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3-D microwell culture of human embryonic stem cells

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Abstract

Human embryonic stem cells (hESCs) have the ability to proliferate indefinitely and differentiate into each of the embryonic cell lineages. Great care is required to maintain undifferentiated hESC cultures since spontaneous differentiation often occurs in culture, presumably resulting from soluble factors, cell–cell contact, and/or cell–matrix signaling. hESC differentiation is typically stimulated via generation of embryoid bodies (EBs) and lineage commitment of individual cells depends upon numerous cues throughout the EB environment, including EB shape and size. Common EB formation protocols, however, produce a very heterogeneous size distribution, perhaps reducing efficiency of directed differentiation. We have developed a 3-D microwell-based method to maintain undifferentiated hESC cultures for weeks without passaging using physical and extracellular matrix patterning constraints to limit colony growth. Over 90% of hESCs cultured in microwells for 2–3 weeks were viable and expressed the hESC transcription marker Oct-4. Upon passaging to Matrigel-coated tissue culture-treated polystyrene dishes (TCPS), microwell cultured hESCs maintained undifferentiated proliferation. Microwell culture also permits formation of hESC colonies with a defined size, which can then be used to form monodisperse EBs. When cultured in this system, hESCs retained pluripotency and self-renewal, and were able to be passaged to standard unconstrained culture conditions.

Keywords: Human embryonic stem cells; Microcontact printing; Microwells; Embryoid body; Self-assembled monolayer

1. Introduction

Human embryonic stem cells (hESCs), cell lines derived from the inner cell mass of blastocyst stage human embryos, are capable of unlimited proliferation and differentiation into the three embryonic germ layers [1–3]. To enable self-renewal, hESCs are typically cocultured with mouse embryonic fibroblast (MEF) feeder cells, or cultured in MEF-conditioned medium on a Matrigel extracellular matrix. Cell microenvironment has long been known to influence differentiation [4,5]. Spontaneous differentiation of hESC cultures occurs along seemingly random pathways during normal cell culture conditions, especially as colony density and size expand [2,6].

hESC differentiation is stimulated through either coculture with various differentiated cells or via generation of embryoid bodies (EBs) [2]. EBs are formed from hESC colonies chemically or mechanically detached from their substrate then cultured in suspension in the absence of MEFs or MEF-conditioned medium [7–11]. After several days in suspension, EBs are plated to promote proliferation and further cell differentiation. Cell lineages obtained during EB differentiation vary dramatically between distinct EBs in the same culture. Presumably, subtle microenvironment differences present in individual EBs affect differentiation of a population of cells in the EB, which then may further guide differentiation of other cells by cell–cell contact or secretion of soluble differentiation factors [6]. One factor that may regulate lineage commitment is EB size [12]. For example, over 500 hESCs were required to seed EBs for efficient generation of hematopoietic cells, though the actual number of hESCs aggregating to form these EBs was not known [13]. Smaller EBs preferentially differentiated along other lineages. To further investigate the size dependence of EBs on hESC

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differentiation, we developed a system that utilizes a combination of chemical and physical restraints to control hESC colony, and resulting embryoid body, size.

Many cell types, including 3T3 fibroblasts [14–17], capillary endothelial cells [18–20], mouse melanoma cells [17], and buffalo rat liver cells [17] have been effectively constrained to 2-D monolayer patterns. Micro-contact printing allows micron-scale organization of a combination of self-assembled monolayers (SAMs) of alkanethiols on a gold surface to both attract and repel extracellular matrix (ECM) proteins in desired spatial configurations [16–24].

SAMs of alkanethiols spontaneously form on gold substrates. SAMs assemble by a linkage of the terminal sulfur group to the three-fold sites of the gold substrate and reach equilibrium within a period of 1–5 h [19]. Alkanethiols used to construct SAMs typically contain an 11–18 carbon chain and are capped with a functional group. The terminating functional group gives rise to the SAM interfacial properties, including susceptibility to protein adsorption [16,19,21].

Several terminal groups have been utilized for cell and protein repulsion and attraction. The most commonly used SAM for protein repulsion is a polyethylene glycol terminated alkanethiol containing three to six ethylene glycol groups [16,20]. Triethylene glycol (EG3)-terminated alkanethiols can resist protein and ECM adsorption for approximately 8 days, but begin to break down under typical cell culture conditions thereafter [16]. Several alkanethiols, including methyl and amine-terminated molecules, have been used to attract ECM proteins [19,21,22,25–27]. By micro-contact printing an EG3-terminated SAM, fibrinogen was effectively patterned on a hexadecanethiolate SAM. Subsequently, 3T3 fibroblasts attached only to regions occupied by fibrinogen [16]. Similarly, bovine capillary endothelial cells have been patterned using both EG3-terminated and an attractive hexadecanethiol-terminated SAMs to organize fibronectin [18,20]. Combinations of vitronectin and fibronectin were concurrently patterned on an octadecylmercaptan-terminated SAM for growth of mouse B16F1 melanoma cells, buffalo rat liver cells, and 3T3 fibroblasts [17].

Application of 2-D microcontact printing to hESCs is limited because of their growth nature. hESCs do not grow to confluence as monolayers, but build upon themselves to form cell aggregates [28], spreading beyond the 2-D system afforded by micro-contact printing. Constructing ECM patterns with a physical constraint in the *z*-dimension may facilitate longer culture periods of cells that are not contact inhibited, such as hESCs. In such a system, cells could be cultured in micron scale wells consisting of a material that adsorbs protein, with the substrate surrounding wells functionalized to prevent ECM adsorption and cell attachment. Thus, cells could grow in the well, but not outside of the well. This approach has been used to culture single epithelial cells [29], adult hippocampal progenitor cells [30], and 3T3 fibroblasts [15].

This study describes development of microwell arrays that facilitate hESC culture and EB formation. Soft lithography-formed PDMS stamps mold polymer substrates whose surfaces are then functionalized with protein resistant SAMs to inhibit protein adsorption and cell attachment. The surfaces of the wells, however, adsorb ECM proteins that promote cell adhesion and proliferation. This method yields defined geometries that permit undifferentiated hESC growth in monodisperse colonies for several weeks without loss of pluripotency. Additionally, these arrays facilitate control of EB size, which may be useful for controlling cell differentiation.

2. Materials and methods

2.1. hESC culture

hESC lines H1 and H9 (passage 20–45) were used throughout all experiments [3]. hESCs were cultured in microwells coated by Matrigel (Beckton-Dickinson Bioscience, Medford, MA, USA) using medium conditioned on MEFs for 24 h, supplemented with 4 ng/ml bFGF. MEF medium, hESC medium without bFGF (UMF−) and MEF-conditioned hESC medium with bFGF (CMF+) were prepared as described elsewhere [31]. hESCs were passaged from microwells using 2 ml/well pre-warmed 10 mg/ml dispase in DMEM/F12 per microwell slide. Plates were incubated 15–25 min at 37 °C and cells were washed from microwells by pipeting.

