



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.

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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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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

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 μ L 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 CO₂ incubator (MCO-170AIC, Panasonic, Kadoma, Osaka, Japan) under 95% air and 5% CO₂ 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 1.7×10^5 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 μ m, and larger than 300 μ m. 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 (8.6×10^4 , 1.7×10^5 , and 2.6×10^5 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

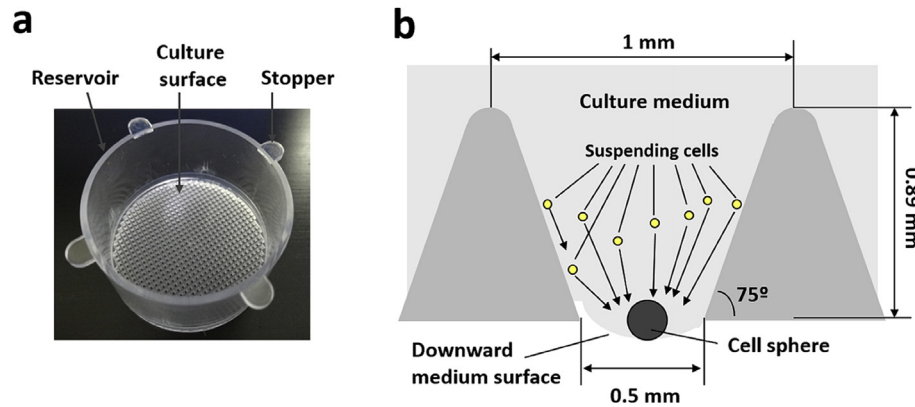


Fig. 1. (a) Gross appearance of the prototype insert (MP device). (b) Lateral view of the micro-funnel structure. The suspended cells are deposited to the downward medium surface and aggregate as 3D cell spheres.

in design, the required volumes of culture medium are also different. According to the manufacturer's recommendations, 2.7 mL and 2 mL of culture media were used in EZ and AW devices, respectively. However, the mouse ES suspension was prepared as 500 cells/micro-well for all devices ($n = 3$). These devices were placed on a metallic tray and cultured in an incubator under 95% air and 5% CO₂. After a 3-day culture period, cell spheres within the devices were observed using the stereomicroscope. Finally, the cell spheres were collected, and the devices post-harvest were examined again.

2.6. Differentiation of EBs

The spontaneous differentiation of EB, produced using the MP device and suspension culture, toward neural lineage was examined. Mouse ES cells were seeded into the MP devices (3.4×10^5 cells in 4 mL ES medium without LIF for one insert) or into a 35-mm Petri dish (5×10^5 cells in 6 mL ES medium without LIF) for suspension culture. After culturing for 24 h, the culture medium was replaced with a differentiation medium, which was composed of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 15% StemSure Serum Replacement (SSR; Wako Pure Chemical Industries, Ltd., Osaka, Japan), 1% bovine serum albumin (BSA; Wako Pure Chemical Industries, Ltd., Osaka, Japan), 1 mM glutamine, 1% non-essential amino acids, mercaptoethanol (5 μ L/1000 mL), and 1% penicillin/streptomycin. The differentiation medium was replaced every 2 days, and the differentiated cells were harvested every 5 days for further analysis.

Endodermal differentiation, induced by retinoic acid (RA) supplement, was examined, and the results were compared among EBs produced by the hanging drop method, suspension culture, and the MP device. ES cell suspensions were seeded into the MP devices or 35-mm Petri dishes as mentioned in the previous section. For the hanging drop method, EBs were produced by laying drops, which comprised 500 cells per 20 μ L of the medium, inside the lid of a 100-mm Petri dish, followed by gently inverting it in a dish filled with phosphate buffered saline (PBS). EBs produced by the three different methods were harvested after 24 h and transferred to 35-mm tissue culture dishes pre-coated with 0.1% gelatin. Then, an endodermal differentiation medium composed of DMEM supplemented with 1% BSA, insulin–transferrin–selenium supplement (ITS-G; Invitrogen, Carlsbad, CA, USA), 1 mM glutamine, 1% non-essential amino acids, mercaptoethanol (5 μ L/1000 mL), and 1% penicillin/streptomycin was provided. The EBs were allowed to differentiate for 18 days. To

improve endoderm differentiation, 10^{-6} M RA (Sigma–Aldrich, St. Louis, USA) was supplied two days after the transfer, and the RA-containing medium was changed every two days [24]. At the end of the 18-day culture period, cells were harvested for further analysis.

2.7. Quantitative RT-PCR

Total RNA of the cells was extracted (ISOGENII, Nippon Gene Co. Ltd., Tokyo, Japan), and the RNA was transcribed into cDNA (VersoTM cDNA Kit, Thermo Fisher Scientific Inc., Waltham, MA, USA) using a reverse-transcription PCR thermal cycler. Real-time quantitative PCR analysis was conducted (StepOnePlus Real-Time PCR system, Applied Biosystems, Foster City, USA) using the SYBR system (SYBR Premix Ex Taq TM II, Takara Bio Inc., Shiga, Japan). Neural cell markers [nestin and neuron-specific class III beta-tubulin (Tuj1)] and endodermal markers [pancreatic and duodenal homeobox 1 (Pdx-1) and insulin I] were analyzed with reference to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of differentiated cells (Table 1). cDNA was amplified with the following cycling parameters: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The mRNA expression of each target gene was normalized to that of GAPDH.

2.8. Statistical analysis

The data obtained from each group were expressed as mean \pm standard deviation (SD). Statistical analyses were conducted by ANOVA with *post-hoc* Dunnett's multiple comparison tests. Difference was considered significant when the *p*-value was less than 0.05.

Table 1
Primer sequences for real-time PCR.

