

Co-culture of human embryonic stem cells with murine embryonic fibroblasts on microwell-patterned substrates

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Abstract

Human embryonic stem (hES) cells are generally cultured as cell clusters on top of a feeder layer formed by mitotically inactivated murine embryonic fibroblasts (MEFs) to maintain their undifferentiated state. This co-culture system, which is typically used to expand the population of undifferentiated hES cells, presents several challenges since it is difficult to control cell cluster size. Large cell clusters tend to differentiate at the borders, and clusters with different sizes may lead to heterogeneous differentiation patterns within embryoid bodies. In this work, we develop a new approach to culture hES cells with controlled cluster size and number through merging microfabrication, and biomaterials technologies. Polymeric microwells were fabricated and used to control the size and uniformity of hES cell clusters in co-culture with MEFs. The results show that it is possible to culture hES cells homogeneously while keeping their undifferentiated state as confirmed by the expression of stem cell markers octamer binding protein 4 (Oct-4) and alkaline phosphatase (ALP). In addition, these clusters can be recovered from the microwells to generate nearly homogeneous cell aggregates for differentiation experiments.

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1. Introduction

Human embryonic stem (hES) cells are a potentially valuable source of cells for transplantation and tissue engineering since they can be expanded in vitro without an apparent limit and differentiated into derivatives of all three germ layers (ectoderm, endoderm and mesoderm) [1–7]. Currently there are two main methods to culture hES cells. The first approach involves the co-culture of hES cells on top of a feeder layer comprising mitotically inactivated

murine embryonic fibroblasts (MEFs) [8,9]. In this approach, MEFs provide a microenvironment for maintenance and growth of undifferentiated hES cells. To eliminate the possibility of pathogen transmission from the mouse feeders, recent studies have reported the use of human feeders including human foreskin fibroblasts [10,11], or human adult marrow cells [12]; however, MEFs are still the most common approach to culture hES cells. The second approach involves the use of feeder free conditions. For example, extracellular matrix substrates including matrigel (soluble basement membrane extract of the Engelbreth–Holm–Swarm mouse tumor), laminin, and fibronectin together with mouse embryonic fibroblast-conditioned medium (MF-CM) containing bFGF or other replacements [13–15]. More recently, improved feeder free conditions have been derived [16,17]; however, significant

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variations in the production of MEFs and harvesting of MF-CM, and lack of long-term genetic stability of hES cells in these cultures have hampered the reproducibility of these conditions to culture hES cells stably [18].

The adherence of hES cells to each other, although critical during embryonic development [19], has presented several challenges in the attempt to passage the cells in a consistent manner and to standardize culture conditions [20]. There are two major procedures to passage the cells including mechanical and enzymatic processes. Unfortunately both methods generate variable size clusters of cells. Large cell clusters tend to differentiate at the borders while very small cell clusters tend to hinder the proliferation and recovery of hES cells in culture. In addition, variable cell cluster size may have a significant effect on the differentiation pattern of these cells. Differentiation of hES cells can be induced by removing the cells from the feeder layer and growing them in suspension to form embryoid bodies (EBs). Therefore, an approach that allows control over the size of hES cell clusters in co-culture with MEF feeder cells may be beneficial for controlling the homogeneity of the cultures.

Microscale engineering approaches may be a potentially powerful tool for controlling the cellular microenvironment [21]. For example, through immobilizing cells on micropatterned surfaces [22,23], cell shape [24] and differentiation [25] can be controlled. In addition, microscale technologies can be used to perform high-throughput experiments to analyze cell–biomaterials as well as combinatorial experiments [26,27]. Microscale technologies have also been used to control cell–cell interactions. Patterned co-cultures have been used to control the degree of homotypic and heterotypic cell–cell interactions on two-dimensional surfaces [28–31]. Despite the potential of this technology, its inability to control the three-dimensional structure of the resulting cell–cell interactions has limited this technique to monolayers of cells. In this work, we present a method to culture hES cells with controlled cluster sizes for maintenance and subsequent differentiation. Specifically, co-cultures of MEFs and hES cells were formed on microwell-patterned poly(dimethylsiloxane) (PDMS) surfaces. The results demonstrate that it is possible to culture these cells homogeneously while maintaining their undifferentiated state as confirmed by the expression of stem cell markers Oct-4 and alkaline phosphatase (ALP). In addition, the cell clusters can be retrieved to generate nearly homogenous cell aggregates for differentiation studies.

2. Materials and methods

2.1. hES and MEF cell culture

Cells were manipulated under sterile tissue culture hoods and maintained in a 5% CO₂ humidified incubator at 37 °C. MEFs (Cell Essential, Boston, MA) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone). Once the cells were confluent, they were trypsinized

(0.25% in EDTA, Sigma) and passaged at a 1:4 subculture ratio. Undifferentiating hES cells (H9, passages 25–50; WiCell, Wisconsin) were grown on an inactivated MEF feeder layer, as previously described [32], and maintained on hES cell medium consisting of 80% knockout-DMEM supplemented with 20% knockout-serum, 0.5% L-glutamine (200 mM in 0.85% NaCl), 1% non-essential amino acids, 0.2% mercaptoethanol (55 mM in PBS) and 5 ng/mL of basic fibroblast growth factor (bFGF) (all from Invitrogen). hES cells were fed daily and passaged every 4 days using collagenase type IV (2 mg/mL, Invitrogen) for 30–40 min and then scrapping the petri dish containing the cells. Recovered cell aggregates were centrifuged to remove the collagenase solution, resuspended in fresh medium, and pipetted onto new MEF-seeded dishes at a subculture ratio of 1:3. hES cells grown on flat PDMS surfaces were passaged in the same way.

