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Full Length Research Paper

Round-bottomed Honeycomb Microwells: Embryoid body shape correlates with stem cell fate

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The differentiation of embryonic stem cells (ESC) into tissue-specific cells utilizes either monolayer cultures or three-dimensional cell aggregates called embryoid bodies (EB). However, the generation of a large number of EB of controlled sizes can be challenging and labor intensive. Our laboratories have developed a simple, robust, ultra-rapid, and inexpensive design of Honeycomb Microwells for generation of EB. Here, we compare EB generated using (1) Honeycomb Microwells, (2) the commercially available AggreWell™400, and (3) the more traditional Hanging Drop method. We compared the efficiency, viability, quality, and control of EB sizes. Results indicate that the Honeycomb Microwell and AggreWell™400 efficiently generate small EB at approximately 500 cells per EB. However, the cone-bottomed AggreWell plate generates cone-shaped EB at 1000-2000 cells per EB. Moreover, the cone-shape correlates with a reduction in the formation of the primitive endoderm GATA-4+ cells (1% compared with 6-8% in spherical EB), but does not significantly affect mesoderm or ectoderm development. We conclude that the non-spherical EB shape correlates with a reduction in the development of primitive endoderm, and that use of these AggreWell plates should be avoided in deriving endoderm tissue products.

Key words: Embryonic stem cells, embryoid bodies, Microwell, AggreWell, hanging drop, primitive endoderm, GATA-4.

INTRODUCTION

Embryonic stem cells (ESC) and induced-pluripotent stem cells (iPSC) are both excellent *in vitro* cell culture systems for studying stem cell fate. These cells are especially attractive due to their unlimited *in vitro* expansion potential (Amit et al., 2000) while also maintaining the capacity to differentiate into a variety of cell types (Odorico et al., 2001). Moreover, once differentiation methodologies are developed and fully optimized, these stem cell sources could provide the specialized tissue-specific cells needed for a variety of applications in regenerative medicine. The most common methods of ESC differentiation require either the induction on monolayer cultures or the generation of 3-D structures, called embryoid bodies (EBs) (Itskovitz-Eldor et al., 2000), which recapitulate various aspects of embryogenesis. Subsequently, many methodologies for initial induction of ESC towards different tissue-specific cell fates require EB formation, reviewed in (Desbaillets et al., 2000).

Unfortunately, the generation of high numbers of reproducible EB is not trivial. EB can vary in size when formed using the traditional liquid suspension or methylcellulose culture (Kurosawa, 2007) methods for EB generation, and thus, affect the downstream stem cell differentiation products (Park et al., 2007). For the production of larger numbers of EB, stirred-suspension cultures using spinner flasks and bioreactors are usually employed (Dang et al., 2004; Dang and Zandstra, 2005; Hwang et al., 2009a; Lock and Tzanakakis, 2009;

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Schroeder et al., 2005) even though these suspension cultures often gives rise to EB populations which are also heterogeneous in morphology and thus, differentiation capacity (Choi et al., 2010; Hwang et al., 2009b; Valamehr et al., 2008). The labor intensive hanging drop can yield relatively uniform EB, but this method requires culturing EB in droplets of medium that are less than 20 µL in size, making medium exchange and hence, long-term culture extremely difficult (Dang et al., 2004). The 96-well plate has also been used successfully to generate EB (Koike et al., 2005). This well-plate remains labor intensive, but does allow medium exchange for longer-term cultures. Lastly, it should be noted that the generation of uniform EB from human ESC is additionally challenging compared with mouse ESC due to the apoptotic nature of human ESC when dispersed into single cell suspensions.

Recently, STEMCELL Technologies introduced the AggreWell™400, the first commercially available plate for the generation of standardized EB from ESC and iPS cells. This product was followed by the AggreWell™800, which is a plate containing larger sized-wells for generating larger EB. Together, these plates allow the generation of large quantities of uniform-sized EB. Although the wells of the AggreWell are cone-shaped, and not spherical, the system allows very nice control over the size of EB by altering the number of cells added to each well.

In differentiation studies using the AggreWell™400, it was observed the larger EB would retain the cone-shaped morphology after transfer from the well plate into suspension culture. We suspected that this shape might affect the stem cell fate of the EB, and therefore, set out to study the germ layer development in the cone-shaped EB compared with the traditional hanging drop. We also include our laboratory's round-bottomed Honeycomb Microwells (Chen et al., 2008; Nguyen et al., 2009) and compared the efficiency, viability, aspect ratio, circularity, and control over EB sizes between the three methods of EB generation.

Results will show that the Honeycomb Microwell and the AggreWell plates are both able to efficiency generate EB of well-controlled sizes, but the round-bottomed Honeycomb Microwell generates more circular-shaped EB. Additionally, the cone-shaped EB from the AggreWell plate exhibit a significant reduction in the formation of the primitive endoderm in the EB.

METHODS

Murine embryonic stem cell culture

E14 mouse ESC transfected with green fluorescence protein (GFP) expression linked to the myosin heavy chain promoter (courtesy of Conklin Lab, UCSF) were maintained in Knockout Dulbecco's modification of Eagle Medium (DMEM: Gibco) supplemented with 15% Knockout Serum Replacement (KSR; Gibco), 100 mg/mL of penicillin-streptomycin (Invitrogen), 1 mM L-glutamine (Gibco), 0.1 mM nonessential amino acids (NEAA; Invitrogen), 0.1 mM betamercaptoethanol (Calbiochem) and 1000 U/mL leukemia inhibitory factor (LIF; Chemicon) and plated on tissue cultured plates coated with 0.1% Gelatin (Sigma-Aldrich). When the mouse ESC on the tissue culture plates were 80% confluent, they were detached using trypsin/EDTA (0.1%/1 mM), and dissociated into single cells using a pipette to assure uniform distribution of cells during the loading process. Mouse ESC were then spun down and re-suspended in differentiation medium, which has the same composition as ESC medium with the exclusion of LIF and supplemented with 20% KSR and 10 ng/ml bone morphogenic protein4 (BMP4; R&D System).

