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Effect of separation distance on the growth and differentiation of mouse embryoid bodies in micropatterned cultures

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Embryoid body (EB) culture has been widely used for *in vitro* differentiation of embryonic stem (ES) cells. Micropatterning of cultures is a promising technique for regulating EB development, because it allows for controlling the EB size and the distance between neighboring EBs. In this study, we examined the relationship of EB separation distance to their growth and differentiation using a micropatterned chip. The basic chip design consisted of 91 gelatin spots (300 μm in diameter) in a hexagonal arrangement on a glass substrate that served as the cell adhesion area; the region without gelatin spots was modified with polyethylene glycol to create the non-adhesion area. Two similar chips were fabricated with distances between gelatin spots of 500 and 1500 μm. Mouse ES cells adhered on the gelatin spots and then proliferated to form EBs. When the EB–EB distance was at 1500 μm, their size and the expression of developmental gene markers were almost the same for all EBs on the chip. This indicated that interference between neighboring EBs was avoided. In contrast, when the EB–EB distance was at 500 μm, the size of EBs located in the inside region of the chip was smaller than that in the outside region. Additionally, in the inside region, hepatic differentiation of EB cells was increased over cardiac and vascular differentiation. These results indicate that the distance between EBs is an important factor in the regulation of their growth and differentiation.

[**Key words:** Mouse embryonic stem cells; Embryoid body; Micropatterned culture; Cell proliferation; Cell differentiation]

Embryonic stem (ES) cells established from inner mass of blastocyst are pluripotent and capable of self-renewal. The formation of three-dimensional cell aggregates called embryoid bodies (EBs) that resemble embryo structure is a principal method for *in vitro* differentiation of ES cells (1–4). The EB formation leads to the generation of multi-cellular interactions such as intercellular signaling, cell–cell contact, and mechanical force, and these interactions are affected by the differences in EB sizes (5–7). Consequently, it is known that EB size affects the fate of differentiating ES cells (8–11). Thus, in the EB culture, control of EB size is important to regulate ES cell differentiation in a reproducible manner.

Among the various techniques used to generate EBs, recent biofabrication techniques, such as photolithography, microcontact printing, microstencils, and microspotting, allow the micropatterning of uniformly sized EBs (12–16). On a cell-culture chip where adhesion and non-adhesion areas are micropatterned on the surface, ES cells first form a monolayer on the adhesion spots and then proliferate to form homogeneous EBs. We have demonstrated that micropatterned EBs have similar differentiation characteristics to those generated by more conventional methods, such as hanging drop or U-type microplate culture (13). A further advantage of micropatterned cultures is that EB size can be controlled by varying the diameter of the cell adhesion spot, and this can also affect the fate of differentiating cells in EBs. For example, Park et al. (14) reported that EBs generated on smaller cell adhesion spots showed increased expression of ectoderm genes, while those generated on larger adhesion spots favored mesoderm and endoderm gene expression. Furthermore, it was reported that adhesion spots of 120 and 200 μm in diameter favor pancreatic and cardiac differentiation respectively in EBs (15,16). The results of these studies have shown that micropatterned cell culture is a promising technique for the study of EB differentiation. Although previous studies have focused on the relationship between EB size and their developmental properties, the properties of EBs in micropatterned cultures may be affected, not only EB size, but also by the interactions between neighboring EBs. However, to our knowledge, there has been no study regarding such interaction effects.

Thus, we made EB–EB interaction the focus of our study, in which we evaluated the effect of EB separation distance on their growth and differentiation. We designed chips micropatterned with gelatin spots that had pitches (distance between spots) of 500 and 1500 μm. We then investigated how the growth and differentiation of mouse EBs on these chips was affected by the different distances between neighboring spots (one EB per spot). In addition, the expression of hypoxia gene markers was evaluated to gain further insight into these effects. The goal of the study was to examine the phenomena induced by EB–EB interaction.

MATERIALS AND METHODS

Preparation of EB micropatterned chips The micropatterned chip contained 91 gelatin spots (300 μm in diameter) in a hexagonal arrangement on a glass substrate (24 × 24 mm) that served as the cell adhesion area; the region lacking the

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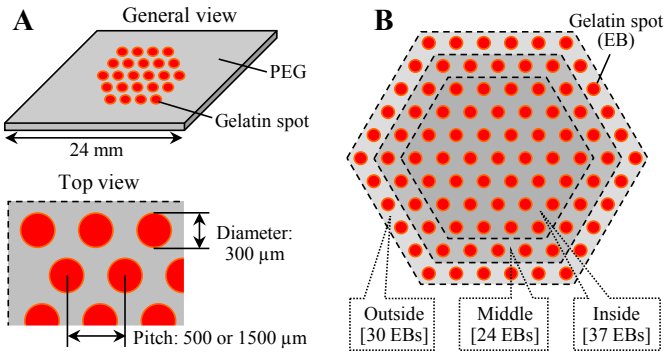

FIG. 1. Schematic diagram of the micropatterned chip (A) and classification of the location of embryoid-bodies (EBs) on the chip (outside, middle, and inside regions) (B).

gelatin spots was modified with polyethylene glycol (PEG) to create the non-adhesion area (Fig. 1A). Two similar chips with different pitches of 500 (chip 500) and 1500 (chip 1500) μm were designed to investigate the effect of separation distance on EBs.

