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Differentiation of mouse iPS cells is dependent on embryoid body size in microwell chip culture

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A microwell chip possessing microwells of several hundred micrometers is a promising platform for generating embryoid bodies (EBs) of stem cells. Here, we investigated the effects of initial EB size on the growth and differentiation of mouse iPS cells in microwell chip culture. We fabricated a chip that contained 195 microwells in a triangular arrangement at a diameter of 600 μm. To evaluate the effect of EB size, four similar conditions were designed with different seeding cell densities of 100, 500, 1000, and 2000 cells/EB. The cells in each microwell gradually aggregated and then spontaneously formed a single EB within 1 d of culture, and EB size increased with further cell proliferation. EB growth was regulated by the initial EB size, and the growth ability of smaller EBs was higher than that of larger EBs. Furthermore, stem cell differentiation also depended on the initial EB size, and the EBs at more than 500 cells/EB promoted hepatic and cardiac differentiations, but the EBs at 100 cells/EB preferred vascular differentiation. These results indicated that the initial EB size was one of the important factors controlling the proliferation and differentiation of stem cells in the microwell chip culture.

[**Key words:** Mouse iPS cells; Microwell chip; Embryoid body; Embryoid body size; Cell proliferation; Cell differentiation; Hypoxia]

Embryoid bodies (EBs), which are generated by aggregation of stem cells such as embryonic stem (ES) cells or induced pluripotent stem (iPS) cells, can promote the initial differentiation of stem cells (1–4). Therefore, EB culture has been widely used as a trigger for the initial process of stem cell differentiation. In the EB culture, a special internal environment exists within the EBs that allows multi-cellular interactions through cell–cell contact and mechanical stress resulting in the formation of cellular aggregates (5–8). Furthermore, a concentration gradient of substances (oxygen, nutrients, and wastes) from periphery to the center of the EB occurs, because the EB is without vessel structure (8–10). These internal environments vary by EB size, and changes in the internal environments of EBs are closely related with the determination of differentiation fates of stem cells. In fact, many researchers have reported on the relationships between the EB size and differentiation fate. For example, the differentiation of erythroid cells from stem cells was highest in the EBs that consisted of 1000 cells in their initial stage (11). Moreover, Koike et al. reported that the EBs forming from 1000 to 4000 cells promoted cardiac and hepatic differentiations, respectively (12). The results of these studies have shown that the initial size of EBs is an important parameter for regulating the differentiation process of stem cells.

In the previous studies, the effects of EB size on the stem cell differentiation utilized conventional methods such as hanging drop, U-shaped 96-well plate, and agitation cultures. Alternatively, we have developed a microwell chip, in which microwells of several hundred micrometers were regularly fabricated on a culture substratum, as a promising platform for generating EBs (13,14). It allowed the production of a large number of homogenous EBs of desired size. In previous studies, we demonstrated that the microwell chip culture promoted hepatic differentiation of the EB better than in traditional hanging drop culture, and that the proliferation and differentiation rate of EBs changed depending on the microwell size of the chip (15,16). These phenomena indicated that EB differentiation in the microwell chip culture was different compared to the traditional culture methods and also varied by the chip parameters. However, our previous studies are limited to the relationship between the microwell size and EB differentiation, and there are no studies regarding the EB size in the microwell chip culture.

In this study, we focused on the effect of EB size which is one of the parameters affecting microwell chip culture. The EB size in the initial stage of culture can be controlled by the cell density of seeding. Therefore, EBs with various sizes were produced by varying the seeding cell densities with ranges of 100–2000 cells/microwell, and the changes in proliferation and cell density of the EBs were evaluated. Furthermore, the differentiation patterns of EBs were compared between the microwell chip and traditional hanging drop cultures. This study aimed to clarify the effect of EB size on the EB properties in microwell chip culture.

MATERIALS AND METHODS

Microwell chips The microwell chip was prepared as described in our previous reports (13–16). It contained 195 microwells in a triangular arrangement at a pitch of 660 μm on a 24 × 24-mm^2 poly-methylmethacrylate plate (Fig. S1). Each

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microwell was 600 μm in diameter and 600 μm in depth. Its surface was modified with polyethylene glycol so as to be non-adhesive to cells. The chip was immersed in the culture medium until use.