Human embryonic stem cell culture

H9 human ESC were maintained in mouse embryonic fibroblast (MEF)-conditioned medium supplemented with 5 ng/ml basic fibroblast growth factor (bFGF, Sigma) and plated on tissue cultured plates coated with Matrigel (Becton Dickenson). When the human ESC on the tissue culture plates were 80% confluent, they were detached using Accutase (Invitrogen), and dissociated into single cells using a pipette to assure uniform distribution of cells during the loading process. The human ESC were then spun down and re-suspended in high glucose Dulbecco's Modification of Eagle Medium (DMEM; Gibco) supplemented with 20% Knockout Serum Replacement (KSR; Gibco), and 25 ng/ml bone morphogenic protein-4 (BMP-4; R&D System). The Y-27632 ROCK inhibitor, final concentration of 10 µM, was added during EB formation to enhance cell survival during EB formation.

Fabrication of Honeycomb Microwells

Our ultra-rapid fabrication method for generating Honeycomb Microwells has been previously published (Chen et al., 2008; Nguyen et al., 2009). Briefly, a laser-jet printer was used to print desired patterns onto pre-stressed polystyrene (PS) sheets. These sheets were then heated to 155°C for approximately 5 m to induce shrinkage, forming closely arrayed Microwells (Honeycomb Microwells) of tunable sizes, and PDMS molded onto the PS master forming Microwells. After removing PDMS from micromolds, the Microwells were bonded to glass slides (to prevent floating) and inserted into standard 24-well culture plates.

Generation of embryoid bodies using Honeycomb Microwell

To load cells, the bottom of the Microwell was bonded to a piece of cover glass (Fisherbrand) using an O_2 plasma machine (SPI Supplies) and placed into each well of a standard 24-well plate containing 500 µL of differentiation medium. The initial 500 µL assisted in preventing air bubbles within the well and enhanced the adherence of the cover glass to the plate. Next an additional 500 µL of differentiation medium was placed into the well and was pipetted gently to remove any remaining air bubbles on the PDMS surface. ESC was added at concentrations of 1.75×10^5 cells/ml concentration to the 200, 300 and 400-µm Honeycomb Microwells for EB approximately 150, 250, and 500 cells each. For larger EB with 1000 and 2000 each, we used 3×10^5 cells per ml and 6×10^5 cells per ml concentration respectively in the 400-µm Honeycomb Microwell. The 1.75×10^5 cells/ml concentration was also used for the 200 and 300 µm Honeycomb Microwells. To achieve uniform EB size, 1 mL of the ESCs were then gently pipetted and dispensed drop-wise into each well of a 24-well plate (Chen et al., 2008; Nguyen et al., 2009). To prevent convective effects within each well of the 24-well plate, which may disrupt the uniform distribution, ESC were allowed to settle into the Honeycomb Microwells at room

temperature for 15–30 m before being transferred into the incubator.

Generation of embryoid bodies using AggreWell™

The manufacturer's instructions from the AggreWell manual version 1.0.0 were initially followed for generating EB. Accordingly, 1 mL of differentiation medium was placed into each well of an AggreWell™400 plate (Stem Cell Technologies) and then centrifuged the AggreWell™400 plate at 3000g for 10 m in a swinging bucket rotor that was fitted with a plate holder to remove any small bubbles from the AggreWell. ESC were then added at concentrations of 3×10^5 cells/ml, 6×10^5 cells/ml, and 1.2×10^6 cells/ml to each well to make EBs with 500, 1000, and 2000 cells respectively. The AggreWell™400 plate was centrifuged at 200 g for 3 m to capture the cells in the wells. According to instructions, aggregates should be harvested 24 h after adding the ESC to the AggreWell™400 plate. Because some aggregates tended to break up if transferred to suspension culture after only 24 h, we increased this time to 48 h in the AggreWell plate before transfer to suspension culture.

Generation of embryoid bodies using hanging drops

For the generation of EB with 500, 1000, and 2000 cells using hanging drops, approximately 100,000, 200,000, and 400,000 ESC were suspended in 3 mL of differentiation medium without LIF respectively. Using a micro-multi-channel pipette, micro-drops were pipetted onto the lid of a culture dish. The lid was inverted and placed on the culture dish. Within 24 h, the ESC in the drops aggregated into the initial stage of EB formation.

Verification of the number of cells per EB

On day 2, 100 EB were collected from each Honeycomb Microwell, AggreWell, and Hanging Drop plates. The 100 EB in each group were pooled together and then dissociated into single cells. The total number of cells from each of the 100 EB-pooled samples was counted using a hemacytometer, and then this number was divided by 100 to calculate the average number of cells per EB.

Characterization of EB size and shape

EB were formed via Microwell, AggreWell, and Hanging Drop methods and imaged at 24 and 48 h after seeding. The EB were imaged in live mode with a digital camera attached to an inverted microscope (Fisher Scientific) and operated by imaging software (Micron, Westover Scientific). From these images, the diameters and perimeters were measured from 100 randomly selected EB formed from each method. From this data, the shape factor, aspect ratio, and circularity of the EB were also calculated.

Cell viability

EB formed by Microwell, AggreWell, and Hanging Drop methods were harvested after 48 h. Cell viability was determined by using a live/dead kit for mammalian cells (Invitrogen). Both whole EB and single cells dissociated from the EB were incubated in 2 M calcein-acetoxymethyl ester (AM) and 4 M ethidium homodimer in phosphate buffer saline (PBS) for 20 min at 37°C. After the incubation period, the stained cells were analyzed by Flow Cytometer LSR II (Becton Dickenson) and whole EBs imaged by laser scanning confocal microscopy. Live and dead cells were indicated by calcein AM (green) and ethidium homodimer (red) respectively.

Confocal microscopy

Laser scanning confocal microscopy was performed on a Nikon digital eclipse C1 confocal microscope equipped with a Nikon eclipse TE2000U inverted microscope using a 20× air objective for imaging. Laser beams with 488 nm excitation wavelengths and 515/30 nm band pass (BP) emission filters were used for Calcein AM and GATA-4 labeled cells. The EBs were also stained with mouse anti-GATA 4-Alexa Fluor® 488 following permeablization with 0.7% Triton X100 for 15 m. Laser beams with 543nm excitation wavelengths and 590/50 nm BP emission filters were used for ethidium homodimer-labeled EBs. Laser beams with 633 nm excitation wavelengths and 650 nm long pass emission filters were used for Draq5 labeled EBs. For EBs approximately 180 µm in diameter, typically 45 images were acquired at 4 µm slice intervals, each slice being the average of three laser scans. Z-stacked images were processed by using the Java-based image analysis program ImageJ (http://rsb.info.nih.gov/ij/). Z-projected images were assembled for each time point to produce a single image based on the sum of pixel brightness values through the image stack (ImageJ: z-project).

