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Manufacturing processes

# Suspension culture on microcarriers and as aggregates enables expansion and differentiation of pluripotent stem cells (PSCs)

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ABSTRACT

*Background aims:* Human pluripotent stem cells (PSCs) hold a great promise for promoting regenerative medical therapies due to their ability to generate multiple mature cell types and for their high expansion potential. However, cell therapies require large numbers of cells to achieve desired therapeutic effects, and traditional two-dimensional static culture methods cannot meet the required production demand for cellular therapies. One solution to this problem is scaling up expansion of PSCs in bioreactors using culture strategies such as growing cells on microcarriers or as aggregates in suspension culture.
*Methods:* In this study, we directly compared PSC expansion and quality parameters in microcarrier- and aggregate-cultures grown in single-use vertical-wheel bioreactors.
*Results:* We showed comparable expansion of cells on microcarriers and as aggregates by day 6 with a cell density reaching $2.2 \times 10^6$ cells/mL and $1.8 \times 10^6$ cells/mL and a fold-expansion of 22- and 18-fold, respectively. PSCs cultured on microcarriers and as aggregates were comparable with parallel two-dimensional cultures and with each other in terms of pluripotency marker expression and retention of other pluripotency characteristics as well as differentiation potential into three germ layers, neural precursor cells and cardiomyocytes.
*Conclusions:* Our study did not demonstrate a clear advantage between the two three-dimensional methods for the quality parameters assessed. This analysis adds support to the use of bioreactor systems for large scale expansion of PSCs, demonstrating that the cells retain key characteristics of PSCs and differentiation potential in suspension culture.



## Introduction

Human pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have a great potential for regenerative therapies as the result of their capacity for self-renewal and differentiation into multiple mature cell types. Many clinical trials involving iPSC-based cell therapy products are underway in Europe, Japan, China and the United States (reviewed in Kim *et al.* [1]). Many of these cell therapy products require extensive cell numbers, which is made possible only through large-scale expansion of stem cells. Conventionally, stem cells are grown either on feeder cells or on extracellular matrix as colonies in a two-dimensional (2D) culture. These methods are convenient, easy to maintain and provide a homogeneous population. However, conventional 2D methods of PSC cell culture are labor intensive and not capable of generating the required cell numbers for clinical products in a cost-effective manner. In addition to scalability limits, 2D methods are potentially more prone to contamination issues, since they require many open manipulations.

To overcome the limitations of 2D systems, novel three-dimensional (3D) culture methods have been explored in the past few years to expand PSCs in suspension. 3D methods of cell culture offer enhanced surface-to-volume ratio, enabling scale-up of PSCs. Multiple types of single-use bioreactors, such as stirred-tank bioreactors with horizontal impellers, hollow fiber, rocking motion and vertical-wheel bioreactors, are being investigated for culture of stem cells (reviewed in Nogueira *et al.* [2]). Generally, for expansion in suspension culture, PSCs are grown on matrix-coated microcarriers or directly as aggregates. Microcarriers are spherical particles, approximately 100–250 $\mu$m in diameter and made of various materials like plastic, cellulose and polyester (reviewed in Tavassoli *et al.* [3]).

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Because of their larger surface area-to-volume ratio, microcarriers provide a large area for attachment of the cells. Alternately, when inoculated directly into culture medium in the absence of a coated surface or coated microcarriers, PSCs assemble into 3D aggregates. Aggregates may provide more physiologically relevant cell–cell interactions that might favor growth and differentiation in suspension culture. Multiple studies have demonstrated the feasibility of expansion of PSCs either solely on microcarriers or as aggregates in scale-up systems using bioreactors [4–16], but few reports have provided any direct comparison between the two methods. As each method has its own advantages and limitations, it is important to understand how each method supports quality, expansion and differentiation of PSCs in comparison with each other when cultured in parallel under similar conditions.

In this study, we sought to compare the quality parameters of cells expanded on microcarriers and as aggregates in vertical-wheel bioreactors. ESCs and an iPSC line were expanded in suspension culture and examined for various quality parameters in relation to the cultures grown as traditional 2D cultures. We studied fold-expansion, pluripotency marker expression and differentiation potential of the 3D suspension cultures. In addition, we also examined the ability of the cells to differentiate into clinically relevant cell types by differentiating them into neural precursor cells and cardiomyocytes. Our data suggest that cell grown in suspension on microcarriers and as aggregates shows similar expansion and are comparable with 2D cultures with respect to PSC characteristics and differentiation potential.

## Methods

### *Human PSC lines and 2D cell culture*

Human ESC line (WA09/H9) was obtained from WiCell (Madison, WI, USA). The iPSC EB1 line was generated at the National Institutes of Health Stem Cell Unit as described [17]. PSCs were cultured on vitronectin (A14700; Thermo Fisher Scientific, Waltham, MA, USA)-coated plates in mTeSR Plus media (100-0276; Stem Cell Technologies, Vancouver, Canada). Cells on 2D plates were passaged as cell clumps using ReLeSR (05872; Stem Cell Technologies).

### *3D cell culture on microcarriers and as aggregates*

Plastic microcarriers (P-221-020, size range 125-212 $\mu$m; Solo-Hill, Ann Arbor, MI, USA) were used at the rate of 500 mg/100 mL media and coated with vitronectin at 0.5 $\mu$g/cm$^2$ and left at room temperature for 2 h with intermittent mixing. The microcarriers were equilibrated in mTeSR Plus media at 37°C for at least 30 minutes before inoculation. On the day of inoculation, cells on 2D plates are harvested using TrypLE Express (12604013; Thermo Fisher Scientific) as single cells, strained through 40-$\mu$m strainers and counted. Cells were inoculated in 50 mL of mTeSR Plus media along with 10 $\mu$m of Rho-associated protein kinase (i.e., ROCK) inhibitor, Y27632 (S1049; Sellek Chemical, Houston, TX, USA) at 10 million cells per 100 mL each into 0.1 Single-Use vertical-wheel bioreactors (FA-0.1-D-001; PBS Biotech, Camarillo, CA, USA) with or without coated microcarriers. The bioreactors were left static for an hour with intermittent mixing by turning on the rpm to 40 rpm for 30 seconds every 15 minutes, after which the agitation was started at 40 rpm. The bioreactors were placed in an incubator at 37°C with 5% $CO_2$. On day 2, 80% (80 mL of 100 mL) media was removed and replaced with fresh 80 mL of mTeSR Plus media. From day 3, 80% media were exchanged every day until the day before harvest. Cells in 3D suspension culture were harvested by incubating with TrypLE express for 10 minutes. Cells were then strained through a 40-$\mu$m strainer and counted or used for further experiments. To determine cell growth and fold-expansion, cell counts were done using the TC20 Cell Counter (Bio-Rad, Hercules, CA, USA).

### *Trilineage differentiation*

A trilineage differentiation kit (05230; Stem Cell Technologies) was used to differentiate PSCs into three germ layers, ectoderm, endoderm and mesoderm, according to manufacturer's protocol.

### *Neural induction*

PSCs were differentiated into neural progenitor cells using the STEMdiff Neural Induction media (Stem Cell Technologies).

### *Cardiomyocyte differentiation*

Cardiomyocyte differentiation was carried out as described [18]. Cells were expanded on microcarriers and as aggregates as described previously until day 6, which was considered day 0 of cardiomyocyte differentiation.

### *RNA extraction, cDNA preparation and reverse transcription (RT) polymerase chain reaction (PCR)*

Total RNA was extracted from cell pellets from cultured PSCs (in 2D, on microcarriers and aggregates), differentiated cells including, ectoderm, mesoderm, endoderm and neural progenitor cells using the QIAGEN RNeasy Mini Kit and from cardiomyocyte cell pellets using the QIAGEN RNeasy Fibrous Tissue Mini Kit (74704; QIAGEN, Hilden, Germany), according to the manufacturer's instructions. For cDNA synthesis, a concentration of 2 $\mu$g of total RNA was used for synthesis of the first-strand cDNA with the Applied Biosystems cDNA synthesis kit (Waltham, MA, USA) in a total reaction volume of 20 $\mu$L according to the manufacturer's instructions. For quantitative PCR, SYBR Green supermix (1725274; Bio-Rad) was used. Transcripts were quantified by RT-PCR and Qbase software [19] was employed, and the reference housekeeping genes shown in Table 1 were used as optimal comparators for calculating $2^{-\Delta Ct}$ values for genes of interest. The PCR primers are listed in Table 1.