Nestin	F: 5'-AGATCGCTCAGATCCTGGAA-3' R: 5'-GAGTTCTCAGCCTCCAGCAG-3'
Tuj1	F: 5'-CAGTGGCGCAACCAGATAG-3' R: 5'-GCAGGTCTGAGTCCCCTACA-3'
Pdx-1	F: 5'-GAT GAA ATC CAC CAA AGC TCA CGC-3' R: 5'-GGG TGT AGG CAG TAC GGG TCC TC-3'
Insulin I	F: 5'-CTTCAGACCTTGGCGTTGGA-3' R: 5'-ATGCTGGTGCAGCACTGATC-3'
GAPDH	F: 5'-GCTACTACTAGGACCAGGTGTGC-3' R: 5'-AGCCGTATTCATTGTCATACCAGG-3'

3. Results

3.1. Formation and harvest of cell spheres in the MP device and suspension culture

After plating the cell suspensions (1.7×10^5 cells/mL) in an optimum volume of medium (4 mL) into the MP device, cells deposited at the downward medium surface toward the bottom opening. Cell spheres with a round and smooth contour were formed in the center of each micro-funnel after culturing for two days (Fig. 2a). For the harvest procedure, cell spheres could be easily and quickly collected by putting the bottle of culture insert in contact with the surface of the culture medium in another Petri dish. The entire medium with cell spheres within the device was eluted into the Petri dish through the bottom opening by hydrostatic pressure (Fig. 2b). The number of harvested cell spheres was counted, and 604 ± 41 spheres were obtained from one insert (efficiency of sphere formation is $88.85 \pm 5.95\%$).

Both the size and shape of the cell spheres obtained from the MP device (Fig. 2c) were relatively uniform when compared to those of the spheres produced by suspension culture (Fig. 2d). The mean diameter of the cell spheres harvested from the MP device was $163.4 \pm 17.2 \mu\text{m}$, whereas the size of cell spheres obtained from suspension culture (static spheroid culture) was $77.9 \pm 26.0 \mu\text{m}$. As shown in Fig. 2e, the size was normally distributed around the mean value in the MP device. However, F-distribution with a rightward stretching was noticed, and many small cell aggregates were found in the suspension culture.

3.2. Cell density and the size of cell spheres

Different cell densities yielded different sphere sizes, and a high seeding cell density resulted in a large cell sphere. For the size

distribution of the spheres, about 62.02% of cell spheres ranged between 100 and 150 μm and other 30.98% located between 150 and 200 μm when 500 cells were seeded. For the cell density of 1000 cells per micro-funnel, 30.00% of the cell spheres ranged between 125 and 150 μm , 30.95% ranged between 150 and 175 μm , and other 30.95% ranged between 175 and 200 μm . For the cell density of 1500 cells, a relative wide distribution in the sphere diameter was observed. However, majority of cell spheres (50.8%) ranged between 175 and 225 μm (Fig. 3). The mean diameters of the cell spheres for cell densities of 500, 1000, and 1500 cells per micro-funnel were 142.7 ± 49.9 , 185.7 ± 31.3 , and $225.0 \pm 52.5 \mu\text{m}$, respectively.

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

While using the EZ and AW devices, some air bubbles were formed in the micro-wells when the medium was added into the devices. The residual bubbles influence cell seeding as well as cell distribution for these two vessels. Therefore, an additional preparation was required (pipetting for EZ device and centrifugation for AW device) to remove air bubbles before cell seeding, whereas our MP device did not require any such additional procedure.

Basically, one cell sphere formed in one micro-well for the MP and AW devices during culture periods (Fig. 4a). Although rare, two cell spheres can be found in one micro-well to these two devices (Fig. 4i). However, the cell spheres on EZ devices were noticed to be float, and several cell spheres were found in one micro-well during cultivation (Fig. 4e). For the MP devices, the cell sphere was retained within each micro-funnel even when the device was moved from the incubator to a clean bench for operation or under the microscope for observation (Fig. 4b). However, the movements of cell spheres were frequently observed in cultivation for these