2.2. EB culture

EBs were formed from hESC colonies removed from microwells and cultured in suspension using 30 ml UMF− in an upright T75 flask for 1 week to facilitate differentiation. EBs were plated on 0.1% gelatin-coated 6- or 12-well cell culture plates, cultured in UMF− supplemented with 5% FBS to facilitate attachment, and allotted 8 days to grow prior to fixation and characterization.

2.3. Microwell formation

Microwells were created in three steps. First, photolithography and plasma etching techniques similar to those used by Chen et al. [22] yielded silicon masters with depressions of desired patterns. Masters were fluorinated with (tridecafluoro-1,1,2,2,-tetrahydrooctyl)-1-trichlorosilane vapor to passivate the surface and allow detachment of PDMS (Sylgard 184 Silicon Elastomer, Ellsworth Adhesives, Germantown, WI, USA) in step 2. Second, PDMS stamps were manufactured by pouring 10:1 ratio elastomer prepolymer and curing agent over silicon masters. The mixture was degassed under vacuum and incubated overnight at 60 °C to promote polymerization. Finally, PDMS stamps were clipped on two sides to glass microscope slides separated by 250 μm spacers. Norland optical adhesive 61 (Norland Products Inc., Cranbury, NJ, USA) polyurethane prepolymer was fed to one end of the clipped stamps and distributed via capillary action. After crosslinking under UV light for 2 h, stamps and spacers were removed, yielding patterned microwells.

2.4. Self-assembled monolayer formation

Microwells were coated with gold by e-beam evaporation to allow SAM assembly. Oblique angles (>45°) restricted gold evaporation to the areas surrounding the wells and the sides of the wells. Two evaporations were performed, with slides rotated 90° between evaporations. A 80 Å titanium layer preceded the 200 Å gold layer evaporation. Resulting gold-coated microwells were semi-transparent, allowing use of light microscopy

during culture. Prior to immersion in SAM solution, microwells were washed in 100% ethanol and sterilized under UV light for 1 h. Slides were placed in individual wells of a six-well culture dish with 2 ml/well of a 2 mM tri-ethylene glycol terminated (Prochimia, Poland) ethanoic SAM solution. Slides were incubated at room temperature for 2 h and washed in 100% ethanol prior to coating with Matrigel. All SAM solutions were stored at 4 °C and used within 1 week.

2.5. Microwell cell seeding

Functionalized microwells were coated with growth factor reduced Matrigel by resuspending 2 mg frozen Matrigel in 12 ml cold DMEM/F12. About 1 ml of cold Matrigel solution was aliquoted to each microwell array and an additional 1 ml of DMEM/F12 was added to each sample. After 1 h incubation at 37 °C, Matrigel coated microwells were washed once in PBS and then transferred to non-cell culture treated six-well plates to prevent cell attachment on cell culture dish surrounding microwell slides. hESCs were passaged from 1 well of a six-well plate to one to two microwell samples. hESCs were passaged to 50 and 100 μm/side microwells using 1 ml/well trypsin (Invitrogen, Carlsbad, CA, USA) pre-warmed to 37 °C. To prevent hESC colony dissociation to single cells, plates were monitored under a microscope and trypsin was neutralized with 2 ml/well MEF medium when hESCs at colony edges began dissociating. hESCs were gently washed from plates and pelleted. The pellet was resuspended in 0.75 ml/sample CMF+, and aliquoted to the top of each array of microwells, maintaining the cell solution on top of arrays. Samples were incubated 30 min at 37 °C to allow hESCs to settle into the microwells before adding 1.5 ml/well CMF+. hESC medium was changed daily thereafter.

2.6. Microscopy

Phase contrast and epifluorescence images of differentiation data were obtained on an Olympus IX70 model microscope (Leeds Precision Instruments, Minneapolis, MN, USA) using MetaVue 5.0r1 imaging software. Phase contrast, brightfield, and epifluorescence images of hESC localization and viability were obtained on a Leica DM ARB microscope (Leica Microsystems, Inc., IL, USA).

2.7. Immunohistochemistry and epifluorescence

2.7.1. hESC localization

hESC localization within microwells was visualized using Hoechst nuclear stain (Sigma-Aldrich, St. Louis, MO, USA). hESCs within microwells were fixed in 4% paraformaldehyde for 15 min at 22 °C and then washed 3× in PBS. Stock Hoechst DNA-binding dye (10 mg/ml aqueous solution) was diluted 1:1000 in PBS and aliquoted to microwell slides for a 5 min incubation at 22 °C. Microwell slides were washed 3× and stored in PBS for analysis.

2.7.2. hESC viability within microwells

Viability data within microwells were determined from Calcein AM live cell marker (Molecular Probes, Carlsbad, CA, USA). Stock Calcein AM was diluted 1:1000 in PBS, aliquoted to microwells, and incubated 30 min at 37 °C. Microwell slides were washed 3× and stored in PBS for analysis.

2.7.3. Differentiation analysis

hESCs were fixed for 15 min in 4% paraformaldehyde in PBS prior to performing differentiation analysis. After blocking in 5% milk in PBS+0.4% Triton X-100 for 1 h at 22 °C, primary antibodies were prepared as a 1:200 dilution in PBS+0.4% Triton X-100 and incubated overnight at 4 °C. Samples were washed five times in PBS before addition of secondary antibodies diluted 1:500 in PBS+0.4% Triton X-100. After 1 h incubation at 22 °C, samples were washed three times in PBS. Hoechst nuclear stain was prepared as a 1:1000 dilution of 10 mg/ml aqueous stock in PBS and aliquoted to each sample for 5 min incubation at 22 °C. hESCs were washed 2× and stored in PBS for analysis. The primary antibodies used for differentiation analysis were Oct3/4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for undifferentiated hESCs, brachyury (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for mesodermal cells, nestin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for ectodermal cells, and α-fetoprotein (Biodesign International, Saco, ME, USA) for endodermal cells. Alexa fluor 488 or 594 conjugated secondary antibodies (Molecular Probes, Carlsbad, CA, USA) were used in all cases.