2.2. PDMS fabrication

PDMS molds were fabricated by pouring a silicone elastomer (Sylgard 184, Essex Chemical) solution containing 10% (w/w) curing agent onto SU-8-coated silicon masters and cured at 60 °C for 4 h. The patterns on the silicon masters had protruding cylinders 200 μm in diameter and 120 μm in height, resulting in PDMS replicas with holes 200 μm in diameter and 120 μm in depth. The PDMS molds were then peeled from the silicon surfaces and cut prior to use (2.5 × 2.5 cm = 6.25 cm²; each mold containing 2400 wells). Before use, these micropatterned substrates were sterilized in 70% (v/v) ethanol for 10 min and then washed in PBS overnight.

2.3. Seeding MEFs on microwell-patterned substrates

To generate a monolayer of MEF feeder cells on PDMS microstructures, MEF cells were trypsinized and resuspended in medium at a concentration of $\sim 2.5 \times 10^5$ cells/mL (4 mL of this cell suspension were used per each PDMS mold). Just prior to seeding, the PDMS molds were coated with fibronectin (Sigma, 50 μg/mL in PBS) over the entire surface for 5 min at room temperature and then rinsed twice with PBS. The cells were then plated and allowed to settle overnight. After 3 days, the MEFs monolayers were inactivated by mitomycin C (8 ng/mL, in DMEM) for 2 h. After 1 day, these inactivated MEF layers were used for hES cell seeding.

2.4. Seeding hES cells on MEF-coated surfaces

hES cell aggregates removed from MEF feeder layer after 2 h incubation with collagenase type IV (2 mg/mL) were dissociated by a non-enzymatic cell dissociation solution (Sigma) into single cells and resuspended in hES cell media ($\sim 2 \times 10^6$ cells/mL; 1 mL of this cell suspension was used per PDMS substrate). This cell suspension was seeded into the microwells of the MEF-layered PDMS. To minimize surface adhesion outside of microwells, the cells were pipetted to create a flow that would carry them off the surface, if they did not fall within wells. This process was repeated ~ 5 times to obtain a reasonable number of cells in the wells. To determine the percentage of hES cells retained in the microwells, hES cells removed from each PDMS substrate were collected and counted using a hemacytometer, and this number was subtracted from the number of hES cells seeded initially. To determine the number of cells per well, hES cells were pre-stained (see “cell labelling and immunostaining” below). At specific culture times, the cells were rinsed with PBS, fixed with 4% (w/v) paraformaldehyde, and the nucleus of cells stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma). hES cells were differentiated from MEFs by the cytoplasmatic staining and individually identified by the nuclear staining. To determine the percentage of cell aggregates recovered from the microwells, the hES–MEF co-cultures were treated with type IV collagenase at 2 mg/mL for 2 h, the cell aggregates counted and the number divided by the number of cell aggregates initially present.

2.5. Quantitative analysis of hES aggregate size and number

Percentage of microwell occupancy by hES cells was determined by imaging the individual microwells, sizing the surface area occupied by hES aggregates in each microwell, and dividing it by the surface area of the microwell (200 μm in diameter, or 31,416 μm^2 in area). The area of hES aggregates was assessed in more than 20 microwells in the same PDMS sample on day 1 and day 6 and in approximately 80 microwells from 3 different samples on day 8. The number of hES aggregates per mm^2 unit area on the PDMS mold (either flat or microwell-patterned) on day 8 was counted for 40 random fields at $\times 5$ magnifications (corresponding to an area of $\sim 2.3 \times 10^7 \mu\text{m}^2$) per sample (3 samples per condition).

2.6. Cell viability analysis

Cell viability of hES–MEF co-cultures was determined using a LIVE/DEAD kit (Molecular Probes) containing calcein AM (2 $\mu\text{g}/\text{mL}$ in PBS) and ethidium homodimer (4 $\mu\text{g}/\text{mL}$ in PBS). Samples were treated with the kit solution for 20 min and visualized under a fluorescent microscope (Axiovert 200, Zeiss). This kit measures the membrane integrity of cells—viable cells fluoresce green through the reaction of calcein AM with intracellular esterase, whereas non-viable cells fluoresce red due to the diffusion of ethidium homodimer across damaged cell membranes and binding with nucleic acids. Percent viability values were estimated by counting the number of live (green) cells and the number of dead (red) cells within microwells in a number of random fields at $\times 50$ magnifications and dividing the number of live cells by the total number of cells (live plus dead).

2.7. Scanning electron microscopy (SEM) analysis

Micropatterns containing hES cells were washed with PBS, fixed with 2% (w/v) paraformaldehyde, and rinsed with distilled water before being freeze-dried for 24 h. The samples were subsequently mounted onto aluminum stages and sputter coated with gold to a thickness of 200 \AA . SEM images were recorded by a field emission SEM (JEOL 6320FV) at 5 kV.

