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Original Article

Visualization of morphological categories of colonies for monitoring of effect on induced pluripotent stem cell culture status

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ABSTRACT

From the recent advances, there are growing expectations toward the mass production of induced pluripotent stem cells (iPSCs) for varieties of applications. For such type of industrial cell manufacturing, the technology which can stabilize the production efficiency is strongly required. Since the present iPSC culture is covered by delicate manual operations, there are still quality differences in produced cells from same culture protocols. To monitor the culture process of iPSCs with the quantified data to evaluate the culture status, we here introduce image-based visualization method of morphological diversity of iPSC colonies. We have set three types of experiments to evaluate the influential factors in iPSC culture technique that may disturb the undifferentiation status of iPSC colonies: (Exp. 1) technical differences in passage skills, (Exp. 2) technical differences in feeder cell preparation, and (Exp. 3) technical differences in maintenance skills (medium exchange frequency with the combination of manual removal of morphologically irregular colonies). By measuring the all existing colonies from real-time microscopic images, the heterogenous change of colony morphologies in the culture vessel was visualized. By such visualization with morphologically categorized Manhattan chart, the difference between technical skills could be compared for evaluating appropriate cell processing.

1. Introduction

Induced pluripotent stem cells (iPSCs) are defined by their unique capacity to differentiate into multiple lineages [1]. Growing expectations are accumulating for their usage both in drug discoveries and clinical applications [2–5]. For wider distribution of iPSCs for various applications, technological development to enable the industrial cell manufacturing, such as their undifferentiated expansion culture, is strongly required to satisfy massive needs [6–8]. However, the present iPSC manufacturing process is mainly covered by manual operation supported by the experience-based skills and memory-based decisions. Therefore it has been considered that the qualities of produced cells may vary [9,10], and the quality control method for massive iPSC culture is an important technological issue.

Commonly, when cells, including iPSCs, are manufactured for further applications, the final product cells are required to be intact. Therefore, in the advancing manufacturing technologies for iPSCs, non-invasive quality monitoring technology is becoming an important enabling technology. To check and evaluate the culture process of undifferentiated iPSCs non-invasively, the manual microscopic observation is the major solution in most of the facilities. Because, it is known that morphological character of cultured iPSCs is an important signature to monitor the culture status, such as the rate and the homogeneity of their undifferentiation status. Commonly, the morphological criteria of undifferentiated iPSCs has been known as; compact colonies that have distinct borders and well-defined edges, and are comprised of cells with a large nucleus with less cytoplasm, such as called ES-cell like colony [11,12].

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Colonies that show irregular morphologies are known as indicator of disturbance of their undifferentiation status in pluripotent stem cells [13]. The disturbance of these cells can lead to consist of differentiated cells or karyotic abnormal cells [14]. Recent studies reported the quality evaluation of iPSCs by their colony morphologies [15–17]. In these works, the morphological characters are linked to some biological phenomenons. In spite of such accumulating data showing correlation between the colony morphology and its undifferentiated status, such morphological evaluation methods are not yet applied to evaluate the culture process. Especially, although "the culture skill" is the background basic factor which can affect the quality of culture process, their effect has not yet been quantitatively evaluated for the standardization of cell culture.

We here propose the evaluation method of undifferentiated iPSC culture process by visualizing the quantitatively measured morphological data of iPSC colonies (Schematic illustration of our concept is shown in Fig. 1). Practically, we measured all colonies in the phase contrast microscopic images of cultured iPSCs, and compared the changes of colony profiles from the aspect of morphological categories. By comparing the Manhattan chart of morphological clusters, the differences between human skills, which can disturb the final quality of the same iPSC culture protocol, could be visualized. For this investigation, we have set three types of experiments to evaluate the influential factors in the iPSC culture skill that may disturb the undifferentiated quality of iPSC colonies: (Exp. 1) technical differences in passage skills, (Exp. 2) technical differences in feeder-cell preparation, and (Exp. 3) technical differences in maintenance skills (medium exchange frequency with the combination of manual removal of abnormal colonies) listed in Table 1.

In Exp. 1, the influence of passage skill was evaluated. The skill of stressless passage is known to be important for maintaining undifferentiated iPSC colonies; however its definition had been ambiguous. We compared the morphologically categorized colony profiles of iPSCs between "before" and "after" 4-repeated times of intentional stressful passages. Practically, in the stressful passage condition, the daily removal of morphologically irregular colonies was neglected during the four passages. By such passage operation, we mimicked to perform the negative influence caused by a careless operator.