2.8. Flow cytometry

hESCs were detached from microwells using a 0.05% trypsin, 0.53 mM EDTA, 2% chicken serum solution. Cells were incubated 10 min at 37 °C and trypsin neutralized using 2 ml/well FACS buffer (PBS without Ca/Mg^{2+}, 2% FBS, 0.1% NaN_3). hESCs were prepared and Oct-4 expression was quantified via flow cytometry as described elsewhere [32]. hESC metabolic activity via Calcein AM reduction was also quantified by flow cytometry. After trypsinization, cells were filtered through a 40 μm mesh to eliminate aggregates and washed in FACS buffer (PBS without Ca/Mg^{2+}, 2% FBS, 0.1% NaN_3). Cells were then resuspended in 200 ml FACS buffer with a 1:1000 dilution Calcein AM and incubated 30 min at room temperature. Cells were washed in FACS buffer and then collected for analysis. Data were collected on a FACScan flow cytometer (Beckton Dickinson) and analysis was performed on CellQuest (Beckton Dickinson) and WinMDI software. All cells were gated according to light scatter and Oct-4 expression or Calcein AM fluorescence.

2.9. Statistics

Error bars were calculated as the standard deviation of sample sets and p-values were computed using a two-tailed Student's t-test, assuming unequal variance.

3. Results

Several design parameters are crucial for development of a 3-D microwell-based array for hESC culture. First, hESC colonies must localize to the microwells but not be able to span across multiple microwells. Also, hESCs must remain viable and undifferentiated after extended culture periods in microwells, and pluripotency must be maintained. Finally, undifferentiated hESC colonies must be recoverable from microwells and have the capacity to transition to standard culture conditions. To address these issues, we constructed surfaces containing square microwells of two lateral sizes, 50 and 100 μm, and two microwell depths, 50 and 120 μm, and assessed hESC growth and differentiation in these wells.

3.1. Microwell fabrication and hESC localization

Microwells were constructed using a PDMS stamp to shape a UV-crosslinkable polymer. The polymer surface between wells was coated with gold at an oblique angle and a SAM of EG3-terminated alkanethiols was assembled to resist protein adsorption on the gold-covered surfaces outside the wells. A Matrigel solution was then incubated over the surface to promote cell adhesion inside the wells, where gold was not deposited (Fig. 1). Fig. 2A demonstrates that microwell fabrication yielded a homogeneous distribution of wells of identical size and shape. hESC

localization to only the insides of the wells was illustrated via two methods: phase contrast microscopy visualizing hESCs inside microwells (Fig. 2B); and Hoechst DNA-binding dye staining (Fig. 2C). The microwells shown in Fig. 2 were 100 μm/side, though desired hESC localization was also obtained at dimensions ranging from 50 to 600 μm/side (data not shown).

Cell localization inside the wells occurred within 1 day of cell seeding and cells typically reached confluence within 1 week. Fig. 2 shows cultures 21 days after cell seeding and hESCs remained restricted to the wells. After several days in culture, bubbles appeared in the polyurethane substrate, as observed between the wells in Fig. 2C. However, the microwell integrity remained intact.

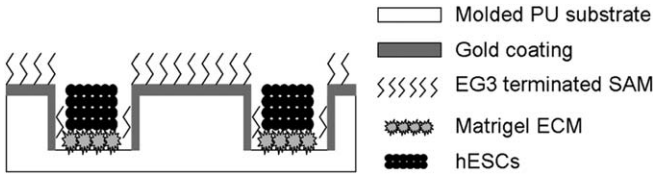

Fig. 1. Schematic of microwell fabrication depicting spatial localization of physical and chemical constraints to hESC attachment and propagation. Microwells are formed by crosslinking polyurethane (PU) prepolymer. Gold is evaporated on the surfaces surrounding the wells and the sides of the wells at an oblique angle, but the bottoms of the wells are shielded from gold. A triethylene glycol-terminated alkanethiol self-assembled monolayer (EG terminated SAM) is then assembled on the gold surface to repel extracellular matrix (ECM) proteins and cells. The wells are then treated with Matrigel which adsorbs to the bare PU at the bottoms of the wells. Cells are cultured in the Matrigel-coated wells. Structures are not to scale.

3.2. hESCS remain viable and undifferentiated for weeks within microwells

Once protein and hESC localization within microwells was established, we characterized the viability and differentiation state of constrained cells. hESC viability was determined by intracellular esterase activity. Calcein AM, which readily permeates cell membranes, was diluted in PBS and added to confluent hESC cultures in microwells, then incubated for 30 min at 37 °C. Live cells with constitutive intracellular esterase activity convert Calcein AM to the polyanionic dye Calcein, which is retained within live cells and detectable via fluorescence microscopy. To verify that hESCs were undifferentiated, cells were also fixed and stained for Oct-4 expression by immunocytochemistry.

hESC viability and Oct-4 expression following 19 days of culture in 120 μm deep microwells at two lateral dimensions, 50 and 100 μm/side, are shown in Fig. 3. All microwells containing cells detectable by phase contrast microscopy also exhibited Calcein fluorescence. The phase dark regions within microwells are confluent cell colonies that replicated to fill the microwells. The inability to focus on a single layer of cells in the microwells resulted in very bright non-localized staining of the corresponding fluorescence images, where fluorescence from several cell layers blended together.

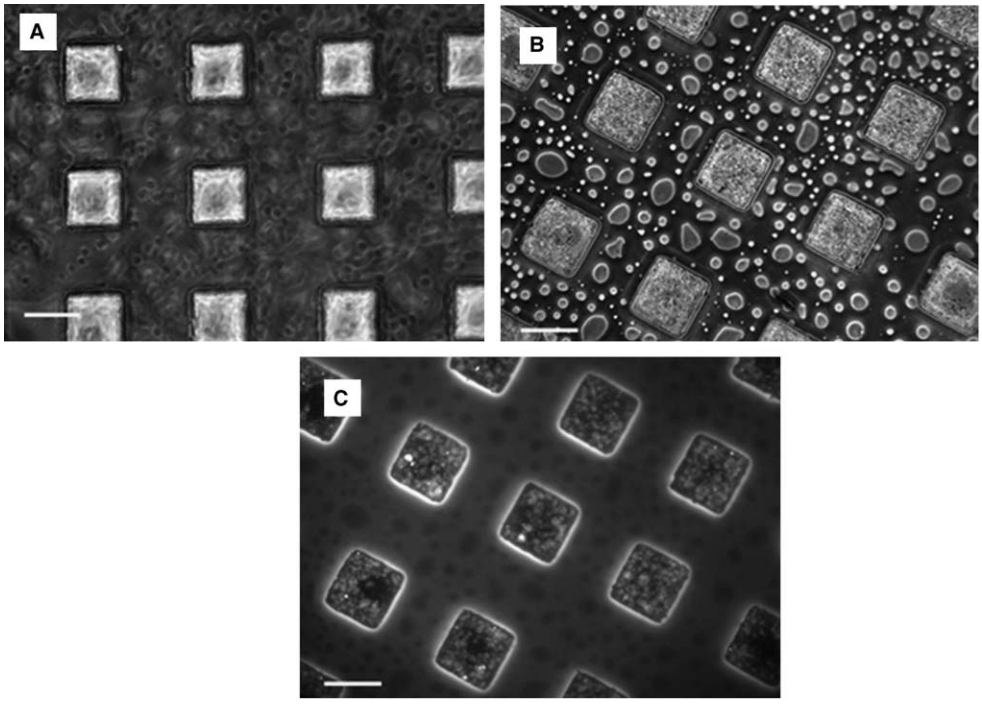

Fig. 2. Localization of hESCs to microwells. Microwells were 120 μm deep and 100 μm/side. (A) Phase contrast images of microwells prior to cell seeding. (B) and (C) hESC localization within microwells after 21 days of culture on Matrigel in CMF+ visualized by: (B) phase contrast; (C) Hoechst nuclear stain. Scale bars: 100 μm.