The chip was fabricated by microspotting. First, the surface of the chip (glass substrate) was coated with a 6-nm-thick layer of platinum (Pt) using an ion sputtering unit (Hitachi High-Tech Science Systems, Japan). To create the cell adhesion area, a 0.1% gelatin solution (EmbryoMax; Millipore Japan, Japan) was applied to the Pt-coated chip using a microspotter system (ML-5000XII, Musashi Engineering, Japan). Subsequently, the chip was immersed in an ethanol solution of 5 mM PEG carrying a thiol group (PEG-SH; molecular weight, 30,000; NOF, Japan) to create the non-adhesion area around the gelatin spots. The gelatin/PEG-modified chip was thoroughly rinsed with deionized distilled water, and then rinsed in 50% ethanol for sterilization and the removal of the unbound PEG-SH. Finally, the chip was immersed in culture medium prior to use.

The EBs were classified according to their chip location for investigating the effect of EB location (Fig. 1B). The outside EBs represented 30 EBs that were located on the outer hexagonal ring of spots, the middle EBs represented 24 EBs that were located on the next ring in, and the inside EBs represented 37 EBs that were located in the center region, that covered all spots to the inside of the middle region.

Mouse ES cell culture Mouse ES cells (129SV; DS Pharma Biomedical, Japan) were cultured on an inactivated mouse embryonic fibroblast (ReproCELL, Japan) feeder layer in a 100-mm, gelatin-coated dish (BD Biosciences, CA, USA) containing 10 mL Dulbecco's modified Eagle's medium (DS Pharma Biomedical) supplemented (supplements from DS Pharma Biomedical) with 15% fetal bovine serum, 1% nonessential amino acids, 1% nucleosides, 110 μM 2-mercaptoethanol, 1% glutamine, 1% penicillin, 1% streptomycin, and 1000 U/mL leukemia inhibitory factor (LIF; Wako Pure Chemical Industries, Japan).

An ES cell suspension was obtained by treating the confluent monolayer formed on the gelatin-coated dish with 0.25% trypsin (DS Pharma Biomedical). 5×10^5 cells were seeded onto the chip that was then immersed in a polystyrene dish (diameter, 35 mm) containing 2 mL of culture medium. After approximately 4 h in culture, the cell-seeded chip was transferred to another polystyrene dish containing 2 mL of fresh culture medium in order to remove the cells that did not adhere to the gelatin spots. 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.

EB size and morphology To evaluate changes in EB size over time, images of EBs formed on the micropatterned chip were obtained using a phase-contrast microscope. EB sizes were measured using a 2-dimensional image analysis program (Win ROOF; Mitani, Japan). Each EB size was calculated by the equivalent-circle diameter method. The three-dimensional conformation of EB on the chip was analyzed using a scanning electron microscope (VE-8800; Keyence, Japan).

Real-time polymerase chain reaction analysis The EBs of each chip region were scraped off using a spatula after 10 d, and they were then collected for gene expression analysis. Total RNA of each sample was extracted using a spin column (NucleoSpin RNA II; 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 polymerase chain reaction (qPCR) analysis. qPCR was performed on an Applied Biosystems StepOne Real-Time PCR system using TaqMan Gene Expression Assay Kits (Applied Biosystems, CA, USA). Typical gene markers of undifferentiated, endodermal, and mesodermal cells were evaluated (Table S1), because it is known that the presence of FBS in the culture medium inhibits differentiation into ectodermal cells (neural lineages) (17). 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 glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Using the gene expression levels in the initially prepared ES cells as a reference, the expression levels of differentiation-related genes in samples were calculated (the expression levels of the initially prepared ES 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.

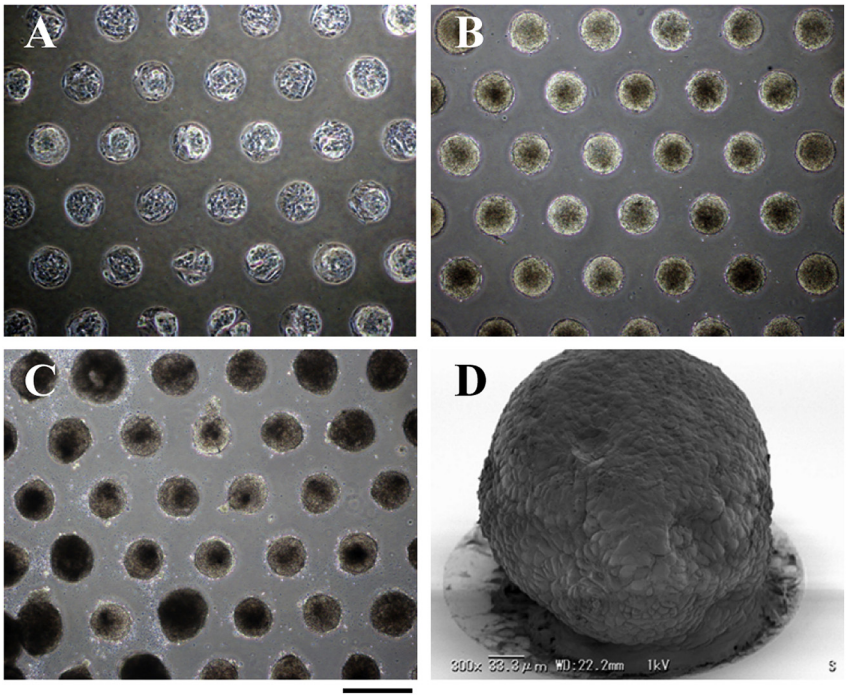

FIG. 2. Formation of embryoid bodies (EBs) by mouse embryonic stem (ES) cells on the chip with gelatin spots spaced 500 μm apart (chip 500) at 1 d (A), 3 d (B), and 7 d (C) of cultures. The scale bar represents 500 μm. SEM image of mouse embryoid body on the chip (D).