentrations of lactic acid were added to the culture medium, and cells were evaluated. Lactic acid was found to have negative effects on hiPSC growth, and μ^{app} decreased with increasing lactic acid concentrations in medium in adherent culture (**Fig. 3.2A**). More than 1 g L^{-1} of lactic acid had toxic effects, and 1.5 g L^{-1} of lactic acid completely inhibited the growth of hiPSCs. Lower frequency of TRA-1-60 positive cells (50.1% ± 3.0%) were also observed in culture with medium containing 1.0 g L^{-1} of lactic acid, compared to that in the control (62.6% ± 6.0%) (**Fig. 3.2B**). As the growth rate and frequency of TRA-1-60 decreased due to the toxic effects of lactic acid, removal of lactic acid from the culture medium might be beneficial for the expansion of hiPSC.

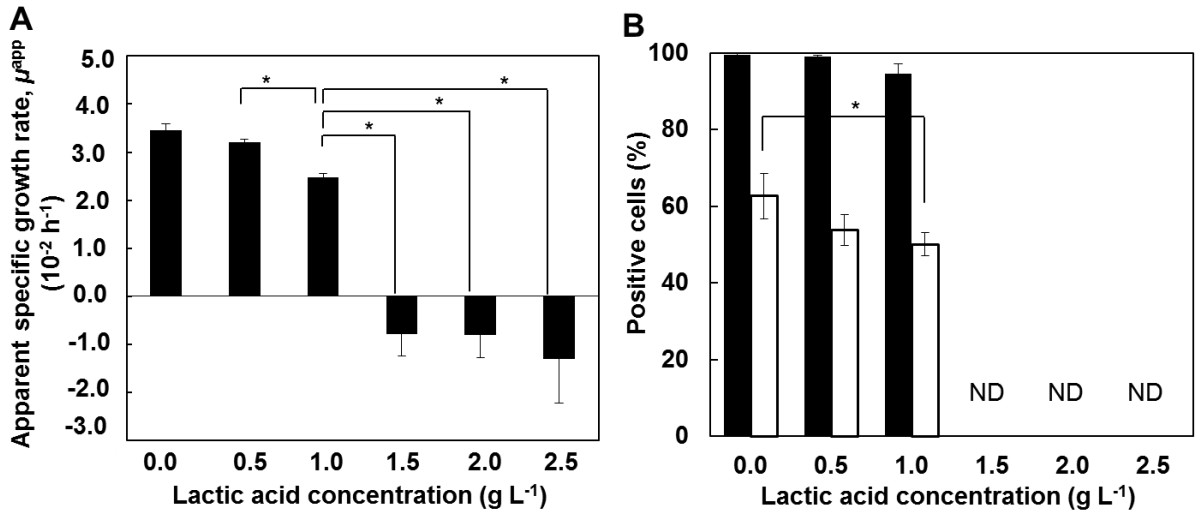


Fig. 3.2 Effect of lactic acid on hiPSCs growth and pluripotency. **(A)** Apparent specific growth rate of hiPSCs, and (**B**) frequency of OCT 3/4 (close bar) and TRA-1-60 (open bar) at different concentrations of lactic acid in adherent culture. *$p < 0.01$.

The medium refinement system, which was set to refresh 100 mL of used suspension culture medium with 600 mL of basal medium by dialysis, was able to remove lactic acid (1.18 ± 0.07 g L^{-1}) and ammonium (0.01 ± 0.00 g L^{-1}) from the used medium, and maintain a high concentration of glucose (3.20 ± 0.05 g L^{-1}) in the refined medium (**Table 3.1**).

3.3.2 Cytokines in the refined medium

Since the MWCO of the dialysis filter (10 kDa) was smaller than the size of most cytokines supplemented in hiPSC culture medium, almost all of the cytokines were considered to be retained after dialysis in the refined medium. In fact, 87% of bFGF and 80% of TGF-β1 remained in the refined medium after dialysis (**Table 3.1**). However, the concentration of bFGF in the refined medium was lower (1.2 ± 0.4 μg L^{-1}) than in the fresh medium (76.8 ± 5.1 μg L^{-1}). Further investigation of bFGF revealed that bFGF was thermally unstable at 37°C without heparin (Chen *et al*., 2012) (**Fig. 3.3**). However, low concentrations of bFGF (13.9 ± 1.1 μg L^{-1})

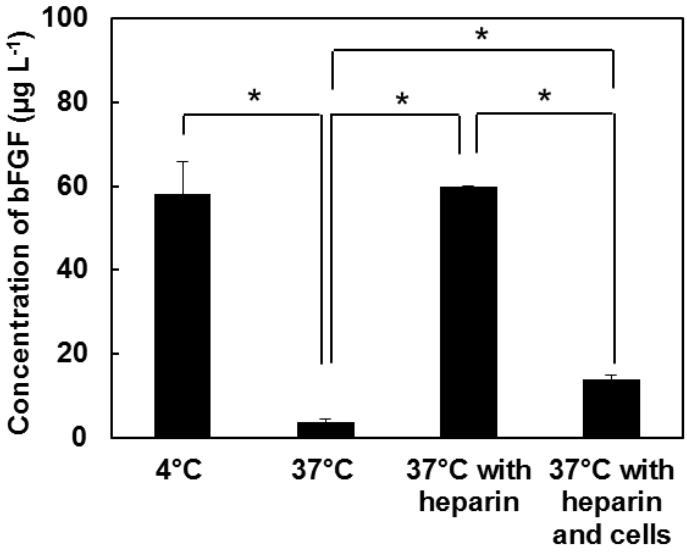


Fig. 3.3 Different storage conditions of fresh medium for checking bFGF aggregation, stabilization with heparin and consumption by cells after 24 h at 37°C. *$p < 0.01$.

were detected in the hiPSC suspension culture medium even when the medium was supplemented with heparin (**Fig. 3.3**). From this result, consumption of bFGF by hiPSC was confirmed in suspension culture.

Table 3.1 Concentration of medium components in fresh, dialysis, and used media

Medium components	Fresh medium (mTeSR™1)	Dialysis medium (DMEM/F-12)	Used medium	
			Before dialysis	After dialysis
Glucose (g L^{-1})	3.25 ± 0.06	3.40 ± 0.10	1.74 ± 0.55	3.20 ± 0.05*
Lactic acid (g L^{-1})†	N. D.	N. D.	1.18 ± 0.07	N. D.*
Ammonium (g L^{-1})††	N. D.	N. D.	0.01 ± 0.00	N. D.*
bFGF (μg L^{-1})	76.8 ± 5.1	N. D.	1.32 ± 0.32	1.16 ± 0.37
TGF-β1 (μg L^{-1})	1.40 ± 0.17	N. D.	0.25 ± 0.04	0.20 ± 0.04

*$p < 0.05$, N. D.: not detected, †Minimum detection limit: 0.2 g L^{-1}, †† Minimum detection limit: 0.004 g L^{-1}

3.3.3 Adherent culture with refined medium

After preparing refined medium by dialysis, the medium was screened for its ability to potentiate the proliferation of hiPSC in adherent culture. hiPSC showed a higher α (1.1 ± 0.1), and μ^{app} ((0.6 ± 0.2) × 10^{-2} h^{-1}) in the refined medium than in the unrefined medium (**Fig. 3.4A and B**), although μ^{app} in the refined medium was found to be lower than that in fresh medium ((3.7 ± 0.2) × 10^{-2} h^{-1}). When the refined medium was supplemented with bFGF, similar α (1.1 ± 0.2) and μ^{app} ((3.6 ± 0.2) × 10^{-2} h^{-1}) were observed as in the fresh medium. hiPSCs cultured in the refined medium supplemented with bFGF showed similar frequency of OCT 3/4 (98.6% ± 0.4%),

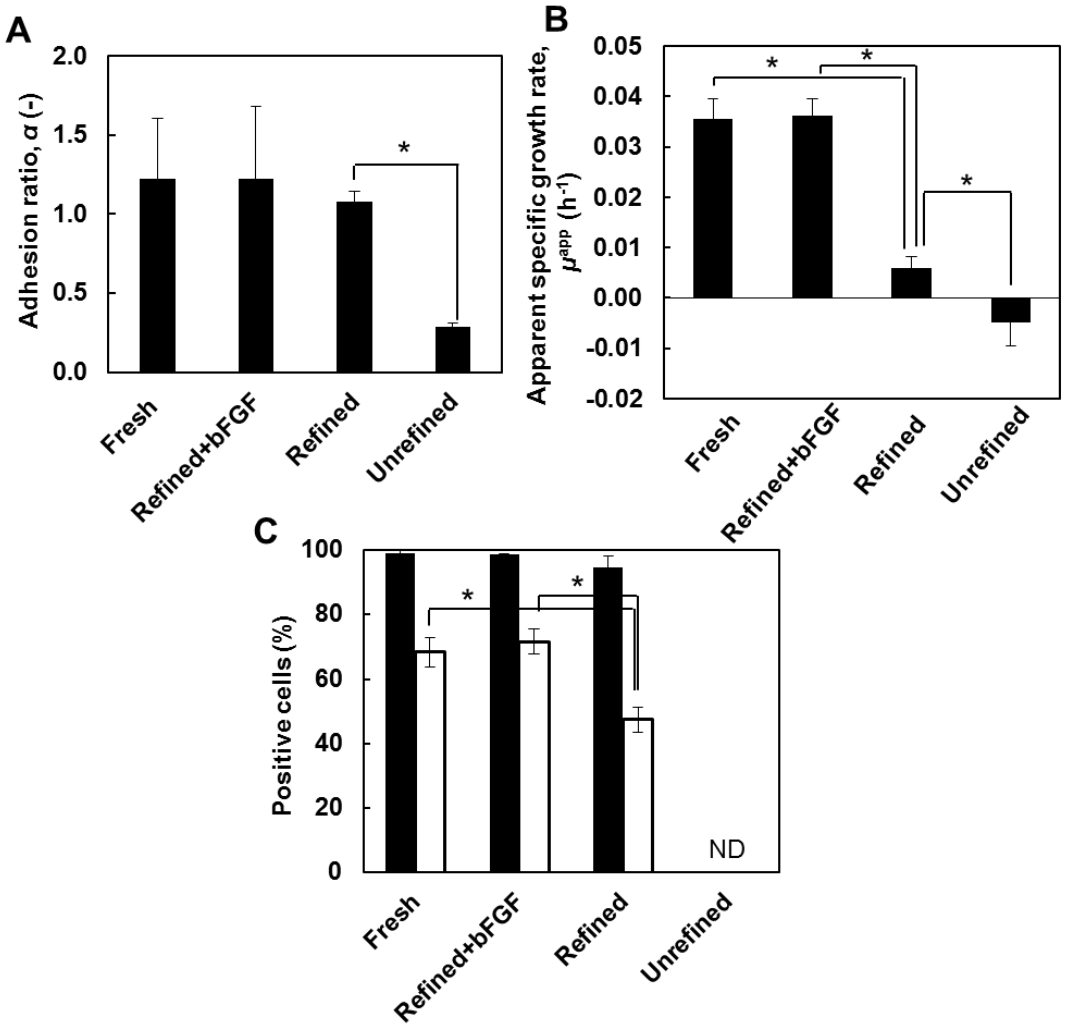


Fig. 3.4 Adherent culture of hiPSCs with the refined medium. (**A**) Adhesion ratio, (**B**) apparent specific growth rate, (**C**) Frequency of OCT 3/4 (close bar) and TRA-1-60 (open bar). *p < 0.01, ND.: Not determined.

and TRA-1-60 (71.6% ± 4.0%) as fresh medium, whereas, refined medium without bFGF showed lower frequency of TRA-1-60 (47.4% ± 3.8%) (**Fig. 3.4C**).