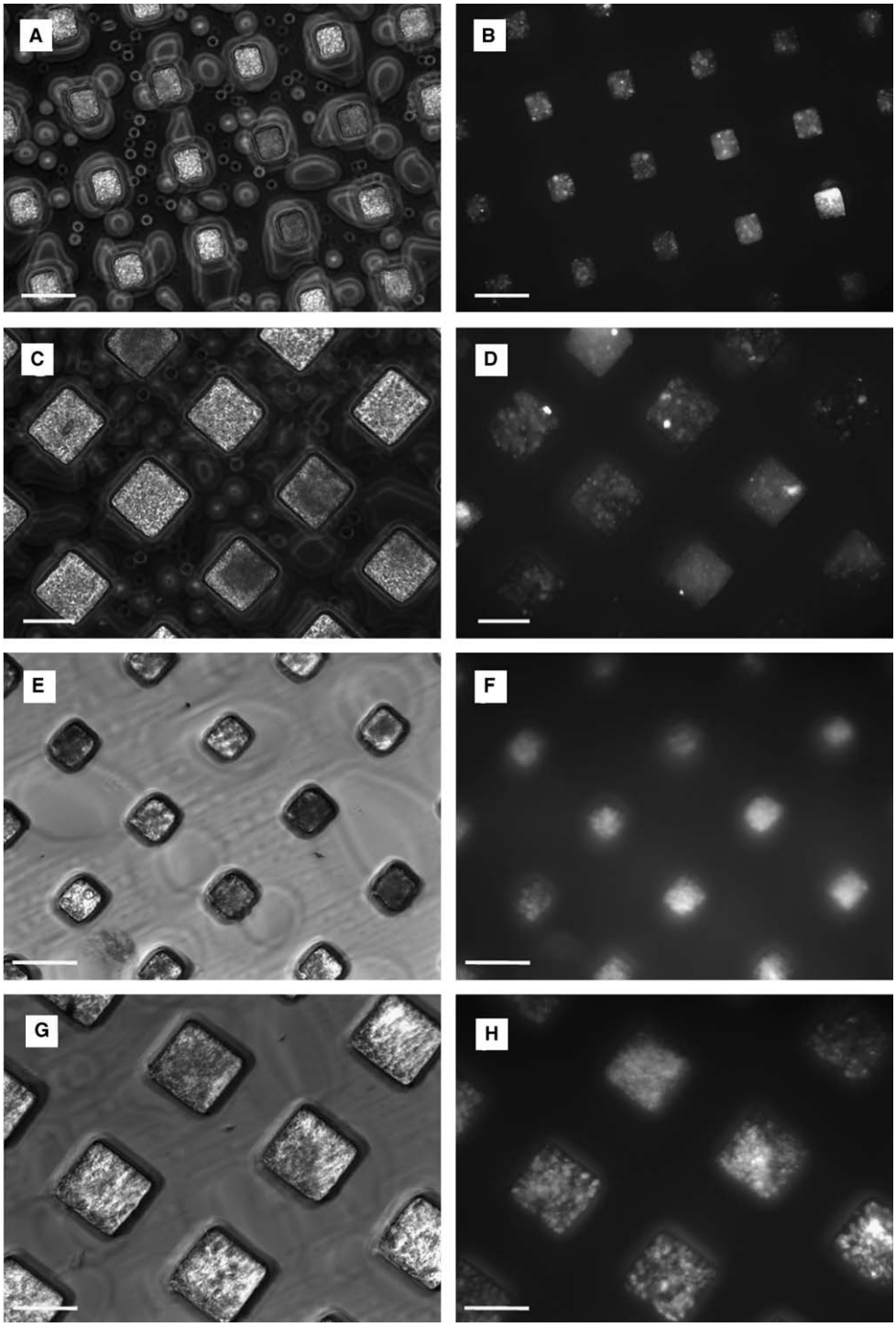

Fig. 3. hESC viability and Oct-4 expression after 19 days of culture on Matrigel-coated microwells in CMF+. Microwells measured 120 μm deep and either 50 μm/side (A and B, E and F) or 100 μm/side (C and D, G and H). Viability was determined by Calcein AM metabolism/intracellular esterase activity. Oct-4 expression was assessed by immunocytochemistry. (A)–(D): Phase contrast (A, C) and corresponding epifluorescence images of Calcein AM (B, D) illustrate hESC viability within microwells. (E)–(H): Phase contrast (E, G) and corresponding epifluorescent images of Oct-4 expression (F, H). Scale bars: 100 μm.

Expression of the transcription factor Oct-4, a marker of undifferentiated hESCs, was used to assess cell differentiation state in the microwells (Fig. 3E–H). Qualitatively, these images show that most cells localized within microwells expressed Oct-4. The images depict several microwells each containing multiple layers of cells that are difficult to discern by phase or fluorescence microscopy.

To quantify differentiation state of hESCs in microwells, cells were harvested following 13 and 18 days of microwell culture (Fig. 4). Metabolic activity of individual cells was