### *Flow cytometry analysis*

For cell-surface marker expression, cells were suspended in 2% fetal bovine serum/phosphate-buffered saline (FBS/PBS) for 30 minutes at room temperature and stained with antibodies for 45 minutes. Cells were then washed twice with 2% FBS/PBS and resuspended in PBS for flow analysis.

For internal markers, cells were fixed in Cytofix/Cytoperm fixation and permeabilization buffer (BD Biosciences, Franklin Lakes, NJ, USA) for 15 minutes on ice, washed twice with Perm/Wash buffer (BD Biosciences) and incubated in 2% FBS/PBS for 30 minutes. Cells are then stained with antibodies for 45 minutes at room temperature, washed twice with perm/wash buffer and resuspended in PBS for flow analysis.

Flow cytometry was done using following antibodies: OCT4 (560186; BD Biosciences), PAX6 (IC8150P), SSEA4 (560128; BD Biosciences), SSEA1 (560142; BD Biosciences), Tra-1-60 (560193; BD Biosciences) and Tra-1-81 (560161; BD Biosciences). For cadherin expression, the following antibodies were used: E-cadherin (562870; BD Biosciences) and N-cadherin (561554; BD Biosciences). For quantification of $\gamma$H2AX-positive cells, $\gamma$H2AX antibody (560445; BD Biosciences) or an isotype control (557702; BD Biosciences) were used. For flow cytometry of DNA content for cell cycle analysis, FxCycle Far Red Stain (F10348; Thermo Fisher Scientific) was used according to the manufacturer's instructions.

**Table 1**
Primers used for RT-PCR analysis.

| Gene category | Gene name | Forward sequence | Reverse sequence |
|---|---|---|---|
| Pluripotency | *POU5F1* | TCGAGAACCGAGTGAGAGG | GAACCACACTCGGACCACA |
| | *SOX2* | TACAGCATGTCCTACTCGCAG | GAGGAAGAGGTAACCACAGGG |
| | *REX1* | CCTGCAGGCGGAAATAGAAC | GCACACATAGCCATCACATAAGG |
| | *LIN28A* | CACGGTGCGGGCATCTG | CCTTCCATGTGCAGCTTACTC |
| | *ESRG* | CCCCGCAGACCATCATGGA | ACAGGCTTTGTGTGAGCAACA |
| | *ZSCAN10* | TCACCATGGCCAGAGGAGAG | GGCACATCCTCAAAGGTCAGG |
| Cadherin pathway | *E-Cadherin* | CAATGCCGCCATCGCTTAC | ATGACTCCTGTGTTCCTGTTAATG |
| | *N-Cadherin* | GACAATGCCCCTCAAGTGTT | CCATTAAGCCGAGTGATGGT |
| | *Fibronectin* | ACCAACCTACGGATGACTCG | GCTCATCATCTGGCCATTTT |
| | *Vimentin* | GAGACTTTGCCGTTGAAGC | TCCAGCAGCTTCCTGTAGGT |
| | *Snail1* | GCTCCACAAGCACCAAGAGT | ATTCCATGGCAGTGAGAAGG |
| | *Snail2* | CTTTTTCTTGCCCTCACTGC | ACAGCAGCCAGATTCCTCAT |
| | *Twist1* | TGCATGCATTCTCAAGAGGT | GTTTTGCAGGCCAGTTTGAT |
| | *Trim28* | TGTTTCCACCTGGACTGTCA | CCAGCAGTACACGCTCACAT |
| Primed | *DUSP6* | TTCCCTGAGGCCATTTCTTT | AGTGACTGAGCGGCTAATG |
| | *OTX2* | CACTTCGGGTATGGACTTGC | GGTACCGGGTCTTGGCAAA |
| | *ZIC2* | GATGTGCGACAAGTCCTACAC | TGGACGACTCATAGCCGGA |
| Naïve | *KLF17* | CTGCAACTACGAGAACTGCG | GCAAGAATATGGCCTCTACC |
| | *KLF4* | GCTGCCGAGGACCTTCTG | GCGAACGTGGAGAAAGATGG |
| | *TFCP2L1* | TTTGTGGGACCCTGCGAAG | TGCTTAAACGTGTCAATCTGGA |
| | *DPPA3* | AGACCAACAAACAAGGAGCCT | CCCATCCATTAGACACGCAGA |
| | *DNMT3L* | CTGCTCCATCTGCTGCTCC | ATCCACACACTCGAAGCAGT |
| | *HMBS* | AGGAGTTCAGTGCCATCATCCT | CACAGCATACATGCATTCCTCA |
| Ectoderm | *PAX6* | AACGATAACATACCAAGCGTGT | GGTCTGCCCGTTCAACATC |
| | *Nestin* | CAGCGTTGGAACAGAGGTTGG | TGGCACAGGTGTCTCAAGGGTAG |
| | *OTX2* | CAAAGTGAGACCTGCCAAAAAGA | TGGACAAGGGATCTGACAGTG |
| | *NCAM1* | GGCATTTACAAGTGTGTGGTTAC | TTGGCGCATTCTTGAACATGA |
| Endoderm | *HNF3B* | GACAAGTGAGAGAGCAAGTG | ACAGTAGTGGAAACCGGAG |
| | *FoxA1* | GGAAGCCCAAGAACCTGAAT | GTTGCTGGAGTTGCTGGAAG |
| | *HNF4A* | CGAAGGTCAAGCTATGAGGACA | ATCTGCGATGCTGGCAATCT |
| | *GSC* | TCTTCCAGGAGACCAAGTACC | GATGAGGACCGCTTCTGC |
| | *CXCR4* | ACGCCACCAACAGTCAGAG | AGTCGGGAATAGTCAGCAGGA |
| Mesoderm | *IGF2* | AGACGTACTGTGCTACCCC | TGCTTCCAGGTGTCATATTGG |
| | *Brachyury* | CAACCTCACTGACGGTGAAAAA | ACAAATTCTGGTGTGCCAAAGTT |
| | *ACTA2* | GTGTTGCCCCTGAAGAGCAT | GCTGGGACATTGAAAGTCTCA |
| | *RUNX1* | TCTTCACAAACCCACCGCAA | CTGCCGATGTCTTCGAGGTTC |
| Neuroderm | *Pax6* | AACGATAACATACCAAGCGTGT | GGTCTGCCCGTTCAACATC |
| | *Neurog2* | AGAGCCAACTAAGATGTTCGTCA | CGATCCGAGCAGCACTAACA |
| | *MAP2* | GACTGCAGCTCTGCCTTTAG | AAGTAAATCTTCCTCCACTGTGAC |
| Cardiomyocyte | *GATA4* | TAGACCGTGGGTTTTGCATTG | CATCCAGGTACATGGCAAACAG |
| | *Nkx2.5* | ACCCTGAGTCCCCTGGATTT | TCACTCATTGCACGCTGCAT |
| | *CTNN1* | CCAACTACCGCGCTTATGC | CTCGCTCCAGCTCTTGCTTT |
| | *hcTnT* | TTCACCAAAGATCTGCTCCTCGCT | TTATTACTGGTGTGGAGTGGGTGTGG |
| | *hMYH7* | TCGTGCCTGATGACAAACAGGAGT | ATACTCGGTCTCGGCAGTGACTTT |
| | *hMYL2* | TGTCCCTACCTTGTCTGTTAGCCA | ATTGGAACATGGCCTCTGGATGGA |
| | *hMYL7* | ACATCATCACCCATGGAGACGAGA | GCAACAGAGTTTATTGAGGTGCCC |
| Housekeeping | *rpl13a* | GCCATCGTGGCTAAACAGGTA | GTTGGTGTTCATCCGCTTGC |
| | *GAPDH* | CGCTGAGTACGTCGTGGAGT | GGGCAGAGATGATGACCCTTT |
| | *RPS26* | TAACTGTGCCCGATGCGTG | GCTCGCTTCAGAAATGTCCC |
| | *RPS29* | AATATGTGCCGCCAGTGTTT | CCCGGATAATCCTCTGAAGG |
| | *RPL39* | CATTCCCCAGTGGATTCGGAT | GACCCAGCTTGGTTCTTCTCC |
| | *RPL4* | GCC TGC TGT ATT CAA GGC TC | GGT TGG TGC AAA CAT TCG GC |
| | *RPS10* | CTGCGAGACTCACAAGAGGG | CCGTCTGTAGGTATCTCTGTCAG |

*Immunostaining*

For immunostaining, cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.5% Triton X-100 on ice for 5 min and blocked with 3% bovine serum albumin for 30 min. The following antibodies were used: anti-alpha smooth muscle actin antibody (A2547; Millipore, Burlington, MA), FOXA2 (Ab108422; Abcam, Cambridge, United Kingdom), anti-tubulin antibody (MAB1637; Millipore) and Oct3/4 (sc-5279; Santa Cruz Biotechnology, Dallas, TX, USA).