3.3.4 Suspension culture with the medium refinement system

Suspension culture of hiPSCs was performed using both fresh medium and the medium refinement system in a stirred suspension bioreactor. In the conventional culture using fresh medium, a final cell density of (8.1 ± 2.2) × 10^5 cells mL^{-1} was obtained at 96 h (**Fig. 3.5A**), and the production of lactic acid and consumption of glucose were observed (**Fig. 3.5C and D**).

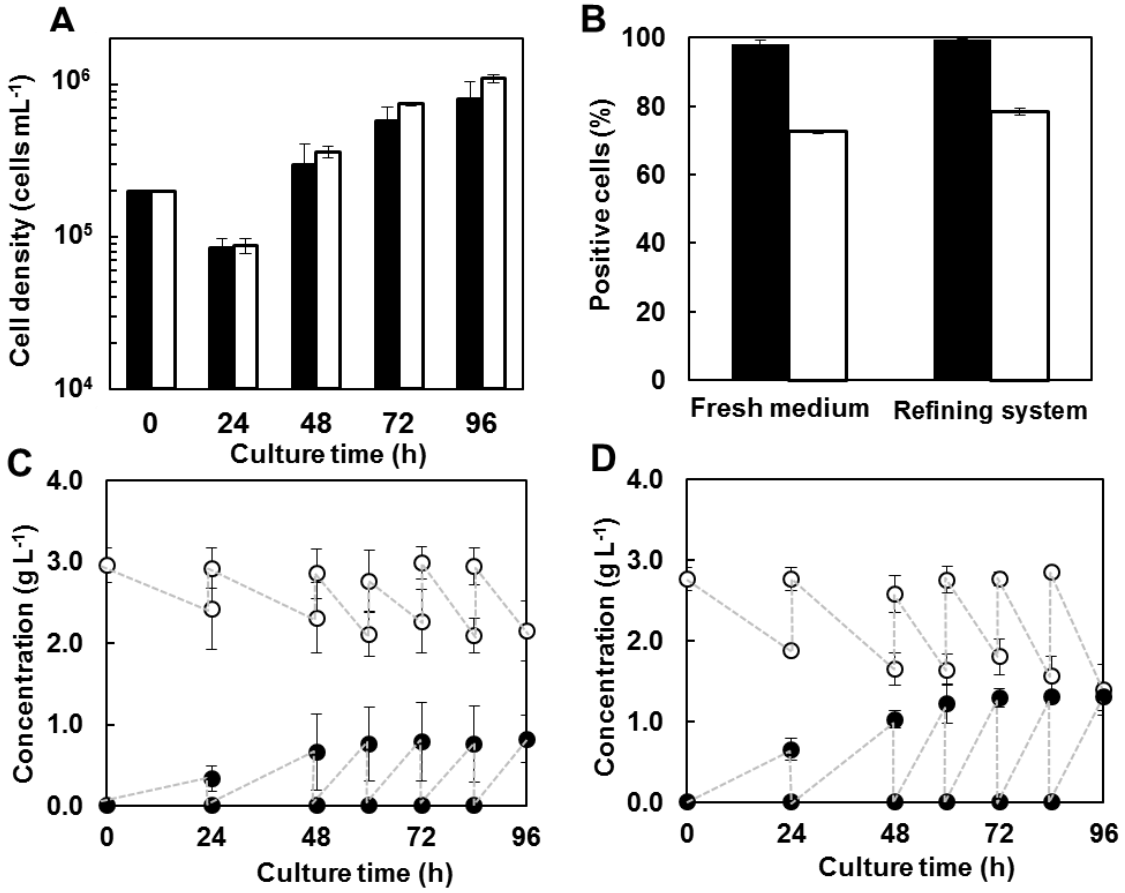


Fig. 3.5 Suspension culture of hiPSC with the medium refinement system. (**A**) Growth profile of hiPSC with fresh medium (close bar), and the medium refining system (open bar). (**B**) Frequency of OCT 3/4 (close bar) and TRA-1-60 (open bar). Concentration of lactic acid (close circle), and glucose (open circle) during culture with (**C**) fresh medium and (**D**) with the medium refining system.

In the culture with the medium refinement system, a final cell density, $X_f = (1.1 \pm 0.1) \times 10^6$ cells mL^{-1} was obtained when the medium was refined from t = 48 h (**Fig. 3.5A**), with the production of lactic acid and consumption of glucose in suspension culture (**Fig. 3.5C and D**). In addition, hiPSC in the culture with the medium refinement system showed a similar growth rate ($(3.5 \pm 0.2) \times 10^{-2}\ h^{-1}$) as that in conventional culture ($(3.1 \pm 0.2) \times 10^{-2}\ h^{-1}$). Flow cytometry analysis showed the frequency of OCT 3/4 and TRA-1-60 in conventional culture were 98.1% ± 0.9% and 69.2% ± 2.1%, respectively, whereas, the respective levels in the culture with the medium refinement system were 99.5% ± 0.2% and 78.3% ± 1.1% (**Fig. 3.5B**). When the used medium was investigated for autocrine factors, a higher concentration of bFGF was detected at t = 96 h of culture (50.1 ± 0.5 μg L^{-1}, and 64.4 ± 5.6 μg L^{-1}, in the conventional culture and culture with the medium refinement system, respectively) than at t = 24 h in the used culture medium (30.2 ± 2.6 μg L^{-1}, and 29.2 ± 5.4 μg L^{-1}, respectively) (**Fig. 3.6A**).

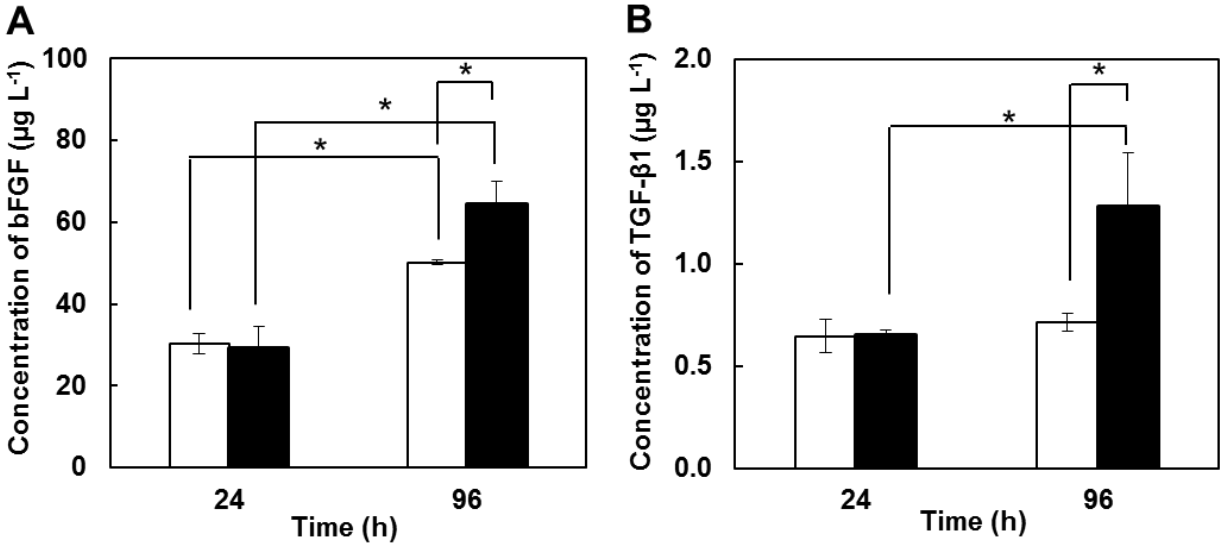


Fig. 3.6 Remaining amount of cytokines in used culture medium. Concentration of (**A**) bFGF, and (**B**) TGF-β1 during culture with fresh medium (open bar) and medium refinement system (close bar). * $p < 0.05$.

Higher concentrations of TGF-β1 were also detected in medium after 96 h of culture (1.3 ± 0.3 μg L^{-1}) than after 24 h (0.7 ± 0.0 μg L^{-1}) in the culture with medium refinement system (**Fig. 3.6B**). The concentrations of bFGF and TGF-β1 at 96 h in the used medium were higher in the culture with medium refinement system than in the conventional culture. By applying the medium refinement system, the use of costly medium components was reduced for the expansion of hiPSCs in suspension culture (**Table 3.2**).

Table 3.2 Theoretical amount of medium and other components required for conventional suspension culture and culture with the medium refinement system at a 100 -mL-scale

Required items	Conventional culture	Culture with the medium refinement system
Medium		
Fresh medium (mTeSR™1)	0.6 L	0.2 L
Dialysis medium (DMEM/F12)		2.4 L
Medium components		
bFGF	60 μg	60 μg
TGF-β1	1.2 μg	0.6 μg
Insulin	11.6 mg	3.9 mg
Transferrin	6.4 mg	2.1 mg
L-ascorbic acid	38.4 mg	12.8 mg
Selenium	8.4 μg	2.8 μg

3.4 Discussion

As high concentrations of toxic metabolites accumulate in the culture medium, frequent changing of medium is required to maintain proliferation of hiPSC in conventional suspension culture. In this study, to reduce the frequency of changing medium, a refinement system was constructed with a dialysis filter to remove toxic metabolites and to recycle macromolecules (including autocrine factors) in the culture medium, after determining the minimum inhibitory levels of lactic acid for hiPSC. The refinement system removed lactic acid and ammonium from the used medium, supplied high concentrations of glucose, and retained bFGF and TGF-β1 in the refined medium after dialysis. The refined medium promoted the growth of hiPSC after supplying bFGF in the adherent culture, since most of the bFGF had been consumed by the cells. When the refinement system was applied to suspension culture, a high cell density as well as high levels of pluripotency markers expression was obtained.