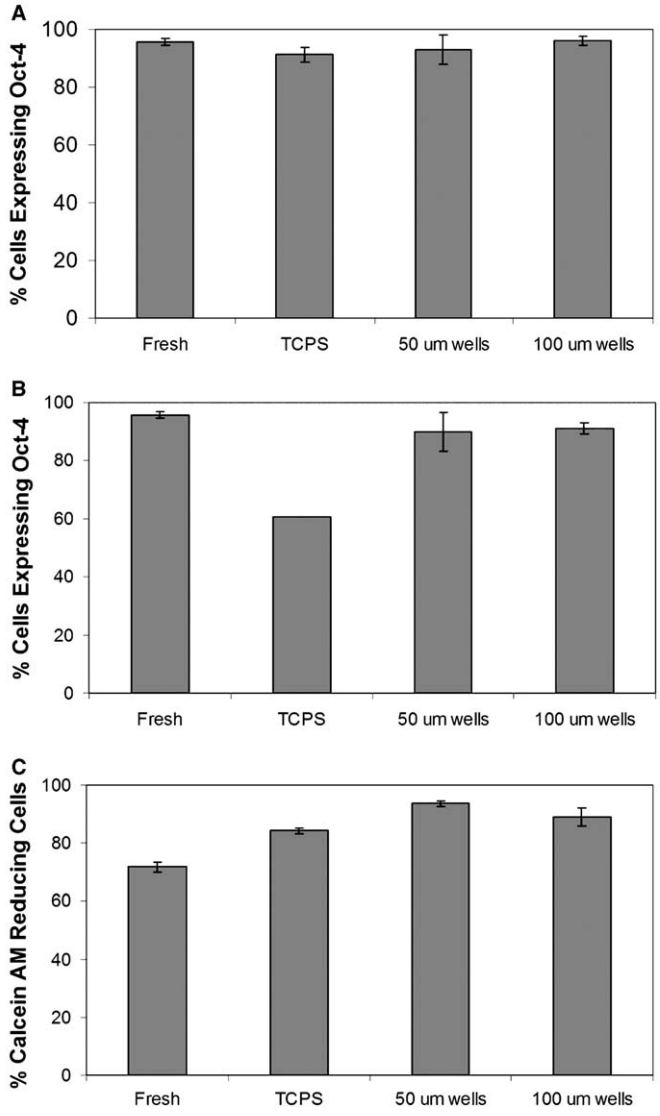

Fig. 4. Comparison of hESC differentiation state and metabolic activity following prolonged culture in microwells versus standard culture in a TCPS dish. Flow cytometric analysis of cells harvested after: (A) 13 days, (B) 18 days, and (C) 20 days. Sample labels "50 μm wells" and "100 μm wells" denote lateral well dimension. *Y*-axis denotes percentage of total cells expressing detectable levels of Oct-4 (A, B) or Calcein AM (C). TCPS dish cultures were sustained on Matrigel in CMF+ concurrently with microwell samples. Fresh controls were day 5 hESC cultures on Matrigel in CMF+. Error bars indicate standard deviation.

quantitatively assessed following 20 days culture in microwells. Cells were removed from the microwells using dispase and trypsin, then treated with Calcein AM or fixed for Oct-4 immunocytochemistry. The fraction of cells able to reduce Calcein AM and the fraction expressing Oct-4 were quantified by flow cytometry. At each microwell depth, two lateral dimensions were analyzed, 50 and 100 μm. Oct-4 expression and Calcein AM reduction in hESCs plated on Matrigel and cultured in CMF+ under typical conditions, without passaging, was also assessed to compare differentiation and viability in microwell culture to differentiation and viability in standard culture conditions. hESCs plated on Matrigel and cultured in CMF+ 5 days prior to fixation were used as positive controls for Oct-4 expression and viability measurements.

Fig. 4A shows little difference in Oct-4 expression between microwell-cultured cells, tissue culture-treated polystyrene dishes (TCPS)-cultured cells, and fresh hESCs at 13 days. Over 90% of cells in each of these culture systems expressed Oct-4. After 18 days, however, clear differences appeared between hESCs cultured in a TCPS dish and those obtained from microwells (Fig. 4B). Oct-4 expression from 50 μm deep × 50 and 100 μm lateral microwells were 90% and 91%, respectively, compared with 96% for fresh controls. The difference in Oct-4 expression between microwell cultures and fresh controls was not statistically significant ($p > 0.10$). Additionally, hESCs cultured on Matrigel-coated TCPS dishes appeared unhealthy and colonies fragmented, leaving many dead cells floating in medium (data not shown). Likewise, 94% of cells in 50 μm wells and 89% of cells in 100 μm wells remained viable after 20 days culture, assessed by Calcein AM reduction, compared with 84% of metabolically active cells in TCPS cultured samples (Fig. 4C). With *p*-values of < 0.0001 and 0.004 for 50 and 100 μm microwells versus TCPS samples, and *p*-values < 0.0001 for both 50 and 100 μm microwells versus fresh Matrigel cultures, there is strong evidence that a larger proportion of hESCs within microwells remain metabolically active than under standard culture conditions. Together, these results indicate that hESCs constrained to microwell geometries remained viable and undifferentiated for extended periods of time.

3.3. *hESCS passaged from microwells to standard cultures maintain undifferentiated replication*

In addition to demonstrating that hESCs remained viable and undifferentiated for extended time periods in microwells, we investigated whether undifferentiated replication occurred after passaging hESCs harvested from microwells to standard culture conditions, e.g. in a TCPS dish on Matrigel or MEFs. To passage hESCs, 18-day microwell cultures were enzymatically detached using dispase. Each slide of microwells was split to one well of a six-well plate coated with Matrigel and cultured for 5 days in CMF+ prior to immunocytochemical analysis of Oct-4 expression.

hESCs were successfully passaged out of 50 μm deep × 50 and 100 μm lateral, and 120 μm deep × 100 μm lateral microwells. Phase contrast images illustrate that the unconstrained colonies were much larger than the microwell features after 5 days and possessed morphology typical of colonies continuously cultured in a TCPS dish (Fig. 5). Epifluorescent images of Hoechst nuclear dye depicted the location of all cells within and surrounding the colony. Finally, epifluorescent images of Oct-4 expression demonstrated that the vast majority of cells harvested from microwells then cultured in a TCPS dish remained undifferentiated.