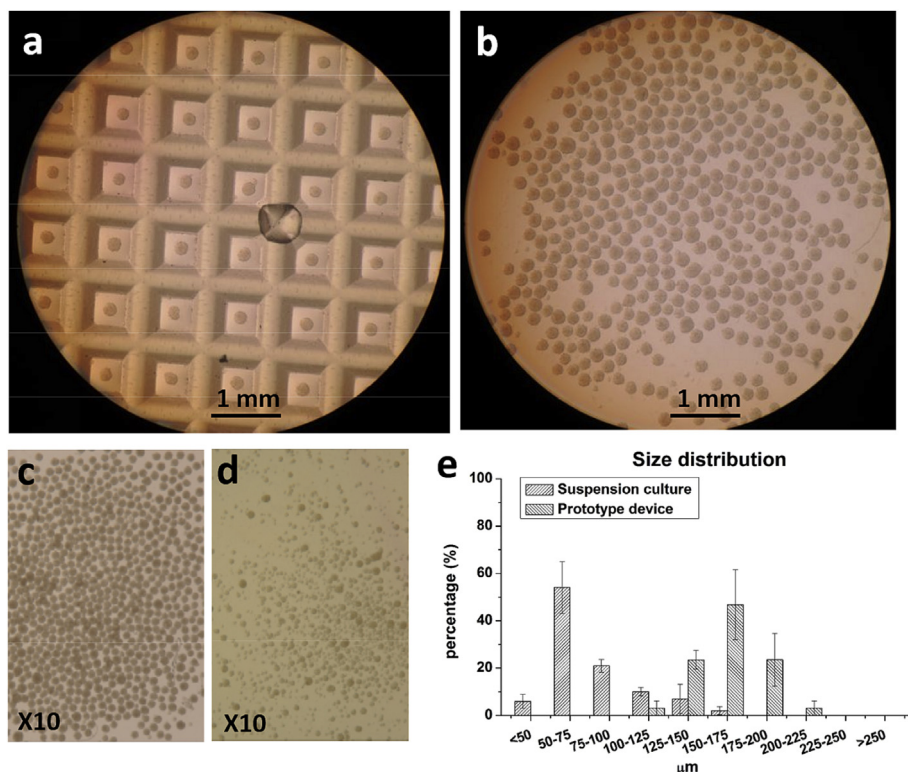


Fig. 2. Cell spheres produced using the MP device and suspension culture. (a) Cell spheres were formed at the center of each micro-funnel after culturing for two days. (b) The harvested cell spheres were intact and uniform. The size and shape of the cell spheres obtained from the MP device (c) are relatively uniform compared to those of the suspension culture (d). (e) Size distribution of the cell spheres obtained by the MP device and suspension culture method.

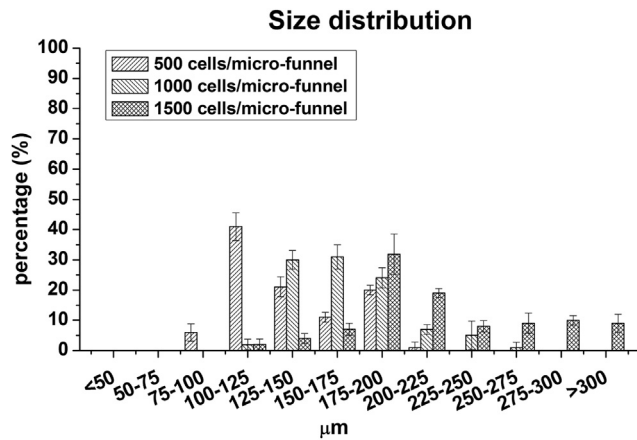


Fig. 3. Cell density and size distribution of the cell spheres.

two commercial vessels. For the EZ devices, the cell spheres in the peripheral two to three rows easily moved to central wells during transportation (Fig. 4e), and these cell spheres stacked together within one micro-well (Fig. 4f). The AW device (Fig. 4i) also had a similar issue, but the movements of cell spheres were randomly (Fig. 4j). Although the number of the migrated cell spheres was less than that of the EZ devices, some of these cell clusters on AW devices were not compact and shall be ignored (Fig. 4k). For the harvest procedure, cell spheres were collected by repeated pipetting and suction for EZ and AW devices. Both the MP device (Fig. 4c) and the EZ device (Fig. 4g) produced uniform cell spheres. However, cell spheres with non-uniform size and shape were found in the AW device (Fig. 4k). No cell spheres remained on the MP device after harvesting (Fig. 4d), while a few cell spheres remained in the micro-wells of the EZ (Fig. 4h) and AW devices (Fig. 4l). Although multiple washing/pipetting processes can improve cell sphere collection from these two commercial devices, the repeated

procedure can break cell spheres. In contrast to our MP device, it is difficult to determine the efficiency of sphere formation for EZ and AW devices since some cell spheres migrated/adhered together during cultivation, and some spheres remained on the devices after harvest procedure.

3.4. Differentiation of EB

Spontaneous neural differentiation of EB was examined to highlight the differences in differentiation potentials to those obtained by suspension culture and the MP device. EBs of various sizes and shapes were formed by static spheroid culture, whereas the size and shape were fairly uniform in the MP device at day 10 (Fig. 5a). Although the size of the EB increased with the culture periods in both methods, the growth of ES cells in the MP device caused an increase in the sphere size because of the stability of cells in each micro-funnel, whereas irregular cell aggregates with various sizes were noticed under suspension culture condition. The marker of neural differentiation nestin increased significantly in the MP device compared to that in the suspension culture at day 10 and 15 (Fig. 5b). Similarly, EB in the MP device also exhibited a relatively higher Tuj1 level at day 10, 15, and 20 (Fig. 5c). However, there was no significant difference in the markers after day 25 between these two groups.

RA was used to induce endodermal differentiation to EB obtained by the hanging drop method, suspension culture, and MP device. Both the hanging drop method and MP device produced EB with good uniformity in size and shape, whereas EBs of various sizes and shapes were formed by the suspension culture (Fig. 6a). Although there was no significant difference in the absence of RA induction, the expression of endodermal markers Pdx-1 (Fig. 6b) and insulin I (Fig. 6c) improved with RA treatments, regardless of the production methods of EB at day 18. However, EB produced by the hanging drop method and MP device exhibited relatively higher