2.8. Cell labeling and immunostaining

To distinguish between MEF cells and hES cells in co-culture experiments, MEF cells were stained with the green membrane dye carboxyfluorescein diacetate succinimidyl ester (CFSE, Sigma) before being seeded onto the PDMS mold, and hES cells were stained with the red Vybrant[®] DiD (Molecular Probes) cell-labeling solution before being seeded onto the now MEF-seeded PDMS mold. The staining procedure involves harvesting cells from culture flasks or petri dishes, centrifuging them to remove culture media, rinsing them in PBS, centrifuging them again to remove PBS, resuspending them to a concentration of 1×10^6 cells/mL within the staining solution (10 $\mu\text{g}/\text{mL}$ CFSE in PBS or 20 $\mu\text{g}/\text{mL}$ Vybrant[®] DiD in PBS), and incubating them for 10 min at room temperature. Stained cells were then rinsed in PBS twice before being seeded onto the PDMS mold for experiments. For confocal microscopy, co-cultures of CFSE-stained MEF cells and Vybrant[®] DiD-stained hES cells were mounted in Fluoromount-G, covered with a No. 1 thickness coverslip, and visualized at $40\times$ magnifications through a FITC and Rhodamine filter with a maximum focal depth of 248 μm . The number of hES cells in microwells was estimated by imaging the microwells at different depths under confocal microscopy and counting the number of Vybrant[®] DiD-stained cells visualized.

For Oct-4 and ALP staining, samples of hES–MEF co-cultures were fixed with 4% (w/v) paraformaldehyde solution for 30 min at room temperature. After blocking with 3% (w/v) bovine serum albumin (BSA) solution, samples were stained for 1 h with rabbit anti-Oct-4 polyclonal antibody (10 $\mu\text{g}/\text{mL}$ in PBS, BioVision Inc.) or monoclonal mouse anti-human ALP (supernatant diluted 1:10 in PBS, Developmental Studies

Hybridoma Bank, B4-78). In each immunofluorescence experiment, a parallel set of cells was stained with the corresponding isotype-matched IgG as negative control. After the 1-h incubation with the primary antibody solutions, samples were rinsed with PBS and stained with the appropriate secondary antibody solutions—FITC-conjugated goat anti-rabbit IgG (diluted 1:20 in PBS, Sigma) for detection of Oct-4 expression or PE-conjugated goat anti-mouse IgG1 (1 $\mu\text{g}/\text{mL}$ in PBS, Molecular Probes) for detection of ALP expression. After 30 min of incubation in secondary antibody solutions, samples were rinsed with PBS and examined under a fluorescence microscope.

2.9. Flow cytometry analysis

Samples of hES–MEF co-cultures were incubated with type IV collagenase at 2 mg/mL for 2 h to remove hES cell aggregates from MEF monolayers. hES aggregates were then dissociated with non-enzymatic cell dissociation solution for 10–15 min. Single cell suspensions were rinsed in PBS containing 5% (v/v) FBS, filtered through a 85 μm mesh strainer to remove any remaining clumps, collected into a 15 mL centrifuge tube, centrifuged to remove the PBS, and resuspended to a concentration of 5×10^5 cells/mL in a solution of monoclonal mouse anti-human ALP (supernatant diluted 1:10 in PBS, Developmental Studies

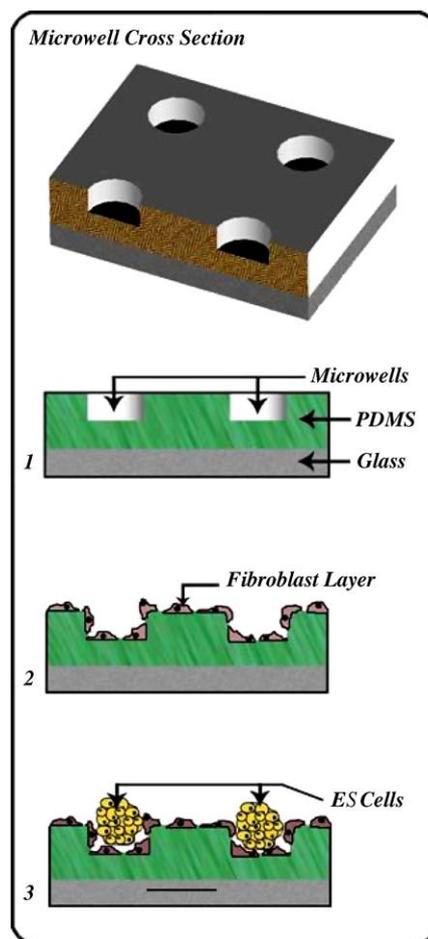


Fig. 1. Schematic representation of the co-culture system formed by hES and MEF cells. PDMS was cured on a silicon master to produce microwell-patterned surfaces. Surfaces were treated with fibronectin and seeded with MEF cells, which grew into a monolayer. The confluent monolayer was inactivated with mitomycin C, then hES cells were seeded inside the microwells, where they formed aggregates.

