Micropatterned Co-cultures of Human Embryonic Stem Cell with Murine Embryonic Fibroblasts on Microwell Patterned Substrates

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Abstract

Human embryonic stem (hES) cells are generally cultured on top of a feeder layer formed by mitotically inactivated murine embryonic fibroblasts (MEFs) to maintain their undifferentiated state. The culture of hES cells in this co-culture system 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 heterogenous 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 Oct-4 and alkaline phosphatase. In addition, these clusters can be recovered from the microwells to result in the generation of uniform cell clusters for differentiation experiments.

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 major ways to culture hES cells. The first approach involves the co-culture of hES cells on top of a feeder layer comprised of 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], 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 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 mechanic 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 hamper the proliferation and recovery of hES cells in culture. In addition, variable cell cluster size may have a significant effect in 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. 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 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 3D 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 murine embryonic fibroblasts 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 octamer binding protein 4 (Oct-4) and alkaline phosphatase (AP). In addition, the cell clusters can be retrieved to generate nearly homogenous cell aggregates for differentiation studies.

Materials and Methods

hES and MEF cell culture: Cells were manipulated under sterile tissue culture hoods and maintained in a 5% CO_2 humidified incubator at 37 ºC. 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 trypsined (0.25% in EDTA, Sigma) and passaged at a 1:4 subculture ratio. Undifferentiating hES cells (H9, passages 25 to 50; WiCell, Wisconsin) were grown on an inactivated MEF feeder layer, as previously described [32], and maintained on hES cell medium consisting on 80% knockout-DMEM supplemented with 20% knockout-serum, 0.5% L-glutamine (200 mM in 0.85% NaCl), 1% nonessential aminoacids, 0.2% mercaptoethanol (55 mM in PBS) and 5ng/mL of basic fibroblast growth factor (bFGF) (all from Invitrogen). The cells were fed daily and passaged every 4 days using collagenase type IV (2mg/mL, Invitrogen) for 30-40 min and then scrapping the petri dish containing the cells. A subculture ratio of 1:3 was generally used to propagate these cells.

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 patterned silicon masters and cured at 60°C for 4 hours. The PDMS molds were then peeled from the silicon surfaces and cut prior to use (25 × 25 mm; each mold containing 2400 wells). In most experiments PDMS molds were generated with microwells that were 200 μm in diameter and 120 μm deep. Before use, these micropatterned substrates were sterilized in 70% (v/v) ethanol for 10 min and then washed in PBS overnight.

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 ~0.25×10^6 cells/mL (4 mL of this cell suspension were used per each PDMS mold). Just prior to seeding, the PDMS molds were treated with fibronectin (50 μg/mL, in PBS) for 5 min at room temperature and then washed 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.

hES cell seeding on MEF coated surfaces: hES 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 (~2×10^6 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, 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.

Cell viability analyses: Cell viability of micropatterned hES cells was determined using a LIVE/DEAD kit (Molecular Probes) containing calcein AM (2 μg/mL, in PBS) and ethidium homodimer (4 μg/mL, in PBS). The micropatterns containing hES cells were placed in 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.

Scanning electron microscopy analyses: 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 Å. SEM images were recorded by a field emission scanning electron microscopy (JEOL 6320FV) at 15 kV.

Cell labelling and immunostaining: Cells were stained with the membrane dye, carboxyfluorescein diacetate succinimidyl ester (CFSE, Sigma), as well as Vybrant® DiD (Molecular Probes) cell-labelling solution. Trypsinized cells were resuspended to a concentration of 1×10^6 cells/mL within the staining solution (10 μg/mL in PBS for CSFE and 20 μg/mL in PBS for Vybrant® DiD) and incubated for 10 min at room temperature. The cells were rinsed in PBS twice before being used for experiments. For confocal microscopy, CFSE-stained and Vybrant® DiD-stained cell samples were fixed with Fluoromount-G and covered with a No. 1 thickness coverslip. Confocal images were taken at 40× magnifications through a FITC and Rhodamine filter with a maximum focal depth of 248 μm.

For Oct-4 and AP staining, the cells were fixed with 4% (w/v) paraformaldehyde solution for 30 min at room temperature. After blocking with 3% (w/v) bovine serum albumin solution (BSA), the cells were stained for 1 h with rabbit anti-Oct-4 polyclonal antibody (10 μg/mL, BioVision Inc.) or monoclonal mouse anti-human alkaline phosphatase (supernatant diluted 1:10, Developmental Studies Hybridoma Bank, B4-78). In each immunofluorescence experiment, an isotype-matched IgG control was used. Binding of primary antibodies to specific cells was detected with PE-conjugated goat anti-rabbit IgG (diluted 1:20, Sigma) or PE-conjugated goat anti-mouse IgG1 (1 μg/mL, Molecular Probes). After the indirect labelling the cells were examined with a fluorescence microscope.

