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Original Article

Expansion and Differentiation of Human Embryonic Stem Cells to Endoderm Progeny in a Microcarrier Stirred-Suspension Culture

Lye T. Lock, B.S.,[1] and Emmanuel S. Tzanakakis, Ph.D.[1,2]

Embryonic stem cells (ESCs) with their abilities for extensive proliferation and multi-lineage differentiation can serve as a renewable source of cellular material in regenerative medicine. However, the development of processes for large-scale generation of human ESCs (hESCs) or their progeny will be necessary before hESC-based therapies become a reality. We hypothesized that microcarrier stirred-suspension bioreactors characterized by scalability, straightforward operation, and tight control of the culture environment can be used for hESC culture and directed differentiation. Under appropriate conditions, the concentration of hESCs cultured in a microcarrier bioreactor increased 34- to 45-fold over 8 days. The cells retained the expression of pluripotency markers such as OCT3/4A, NANOG, and SSEA4, as assessed by quantitative PCR, immunocytochemistry, and flow cytometry. We further hypothesized that hESCs on microcarriers can be induced to definitive endoderm (DE) when incubated with physiologically relevant factors. In contrast to embryoid body cultures, all hESCs on microcarriers are exposed to soluble stimuli in the bulk medium facilitating efficient transition to DE. After reaching a peak concentration, hESCs in microcarrier cultures were incubated in medium containing activin A, Wnt3a, and low concentration of serum. More than 80% of differentiated hESCs coexpressed FOXA2 and SOX17 in addition to other DE markers, whereas the expression of non-DE genes was either absent or minimal. We also demonstrate that the hESC-to-DE induction in microcarrier cultures is scalable. Our findings support the use of microcarrier bioreactors for the generation of endoderm progeny from hESCs including pancreatic islets and liver cells in therapeutically useful quantities.

Introduction

Human embryonic stem cells (hESCs) hold a great promise as a renewable source of cellular material for regenerative therapies against maladies that were considered incurable before. One such disease is type 1 diabetes characterized by loss of pancreatic insulin-producing cells due to autoimmune destruction. Frequent administration of insulin is essential for survival of diabetes patients, but it does not cure diabetes nor does it prevent its long-term complications. In contrast, islet transplantation has afforded remarkable benefits, including liberation from insulin dependence for more than 5 years.[1] Yet, wide application of this therapeutic modality is impractical, given the severe shortage of donor islets. Now recent advances in the differentiation of stem cells to pancreatic islet hormone-expressing cells[2–4] suggest that hESCs may be used to derive transplantable islets.

Translating these advances to therapies requires the development of technologies for large-scale generation of islet cells from stem cells. Maintenance and differentiation of hESCs are typically carried out in static cultures (e.g., dishes) that are exceedingly difficult to scale up. Improved expansion of hESCs is possible in perfusion cultures featuring gas-permeable substrata[5] although the number of cells is still tied to the surface area available for growth. Human ESCs can also be cultured in suspension bioreactors as embryoid bodies (EBs).[6,7] These culture systems have a simple design, are amenable to scale up, and allow for better monitoring/control of the culture environment through automation. Nevertheless, ESCs differentiate within EBs via nonselective and poorly understood mechanisms.

To this end, hESCs in monolayers can be coaxed with high efficiency to become definitive endoderm (DE)[8] and eventually islet-like cells[2] when treated with physiologically relevant factors recapitulating stages of embryonic pancreas development. An important observation is that efficient induction to DE and beyond relies on avoidance of EB formation. Unlike in EBs, all hESCs in monolayers are exposed to soluble factors stimulating differentiation.

[1]Department of Chemical and Biological Engineering, State University of New York at Buffalo, Buffalo, New York.
[2]New York State Center of Excellence in Bioinformatics and Life Sciences, Buffalo, New York.

It is implied then that stirred-suspension vessels for the culture of hESCs as EBs may not be optimal for directed differentiation to endoderm lineages. Still, we hypothesize that such bioreactors can be used in conjunction with microcarriers for propagating hESCs and then coaxing them to endoderm progeny. In addition to the advantages of stirred bioreactors, microcarrier systems exhibit high surface-to-volume ratio, which can be easily adjusted. In principle, all cells growing on the surface of (non-macroporous) microbeads are exposed to the bulk medium, thus allowing for better control of stem cell differentiation to a specific lineage. Expansion of mouse ESCs (mESCs) in microcarrier bioreactors has been reported,[9,10] but translation of these findings to the microcarrier culture of hESCs is challenging mainly due to differences between the culture methods of mESCs and hESCs. For example, unlike mESCs, hESCs are typically passaged as small clumps and not as single cells. Moreover, a single factor (leukemia inhibitory factor) is commonly used to maintain mESCs undifferentiated, whereas such a factor is not presently available for the self-renewal of hESCs.

We set out to explore if hESCs can be grown and differentiated toward DE cells on microcarriers in a stirred-suspension culture. Human ESCs cultured on microbeads grew under suitable culture conditions (mainly the stirring speed and seeding cell concentration) while expressing pluripotency markers. Then, hESCs expanded on microbeads were incubated in media known to induce DE formation in hESC monolayers. Differentiated cells were probed for DE markers. Our results support the use of scalable microcarrier suspension cultures for the culture and directed differentiation of hESCs.

Materials and Methods

Human embryonic stem cell culture

The hESC lines H1 (passages 29–43) and H9 (passages 24–45) were obtained from the WiCell Research Institute (Madison, WI). The use of the cells in this project has been approved by the Committee for Stem Cell Research Oversight at SUNY–Buffalo. Cells were maintained on mouse embryonic fibroblasts (mEFs) treated with mitomycin C (Sigma-Aldrich, St. Louis, MO). The culture medium consisted of 20% KnockOut serum replacement (Invitrogen, Carlsbad, CA) in Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F12) with 2 mM L-glutamine, 1 mM nonessential amino acids, 100 U/mL penicillin/100 μg/mL streptomycin (all from Invitrogen), and 4 ng/mL basic fibroblast growth factor (bFGF; Sigma-Aldrich). Medium was replaced every day, and the cells were subcultured every 6–7 days by enzymatic dissociation with collagenase type IV (Invitrogen). Cultured hESCs are routinely checked for normal karyotype (Spectral Karyotyping Analysis Facility, Roswell Park Cancer Institute, Buffalo, NY).

Conditioned medium (CM) was prepared as described before.[11] The CM was filter-sterilized and supplemented with bFGF before its addition to hESCs. For mEF-free propagation, hESCs were plated on dishes coated with Matrigel (BD Biosciences, San Jose, CA) and grown in CM in 5% CO_2/95% air at 37°C. The medium was replaced daily.

Seeding of microcarrier bioreactors with hESCs

Collagen-coated microcarriers (Hyclone, Logan, UT) were suspended in PBS and autoclaved at 121°C for 30 min before their use. The beads were then coated with Matrigel at room temperature for 1 h, washed twice with DMEM/F12, and further equilibrated in CM for 1 h. Five hundred mg of beads ($\sim$180 cm^2 surface area) per 50 mL of culture medium was utilized for the experiments reported here. The microcarriers were transferred to Petri dishes with $2.5–10\times10^6$ hESCs ($\sim$12–50 cells/bead) and placed in a 5% CO_2 incubator at 37°C for 4 h to allow cell attachment to beads. Subsequently, the cell/bead suspension was transferred to ProCulture spinner flasks (Corning, Corning, NY). The total volume of CM was brought to 50 mL, and intermittent stirring at 45 rpm (1–2 min every 30 min) was performed for 3 h. The medium was exchanged once to remove nonadherent cells, and the agitation rate was set to 45–80 rpm as noted for the duration of each run. The cultures were maintained at 37°C in 5% CO_2/95% air. Culture medium (half-volume) was replaced every 2 days.

