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Comparison of growth kinetics between static and dynamic cultures of human induced pluripotent stem cells

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Understanding the fundamental mechanisms that govern the growth kinetics of human induced pluripotent stem cells (hiPSCs) contributes to culture design strategies to improve large-scale production. Two hiPSC lines (Tic and 253G1) were cultured under static and dynamic suspension conditions, and growth kinetics were compared during early (24–48 h), middle (48–72 h), and late (72–96 h) stages. In 2D static culture, similar growth profiles were observed for both hiPSC lines. However, there were significant differences in growth profile patterns and aggregate morphologies between hiPSC lines grown in 3D static and dynamic cultures. Based on immunostaining comparing the two hiPSC lines, surface distribution of collagen type I was observed in aggregates of the Tic line, but not in those of the 253G1 line. Compared to that in 3D static culture, the numbers of cells at 96 h were significantly decreased in 3D dynamic culture. The apparent specific growth rate (μ^{app}) of the Tic line was maintained continuously throughout culture, whereas that of the 253G1 line decreased gradually with culture until the late phase, at which time this parameter was reduced to $\mu^{app} = (0.85 \pm 0.71) \times 10^{-2}$ h^{-1}. This indicates that during the growth of hiPSCs in 3D dynamic culture, cells were damaged by liquid flow, which disrupted the cell-synthesized extracellular matrix (ECM). These results demonstrate that cell-synthesized ECM is an important factor affecting cell growth and morphology, and that changes to the ECM within aggregates lead to reduced growth abilities in dynamic culture.

[**Key words:** Human induced pluripotent stem cells; Static culture; Dynamic culture; Growth kinetics; Aggregate formation; Extracellular matrix]

Human induced pluripotent stem cells (hiPSCs) have great potential for cell-based therapy, drug discovery, and other translational applications (1,2). The self-renewal capacity of hiPSCs enables rapid cell expansion without a loss of pluripotency, which is critical for generating sufficient cell quantities for large-scale cell-based applications. Recently, strategies have been developed to overcome the problems and limitations associated with existing culture systems. In conventional two-dimensional (2D) culture, cells are grown on a variety of planar substrates with extracellular matrix (ECM) coatings (3,4). Current 2D cell culture platforms—which suffer from inherent heterogeneity and limited scalability and reproducibility—are emerging as a bottleneck for producing sufficient numbers of high-quality cells for downstream applications. An attractive approach for scaling up production is to shift cell culture from 2D to three dimensional (3D) platforms, and accordingly several methodologies for hiPSC culture have been assessed for cell production, including cell aggregates, culture on Matrigel-coated microcarriers, and growing the cells in alginate microencapsulates (5–7). However, scaling up dynamic suspension cultures is not appropriate for some hiPSC culture applications, because the high shear stresses encountered by cells can damage them. These culture strategies are associated with limited productivity, might have differential standards, and could yield substantial line-to-line and batch-to-batch variability. Differences between cell lines and between culture systems are a common problem and this presents technical limitations for the design of the culture process. Such fluctuations might pose potential regulatory problems regarding the validation of manufacturing-scale processes. However, some studies have focused on understanding the growth properties in static (8,9) and dynamic (10–14) culture environments for growing various hiPSC lines.

Cell aggregate culture has been developed as a scalable expansion platform for hiPSCs, and this method exploits the fact that hiPSCs form aggregates in suspension culture (10–14). This property is regulated by intercellular adhesion and ECM synthesis (15,16). Cell–cell or cell–matrix adhesions are primarily mediated by cell adhesion molecules including cadherins and integrins, and these functions have been shown to be important for many aspects of cell survival or growth (17,18). Intercellular adhesion plays an important role in aggregate formation by controlling the assembly of individual cells into 3D aggregates. Many studies have shown that hiPSCs tend to self-assemble and spontaneously form 3D aggregates in vitro in the absence of an adherent surface, under mechanical force (10–14), or within confined spaces (8,9). Cell-synthesized ECM proteins also play important roles in hiPSC aggregation and affect aggregate stabilization and compactness (19). The binding of integrins to their cell-derived ECM protein ligands elicits intracellular signaling that leads to the tyrosine phosphorylation of focal adhesion components, the formation of focal adhesion complexes, and the rearrangement

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of the actin cytoskeleton (20). This dynamic structure physically connects neighboring cells and couples intercellular adhesive contacts with the cytoskeleton. The interaction between cell–cell and cell–matrix associations plays a central role in the regulation of cell behavior. However, the mechanisms through which hiPSCs organize into aggregates and the effect of such structures on cell behaviors are only beginning to be investigated.