Flow cytometry analysis: hES cells were incubated with type IV collagenase for 2 h at 2 mg/mL to remove hES cell aggregates from the MEF monolayers. hES aggregates were then dissociated with

non-enzymatic cell dissociation solution for 10-15 min. Single cell suspensions were washed with PBS containing 5% (v/v) FBS and filtered through 85 μm mesh strainer to remove remaining clumps. The single cell suspensions were aliquoted (1.25-2.5 $\times$ 10^5 cells), fixed and permeabilized using an intrastain kit (Dako). Afterwards, the cells were stained with either Oct-4 or the corresponding isotype control for 30 min and then the monoclonal antibodies conjugated with the corresponding PE-secondary antibodies (see above). The stained cells were analyzed on a FACScan (Becton Dickinson) and the data analysis was carried out using CellQuest software.

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 $P \leq 0.05$.

Results and Discussion

MEFcell 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 standard co-culture approach. This approach consisted of seeding cells on microwell patterned elastomeric, biocompatible polymeric surfaces. These surfaces were coated with fibronectin and seeded with MEFs to form a monolayer and subsequently seeded with hES cells (Figure 1).

MEFs seeded on fibronectin-treated micropatterned PDMS formed monolayers as indicated in Figure 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 ~5×10^4cells/cm^2, and formed a confluent monolayer 3 days. It was found that high MEF seeding densities resulted in filling of the microwells and thus prevented their subsequent use.

To determine potential microwell sizes that could be used to generate the patterned co-cultures various size microwell patterned surfaces were analyzed (data not shown). In general, it was found that microwell diameters of less than 100 μm 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 μm and a depth of ~120 μm were selected. Using this geometry, few MEF cells adhered to the vertical surfaces (Figures 2E and 2F). This likely resulted from a limited exposure of

the cells to microwell walls during cell settling and an inability of the mitomycin C treated MEFs to navigate steep (90^{o}) substrate topography.

hES cell seeding onto micropatterns

To assess the capability of the technique as a standard technique 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. Using this procedure we were able to retain ca. 5% of the hES cells 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 Figure 3. Fluorescent images during the first two days (Figures 3A-3D) indicate hES cells (red) were localized in the wells and MEFs (green) on the surrounding surface. At day 1, there were ca. 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 (Figure 3G). By day six, defined aggregates had formed consistently over a large surface area (Figures 3G and 3H). Figure 4A shows that the area occupied by the hES cells in the microwells increased over time and thus showing that they can proliferate inside of the microstructures.

Microwells Versus Flat Surfaces

To assess the potential advantages of this approach, the microwell method of hES-MEF co-culture was compared with a more traditional flat co-culture. hES-MEF co-cultures were analyzed

at day 8 for aggregate area and number of aggregates per unit area. According to Figure 4, the averaged area for aggregates in the microwells is statistically lower ($P< 0.001$) than aggregates in flat surfaces. At this time, the averaged area of the hES aggregates (25,690 ± 385) in the microwells is close to the area of these microstructures (31,140 μm^2), and thus showing that the microwells were almost covered by hES cells. Furthermore, the homogeneity of hES aggregates in the microwells was superior to the ones on flat surface. The standard deviation of areas within patterned co-culture was determined to be statistically smaller than that of flat co-culture (8,300 μm^2 versus 46,000 μm^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 small ones before seeding them again on top of MEF feeder layers. Therefore, the heterogeneity in size of these initial aggregates will be constant over time. Furthermore, during the culture of these aggregates in flat surfaces, some of them agglomerate over time increasing even more their size and heterogeneity.

With regard to number of hES aggregates per unit area, the two methodologies produced relatively similar results (~4 aggregates/mm^2), again with greater homogeneity in the microwell case (standard deviation of 0.2 aggregates/mm^2 versus 1.1 aggregates/mm^2; $P<0.01$). The similar number, however, is coincidental with the choice of microwell separation. In our system, the microwells were separated by each other by 350 μm. If more microwells were present per unit area, then the number of aggregates should increase accordingly. Therefore, using our microwell system we may achieve a high number of hES cell aggregates per unit area than culturing the aggregates in flat surfaces.

Given that we are proposing a new system that may be applied for the expansion of hES cells, it was imperative to demonstrate the effect of the microwells on the maintenance of hESC in an undifferentiated state. Figure 5 shows that ca. 90-95% of the cells remain viable after 8 days in the microwells. In addition, the hES cells express octamer binding protein 4 (Oct-4) and alkaline phosphatase (ALP), two well-known markers of undifferentiated hES cells [9, 20, 33, 34]. The expression of ALP was also quantified by flow-activated cell sorting (FACS) for both culture systems (Figure 5). Similar levels of ALP were founded in both culture systems, not significantly different from the ALP levels found on undifferentiated stem cells at day zero (data not shown), showing that apparently both cells present the same level of pluripotency.