*Supernatant analysis of suspension culture media*

A BioProfile FLEX 2 analyzer (Nova Biomedical, Waltham, MA, USA) was used for supernatant analysis in spent media of suspension cultures. Triplicate samples were analyzed every day from day 3 onwards before changing culture media. As a control, mTeSR plus medium placed in a six-well plate in parallel with suspension cultures in the incubator was used.

*Plating cells from suspension culture onto 2D*

Single cells harvested from suspension culture were plated on vitronectin-coated plates or coverslips at a density of 500 000 cells per well of a six-well plate in mTeSR plus medium along with Rock inhibitor. The medium was replaced after 24 h and changed every day.

*Statistical analysis*

All the data presented in graphs are expressed as mean ± standard deviation except for Figure 1B, which is expressed as mean ± standard error of mean. Statistical analysis was done using GraphPad Prism 7 (GraphPad, San Diego, CA, USA). Statistical significance was

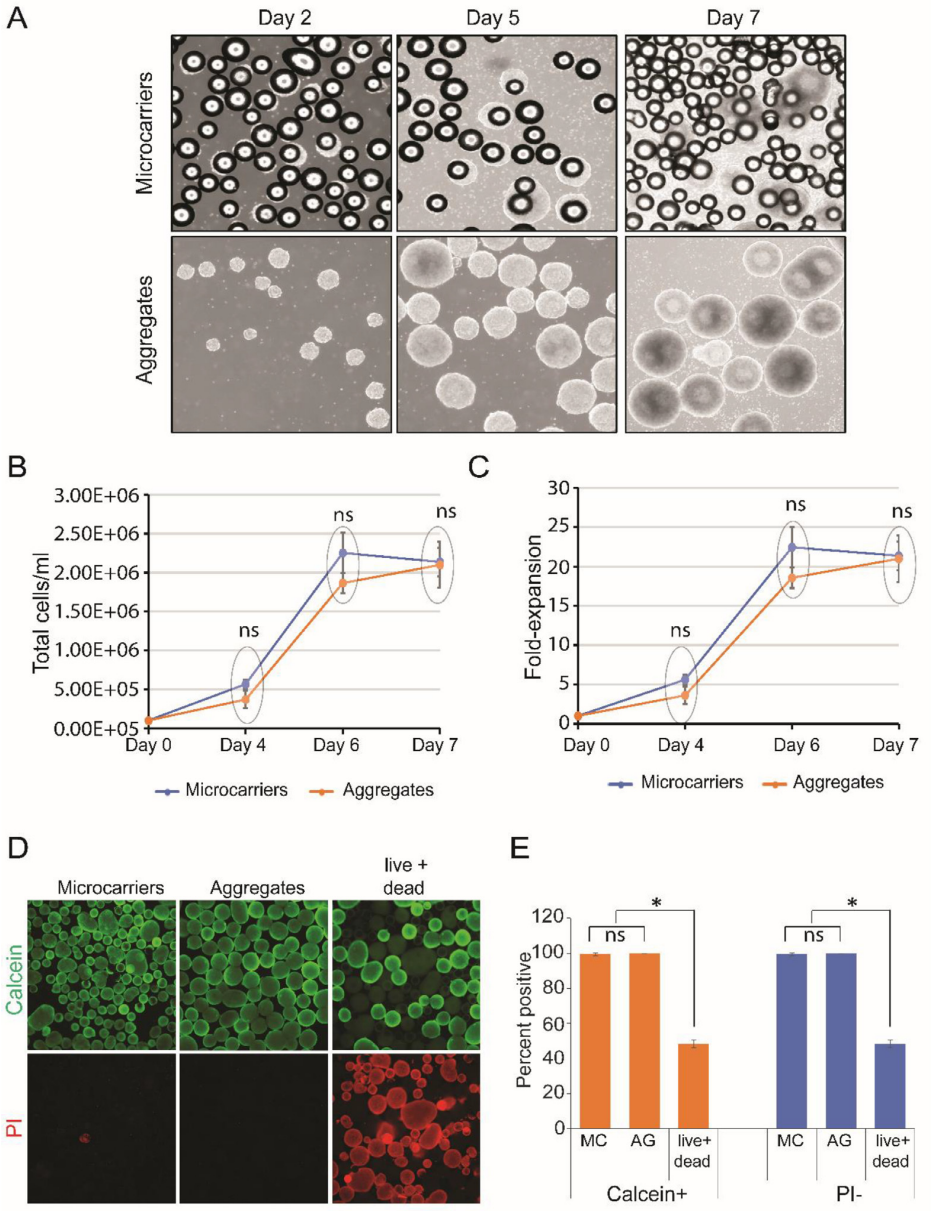


**Figure 1.** Growth and expansion of ESCs on microcarriers and as aggregates in vertical-wheel bioreactors. (A) Representative images of cells grown on microcarriers and as aggregates on denoted days. (B) Cell density (total cells/mL) and (C) fold-expansion of cells grown on microcarriers and as aggregates. Data represents mean ± standard error of the mean from three replicates. (D) Microcarrier and aggregate cultures stained with calcein AM and propidium iodide (PI). Live+dead is a mixture of live and heat-killed microcarrier/aggregate cultured cells, used as a control for staining. (E) Quantitative analysis of calcein positive (Calcein+) and PI negative (PI−) microcarrier (MC) and aggregate (AG) cultured cells, n = 3. *denotes $P$ value <0.05, and ns denotes not significant with $P$ value >0.05.

assessed by analysis of variance followed by Tukey's multiple comparisons test. A value of $P < 0.05$ is considered statistically significant.

## Results

### *Vertical-wheel bioreactors support robust expansion of PSCs on microcarriers and as aggregates*

We initially investigated the use of two types of bioreactors, spinner flasks and vertical-wheel bioreactors. However, our preliminary analysis showed that spinner flasks did not support growth of the aggregates under our culture conditions (data not shown). Since our main goal was to compare cells grown in two different 3D conditions and not two different bioreactors, we limited our further studies to the vertical-wheel bioreactor with the variable of cell expansion as aggregates or on microcarriers. Cells were inoculated onto coated microcarriers or as aggregates as described in the Methods. Cells formed visible clusters around the microcarriers, whereas those directly inoculated formed small round aggregates by day 2 (Figure 1A). By days 5 and 7, the cells expanded to form larger clusters and aggregates (Figure 1A). Cells reached a density of $2.2 \times 10^6$ cells/mL and $1.8 \times 10^6$ cells/mL and a fold-expansion of 22- and 18-fold on microcarriers and aggregates, respectively by day 6 with no

