Brazilian Journal of Medical and Biological Research (2009) 42: 515-522
ISSN 0100-879X

Successful scale-up of human embryonic stem cell production in a stirred microcarrier culture system

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Future clinical applications of human embryonic stem (hES) cells will require high-yield culture protocols. Currently, hES cells are mainly cultured in static tissue plates, which offer a limited surface and require repeated sub-culturing. Here we describe a stirred system with commercial dextran-based microcarriers coated with denatured collagen to scale-up hES cell production. Maintenance of pluripotency in the microcarrier-based stirred system was shown by immunocytochemical and flow cytometry analyses for pluripotency-associated markers. The formation of cavitated embryoid bodies expressing markers of endoderm, ectoderm and mesoderm was further evidence of maintenance of differentiation capability. Cell yield per volume of medium spent was more than 2-fold higher than in static plates, resulting in a significant decrease in cultivation costs. A total of 10^8 karyotypically stable hES cells were obtained from a unitary small vessel that needed virtually no manipulation during cell proliferation, decreasing risks of contamination. Spinner flasks are available up to working volumes in the range of several liters. If desired, samples from the homogenous suspension can be withdrawn to allow process validation needed in the last expansion steps prior to transplantation. Especially when thinking about clinical trials involving from dozens to hundreds of patients, the use of a small number of larger spinners instead of hundreds of plates or flasks will be beneficial. To our knowledge, this is the first description of successful scale-up of feeder- and Matrigel™-free production of undifferentiated hES cells under continuous agitation, which makes this system a promising alternative for both therapy and research needs.

Key words: Human embryonic stem cells; Scale-up; Microcarriers; Spinner bioreactors

Research supported by FAPERJ, CNPq, Fundação Ary Frauzino para Pesquisa e Controle do Câncer (FAF), CAPES, as well as Ministério da Saúde, the Academy of Sciences of the Developing World (TWAS), and Pew Latin American Fellows Program in Biomedical Sciences.

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Received February 15, 2009. Accepted April 13, 2009

Introduction

Human embryonic stem (hES) cells have an unlimited capacity for self-renewal and the ability to differentiate into all cell types (1). These cells are of great interest for cell therapy and regenerative medicine (2,3) as well as for high throughput drug screening (4,5). Both applications, however, still represent a challenge, mainly because of technical limitations in scaling-up stem cell cultures (6,7). Stirred culture systems, such as the rotary RCCS™ bioreactor used by Chen et al. (8) and the spinner flasks employed by Fok and Zandstra (9), represent a significant improvement in culture techniques compared to static systems, as they offer several advantages in the scale-up process, includ-

ing: 1) a controlled environment leading to a homogeneous culture, 2) simplicity of handling, and 3) less susceptibility to contamination (10). Indeed, bioreactors have been used in a variety of bioprocesses, such as cell expansion (11-14) and protein and virus production (15-17).

We investigated here the cultivation of hES cells in a stirred system with commercial dextran-based microcarriers coated with denatured collagen (Cytodex™ 3), which provide an increased surface area for cell adhesion (18). Although microcarriers have been described for culturing stem cells from both animals (12,19) and humans (20-22), to our knowledge, this is the first description of the use of microcarriers, without coating with mouse fibroblasts or Matrigel™, that successfully scaled-up human embryonic stem cell production in a stirred culture system. The cultivation method presented here will facilitate expansion of hES cells needed for cell therapy, research and industrial applications.

Material and Methods

Undifferentiated H9 human embryonic stem cells (WiCell Research Institute, USA), which are so far the most frequently used hES cell line (23), were initially cultured in 6-well plates on inactivated mouse embryonic fibroblasts (MEFs) (Hygeia Biotech, Brazil) in hES cell medium, which consisted of high-glucose DMEM/F12 medium supplemented with 20% Knockout™ Serum Replacement (KSR), 200 mM 2% glutamine, 55 mM 0.2% 2-mercaptoethanol, 1% non-essential amino acids (all from Gibco Invitrogen Corporation, USA), and 8 ng/mL fibroblast growth factor-2 (FGF-2, R&D Systems, UK).

For the stirred cultures, spinner flasks of 125-mL nominal volume (Techne, USA) were used. Cytodex™ 3 microcarriers (GE Healthcare, UK) were prepared according to manufacturer instructions. In each spinner, 18 mL MEF-conditioned medium (MEF-CM) supplemented with 8 ng/mL FGF-2 and a total of 1.2-1.5 x 10^7 cells dissociated from plates with TrypLE™ (Gibco Invitrogen Corporation) were inoculated onto 0.18 g of microcarriers suspended in 6 mL PBS remaining from the washing steps. hES cells adhered to the beads overnight under an intermittent agitation regime (cycles of 3 min/40 rpm followed by 27 min with no agitation). Then, 42 mL MEF-CM supplemented with 8 ng/mL FGF-2 was added, resulting in a final volume of 66 mL, a microcarrier concentration of 2.7 g/L and an inoculum density of 2.0-2.5 x 10^5 cells/mL. MEF-CM was obtained using hES cell medium without FGF-2, conditioned for 24 h by confluent MEFs in 75-cm^2 T flasks. hES cells were then cultured under continuous agitation (60 rpm) for 15 days, with daily exchange of 30 mL (45%) of medium. To exchange the medium, agitation was stopped for a short period to allow the microcarrier beads to settle and 30 mL of supernatant was easily withdrawn. Daily aliquots of 1 mL were taken under agitation, centrifuged to remove the supernatant, incubated with crystal violet solution (1 h), and vortexed vigorously, followed by counting the released nuclei using a hemocytometer, as described previously for microcarrier cultures (24).