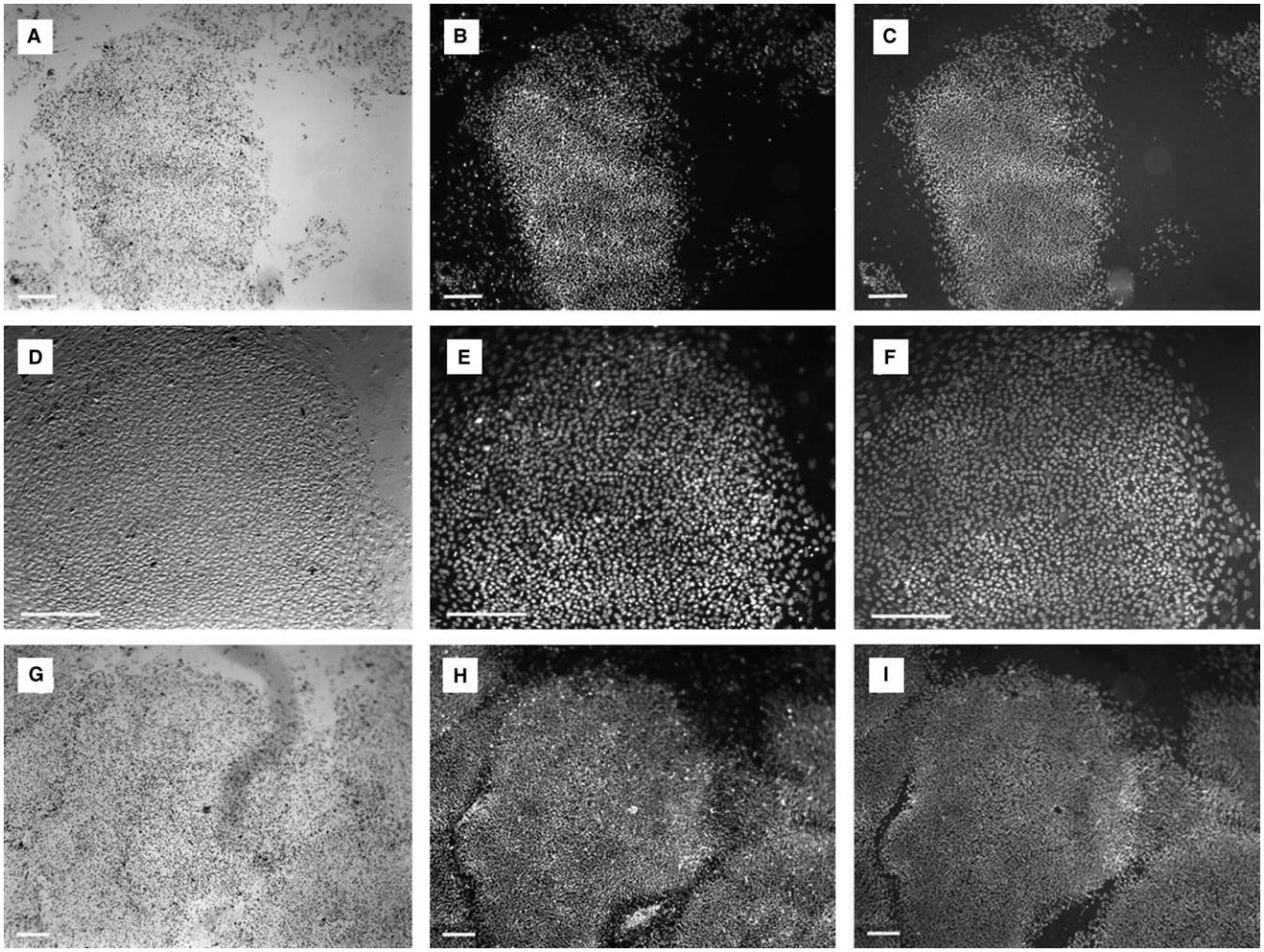

Fig. 5. Transfer of hESCs from microwells to unconstrained growth in a TCPS dish. Phase contrast (A, D, G), Hoechst nuclear dye (B, E, H), and Oct-4 immunocytochemistry (C, F, I) of hESCs harvested after 18 days of culture in microwells, then cultured 5 days in TCPS dishes on Matrigel in CMF+. hESCs were harvested from 50 μm deep, 50 μm/side microwells ((A)–(C)); 50 μm deep, 100 μm/side microwells ((D)–(F)); and 120 μm deep, 100 μm/side microwells ((G)–(I)). Scale bars: 300 μm.

For each microwell size and depth measured, undifferentiated hESC colonies were able to be passaged to unconstrained TCPS culture with little cell differentiation. Typically, hESC colony differentiation on Matrigel begins in the colony interior and spreads radially as the colony grows. The low level of differentiation observed in Fig. 5 typically occurred at the colony edges. The differentiation levels observed in Fig. 5 appear no higher than the 4% typically observed in standard hESC cultures on Matrigel (Fig. 4).

3.4. Homogeneous EBs generated from microwell-cultured hESC aggregates

hESCs cultured in microwells were typically constrained to the well boundaries. However, if care was taken to minimize shear during medium exchange, colony growth expanded into the medium above the microwell. These hESC aggregates growing above the surface could be easily sheared into the medium by gentle pipetting, leaving behind a confluent layer of hESCs within the microwells. This base layer of cells then replicated to fill the microwell and form a new aggregate.

hESC aggregates cultured for 11 days in 50 μm deep × 100 μm lateral microwells grew as relatively monodisperse hESC colonies while attached to microwells (Fig. 6A) and were harvested by gentle pipetting to release them into the medium. To assess viability and differentiation state, aggregates were plated on Matrigel-coated TCPS plates and cultured for 6 days in CMF+. All aggregates attached to the Matrigel substrate and began replication within 1 day (Fig. 6B). Rates of hESC growth were consistent with standard hESC cultures and colonies reached passaging confluence within 6 days. To verify hESCs were undifferentiated, colonies were fixed on the sixth day and analyzed via immunocytochemistry for Oct-4 expression. Very little differentiation occurred around colony perimeter, with no visible differentiation in colony interior, similar to results obtained for enzymatic harvest of entire hESC colonies from microwells.