Cell viability and microcarrier colonization

Cell/microcarrier samples withdrawn from each bioreactor were stained with 20 μg/mL fluorescein diacetate (FDA-live cells; Sigma-Aldrich) and 10 μg/mL propidium iodide (PI-dead cells; Sigma-Aldrich) in PBS for 3–5 min. Cells were washed with PBS twice, placed on a fluorescence-inverted microscope (Nikon Diaphot; Nikon, Melville, NY) and images were obtained with a digital camera. The total number of beads and the number of beads that were colonized with cells were counted, and the percentage of colonized beads was calculated. Cells on beads were further treated with TrypLE, stained with the Trypan Blue dye (all from Invitrogen), and counted in a hemacytometer.

Supernatant samples were also collected, and the lactate dehydrogenase (LDH) activity was determined with a cytotoxicity detection kit (Roche, Indianapolis, IN) according to the manufacturer's instructions.

Definitive endoderm differentiation

Human ESCs were coaxed to DE by implementing a previously described protocol[8] with modifications. Cells were seeded at 4×10^4 cells/cm^2 on Matrigel-coated dishes or glass slides with chambers (Nagle Nunc International, Rochester, NY), and differentiation was initiated after the cells reached 80% confluence by washing with PBS and incubating in RPMI (Invitrogen) containing 100 ng/mL activin A and 25 ng/mL Wnt3a (R&D Systems, Minneapolis, MN) for 24 h. Then, the cells were treated with 0.2% FBS (Sigma-Aldrich) and 100 ng/mL activin A in RPMI for 24 h. At 48 h onward, cells were maintained in RPMI containing 2% FBS and 100 ng/mL activin A. Cultured cells were characterized on day 4 or 5 of differentiation. Control cultures were treated with the same media but without activin A/Wnt3a.

For cells grown on microcarriers, differentiation was initiated when the cell concentration peaked. The cells were washed twice with RPMI basal medium before incubated with 100 ng/mL activin A and 25 ng/mL Wnt3a in RPMI for 24 h. Next, half of the medium was replaced with the same

volume of RPMI with 200 ng/mL activin A and 0.4% FBS. The same procedure was repeated the next day to bring the final concentration to 2% FBS/RPMI with 100 ng/mL activin A. Differentiations were carried out under constant agitation.

Primitive gut tube differentiation

Human ESCs on beads induced to differentiate into DE cells were harvested with collagenase and seeded on Matrigel-coated glass slides with chambers. Differentiation to primitive gut tube (PGT) was induced in RPMI with 2% FBS and 50 ng/mL keratinocyte growth factor (R&D Systems). Three days later the cells were probed for PGT markers.

Immunocytochemistry

Cells plated on glass slides were fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS and blocked/permeabilized in PBS with 0.1% Triton X-100 (Mallinckrodt Baker, Phillipsburg, NJ) and 1% bovine serum albumin for 30 min. Samples were incubated overnight at 4°C with the following primary antibodies: rabbit OCT3/4A and goat HNF1B (both from Santa Cruz Biotechnology, Santa Cruz, CA), goat NANOG, mouse SOX17, and goat FOXA2 (all from R&D Systems), and mouse TRA-1–81 and mouse SSEA4 (both from Abcam, Cambridge, MA). After three washes with PBS, cells were incubated with appropriate secondary antibodies for 1 h at room temperature. Donkey anti-rabbit, -goat, and -mouse secondary antibodies conjugated with FITC or Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) were used. Nuclear DNA was stained with DAPI (Sigma-Aldrich). Mounting medium was added (Invitrogen), and the slides were sealed with coverslips. Fluorescence images were acquired on a microscope (Nikon) equipped with a QImaging Retiga CCD camera (Burnaby, BC, Canada).

For cell staining on beads, 1-mL samples were transferred to 1.5 mL microcentrifuge tubes and allowed to settle before rinsing with PBS. Cells on microcarriers were fixed, permeabilized, and stained with antibodies as described above for plated cells except that nuclear DNA was stained with TO-PRO-3 iodide (Invitrogen). Fluorescence images were acquired with a Zeiss LSM 510 Meta NLO confocal microscope (Carl Zeiss, Thornwood, NY) and processed with the AxioVision software.

Flow cytometry

Cells on microcarriers were incubated with TrypLE before passing the suspension through 100-μm mesh strainers (BD Biosciences) to separate the cells from the beads. Cells were collected by centrifugation at 500 *g* for 5 min and fixed for 10 min with 3.7% formaldehyde solution (Sigma-Aldrich). Cells were washed with PBS, blocked with 3% normal donkey serum for 20 min, and incubated with primary antibodies: rabbit OCT3/4A, mouse SOX17, goat FOXA2, and rabbit BRACHYURY (Abcam) for 1 h at room temperature. Cells were washed again and incubated with appropriate Cy3- or FITC-conjugated donkey secondary antibodies for 40 min at room temperature. For SSEA4 staining, cells were incubated with a mouse SSEA4 (Abcam) antibody for 40 min and with a secondary antibody (Jackson ImmunoResearch Laboratories) at room temperature for 30 min. Sample analysis was carried out in a FACS Calibur flow cytometer with the CellQuest software (Becton Dickinson, Franklin Lakes, NJ). Cells were registered as positive for a particular antigen if their emitted fluorescence level was higher than 98% of that of samples stained only with the corresponding secondary antibodies.

RT-PCR and quantitative PCR

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions, and reverse transcription (RT) was performed using the ImPromII reverse transcriptase (Promega, Madison, WI). Subsequent PCRs were performed for 30–35 cycles at an annealing temperature of 58–60°C depending on the primer set (Supplemental Table S1).

Quantitative PCR (qPCR) was performed on an iCycler machine (Bio-Rad, Hercules, CA) using the Dynamo qPCR Mix (Finnzymes, Espoo, Finland) under the following conditions: denaturation and polymerase activation at 95°C for 2 min; amplification for 40 cycles at 95°C for 15 s, 58–60°C for 30 s, and 72°C for 1 min. All reactions were run in triplicates. Amplification specificity was verified by the melting curve method and gel electrophoresis. Relative gene expression was calculated by normalizing to the expression of endogenous β-*ACTIN*, using the $\Delta\Delta C_T$ method.[12] The C_T for the housekeeping gene did not vary under different experimental conditions when equal amounts of RNA were used. PCR efficiencies for different primer pairs were determined by a standard curve method on serially diluted templates and found to be ~ 1.