In summary, the results of these co-culture experiments imply that hES-MEF co-culture on micropatterned PDMS surfaces has similar characteristics to flat co-culture 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 on effects that may be dependent on aggregate size, like differentiation and protein synthesis. Control over separation, combined with microfluidics, can allow selective treatment of individual aggregates or parts of aggregates in a high throughput manner [21]. It is also conceivable that shaped microwells could be used for control over aggregate morphology.

The use of microppaterned-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 3-dimenstional cell spheroids called embryoid bodies (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 nearly all of the aggregates could be recovered from the microwells (~100%). Approximately 26% of the EBs had a size between 130 and 200 μm; which shows that it is possible to generate EBs with controllable sizes (Figure 6). 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 (Figure 6).

Conclusions

We have developed a platform to culture hES cells on MEF feeder layers with control of hES cell cluster size and number per surface area. For that purpose, MEFs were seeded on micropatterned PDMS followed by culture of hES cells inside of each microwell. The hES cell aggregates obtained from these microwells at day 8 had an average area of 26,000 ± 8,300 μm^2. The hES cells cultured inside of the microwells maintained their undifferentiated state as confirmed by the expression of Oct-4 and alkaline phosphatase stem cell markers. 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 different cell cluster size in their differentiation profile through an embryoid stage.

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References

1. Chadwick K, Wang L, Li L, Menendez P, Murdoch B, Rouleau A, et al. Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. Blood 2003 Aug 1;102(3):906-915.
2. Kaufman DS, Hanson ET, Lewis RL, Auerbach R, Thomson JA. Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 2001 Sep 11;98(19):10716-10721.
3. Levenberg S, Golub JS, Amit M, Itskovitz-Eldor J, Langer R. Endothelial cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 2002 Apr 2;99(7):4391-4396.
4. Mummery C, Ward D, van den Brink CE, Bird SD, Doevendans PA, Opthof T, et al. Cardiomyocyte differentiation of mouse and human embryonic stem cells. J Anat 2002 Mar;200(Pt 3):233-242.
5. Reubinoff BE, Itsykson P, Turetsky T, Pera MF, Reinhartz E, Itzik A, et al. Neural progenitors from human embryonic stem cells. Nat Biotechnol 2001 Dec;19(12):1134-1140.
6. Xu C, Police S, Rao N, Carpenter MK. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. Circ Res 2002 Sep 20;91(6):501-508.
7. Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 2001 Dec;19(12):1129-1133.
8. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 2000 Apr;18(4):399-404.
9. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998 Nov 6;282(5391):1145-1147.
10. Amit M, Margulets V, Segev H, Shariki K, Laevsky I, Coleman R, et al. Human feeder layers for human embryonic stem cells. Biol Reprod 2003 Jun;68(6):2150-2156.
11. Hovatta O, Mikkola M, Gertow K, Stromberg AM, Inzunza J, Hreinsson J, et al. A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. Hum Reprod 2003 Jul;18(7):1404-1409.
12. Cheng L, Hammond H, Ye Z, Zhan X, Dravid G. Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. Stem Cells 2003;21(2):131-142.
13. Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, et al. Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 2001 Oct;19(10):971-974.
14. Xu C, Rosler E, Jiang J, Lebkowski JS, Gold JD, O'Sullivan C, et al. Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. Stem Cells 2005 Mar;23(3):315-323.
15. Xu RH, Peck RM, Li DS, Feng X, Ludwig T, Thomson JA. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods 2005 Mar;2(3):185-190.
16. Ludwig TE, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, Frane JL, et al. Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol 2006 Mar;24(2):185-187.
17. Levenstein ME, Ludwig TE, Xu RH, Llanas RA, Vandenheuvel-Kramer K, Manning D, et al. Basic FGF Support of Human Embryonic Stem Cell Self-Renewal. Stem Cells 2005 Nov 10.
18. Draper JS, Smith K, Gokhale P, Moore HD, Maltby E, Johnson J, et al. Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat Biotechnol 2004 Jan;22(1):53-54.