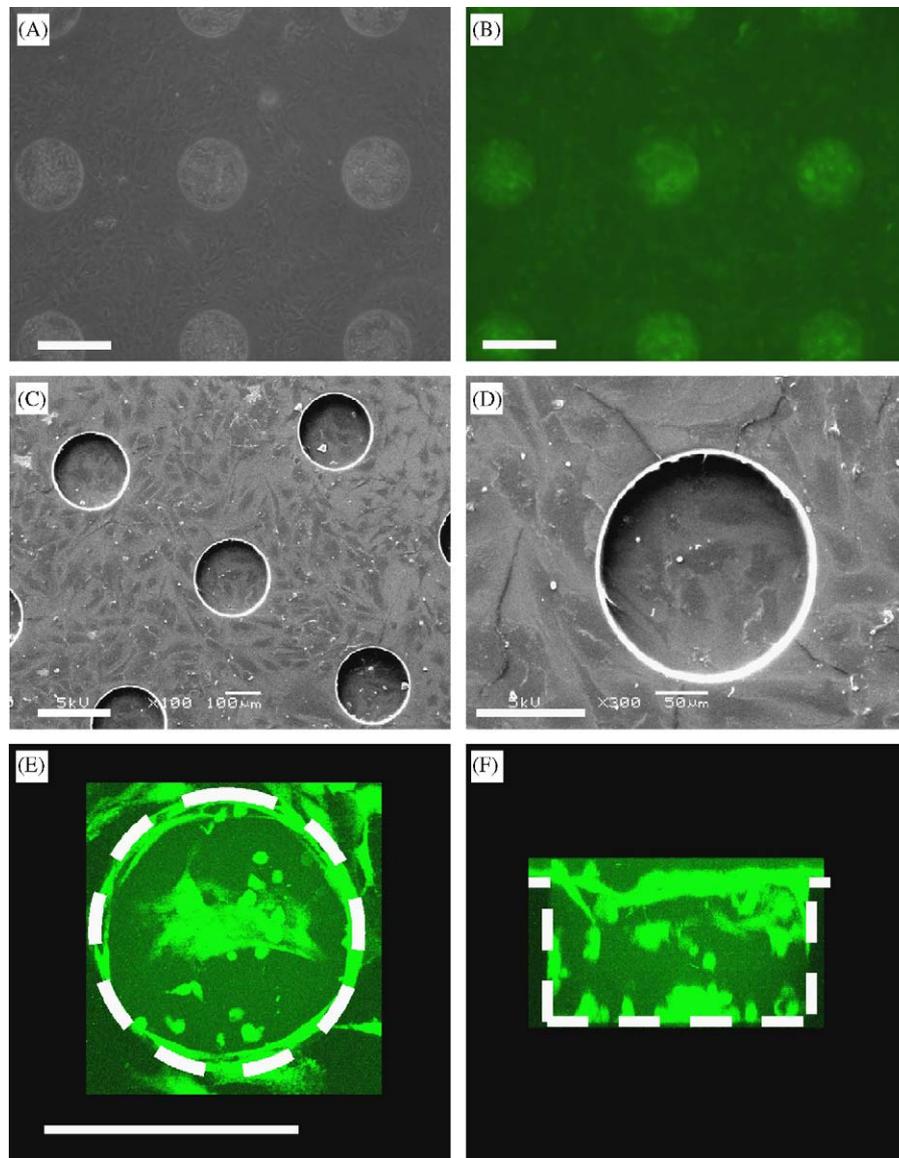


Fig. 2. Formation of MEF monolayers. (A, B) Transmission (A) and fluorescent (B) images of inactivated MEF monolayers with mitomycin C. MEFs were labeled with CFSE. (C, D) SEM micrographs showing the spreading of MEF cells within the wells and along the surface. (E, F) Confocal microscopy images showing the three-dimensional contour of the monolayer. In all figures, scale bars correspond to 200 μm .

Hybridoma Bank, B4-78). A parallel set of cells was stained with the corresponding isotype-matched IgG as negative control. After 1 h of incubation on ice, cells were centrifuged to remove the primary antibody staining solution, rinsed in PBS, centrifuged again to remove the PBS, and resuspended in a solution of PE-conjugated goat anti-mouse IgG1 (1 $\mu\text{g}/\text{mL}$ in PBS, Molecular Probes). After 30 min of incubation on ice, cells were centrifuged to remove the secondary antibody staining solution, resuspended in PBS, and analyzed for ALP expression based on PE fluorescence on a FACScan (Becton Dickinson) instrument. Data analysis was carried out using CellQuest software.

2.10. Statistical analysis

Unless stated, the data described in this work is representative of 3 independent experiments. Statistical significance was determined using an unpaired Student *t*-test. Results were considered significant when $P \leq 0.05$.

3. Results and discussion

3.1. MEF cell seeding onto micropatterns

Long-term proliferation of hES cells is currently achieved by co-culture with mitotically inactivated MEFs. It is generally thought that MEFs secrete factors that enrich the medium, adhere to the extracellular matrix or interact with membrane-bound proteins, enabling the hES cells to remain undifferentiated. In this work we aim to develop a new method of culturing hES cells that may provide specific advantages in comparison with the standard co-culture approach. This approach consisted of seeding cells on microwell-patterned elastomeric, biocompatible polymeric surfaces. These surfaces were coated with