In the present study, hiPSCs were observed to be more sensitive to lactic acid than other cell types (**Fig. 3.2**A) (Hassell *et al*., 1991; Ozturk *et al*., 1992; Lao and Toth, 1997; Patel *et al*., 2000; Ouyang *et al*., 2007; Schop *et al*., 2009; Chen *et al*., 2010; Luo *et al*., 2012). As a consequence, 1 g L^{-1} of lactic acid inhibited the growth of hiPSCs, and caused lower expression levels of pluripotency markers, although other types of cells can tolerate more than 1 g L^{-1} of lactic acid. Human mesenchymal stem cells (hMSCs) and mouse hybridoma cells have been reported to tolerate more than 3 g L^{-1} of lactic acid (Ozturk *et al*., 1992; Patel *et al*., 2009), whereas, human hematopoietic cells, Chinese hamster ovary (CHO) cells, and monkey kidney cells can tolerate about 2 g L^{-1} of lactic acid (Hassell *et al*., 1991; Lao and Toth, 1997; Patel *et al*., 2000; Luo *et al*., 2012). Moreover, lactic acid had direct effects on hiPSC as the growth rate

significantly decreased with the increase in lactic acid concentration, even though the initial pH of the medium had been adjusted. Ouyang *et al*. reported that 1.5 g L^{-1} or more of lactic acid had toxic effects on mouse embryonic stem cells (mESCs) and could induce spontaneous differentiation (Ouyang *et al*., 2007). In addition, Chen *et al*. reported that the frequency of a hESC pluripotency marker (TRA-1-60) was reduced significantly in cultures with high concentration of lactic acid (1 g L^{-1}) (Chen *et al*., 2010). On the other hand, the specific production rate of lactic acid in hPSC was higher than it was in other types of cells. The specific production rate of lactic acid in mESC, hESC and hiPSC has been reported to be 0.09, 0.07, and 0.08 (present study) g h^{-1} 10^{-9} cells, respectively (Terstegge *et al*., 2007; Chen *et al*., 2010), whereas, it was 0.002, 0.04, 0.01, and 0.04 g h^{-1} 10^{-9} cells for CHO, hybridoma, hematopoietic, and Vero cells, respectively (Ozturk *et al*., 1992; Patel *et al*., 2000; Quesney *et al*., 2003; Luo *et al*., 2012). From this result, higher production of lactic acid was confirmed to be the major limiting factor for the proliferation of hiPSC, since hiPSC showed lactic acid toxicity at a lower concentration (1 g L^{-1}) than in other types of cells. To reduce the toxic effects of lactic acid, frequent removal of lactic acid from culture medium is advantageous for the proliferation of hiPSC.

Our proposed medium refinement system was efficient in removing lactic acid from the used medium and maintaining a high concentration of glucose in the refined medium (**Table 3.1**). It also removed ammonium from the used medium, although only trace amounts of ammonium were produced by cells which did not exceed the minimum inhibitory level (Chen *et al*., 2010). This was important because high concentrations of ammonium can be toxic to cells, causing cytosol vacuolization and subsequent cell death (Silvac *et al*., 2010). Based on these results, it

appears that the medium refining system could replace conventional medium change for the expansion of hiPSC in suspension culture.

The retention of bFGF and TGF-β1 in the refined medium showed that the refining system allowed efficient recycling of costly macromolecules including autocrine factors (**Table 3.1**). The concentration of bFGF detected in the refined medium was lower than that in fresh medium (100 μg L^{-1}) (Levenstein *et al*., 2006; Ludwig *et al*., 2006), and most of the bFGF was found to be consumed by cells (**Fig. 3.3**). Previously, it was assumed that thermal instability was the major cause for low concentrations of bFGF in medium after 24 h of culture, as bFGF can aggregate in an inactive form at 37°C (Chen *et al*., 2012). By stabilizing bFGF with heparin (Furue *et al*., 2008; Chen *et al*., 2012), we confirmed that not only thermal aggregation; but also consumption by cells were the major causes of low bFGF concentrations in the used, 24 h suspension culture medium. However, a similar growth rate and expression of pluripotency markers in both fresh and refined medium supplemented with bFGF showed that the refined medium could be used effectively for the expansion of hiPS cells in adherent culture (**Fig. 3.4**).

As high concentrations of toxic metabolites accumulate during daily medium change in conventional suspension culture, Fernandes-Platzgummer *et al*. reported that periodic removal (every 12 h) of these metabolites was beneficial for cell growth when culturing mESCs in a microcarrier-immobilized stirred tank reactor (Fernandes-Platzgummer *et al*., 2014). In this study, when medium was exchanged every 12 h after day 2, a final cell density of (8.1 ± 2.2) × 10^5 cells mL^{-1} with the expression of pluripotency markers were obtained on day 4 in conventional suspension culture (**Fig. 3.5**). Human iPSC cultured with the refining system, in

which DMEM/F12 was used as a dialysis medium, showed a similar cell density ((1.1 ± 0.1) × 10^6 cells mL^{-1}), and metabolic activity when compared with the conventional suspension culture. In suspension culture with the medium refining system, low molecular weight components of the fresh medium (insulin (5.8 kDa), selenium (0.08 kDa), L-ascorbic acid (0.18 kDa), pipecolic acid (0.13 kDa), gamma-aminobutyric acid (GABA) (0.1 kDa), etc. in mTeSR™1), which might pass through the dialysis membrane (MWCO: 10 kDa), were removed from the used culture medium. Only high molecular weight components (bFGF (18 kDa), TGF-β1 (25 kDa), transferrin (80 kDa), bovine serum albumin (BSA) (66 kDa) and autocrine factors) were retained in the refined medium after dialysis (**Table 3.3**).

Although insulin, selenium, and L-ascorbic acid play prominent roles in cell survival, self-renewal, and proliferation of hPSCs during multiple passages (Chen *et al*., 2011), the refined medium promoted the growth of hiPSCs without these components. It was assumed that the roles of these removed-components were fulfilled by the retained-components of the refined medium, especially bFGF and TGF-β1, as well as other autocrine factors that have overlapping functions of promoting self-renewal and undifferentiated proliferation of hPSCs (Amit *et al*., 2004; James *et al*., 2005; Xu *et al*., 2005). During suspension culture with the medium refinement system, higher concentrations of bFGF and TGF-β1 were detected in the 96 h used culture medium, than that in the conventional suspension culture (**Fig. 3.6**). This remaining amount of bFGF and TGF-β1 were possibly due to less consumption by cells, or recycling by the medium refining system, or secretion from cells in suspension culture. hESCs were reported to secrete bFGF, and stimulate the production of TGF-β1 and insulin as autocrine factors in adherent culture (Dvorak *et al*., 2005; Bendall *et al*., 2007; Greber *et al*., 2007; Montes *et al*., 2009).

Table 3.3 Comparison of medium components between conventional culture and medium refinement system

Medium components	Conventional culture	Medium refinement system
DMEM/F12	√	√
L-Ascorbic acid	√	-
Selenium	√	-
Transferrin	√	*
$NaHCO_3$	√	-
Glutathione	√	-
L-Glutamine	√	-
Defined lipids	√	-
Thiamine	√	-
Trace element B	√	-
Trace element C	√	-
BSA	√	*
bFGF	√	√
TGF-β1	√	√
Insulin	√	-
Pipecolic acid	√	-
LiCl	√	-
GABA	√	-
Autocrine factors		*

√ : Included in medium
* : Expected to be contained in the refined medium
- : Expected not to be contained in the refined medium
Blank: Not included in medium

In the conventional suspension culture, autocrine factors are removed from culture medium during daily medium change. As suspension culture of hiPSC requires large volume of medium supplemented with high concentration of growth factors (macromolecules) (Tian *et al*., 2004; Ludwig *et al*., 2006), removal of autocrine factors during daily medium change results in high consumption of macromolecules. During culture with the medium refining system, additional supplementation with macromolecules was not needed, except for bFGF and TGF-β1 (50% less supplementation) (**Table 3.2**). Therefore, the established medium refinement system may provide a platform for saving growth factors for the expansion of hiPSCs by recycling macromolecules, as well as autocrine factors in suspension culture.

In conclusion, a culture medium refinement method by dialysis was established in which toxic metabolites were removed, and macromolecules of medium (bFGF, TGF-β1, etc.) including autocrine factors were recycled efficiently for the expansion of hiPSCs in suspension culture. As large numbers of cells with high levels of pluripotency markers expression were obtained by exchanging fresh medium only once, and further refinement of medium with the refinement system, this system is proposed to provide a cost-effective platform by reducing the consumption of growth factors for the expansion of hiPS cells in suspension culture.

3.5 Summary

hiPSCs secrete essential autocrine factors that are removed along with toxic metabolites when the growth medium is exchanged daily. In this study, after determining the minimum inhibitory level of lactic acid for hiPSCs, a medium refinement system was constructed by which toxic metabolites were removed from used culture medium, and autocrine factors as well as other growth factors were recycled. Specifically, about 87% of bFGF and 80% of TGF-β1 were retained in the refined medium after dialysis. The refined medium efficiently potentiated the proliferation of hiPS cells in adherent culture. When the refinement system was used to refresh medium in suspension culture, a final cell density of $(1.1 \pm 0.1) \times 10^6$ cells mL^{-1} was obtained, with 99.5% ± 0.2% OCT 3/4 and 78.3% ± 1.1% TRA-1-60 expression, on day 4 of culture. These levels of expression were similar to those observed in conventional suspension culture. With this method, culture medium refinement by dialysis was established to remove toxic metabolites, recycle autocrine factors, as well as other growth factors, and reduce the use of macromolecules for the expansion of hiPSCs in suspension culture.