Flow cytometry

Individual cells were isolated from EB with cell dissociation buffer and stained for calcein-AM (green) and ethidium homodimer (red) for analyzing live and dead cells within the EB. Cells were also stained with mouse anti-nestin-PE (R&D System), mouse anti-brachyury-PE (R&D System) and mouse anti-GATA 4-Alexa Fluor® 488 (Becton Dickenson) for 60 m after fixation and permeablization with 0.7% Triton X100 for 15 m.

Statistics

For statistical significance, data was acquired and pooled from 100 EB for each treatment group and all treatment groups for generating EB were repeated at least 3 times each. Statistical significance was measure using a Student's T-test individually between all groups.

RESULTS

According to the technical manual version 1.0.0 for the AggreWell plate, EB may be harvested from the AggreWell plate after 24 h. However, after only 24 h, the cells had not yet aggregated sufficiently to be removed as EB (Figures 1A and B) and often broke apart into smaller clusters once transferred to suspension cultures (not shown). Therefore, we also examined the ESC aggregates after 48 h in both AggreWell and Honeycomb Microwell and found that, after 48 h, the cells had aggregated enough to be removed and transferred into suspension cultures without breaking apart (Figures 1C and D). Additionally, we calculated aspect ratio of the EB in the wells using the diameters of the long- and short-axes for both the AggreWell and Honeycomb Microwell (Figure 1E). Both the AggreWell and Honeycomb

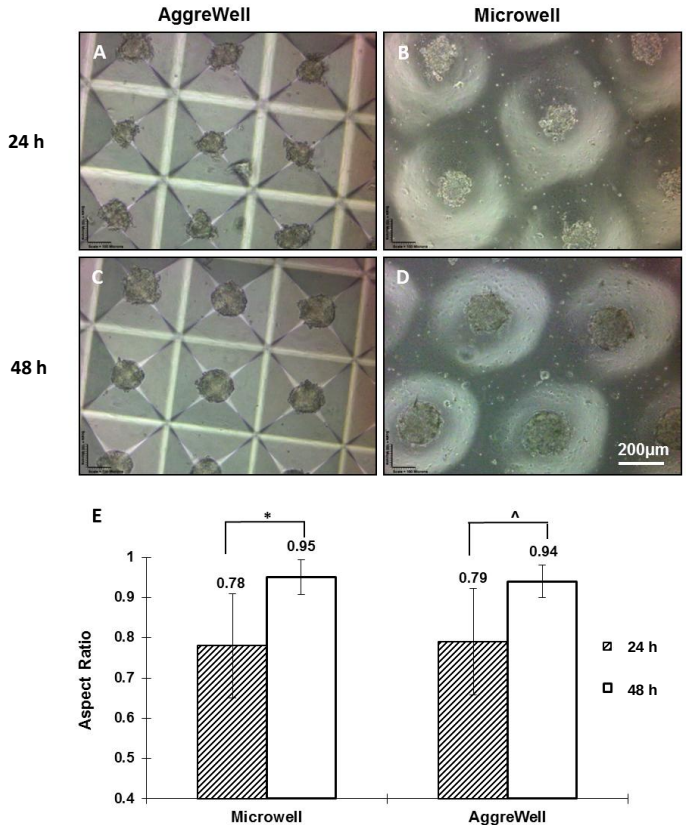


Figure 1. The aspect ratios of EB formed in the Honeycomb Microwell and AggreWell plates increase after 48 h compared with after only 24 h. Images of the EB formed in the AggreWell plates and the 400-µm sized Honeycomb Microwells. (**A)** Cell aggregates in AggreWell 24 h after seeding. (**B)** Cell aggregates in Honeycomb Microwell 24 h after seeding. (**C)** Cell aggregates in AggreWell 48 h after seeding. (**D)** Cell aggregates in Honeycomb Microwell 48 h after seeding. Note that the cells seeded 48 h prior formed larger aggregates that appear to be more circular compared with after only 24 h. (**E)** Aspect ratios of the EBs were also calculated. The long- and short-axis diameters were measured from EBs formed via AggreWell and Honeycomb Microwell. No significant differences were observed between the calculated aspect ratios for AggreWell and Honeycomb Microwell. Note that after 48 h, the aspect ratios of the EBs formed using both Honeycomb Microwell and AggreWell are both approaching 1, indicating a more circular formation compared with after only 24 h (* and ^ indicate comparisons with $P < 0.0001$).

Microwell are able to generate equivalent EB, with the EB generated from both methods exhibiting a more circular shape after 48 h compared with 24 h ($P < 0.0001$).

Circularity of EB

After transferring EB into suspension culture, we noticed morphological differences for larger sized EB ~ 1000 cells per EB (Figure 2). Specifically, the larger EB formed using the AggreWell plate appeared to retain the cone-shape from the bottom of the well plate (Figure 2E). Moreover, the X-large EB in the AggreWell (~2000 cells per EB) were even more densely packed with increased cone-shapes (Figure 2H).

We then calculated the circularity, C, a more sensitive measurement than aspect ratio, for the 2 day old EB following transfer into suspension culture. The cross-sectional area and perimeter of each cell aggregate was acquired using Image J. From each image (Figure 3A), a high contrast black and white image (Figure 3B) was generated. Image J then created a high resolution outline of the cell aggregates (Figure 3C), and running this outline through a mean filter then smoothed the outlines of the cell aggregates (Figure 3 D and E). A radius of 20 pixels was used in order to remove excess surface aberrations in the aggregate outlines. This allows one to measure the general shape of the cell aggregate without incorporating the natural surface topology of the EB. Cross-sectional area, A, and perimeter, P, values were finally measured from these smoothed outlines and then used to calculate the circularity of individual cell aggregates (Figure 3 F and G).

$$C = 4\pi * [A/(P*r^2)] \quad (1)$$

The circularity calculations indicate that the Honeycomb Microwell and AggreWell plates generate more circular-shaped EB compared with the Hanging Drop for the smaller sized-EB, but that the Honeycomb Microwell generated more circular EB of the larger sized EB.