Statistical analysis

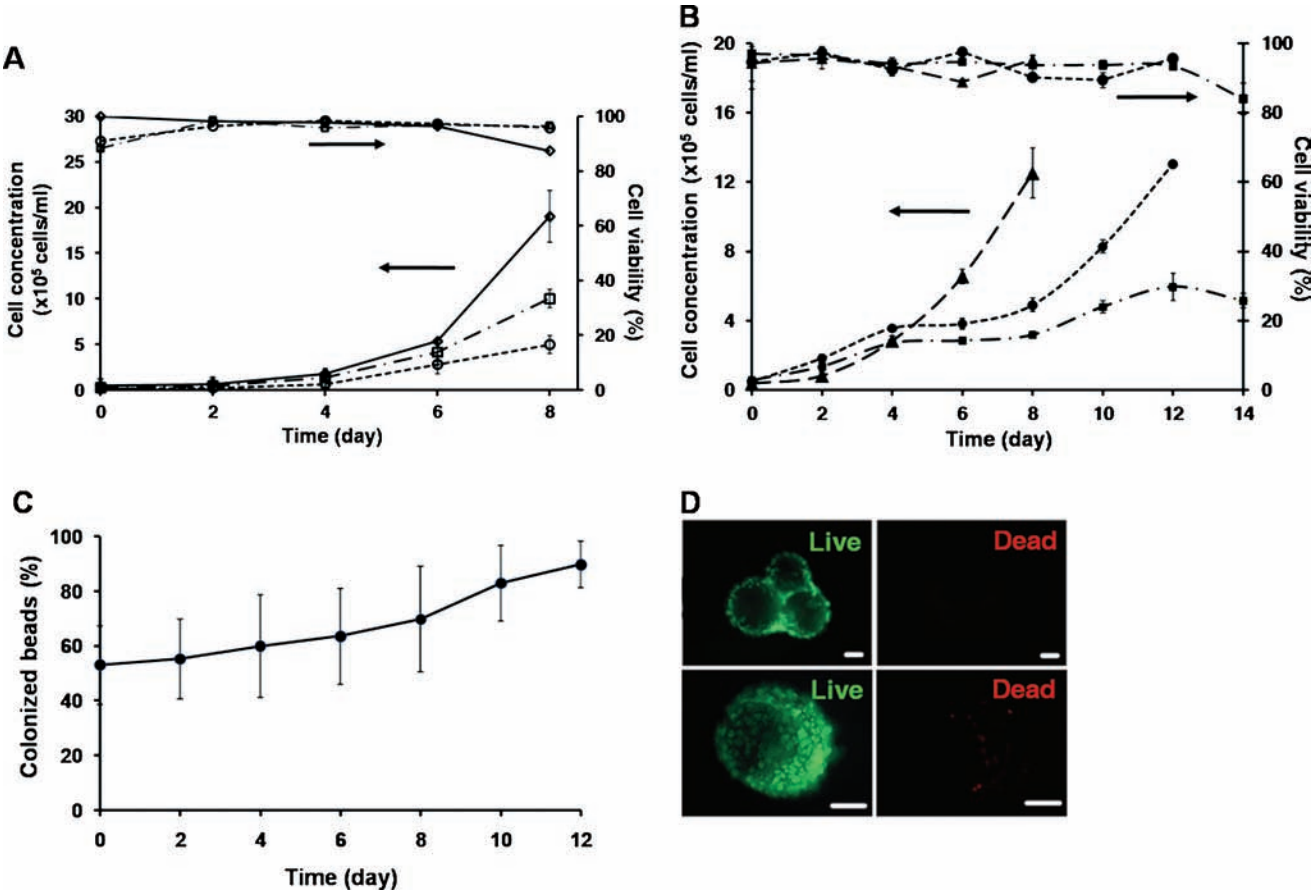
Data are presented as mean $\pm$ SD from at least three independent experiments. Statistical analysis, including ANOVA, was performed using Minitab (Minitab, State College, PA). *p*-Values less than 0.05 were considered significant.

Results

Expansion of hESCs in a microcarrier suspension bioreactor

Human ESCs are typically maintained in dishes with feeder cell layers (e.g., mEFs) or coated with Matrigel. We developed a method for cultivation of hESCs on microcarriers in stirred-suspension vessels. Cells were cultured on Matrigel-coated plates for 1–2 passages to eliminate residual growth-arrested mEFs. After enzymatic dissociation, hESC clumps were transferred first to Petri dishes for initial attachment on Matrigel-coated microcarriers and then to spinner flasks.

The number of cells attached on beads at this stage depends to a large extent on the seeding cell density. For this study, the total amount (and number) of beads was kept constant while the cell inoculum concentration was varied. Cells were loaded onto microcarriers at 5, 10, and 20×10^4 hESCs/mL (i.e., 12.5, 25, and 50 hESCs/bead), whereas higher hESC seeding concentrations resulted in the formation of EB-like structures. The agitation rate was set at 45 rpm (Fig. 1A) for the duration of the run. The fraction of seeded



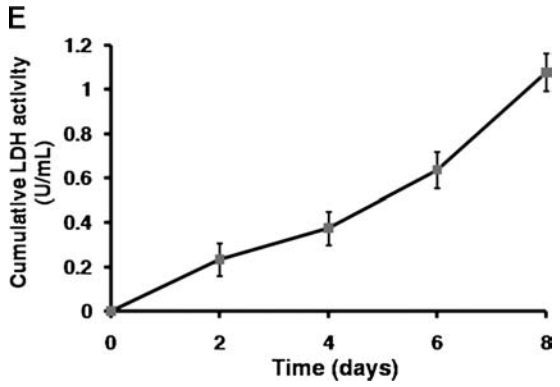


FIG. 1. Culture of hESCs in a microcarrier suspension. (**A**) H9 cells were seeded at 5×10^4 (-◇-), 10×10^4 (-■-), and 20×10^4 (-◆-) hESCs/mL and cultured at 45 rpm. (**B**) Human ESCs were seeded at 1×10^5 cells/mL and propagated at 45 (-▲-), 60 (-●-), and 80 (-■-) rpm. (**C**) Colonization of beads by hESCs cultured at 60 rpm. (**D**) FDA/PI staining of hESCs on beads at 45 rpm (day 8). (**E**) Cumulative LDH activity for hESCs cultured at 45 rpm. Bars in (**D**): 50 μm. Color images available online at www.liebertonline.com/ten.

Table 1. Seeding Efficiency and Doubling Time of H9 hESCs Cultured with Microcarriers at Different Seeding Densities and at 45 rpm

	Initial cell concentration (hESCs/mL)		
	5×10^4	*10×10^4*	*20×10^4*
Seeding efficiency (%)	30 ± 2.5	22.8 ± 4.2	24.3 ± 6.6
Doubling time (h)	38.1 ± 1.5	35.1 ± 2.1	36.4 ± 3.83

See also fig. 1A.

cells attached on the microcarriers (seeding efficiency) ranged from 23–30% (Table 1). However, the cells grew at comparable rates resulting in 34–45-fold increase in hESC concentration over 8 days.

Subsequently, we examined the growth of hESCs on microcarriers under different agitation rates. For this purpose, 1×10^5 hESCs/mL were seeded with microcarriers and cultured in spinner flasks at 45, 60, and 80 rpm. A peak concentration of $\sim1.25\times10^6$ live hESCs/mL was reached at 8 days (doubling time $t_d = 36.7 \pm 1.3$ h) when the cells were cultured at 45 rpm (Fig. 1B). At 60 rpm, a maximum concentration of 1.3×10^6 hESCs/mL was observed at 12 days ($t_d \sim 49$ h). Nonetheless, cells had grown only up to 0.6×10^6 hESCs/mL after 12 days at 80 rpm.

Peak hESC concentrations at 45 and 60 rpm coincided with colonization of almost 95% of the beads (Fig. 1C, D) as tracked by live/dead cell staining. Bead agglomerates covered with cells were also observed. The average number of populated beads increased over time, suggesting that initially empty microcarriers were utilized by proliferating hESCs.

The viability of cells attached on the microcarriers was also determined by Trypan Blue dye exclusion. For the conditions described here, hESC viability was consistently above 85%. We also determined the LDH activity in the medium to detect possible cell lysis (Fig. 1E). Cumulative LDH increased over time, indicating that cell lysis occurred to some extent although the maximum LDH activity observed was substantially lower than values reported previously for hESCs[7] (50–150 U/mL) cultured in suspension. Hence, these results support that hESCs can be grown in a microcarrier culture.

Gene expression of hESCs propagated in a microcarrier bioreactor culture

Next, we examined if hESCs in the bioreactor retain the expression of pluripotency markers. The expression of *POU5F1* (denoted as *OCT3/4A* for targeting specifically its segment corresponding to the long protein isoform OCT3/4A[13–15]), *NANOG*, and *REX1* (also known as *ZFP42*) was analyzed with RT-PCR (Fig. 2A). Expression of pluripotency markers was constant during the culture in agreement with qPCR results (Fig. 2B). We also probed the expression of genes suggestive of tri-lineage differentiation. The expression of these genes was either minimal (e.g., *FOXA2*, *PAX6*, and *BRY*) or absent (e.g., *SOX17*, *SOX1*, and *NKX2.5*).