19. Bloor DJ, Metcalfe AD, Rutherford A, Brison DR, Kimber SJ. Expression of cell adhesion molecules during human preimplantation embryo development. Mol Hum Reprod 2002 Mar;8(3):237-245.
20. Hoffman LM, Carpenter MK. Characterization and culture of human embryonic stem cells. Nat Biotechnol 2005 Jun;23(6):699-708.
21. Khademhosseini A, Langer R, Borenstein J, Vacanti JP. Microscale technologies for tissue engineering and biology. Proc Natl Acad Sci U S A 2006 Feb 13;103(8):2480-2487.
22. Whitesides GM, Ostuni E, Takayama S, Jiang XY, Ingber DE. Soft lithography in biology and biochemistry. Annual Review of Biomedical Engineering 2001;3:335-373.
23. Khademhosseini A, Jon S, Suh KY, Tran TNT, Eng G, Yeh J, et al. Direct Patterning of protein- and cell-resistant polymeric monolayers and microstructures. Advanced Materials 2003 DEC 3;15(23):1995-2000.
24. Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Geometric control of cell life and death. Science 1997 May 30;276(5317):1425-1428.
25. McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell 2004 Apr;6(4):483-495.
26. Flaim CJ, Chien S, Bhatia SN. An extracellular matrix microarray for probing cellular differentiation. Nature Methods 2005 FEB;2(2):119-125.
27. Anderson DG, Levenberg S, Langer R. Nanoliter-scale synthesis of arrayed biomaterials and application to human embryonic stem cells. Nat Biotechnol 2004 Jul;22(7):863-866.
28. Khademhosseini A, Suh KY, Yang JM, Eng G, Yeh J, Levenberg S, et al. Layer-by-layer deposition of hyaluronic acid and poly-l-lysine for patterned cell co-cultures. Biomaterials 2004 Aug;25(17):3583-3592.
29. Bhatia SN, Balis UJ, Yarmush ML, Toner M. Effect of cell-cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells. Faseb J 1999;13(14):1883-1900.
30. Bhatia SN, Balis UJ, Yarmush ML, Toner M. Probing heterotypic cell interactions: hepatocyte function in microfabricated co-cultures. J Biomater Sci Polym Ed 1998;9(11):1137-1160.
31. Bhatia SN, Yarmush ML, Toner M. Controlling cell interactions by micropatterning in co-cultures: hepatocytes and 3T3 fibroblasts. J Biomed Mater Res 1997;34(2):189-199.
32. Schatten G, Smith J, Navara C, Park JH, Pedersen R. Culture of human embryonic stem cells. Nat Methods 2005 Jun;2(6):455-463.
33. Li Y, Powell S, Brunette E, Lebkowski J, Mandalam R. Expansion of human embryonic stem cells in defined serum-free medium devoid of animal-derived products. Biotechnol Bioeng 2005 Sep 20;91(6):688-698.
34. Wang G, Zhang H, Zhao Y, Li J, Cai J, Wang P, et al. Noggin and bFGF cooperate to maintain the pluripotency of human embryonic stem cells in the absence of feeder layers. Biochem Biophys Res Commun 2005 May 13;330(3):934-942.
35. 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 Sep 1;106(5):1601-1603.

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Figure 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.

Figure 2- Formation of MEF monolayers. A,B) Transmission (A) and fluorescent (B) images of inactivated MEF monolayers with mitomycin C. MEFs were labelled 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.

Figure 3- hES-MEF co-cultures at various time intervals. MEFs were stained with CFSE (green) and hES cells with Vybrant® DiD (red). A, B, C, D, E, F) Light and fluorescent images of the hES-MEF co-culture after one (A,B), two (C,D), or six days (E,F) after hES cell seeding. G) Confocal images of hES cells within a microwell as rotated in 45º intervals. In all figures, bar corresponds to 200 μm.

Figure 4- Quantitative analysis of hES aggregate size and frequency. A) Percentage of microwell occupancy by hES cells over time. The area of hES aggregates at day 1 and day 6 was assessed in more than 20 microwells in the same PDMS sample. B) Area of 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, on flat or microwell patterned substrates. The number of aggregates was calculated on 40 random fields at ×5 magnifications (corresponding to an area of ~2.3×10^7

μm^2) per sample (3 samples per condition). In all graphs, values indicate average ± S.D., from 3 independent experiments. * Denote statistical significance (P<0.001).

Figure 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 size (A,B; transmission ×5), viability (C,D; green: live; red: dead; ×10), and the expression of hES cell markers including Oct-4 (E,F; ×10) and alkaline phosphatase (G,H; ×10). (I, J) Indicates the expression of alkaline phosphatase as measured by FACS. Percent of positive cells were calculated based in the isotype controls (gray plots) and are shown in each histogram plot.

Figure 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. Bar corresponds to 100 μm. C,D) Light microscopy image (C) and size distribution (D) of EBs formed from a flat surface. Bar corresponds to 200 μm. In both systems the area distribution was assessed in more than 50 aggregates from two different samples.

