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Original Article

# A multiple-funnels cell culture insert for the scale-up production of uniform cell spheroids

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ABSTRACT

*Introduction:* Formation of cell spheres is an important procedure in biomedical research. A large number of high-quality cell spheres of uniform size and shape are required for basic studies and therapeutic applications. Conventional approaches, including the hanging drop method and suspension culture, are used for cell sphere production. However, these methods are time consuming, cell spheres cannot be harvested easily, and it is difficult to control the size and geometry of cell spheres. To resolve these problems, a novel multiple-funnel cell culture insert was designed for size controlling, easy harvesting, and scale-up production of cell spheres.

*Methods:* The culture substrate has 680 micro-funnels with a 1-mm width top, 0.89 mm depth, and 0.5 mm square bottom. Mouse embryonic stem cells were used to test the newly developed device. The seeded embryonic stem cells settled at the downward medium surface toward the bottom opening and aggregated as embryoid bodies (EBs). For cell sphere harvest, the bottom of the culture insert was put in contact with the medium surface in another culture dish, and the medium in the device flowed down with cell spheres by hydrostatic pressure.

*Results:* Compact cell spheres with uniform size and shape were collected easily. The diameter of the spheres could be controlled by adjusting the seeding cell density. Spontaneous neural differentiation (nestin and Tju1) and retinoic acid-induced endodermal differentiation (Pdx-1 and insulin I) were improved in the EBs produced using the new insert compared to those in EBs produced by suspension culture.

*Conclusions:* This novel cell culture insert shall improve future studies of cell spheres and benefit clinical applications of cell therapy.



## 1. Introduction

Three-dimensional (3D) cell spheres are an emerging structure in biomedical research. Cell sphere is a common method used to examine cell biology in stem cell and cancer cell [1–3]. Formation of embryoid bodies (EBs) can induce spontaneous differentiation to embryonic stem (ES) cells, which can be used to study embryogenesis [4]. EBs are also used to predict embryotoxicity *in vitro* [5]. Cancer cell spheroid is a well-accepted model for cancer research, particularly in breast cancer [3]. The multicellular tumor model is a useful platform to facilitate high-throughput anti-cancer drug

*Abbreviations:* DMEM, Dulbecco's Modified Eagle Medium; EBs, embryoid bodies; ES cells, embryonic stem cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LIF, leukemia inhibitory factor; MSC, mesenchymal stem cell; MEFs, mouse embryonic fibroblasts; PBS, phosphate buffered saline; PCR, polymerase chain reaction; Pdx-1, pancreatic and duodenal homeobox 1; RA, retinoic acid; RPMI, Roswell Park Memorial Institute; RT-PCR, real time polymerase chain reaction; SD, standard deviation; Tuj1, neuron-specific class III beta-tubulin.

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screening. Likewise, *in vitro*, 3D spheroid culture is used to demonstrate adipocyte inflammation [6]. Similarly, neural cell spheroid is applied in cortical studies [7].

Cell spheroids are also critical to regenerative medicine and therapeutic applications. Formation of 3D human mesenchymal stem cell (MSC) spheroids is a proposed strategy for the large-scale production of cell sources [8]. Cell sphere processing also modulates physiological functions of cells such as hepatocytes and pancreatic β-cells [9,10]. Hybrid cell spheroids are suggested for use in cell therapy [11]. Compared to single cells, cell spheres can improve engraftment and efficacy of the transplanted cells [12]. Currently, static methods, including hanging drop, suspension culture, and low-attachment plates, are used for 3D cell sphere fabrication. Moreover, dynamic approaches such as spinner flask culture, rotary culture, and electric, magnetic, or acoustic force cell aggregation have also been developed [13]. Although cell spheres can be obtained using these approaches, it may be difficult to control the size and geometry of the spheres, and the approaches are inefficient and time consuming. Using a non-adhesive culture substrate, cancer stem cell spheres can be isolated from oral squamous cell carcinoma or hepatoma cell lines, but the size of the sphere is not uniform [14,15]. In addition, the size of colonies and EB can influence the differentiation fate [16,17]. Uniform sphere size is important for other cell types. For example, a relatively smaller pancreatic β-cell sphere is favorable in terms of insulin secretion and cell survival [18,19]. Therefore, a reliable method to prepare uniform cell spheres with controllable size is important.

To produce uniform cell spheres, micro-patterned culture substrates such as concave and cylindrical micro-wells have been designed [20,21]. Culture substrates with various sizes and depth of micro-wells have been developed; some of them are commercially available. Although a micro-patterned substrate with shallow wells enables easy harvest of cell spheres, the spheres may elute and fuse with other spheres [20]. In contrast to shallow wells, deep wells may steadily maintain the spheres, but present difficulty in harvesting. Therefore, we invented a novel multiple-funnels culture insert that allows secure sphere maintenance, scaled-up production, and easy harvest of cell spheres. The performance of this cell sphere culture device was examined using mouse ES cells, and the differentiation potentials of the obtained cell spheres (EBs) were compared to those produced by the hanging drop or suspension culture method.