Pluripotency markers in cultured hESCs were also probed by immunocytochemistry and flow cytometry. Cells dissociated from beads were plated on Matrigel-coated dishes forming colonies typical of undifferentiated hESCs (data no shown) and were positive for TRA-1–81, NANOG, OCT3/4A, and SSEA4 (Fig. 2C–E). This was further corroborated by flow cytometry analysis of hESCs cultured for 8 days in the bioreactor (Fig. 2F, G). Approximately $81.36 \pm 0.48\%$ was OCT3/4A$^+$ compared to $89.74 \pm 0.79\%$ of hESCs grown in Matrigel-coated dishes. Similarly, $84.77 \pm 0.33\%$ of hESCs in microcarrier bioreactors was SSEA4$^+$ ($98.54 \pm 0.04\%$ in static cultures). The difference in the presentation of surface SSEA4 may be in part due to the enzymatic dissociation of cells from beads during sample preparation.

Our findings show that pluripotency markers do not deteriorate significantly during hESC propagation in microcarrier cultures.

Differentiation of hESCs to definitive endoderm in a microcarrier suspension culture

We then set out to investigate if directing hESCs to endoderm progeny while cultured on microcarriers was feasible. Approximately 1×10^5 hESCs/mL were seeded on microcarriers in CM and expanded at 45 rpm. When a concentration of 1×10^6 hESCs/mL was reached after 8 days (Fig. 3A), the CM was replaced with differentiation medium containing activin A, Wnt3a, and serum at 0.2–2%. Human ESCs maintained in dishes and treated with these media were previously shown to become DE.[8] The cell concentration increased during the expansion phase, but a decline was noted when differentiation was commenced. The drop in cell number was reflected by the increase in cumulative LDH activity (Fig. 3B) although the viability of cells attached to microcarriers remained above 85%. During the differentiation though, a stable number of cells on beads was maintained ($\sim4\times10^5$ hESCs/mL). Control bioreactors running in parallel showed a similar decline in cell concentration when CM was replaced with differentiation medium excluding activin A/Wnt3a.

Characterization of differentiated hESCs

We monitored the dynamics of gene expression of hESCs differentiated in the bioreactor. Stem cell markers *OCT3/4A* and *NANOG* decreased after the removal of CM and the addition of differentiation medium (Fig. 4A). Activin and Wnt signaling in low serum medium induce the transition of hESCs to DE in dishes.[8,16] Thus, we probed the cells cultured on microcarriers for the expression of DE markers such as *FOXA2*,[17,18] *SOX17*,[19] *GSC* (goosecoid),[20] and *CER1* (cerberus 1).[21–23] A substantial upregulation of these genes was noted 24 h after treatment with activin A/Wnt3a began (Fig. 4B). In contrast, cells from control cultures exhibited very low expression of *SOX17*.

However, the expression of these genes is not confined to DE, and there is no known marker that is uniquely expressed in DE. Therefore, we looked for markers that are present in primitive/visceral/parietal endoderm but not in DE. One such gene is *SOX7*,[19] which was detected in samples from control cultures, but its expression was minimal in cells treated with activin A and Wnt3a. Similarly, there was very low expression of α-fetoprotein (*AFP*; visceral endoderm), *SOX1*[24] (ectoderm), *ZIC1*[25] and *CDX1*[26] (ectoderm/mesoderm), and *NKX2.5*[27] (cardiac mesoderm) in treated cells although their expression (except for *SOX1*) was apparent in cells cultured in control bioreactors (Fig. 4C).

Differentiated hESCs on microcarriers were analyzed for their immunoreactivity to FOXA2 and SOX17. The majority of cells coexpressed these antigens as evidenced by immunocytochemistry (Fig. 5A, B) and flow cytometry (Fig. 5C–E). Approximately, $84.25 \pm 2.35\%$ of H9 cells treated with activin A/Wnt3a on microcarriers were both FOXA2$^+$ and SOX17$^+$ after differentiation, whereas only $23.2 \pm 2.4\%$ of control cells coexpressed FOXA2 and SOX17. Differentiation of H9 cells in dishes resulted in $63.2 \pm 0.48\%$ of FOXA2$^+$/SOX17$^+$ cells. Induction of other hESC lines[8] to DE in dish cultures reportedly resulted in 80% (or more) FOXA2$^+$/SOX17$^+$ cells.

Given the different proclivities for endoderm specification of various hESC lines,[28] we also subjected H1 cells to differentiation with the same protocol (Supplemental Fig. S1). Expansion of 1×10^5 H1 cells/mL on microcarriers (45 rpm) led to 10.8×10^5 hESCs/mL in 8 days. After 4 days of differentiation, $82.23\pm6.56\%$ of the cells was positive for both FOXA2 and SOX17 compared to 67.5% in static cultures. Thus, the microcarrier system may be utilized independently of the hESC lines cultured for directed differentiation.

Treatment of ESCs[2,29–31] with activin A causes their transition through a mesendoderm intermediate with a characteristic expression profile for BRACHYURY. We also observed a pronounced expression of BRACHYURY (Fig. 5F) with more than 90% of the cells being positive at 24 h after exposure to activin A and Wnt3a. This fraction decreased to less than 3.5% at 96 h (Fig. 5G) with a concomitant increase in $SOX17^+$ (and $FOXA2^+$) cells indicating a mesendoderm transition.

Our results strongly support that hESCs can be directed to DE-like cells in a microcarrier suspension bioreactor.

Scalability of directed differentiation of hESCs cultured in a microcarrier suspension

After differentiation, there were 20×10^6 cells/50 mL in microcarrier culture (Fig. 3A) compared to 3.2×10^6 cells/4 mL in 6-cm dishes. If the DE cell population is represented by cells coexpressing FOXA2 and SOX17, then there are at least 16.9×10^6 DE cells (84.25%) in the bioreactor compared to