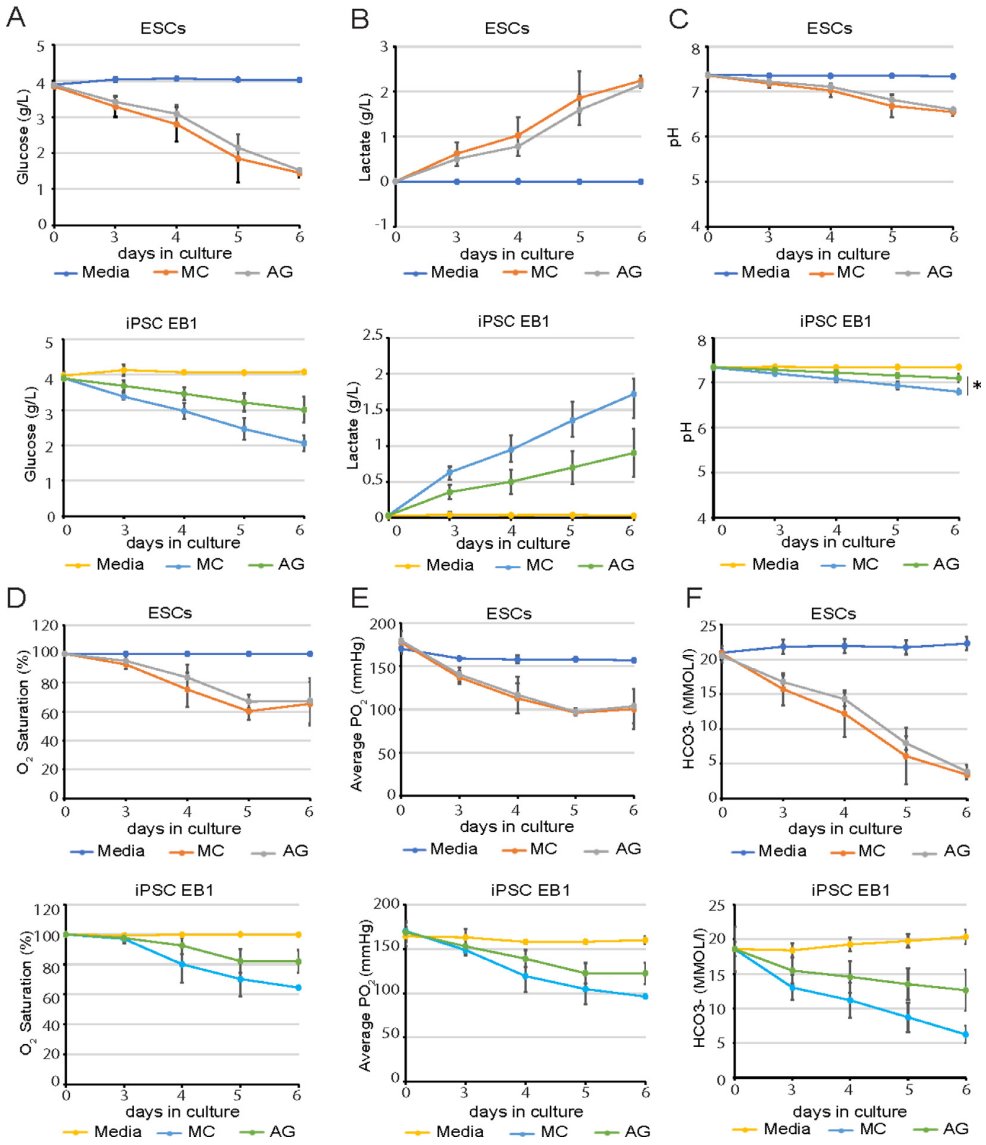


**Figure 2.** Assessment of nutrient, metabolite and process parameter profiles of cells cultured on microcarriers (MC) and as aggregates (AG) in suspension culture media. Analysis of various parameters: glucose (A), lactate (B), pH (C), $O_2$ saturation (D), average partial pressure of oxygen ($PO_2$) (E), and HCO3− concentration (F) was done by sampling the spent media on days shown in the graph just before medium exchange in cultures grown on microcarriers or as aggregates in ESC (n = 3) and iPSC EB1 lines (n = 3). Media alone placed in parallel with the suspension cultures in six-well plate was used a control. *Denotes *P* value <0.05.

significant differences between them (Figure 1B,C). Staining with calcein AM, a live cell stain, and propidium iodide, a dye that stains non-viable cells, showed that the cells on microcarriers and as aggregates were viable with above 99% microcarrier and aggregate clusters being positive for calcein (Figure 1D,E).

*BioProfile FLEX 2 supernatant analysis of cells cultured in 3D suspension culture*

Growing cells as 3D structures at an increased scale in suspension culture could affect nutrient consumption and metabolite release, which in turn could affect the quality of the PSCs. We monitored various nutrients, metabolites and other process parameters in suspension culture media sampled from day 3 onwards every day before media exchange using a cell-culture analyzer, the BioProfile FLEX2 Analyzer (Nova Biomedical). Average glucose values decreased with a concomitant increase of lactate as days in cell culture increased in both ESC and iPSC EB1 cell lines with no significant differences between microcarrier and aggregate cultures (Figure 2A,B). pH levels decreased in both microcarrier and aggregate cultures in both lines reaching a low of 6.6 on day 6 compared with medium alone of 7.3 (Figure 2C). We observed a significantly lower pH in microcarrier culture (6.7) than aggregate culture (7.0) on day 6 in iPSC EB1 line but no significant differences in pH in ESC line. $O_2$ saturation levels dropped from 100% on day 0 to close to 60% on day 6 (Figure 2D), with a similar drop in partial $O_2$ pressure (Figure 2E) in microcarrier and aggregate cultures in both cell lines. There was a gradual drop in HCO3− concentration as the days in culture progressed to day 6 in both microcarrier and aggregate cultures (Figure 2F). Our data show that quality parameters of supernatant medium dropped with increase in culture time commensurately in both microcarrier and aggregate cultures in terms of increased lactate, reduced glucose, pH,

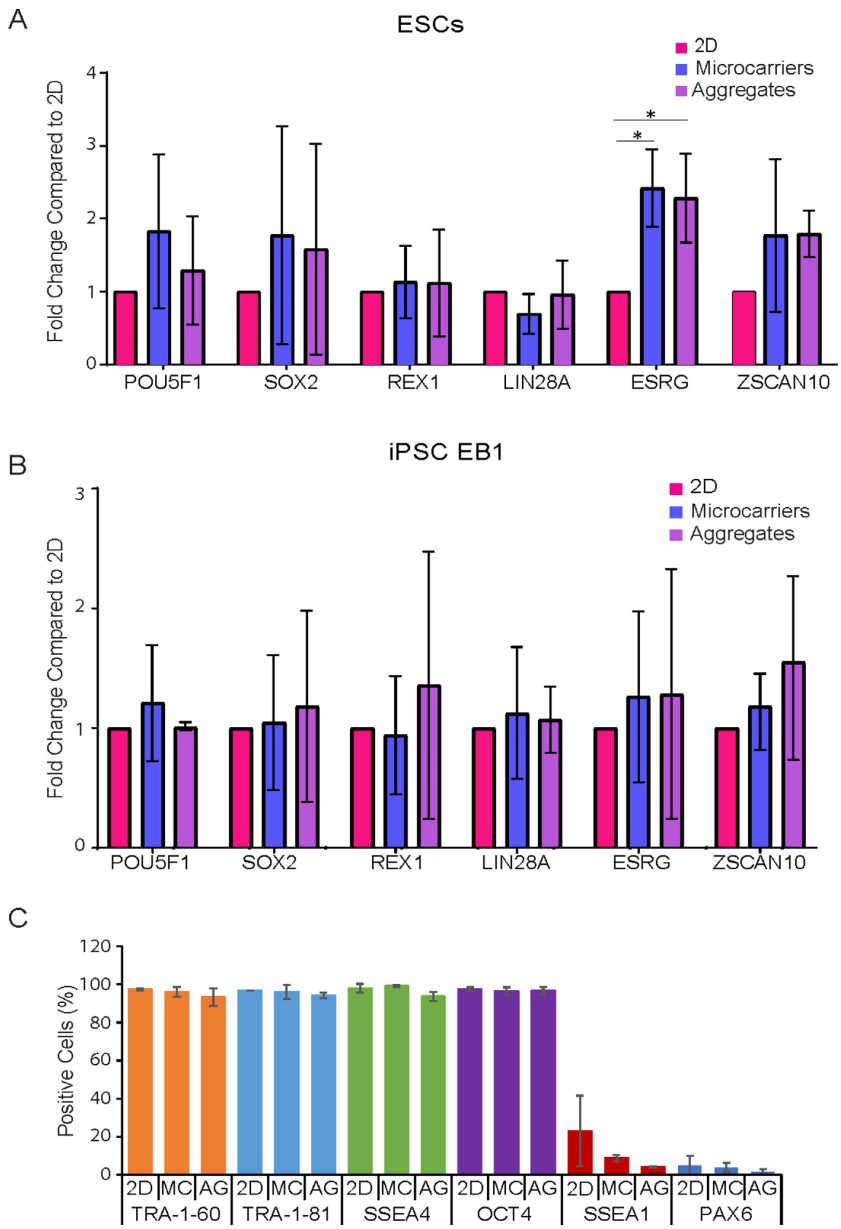


**Figure 3.** Pluripotent marker expression of cells grown on microcarriers and as aggregates in comparison with 2D cultured cells. The gene expression of pluripotency-associated markers, POU5F1, SOX2, REX1, LIN28, ESRG and ZSCAN10, expressed as fold change compared with 2D, in (A) ESC (n = 3) and (B) iPSC EB1 (n = 3) cell lines. (C) Flow cytometry analysis of pluripotency-associated markers TRA-1-60, TRA-1-81, SSEA4, OCT4 and differentiation markers SSEA1 and PAX6 in 2D, microcarrier (MC) and aggregate (AG) cultures of ESC line, n = 3. *Denotes *P* value <0.05.