Chapter 4

Suspension culture of hiPSCs with medium refinement and aggregate break-up for high-density and high-yield

4.1 Introduction

After the first isolation of human induced pluripotent stem cells (hiPSCs) by Yamanaka group (Takahashi *et al*., 2007), application of these cells are increasing day by day as regenerative medicine for cell replacement therapies (Schulz *et al*., 2012), tissue engineering (Badylak *et al*., 2011), and drug discovery (Wernig *et al*., 2007) due to their pluripotency and self-renewal capacity. To realize their potential, large-scale expansion of hiPSCs is important as practically more than 10^9–10^{10} cells per patient are needed for clinical application (Lock and Tzanakakis, 2007; Jing *et al*., 2008; Kehoe *et al*., 2010; Olmer, *et al*., 2010; Serra *et al*., 2010). Compared to adherent culture, suspension culture of hiPSC aggregate is promising to obtain such numbers of cells, as a higher cell density, and better control of process parameters can be achieved in a stirred suspension bioreactor culture (Zweigerdt *et al*., 2011; Olmer *et al*., 2012; Haraguchi *et al*., 2015).

During the initial seeding in suspension culture, single hiPSCs are inoculated into culture vessel, and cells agglomerate to form sphere shape aggregates by encountering and connecting with neighboring cells which is mediated by E-cadherin (Sugawara *et al*., 2010; Li *et al*., 2012). The size of aggregate increases with the increase of culture time and some of the aggregates simultaneously fuse to form an un-uniform shape (Bauwens *et al*., 2008; Otsuji *et al*., 2014). The prolongation of culture time leads to undesired differentiation, as well as cell necrosis inside the aggregate which also cause the difficulty for maintaining proper growth in suspension culture (Sachlos *et al*., 2008). Moreover, accumulation of toxic components increases with the increase

of cell density in suspension culture which inhibits the growth of hiPSC (Chen *et al*., 2010). Production of lactic acid is higher in human pluripotent stem cell (hPSC) culture than it is in other cell types (Varum *et al*., 2011), and lactic acid has potential toxic effects on the proliferation of hPSC (Ouyang *et al*., 2007; Chen *et al*., 2010). As a result, frequent changing of culture medium is required to remove toxic metabolites (Fernandes-Platzgummer *et al*., 2014). On the other hand, hPSCs secrete various autocrine factors in the culture medium that maintain culture homeostasis, and potentiate the growth of cells (Bendall *et al*., 2007; Montes *et al*., 2009). These autocrine factors are removed from culture medium along with toxic metabolites during daily medium changes in conventional suspension culture. Moreover, the cost of culturing hiPSC increases with increasing medium consumption, as the culture medium contains many costly macromolecules including bFGF, TGF-β1, and insulin (Tian *et al*., 2004; Ludwig *et al*., 2006). Therefore, maintaining small size aggregates and recycling costlier macromolecules is important to obtain high cell density as well as for reducing the culture cost.

In this study, an improved operational design of hiPSC culture was applied for obtaining high cell density by breaking up of hiPSCs aggregates into small sizes, and integration of medium refinement system for efficient recycling of autocrine factors and other growth factors in suspension culture. This integrated system was also investigated for high-yield on medium in suspension culture.

4.2 Materials and methods

4.2.1 Culture of hiPSCs

hiPSCs were obtained from Japanese Collection of Research Bioresources (Tic, JCRB number: 1331). For subculture, single cells (1.0×10^4 cells cm^{-2}) were seeded with 10 μM ROCK inhibitor (Tocris Bioscience, UK) in a 55 cm^2 culture dish (Corning Inc., USA) coated with laminin fragment (iMatrix™-511, Nippi Inc., Japan), with hiPSC expansion medium, mTeSR™1 (STEMCELL Technologies, Canada), 2 mm in depth. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2, and medium was exchanged daily with fresh medium. On day 4, when cells reached 80－90% confluence, cells were subcultured. For routine passage, hiPSCs were treated with 5 mM EDTA/PBS for 7 min at room temperature with 10 μM ROCK inhibitor. After that, dissociation reagent (TrypLE Select™, Invitrogen, USA) with10 μM ROCK inhibitor was applied for another 7 min at room temperature. After dissociating the hiPSC colony into single cells, 1.0×10^4 cells cm^{-2} were seeded with 10 μM ROCK inhibitor into a new culture dish.

4.2.2 Medium refinement system

The medium refinement system was designed with a cross flow dialysis membrane filter by which used suspension culture medium was refreshed by using DMEM/F12 (Sigma-Aldrich, USA) as the dialysate. The cross flow dialysis membrane filter contained 300 hollow fibers, and had a 200 μm internal diameter, a MWCO of 10 kDa, and a surface area of 150 cm^2 (AKH-5S, Toyobo) (**Fig. 4.1**). Aseptic connections were maintained using silicone tubing. The flow rate of used medium was 2.6 mL min^{-1}, whereas, the cross flow rate of fresh basal medium

(DMEM/F12), which was considered to be equivalent to the basal component in expansion medium, was 6.6 mL min^{-1}.

4.2.3 Suspension culture with the medium refinement and aggregate break-up

Suspension culture of hiPSC aggregates was performed by seeding single cells at a density of 2.0×10^5 cells mL^{-1} in a stirred suspension bioreactor (working volume: 120 mL, BWV-S10A, Able Co., Japan) with ROCK inhibitor (**Fig. 4.1**). In the expansion medium, dissolved oxygen (DO, 3.2 mg L^{-1}) was regulated by air and N_2 gas. Moreover, pH was regulated by CO_2 and the agitation rate was maintained at 40 rpm. In the control culture, medium was exchanged with fresh one every 24 h during the first 48 h and every 12 h thereafter. During culture with the medium refining system, spent medium was exchanged with fresh medium only at t = 24 h to remove the ROCK inhibitor. At t = 48 h, 100 mL of used medium was transferred to another bioreactor which was set with the medium refinement system. Further culture was performed by dialyzing the used medium with the basal medium (600 mL at each time) supplemented with bFGF (100 μg L^{-1}) every 12 h. After finishing the medium refinement at each time, the refined medium was transferred to the original bioreactor. At 87 h, HA (40 nM, because the aggregates were difficult to break with 20 nM in the refined medium) was added to the culture medium, and kept for 9 h. Aggregates were broken into small sizes by pipetting, and all of the cells were re-seeded into the bioreactor with 10 μM ROCK inhibitor and cultured for additional 96 h. In the control culture, aggregates were not treated with HA, as well as not broken into small sizes by pipetting.

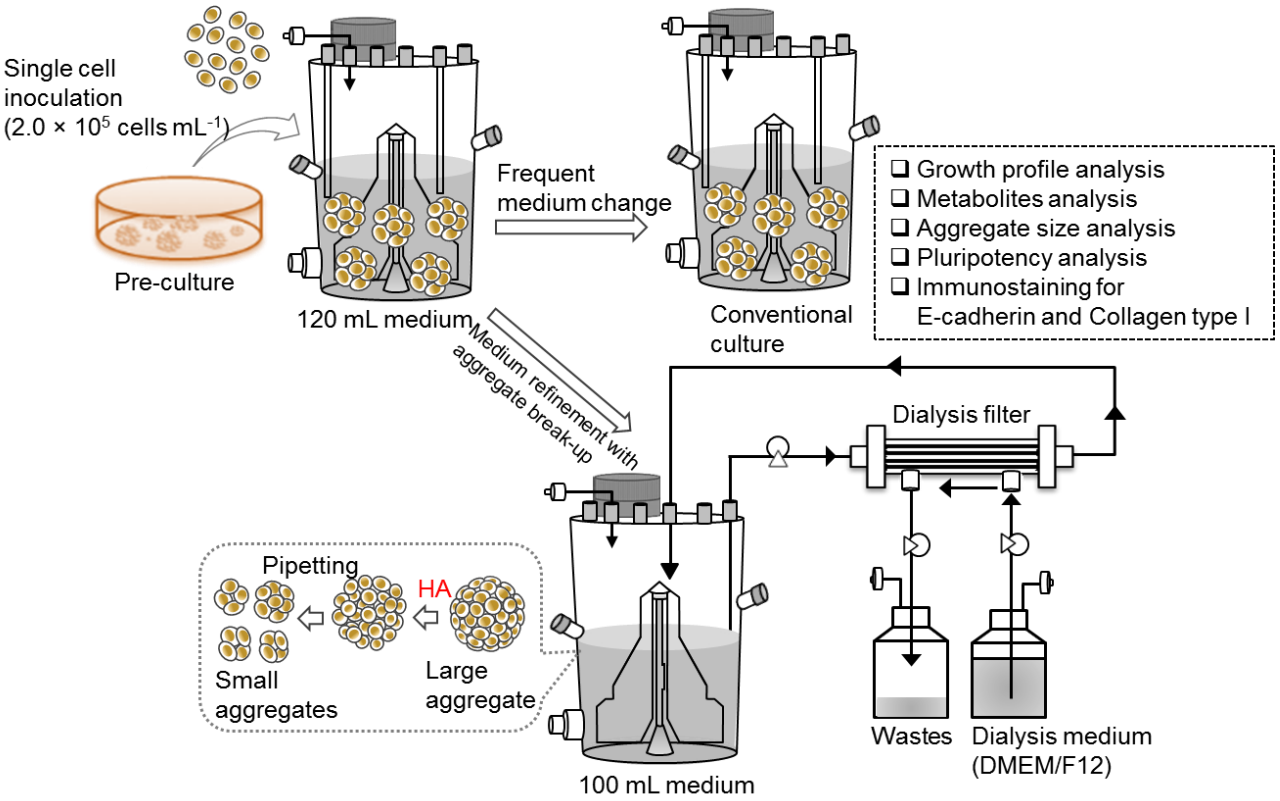


Fig. 4.1 Schematic diagram showing the experimental methods for integrating the medium refinement system with aggregate break-up by HA for obtaining high-density of hiPSCs in suspension culture.

4.2.4 Kinetic Analysis of Growth Properties of hiPSC Aggregates

To determine apparent specific growth rate, μ^{app} (h^{-1}), hiPSC aggregates were collected from the bioreactor (3 samples each time) at every 24 h, and dissociated into single cells by using TrypLE Select™. After centrifugation, supernatant was discarded, and cells were re-suspended in fresh medium. Then live cells were counted by using the cell counter. Initial live cell ratio, α (-), was determined by $\alpha = X_{24}/X_0$, where, X_0 is the cell density at seeding, and X_{24} is the cell density at t = 24 h. Apparent specific growth rate, μ^{app} (h^{-1}), was calculated by the following equation: $\mu^{app} = \ln(X_f/X_i)/(t_f - t_i)$, where, X_f is the final cell density at t_f = 87 or 192 h, and X_i is the initial cell density at t_i = 24 or 120 h. Expansion fold (-) was calculated by X_f/X_i, where, X_f is the final cell density at t_f = 87 or 192 h, and X_i is the initial cell density at t_i = 0 or 96 h. Yield on

culture medium, Y (cells mL^{-1}) was calculated by the following equation, $Y = N_t/V_m$, where, N_t is the total number of cells (cells), and V_m is the total volume of medium consumption (mL). To determine the aggregate size, images were captured at every 24 h by using an image analyzer with a 4× objective lens (IN Cell Analyzer 2000; GE Healthcare, USA), and aggregate diameter was measured by using Image-Pro Plus software (Version 6.0; Media Cybernetics, USA).