Controlling EB sizes

The diameters of the EB were measured after 2 and 7 days for the three different sized Honeycomb Microwells (Figure 4A), and using the Honeycomb Microwell, AggreWell, and Hanging Drop to generate both the smaller EB (500 cells; Figure 4B) and the larger EB (1000 cells; Figure 4C). All three methods of EB formation generated EB with well-controlled sizes (Figures 4A and B) as evident from the small standard deviations in these EB. The EB were then moved to suspension cultures and allowed to grow and differentiate for another 5 days. The diameters of the EB were measured again after 7 total days in culture. After 5 days with unconstrained growth, the variation in sizes of the EB had increased, but has remained proportional to the original EB sizes.

Cell Viability in EB

Cell viabilities in EB after 2 days of growth in the Honeycomb Microwell, AggreWell, and Hanging Drop were measured using a live/dead (red/green) staining kit. Z-stacked confocal microscopy images revealed a high number of live cells (green) compared with dead (red) cells (Figures 5A, B, and C). The EB were then

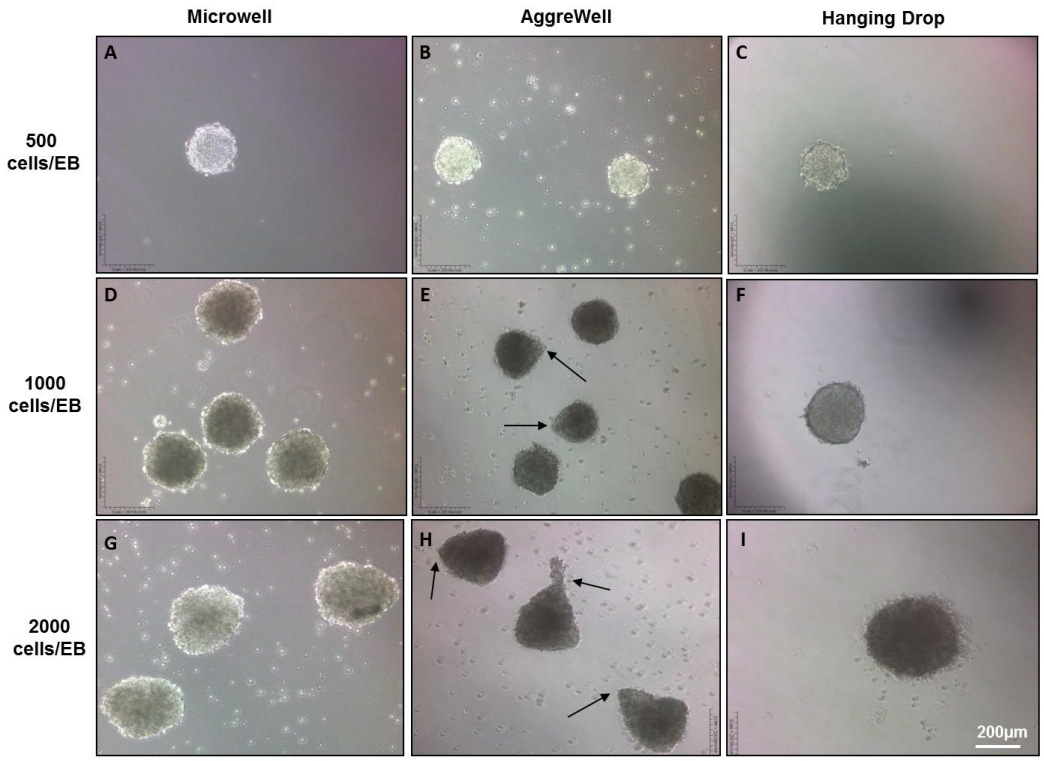


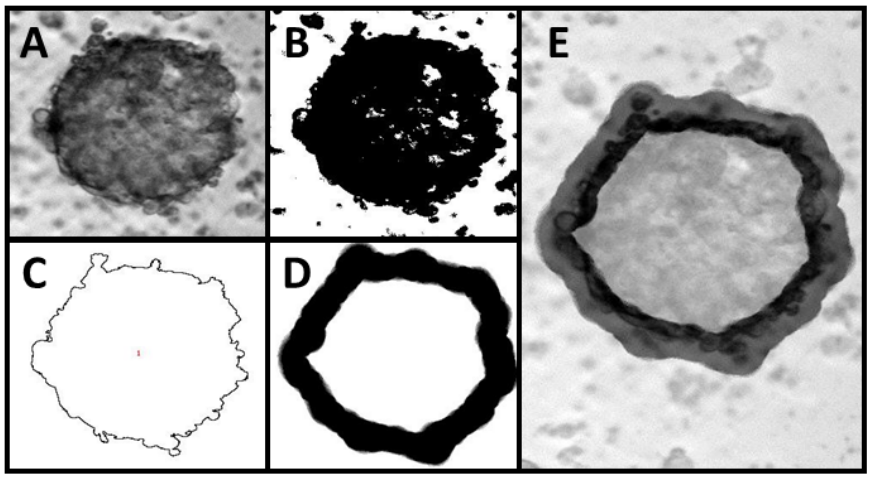
Figure 2. Representative images depicting the morphology of EB flipped into suspension after 48 h. The methods for generating EB with 500- and 1000-cell EB were optimized for the Honeycomb Microwell, AggreWell and Hanging Drop and imaged after transferring cells into suspension culture after 48 h. Images depict EB at 500 cells per EB generated using the A) Honeycomb Microwell, B) AggreWell and C) Hanging Drop, and the larger EB at 1000 cells per EB generated using the D) Honeycomb Microwell, E) AggreWell and F) Hanging Drop. The smaller EB with 500-cell EB appear to be approximately equivalent in size, shape, and cell density for all three generation methods, whereas the EB containing 1000 cells per EB generated using the AggreWell appear to be more densely packed, smaller in size, and exhibit cone-shaped peaks (indicated by arrows) that reflect the cone-shaped morphology of the wells in the AggreWell plate. EB were also generated at 2000 cells per EB in G) Honeycomb Microwells, H) AggreWell plates, and I) Hanging Drops in order to test the limits of the well plates. Note that at 2000 cells per EB, the AggreWell plate generated smaller, denser, and more distinctly cone-shaped (indicated by arrows) EB compared with the other methods.

disaggregated, stained and analyzed by flow cytometry, plotted as histograms expressing the calcein fluorescent dye indicating live cells (not shown). The data indicates that after 48 h, the EB formed in the AggreWell and Honeycomb Microwell still contained greater than 95% live cells (Figure 5D).