$O_2$ saturation and HCO3− concentration. No significant differences were found between microcarrier and aggregate cultures, indicating no advantage of one method over the other in this respect. Concentrations of $Na^+$, $K^+$ and $Ca^{++}$ were unchanged throughout the culture period in both conditions (supplementary Figure 1).

*PSCs cultured on microcarriers and as aggregates show pluripotent marker expression comparable to 2D*

To examine the quality of cells grown in suspension culture, we conducted pluripotent marker expression analysis on cells cultured in 2D and in suspension. We analyzed gene expression for the pluripotency-associated markers POU5F1, SOX2, REX1, LIN28, ESRG and ZSCAN10 using RT-PCR. Our analysis of 3D cultured cells from two cell lines, ESC and iPSC EB1, showed no significant differences between transcript expression of markers between 2D and both microcarrier and aggregate suspension cultures, except for ESRG in the ESC line, which had greater expression in suspension culture (Figure 3A,B). Three differentiation markers, Nestin (ectoderm), HNF3B (endoderm) and IGF2 (mesoderm), were expressed at very low levels, with Cq (quantification cycle at which fluorescence is detected) values greater than the pluripotency marker POU5F1 and one of the housekeeping genes rpl13a (supplementary Figure 2). We further conducted flow cytometry analysis for pluripotency-associated cell surface markers TRA-1-60, TRA-1-81, SSEA4 and the intracellular OCT4 marker. Greater than 85% cells were positive for each

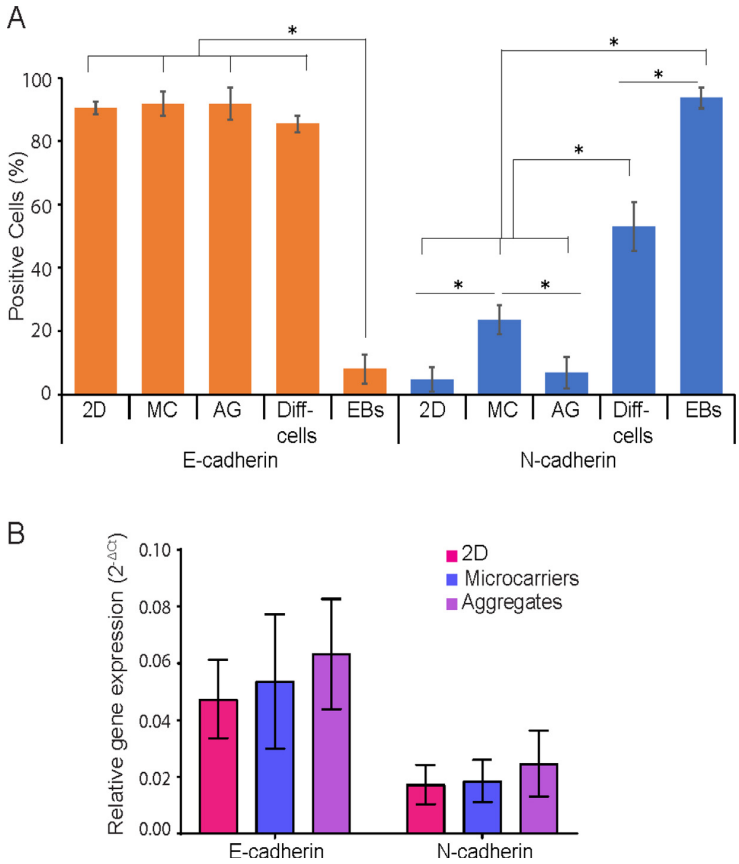


**Figure 4.** Analysis of expression of cadherins in 2D, microcarrier and aggregate cultures. (A) Flow cytometric analysis of E-cadherin and N-cadherin expression in ESCs cultured on 2D (2D), on microcarriers (MCs) and as aggregates (AG) (n = 3). Cells differentiated for 4 days (Diff-cells) or EBs were used as differentiation cell controls. (B) Gene expression analysis of E-cadherin and N-cadherin in 2D, microcarrier and aggregate ESC cultures, n = 3. *Denotes *P* value <0.05.

marker tested with no significant differences between 2D, microcarrier and aggregate cultures in ESC line (Figure 3C). Further, there was very low expression of differentiation markers, SSEA1 and PAX6, suggesting the absence of differentiation.

Human PSCs exist in naïve and primed states representing early- and late-stage human epiblasts respectively. Primed cells can be converted to naïve cells by manipulating culture conditions using inhibitors and growth factors [20]. We asked whether culturing cells in suspension as 3D structures on microcarriers or as aggregates could induce naïve state from the current primed state by using RT-PCR to measure the gene expression of naïve- and prime- specific genes. We examined DUSP6, OTX2 and ZIC2 as markers for the primed state and KLF17, KLF4, TFCP2L1, DPPA3, DNMT3L and HMBS as naïve markers [21]. No significant difference was observed in primed marker expression among 2D, microcarrier and aggregate cultures, and there was no detectable expression of the naïve markers tested (supplementary Figure 3).

*Expression of E-cadherin (E-cad) and N-cadherin (N-cad) in suspension culture*

E-cad and N-cad proteins are cell surface glycoproteins involved in cell–cell adhesion processes. E-cad is expressed at high levels on ESCs, and differentiation is associated with loss of E-cad expression and increase of N-cad expression [22]. This is also a defining characteristic of the epithelial–mesenchymal transition event that occurs during embryonic development and is implicated in tumor cell invasion and metastasis [23,24]. To determine whether culturing cells in 3D suspension affects cell–cell adhesion protein expression and if expression of cadherins could be used as potential markers of pluripotency and differentiation in suspension, we examined the expression of E-cad and N-cad by flow cytometry in 2D, microcarrier and aggregate cultures. For monitoring changes in E-cad and N-cad expression in differentiated cells we used two types of differentiated cells as controls. One was to grow aggregate cultures in differentiation medium for four days in suspension. The second control was differentiating PSCs into embryoid bodies (EBs) in ultra-low attachment plates in differentiation medium in parallel with suspension cultures until harvest. Greater than 90 % of cells were positive for E-cad in 2D, microcarrier and aggregate cultures. Low numbers of cells were positive for N-cad in 2D (4.7%) and aggregate cultures (6.8%). However, we noticed a significantly greater number of N-cad–positive cells in microcarrier culture (23.6%) with no significant change in E-cad expression (Figure 4A). Cells differentiated for 4 days showed a slightly lower, but non-significant, percentage of cells positive for E-cad. However, a significant increase in cells positive for N-cad (50%) was observed in the 4-day differentiated cells compared with 2D, microcarrier and aggregate cultures. In contrast, EBs had a very low number of cells positive for E-cad and around 96% cells positive for N-cad, supporting the observation that differentiation leads to decrease in E-cad and increase in N-cad expression [22]. RT-PCR analysis of E-cad and N-cad expression showed no differences in expression in 2D and suspension cultures (Figure 4B). Other markers in the epithelial–mesenchymal transition pathway including fibronectin, vimentin and Trim 28 did not show any differential transcript expression between 2D and suspension cultures whereas the transcription