4.2.5 Analysis of Medium Components

To analyze the components of culture medium associated with cell cultivation, medium was collected at every time during medium change, and analyzed for glucose and lactic acid by using a biochemical analyzer (Bioprofile 400; Nova Biomedical, USA). To determine the amounts of remaining growth factors, specifically bFGF and TGF-β1 in the culture medium, used medium was collected at t = 24, 87, and 192 h, and analyzed by an ELISA kit (R&D Systems, UK).

4.2.6 Flow Cytometry

To perform flow cytometry, cells were collected at t = 192 h for checking the expression of pluripotency markers, OCT 3/4 and SSEA 4. The procedure for flow cytometry was similar to that described previously. Briefly, after preparing single cells, cells were treated with the Cytofix/Cytoperm™ permeabilization kit (BD Biosciences, USA) and incubated with primary antibody, mouse monoclonal OCT 3/4 (Santa Cruz Biotechnology, USA), and secondary antibody, APC anti-mouse IgG (BD Biosciences). Direct staining of SSEA 4 was performed by using human/mouse SSEA 4 fluorescein (R&D Systems, USA). Flow cytometry was performed with a flow cytometer (Sysmex), and data were analyzed by commercially available software (FlowLogic, Inivai Technologies, Australia).

4.2.7 Fluorescence Staining

The procedure for staining was similar to that described previously. Briefly, hiPSC aggregates were washed with PBS, and then embedded in optical cutting temperature compound (Tissue-Tek; Sakura Fine Chemical Co., Ltd.), and sectioned into 20-μm- thick slices by using a cryostat microtome (Leica). The sections were then washed with PBS, and fixed with 4% paraformaldehyde (Wako Pure Chemical Industries) for 10 min at room temperature. After washing with PBS, the specimens were permeabilized with PBS containing 0.5% Triton X-100 (Wako Pure Chemical Industries) for 5 min, then washed twice with PBS containing 0.1% Tween 20 (Sigma-Aldrich), and blocked in Block Ace (DS Pharma Co. Ltd.) for 90 min at room temperature. The sections were washed with Tris-buffered saline (TBS; Dako), and then probed with anti-E-cadherin antibody (Santa Cruz Biotechnology, USA), and anti-collagen type I antibody (Abcam, USA), and kept overnight at 4°C. Next, they were washed twice with TBS, and immuno-labeled with Alexa Fluor 488-conjugated goat anti-rabbit (Santa Cruz Biotechnology, USA) or Alexa Fluor 594-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) for 60 min at room temperature. The cell nuclei were stained with 4´, 6-diamidino-2-phenylindole (DAPI; Life Technologies) for 20 min at room temperature. Images were captured by using a confocal laser scanning microscope (FV 1000; Olympus), with 20× and 60× objective lens.

4.2.8 Statistical Analysis

Data presented in this study were obtained from two independent experiments and expressed as mean ± standard deviation (SD). Statistical comparisons were evaluated using the Student's t-test and values of $p < 0.01$ and $p < 0.05$ were considered to be significant.

4.3 Results

To obtain high-density of hiPSCs, aggregates were exposed to HA for 9 h, and broken up into small sizes at t = 96 h by pipetting. Lower expression of E-cadherin was observed after 9 h of HA addition which indicated the weakening of cell-cell connections by disrupting the E-cadherin located in the intercellular junctions during culture with the integrated system (**Fig. 4.2**). hiPSC aggregates showed higher expression of collagen type I at the periphery before HA addition at t = 87 h, whereas, the expression decreased at the aggregate periphery after exposure to HA at t = 96 h.

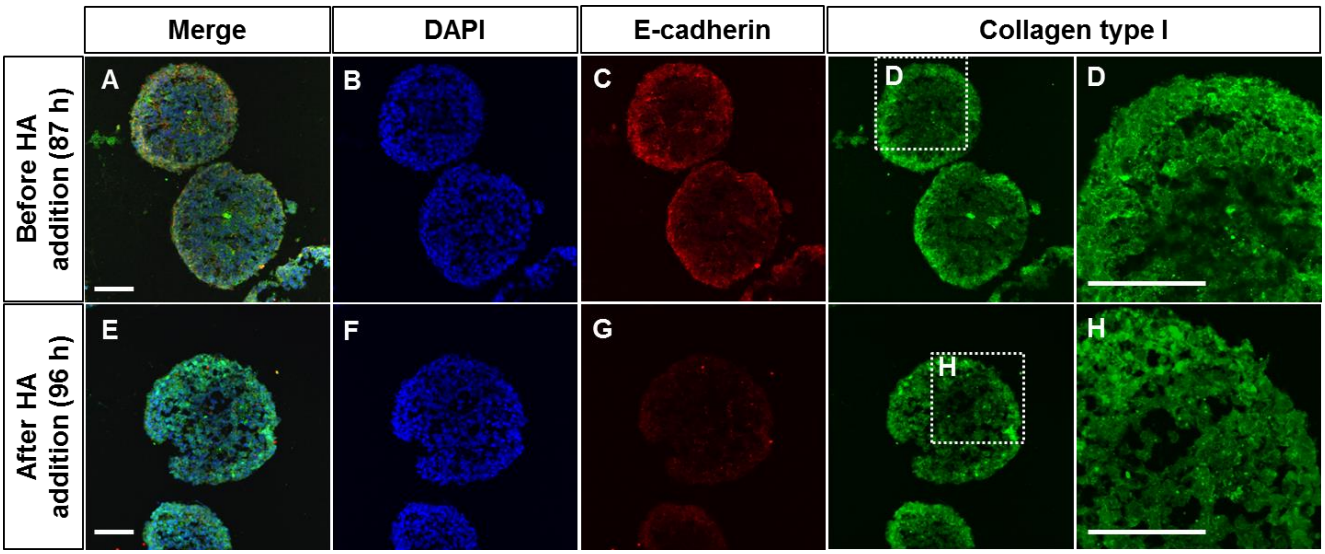


Fig. 4.2 Fluorescent images of hiPSC aggregates for DAPI (blue), E-cadherin (red), and collagen type I (green) before (A-D), and after (E-H) adding HA at 87 and 96 h, respectively. Panels D and H show magnified views of the boxed areas. Scale bars: 100 μm.

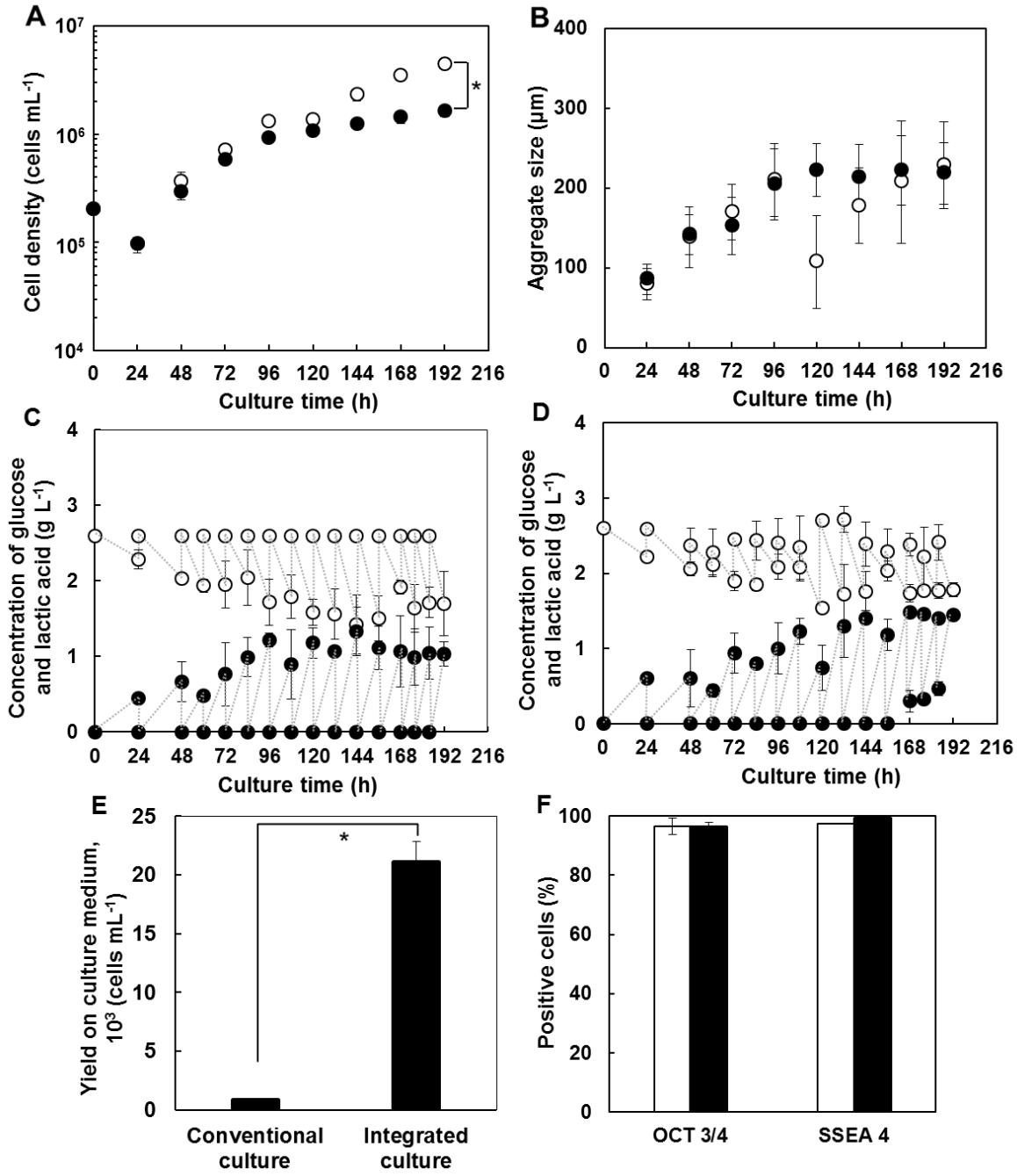


Fig. 4.3 High-density culture of hiPSCs by aggregate break-up and medium refinement. (**A**) Growth profile, and (**B**) aggregate size of hiPSCs in the conventional culture without aggregate break-up (close circle), and integrated culture (open circle). Glucose consumption (open circle), and lactic acid production (close circle) in the (**C**) conventional culture without aggregate break-up, and (**D**) integrated culture with aggregate break-up. (**E**) Yield on culture medium. (**F**) Flow cytometry for OCT 3/4 and SSEA 4 in the conventional culture (open bar), and integrated culture (close bar). *$p < 0.01$.