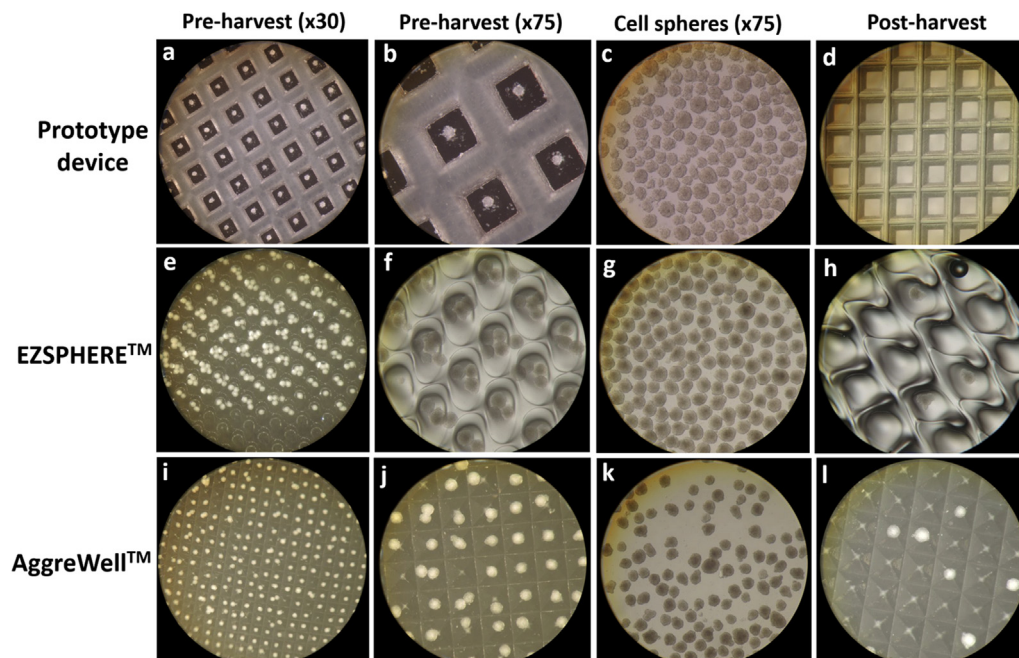


Fig. 4. Comparison of cell spheres produced by the MP device and commercial culture plates. (a and b) For the MP devices, the cell sphere was retained within each micro-funnel when the device was moved. (e) Cell spheres in the peripheral area of EZ device easily moved to the adjacent micro-wells during transportation (f) and several cell spheres were stacked in one micro-well. (i) AW device also had the same issue (j), but the number of migrated cell spheres was less than that of the EZ device. (c) The MP and (g) EZ devices produced uniform cell spheres. (k) Cell spheres with non-uniform size and shape were found in the AW device. (d) No cell spheres remained on the MP device after harvesting. A few cell spheres remained in the micro-wells of (h) the EZ and (l) AW devices.

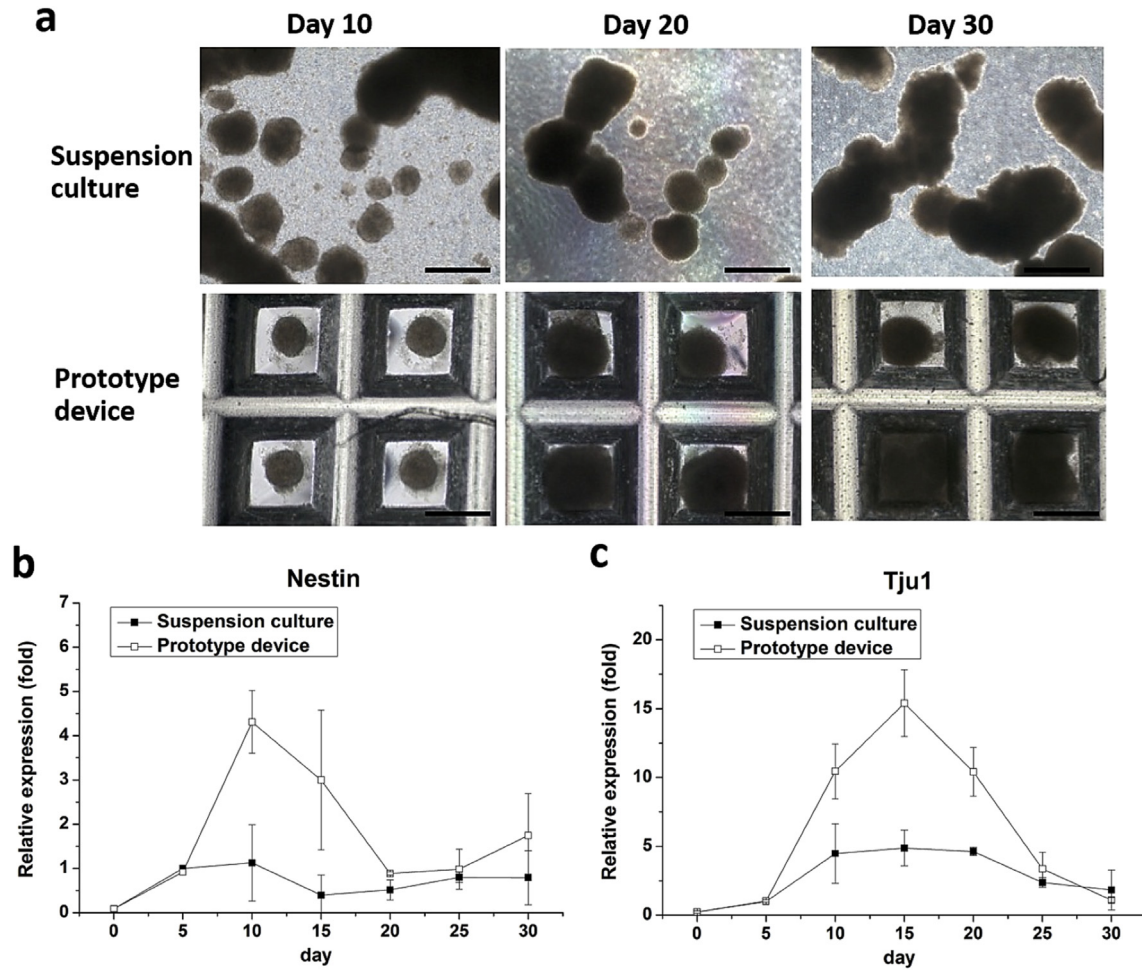


Fig. 5. (a) EB produced by suspension culture and the MP device. Scale bar: 500 μm . (b) The level of the marker of neural differentiation nestin increased significantly in the MP device compared to that of suspension culture at day 10 and 15. (c) EB produced by the MP device also had a relatively higher Tju1 level at day 10, 15, and 20.

Pdx-1 and insulin I levels relative to those of the suspension culture.