Figure 1

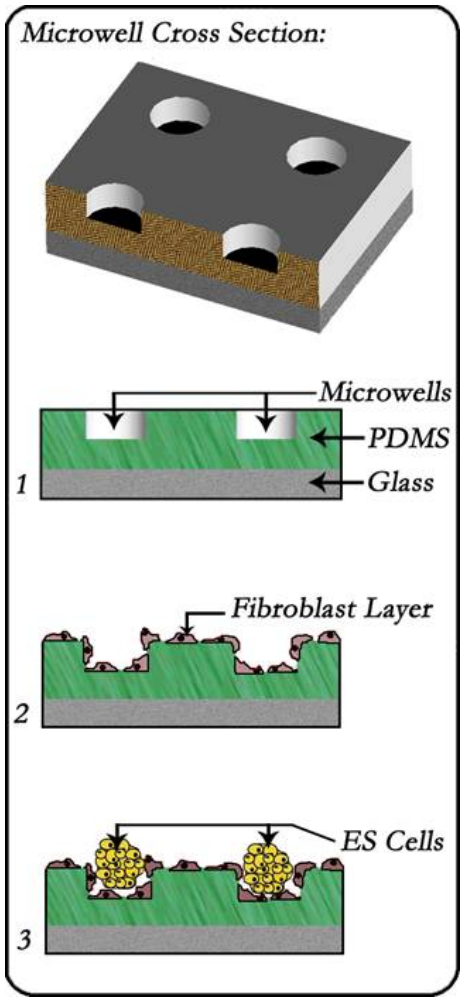

Microwell Cross Section:
Microwells
PDMS
Glass
1
Fibroblast Layer
2
ES Cells
3

Figure 2