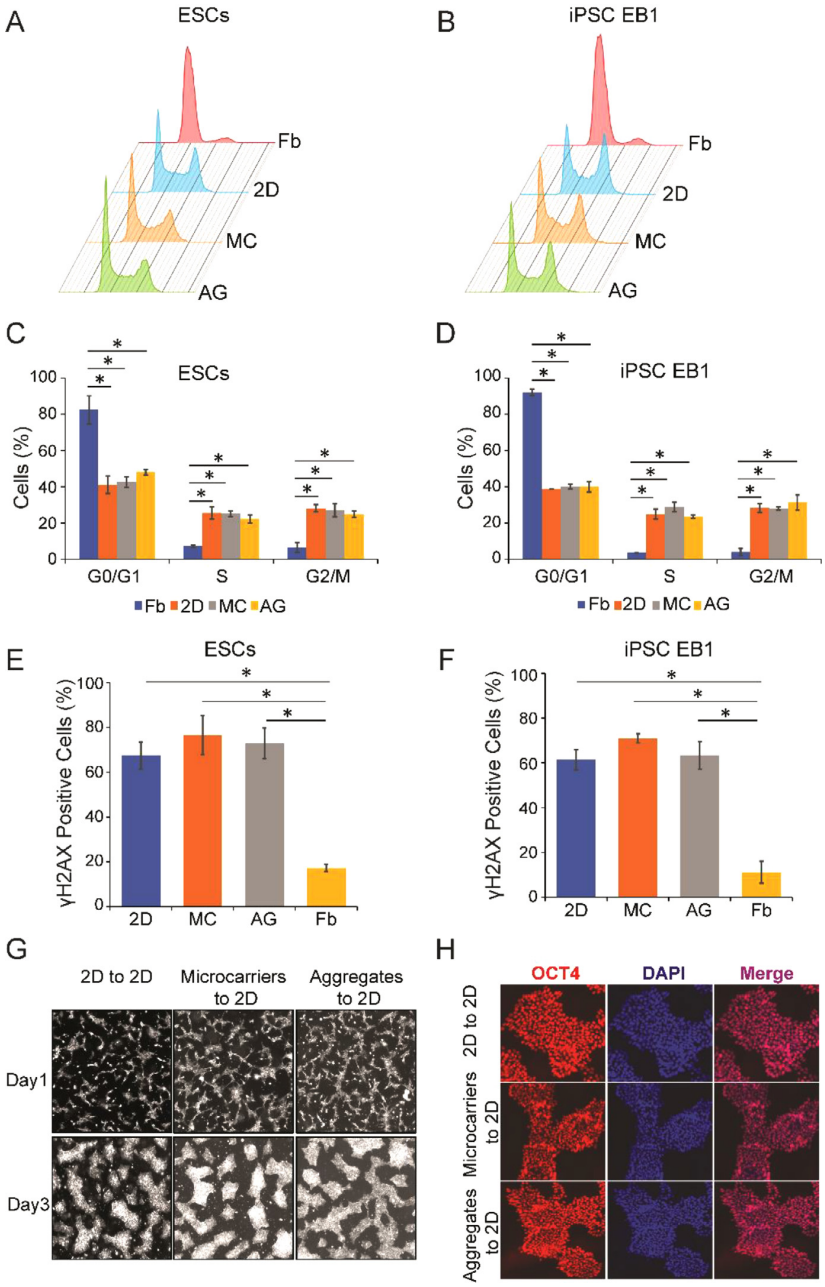


**Figure 5.** Analysis of PSC-specific characteristics in cells cultured on microcarriers (MC) and as aggregates (AG) in comparison with 2D. Cell-cycle profiles of fibroblasts (Fb), cells cultured on 2D, on microcarriers and as aggregates for ESC line (A) and iPSC EB1 line (B). Quantitative analysis of percent cells in different phases of cell cycle, G0/G1, S and G2/M phases for fibroblasts, cells cultured on 2D, on microcarriers and as aggregates for ESC line, n = 3 (C) and iPSC EB1 line, n = 2 (D). Analysis of γH2AX-positive cells in cells cultured on 2D, on microcarriers, as aggregates and fibroblasts for ESC line, n = 3 (E) and iPSC EB1 line, n = 3 (F). (G) Representative images on day 1 and day 3 of single cells harvested from 2D, microcarrier and aggregate cultures and plated on vitronectin coated plates. (H) Representative images of OCT4 staining of single cells harvested from 2D, microcarrier and aggregate cultures and replated on 2D until day 3. *Denotes *P* value <0.05.

factors Snail 1, Snail 2 and Twist1 showed very low expression in all samples (supplementary Figure 4).

*PSCs grown on microcarriers and as aggregates retain typical PSC cell-cycle profiles and morphology*

PSCs have distinct cell-cycle profiles and cell morphologies that distinguish them from differentiated cells. To ask whether cells grown on microcarriers and as aggregates retained PSC-specific cell-cycle profiles, we compared their cell cycle profiles with 2D-cultured cells. Cell cycle profiles of the ESC and iPSC EB1 lines cultured in 2D and 3D were very similar and very distinct from the fibroblast cells (Figure 5A,B), with the average number of cells at each cell-cycle stage similar between 2D and suspension cultures (Figure 5C,D). Our data show that growing cells on microcarriers or as aggregates did not alter the cell-cycle parameters of PSCs.

PSCs have high basal levels of γH2AX, a variant of Histone H2A phosphorylated at serine 169 in response to DNA damage. Our

laboratory has shown that γH2AX in PSCs correlates with replication markers and that the high basal levels of γH2AX observed in PSCs associate with the rapid replication rate of these cells [17]. We asked whether culturing PSCs in 3D suspension affected the expression of γH2AX. ESCs cultured on 2D, microcarriers and as aggregates showed mean % γH2AX positive cells of 67, 76 and 72, respectively, whereas slow-dividing fibroblasts had close to 17% of γH2AX-positive cells (Figure 5E). iPSC EB1 cells cultured in 2D, on microcarrier and as aggregates showed a mean % γH2AX-positive cells of 63, 70 and 61, whereas fibroblasts showed 12% γH2AX-positive cells (Figure 5F). The percent of γH2AX-positive cells in suspension and 2D cultured cells showed no significant differences, indicating that suspension culture supported the retention of PSC replication characteristics.

PSCs have a distinct morphology, growing as colonies when plated on 2D. We plated cells harvested as single cells from suspension culture on day 6 on vitronectin coated 2D culture plates to test if cells maintained the characteristic growth pattern after growing in 3D suspension culture. Images taken on day 1 after plating cells showed that they form small colonies and by day 3 expanded to form larger colonies similarly to 2D (Figure 5G). Staining for the pluripotency marker, OCT4, on day 3 cells plated on 2D showed high OCT4 expression in cells plated onto 2D from microcarriers and aggregates (Figure 5H). This shows that the cells harvested from 3D cultured cells retained both pluripotency and their typical growth patterns.

*PSCs grown on microcarriers and as aggregates show differentiation potential similar to 2D cultures*

In addition to expression of pluripotency markers, PSCs are characterized by their ability to differentiate into three germ layers. Hence large-scale expansion methods of PSCs should support retention of their differentiation potential. To check the differentiation potential of 3D cultured cells, we plated cells harvested from 2D and 3D cultures from ESC and iPSC EB1 lines onto vitronectin coated plates and differentiated them separately into ectoderm, endoderm and mesoderm using the Stemdiff trilineage differentiation kit.

Differentiation potential was assessed using RT-PCR to interrogate transcripts specific for each of the three germ layers, ectoderm (PAX6, Nestin, OTX2 and NCAM1), endoderm (HNF3B, FoxA1, HNF4A, GSC and CXCR4) and mesoderm (IGF2, Brachyury, ACTA2 and RUNX1). Similar expression of all the markers of ectoderm (Figure 6A), endoderm (Figure 6B) and mesoderm (Figure 6C) was observed in 3D cultured cells as compared with 2D in both ESC and iPSC lines, suggesting that the cells cultured on microcarriers and as aggregates retain their differentiation potential. Further, the expression of pluripotency markers (POU5F1 and REX1) decreased after differentiation into three lineages indicating our differentiation protocol was successful (supplementary Figure 5).

*PSCs cultured in 3D suspension can be effectively differentiated into neural progenitor cells and cardiomyocytes*

Therapeutic applications of PSCs require that they be successfully differentiated into large numbers of clinically relevant cell types. Since differentiated cells have low proliferation rates, to obtain large numbers of cells required for clinical applications, most sponsors use large-scale expansion of the rapidly dividing PSCs before differentiation. We examined the potential of cells expanded in 3D suspension to differentiate into two commonly used clinically relevant cell types, neural progenitor cells and cardiomyocytes. Neural progenitor cells were generated using STEMdiff Neural Induction Medium (Stem Cell technologies) and analyzed by RT-PCR for Pax6, Neurog2 and MAP2 neural markers. The expression of all the three markers was comparable with 2D-cultured cells, suggesting that PSCs cultured on microcarriers, and as aggregates can be successfully differentiated to neural progenitor cells (Figure 7A). There was very low expression of pluripotency-associated markers POU5F1 and REX1 (supplementary Figure 6A). Next, we examined whether PSCs expanded on microcarriers, and aggregates can be directly differentiated into cardiomyocytes in the bioreactors. On day 6 of expansion of PSCs in the bioreactors, cells were directly subjected to differentiation using cardiomyocyte differentiation protocol [18]. A 2D culture was also differentiated in parallel as a control. RT-PCR was conducted on cells collected on days 18-20 of differentiation using cardiomyocyte-specific markers GATA4, Nkx2.5, CTNN1, hcTnT, hMYH7, hMYL2 and hMYL7. No statistically significant differences were observed between 2D and suspension cultures except for Nkx2.5 (Figure 7B). Cells in 2D culture (supplementary Video 1), on microcarriers (supplementary Video 2) and as aggregates (Supplemental video 3) showed contraction typical of cardiomyocytes. The pluripotency-associated markers, POU5F1 and REX1, showed very low expression indicating efficient cardiomyocyte differentiation (supplementary Figure 6B).