Table 4.1 Comparison of growth properties of hiPSCs in conventional culture and integrated culture.

	Conventional culture		Integrated culture	
	Early-stage (0–96 h)	Late-stage (96–192 h)	Early-stage (0–96 h)	Late stage (96–192 h)
Apparent specific growth rate, μ^{app} (10^{-2} h^{-1})	3.2 ± 0.2	1.0 ± 0.3	3.2 ± 0.1	2.0 ± 0.1*
Final cell density, X_f (10^5 cells mL^{-1})	9.2 ± 0.5	16.3 ± 1.9	1.3 ± 0.1	44.1 ± 3.5*
Expansion fold (-)	4.5 ± 0.2	1.5 ± 0.1	6.5 ± 0.5	3.3 ± 0.1*
Yield on culture medium (0-192 h) (10^4 cells mL^{-1})	0.1 ± 0.01		2.1 ± 0.02*	

*$p < 0.01$

hiPSC showed higher cell density ($(4.3 \pm 0.3) \times 10^6$ cells mL^{-1}) in the integrated culture in comparison to that in the conventional culture without aggregate break-up (**Fig. 4.3A)**. When aggregates were broken into small sizes with HA, small size of aggregates was observed at t = 120 h, and the size of aggregates increased gradually thereafter (**Fig. 4.3B)**. However, aggregate size did not change significantly during late-stage of conventional culture. Higher consumption of glucose and production of lactic acid was observed in hiPSC in the integrated culture compared to conventional culture (**Fig. 4.3C and D**). Lactic acid was not completely removed from the used medium in the integrated culture. However, hiPSC showed higher yield on culture medium in the integrated culture compared to the conventional culture during the late-stage (**Fig. 4.3 E**). Moreover, similar frequency of OCT 3/4 and SSEA 4 were observed in the integrated culture and the conventional culture without aggregate break-up (**Fig. 4.3 F**).

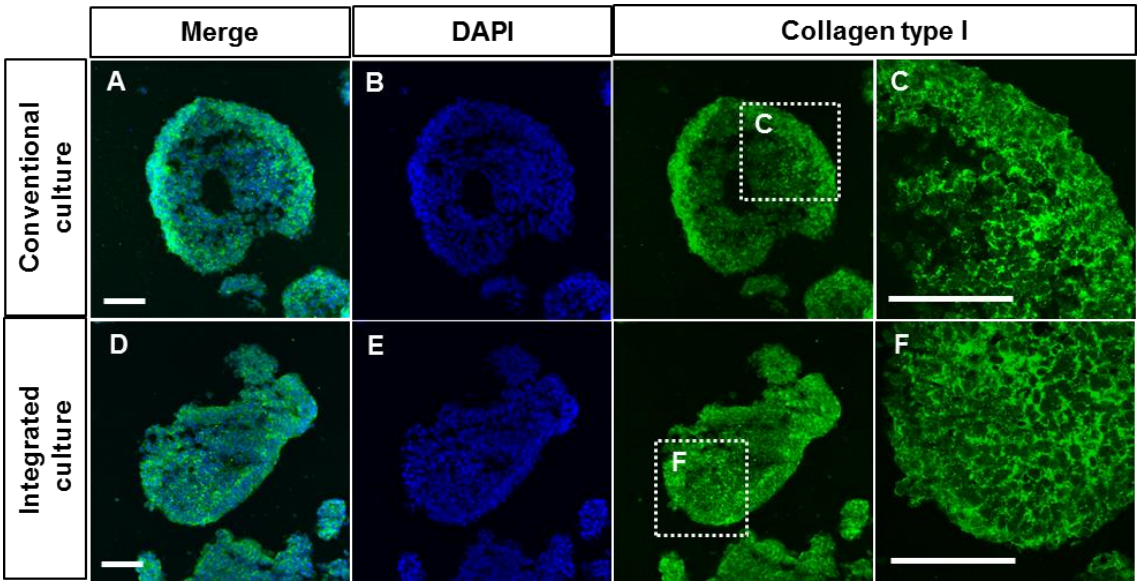


Fig. 4.4 Fluorescent images of hiPSC aggregates for DAPI (blue) and collagen type I (green) after t = 192 h in conventional culture (A-C), and integrated culture (D-F). Panels C and F show magnified views of boxed areas. Scale bars: 100 µm.

hiPSCs showed higher specific growth rate ($(2.0 \pm 0.1) \times 10^{-2}$ h^{-1}) in the integrated culture compared to the conventional culture without aggregate break-up ($1.0 \pm 0.3) \times 10^{-2}$ h^{-1}) during late-stage of culture (**Table 4.1**). In addition, more than 21-times higher expansion folds of hiPSCs were obtained by the integrated culture in comparison to the conventional culture without aggregate break-up. Higher accumulation of collagen type I was observed on the aggregate periphery at t = 192 h in the conventional culture in comparison to the integrated culture (**Fig. 4.4**).

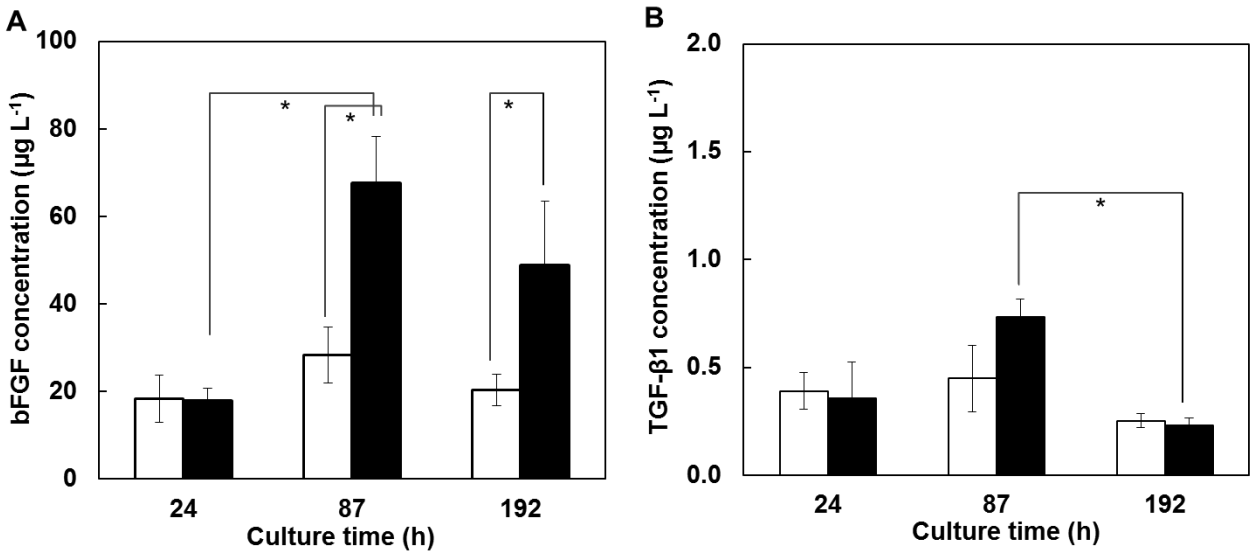


Fig. 4.5 Remaining amount of cytokines in the used culture medium. Concentrations of (**A**) bFGF, and (**B**) TGF-β1 in the conventional culture (open bar), and integrated culture (close bar). * $p < 0.01$.

Higher concentrations of bFGF was detected at t = 87, and t = 192 h in the used medium of integrated culture compared to the conventional culture (**Fig. 4.5A**). The concentration of TGF-β1 was similar in both conventional culture and culture with the integrated system during 24-87 h (**Fig. 4.5B**). However, the concentration of TGF-β1 decreased significantly at t = 192 h in the integrated culture. During expansion of hiPSCs with the integrated culture, additional supplementation of macromolecules (growth factors) was not needed, except for bFGF (**Table 4.2**). Therefore, supplementation of a large amount of costlier macromolecules was reduced, especially TGF-β1 and insulin during culture with the medium refinement system.

Table 4.2 Theoretical amounts of medium and other components required for conventional suspension culture and integrated culture for 100-mL-scale. The estimated amounts are for a single experiment.

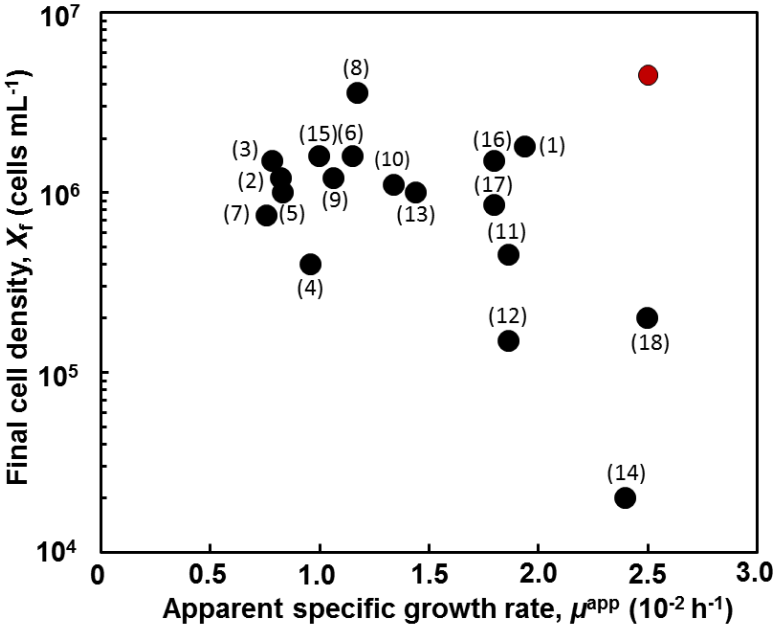
Required items	Conventional culture	Integrated culture
Medium		
Fresh medium (mTeSR™1) (L)	1.6	0.2
Dialysis medium (DMEM/F12) (L)		8.0
Medium components		
bFGF (μg)	160	160
TGF-β1 (μg)	3.2	0.6
Insulin (mg)	30.9	3.9
Transferrin (mg)	17.1	2.1
L-ascorbic acid (mg)	102.4	12.8
Selenium (μg)	22.4	2.8

4.4 Discussion

In this study, an integrated culture was established by combining the medium refining system with the aggregate break-up by HA for obtaining high-density of hiPSCs with low supplementation of growth factors as shown in **Fig. 4.7**. The integrated culture was efficient in providing high yield on culture medium, as well as recycling the autocrine factors for reducing the consumption of growth factors in suspension culture.