Primitive mesoderm, ectoderm, and endoderm in EB

While evaluating our phase contrast images, we also noticed that the large and extra-large EB formed using the cone-shaped bottomed AggreWell plate (Figures 2E and H respectively) did not generate the same distinct primitive endoderm layer on the exterior surface seen in the small EB (Figures 2A to C) or the larger EB formed in the Microwell (Figure 2D) and the Hanging Drop (Figure 2F). Therefore, we stained the large EB for the presence of primitive endoderm marker, GATA-4, on 2 day EB and found that the EB formed by AggreWell (Figure 6B) expressed endoderm marker GATA-4 only at the cone-shaped tips while the EB formed by Microwell and Hanging Drop expressed the primitive endoderm maker evenly throughout the surface of the EB (Figures 6A and C).

We proceeded to quantitatively examine the development all three germ layers at day 2 and 7, using nestin to identify the primitive ectoderm and brachyury for mesoderm differentiation. The percentage of cells expressing the early mesoderm marker, brachyury, ranged from 42-47%, but did not differ significantly between EB formed from Microwell, AggreWell, or Hanging Drop (Figures 6D and E). The percentage of cells expressing, nestin, the marker for primitive ectoderm, was less than 50% in the AggreWell on day 2, compared with the Microwell and Hanging Drop



F	Microwell	AggreWell	Hanging Drop
500 cells per EB	505 ± 16	51 2± 9	502 ± 33
Seeding Concentration (cells/ml)	1.75×10^5	3×10^5	0.33×10^5
Circularity	0.82 ± 0.03	0.80 ± 0.06	0.77 ± 0.011
1000 cells per EB	969 ± 2	1200 ± 28	967 ± 47
Seeding Concentration (Cells/ml)	3×10^5	6×10^5	0.67×10^5
Circularity	0.83 ± 0.04	0.73 ± 0.05	0.76 ± 0.10

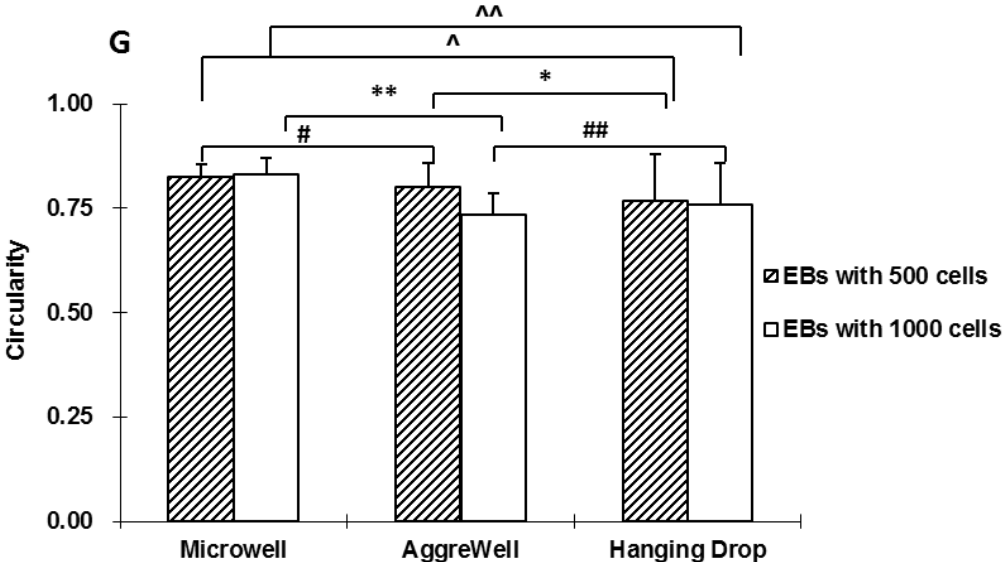


Figure 3. The EB formed in the Honeycomb Microwell are more circular compared with EB formed in AggreWell and Hanging Drop. Images of EB were further processed for determining the circularity, a more sensitive measurement of roundness, using ImageJ software. Images include: A) bright field image, B) high contrast image, C) rendered outline of cell aggregate, D) outline modified with mean filter, and E) composite image of original bright field image with modified outline overlay. Scale bar is 50 μm. F) Tables indicating cell numbers (with standard deviations in cell numbers), seeding concentration and circularity values for the targeted 500 cells/EB and 1000 cells/EB. G) Graph depicting the circularity values calculated from the area and perimeter measurements of the inside of the modified outline. The data indicates that at 500-cell EB, the Microwell (comparison noted by ^, P<0.0001) and AggreWell (*, P< 0.0001) are both superior to the Hanging Drop, and the Microwell is superior to the AggreWell (#, P<0.01) while at 1000 cells per EB, the Microwell forms more circular EB compared with both the AggreWell (**, P<0.0001) and Hanging Drop (^^, P < 0.0001), but the Hanging Drop is superior to the AggreWell (##, P <0.01).