4. Discussion

Conventional approaches to prepare cell spheres in biomedical research present shortcomings such as difficulty in controlling the sphere geometry, difficulty in harvesting, and long processing time. Moreover, the quality of the cell spheres influences the differentiation potential of stem cells as well as the biological functions of somatic cells. Accordingly, we designed a novel multiple-funnels cell culture insert for the production of a high number of uniform cell spheres.

In the multiple-funnels cell cultured device designed by us, the medium containing suspended cells is maintained within the culture surface owing to the balance between the hydrostatic pressure ($P = \rho H$) and surface tension (γ_L) of the downward medium surface (Fig. 7a).

From Laplace equation,

$$\Delta P = \gamma_L(1/r_1 + 1/r_2)$$

where r_1 and r_2 are the curvature radii of the downward medium surface, which is a convex.

In theory, the downward medium surface is disrupted when r_1 and/or r_2 values are lower than the estimated radius of the bottom

opening. Considering the contact angle of the medium and the underside surface of the device, the downward medium surface can also be disrupted when the angle (θ in Fig. 7a) between the downward medium surface and under surface of the device exceeds the contact angle. In this case, the medium spreads on the underside surface and the surface tension of the downward medium surface cannot sustain the medium pressure. Because the volume of the cell suspensions correlates with the downward medium pressure, the total volume of the medium should be controlled.

For example, when 4 mL of cultured medium was added into one MP device (Fig. 7b),

$$\text{The bottom area of device: } \pi r^2 = 3.14 \times 1.5^2 = 7 \text{ (cm}^2\text{)}$$

$$\text{The pressure is: } \text{depth} \times \rho \times G = (4/7) \times 1 = 0.57 G \text{ (dyn/cm}^2\text{)}$$

$$\text{When } r_1 = r_2, \Delta P = 2\gamma_L/r, \text{ and thus } r = 2\gamma_L/\Delta P$$

$$\text{The surface tension is: } 7 \times 10^{-2} \text{ (N/m)} = 70 \text{ (dyn/cm)}$$

$$\text{While } G = 9.8 \text{ (m/s}^2\text{)} = 980 \text{ (cm/s}^2\text{)}$$

$$\text{Therefore, } r = 2 \times 70 / 0.57 \times 980 = 0.25 \text{ (cm)} = 2.5 \text{ (mm)} \text{ (Fig. 7c)}$$

(Numbers are approximated)

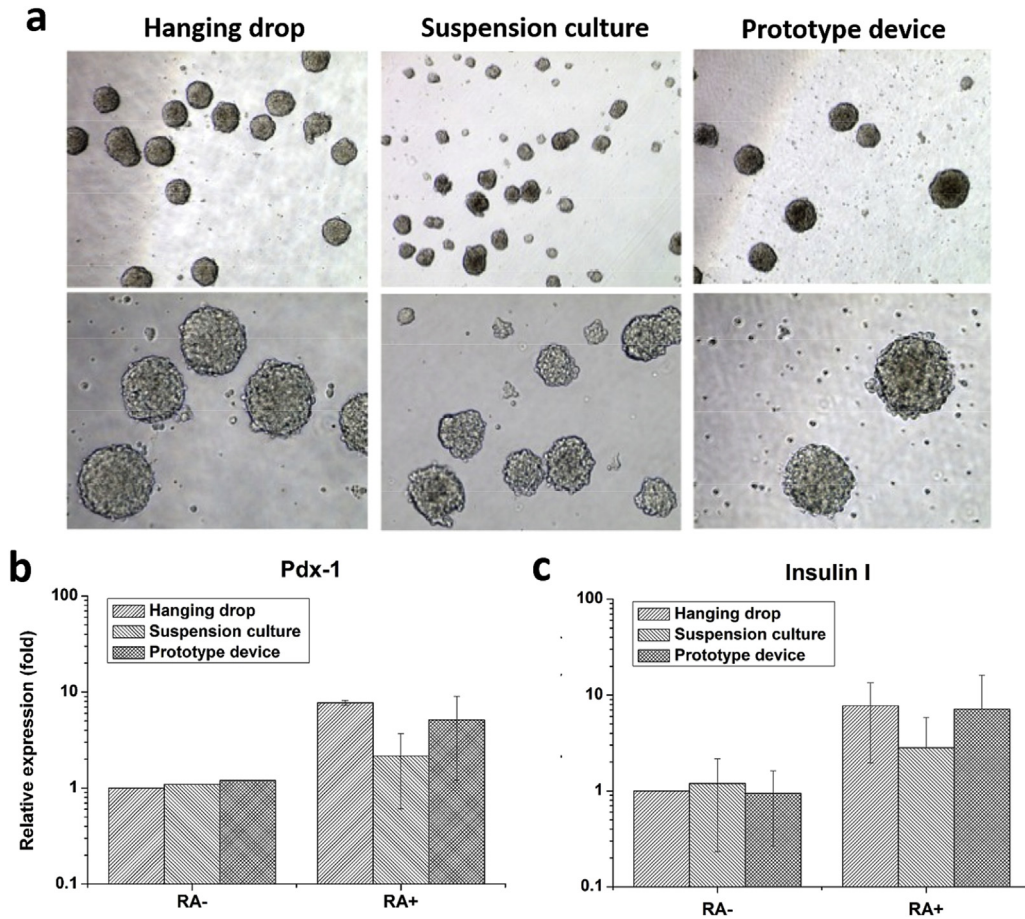


Fig. 6. (a) Microscopic view of EB produced by the hanging drop method, suspension culture, and MP device. Under RA induction, the expression of endodermal markers (b) Pdx-1 and (c) insulin I were upregulated for EB produced by the hanging drop method and MP device compared to suspension culture at day 18.