In the integrated culture, after adding HA, compact aggregates with smooth edge turned into fragile aggregates with rough edge which was further broken into small sizes by pipetting after 9 h of HA addition (**Fig. 4.2**). Generally, cell-cell connections in hiPSC aggregates are mediated by E-cadherin (Chen *et al*., 2010; Li *et al*., 2012). While hiPSC aggregates were exposed to HA,

cell-cell connections were loosened by directly disrupting the E-cadherin located in the intercellular barrier of hiPSCs aggregate. Thus, lower expression of E-cadherin was observed throughout the aggregate after 9 h of HA addition (**Fig. 4.2G**). HA was reported to disrupt the E-cadherin mediated cell-cell connections in different types of cells in adherent culture (Sugawara *et al*., 2010; Lee *et al*., 2014; Kim *et al*., 2017). Moreover, the distribution of collagen type I in the aggregate periphery changed significantly after 9 h of HA addition (**Fig. 4.2D and H**). While aggregates were exposed to HA, the E-cadherin mediated cell-cell connections were disrupted which was considered to promote higher migration of cells in the aggregate. High migration of cells might result in the disruption of organized collagen type I skeleton in the aggregate after 9 h of HA addition in the culture medium.



Culture systems	References
Stirred suspension Bioreactor culture	Amit *et al*., 2011 (1)
Stirred suspension Bioreactor culture	Abbasalizadeh *et al*., 2012 (2)
Stirred suspension Bioreactor culture	Olmer *et al*., 2012 (3)
Stirred suspension Bioreactor culture	Matsuura *et al*., 2012 (4)
Stirred suspension Bioreactor culture	Haraguchi *et al*., 2013 (5)
Stirred suspension Bioreactor culture	Wang *et al*., 2013 (6)
Stirred suspension bioreactor culture	Miranda *et al*., 2016 (7)
Stirred suspension Bioreactor culture	Kropp *et al*, 2016 (8)
Stirred suspension Bioreactor culture	Kwok *et al*., 2017 (9)
Stirred suspension Bioreactor culture	Kino-oka lab (10)
Stirred suspension culture in tube	Elanzew *et al*., 2015 (11)
Suspension culture in plate	Olmer *et al*., 2010 (12)
Hydrogel culture system	Lei *et al*., 2014 (13)
Bag culture	Otsuji *et al*., 2014 (14)
96-well V-bottom plate	Kino-oka lab (15)
Micropatterned plate	Kino-oka lab (16)
Flow device	Kino-oka lab (17)
Adherent culture in dish	Kino-oka lab (18)

Fig. 4.6 Comparison of final cell density and apparent specific growth rate of hiPSCs reported by other researchers (black circle) and current result obtained in this study (red circle) in stirred suspension bioreactor culture.

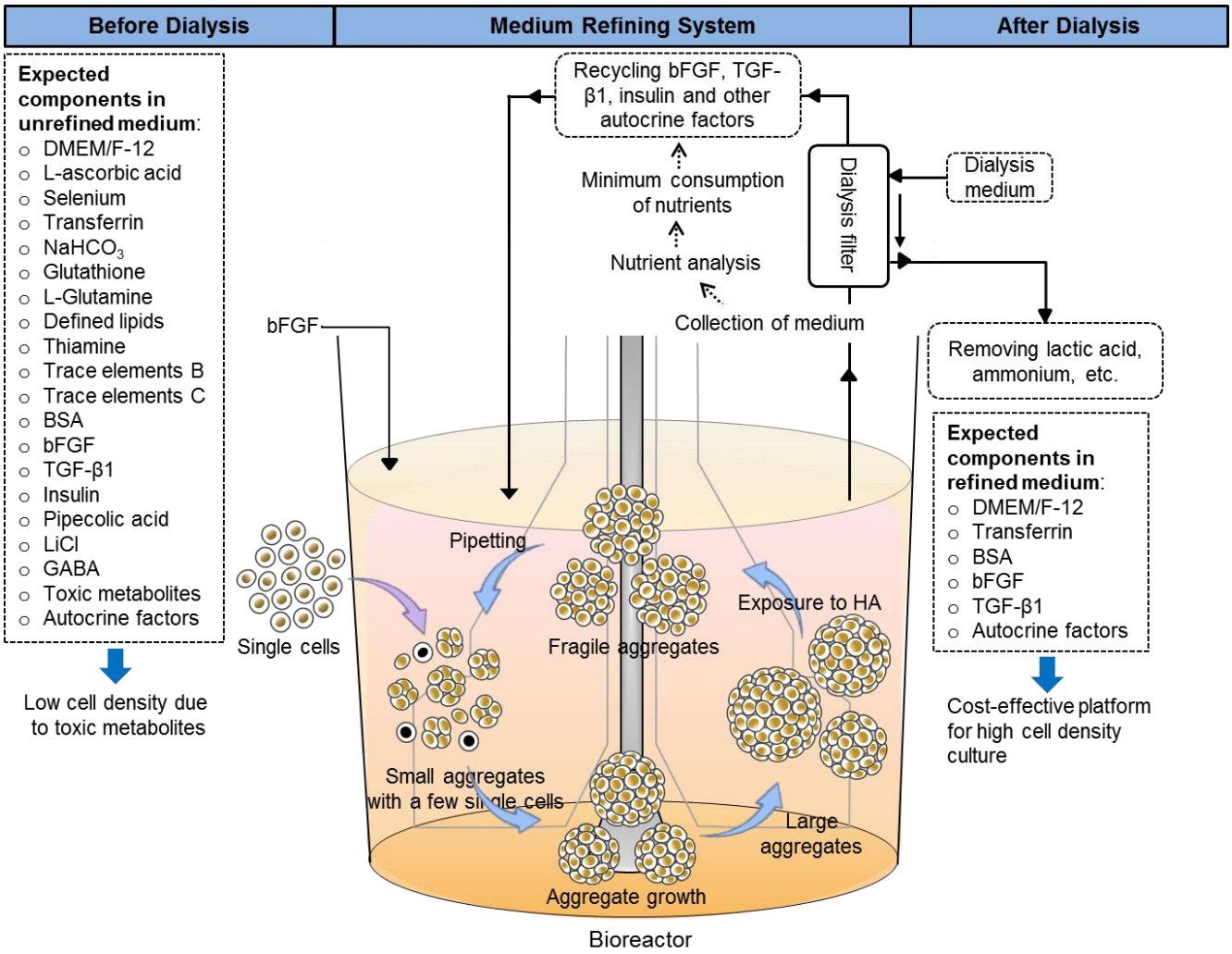


Fig. 4.7 Schematic interpretation of the current study.

Moreover, the fluid shear force in the bioreactor also contribute to wash out the collagen type I from the aggregate periphery which resulted in lower accumulation of collagen type I after 96 h in comparison to 87 h of HA addition in the culture medium.

Combining our previously established medium refinement system and aggregate break-up in the late-stage of culture resulted in high cell density which was three times higher than the control culture without aggregate break-up (**Fig. 4.3A**). Several recent studies have also been

reported to obtain high density of hPSCs (2.0×10^6 cells mL^{-1}) in the conventional suspension culture (Amit *et al*., 2010; Abbasalizadeh *et al*., 2012; Olmer *et al*., 2012) which are significantly lower density in comparison to the current study (**Fig. 4.6**). By using the integrated culture system, high cell density as well as high growth rate was obtained which is promising for large-scale expansion of hiPSCs in suspension culture. Although hiPSCs can be expanded to a high-density (3.6×10^6 cells mL^{-1}) by continuous perfusion (Kropp *et al*., 2016), maintaining such density in aggregate suspension culture is still challenging due to aggregate size limitation. Moreover, culturing hiPSCs for a prolonged period requires higher volume of medium which is significantly cost-intensive. By using our established integrated culture, high yield on medium was obtained by recycling costlier macromolecules as well as other autocrine factors to promote a cost-effective platform in suspension culture (**Fig. 4.3E**).

The proliferation of hiPSC aggregate reduced significantly during the late-stage of conventional culture due to large size of aggregates, and showed lower apparent specific growth rate in comparison to the integrated culture with aggregate break-up (**Table 4.1**). Large size of aggregates have been reported to show undesired differentiation, as well as cell necrosis in the center region of aggregate as collagen-rich shell forms on the outer surface of aggregate which hinders the diffusive transport of oxygen and nutrients during late-stage of culture (Sachlos *et al*., 2008). In this study, lower growth rates in the conventional culture were considered due to higher accumulation of collagen type I on the aggregate periphery during late-stage of culture (**Fig. 4.4**). However, higher expansion fold of cells and higher yield on culture medium was obtained with the integrated system which was higher than those reported for hPSC expansion (2–4 folds) during long-term suspension culture (Abbasalizadeh *et al*., 2012; Chen *et al*., 2012; Olmer *et al*., 2012; Hunt *et al*., 2014).

Higher concentrations of bFGF was detected in the used medium of integrated culture at 87, and 192 h which was considered due to less consumption by cells, or recycling by the medium refinement system, or secretion from cells in suspension culture (**Fig. 4.5A**). hPSCs were reported to secrete bFGF, and stimulate the production of TGF-β1 and insulin as autocrine factors in adherent culture (Dvorak *et al*., 2005; Bendall *et al*., 2007; Greber *et al*., 2007; Montes *et al*., 2009). In this study, although TGF-β1 was not supplemented in the medium of integrated culture, higher concentration of TGF-β1 was detected at 87 h which was considered due to the secretion from cells as autocrine factors (**Fig. 4.5B**). Since additional supplementations with macromolecules (growth factors) was not needed in the integrated culture except for bFGF (**Table 4.2**), the established integrated culture provided a cost-effective platform for saving growth factors for the expansion of hiPSCs by recycling macromolecules, as well as other autocrine factors in suspension culture.

Moreover, the supplementation of TGF-β1 and heparin (for stabilization of bFGF) was not needed in this time which showed more cost-effective way of saving growth factors in comparison to the previous medium refinement system (described in chapter 3) (**Table 4.3**). This successful performance suggests the integrated culture as a promising platform for obtaining high cell density with high-yield on medium which will pave the way to establish an automatic and cost-effective platform for large-scale expansion of hiPSCs in suspension culture.