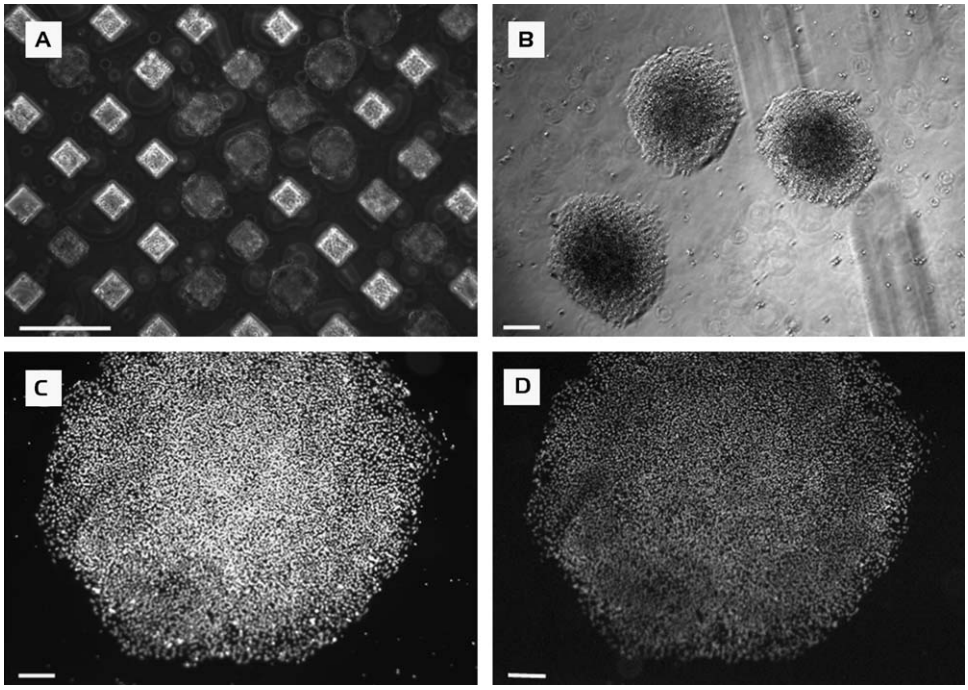

Fig. 6. Undifferentiated hESC aggregates formed on microwells were passaged to TCPS dishes. (A) Phase contrast image of hESCs cultured in 50 μm deep, 100 μm/side microwells for 11 days in CMF+ medium with gentle pipetting to avoid shearing aggregates from microwells. (B) Phase contrast image of undifferentiated aggregates 1-day after removal from microwells by gentle pipeting and cultured on Matrigel in CMF+. (C) and (D) Hoechst nuclear stain and Oct-4 immunocytochemistry of a hESC aggregate harvested from microwells then cultured on Matrigel in a TCPS dish for 6 days. Scale bars: 300 μm.

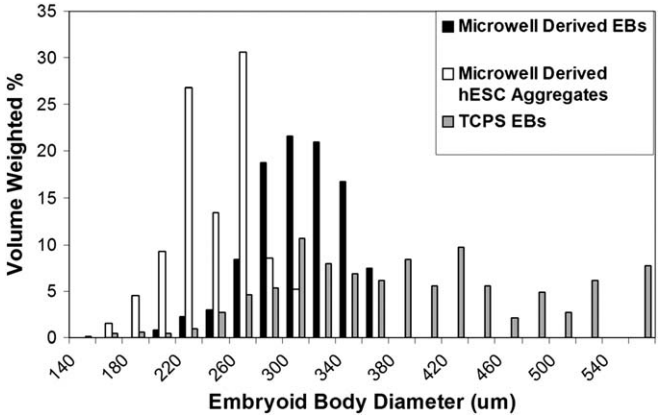

Fig. 7. hESC colony and embryoid body size distributions from microwell-cultured hESCs and from MEF cultured hESCs. *Y*-axis: percentage of cells found in EBs of a specific diameter normalized to total EB count (%). *X*-axis: embryoid body diameter (μm). TCPS EBs were obtained from hESCs cocultured 7 days in UMF+ with MEF feeder cells and then cultured 10 days in suspension to induce differentiation. Microwell-derived EBs were cultured 14 days in 50 μm deep × 100 μm lateral microwells in CMF+ and then cultured 8 days in suspension in UMF− to induce differentiation. Microwell harvested hESC aggregates were collected after 11 days culture in 50 μm deep × 100 μm lateral microwells in CMF+. Histogram consisted of 121 TCPS EBs, 73 microwell-derived EBs, and 42 microwell harvested hESC aggregates.

Fig. 7 quantifies the size distributions of EBs generated from microwell-cultured hESC colonies and TCPS-harvested hESC colonies. The TCPS EBs were formed from hESCs cultured on a MEF feeder layer in UMF+ for 7 days then cultured in suspension to induce differentiation and EB formation for 10 days. The microwell-derived EBs were generated from hESC aggregates cultured 14 days in 50 μm deep × 100 μm lateral microwells in CMF+ before 8 days suspension culture in UMF− to induce differentiation. Individual EB diameter counts were normalized to total number of EBs and weighted by EB volume, such that data represent the percent of cells found in an EB of a particular diameter. Microwell-derived EBs ranged in diameter from 200 to 359 μm, with a majority of cells (78%) found in EBs with diameters between 280 and 359 μm. TCPS-derived EBs, however, were dispersed between 140 and 579 μm diameter, with only 31% of the total cell population found in EBs between 280 and 359 μm in diameter. Microwell-derived EBs yielded a significantly more homogeneous size distribution than EBs derived from hESCs cocultured with MEFs on TCPS. hESC aggregates harvested from microwells also possessed a homogeneous size distribution prior to EB formation. hESC aggregate diameter ranged from 160 to 299 μm, with 80% of hESCs found in aggregates with diameters between 200 and 259 μm.

hESCs within microwells and aggregates released into the medium remained viable, undifferentiated, and able to be passaged. Pluripotency assessment of hESCs cultured in microwells was investigated using hESC aggregates similar to those depicted in Fig. 6A. We demonstrated pluripotency by culturing EBs in two fashions. First, we followed standard protocols and differentiated hESC

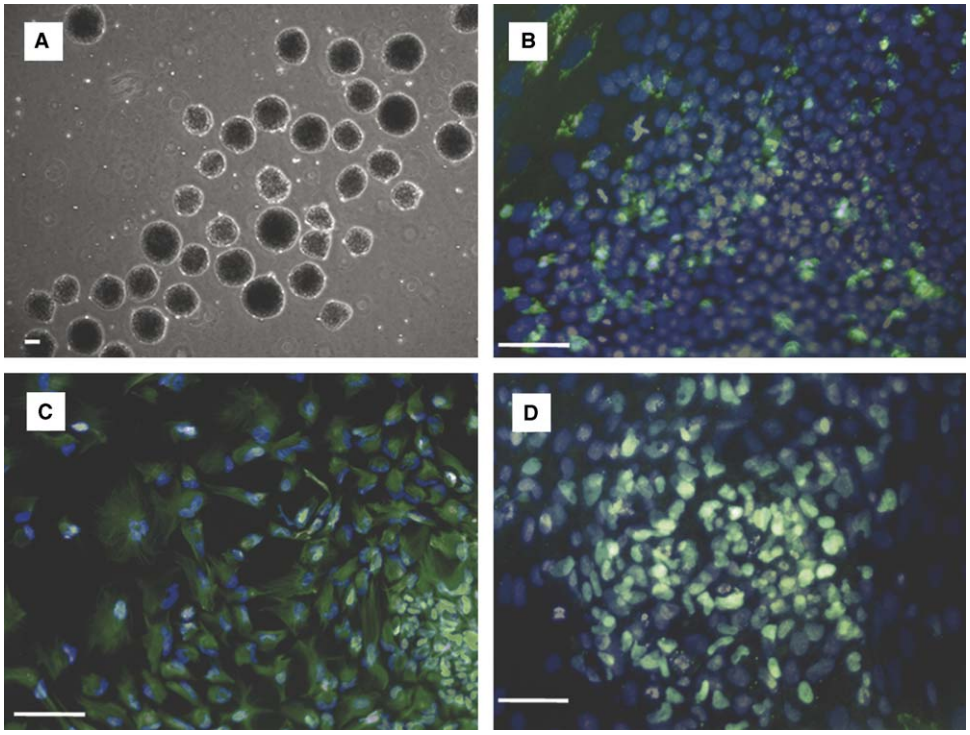

Fig. 8. Embryoid bodies from hESCs cultured for 11 days in 50 μm deep, 100 μm/side microwells retain pluripotency. EBs were cultured in suspension 8 days in UMF− to allow differentiation before plating on gelatin and culturing 8 days in UMF− medium with 5% FBS. (A) Day 0 embryoid bodies on gelatin surface prior to attachment and differentiation. Pluripotency was determined through identification cells in each of the three embryonic germ layers via Hoechst nuclear dye staining (blue) and immunocytochemistry (green) targeting: (B) α-fetoprotein (endoderm); (C) nestin (ectoderm); and (D) brachyury (mesoderm). Scale bars: 100 μm.

