Journal of Bioscience and Bioengineering
VOL. 115 No. 5, 571–574, 2013

www.elsevier.com/locate/jbiosc

NOTE

Effects of hanging drop culture conditions on embryoid body formation and neuronal cell differentiation using mouse embryonic stem cells: Optimization of culture conditions for the formation of well-controlled embryoid bodies

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Received 21 August 2012; accepted 20 November 2012
Available online 29 December 2012

Hanging drop (HD) cultures were carried out with a drop volume of either 20 or 30 μl. An incubation period of 3 days was determined to be appropriate for the formation of well-controlled embryoid bodies (EBs), and the initial cell number was identified as the most critical factor in the growth and neuronal cell differentiation of EBs.

[**Key words:** Hanging drop; Embryonic stem cells; Embryoid body; Cell density; Neurogenesis]

The formation of embryoid body (EB) is an experimental procedure used to differentiate embryonic stem (ES) cells, resulting in the formation of three-dimensional multicellular aggregates. There are various methods to aggregate ES cells for inducing EB formation (1). The hanging drop (HD) method is conventionally and widely used to form EBs from mouse ES cell lines (2) and mouse embryonal carcinoma cell lines (3). In the HD method suspension culture is performed in small droplets hung from the lid of Petri dish. The HD method is advantageous in that the initial cell number contained in a hanging drop can be controlled so that EBs can be obtained from a predetermined number of cells. It is known that the initial cell number to form an EB affects the growth and differentiation of EBs (4–6). As shown in Table 1, however, the initial cell number and other culture conditions have been varied from studies to studies for the differentiation of the same cell type, for example, neuronal cell or cardiomyocyte (7–14). In the EB formation for neuronal cell differentiation, the initial cell numbers in hanging drops ranged from 400 to 1000 cells, the initial cell densities ranged from 20 to 50 cells/μl (7–10), and the culture period varied from 2 to 4 days. The initial cell number and culture period are expected to influence the growth and differentiation of EBs. Previous studies have investigated the optimal HD culture conditions for cardiomyocyte differentiation (15). The effects of initial cell number and culture period on EB differentiation into cardiomyocytes has been reported, however, the influence of drop volume remains to be determined. This prompted us to investigate the optimal HD culture conditions (initial cell number, drop volume, and culture period) for neuronal cell differentiation.

In the present study, we investigated the HD culture conditions for the formation of EBs destined to neuronal cell differentiation using mouse embryonic stem cells from the viewpoint of quality control.

Mouse ES (mES) cells of the cell line 129/SV (Millipore, Billerica, MA, USA) were maintained on STO fibroblast feeder layer cells (RCB0536; RIKEN BRC), as previously described (16). Briefly, the mES cells were maintained at 37°C in humidified air with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; high glucose, Invitrogen, Carlsbad, CA, USA) supplemented with 15% KnockOut™ Serum Replacement (KSR; Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma, St. Louis, MO, USA) 50 U/ml penicillin (Invitrogen), 50 μg/ml streptomycin (Invitrogen), and 1000 U/ml murine leukemia inhibitory factor (mLIF; Chemicon, Temecula, CA, USA) on a 0.1% gelatin-coated dish with feeder layer cells. mES cell colonies were dissociated into single cells using 0.05% trypsin-EDTA (Invitrogen). The dissociated mES cells were then resuspended in DMEM supplemented with 20% fetal bovine serum (FBS; Invitrogen), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin (EB medium). Hanging drops, which contained 100, 500, or 1000 dissociated ES cells in 10, 20, 30, or 50 μl of EB medium were placed onto the undersurface of the lids of 100-mm culture dishes. The lids were then inverted and placed onto culture dishes filled with 10 ml of phosphate-buffered saline (PBS). After 3, 5, and 7 days, EBs, having formed as cell aggregates in hanging drops, were transferred into bacteriological grade dishes to capture phase-contrast images using a digital microscope system (Moticam 2000; Shimadzu Rika Co., Tokyo, Japan). Eight EBs were randomly chosen for morphological measurement. The mean size of EBs represents the average of the smallest and largest diameters of eight EBs.

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2012.11.016

TABLE 1. Hanging drop culture conditions for the formation of embryoid bodies using mouse embryonic stem cells.

Differentiation	Initial cell number (cells/drop)	Drop volume (μl)	Cell density (cells/μl)	Period of hanging drop culture (d)	Refs.
Neuronal cell	400	20	20	2	7
Neuronal cell	500	20	25	4	8
Neuronal cell	900	30	30	2	9
Neuronal cell	1000	20	50	3	10
Cardiomyocyte	300	30	10	2	11
Cardiomyocyte	400	20	20	2	12
Cardiomyocyte	400	20	20	5	13
Cardiomyocyte	800	20	40	2	14

The EBs obtained on the third and fifth day of HD cultures – referred to as 3-day-old EBs and 5-day-old EBs, respectively – were transferred onto bacteriological grade dishes. One EB at a time was carefully collected using a pipette and placed in a 24-well plate coated with 0.1% gelatin containing 1 mL/well of medium. Using two 24-well plates, adhesion cultures of 48 EBs were performed for neuronal cell differentiation. The composition of the medium used for neuronal cell induction was the same as the EB medium, but was supplemented with 20% KSR instead of FBS. The adhesion culture of 3-day-old EBs was performed for 9 days. The adhesion culture of 5-day-old EBs was carried out over a period of 7 days. After a total of 12 days differentiation, microscopic observation and qRT-PCR analysis were performed to verify the generation of neuron-like cells. Generation efficiency of neuron-like cells was defined as the percentage of wells in which neuron-like cells were observed under a microscope.