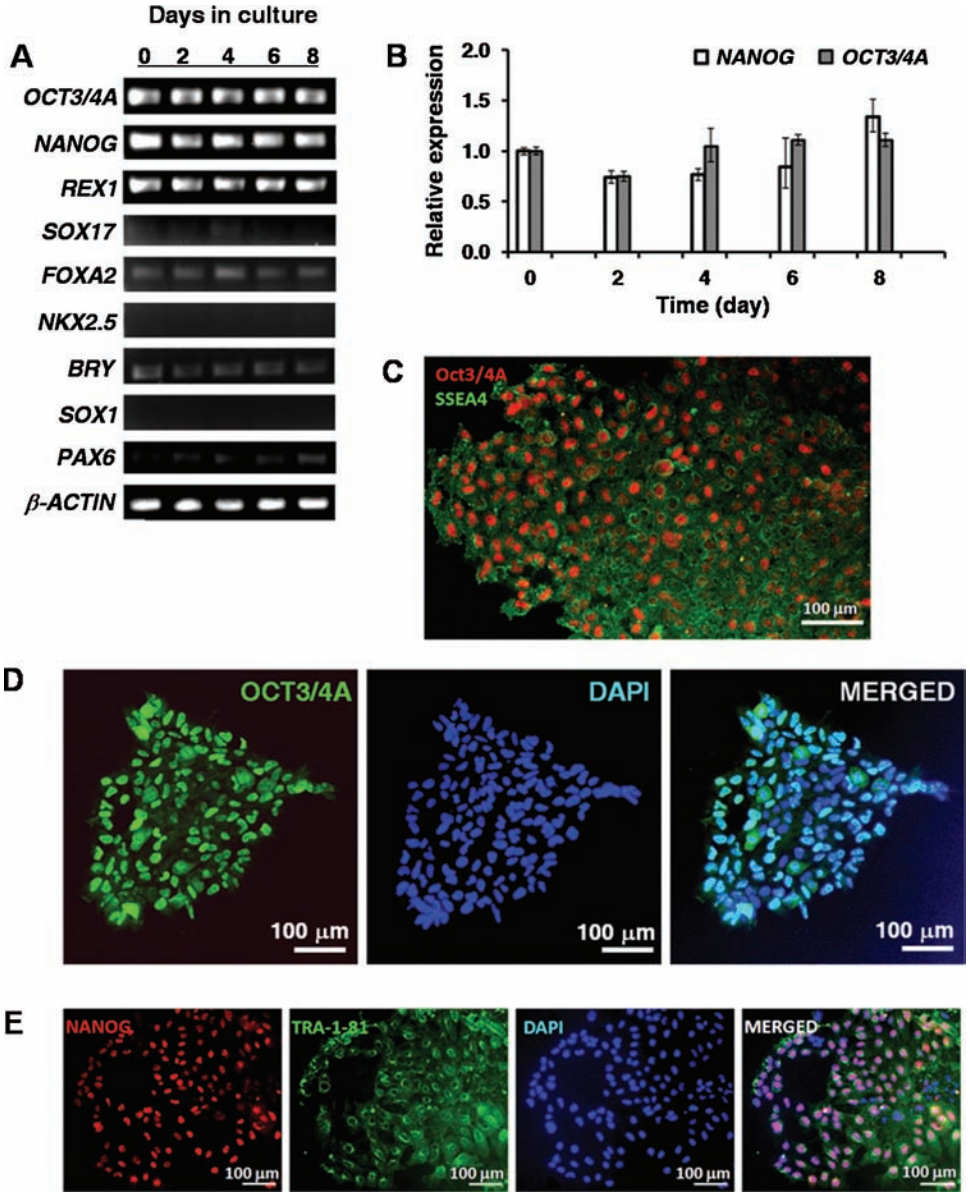


FIG. 2. Human ESCs expanded on microcarriers retain the expression of pluripotency markers. The expression profile of cultured hESCs was analyzed by (**A**) RT-PCR, (**B**) qPCR, (**C–E**) immunocytochemistry, and (**F, G**) flow cytometry. In (**A**) gene expression suggestive of tri-lineage differentiation was minimal. Black curves in (**F, G**) represent controls. The data shown were obtained from samples from a representative run at 45 rpm. Color images available online at www.liebertonline.com/ten.

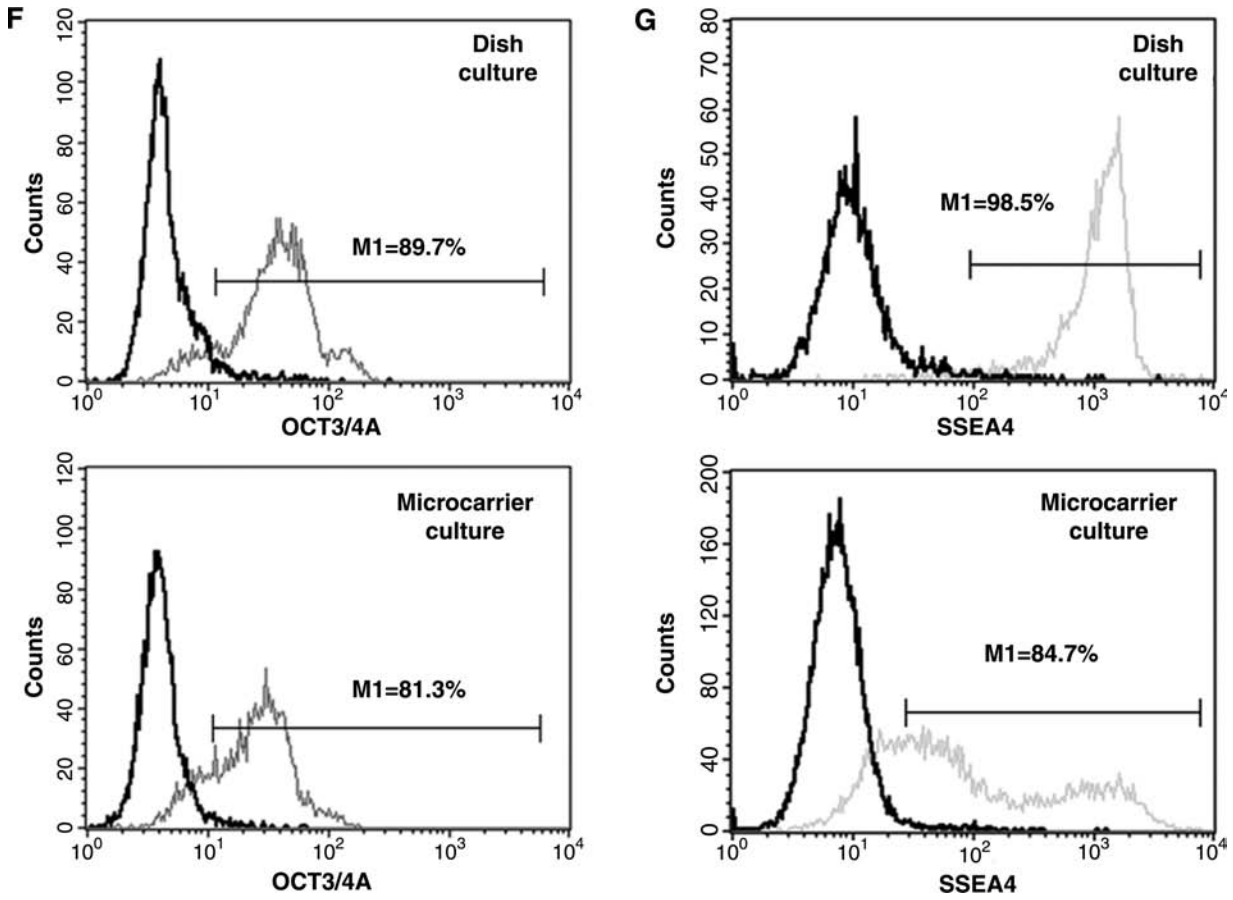


FIG. 2. (Continued)

2×10^6 DE cells (63.2%) in dishes. The overall surface area that is available for growth is $\sim180\,cm^2$ for 500 mg of beads and $20\,cm^2$ for a 6-cm dish. Then, 9.4×10^4 DE cells/cm^2 are generated in the bioreactor versus 10×10^4 DE cells/cm^2 in dishes. Therefore, differentiation of hESCs cultured in the microcarrier bioreactor and in dishes yields comparable numbers of $FOXA2^+/SOX17^+$ cells per unit of surface area under the conditions described here.

Given the higher fraction of $FOXA2^+/SOX17^+$ cells in the bioreactor compared to static cultures, we decided to combine the cells/microcarriers from two spinner flasks in a single vessel keeping the volume constant (50 mL), thereby doubling the surface area. We chose to combine the two bioreactors 24 h after the beginning of differentiation because then the decline in cell concentration subsides and the number of cells stabilizes (Fig. 3A).

Combination of 7.6×10^5 and 5.2×10^5 cells/mL in a single spinner flask with 50 mL of medium (Fig. 6) resulted in 10.7×10^5 cells/mL 3 days later. Almost 80% of the cells or 8.56×10^5 cells/mL were $FOXA2^+$ and $SOX17^+$ compared to 5×10^5 cells/mL in dishes. The advantage of the microcarrier system for directed differentiation of hESCs becomes even clearer if one considers the number of DE cells per mL of differentiation medium spent. Here, we replaced half of the medium every day after combining the cells/beads from the two bioreactors. Then, 2.14×10^5 and 1.25×10^5 DE cells per mL of differentiation medium spent are generated in microcarrier and in dish cultures, respectively (Supplemental Table S2).

Finally, cells subjected to DE differentiation were separated from beads and plated for further differentiation to PGT cells. Upon treatment with keratinocyte growth factor (FGF7), cells expressing the transcription factor HNF1B were detected (Supplemental Fig. S2). Our findings show that microcarrier bioreactors can be used for guiding the commitment of hESCs to DE in a scalable fashion.

Discussion

We showed that hESCs could be cultured on microcarriers in stirred suspension. Depending on culture conditions (e.g., agitation rate), cell proliferation was comparable to that of hESCs in dishes. The hESCs grown on microbeads were also positive for stemness markers. While cultured on microcarriers, hESCs were treated with soluble factors known to induce DE differentiation.[2] The hESCs in the bioreactor transitioned to DE with high efficiency and in a scalable manner.