## Discussion

Large-scale expansion of PSCs is critical to generate the extensive numbers of differentiated cell types necessary for clinical applications. 3D suspension methods offer a greater advantage over 2D culture in terms of time, labor, and cost. Previous studies have tested various 3D culture strategies including growth on microcarriers, or as aggregates individually in different kinds of bioreactors [5,11,12,14,25-28]. However, each condition was examined individually, such that a direct comparison of these two conditions could not be made. In this report, we provide a parallel examination of expansion, pluripotency and differentiation potential of PSCs grown on microcarriers and as aggregates in vertical-wheel bioreactors.

Under our culture conditions, by day 6, ESCs on microcarriers and aggregates reached a density of $2.2 \times 10^6$ cells/mL and $1.8 \times 10^6$ cells/mL and a fold-expansion of 22 and 18, respectively, with no significant differences between the two culture conditions. Scaling up by using the same optimized parameters into larger volume bioreactors could lead to further enhanced production of cells. Other reports have shown different fold-expansion values for PSCs cultured on microcarriers and as aggregates under different culture conditions in vertical-wheel bioreactors [2,5,6,15,29]. Table 2 summarizes the culture conditions used and the cell expansion profiles of PSCs grown in vertical-wheel bioreactor from literature. Our analysis of spent media using the BioProfile FLEX 2 Analyzer showed no significant differences between glucose and lactate concentrations between microcarrier and aggregate cultures from day 3 through day 6 in both ESC and iPSC lines. In addition, pH and $O_2$ saturation dropped as days in culture increased with no significant differences between microcarrier and aggregate cultures except for pH on day 6 in iPSC EB1 line. Expansion of cells after 6 days under these culture conditions would potentially lead to poor medium conditions that could affect cell quality. Optimizing parameters such as seeding density, cell culture conditions and serial passaging might make it possible to culture cells for longer times until desired cell density is reached [14,25,27]. Incorporation of supernatant analysis of medium might inform the ideal number of days the cells could be maintained in long-term culture. Further, computer-controlled perfusion bioreactors could help better control medium parameters and nutrients as pH, dissolved $O_2$, glucose and lactate concentrations and thus facilitate long-term expansion in suspension culture [14,30,31].

In addition to cell number, cell quality is an essential factor in the upscaling process of PSCs for therapeutic applications. PSCs cultured on microcarriers and as aggregates showed similar pluripotency transcript and protein expression that was comparable with parallel 2D cultures while maintaining low levels of expression of markers of differentiation. However, expression of pluripotency-associated genes might not be a reliable indicator for detection of spontaneous

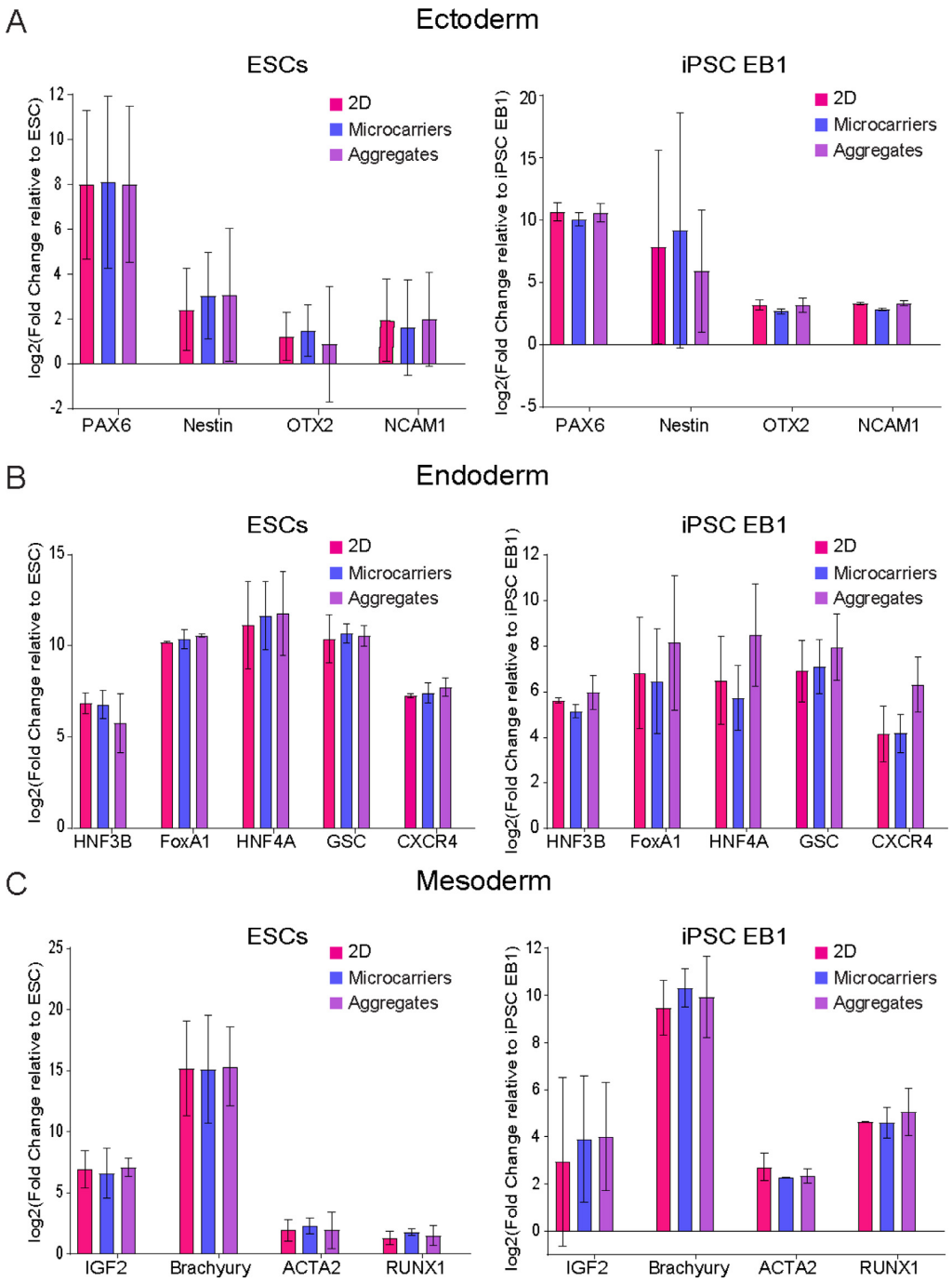


**Figure 6.** Assessment of differentiation potential of 2D, microcarrier and aggregate cultures of PSCs. (A) Analysis of gene expression of ectoderm-specific markers (PAX6, nestin, OTX2 and NCAM) in cells plated on 2D from 2D, microcarrier and aggregate cultures and differentiated into ectoderm for 7 days in ESC line (n = 3) and iPSC EB1 line (n = 3). (B) Gene expression of endoderm-specific markers (HNF3B, FoxA1, HNFA, GSC and CXCR4) in cells plated on 2D from 2D, microcarrier and aggregate cultures and differentiated into endoderm for 5 days in ESC line (n = 3) and iPSC EB1 line (n = 3). (C) Analysis of gene expression of mesoderm-specific markers (IGF2, Brachyury, ACTA2, RUNX1) in cells plated on 2D from 2D, microcarrier and aggregate cultures and differentiated into mesoderm for 5 days in ESC line (n = 3) and iPSC EB1 line (n = 3).

differentiation, as this expression might not be reduced instantaneously and significantly upon differentiation. Hence, finding reliable and sensitive markers for detecting even low levels of differentiated population in undifferentiated cells would be advantageous. In our experiments, 4-day differentiated cells and EBs showed lower E-cad and higher N-cad expression compared with undifferentiated cells, supporting the use of E-cad and N-cad as markers for undifferentiated status of PSCs [32]. We also noted a significant increase in N-cad expression in microcarriers compared with 2D and aggregate cultures, indicative of spontaneous differentiation. However, no notable decrease in E-cad expression or other pluripotent marker expression in microcarrier culture was detected, suggesting that N-cad expression could be a more sensitive marker for spontaneous differentiation than E-cad.