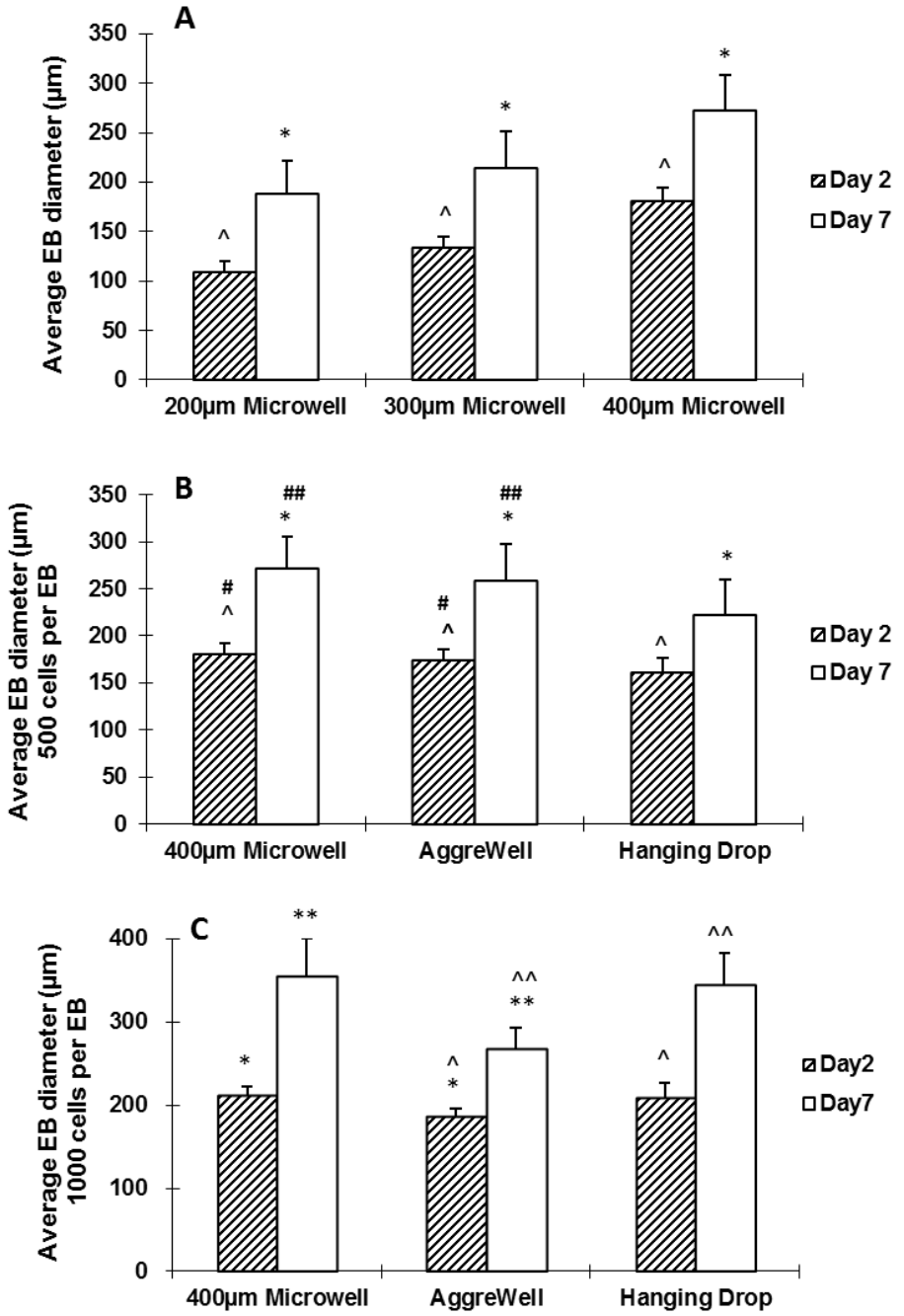


Figure 4. Honeycomb Microwell, AggreWell and Hanging Drop all generate well-controlled sized EB. The diameters of the EB generated in the **A)** 200-, 300- and 400-µm sized Honeycomb Microwells indicate show that the size of the Microwells dictate the size of EBs, **B)** small 500-cell EB and large 1000-cell EB were then measured following generation in the 400 µm sized Honeycomb Microwells, AggreWells, and Hanging Drops. The EB were moved to suspension cultures after 2 days and allowed to grow further. The diameters of the EB were measured again after 7 days. The differences between all diameters were statistically significant (Figure A and B; ^, $P<0.0001$, except differences between the 400µm-sized Microwell and AggreWell (noted by # were significant at $P<0.001$) on day 2. At day 7, all differences were statistic (Figure A and B, *, $P<0.0001$, except differences between the 400µm-sized Microwell and AggreWell were significant at ##, $P<0.1$). More importantly, the small standard deviations in the sizes indicate the ability for all methods to control the EB sizes by adjusting cell numbers. **C)** We also examined the EB sizes for the larger EB using only the largest 400 µm-sized Microwell. We noted that the diameter of the EB generated in the AggreWell plate were smaller than the EB in the Microwell at both day 2 and 7 (* and **, $P<0.0001$) or Hanging Drop (^ and ^^, $P<0.0001$).

