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

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Aggregate culture of human induced pluripotent stem cells (hiPSCs) is a promising method to obtain high number of cells for cell therapy applications. This study quantitatively evaluated the effects of initial cell number and culture time on the growth of hiPSCs in the culture of single aggregate. Small size aggregates ($(1.1 \pm 0.4) \times 10^1$–$(2.8 \pm 0.5) \times 10^1$ cells/aggregate) showed a lower growth rate in comparison to medium size aggregates ($(8.8 \pm 0.8) \times 10^1$–$(6.8 \pm 1.1) \times 10^2$ cells/aggregate) 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 ($(1.1 \pm 0.2) \times 10^3$–$(3.5 \pm 1.1) \times 10^3$ cells/aggregate) 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 the culture of single aggregate.

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[**Key words:** Initial cell number; Culture time; hiPSC aggregate proliferation; Culture of single aggregate; Boundary conditions; Aggregate heterogeneity]

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 (1,2). High cell numbers are generally required for clinical application, requiring robust methods for cell expansion (3,4). Conventional suspension culture of hiPSC aggregates has become a reliable method to obtain high numbers of cells (10^8–10^9 cells) (5–7), in particular after seeding single cells with Rho-associated protein kinase (ROCK) inhibitor which prevents single cell apoptosis (8). 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 (9,10).

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 (11–13). The heterogeneous size of aggregates causes low growth efficiency since micro-environmental stimuli, such as, cell–cell contact and cell–soluble factor interactions are dependent on the aggregate size (14). 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 human pluripotent stem cells (hPSCs) (15–17). Moreover, the size of individual aggregate has been reported to influence the growth and differentiation of hPSCs in aggregate suspension culture (18–21). 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 (22,23). Due to aggregate size limitation as well as extracellular matrix (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 (24). 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.

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MATERIALS AND METHODS

Cells and culture conditions The hiPSC line, Tic, was provided by the Japanese Collection of Research Bioresources (JCRB1331, JCRB Cell Bank, Osaka). Cells were routinely maintained on polystyrene substrate coated with recombinant laminin-511 E8 fragments (iMatrix-511; Nippi Inc., Tokyo) in commercially available medium (mTeSR1; STEMCELL Technologies, Vancouver, Canada). For subculture, single cells were seeded with 10 μM ROCK inhibitor (Y-27632; Wako Pure Chemical Industries, Osaka). Initial seeding was fixed at a viable cell density of 1×10^4 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 as previously described (25). 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, Waltham, MA, 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 (26), cells were re-seeded into a new culture dish.

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., Hercules, CA, 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., Tokyo), by seeding, $N_0 = 4.0 \times 10^1$–5.1×10^3 cells/well in 100 μL of medium/well and cultured for 120 h. 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.

Determination of 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 denotes N_{24}, N_{48}, N_{72}, and N_{120}, respectively.

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 $t = 24$–72 h and $\mu^{app} = \ln(N_{120}/N_{72})/(\Delta t)$ at $t = 72$–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, MA, USA) with a 10× objective lens and D was determined by using an image processing software (Image-Pro Plus, Media Cybernetics Inc., MD, USA).

Fluorescent staining The procedure for staining was similar to that described previously (27). Briefly, hiPSC aggregates were washed with PBS (Sigma–Aldrich, St. Louis, MO, USA) and embedded in optical cutting temperature compound (OCT, Tissue-Tek, Tokyo). Thin sections (20 μm) were prepared using a cryostat microtome (Leica, Wetzlar, 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 (Dainippon Sumitomo Pharma Co. Ltd., Osaka) at 4°C overnight. The sections were then probed with primary antibodies against ki-67 (Abcam, Cambridge, MA, USA) and collagen type I (Abcam) at 4°C overnight. Sections were then washed twice with Tris-buffered saline (TBS: Dako, Tokyo) and immersed in PBS containing 10% Block Ace and Alexa Fluor 488-conjugated secondary antibody (Life technologies, Camarillo, CA, USA) for 60 min at room temperature. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Life Technologies) for 20 min. The specimens were washed with PBS and observed with a confocal laser scanning microscope (FV1000, Olympus, Tokyo).

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, Littleton, MA, 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 mTeSR1 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.

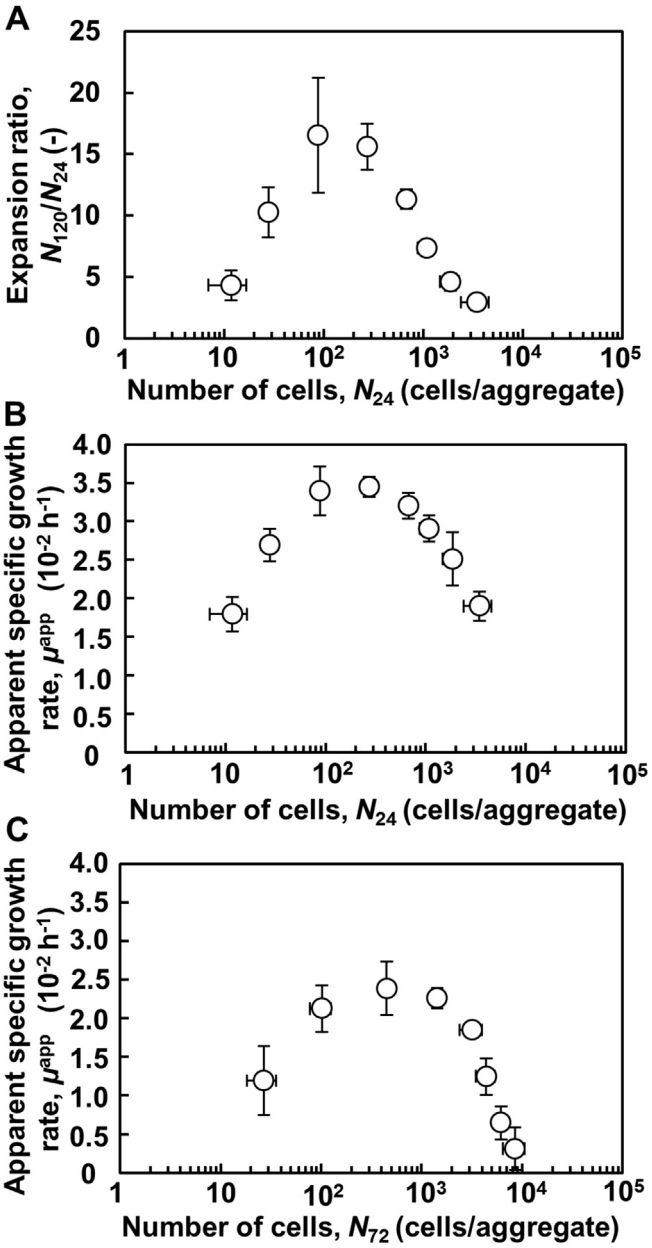

FIG. 1. Growth profiles of single hiPSC aggregates. (A) Expansion ratio of hiPSC after 120 h culture. (B) Growth rate of hiPSC aggregates containing different initial cell numbers from $t = 24$ to 72 h, and (C) from $t = 72$ to 120 h. Data presented as practical number of cells/aggregate.

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.

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,

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