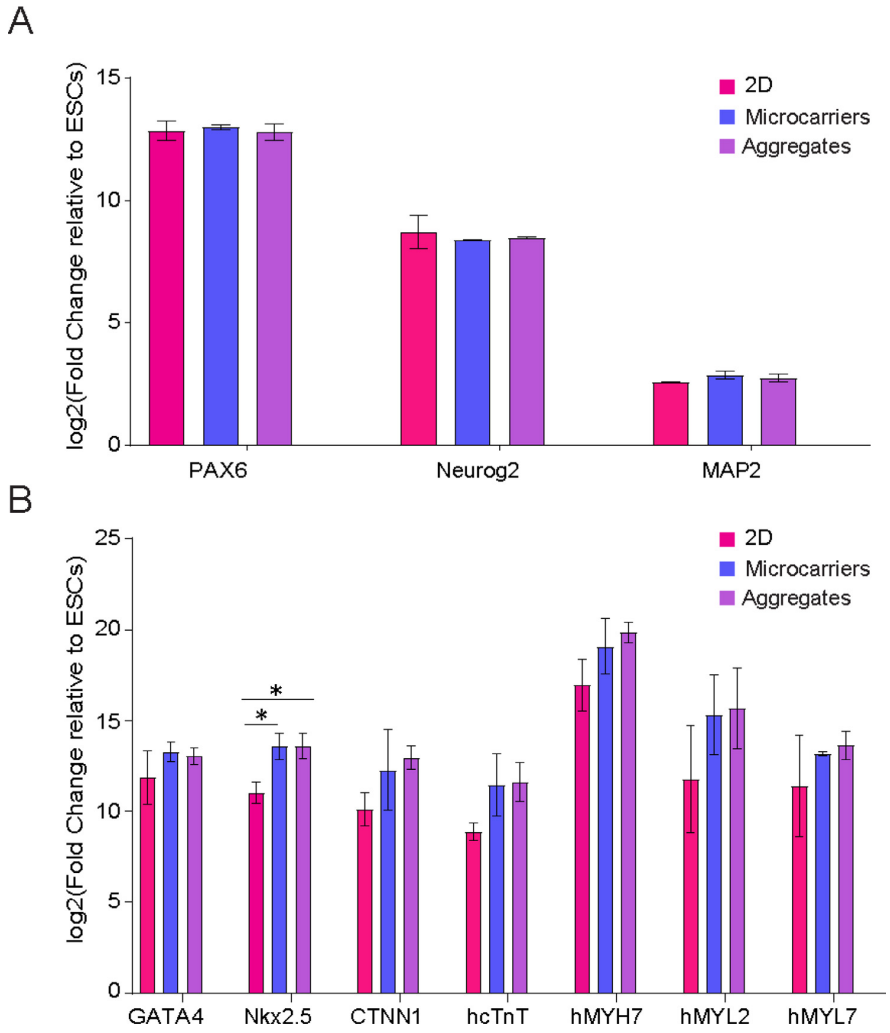


**Figure 7.** Directed differentiation of 2D, microcarrier and aggregate cultures of PSCs into neural precursor cells and cardiomyocytes. (A) Gene expression analysis for neural-specific markers (Pax6, Neurog2 and MAP2) in cells plated on 2D from 2D, microcarrier and aggregate cultures and differentiated into neural progenitor cells for 7 days in ESC line (n = 3). (B) Gene expression analysis for cardiomyocyte markers (GATA4, Nkx2.5, CTNN1, hcTnT, hMYH7, hMYL2 and hMYL7) in cells differentiated into cardiomyocytes in 2D, microcarrier and aggregates cultures for 18–20 days in ESC line (n = 3). *Denotes *P* value <0.05.

Rapid expansion is a critical attribute of PSCs to obtain large cell numbers. PSCs have a characteristic cell cycle with a short G1 phase and with substantial numbers of cells in S-phase compared to differentiated cells [33,34]. PSCs grown on microcarriers, and aggregates maintained this characteristic cell cycle profile with percentages of cells in G0/G1, S and G2/M phases comparable to 2D cultured cells. This suggests that suspension culture supports replication of stem cells similarly to 2D culture. Concomitant with this observation, suspension culture also did not affect the percent cells positive for $\gamma$H2AX expression which in PSCs correlates with their high replication rate.

The main therapeutic utility of PSCs lies in their ability to differentiate into clinically relevant cell types. Many studies conducted individually with either cells cultured on microcarriers or as aggregates showed that they had the ability to differentiate into all three germ layers [12,14,25,29]. Our study simultaneously examined the relative expression of multiple markers for ectoderm, endoderm and mesoderm after differentiation of cells cultured on microcarriers, as aggregates and in 2D and showed they were all similar. Further, we show that PSCs on day 6 can be directly differentiated into neural progenitor cells or continuously differentiated into cardiomyocytes in suspension. The ability to directly differentiate PSCs after scale-up expansion would not only enable large numbers of cells for preparation of final product but also reduces the total time required.

With the advent of new advanced manufacturing technologies, large-scale expansion of PSCs has become feasible. The final quality of the differentiated cells produced is the most critical attribute for clinical manufacturing. The availability of multiple strategies for growing PSCs in suspension makes it of primary importance to understand any differences in cell quality produced under these conditions to make an informed choice of various available systems. Our study demonstrates that PSCs cultured in suspension, either on microcarriers or as aggregates, retained all measured characteristics similarly to contemporary 2D cultures, with no significant differences in expansion potential and cell quality parameters between microcarrier- and aggregate-based cultures, suggesting that choice of the system lies with the end-users. However, we note that microcarriers can pose challenges during downstream processing during cell harvest. Most microcarriers are not biodegradable and must be removed from cultures before administration of the cells. Further, they might lead to impurities in the final product in the form of extractables and leachables, affecting product safety and quality. However, it may be

**Table 2**
Comparison of studies done in vertical bioreactors (0.1 L) for expansion of PSCs.

| Cell type | Culture strategy | Media | Volume and rpm | Starting cell density, cells/mL | Number of days in culture | maximum yield, cells/mL | Maximum fold-expansion | Media change | Reference |
|---|---|---|---|---|---|---|---|---|---|
| hiPSCs | Plastic microcarriers coated with vitronectin | E8 | 80 mL, 10–14 rpm for first 48 h, 22–26 rpm until harvest | $2.5 \times 10^4$ | 6 | $1.2 \times 10^6$ | 6.7 | Replacing 80% medium on a daily basis | [15] |
| hiPSCs | Aggregates | mTeSR1 | 60 mL, 30 rpm | $2.5 \times 10^5$ | 7 | $1.2 \times 10^6$ | 4.8 | 80% medium exchange daily 48 h post-inoculation | [12] |
| hiPSCs | Aggregates | mTeSR1 | 100 mL, 80 rpm | $2 \times 10^4$ | 6 | $6 \times 10^5$ | 30 | Single, 50% media exchange on day 4 | [5] |
| hiPSCs | Aggregates | mTeSR1 | 100 mL, 40 rpm | $2 \times 10^4$ | 6 | ~$6 \times 10^5$ | 32 | Single, 50% media exchange on day 4 | [29] |
| hiPSCs | Aggregates | mTeSR1 | 100 mL, 60 rpm | $2 \times 10^4$ | 7 | ~$7 \times 10^5$ | 27-37 | 50% medium exchange on day 3, 5 and 6 | [6] |
| hiPSCs and ESCs | Plastic microcarriers coated with vitronectin and aggregates | mTeSR PLUS | 100 mL, 40 rpm | $1.0 \times 10^5$ | 6 | $2.2 \times 10^6$ for microcarriers and $1.8 \times 10^6$ for aggregates | 22 for microcarriers and 18 for aggregates | 80% medium exchange daily from day 2 to 5 | This study |

that for certain applications, microcarrier-based cultures provide more complete differentiation or are more suitable to a specific manufacturing strategy. Further analysis with a broad range of differentiation endpoints and scale up targets will help to better understand the quality aspects of these new promising technologies for large scale production of stem cells.

## Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

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## Author Contributions

Conception and design of the study: HV and DAH. Acquisition of data: HV, TS and PS. Analysis and interpretation of data: HV, TS, PS and DAH. Drafting or revising the manuscript: HV and DAH. All authors have approved the final article.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcyt.2023.05.002.

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