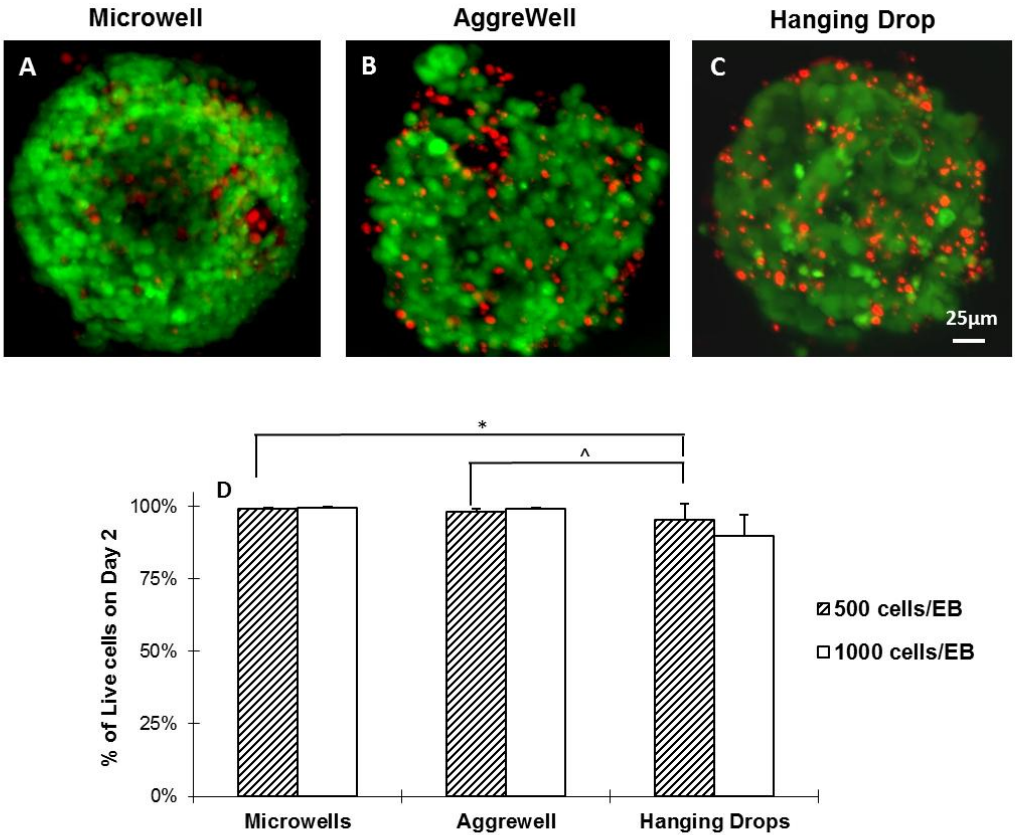


Figure 5. The EB formed in the Honeycomb Microwell and AggreWell, and Hanging Drops contain viable cells. The larger 1000-cell EB generated using A) 400-µm sized Honeycomb Microwells, B) AggreWell plates, and C) Hanging Drops were stained with calcein-acetoxymethyl ester (green) for live cells and ethidium homodimer (red) for dead cells on day 2 and imaged using z-stacked confocal microscopy. In order to quantify the numbers of live and dead cells, the small 500-cell EB and large 1000-cell EB were dissociated into single cells and analyzed using a flow cytometer. D) The viability (% of live cells) in EB formed via AggreWell, Honeycomb Microwell and Hanging Drops was consistently greater than 90% for all methods. The differences in viability of cells in EB between formation methods was not generally significant, but cells in the EB formed in the Microwell (*, P<0.1) and AggreWell (^, P<0.1) both contained more viable cells compared with the cells in the EB formed via Hanging Drop.

expressing over 55% nestin positive cells (Figure 6F). However, the nestin expression by day 7 was comparable in the EB from Microwells and AggreWells at 50%, and slightly lower in the Hanging Drop (Figure 6G). Conversely, the expression of primitive endoderm marker, GATA-4, was significantly lower in the EB formed in AggreWell plates on day 2 (Figure 6G). Although the cone-shaped morphology of the EB in the AggreWell do recover after transfer to suspension cultures (not shown), the GATA-4 development of the primitive endoderm in these EB does not recover and remains below 1% compared with 6-8% expression in EB from Microwell and Hanging Drop (Figure 6I).

EB from human ESC

Perhaps most importantly, the Microwell and AggreWell plates are able to generate EB from single human ESC with greater efficiency than conventional Hanging Drop Methods (Figure 7). This is a significant advancement in the field, as the generation of human EB from single cells is notoriously challenging. We expect the success of these well plates in generating EB from single cells is due to the physical aggregation of individual cells that bring the cells into closer contact with one another compared with a Hanging Drop.

DISCUSSION AND CONCLUSIONS

The formation of the EB is a principal step in the differentiation of ESC. When factors that maintain the undifferentiated state of an ESC are removed from the culture medium and placed in suspension culture, the ESC will spontaneously self-assemble into an EB