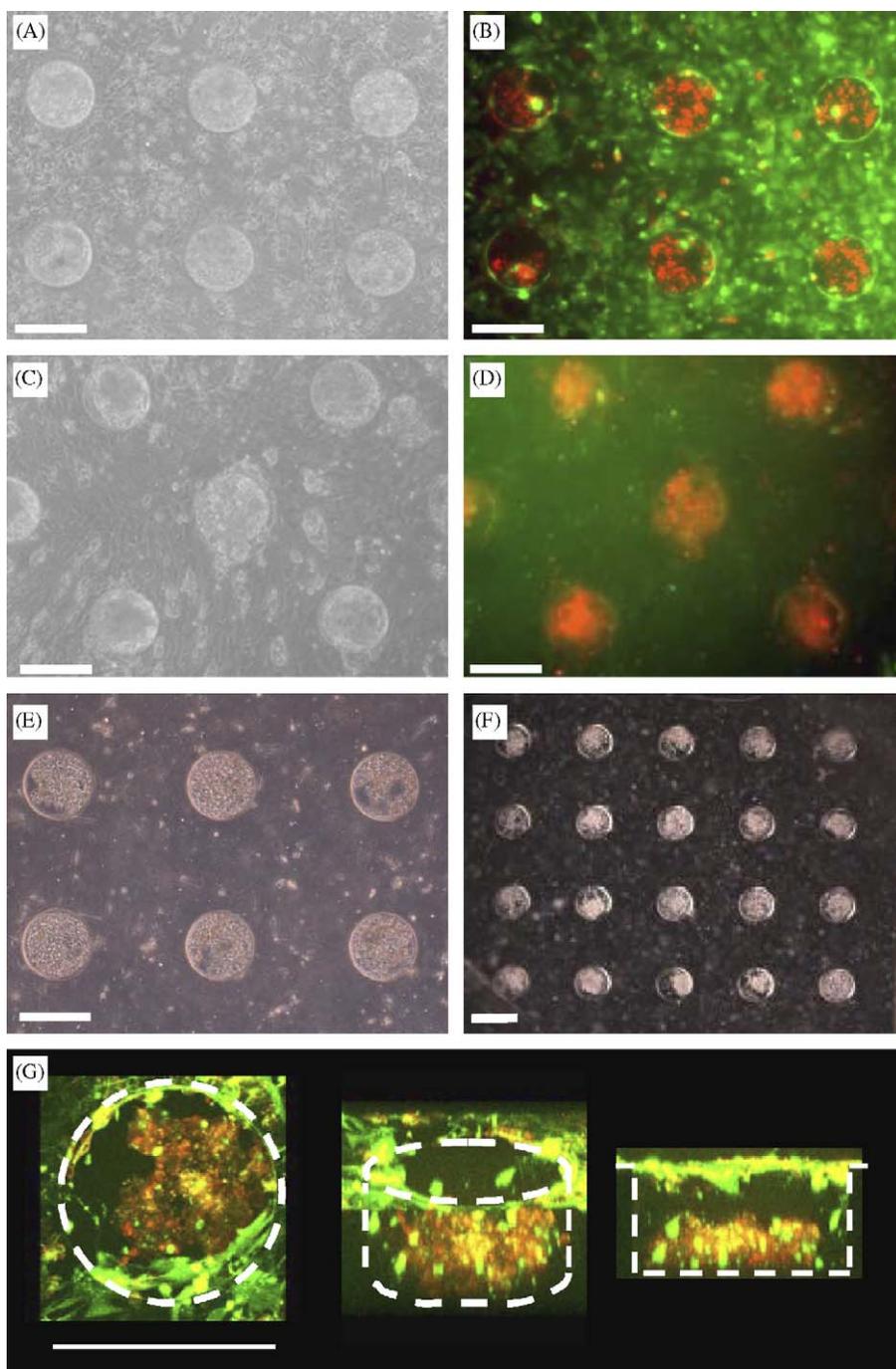


Fig. 3. hES-MEF co-cultures at various time intervals. MEFs were stained with CFSE (green) and hES cells with Vybrant[®] DiD (red). Light (A, C, E, F) and fluorescent (B, D) images of the hES-MEF co-culture after 1 (A, B), 2 (C, D), or 6 (E, F) days after hES cell seeding. (G) Confocal images of hES-MEF co-cultures within a microwell on day 1 as rotated in 45° intervals. In all figures, scale bars correspond to 200 μm.

fibronectin and seeded with MEFs to form a monolayer and subsequently seeded with hES cells (Fig. 1).

MEFs seeded on fibronectin-treated micropatterned PDMS formed monolayers as indicated in Fig. 2. PDMS without fibronectin is a poor substrate for MEF attachment, thus the deposition of an adhesive protein is required to ensure adhesion of cells. Gelatin, which is typically used to promote MEF attachment to polystyrene [32] was not

used in this study since it occluded the microwells due to its inherent viscosity. MEFs were seeded at $\sim 5 \times 10^4$ cells/cm², and formed a confluent monolayer in 3 days. Higher cell seeding densities occluded the microwells and thus prevented their subsequent use.

To determine potential microwell sizes that could be used to generate the patterned co-cultures, various sizes of microwell-patterned surfaces were analyzed (data not