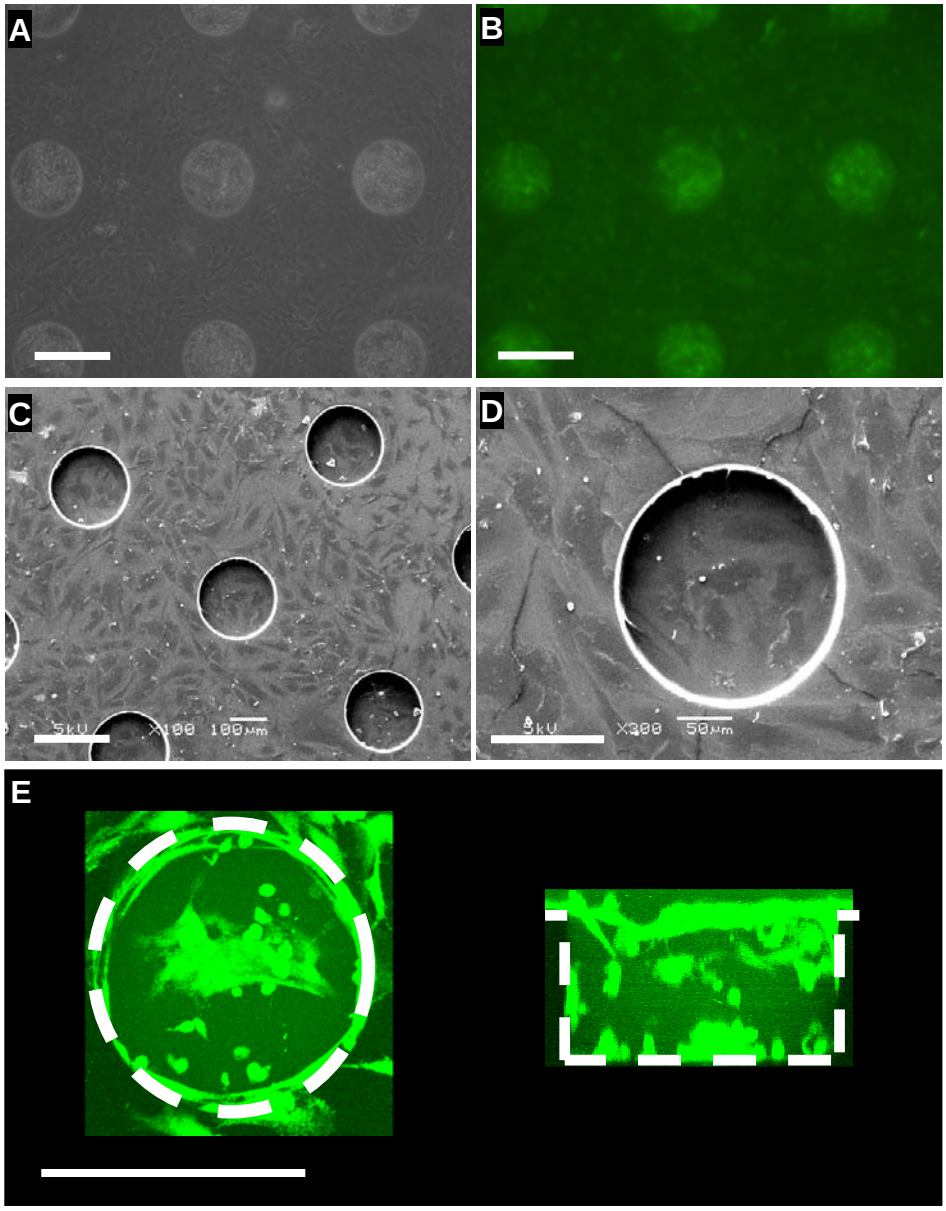


Figure 3

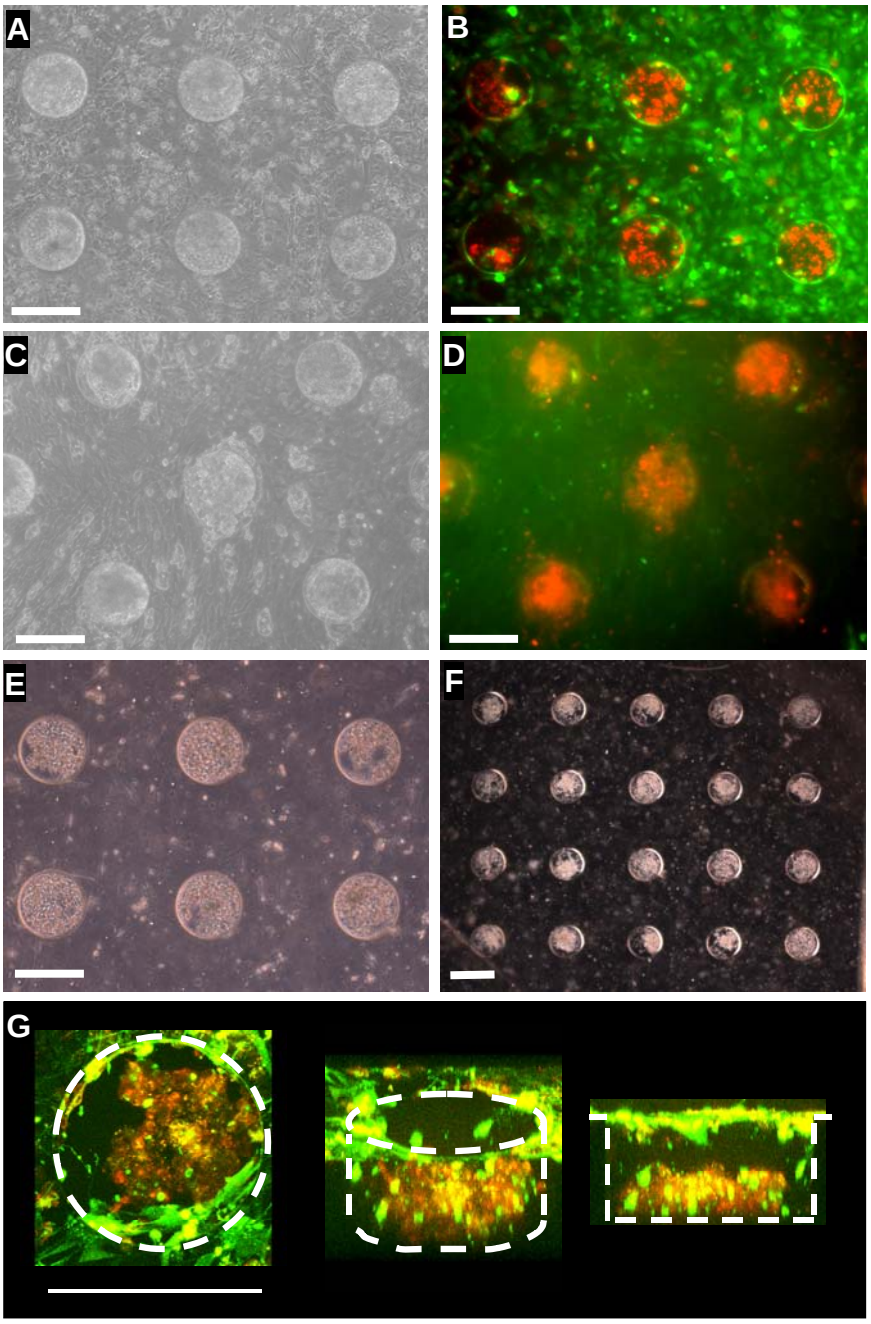


Figure 4

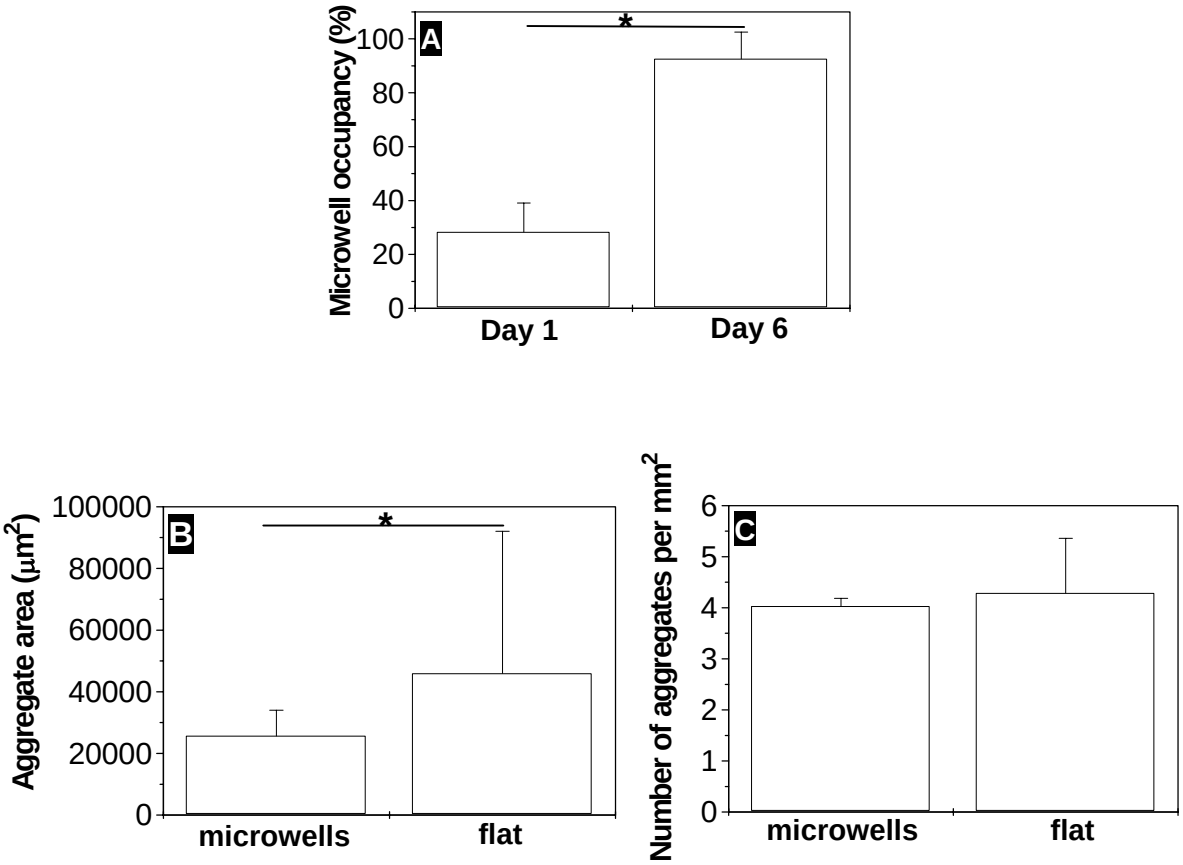

A
Microwell occupancy (%)
0
20
40
60
80
100
*
Day 1
Day 6
B
Aggregate area (μm²)
0
20000
40000
60000
80000
100000
*
microwells
flat
C
Number of aggregates per mm²
0
1
2
3
4
5
6
microwells
flat