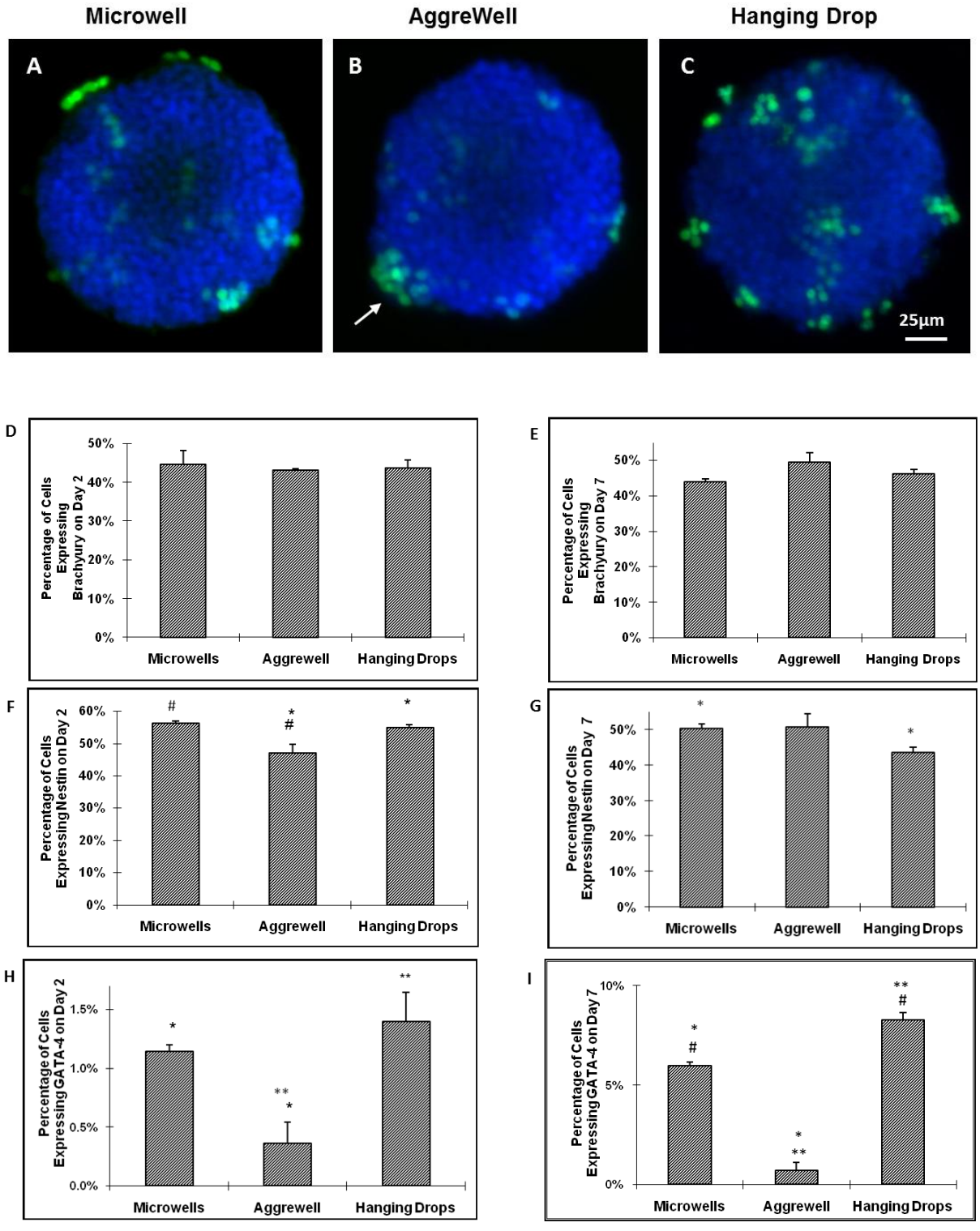


Figure 6. Primitive Endoderm, Ectoderm and Mesoderm in EB. The larger EB generated using A) 400-µm sized Honeycomb Microwells, B) AggreWell plates, and C) Hanging Drops were stained on day 2 with primitive endoderm marker, GATA-4 (green), and cell nucleus, Draq5 (blue) and imaged using z-stacked confocal microscopy. Note that the GATA-4 expression in the EB formed using the AggreWell plate is only present on the cone-shaped tip (indicated by arrow) rather than throughout the surface layer of the EB. These were then quantitatively analyzed by staining germ layers for FACS analysis on both days 2 and 7 with D and E) mesoderm marker, brachyury, (F and G) primitive ectoderm marker, nestin, and (H and I) primitive endoderm marker, GATA-4 . Note that the GATA-4 expression in the EB formed using the AggreWell plate is most significantly reduced compared with the GATA-4 expression in EB formed using the Microwells and Hanging Drops (* and **, $P<0.01$) and (#, $P<0.05$).

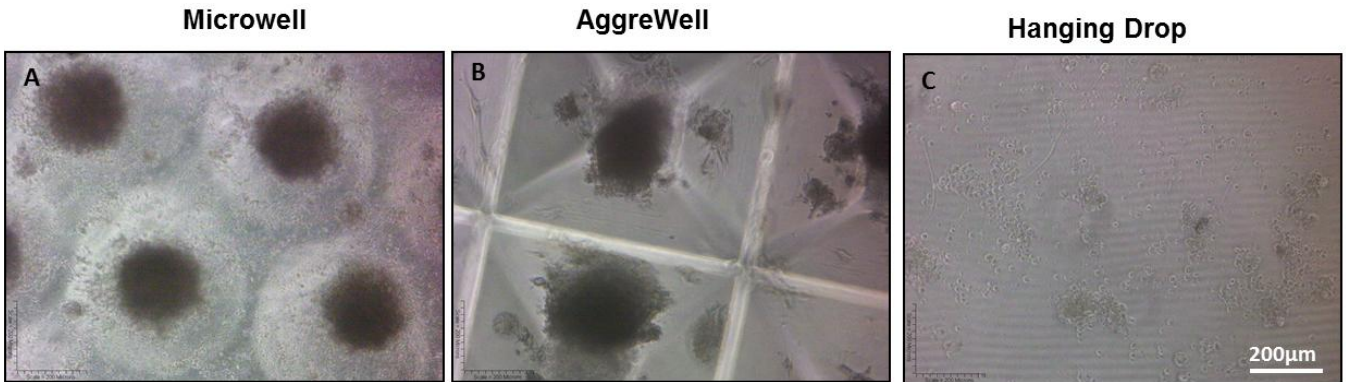


Figure 7. EB generation from human ESC. We were also able to successfully generate EB from single human ESC using both the (A) Microwell and (B) AggreWell plates more readily compared with conventional the (C) Hanging Drop method.

(Kurosawa, 2007). The initial size of the EB is dependent on the number of cells which initially self-assemble via cell–to-cell adhesion receptors (Bratt-Leal et al., 2009). Thereafter, the differentiation of the cells within the EB proceeds. After 2–4 days of suspension culture, the primitive endoderm forms on the surface of the EB, giving rise to a structure called the “simple EB”. After 4 days, a “cystic” EB develops, characterized by the formation of a central cavity and columnar epithelium with a basal lamina (Khoo et al., 2005). Upon continued culture, the EB will give rise to all three germ layers and differentiate into a large variety of cell types.

Because the differentiation of stem cells within an EB is thought to recapitulate, at least in part, the developing embryo, the non-uniform shape of EB in the larger EB from the AggreWell plate is assumed to be an undesirable factor. This manuscript has shown that the endoderm marker, GATA-4, does not develop normally with a cone-shaped EB. On day 2 of development, the GATA-4 is concentrated at the cone-shaped tips of the AggreWell EB and is reduced throughout the remaining surface of the EB. Although the cone-shape of these EB do recover into a spherical shape after transfer from the AggreWell plates into suspension culture, the GATA-4 expression remains significantly reduced in these EB compared with EB formed using the Microwell and Hanging Drop. Most importantly, use of the AggreWell plate for initial EB formation would most likely limit the derivation of endodermal tissue products, like cells in the gastrointestinal and respiratory tracts, and should be avoided in these protocols.

This study is the first indicating that the shape of an EB can influence the fate of the differentiating ESC, however; the initial size of an EB is already known to be an important physical parameter influencing the proportion of cells differentiating towards some specific lineages (Ng et al., 2005; Park et al., 2007). In addition, the shape of individual human mesenchymal stem cells affects their differentiation efficiency towards adipocyte or osteoblast lineages (McBeath et al., 2004). Therefore, it is not surprising that the shape of an EB might play a role in the fate of the differentiating cells within the EB.

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