Total RNA extraction and cDNA synthesis were performed using each standard kit (NucleoSpin RNA XS and PrimeScript II 1st strand cDNA Synthesis kit, Takara, Otsu, Japan) following the manufacturer's instructions. The reaction and analysis for qRT-PCR were performed in a real-time thermal cycling system (Thermal Cycler Dice; Takara) using a SYBR green RT-PCR kit (SYBR Premix EX Taq II; Takara). The primers used for qRT-PCR were as follows: β-Actin, 5′-CATCCGTAAAGACCTCTATGCCAAC-3′ sense and 5′-ATGGAGCCACC GATCCACA-3′ antisense; *Pax6*, 5′-TGCCCTTCCATCTTTGCTTG-3′ sense and 5′-TCTGCCCGTTCAACATCCTTAG-3′ antisense; βIII-tubulin, 5′-CCTATTCAGGCCCGACAACTTTA-3′ sense and 5′-CAGGCAGTCACAATT CTCACACTC-3′ antisense. qRT-PCR was carried out for 50 cycles, with the denaturation steps conducted at 95°C for 5 s. Annealing-elongation step were carried out at 55°C for 30 s. Relative gene expressions were calculated by the comparative Ct method after normalization to an endogenous control (β-actin).

Changes in the size of EBs formed under various HD culture conditions are shown in Fig. 1. Suspended cells aggregated into a single EB in each drop by day 3 under all drop conditions tested. The size of 3-day-old EBs was dependent on the initial cell number. The size of 3-day-old EBs increased with increasing initial cell number regardless of the drop volume. Little variation in size was observed in 3-day-old EBs within each cell number group. On the other hand, when the period of HD culture was prolonged to 5 or 7 days, the sizes of 5- and 7-day-old EBs were affected by both the initial cell number and drop volume. The drop volume of 10 μl failed to support EB growth more than 3 days when initial cell numbers of 500 and 1000 cells were used. The deterioration of medium in hanging drops may have occurred in cultures carried out beyond 3 days because the HD culture is performed without medium change. An increase of the drop volume improved EB growth in long-term cultures. The drop volume of 50 μl supported best the time-dependent growth of EBs for up to 7 days of culture even when initial cell numbers of 500 and 1000 cells were used. However, it was technically challenging to hang 50-μl drops because the surface tension between the droplets and the polystyrene lid is insufficient to maintain them. Therefore, we suggest that a drop volume of 20 or 30 μl is adequate in view of technical limitations. Overall, our results indicate that the three days culture using 20- or 30-μl drop are optimal for the formation of EBs with controlled sizes.

The effects of HD culture conditions on the efficiency of neurogenesis from EBs were also investigated. The 3- and 5-day-old EBs were transferred onto adhesion cultures for inducing neuronal cell differentiation. After 12 days of differentiation including the period of HD culture and adhesion culture, the generation of neuron-like cells from the attached EBs was confirmed based on microscopic observation. A typical aspect of neuron-like cells is represented in Fig. 2a. As shown in Fig. 2b, the 3-day-old EBs exhibited higher generation efficiency in the EBs resulting from higher initial cell number in both drop volumes of 20 and 30 μl. The generation of neuron-like cells was markedly decreased in the 5-day-old EBs as shown in Fig. 2c. The differentiation efficiency was not improved even when the period of adhesion culture was prolonged (data not shown). The expression levels of neuronal differentiation marker genes (*Pax6* and βIII-tubulin) were analyzed by qRT-PCR on day 9 of the adhesion culture of 3-day-old EBs. The expression levels of *Pax6* increased with the cell density, as shown in Fig. 2d. The expression of βIII-tubulin had a similar trend to that

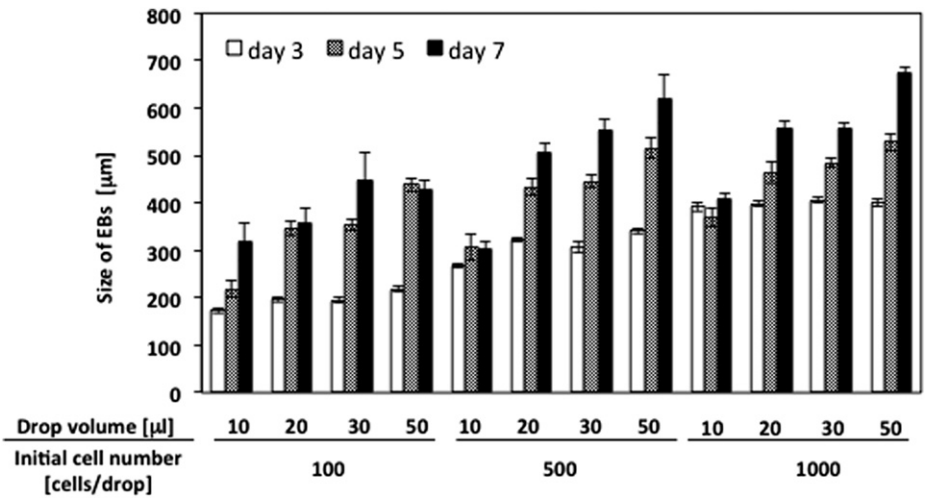

FIG. 1. Effects of drop volume and initial cell number on EB formation. Changes in the size of EBs formed under various HD culture conditions. The mean diameter was calculated from eight EBs on days 3 (open bars), 5 (dotted bars), and 7 (closed bars). Data represent mean ± SD of three independent experiments.