In our preliminary study, up to 5 mL of the medium can be added into the MP device, up to 5 mL of the medium can be added into the MP device without damaging the downward medium surface, and thus 4 mL of the medium was used in our study under calculation. For practice, the MP devices with cells can be placed on a shock absorbing pad in a stainless steel tray to avoid vertical acceleration during the culture period. However, handling with care is most important.

The newly developed multiple-funnels cell culture insert facilitates the formation of 680 spheroids in one insert by simple seeding of cell suspensions. Compared with the time consuming hanging drop method, the MP device affords better cost- and time-efficiency to produce a large number of uniform cell spheres. Compared to the hanging drop method, Kim et al. reported that a multi-well substrate can improve the efficiency of EB formation

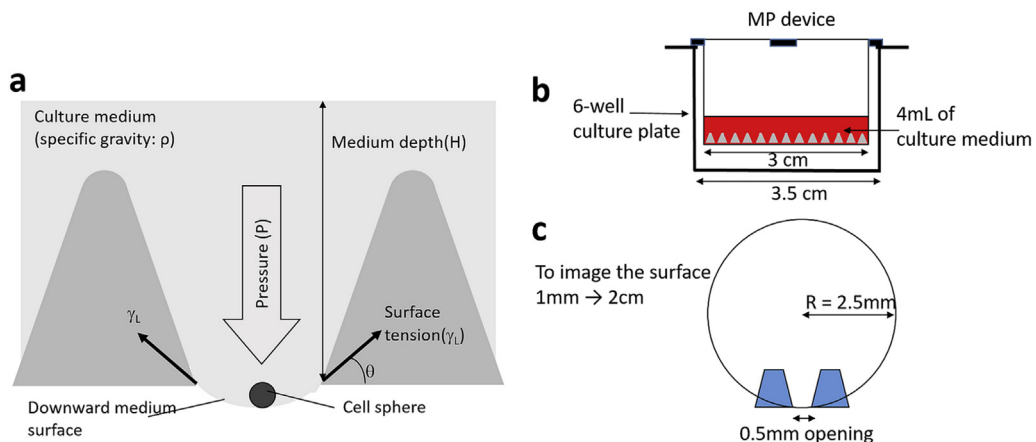


Fig. 7. (a) Illustration of the mechanic balance between the medium pressure and surface tension of the downward medium surface within the micro-funnel device. (b) 4 mL of culture medium was added into one MP device, and (c) a closed view of micro-funnel.

[25]. In addition, cell spheres in other commercial culture plates may move to the adjacent micro-wells easily, and the contacts between multiple EBs resulted in the fusion of cell spheres. The formation of irregular cell aggregates is frequently found during static spheroid culture [26]. This may cause difficulty in replacing the medium and limit long-term culture for producing cell spheres. Furthermore, intact cell spheres can be harvested easily by breaking the downward medium surface in our MP device. Although the micro-well devices can also produce uniform cell spheres, the mechanical stress caused by repeated pipetting, suction, and centrifugation during harvesting may induce stress in the cell spheres [27,28]. In contrast to the micro-wells, our device can collect cell spheres through the bottom opening and may reduce this stress. Furthermore, as shown in the spontaneous differentiation study, EB can be cultured for 30 days in the MP device with multiple medium changes. After careful aspiration of the culture medium, cell spheres were retained in the micro-funnels with the residual medium and subsequent new medium could be added for long-term culture.

The size of cell spheres is an important factor in stem cell differentiation. A previous study has indicated that EB produced by seeding a defined number of human ES cells foster reproducible hematopoietic differentiation [29]. Another study has revealed that small EBs are less likely to form contracting EB, but these contracting EBs are more enriched in cardiomyocytes compared to larger EBs [30]. Thus, micro-well substrates are designed for controlling the size of EB, thereby determining their fate [31]. Hwang et al. fabricated micro-wells with diameters of 150, 300, and 450 μm to restrict EB size and reported that large EB enhance cardiogenesis, whereas small EB increase endothelial cell differentiation [21]. Likewise, Valamehr found that EB with a diameter of 100–300 μm exhibit a higher proliferative rate with a relatively better differentiation potential and a lower apoptosis rate [32]. However, the size of EB is determined by the diameter of micro-wells, and thus, different applications may require EB produced using differently sized micro-wells. However, we can adjust the size of the cell spheres simply by changing the cell density. Furthermore, the size of the cell spheres prepared by the MP device is well controlled.

Regarding the spontaneous neural differentiation of EB, the increasing expression of neuron marker Tuj1 at day 15 was accompanied with a decrease in the neural precursor marker nestin after day 10, which is consistent with the finding of a previous study [33]. Interestingly, EB in the MP device exhibited relatively higher expression of nestin and Tuj1. Previously, Ankam et al. found that the size of EB influences the differentiation toward neuronal or glial lineage, although the mechanism is unclear [34]. Concerning the induction of endodermal differentiation, RA is implicated in embryonic endodermal patterning, particularly during the early period of pancreas formation [35]. Despite the fact that EB produced by the hanging drop method and the MP device exhibited similar morphology, increases in the Pdx-1 and insulin I levels were noticed compared to those of the suspension culture. Sakai et al. used micro-well and micro-patterned chips to culture EB and found that ES differentiation properties were different, although the EB size, cell number, and cell density were almost the same [36]. The detailed mechanism behind this finding shall be studied.

Central necrosis is a major concern for cell spheres because of the limited passive diffusion of oxygen and nutrients. Luther et al. produced pancreatic β -cell spheres by using gelatin-coated dishes and noticed that the rate of apoptosis was upregulated [37]. Similarly, Yang et al. reported serious central apoptosis in cell spheres cultured on ultra-low attachment and bacterial (non-treated polystyrene) dishes [38]. Using an oxygen-permeable material to fabricate a cell spheroid formation device, Anada et al. reported

that hypoxia and central necrosis could be prevented [39]. Likewise, Shinohara et al. fabricated an oxygen-permeable polydimethylsiloxane honeycomb micro-well sheets for the formation of cell spheres, and they found that spherical MIN-6 cellular aggregates had aerobic respiration in their culture system [19]. Moreover, they also reported that the relative larger spheroids have a decreased metabolic response. Since the formed cell sphere is cultured at the downward medium surface of the culture insert, our MP device shall facilitate the transportation of oxygen to cell spheres.