## 2. Materials and methods

### 2.1. Prototype of multiple-funnels cell culture device

The gross appearance of the prototype of multiple-funnels cell culture device (MP device; “MP” represents “multiple pores”) is shown in Fig. 1a. This device was designed as an insert to fit one well of the 6-well culture plate and there were 680 micro-funnels in the bottom side of one device. The main body (reservoir to preserve the cells/culture medium and a stopper to hang the device on the well) was constructed using polycarbonate, and the culture substrate was made of silicone rubber. The static contact angle of the silicone rubber to water was approximately 90°, which was determined by a contact angle meter (CA-X, Kyowa Interface Science Co., Ltd., Niiza, Saitama, Japan). Fig. 1b illustrates the lateral view of the micro-funnel structure (1 mm width top, 0.89 mm depth, and 0.5 mm square bottom). The suspending cells were deposited at the downward medium surface and aggregated as 3D cell spheres. The MP devices used in the present study were manufactured by an injection molding company (Kyowakasei Co., Ltd., Uji, Kyoto, Japan). All fabricated devices were sterilized by autoclaving and dried before usage. This multiple-funnels cell culture insert was patented as “DEVICE FOR FABRICATING SPHEROID, AND SPHEROID RECOVERY METHOD AND MANUFACTURING METHOD (WO2015/129263)”.

### 2.2. Cultivation of mouse ES cells

Mouse ES cells (ES-D3, ATCC® CRL-1934™, Manassas, VA, USA) were cultured in tissue culture dishes pre-coated with 0.1% gelatin and seeded with mitomycin C (Kyowa Hakko Kirin, Inc., Tokyo, Japan)-treated mouse embryonic fibroblasts (MEFs). ES cells were cultured in the ES medium composed of Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 1 mM glutamine, 1% nonessential amino acids, mercaptoethanol (5 μL per 1000 mL), and 1% penicillin/streptomycin. Recombinant leukemia inhibitory factor (LIF, StemSure LIF, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added at a concentration of 1000 U/mL for maintenance of the undifferentiated state of the ES cells. Before further experimentation, the ES cells were maintained on gelatin-coated dishes without MEF in the ES medium, including LIF, for 3 passages to deplete MEF. The cells were then cultured in a $CO_2$ incubator (MCO-170AIC, Panasonic, Kadoma, Osaka, Japan) under 95% air and 5% $CO_2$ 37 °C.

### 2.3. Production and harvest of cell spheres from the MP device and suspension culture

To compare the size and shape of the cell spheres produced by the MP device and conventional suspension culture (static spheroid culture), mouse ES cells at a cell density of $1.7 \times 10^5$ cells/mL were cultured in ES medium without LIF, and 4 mL of cell suspensions were seeded into either the MP device or an untreated 35-mm Petri dish. After culturing for 2 days, the MP device was gently removed from the 6-well culture plate, and its bottom side was put in contact with the medium (0.1 mL) in another 35-mm Petri dish for cell sphere harvest. To ensure complete sphere collection, the bottom of the device was put in contact with the medium surface a few times. The collected cell spheres were observed with a stereomicroscope (SMZ-U, Nikon, Tokyo, Japan), and the images were recorded (COOLPIX P7700, Nikon, Tokyo, Japan). The cell sphere size values were organized into seven groups: smaller than 50, 50–75, 75–100, 100–125, 125–150, 150–175, 175–200, 200–225, 225–250, 250–275, 275–300 μm, and larger than 300 μm. More than 100 cell spheres were measured for each group.

### 2.4. Cell density and the size of cell spheres

Mouse ES cells at different cell density ($8.6 \times 10^4$, $1.7 \times 10^5$, and $2.6 \times 10^5$ cells/mL) were suspended in ES medium without LIF, and 4 mL of cell suspensions were seeded into one MP device. The three different densities of the cell suspensions were corresponded to seed 500, 1000, and 1500 cells into each micro-funnel to demonstrate whether the sphere size could be controlled by changing the number of cells. Cell spheres were harvested, and the diameter was determined as mentioned in the previous section.

### 2.5. Comparison of the cell spheres produced by the MP device and commercial culture plates

3D cell spheroid production of the MP devices was compared with commercial products EZSPHERE™ SP Dish 35 mm (EZ; 4000-900SP, AGC Techno Glass Co., Ltd., Shizuoka, Japan) [22] and AggreWell™400 (AW; Stemcell Technologies Inc., Vancouver, Canada) [23]. For the MP device, 4 mL culture medium was used for cell culture. Because these two commercial vessels are different