aggregates in suspension in UMF− containing 5% FBS before plating to gelatin. Second, we omitted the suspension culture and directly plated hESC aggregates harvested from microwells onto gelatin in UMF− containing 5% FBS. Fig. 8A shows the relatively monodisperse size distribution of EBs created from microwells after 8 days in suspension culture, prior to attachment on the gelatin substrate. Attachment occurred within 2 days of plating and EBs were then cultured 8 days post-attachment on gelatin. Differentiated cultures were fixed and assayed for expression of markers characteristic of the three embryonic germ layers using immunocytochemistry targeting the mesodermal marker α-fetoprotein, the ectodermal marker nestin, and the endodermal marker brachyury. Upregulation of these markers typically occurs at 2 weeks of exposure to differentiation stimuli [33–35], although optimum expression of these markers may not occur at this time point. Fig. 8 illustrates pluripotency of hESCs cultured in microwells via formation of EBs cultured in suspension for 1 week; similar data indicated EBs plated directly to gelatin without culturing in suspension formed cells in each of the embryonic germ layers (data not shown).

4. Discussion

With few exceptions, current literature regarding 2-D microcontact printing focuses primarily on cell attachment and replication to generate confluent monolayers in patterned regions, but does not investigate effects of 3-D confined geometries on long-term health and stability of cell lines that are not strictly contact dependent. hESC attachment to laminin-derived peptides deposited in 750 μm squares through microcontact printing was discussed by Orner et al. [36]. However, the hESCs were only cultured for 2 days before cellular analysis, when significant spontaneous generation is unlikely to occur even in suboptimal conditions, and no cell characterization data (e.g. differentiation or viability) were presented. Although short-term analysis of selective attachment is useful for screening substrates that permit cell adherence and initial replication, several other requirements exist for use as a robust culture technique. hESCs must remain viable, undifferentiated, retain ability for undifferentiated proliferation upon passaging, and remain pluripotent. Because hESC differentiation does not occur immediately, short-term analysis may not accurately represent hESC response to confinement.

Long-term cell culture on microcontact printed substrates has been evaluated for SAM screening and pattern integrity. Luk et al. [16] report tri-ethylene glycol SAMs resist protein and cell adhesion only 6–8 days, after which cells rapidly spread onto EG3-coated areas. Using a modified ethylene glycol SAM, poly L-lysine-g-poly(ethylene glycol), Lussi et al. [14] patterned 3T3 fibroblasts up to 13 days checking for pattern integrity. Cells remained

viable on these microcontact printed surfaces for 13 days; however the cells were not passaged from patterns or characterized in any other fashion. Our initial efforts at 2D microcontact printing of Matrigel on SAM surfaces indicated that this method was not suitable for long-term hESC culture because of substrate instability and the ability of growing colonies to span across unpatterned regions. Thus, we added the additional constraint of physical microwells to the chemical constraint of patterned extracellular matrix.

Prior to this study, 3-D microwells have been used to study effects of confinement on short-term culture of anchorage-dependent cells. First, NIH 3T3 fibroblasts were deposited as single cells in microwells 15 μm deep, 75 μm^2 cross-sectional area [15]. These cells, however, were incubated only 4 h to investigate initial cell attachment and spreading, rather than long-term behavior within microwells. In a separate study, single epithelial cells were deposited in 11 μm deep × 10 μm lateral microwells. However, cell viability after 2 days was determined solely by visual cell replication [29]. These studies demonstrated the possibility of cell attachment within microwells, but did not show a marked improvement over prior microcontact printing techniques which also constrained cells for at least 2 days. After 18 days culture in microwells, over 90% of hESCs expressed Oct-4, and were found to be statistically indistinguishable from fresh Matrigel cultures in terms of differentiation. The ability to sustain high density, undifferentiated hESC cultures for weeks without passaging may have valuable applications to general hESC culture techniques. Additionally, the lack of hESC differentiation after several weeks in constrained culture suggests that differentiation is tightly linked to hESC colony size or shape.

Aside from constraining hESC growth, microwell culture facilitated generation of undifferentiated cell aggregates that were easily passaged or differentiated in suspension to form EB. Lussi et al. [14] also reported formation of 3T3 fibroblast aggregates that maintained pattern boundaries on 2-D microcontact printed substrate by adhering to a base cell layer while growing away from patterns in the *z*-direction. However, cell aggregates were not collected or characterized. EB size has been suggested to influence differentiation fate, although the only reported means of controlling EB size involves enzymatically digesting hESC colonies with trypsin to single cells then centrifuging the desired number of cells to form a pellet [13]. However, trypsin inhibits later hESC aggregation and the cell clump formed by centrifugation is morphologically distinct from typical hESC colonies. Using microwell culture, we can define EB size without compromising cell viability or EB structure.

The development of microwell technology with hESC culture may presumably be merged with many developing technologies, such as high-throughput screening of hESC attachment or differentiation factors. Designing novel biomaterials for hESC adherence and guiding differentiation has been studied on 2-D polymer substrates with protein resistant polymers surrounding islands of various potentially adherent polymer [37]. Microwell technology could be combined with such approaches to identify environmental signals that enhance long-term hESC culture or differentiation toward desired lineages.

5. Conclusions

We have constructed a 3-D microwell system for long-term hESC culture and homogeneous embryoid body formation. hESCs confined to microwells remained viable and undifferentiated after several weeks in culture, as demonstrated by intracellular staining and flow cytometric analysis of hESC markers, and maintained undifferentiated replication when passaged to Matrigel-coated TCPS dishes. Microwell-cultured hESCs retained pluripotency, differentiating to each of the three embryonic germ layers. hESC aggregates released from microwells may be passaged for undifferentiated replication, or differentiated to yield monodisperse EBs. Additionally, the microwell manufacturing process is easily scaled to generate different size and depth microwells, which could aid in dictating EB size. The ability to constrain hESC growth in three dimensions may have several utilities, including more efficient, reproducible culture of undifferentiated cells, high-throughput screening, and guiding hESC differentiation via generation of monodisperse EB.

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