Mouse iPS cells culture Mouse iPS cells (iPS-MEF-Ng-20D-17) were provided by the RIKEN BRC through the Project for Realization of Regenerative Medicine and the National Bio-Resource Project of the MEXT, Japan (17). The iPS cells were sub-cultured on an inactivated mouse embryonic fibroblast (MEF; ReproCELL Inc., Japan) feeder layer in a 100-mm gelatin-coated dish (BD Biosciences, USA) with 10 mL of culture medium. The medium used was Dulbecco's modified Eagle's medium (Millipore Corporation, Germany) supplemented with 15% fetal bovine serum (Millipore), 1% nonessential amino acids (Millipore), 110 μM 2-mercaptoethanol (Millipore), 1% glutamine (Gibco Corporation, USA), 1% penicillin (Gibco), 1% streptomycin (Gibco), and 1000 U/mL leukemia inhibitory factor (LIF; Wako Pure Chemical Industries, Japan).

An iPS cell suspension was obtained by treating the confluent monolayer formed on the gelatin-coated dish with 0.25% trypsin (Millipore). Four similar conditions with different seeding cell densities of 100 (EB-100), 500 (EB-500), 1000 (EB-1000), and 2000 (EB-2000) cells/microwell were designed to evaluate the effect of initial EB size on the growth and differentiation of iPS cells (Fig. S1). The microwell chip including the cells was incubated in a polystyrene dish (diameter, 35 mm) containing 2 mL of culture medium. Hanging drop culture was used as a control condition. In the hanging drop method, culture drops were prepared containing 100 to 2000 cells/20 μL. The same medium, but without LIF, was used for the experiments on EB formation and cell differentiation. The culture medium on the chips was changed every day. The cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C.

Hypoxia evaluation by Lox-1 staining Lox-1 reagent (SCIVAX Life Sciences, Inc., Japan) was used to evaluate the hypoxic state in the EB. It produces fluorescence when the intracellular oxygen concentration is the range of 2.5–10% O_2 (18). Culture medium containing 20 μg/mL Lox-1 reagent was prepared as the assay solution. EBs were incubated in the assay solution for 24 h, and then the stained EBs were examined under a fluorescence microscope (Biorevo BZ-9000; Keyence Corporation, Japan).

EB size and cell number To evaluate the changes in EB size over time, images of 50 EBs formed in the microwell chip were collected using a phase-contrast microscope. Size of the EBs was measured using a 2-dimensional image analysis program (Win ROOF; Mitani Corporation, Japan). The size was calculated by the equivalent circle diameter method.

Cell numbers were determined on days 3, 7, and 10 of culture using a modified DNA-4,6-diamidino-2-phenylindole (DAPI) fluorescence method (19). DNA extracted from EBs was mixed with a DAPI solution at a ratio of 1:1 and the fluorescence was measured using a fluorescence plate reader (excitation wavelength, 355 nm; emission wavelength, 460 nm) (Fluoroskan Ascent; Thermo Electron Corporation, Finland). A standard curve for the DNA content was prepared using values obtained from a cell suspension, and was used to convert the DNA-DAPI fluorescence values into cell numbers.

Real-time polymerase chain reaction analysis The differentiation patterns of EBs were compared between the microwell chip and hanging drop cultures. The expression of typical gene markers of differentiated cells: transthyretin (*Ttr*) and alpha-fetoprotein (*Afp*) as endodermal markers for hepatocytes; early cardiac transcription factor (*Nkx2.5*) and alpha-myosin heavy chain (*aMhc*) as mesodermal markers for cardiomyocytes; fetal liver kinase-1 (*Flk1*) and platelet-derived growth factor receptor beta (*Pdgfrb*) as mesodermal markers of vascular cells; and a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), were analyzed by using real-time polymerase chain reaction (PCR).

The EBs of each condition were collected after 10 d of culture for gene expression analysis. The total RNA of each sample was extracted using a spin column (NucleoSpin RNAII; Nippon Genetics, Japan) according to the manufacturer's instructions. cDNA was synthesized from 0.2 μg total RNA via a high-capacity cDNA reverse transcription kit (Applied Biosystems, Japan). The samples were then stored at −20°C until they were processed for real-time PCR analysis.