Propagation of hESCs as EBs in stirred suspension has been demonstrated,[6,7] but factors keeping the cells within EBs from differentiating spontaneously are still elusive. Although EB culture of ESCs appears to be somewhat effective for commitment to certain cell types,[32,33] poor control over the differentiation process hinders the use of this culture

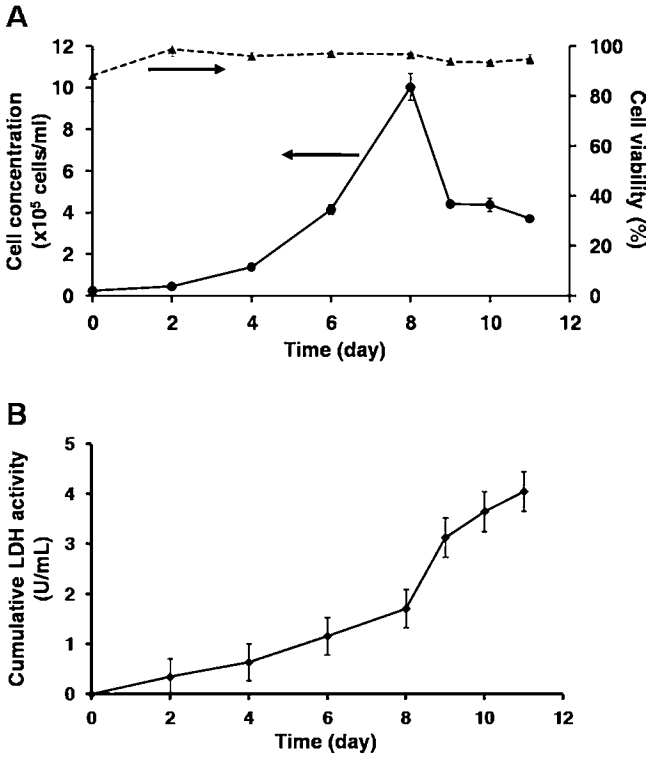


FIG. 3. Combined expansion and differentiation of hESCs in a microcarrier bioreactor. (**A**) H9 hESCs were seeded at 10^5 hESCs/mL and expanded at 45 rpm for 8 days. On day 8, CM was replaced with differentiation medium. The cell concentration and viability during culture are shown. (**B**) Cumulative LDH activity corresponding to the culture in (**A**).

modality for directing hESCs along particular lineages. Specifically, the EB culture is not preferable for efficient hESC-to-DE transition[2] (and subsequent commitment to islet cells) possibly due to the uneven exposure of cells within the aggregates to soluble differentiation agents.

The uneven exposure of cells to differentiation stimuli may be circumvented in a microcarrier culture. Although mESCs have been cultivated in microcarrier bioreactors,[9,10] translating these findings to hESCs has not been successful[34] potentially due to differences in the maintenance of mESCs and hESCs. Unlike mESCs, for example, hESCs are passaged as small clumps. Here, we dispersed hESCs into small clusters before their loading on microcarriers. This may explain in part the relatively low efficiency of hESC attachment on the beads. Another reason is the poor initial colonization, which was suggested in the case of mESC stirred microcarrier cultures.[9,35] Of note is also the fact that the efficiency of subculturing hESCs in static cultures is modest. Even when we varied the number of inoculated cells per bead, the efficiency of cell attachment on the beads did not change significantly. Functionalization[36,37] of the bead surface with extracellular matrix molecules besides Matrigel may increase the efficiency of hESC attachment. In our study, the beads were precoated with collagen to enhance the attachment of hESCs. However, collagen (as well as gelatin) does not support the growth of undifferentiated hESCs as effectively as Matrigel.[38] To that end, we coated the beads with Matrigel before their use because Matrigel supports the growth of undifferentiated hESCs on plates without feeder cells. It is plausible that other microcarrier types may improve the adhesion efficiency of hESCs.

These issues should be considered in developing a strategy for successive passaging of hESCs without loss of their pluripotency in microcarrier bioreactors. In this study, we did not subculture hESCs serially in microcarrier bioreactors. One should note that cells must be dissociated from the beads as clumps instead of as single cells. Further, cell losses should be minimized during the separation and retention of cells from the beads. Various separation methods have been described, including low-speed density centrifugation[39] and tangential flow filtration.[40] Development of improved methods for cell-bead separation and cell retention will be a key aspect of effective deployment of microcarrier systems for the expansion/directed differentiation of hESCs.

Cell proliferation was modulated with stirring but was fairly comparable for different concentrations of inoculated cells cultured at the same agitation rate. Expansion of mESCs on microcarriers has been demonstrated, but potential effects of stirring on cell proliferation were not addressed.[9,10,35] However, a change was noted in the doubling time of mESCs cultured as aggregates in stirred suspension.[10] An important culture variable on the proliferation and differentiation state of hESCs cultivated in a microcarrier suspension is the shear stress, which is a function of the agitation rate. Kolmogorov eddies formed in microcarrier culture vessels affect cells on microcarriers even at lower agitation rates than single cells.[41] Shear may induce signaling responses by cultured ESCs as suggested previously.[42] Further studies are needed to characterize the effect of shear on the self-renewal of cultured hESCs.

Human ESCs grown on microcarriers retained the expression of pluripotency markers such as NANOG, TRA-1–81, OCT3/4A, and SSEA4. After 8 days of culture on microcarriers, more than 80% of the cells were positive for OCT3/4A and SSEA4. These fractions were lower than those of hESCs cultured in plates. This may be in part due to enzymatic dissociation of the cells from beads before flow cytometry analysis. Still, differentiation of a subpopulation of hESCs during their expansion in the bioreactor cannot be ruled out considering the minimal but persistent expression of other tri-lineage genes. Human ESCs cultured in dishes exhibit a similar heterogeneity in their pluripotency state. It should be determined if over successive passages the fraction of pluripotent hESCs is maintained, for example, through selection mechanisms similar to those suggested for mESCs grown in stirred suspension.[10]

Differentiation of hESCs cultured on microcarriers was initiated by exchanging the CM with differentiation medium known to induce DE formation in dishes.[4,8] Although there is no single DE-specific marker or functional assay that can unequivocally identify this cell type, several lines of evidence indicate that a major fraction of the cells in the bioreactor adopted a DE phenotype. The forkhead box A2 gene (*Foxa2*; *Hnf3*β) is expressed in DE as well as in axial mesoderm[17,18] and *Sox17* is expressed in DE and in extraembryonic endoderm.[19] Their coexpression is indicative of DE cells. Over 85% of cells on microcarriers coexpressed FOXA2 and SOX17