In Exp. 2, the influence of feeder-cell preparation skill was evaluated. Feeder cells are known to influence the quality of PSCs [18,19], therefore early passage of cells are commonly recommended. However, the definition of "early" had been ambiguous. The suggested definition of cellular usage by "passage numbers" may result differently, even with immortal cells, because their detailed maintenance conditions including culture skills can be different between facilities. To evaluate such ambiguous facility-specific influential factor, the morphologically categorized colony profiles of the same iPSCs on different feeder-cell conditions ("before" and "after" 8-repeated times of intentional over-passages) were compared.

In Exp. 3, the influence of culture maintenance skill was evaluated. As a maintenance skill, it is known that the control of frequencies/volume of medium exchange can change the cellular condition. Moreover, the removal skill of morphologically irregular colonies is also an important skill. However, its individual effect or their combinational effect on iPSCs' undifferentiated state had been ambiguous. By visualizing the morphologically categorized colony profiles, four types of manners which change the rate of medium change and colony removal were quantitatively compared.

In this work, the image-based quantitation of morphologies of the cultured iPSCs was found to be effective for visualization and understanding of the heterogenic changes in culturing iPSCs. We propose that our method of visualization can provide quantitative approach to evaluate the delicate skill-derived effects in the culture process which were not objectively analyzed before. We consider our method can provide real-time culture record of iPSCs to evaluate, compare, and optimize the ambiguous cell culture operation skills.

2. Methods

2.1. Cells and cell culture

Human iPS cell line, 201B7, (provided by Dr. Shinya Yamanaka, Center for iPS Cell Research and Application, Kyoto University) was used in this study. For feeder cells, SNL 76/7 feeder cells (European Collection of Authenticated Cell Cultures (ECACC), Salisbury, UK) were used for Exp. 1 and 2, and MEF feeder cells (Merck Millipore, Billerica, MA, USA) were used for Exp. 3 (detailed experimental conditions are listed in Table 1). For both types of feeder cells, cells were only used within passage 3 from the first seeding of purchased cells in their usual maintenance. Only for Exp. 2, we over passaged the SNL cells for 8 repeated times (passage was decided by their sub-confluent status) after passage 3. Feeder cells were seeded at a density of 8.0×10^4 cells/well in 6-well plate. For MEF culture, EmbryoMax® 0.1% Gelatin Solution (Merck Millipore) was coated for 1 h in the incubator (37 °C, 5% CO_2 condition). iPSCs were maintained in Knockout DMEM/F-12 (Life Technologies, Carlsbad, CA, USA) containing 0.1 mM Non-aminoessential acid (Life Technologies), 0.1 mM 2-Mercaptoethanol (Life Technologies), 2 mM L-glutamine (Life Technologies), and 20% Knockout serum replacement (Life Technologies). The iPSC colonies were treated by 1 mg/ml Dispase II (Roche Applied Science, Penzberg, Upper Bavaria, Germany) until the colony edge shows slight lift-up (within 3–10 min), After sucking Dispase II, new medium were added 5 ml and colonies were collected with scraper and pipetting. iPSCs aggregates were dispersed by pipetting and split into five portions for each new vessel for passage. The morphologically irregular colonies are removed using cell scraper or sucked by aspirator. In their maintenance culture, medium was changed every day supplemented with 10 ng/ml bFGF (Life Technologies). In usual maintenance culture for cell expansion, above described cell collection protocol was carried out with the cell split ratio (1:4 to 1:10). During the maintenance culture, the irregular colonies are also scraped off in medium change process. Cells were cultured in the 37 °C, 5% CO_2 condition. Prior to the start of each experiment designed in this experiment, colonies were stained with SSEA4, Tra-1-60, and OCT 3/4 to confirm their undifferentiation status (Representative staining images provided in Supplementary information Fig. S1). All cell culture and maintenance were conducted by two operators (Exp 1 and 2: 5 years of culture experiences, Exp 3: 2 years of culture experiences). Both operators were trained until morphologically irregular colonies can be eliminated by their own decision in daily maintenance.

2.2. Image acquisition

Phase contrast microscopic images of iPSCs were obtained by IX81 (Olympus, Tokyo, Japan) with camera C11440-10C (Hamamatsu Photonics, Hamamatsu, Japan). In Exp. 1 and 2, five view fields (center position and four positions 2.2 mm from the center in the well of 6-well plate) were acquired from each well. In Exp. 3, the five positions in the view fields in each well were manually selected and memorized as x–y coordinates before the image acquisition. The time-course image acquisition was done semi-automatically according to the x–y coordinates by the x–y stage (Olympus) regulated by MetaMorph (Molecular Devices, Sunnyvale, CA, USA).