In this study, we compared two types of hiPSCs, which have different growth properties in static 2D culture, and investigated differences in growth kinetics using both 3D static and dynamic suspension cultures. Based on morphological characteristics and growth kinetics, the fundamental mechanisms of cell–cell and cell–matrix interactions are discussed with respect to the growth capacities of hiPSCs, in both types of culture systems.

MATERIALS AND METHODS

Cells and culture conditions The hiPSC lines Tic and 253G1 were provided by the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and RIKEN Bioresource Center (Tsukuba, Japan), respectively. They were cultivated in 55-cm^2 culture dishes (Corning Costar, Cambridge, MA, USA) coated with iMatrix-511 (Nippi Inc., Tokyo, Japan). For subculture, 1.0×10^4 cells/cm^2 were seeded 2-mm deep in mTeSR1 medium (StemCell Technologies, Canada). Cells were incubated at 37°C with 5% CO_2 with a humidified atmosphere, and medium was exchanged every day. On day 4, when cells reached 80–90% confluence, subculture was performed. For routine passage, hiPSCs were treated with 5 mM ethylenediaminetetraacetic acid (EDTA)/phosphate-buffered-saline (PBS) for 7 min at room temperature, and then, a dissociation reagent (TrypLE Select, Invitrogen, Waltham, MA, USA) with 10 μM of a ROCK inhibitor (Y-27632, Wako Pure Chemical Industries, Osaka) was added for 7 min at room temperature. After preparing single cells by pipetting, cells were seeded in fresh culture dishes coated with iMatrix-511.

Experimental platforms Culture platforms were classified as 2D static culture, 3D static suspension culture, and 3D dynamic suspension culture.

2D static culture of hiPSCs was conducted by seeding single dissociated cells at 5×10^4 cells/ml (1×10^4 cells/cm^2) in plates coated with iMatrix-511 (0.25 μg/cm^2) with mTeSR1 medium containing a ROCK inhibitor. During culture, the medium was exchanged with fresh medium without the ROCK inhibitor every 24 h.

Static culture of hiPSC aggregates was conducted by seeding single dissociated cells at 1×10^5 cells/ml, to produce uniform aggregates (2×10^2 cells/aggregate), in 3D culture plates with micro-spaces on the surface (BWV-400P, Kuraray Co., Japan) coated with poly 2-hydroxyethyl methacrylate (Sigma-Aldrich, MO, USA) in mTeSR1 medium with the ROCK inhibitor. During culture, the medium was exchanged for fresh medium without the ROCK inhibitor every 24 h. After culturing for 4 days, aggregates were collected and frozen for immunostaining.

Dynamic suspension culture of hiPSCs was conducted by seeding single dissociated cells at 1×10^5 cells/ml in a 30-ml stirred suspension bioreactor (BWV-S03A, Able Co., Tokyo, Japan) in mTeSR1 medium with the ROCK inhibitor as described previously (21–23). Agitation of the bioreactor can be via a magnetically driven delta shape impeller and controls the rotational speed with 55 rpm. During culture, the medium was exchanged for fresh medium without the ROCK inhibitor every 24 h.

Kinetic analysis of growth properties Cells were counted every 24 h with an automatic cell counter (TC20, Bio-Rad Inc., Hercules, CA, USA). Cell density, X_t (cells/ml), was measured at culture time (t =) 0, 24, 48, 72, and 96 h. For 3D culture, collected aggregates were washed with PBS and dissociated into single cells using Accumax (Innovative Cell Technologies Inc., San Diego, CA, USA) with 10 μM of the ROCK inhibitor. Apparent specific growth rates over time (Δt = 24 h), μ^{app} (h^{-1}), in early (t = 24 h), middle (t = 48 h) and late (t = 72 h) stages of culture were calculated as:

$$\mu^{app} = \frac{\ln(X_{t+\Delta t}/X_t)}{\Delta t} \quad (1)$$

Preparation and immunostaining of frozen sections HiPSC aggregates were washed with phosphate-buffered saline (PBS, Sigma-Aldrich) and embedded in optical cutting temperature compound (OCT, Tissue-Tek, Sakura Finetek Japan Co., Ltd., Tokyo, Japan). The aggregates were cut into 10-μm thick sections using a cryostat (Leica CM1850, Wetzlar, Germany). Thin sections were prepared and fixed with 4% paraformaldehyde (Wako Pure Chemical Industries) for 10 min. After washing with PBS, 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, Japan) at 4°C overnight. The sections were then probed with primary antibodies against collagen type I (Abcam, Cambridge, MA, UK) at 4°C overnight. Next, they were washed twice with tris-buffered saline (TBS) and immersed in PBS containing 10% Block Ace and Alexa Fluor 488-conjugated secondary antibody (Life Technologies, USA) for 60 min. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Life Technologies) for 20 min. Specimens were washed five times with PBS and observed with a confocal laser scanning microscope (model FV-1000, Olympus, Tokyo, Japan).

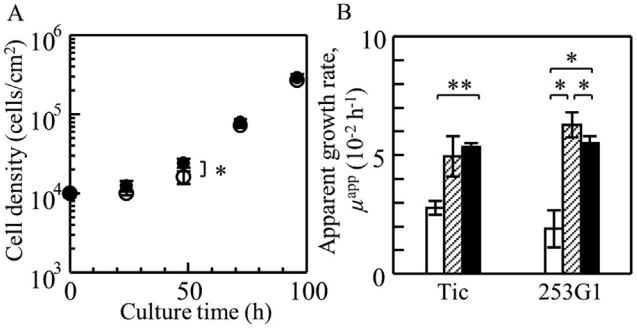

FIG. 1. Growth profiles in 2D static culture using two human induced pluripotent stem cell lines (Tic and 253G1). (A) Cell density and (B) apparent growth rate, μ^{app} (h^{-1}), in early, middle, and late phases. Statistical significance was analyzed by performing two-tailed Student's t-tests; $^*P < 0.01$, $^{**}P < 0.05$ (n = 3 per culture). Error bars represent the standard deviation. Closed circle, Tic line; open circle, 253G1 line. Open bars, early phase; shaded bars, middle phase; closed bars, late phase.

Statistical analysis At least three independent experiments were conducted for each tested condition, and the data were expressed as means with standard deviations. Statistical comparisons were performed using the Student's t-test and values of $P < 0.01$ and $P < 0.05$ were considered significant.

RESULTS

Growth of hiPSCs in 2D static culture To compare the cell morphologies and growth properties of hiPSCs in 2D static culture, two types of hiPSC lines (Tic and 253G1 line) were grown on iMatrix-coated plates for 96 h. All hiPSC lines appeared to be spread out at t = 24 h (Fig. S1). On these surfaces, cells gradually reached confluence as a monolayer, with the formation of colonies at t = 96 h. In addition, cell growth profiles in early, middle, and late phases were evaluated separately during culture of the two hiPSC lines. Cell density of both types of hiPSC lines increased with time, and cell densities of $(2.69 \pm 0.06) \times 10^5$ cells/cm^2 and $(2.89 \pm 0.33) \times 10^5$ cells/cm^2, respectively, were achieved at t = 96 h (Fig. 1A). For this, differences between the two hiPSC lines were not statistically significant. When μ^{app} values were compared for the early (t = 24–48 h), middle (t = 48–72 h), and late (t = 72–96 h) phases, different growth profiles were observed. For the Tic cell line, μ^{app} increased gradually with elapsed culture time until the late phases, with a maximum μ^{app} value of $(5.36 \pm 0.13) \times 10^{-2}$ h^{-1}. In contrast, the 253G1 cell line showed maximum a μ^{app} value in the middle phase ($(6.27 \pm 0.53) \times 10^{-2}$ h^{-1}), and this declined to $(5.51 \pm 0.28) \times 10^{-2}$ h^{-1} in the late phase.

Growth of hiPSCs in 3D static suspension culture To compare the cell morphologies and growth properties of hiPSCs in 3D static culture, cells were dissociated (into single cells) and seeded at a concentration of 1.00×10^5 cells/ml per well in a micro-space cell culture plate. The formation of aggregates from single cells was observed at t = 24 h, and these increased in size considerably during culture (Fig. S2). Each hiPSC line was observed to generate uniform, round aggregates with distinct morphologies, indicating that independent hiPSC lines appear to have noticeable effects on aggregate morphology at t = 96 h. In addition, significant differences were observed between the growth profiles of the two hiPSC lines, although cell density was not significantly affected at t = 96 h (Fig. 2B). The μ^{app} value of

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