Real-time PCR was performed on an Applied Biosystems StepOne Real-Time PCR system using TaqMan Gene Expression Assay Kits (*Ttr*, Mm00443267_m1; *Afp*, Mm00431715_m1; *Nkx2.5*, Mm00657783_m1; *aMhc*, Mm00440354_m1; *Flk1*, Mm01222419_m1; *Pdgfrb*, Mm00435546_m1; *Gapdh*, Mm99999915_g1; Applied Biosystems). The comparative cycle time (ΔΔCT) method was used to quantify gene expression according to the manufacturer's protocol. The expression levels of the target gene were normalized to that of *Gapdh*. Using the gene expression levels in the initially prepared iPS cells as a reference, the expression levels of differentiation-related genes in samples were calculated (the expression levels of the initially prepared iPS cells were set to 1).

Statistical analysis Data are presented as mean ± standard deviation (SD) and correspond to three time points. Statistical analysis was performed using a repeated-measures analysis of variance. $P < 0.05$ was considered statistically significant.

RESULTS

EB formation and Lox-1 staining Fig. 1 shows the typical morphological changes seen in iPS cells in the microwell of the chip. Under all culture conditions, the cells trapped and dispersed in each microwell gradually aggregated and then spontaneously formed a single EB within 1 d of culture. The EB morphology was maintained despite further cell proliferation and the EBs were trapped within each microwell throughout the culture duration.

An interesting morphology was seen in the EB-1000 and 2000 conditions, where a dark region with high-cell density appeared at the center of the EB from 3 d of culture. After 7 d of culture, such a region was observed under all conditions. Furthermore, in the EB-100 condition, the majority of EBs developed into cystic EBs that had a cavity structure in the center of the EB after 7 d of culture.

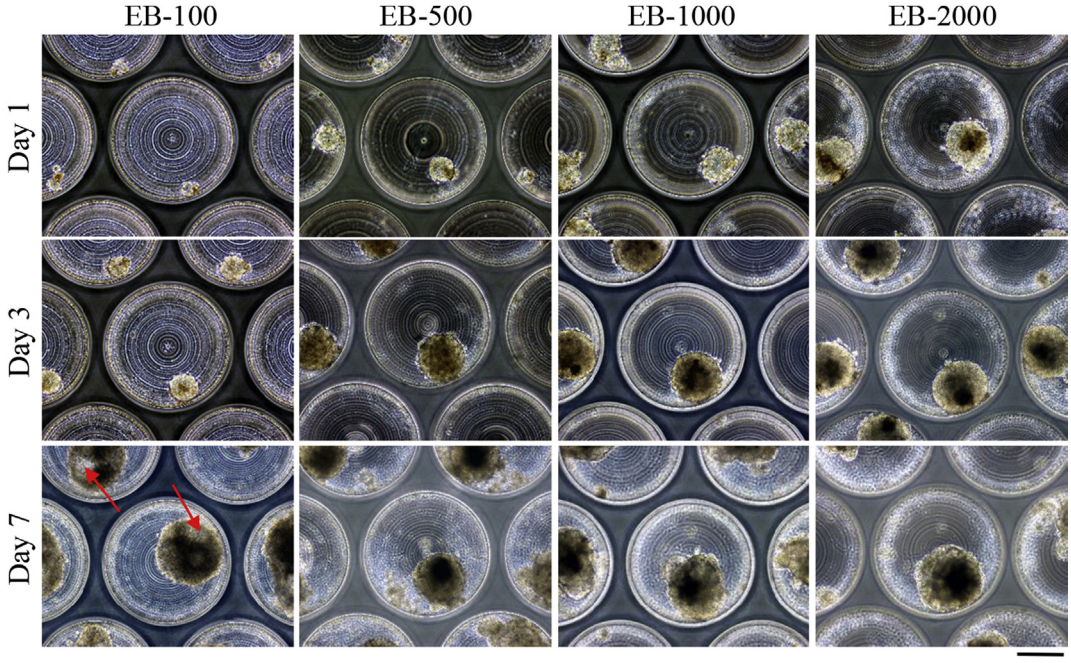

FIG. 1. Typical EB morphological changes with culture time in the mouse iPS cells. Arrow presents cavity structure. Scale bar represents 200 μm.

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