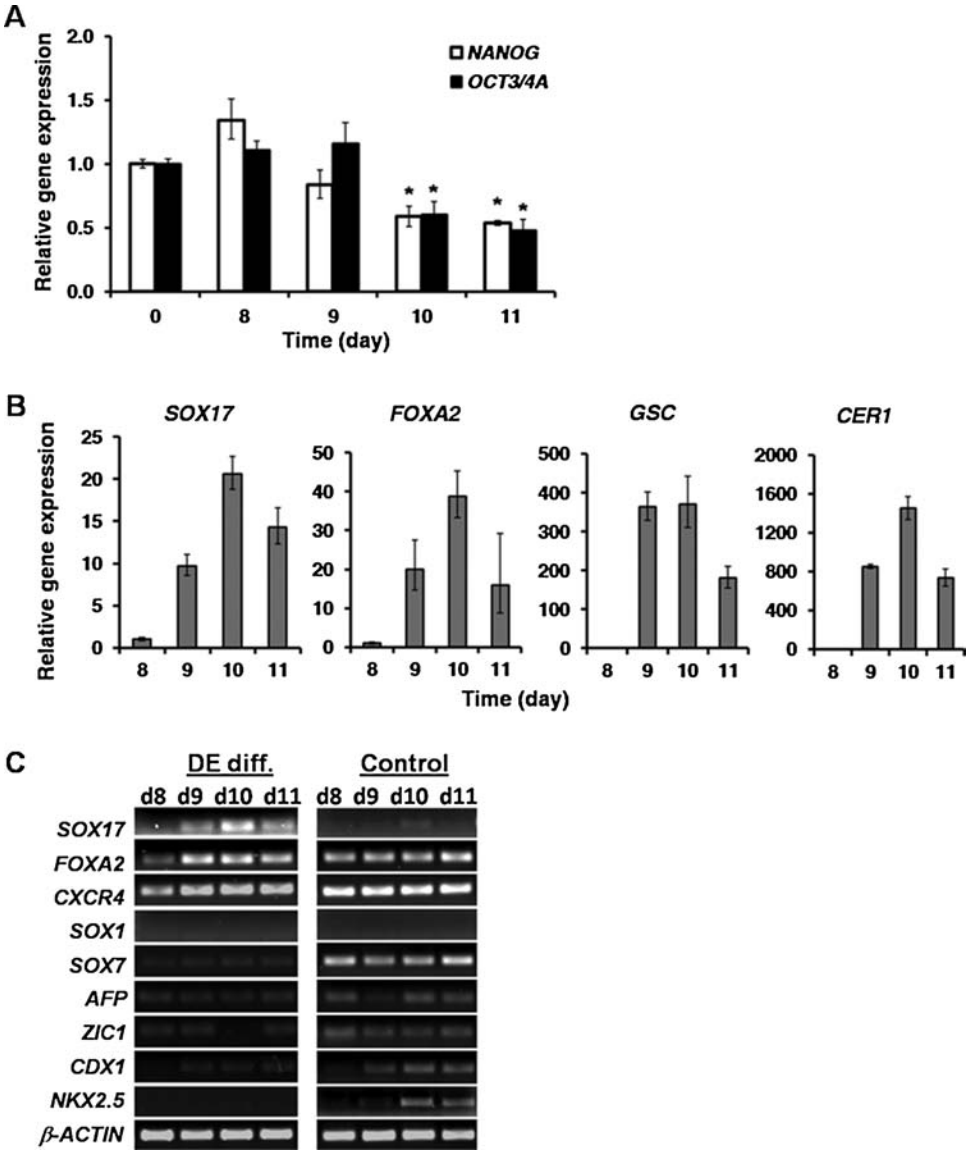


FIG. 4. Gene expression dynamics of hESCs subjected to DE differentiation in a microcarrier bioreactor. (**A**) Pluripotency genes were downregulated, whereas (**B**) DE gene expression increased during differentiation. Expression on day 0 (**A**) or day 8 (**B**) was set to 1. (**C**) The expression of DE and non-DE genes is shown for hESCs directed toward DE and for hESCs treated with differentiation medium but without activin A/Wnt3a (control). *$p < 0.01$ for the expression of corresponding genes compared to that on day 0.

after differentiation. This fraction was higher than that observed for differentiation in dishes for the hESC lines tested here and comparable to hESC monolayers[8] treated with 100 ng/mL activin A in medium with low serum. Of note is the fact that differentiation of either H1 or H9 cells gave rise to similar size populations of FOXA2$^+$/SOX17$^+$ cells. Given the differences in the proclivity of different hESC lines for differentiation,[28] it will be interesting to see if hESCs from these lines transition with similar efficiencies through subsequent stages of differentiation to mature endoderm progeny (e.g. pancreatic or liver cells).

Besides the coexpression of FOXA2 and SOX17, an increase in the expression of *CER1*[21,22] and *GSC*,[20] similar to previous reports,[8,16] was noted 24–48 h after switching to differentiation medium. The generation of Gsc$^+$/Foxa2$^+$/Sox17$^+$ endodermal progenitors from mESCs treated with activin in serum-free media has been demonstrated.[30] Because Gsc is also expressed in some visceral endoderm cells,[20,43] we checked the expression of the visceral endoderm gene *SOX7*[19] in differentiating hESCs, and it was found to be minimal. Similarly, the expression of the caudal homeobox domain 1 (*CDX1*) gene, which is not expressed in DE,[26] was very low. Moreover, α-fetoprotein (*AFP*; visceral endoderm), *SOX1* and *ZIC1* (neuroectoderm), and *NKX2.5* (early cardiac mesoderm) were either absent or expressed at low levels. In contrast, control cells displayed multiple markers suggestive of spontaneous commitment. These cells also failed to give rise to PGT cells characterized by expression of HNF1B when exposed to FGF7 (data not shown). Thus, the majority of cells on microcarriers treated with activin A/Wnt3a adopt a DE phenotype although differentiation to other lineages by a minor fraction of cultured cells cannot be ruled out.

Interestingly, a large fraction of hESCs exposed to activin A/Wnt3a became BRACHYURY$^+$ initially. This fraction decreased with a concomitant increase in SOX17$^+$ (and