Table 4.3 Comparison of medium components among the conventional culture, medium refinement system (Chapter 3), and integrated culture (Chapter 4).

Medium components	Conventional culture	Medium refinement system (Chapter 3)	Integrated culture (Chapter 4)
DMEM/F12	√	√	√
L-Ascorbic acid	√	-	-
Selenium	√	-	-
Transferrin	√	*	*
$NaHCO_3$	√	-	-
Glutathione	√	-	-
L-Glutamine	√	-	-
Defined lipids	√	-	-
Thiamine	√	-	-
Trace element B	√	-	-
Trace element C	√	-	-
BSA	√	*	*
bFGF	√	√	√
TGF-β1	√	√	-
Insulin	√	-	-
Pipecolic acid	√	-	-
LiCl	√	-	-
GABA	√	-	-
Autocrine factors		*	*
Heparin		√	

√ : Included in medium, * : Expected to be contained in the refined medium,
- : Expected not to be contained in the refined medium, Blank: Not included in medium

4.5 Summary

In this study, an integrated culture was established by combining the medium refinement system with aggregate break-up for obtaining high cell density with low supplementation of growth factors in suspension. The integrated culture showed high cell density which was three-times higher than the conventional culture without aggregate break-up. Moreover, higher expansion fold, as well as higher yield on culture medium (21-times) was obtained with the integrated culture in comparison to the conventional culture. Break-up of hiPSCs aggregates with HA during the late-stage of culture reduced the accumulation of collagen type I on the aggregate periphery in comparison to the conventional culture. Moreover, efficient recycling of bFGF and TGF-β1, as well as other autocrine factors provided a cost-effective platform by less supplementation of costlier macromolecules for obtaining high-density of hiPSCs in the integrated culture. This successful performance suggests the integrated culture as a promising platform for reducing the consumption of costlier macromolecules for large-scale expansion of hiPSCs in suspension culture.

General conclusion

In this study, a simple and scalable method for high-density culture of hiPSCs was established by investigating the size- and time-dependent growth properties of aggregates, and controlling aggregate size during late-stage of culture by breaking up the aggregates into small sizes with HA, and reducing the supplementation of costlier macromolecules in suspension culture.

In Chapter 1, hiPSCs showed heterogeneous growth properties depending on aggregate size and culture time in single aggregate culture. Small size aggregates showed low growth rate due to low secretion of autocrine factors, whereas, large size aggregates showed low growth rate in comparison to medium size aggregates due to cellular quiescence during early-stage of culture. Culture time was also important for influencing heterogeneous growth during prolonged period by uneven distribution of inductive biochemicals because of the formation of an ECM-shell on the aggregate periphery. Therefore, considerations for aggregate size and culture time-dependent boundary conditions for maintaining the balance between autocrine factors secretion, and ECM accumulation on the aggregate periphery is important to obtain high growth rate of hiPSCs in suspension culture.

In Chapter 2, a simple method for *in situ* break-up of hiPSC aggregates by HA was established by elucidating the mechanism of E-cadherin mediated cell-cell connections disruption for controlling the aggregate size in suspension culture. Since large size aggregates show low growth rate during late-stage of culture, aggregates broken into small sizes with HA resulted in high cell density in suspension culture. The temporal activity of

HA for disrupting E-cadherin mediated cell-cell connections facilitated the aggregates break-up into small sizes for minimizing the initial cell loss during seeding in suspension culture. This promising method of hiPSC aggregate break-up with HA paved the way to obtain high cell density with high growth rate by simplifying the culture operations without the need for enzymatic treatment or centrifugation in suspension culture.

In Chapter 3, more than 1.0 g L^{-1} of lactic acid was found to be toxic to hiPSCs growth and pluripotency. The medium refinement system was efficient in removing the toxic metabolites including lactic acid and ammonium in suspension culture. This method also allowed recycling of macromolecules (bFGF, TGF-β1, insulin, etc.) including other autocrine factors for the expansion of hiPSCs in suspension culture. As large numbers of cells with high levels of pluripotency markers expression were obtained by exchanging fresh medium only once, and further refinement of medium with the refining system, this system provided a cost-effective platform by reducing the supplementation of growth factors for the expansion of hiPS cells in suspension culture.

In Chapter 4, the medium refinement system was integrated with aggregate break-up during the late-stage of culture which resulted in more than three-times higher cell density than the conventional culture without aggregate break-up in suspension culture. Higher expansion fold of hiPSCs, as well as higher yield on culture medium was obtained with the integrated culture in comparison to the conventional culture. hiPSCs aggregates broken with HA during the late-stage of culture reduced the accumulation of collagen type I on the aggregate periphery in comparison to the conventional culture without aggregate break-up. Furthermore, efficient recycling of bFGF

and TGF-β1, as well as other autocrine factors helps to reduce the supplementation of costlier macromolecules in the integrated culture which provided a cost-effective platform for obtaining high-density of hiPSCs in suspension culture.

Overall, a simple and scalable method for high-density culture of hiPSCs was established by revealing the heterogeneous growth of hiPSCs, and setting up a boundary condition for aggregate size and culture time in suspension. The temporal activity of HA for disrupting E-cadherin mediated cell-cell connections paved the way for controlling the aggregate size, and establishing an *in situ* simple method of hiPSC aggregate break-up without initial cell loss during seeding in suspension culture. High cell density and high growth rate was also obtained by breaking up the aggregates into small sizes during the late-stage of culture in comparison to that in the conventional culture without aggregate break-up. This promising method of aggregate break-up provided the simplification of culture operations without enzymatic treatment or centrifugation in suspension culture. Moreover, the medium refinement system was efficient in removing toxic metabolites, as well as recycling bFGF, TGF-β1, and other autocrine factors which helps to reduce the supplementation of costlier macromolecules in suspension culture. Finally, the integration of medium refinement system with aggregate break-up facilitated high-density of hiPSCs with high-yield on culture medium by reducing the supplementation of growth factors in suspension culture. Thus, the established integrated culture provided a cost-effective platform for obtaining high cell density by efficient recycling of autocrine factors, and simplifying the culture operations for large-scale expansion of hiPSCs in suspension culture.

Proposals for future works

To extend the current findings towards future application, the following proposals should also be considered:

1. Development of an efficient medium collecting filter

The current medium collecting filter used in the medium refinement system (in chapter 3) was not efficient for high-density culture. As a result, the medium collecting filter was excluded during high-density culture with the integrated system (in chapter 4). Although developing circular filter which can be submerged in the culture medium containing aggregate is difficult, further attempts should be taken to choose suitable materials for making filter which can prevent cell attachment on the filter also. Developing a more efficient medium collecting filter will promote better medium refinement in suspension culture.

2. Automation of integrated system for hiPSCs expansion and differentiation

The integration of medium refinement system with aggregate break-up by HA seems to be promising for the establishment of an automatic integrated system for hiPSCs expansion and differentiation in suspension culture. Since the medium refinement system can be automated by using glucose and lactic acid sensors, periodic removal of toxic metabolites is possible to perform automatically in suspension culture. Moreover, spontaneous break-up of aggregates is also possible after treating with HA, and optimizing the agitation rate of impeller in bioreactor, which will help to maintain small size of aggregates during late-stage of culture. Since the medium refinement system can keep the autocrine factors in culture medium, hiPSCs cultured by the integrated system may show high differentiation potential in comparison to the conventional culture. After obtaining a high cell density, differentiation of these cells will result in high

number of desired cells in suspension culture which will lead to a cost-effective platform for large-scale differentiation of hiPSCs by the automatic integrated system.

Nomenclature

t	Culture time	[h]
X	Cell density	[cells mL^{-1}]
X_i	Initial cell density	[cells mL^{-1}]
X_0	Seeding density	[cells mL^{-1}]
X_f	Final cell density	[cells mL^{-1}]
X_t	Cell density at t h	[cells mL^{-1}]
X_{24}	Cell density at 24 h	[cells mL^{-1}]
X_{48}	Cell density at 48 h	[cells mL^{-1}]
X_{72}	Cell density at 72 h	[cells mL^{-1}]
X_{96}	Cell density at 96 h	[cells mL^{-1}]
X_{120}	Cell density at 120 h	[cells mL^{-1}]
X_{192}	Cell density at 192 h	[cells mL^{-1}]
N_0	Initial cell number	[cells]
N_{24}	Cell number at 24 h	[cells]
N_{72}	Cell number at 72 h	[cells]
α	Viable cell ratio	[-]
μ^{app}	Apparent specific growth rate	[h^{-1}]
Nt	Total number of cells	[cells]
Y	Yield	[cells mL^{-1}]
Vm	Total volume of medium	mL
D	Diameter	μm

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
bFGF	Basic fibroblast growth factor
TGF-β1	Transforming growth factor beta 1
IGF	Insulin-like growth factors
BSA	Bovine serum albumin
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
FBS	Fetal bovine serum
TBS	Tris-buffered saline
PBS	Phosphate buffered saline
SD	Standard deviation
CLSM	Confocal laser scanning microscopy
HLA	Human leukocytes antigen
APC	Allophycocyanin
CM	Conditioned medium
SSEA	Stage-specific embryonic antigen
OCT	Octamer-binding transcription factor
DAPI	4',6-diamidino-2-phenylindole
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

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List of Publications

Original Papers:

1. Nath, S.C., Nagamori, E., Horie, M., Kino-oka, M.: Culture medium refinement by dialysis for the expansion of human induced pluripotent stem cells in suspension culture. *Bioprocess Biosyst. Eng.*, **40**, 123-131. doi: 10.1007/s00449-016-1680-z (2016).
2. Nath, S.C., Horie, M., Nagamori, E., Kino-oka, M.: Size- and time-dependent growth properties of human induced pluripotent stem cells in the culture of single aggregate. *J. Biosci. Bioeng*. doi: 10.1016/j.jbiosc.2017.05.006 (in press).

International conferences:

1. Nath, S.C., Kino-oka, M.: High-density culture of human induced pluripotent stem cells through the refinement of medium by dialysis in suspension culture. "Scale-up and manufacturing of cell-based therapies V" conference, San Diego, CA, USA (15^{th}–19^{th} January, 2017).
2. Nath, S.C., Nagamori, E., Horie, M., Kino-oka, M.: Refining of culture medium by dialysis for the expansion of hiPSC in suspension culture. "The 27^{th} annual and international meeting of the Japanese Association for Animal Cell Technology (JAACT)", Kita-Kyushu, Japan (11^{th} –14^{th} November, 2014).

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