In addition to ES cells, we also tested the formation of cell spheres for the mouse pancreatic β -cell line MIN-6, human pancreatic cancer cell line PANC-1, dispersed rat islet cells, rat primary MSC, rat primary hepatocytes, and human primary submaxillary salivary gland cells. Cell spheres of a uniform size and well-rounded shape were obtained after culturing for two days, but the PANC-1 line maintained single cells (data not shown). We assume that the characteristics of each cell type, such as its junctional proteins and adhesion molecules, may be responsible for this result. Moreover, Alimperti et al. found that serum-free spheroid suspension culture can maintain MSC proliferation and differentiation potential [40]. Taken together, our novel multiple-funnels cell culture insert enables formation of cell spheres for various cell types, revealing the wide-ranging applicability of this device. It is expected that studies and practice in regenerative medicine using cell spheres will be accelerated by this novel and efficient cell sphere formation device.

5. Conclusion

Our novel culture insert with multiple micro-funnel surface facilitates efficient formation and harvesting of cell spheres with a uniform size and a well-rounded shape. The size of the spheres is controllable by changing the seeding cell density. Cell sphere production by the MP device is better than that using commercial micro-well plates. Neural differentiation and RA-induced endodermal differentiation were improved in the EB produced by the new insert. This novel cell culture insert could improve future studies of cell spheres and benefit clinical applications of cell therapy.

Conflicts of interest

This study was performed, in part, as a collaborative study with Kuraray Co. Ltd. The prototype insert presented here was modified and, now, they are commercialized as Elplasia™ MPC Type by Kuraray Co., Ltd.

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References

- [1] Fang D, Nguyen TK, Leishear K, Finko R, Kulp AN, Hotz S, et al. A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer Res* 2005;65(20):9328–37.
- [2] Bratt-Leal AM, Carpenedo RL, McDevitt TC. Engineering the embryoid body microenvironment to direct embryonic stem cell differentiation. *Biotechnol Prog* 2009;25(1):43–51.
- [3] Weiswald LB, Bellet D, Dangles-Marie V. Spherical cancer models in tumor biology. *Neoplasia* 2015;17:1–15.