Figure 5

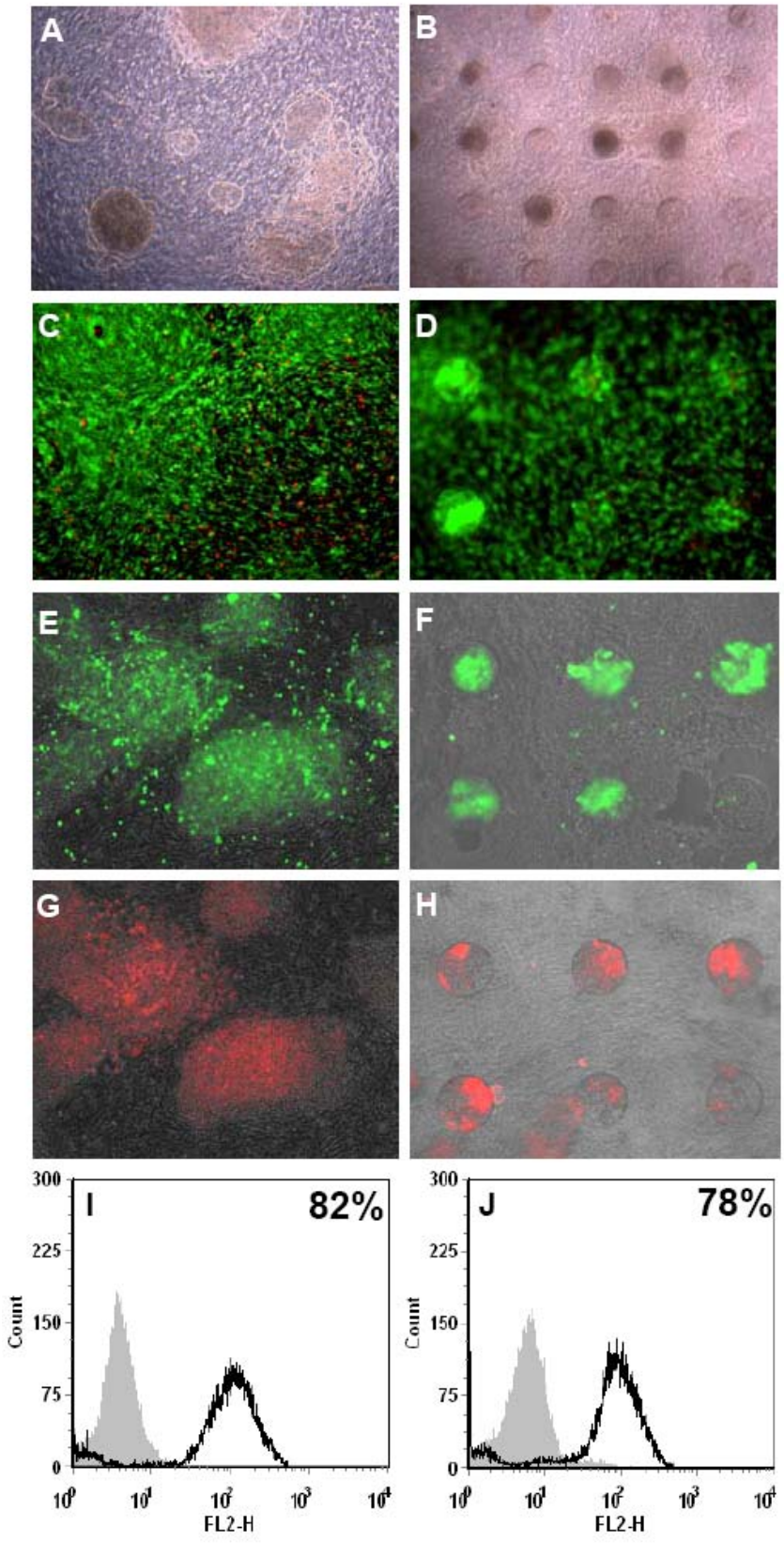
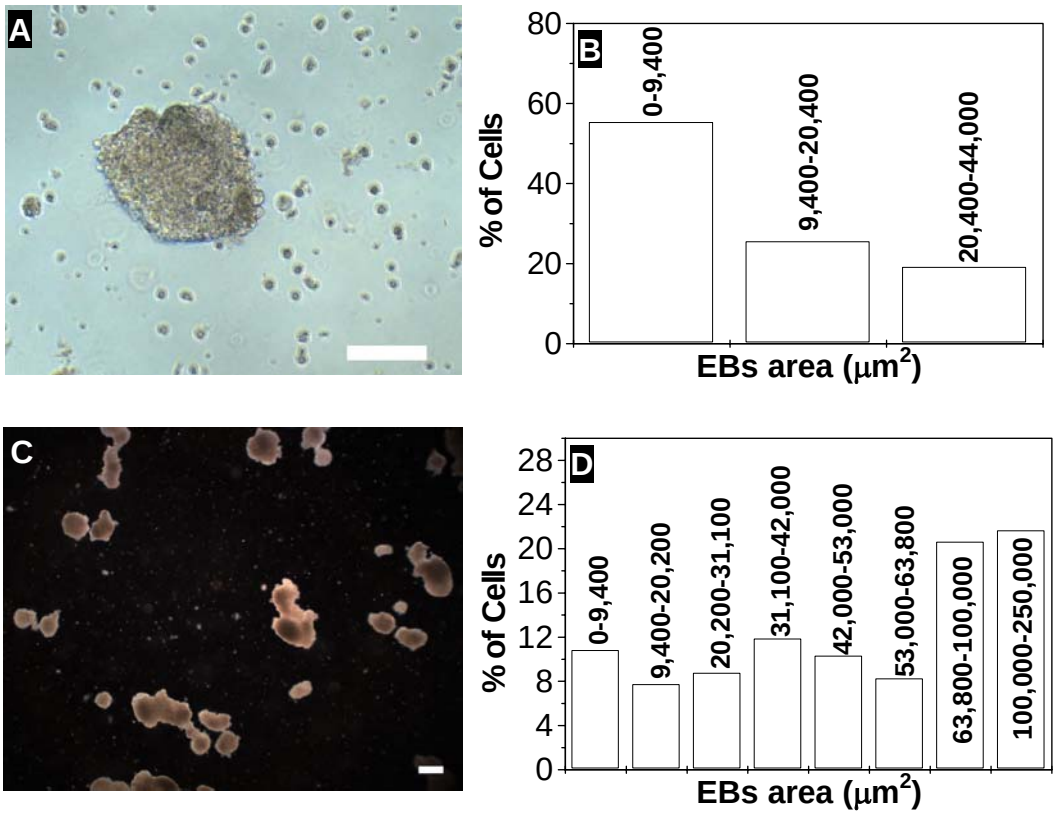

A
B
C
D
E
F
G
H
I
82%
J
78%
300
225
150
75
0
Count
10^0
10^1
10^2
10^3
10^4
FL2-H

Figure 6


A
B
80
60
40
20
0
% of Cells
0-9,400
9,400-20,400
20,400-44,000
EBs area (μm²)
C
D
28
24
20
16
12
8
4
0
% of Cells
0-9,400
9,400-20,200
20,200-31,100
31,100-42,000
42,000-53,000
53,000-63,800
63,800-100,000
100,000-250,000
EBs area (μm²)