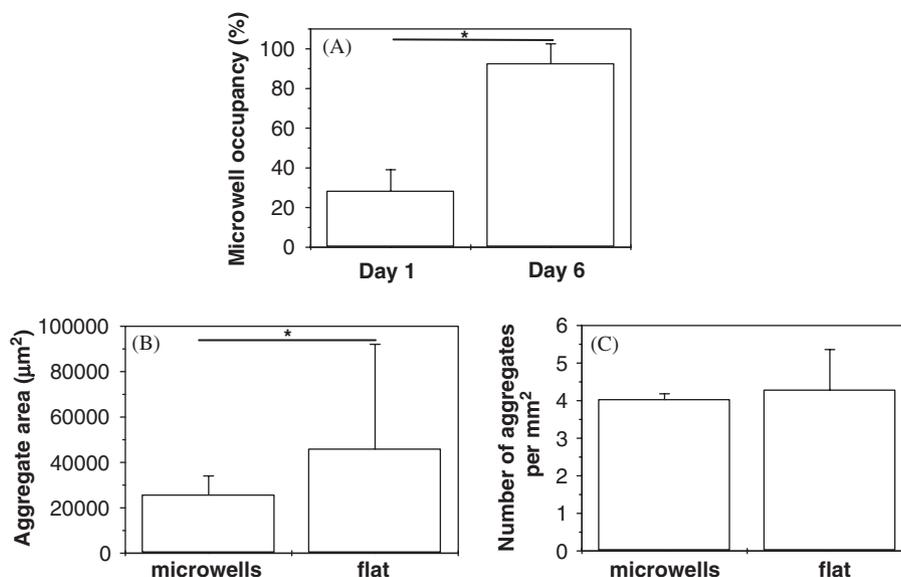


Fig. 4. Quantitative analysis of hES aggregate size and number. (A) Percentage of microwell occupancy by hES cells over time, as determined by imaging the individual microwells, sizing the surface area occupied by hES aggregates in each microwell, and dividing it by the surface area of the microwell. The area of hES aggregates at day 1 and day 6 was assessed in more than 20 microwells in the same PDMS sample. (B) Surface area occupied by hES cell aggregates at day 8, on flat or microwell-patterned substrates. Approximately 80 microwells from 3 different samples were used to calculate the averaged area of hES cell aggregates. (C) Number of hES aggregates (at day 8) per mm^2 unit area on the PDMS mold (either flat or microwell-patterned). The number of aggregates was calculated on 40 random fields at $\times 5$ magnifications (corresponding to an area of $\sim 2.3 \times 10^7 \mu\text{m}^2$) per sample (3 samples per condition). In all graphs, values indicate average \pm S.D., from 3 independent experiments. *Denote statistical significance ($P < 0.001$).

shown). In general, it was found that microwell diameters of less than $100 \mu\text{m}$ permitted individual MEFs to form bridges across the edges of the wells and thus occlude the microwells. Therefore to overcome these difficulties and to create microwells with enough depth for hES cell seeding, microwells with a diameter of $200 \mu\text{m}$ and a depth of $\sim 120 \mu\text{m}$ were selected. Using this geometry, few MEF cells adhered to the vertical surfaces (Figs. 2E and F). This likely resulted from a limited exposure of the cells to microwell walls during cell settling and an inability of mitotically inactivated MEFs (as result of mitomycin treatment) to navigate steep (90°) substrate topography.

3.2. hES cell seeding onto micropatterns

To assess the capability of the approach in stem cell culture, hES cells were seeded onto the microwell containing surfaces that contained a monolayer of inactivated MEFs. To promote settling within microwells, the hES cell suspension was pipetted slowly onto the MEF surface. The cells were allowed to settle within the wells and after a few minutes the cells outside the microwells were removed by gentle washing whereas cells within the shear-protected microwells remained. It is of concern that this washing step may not induce flow evenly across all wells, and we are working to develop a more controlled removal technique. With the current procedure we were able to retain approximately 5% of the hES cells within the microwells using the specified geometries. However, since the cells that

were not seeded within the microwells are recovered in the washing step, we anticipate that the repeated use of this process can be used to achieve much higher overall capturing efficiencies. The results of this process and the subsequent development of hES cell aggregates are presented in Fig. 3. Fluorescent images during the first 2 days (Figs. 3B and D) indicate hES cells (red) were localized in the wells and MEFs (green) on the surrounding surface. At day 1, there were approximately 40 cells per microwell. According to confocal microscopy analyses, hES cells attached to the bottom of the microwell and formed colonies with intimate cell–cell interactions (Fig. 3G). By day 6, defined aggregates had formed consistently over a large surface area (Figs. 3E and F).

3.3. Microwells versus flat surfaces

To assess the potential advantages of this approach, the microwell method of hES–MEF co-culture was compared with the traditional flat co-culture system. HES–MEF co-cultures were monitored in terms of microwell occupancy over time, and it was observed that the % surface area occupied by the hES cells in the microwells increased over time (Fig. 4A, >20 microwells assessed). To determine whether this increase in occupied area correlates with cell proliferation, 3D views of the microwells at different depths under confocal microscopy were used to estimate the number of hES cells present in each microwell, and results suggest an increase in the average number of hES