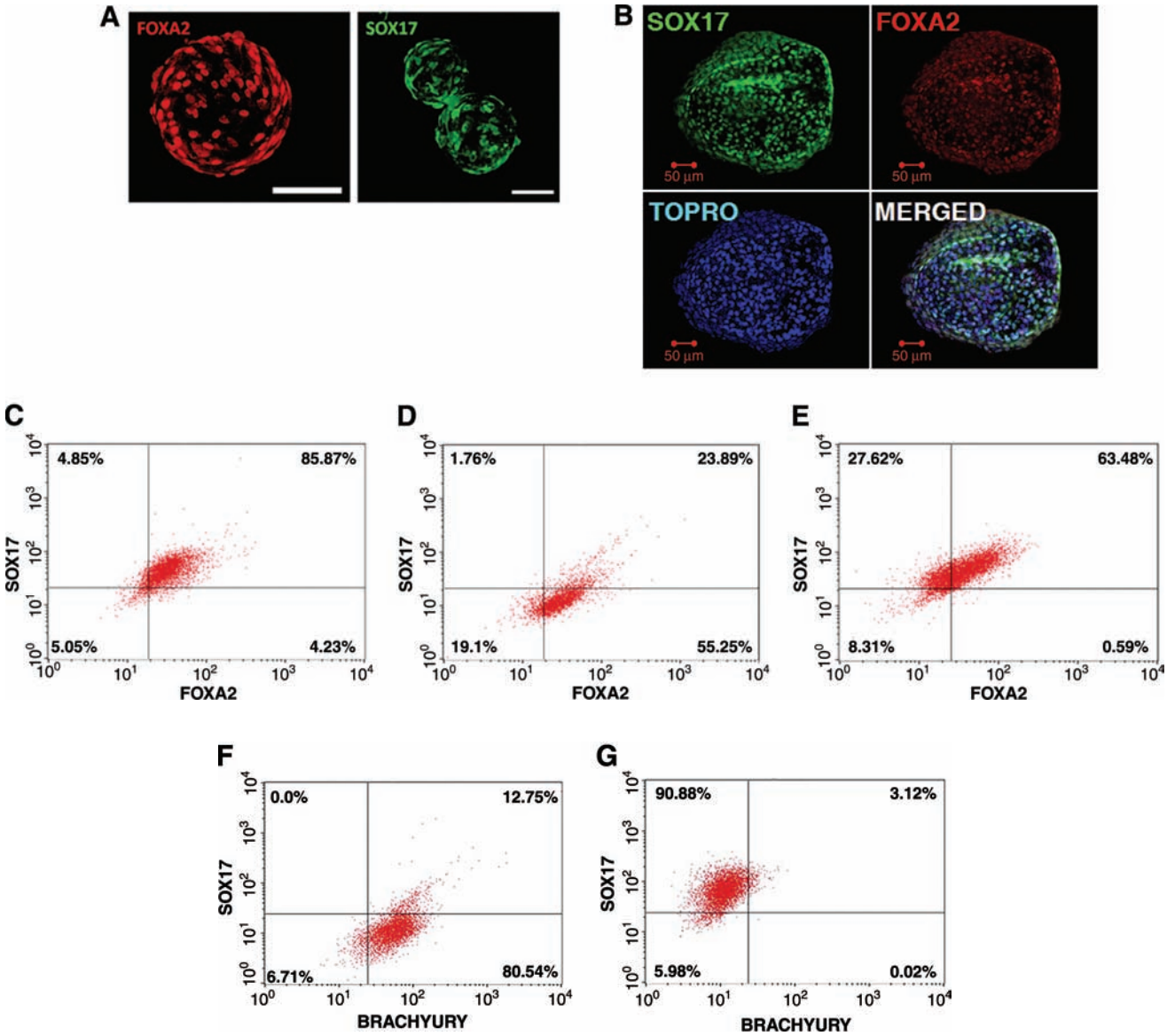


FIG. 5. DE proteins in differentiated H9 cells. (**A, B**) Cells on microbeads expressed FOXA2 and SOX17. The $SOX17^+/FOXA2^+$ cell fraction was determined for hESCs on microcarriers treated in differentiation medium (**C**) with or (**D**) without activin/Wnt3a. (**E**) H9 cells differentiated to DE in dishes. $SOX17^+/BRACHYURY^+$ cells after (**F**) 24 h and (**G**) 4 days of differentiation in a microcarrier bioreactor culture. Bars in (**A**): 100 μm. Color images available online at www.liebertonline.com/ten.

$FOXA2^+$), indicating a mesendoderm transition as described previously for endoderm differentiation of hESCs[2] and mESCs.[29–31] Gadue *et al.*[44] demonstrated that Wnt signaling and TGF-β/Nodal/activin signaling simultaneously are required to generate $Foxa2^+/Brachyury^+$ cells from mESCs. These cells represent an anterior primitive streak population and become committed to endoderm upon sustained activin signaling. We treated hESCs with the same combination of inductive signals and in the same order. Future studies are warranted to determine if hESCs on microcarriers treated with activin A/Wnt3a for mesendoderm formation can be further guided to mesoderm progeny (e.g. cardiomyocytes) by exposure to proper factors (e.g. BMPs[45]).

We observed that the number of hESCs decreased significantly when the differentiation was initiated by switching from CM with 20% KnockOut serum replacement to differentiation medium with 0.2% serum. The shear created by stirring may also contribute to the removal of cells from beads. Nonetheless, the number of cells remained constant after the first 24 h of differentiation. It should be noted that hESC death has also been reported[2] during transition to mesendoderm in dish cultures.

Microcarrier bioreactors are characterized by their superb scalability. Given that the numbers of $FOXA2^+/SOX17^+$ cells per cm^2 in the dish and the microcarrier suspension were similar, we combined the cells/beads from two vessels (50 mL of medium/vessel) into a single spinner flask culture with 50 mL of medium. This led to an almost proportional increase in the cell concentration, surface area available for growth, and the number of $FOXA2^+/SOX17^+$ cells at the end

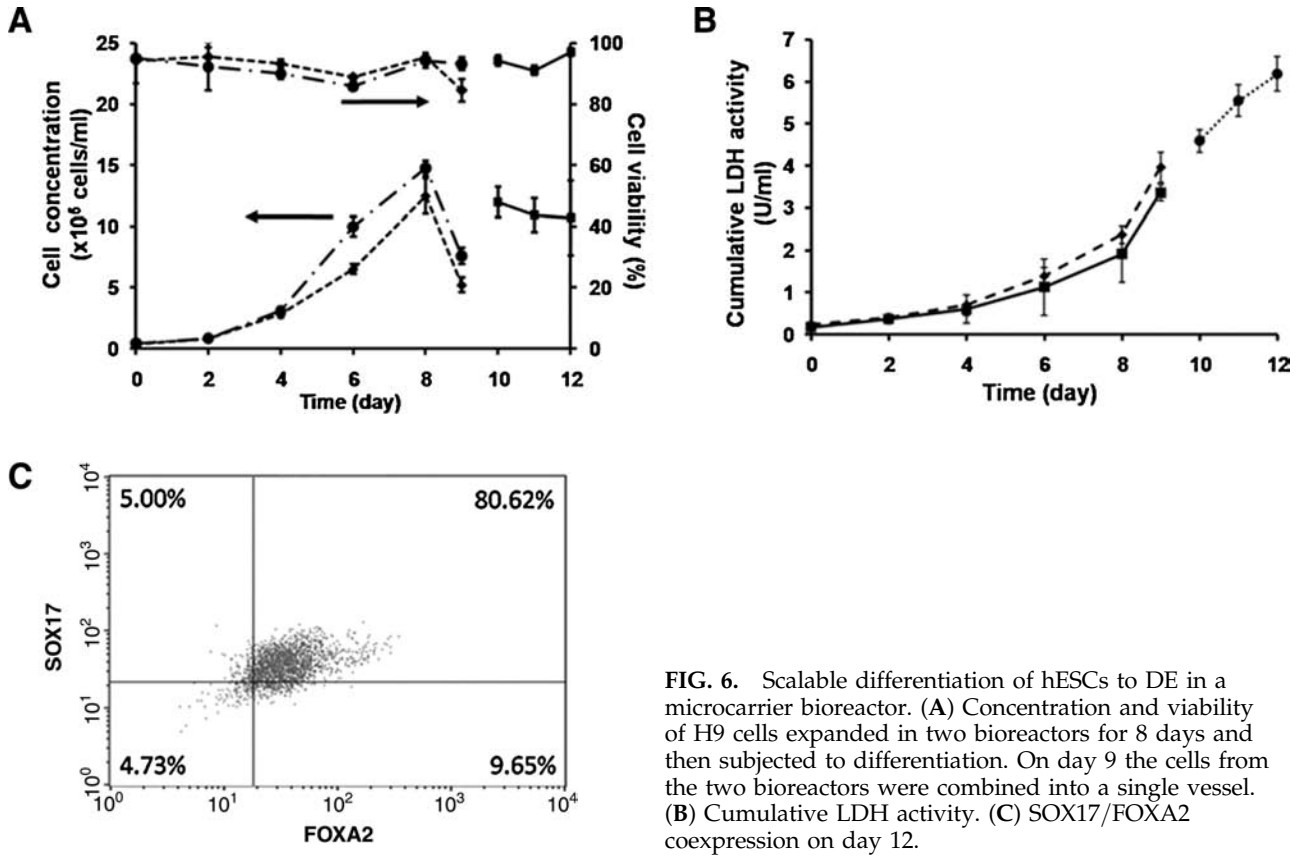


FIG. 6. Scalable differentiation of hESCs to DE in a microcarrier bioreactor. (**A**) Concentration and viability of H9 cells expanded in two bioreactors for 8 days and then subjected to differentiation. On day 9 the cells from the two bioreactors were combined into a single vessel. (**B**) Cumulative LDH activity. (**C**) SOX17/FOXA2 coexpression on day 12.

of the differentiation. The results point to a more efficient utilization of the differentiation medium. This can have a measurable impact on the economics of the bioprocess considering the high cost of ligands used for differentiation.

Our findings collectively show that directed commitment of hESCs to DE in a microcarrier bioreactor is scalable and encourage further investigation for *en masse* derivation of pancreatic islets for diabetes. Fine-tuning of existing protocols developed for the hESC differentiation to endoderm progeny in static cultures will be necessary, before these can be implemented in a microcarrier bioreactor. Nonetheless, this is a promising culture system for the production of therapeutically useful cell types in clinically relevant quantities.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Emmanuel (Manolis) S. Tzanakakis, Ph.D.
Department of Chemical and Biological Engineering
State University of New York at Buffalo
907 Furnas Hall
Buffalo, NY 14260

E-mail: emtzan@eng.buffalo.edu

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