- [4] Desbaillets I, Ziegler U, Groscurth P, Gassmann M. Embryoid bodies: an *in vitro* model of mouse embryogenesis. *Exp Physiol* 2000;85:645–51.
- [5] Seiler AE, Spielmann H. The validated embryonic stem cell test to predict embryotoxicity *in vitro*. *Nat Protoc* 2011;6(7):961–78.
- [6] Turner PA, Tang Y, Weiss SJ, Janorkar AV. Three-dimensional spheroid cell model of *in vitro* adipocyte inflammation. *Tissue Eng Part A* 2015;21(11–12):1837–47.
- [7] Dingle YT, Boutin ME, Chirila AM, Livi LL, Labriola NR, Jakubek LM, et al. Three-dimensional neural spheroid culture: an *in vitro* model for cortical studies. *Tissue Eng Part C Methods* 2015;21(12):1274–83.
- [8] Panchalingam KM, Jung S, Rosenberg L, Behie LA. Bioprocessing strategies for the large-scale production of human mesenchymal stem cells: a review. *Stem Cell Res Ther* 2015;6:225.
- [9] Chiang TS, Yang KC, Zheng SK, Chiou LL, Hsu WM, Lin FH, et al. The prediction of drug metabolism using scaffold-mediated enhancement of the induced cytochrome P450 activities in fibroblasts by hepatic transcriptional regulators. *Biomaterials* 2012;33(21):5187–97.
- [10] Yang KC, Qi Z, Shirouza Y, Lu DH, Yanai G, Lee HS, et al. Cell coupling regulates *Ins1*, *Pdx-1* and *MafA* to promote insulin secretion in mouse pancreatic beta cells. *Process Biochem* 2011;46(9):1853–60.
- [11] Kim JY, Kim HW, Bae SJ, Joo DJ, Huh KH, Fang YH, et al. Hybrid cellular spheroids from hepatocellular carcinoma and insulin-secreting cell lines. *Transpl Proc* 2012;44(4):1095–8.
- [12] Bhang SH, Lee S, Lee TJ, La WG, Yang HS, Cho SW, et al. Three-dimensional cell grafting enhances the angiogenic efficacy of human umbilical vein endothelial cells. *Tissue Eng Part A* 2012;18(3–4):310–9.
- [13] Lin RZ, Chang HY. Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnol J* 2008;3(9–10):1172–84.
- [14] Chen SF, Chang YC, Nieh S, Liu CL, Yang CY, Lin YS. Nonadhesive culture system as a model of rapid sphere formation with cancer stem cell properties. *PLoS One* 2012;7(2):e31864.
- [15] Cao L, Zhou Y, Zhai B, Liao J, Xu W, Zhang R, et al. Sphere-forming cell subpopulations with cancer stem cell properties in human hepatoma cell lines. *BMC Gastroenterol* 2011;11:71.
- [16] Lee LH, Peerani R, Ungrin M, Joshi C, Kumacheva E, Zandstra PW. Micropatterning of human embryonic stem cells dissects the mesoderm and endoderm lineages. *Stem Cell Res* 2009;2(2):155–62.
- [17] Bauwens CL, Peerani R, Niebruegge S, Woodhouse KA, Kumacheva E, Husain M, et al. Control of human embryonic stem cell colony and aggregate size heterogeneity influences differentiation trajectories. *Stem Cells* 2008;26(9):2300–10.
- [18] Fujita Y, Takita M, Shimoda M, Itoh T, Sugimoto K, Noguchi H, et al. Large human islets secrete less insulin per islet equivalent than smaller islets *in vitro*. *Islets* 2011;3:1–5.
- [19] Shinohara M, Kimura H, Montagne K, Komori K, Fujii T, Sakai Y. Combination of microwell structures and direct oxygenation enables efficient and size-regulated aggregate formation of an insulin-secreting pancreatic β -cell line. *Biotechnol Prog* 2014;30(1):178–87.
- [20] Choi YY, Chung BG, Lee DH, Khademhosseini A, Kim JH, Lee SH. Controlled-size embryoid body formation in concave microwell arrays. *Biomaterials* 2010;31(15):4296–303.
- [21] Hwang YS, Chung BG, Ortmann D, Hattori N, Moeller HC, Khademhosseini A. Microwell-mediated control of embryoid body size regulates embryonic stem cell fate via differential expression of *wnt5a* and *wnt11*. *Proc Natl Acad Sci USA* 2009;106(40):16978–83.
- [22] Ebert AD, Shelley BC, Hurley AM, Onorati M, Castiglioni V, Patitucci TN, et al. EZ spheres: a stable and expandable culture system for the generation of pro-
- rosette multipotent stem cells from human ESCs and iPSCs. *Stem Cell Res* 2013;10(3):417–27.
- [23] Antonchuk J. Formation of embryoid bodies from human pluripotent stem cells using AggreWell™ plates. *Methods Mol Biol* 2013;946:523–33.
- [24] Shirouza Y, Yanai G, Yang KC, Sumi S. Effects of activin in embryoid bodies expressing fibroblast growth factor 5. *Cell Reprogr* 2016;18(3):171–86.
- [25] Kim C, Lee IH, Lee K, Ryu SS, Lee SH, Lee KJ, et al. Multi-well chip for forming a uniform embryoid body in a tiny droplet with mouse embryonic stem cells. *Biosci Biotechnol Biochem* 2007;71(12):2985–91.
- [26] Sarvi F, Arbatan T, Chan PPY, Shen W. A novel technique for the formation of embryoid bodies inside liquid marbles. *RSC Adv* 2013;3(34):14501–8.
- [27] Welch WJ. How cells respond to stress. *Sci Am* 1993;268(5):56–64.
- [28] Sinha B, Köster D, Ruez R, Gonnord P, Bastiani M, Abankwa D, et al. Cells respond to mechanical stress by rapid disassembly of caveolae. *Cell* 2011;144(3):402–13.
- [29] Ng ES, Davis RP, Azzola L, Stanley EG, Elefanty AG. Forced aggregation of defined numbers of human embryonic stem cells into embryoid bodies fosters robust, reproducible hematopoietic differentiation. *Blood* 2005;106(5):1601–3.
- [30] Mohr JC, Zhang J, Azarin SM, Soerens AG, de Pablo JJ, Thomson JA, et al. The microwell control of embryoid body size in order to regulate cardiac differentiation of human embryonic stem cells. *Biomaterials* 2010;31(7):1885–93.
- [31] Sasaki D, Shimizu T, Masuda S, Kobayashi J, Itoga K, Tsuda Y, et al. Mass preparation of size-controlled mouse embryonic stem cell aggregates and induction of cardiac differentiation by cell patterning method. *Biomaterials* 2009;30:4384–9.
- [32] Valamehr B, Jonas SJ, Polleux J, Qiao R, Guo S, Gschweng EH, et al. Hydrophobic surfaces for enhanced differentiation of embryonic stem cell-derived embryoid bodies. *Proc Natl Acad Sci USA* 2008;105(38):14459–64.
- [33] Montgomery A, Wong A, Gabers N, Willerth SM. Engineering personalized neural tissue by combining induced pluripotent stem cells with fibrin scaffolds. *Biomater Sci* 2015;3(2):401–13.
- [34] Ankam S, Suryana M, Chan LY, Moe AA, Teo BK, Law JB, et al. Substrate topography and size determine the fate of human embryonic stem cells to neuronal or glial lineage. *Acta Biomater* 2013;9(1):4535–45.
- [35] Kim PT, Hoffman BG, Plesner A, Helgason CD, Verchere CB, Chung SW, et al. Differentiation of mouse embryonic stem cells into endoderm without embryoid body formation. *PLoS One* 2010;5(11):e14146.
- [36] Sakai Y, Yoshiura Y, Nakazawa K. Embryoid body culture of mouse embryonic stem cells using microwell and micropatterned chips. *J Biosci Bioeng* 2011;111(1):85–91.
- [37] Luther MJ, Davies E, Muller D, Harrison M, Bone AJ, Persaud SJ, et al. Cell-to-cell contact influences proliferative marker expression and apoptosis in MIN6 cells grown in islet-like structures. *Am J Physiol Endocrinol Metab* 2005;288:E502–9.
- [38] Yang KC, Wu CC, Yang SH, Chiu CC, Sumi S, Lee HS. Investigating the suspension culture on aggregation and function of mouse pancreatic β -cells. *J Biomed Mater Res A* 2013;101(8):2273–82.
- [39] Anada T, Fukuda J, Sai Y, Suzuki O. An oxygen-permeable spheroid culture system for the prevention of central hypoxia and necrosis of spheroids. *Biomaterials* 2012;33(33):8430–41.
- [40] Alimperti S, Lei P, Wen Y, Tian J, Campbell AM, Andreadis ST. Serum-free spheroid suspension culture maintains mesenchymal stem cell proliferation and differentiation potential. *Biotechnol Prog* 2014;30(4):974–83.