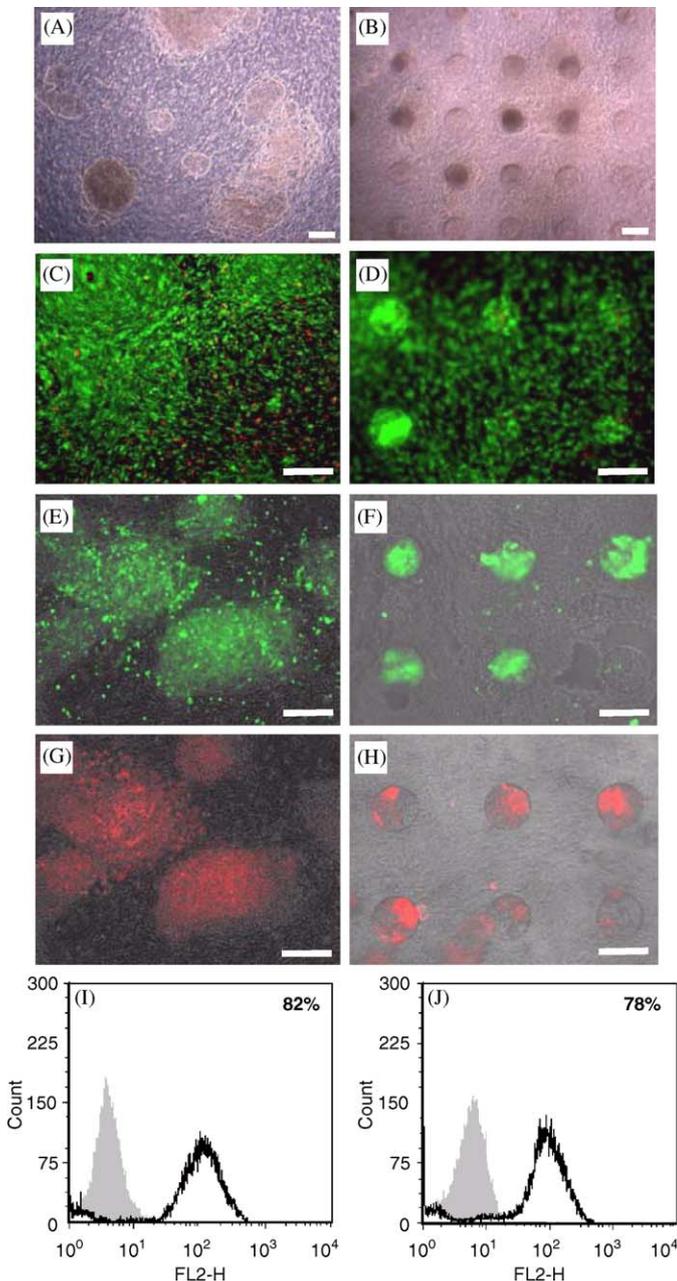


Fig. 5. Cell viability and expression of undifferentiating markers in hES cells cultured either on a flat or microwell-patterned surface. Co-culture of hES cells with mitotically inactivated MEFs on a microwell-patterned surface (B, D, F, H, J) or on a flat surface (A, C, E, G, I) after 8 days of culture. In both systems, the hES cell-colonies were characterized for their aggregate size (A, B; transmission $\times 5$), viability (C, D; green: live; red: dead; $\times 10$) based on calcein AM and ethidium homodimer staining, and the expression of hES cell markers including Oct-4 (E, F; $\times 10$) and alkaline phosphatase (G, H; $\times 10$). (I, J) Indicates the expression of alkaline phosphatase as measured by FACS. Percent of positive cells were calculated based on the isotype controls (gray plots) and are shown in each histogram plot. In all figures, scale bars correspond to 200 μm .

cells per microwell from day 1 to day 6 (data not shown). On day 8, a typical time frame for 2 passages of hES, it was observed that the average area of aggregates was statistically smaller ($P < 0.001$) in microwells than on flat surfaces

(Fig. 4B, ~ 80 microwells assessed). At this time, the averaged area of the hES aggregates ($25,690 \mu\text{m}^2$) in the microwells is close to the surface area of these microstructures ($31,416 \mu\text{m}^2$), and thus showing that the hES cells were nearly confluent within the microwells. Furthermore, the homogeneity of aggregate size of hES cells grown in microwells was superior to that of hES cells grown on flat surfaces. The standard deviation of areas within patterned co-culture was determined to be statistically smaller than that of flat co-culture ($8300 \mu\text{m}^2$ versus $46,000 \mu\text{m}^2$; $P < 0.0001$), indicating a greater level of control over aggregate size.

The higher averaged area and heterogeneity of the hES aggregates in the flat area as compared to the ones formed in the microwells is a consequence of the methodology normally used to passage hES cells [32]. hES cells are passaged after collagenase treatment of hES cell aggregates seeded on top of the MEF feed layer. The collected aggregates are further disrupted into smaller aggregates before seeding them again on top of MEF feeder layers. Therefore, the heterogeneity in size of these initial aggregates is unavoidable. Furthermore, during the culture of these aggregates on flat surfaces, some of them agglomerate over time, increasing their size and heterogeneity even more.

With regard to number of hES aggregates per unit area (Fig. 4C), the two methodologies produced relatively similar results (~ 4 aggregates/ mm^2 , averaged from data taken from 40 random fields at $5\times$ magnification, corresponding to an area of $\sim 2.3 \times 10^7 \mu\text{m}^2$), again with greater level of homogeneity in the microwell case (standard deviation of 0.2 aggregates/ mm^2 versus 1.1 aggregates/ mm^2 ; $P < 0.01$). The smaller variation in the number of aggregates per unit area for the microwell system suggests greater control over localization of hES cells, which may be relevant to study, and regulating cell interactions with their surroundings. The similar average number of aggregates per unit area for the microwell system and for the flat surface, however, is coincidental with the choice of microwell separation. In our system, the microwells were separated from one another by $300 \mu\text{m}$. If more microwells were present per unit area on the PDMS mold, then the number of aggregates should increase accordingly. Therefore, using our microwell system we may achieve a higher number of hES cell aggregates per unit area than culturing the aggregates on flat surfaces.

Given that we are proposing a new system that may be applied for the expansion of hES cells, it is important to examine the effect of microwells on the maintenance of hES cells in an undifferentiated state. hES cells grown in microwells have a superior homogeneity of aggregate size than hES cells grown on flat surfaces (Figs. 5A and B). High cell viability (~ 90 – 95%) was observed after 8 days of culture in microwells (Fig. 5D), based on calcein AM and ethidium homodimer staining, and this level is comparable to that on flat surfaces (Fig. 5C). In addition, these hES cells were shown to express Oct-4 (Fig. 5F) and ALP (Fig.

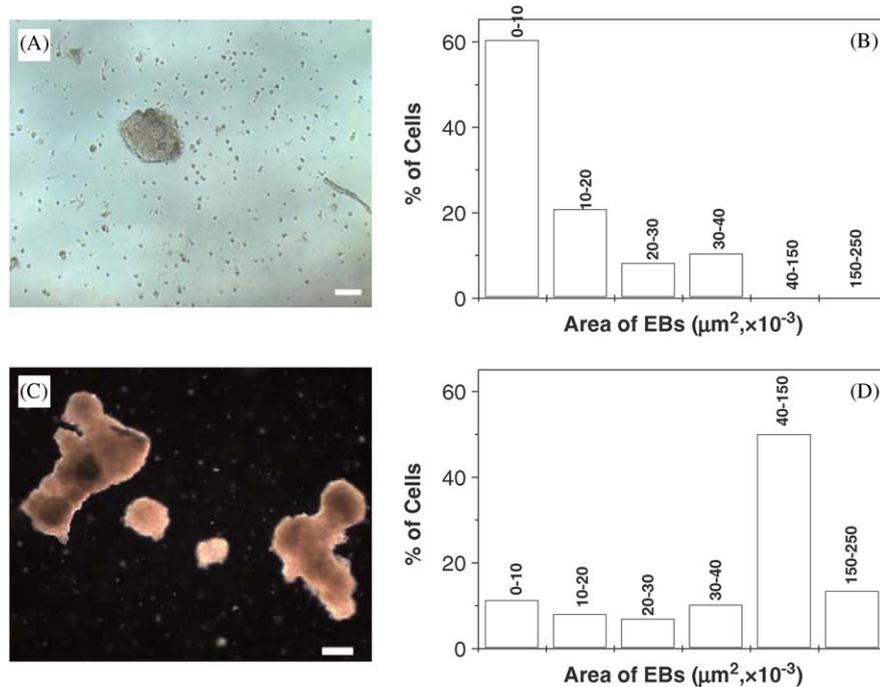


Fig. 6. Formation of EBs with controlled size using the microwells system. (A, B) Light microscopy image (A) and area distribution (B) of EBs formed by the microwell system. (C, D) Light microscopy image (C) and size distribution (D) of EBs formed from a flat surface. In both systems the area distribution was assessed in more than 50 aggregates from two different samples. In all figures, scale bars correspond to 100 μm .

5(H), two well-known markers of undifferentiated hES cells [9,20,33,34], at day 8 of culture in microwells, and the level of expression by qualitative comparison is similar to that of hES cells grown on flat surfaces (Figs. 5E and G, respectively). The expression of ALP was quantified by flow-activated cell sorting (FACS), and similar levels of ALP were observed in both culture systems (Figs. 5I and J), not significantly different from the ALP levels observed in undifferentiated stem cells at day 0 (data not shown), suggesting that hES cells grown in microwells maintain a similar undifferentiated profile as their counterparts grown on flat surfaces.

In summary, the results of these co-culture experiments imply that hES–MEF co-cultures on micropatterned PDMS surfaces have similar characteristics to those of flat co-cultures with two particular advantages: the PDMS microwells provide greater control over size and localization of hES cell aggregates. This type of control permits specific studies to be done on the effect of aggregate size on such cellular processes as differentiation and protein synthesis. Control over separation, combined with microfluidics, may enable selective treatment of individual aggregates or parts of aggregates in a high throughput manner [21]. It is also conceivable that shaped microwells may be used for control over aggregate morphology.

3.4. The use of micropatterned-hES cells to produce embryoid bodies with controlled size

In most cases, the differentiation of hES cells is conducted by removing the cells from MEF layer and

allowing them to form three-dimensional cell spheroids called EBs in medium conditions in the absence of bFGF. EBs can be formed from either single cell suspensions of hES cells or from aggregates of cells. EBs mimic the structure of, and recapitulate many of the stages involved during the differentiation process of, the developing embryo, and clonally derived EB can be used to locate and isolate tissue specific progenitors. One of the potential advantages of the current system is that it can be used to generate EBs with controlled size. This may be particularly important to differentiate the EBs into a particular cell lineage. For instance, it has been reported that efficient blood formation (with the concomitant formation of myeloid and erythroid lineages) in EBs required between 500 and 1000 cells [35]. EBs with higher number of cells did not form the erythroid lineage.

To test the validity of this approach, we generated hES/MEF co-cultures using the microwell system and after 3 days, the hES cell aggregates were removed from the microwells after collagenase treatment. The resulting cell clusters were analyzed for their number and size distribution. It was found that after this treatment approximately 50% of the aggregates could be recovered from the microwells. Approximately 26% of the EBs had an area between 10,000 and 21,000 μm^2 ; which shows that it is possible to generate EBs with controllable sizes (Figs. 6A and B). Despite the fact that further improvements are needed in this process to achieve higher yields of EBs with a specific size, the results obtained are clearly encouraging when compared to the EBs prepared by traditional methodologies. When EBs were prepared from hES

aggregates without the microwell system, they present a larger size and they are less homogeneous than the ones prepared with the microwell system (Figs. 6C and D).

4. Conclusions

We have developed a platform to culture hES cells on MEF feeder layers with control over hES cell aggregate size and localization using microwell-patterned PDMS substrates. The hES cells cultured within microwells maintained their viability and undifferentiated state as confirmed by calcein AM labeling and Oct-4 and ALP immunostaining, respectively. While exhibiting a similar viability and self-renewal profile as that of hES cells grown on flat surfaces, hES cells grown on microwell-patterned substrates show a greater level of homogeneity in aggregate size ($\pm 8300 \mu\text{m}^2$ within microwells versus $\pm 46,000 \mu\text{m}^2$ on flat surfaces in terms of \pm S.D. for $n = 3$, $P < 0.0001$). These micropatterned hES cells can be recovered to form EBs with controllable size, to be used, for example, in studies of how aggregate size may affect such cellular processes as differentiation and protein synthesis. The methodology described in this work is simple and may be scaled up for culture of large numbers of hES cells. For future experiments, it would be necessary to demonstrate that these micropatterned hES cultures can be serially passaged while maintaining their undifferentiated state. Finally, it would be important to study the effect of aggregate size on cell differentiation during the embryoid stage.

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