Control experiments were carried out in 6-well plates (9 cm^2 each well), which are the kind of static plates most frequently used to culture hES cells, using a feeder layer of inactivated MEFs. Each well was initially filled with 5 mL of medium, which was completely exchanged for 4 mL of fresh medium at 48 and 72 h. A sufficient number of wells were inoculated to permit daily sampling of cells from 2 wells by detaching them with TrypLE™. All culture experiments were carried out in duplicate.

In order to assess spontaneous differentiation through embryoid body (EB) formation, cells growing in static plates were dissociated with TrypLE™ and those from spinner cultures were detached from microcarriers also using TrypLE™. Approximately 4 x 10^6 cells were plated onto non-adherent dishes (60 mm) in 7 mL hES cell medium supplemented with 15% KSR without FGF-2 for 7 days, in order to obtain complete EB formation, with medium being changed every other day. Next, EBs were fixed in 4% paraformaldehyde (PF), embedded in Tissue Tek® OCT compound and 10-µm sections were prepared with a cryostat.

Immunocytochemistry against the transcriptional factor Oct-4 (Santa Cruz Biotechnology Inc., USA; Mouse-1:100), the specific cell surface marker SSEA-4 (Chemicon-Temecula, USA; Mouse-1:100), and the proteoglycan recognized by the antibody TRA 1-60 (Chemicon-Temecula; Mouse-1:100) was performed before, during and after submitting cells to the stirred microcarrier culture. The spontaneous EB differentiation assay was performed prior to and after microcarrier culture by re-plating cells detached from microcarriers with TrypLE™ on inactivated MEFs. Immunocytochemistry against markers from the three germ layers was performed in slices of these EBs using antibodies against alphafetoprotein (AFP; Rabbit: 1:100; Chemicon-Temecula), alpha smooth muscle actin (α-SMA; Mouse: 1:400; Sigma-Aldrich Sweden AB) and nestin (Mouse: 1:100; Chemicon-Temecula) for the endoderm, mesoderm and ectoderm, respectively. Briefly, hES cells were fixed with 4% PF for 30 min and permeabilized with Triton X-100. Non-specific epitopes were blocked with 5% bovine serum albumin (BSA) for 1 h. Primary antibodies were incubated for 1 h and the secondary anti-

bodies (Alexa Fluor 546 goat anti-mouse or anti-rabbit IgG, 1:400; Molecular Probes, Gibco Invitrogen Corporation) for 30 min (all at room temperature). Nuclei were counterstained with 4'-6-diamidino-2-phenylindole and images were obtained by fluorescence microscopy (Nikon, Eclipse T300). Cytometric analyses were carried out using a FACScalibur flow cytometer (BD Biosciences). Undifferentiated colonies and EBs were manually dissociated and fixed with 4% PF, washed in FACS buffer (1% BSA and 0.01% sodium azide in PBS), and blocked for 1 h with FACS buffer. Primary and secondary antibody stainings were performed for 1 h. Data acquisition from at least 10,000 events was performed using the CellQuest™ software (BD Biosciences, USA) and the Summit MoFlo software (Dako Cytomation, USA). To analyze specific pluripotentiality markers, the same primary antibodies as used in immunocytochemistry, directed against Oct-4, SSEA-4, and TRA 1-60, plus SOX-2 (Chemicon-Temecula; Rabbit: 1: 200) were used. The same primary antibodies used in immunocytochemistry against AFP, α-SMA and nestin were used for the evaluation of spontaneous differentiation into three germ layers. Secondary antibodies were Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Gibco Invitrogen Corporation; 1:400) and phycoerythrin F(ab')2 fragment donkey anti-mouse IgG (Jackson ImmunoResearch, USA; 1:200).

Cellular ploidy was determined before and after culturing the hES cells in the spinner flasks as previously described (25). For this assay, hES cells were incubated with 1 µg/mL Karyo MAX Colcemid (Gibco Invitrogen Corporation) for 6 h to cause mitotic arrest at metaphase. Then, cells were detached using 0.05% trypsin/EDTA. After incubation in a hypotonic solution of 75 mM KCl for 15 min, swollen cells were fixed overnight with a methanol/acetic acid (3:1) solution and spread onto dry slides. The number of chromosomes was determined in 20 metaphases after G-banding.

Results and Discussion

Human embryonic stem cells were able to adhere to and to proliferate on commercial collagen-coated dextran microcarriers. For the sake of process simplification and elimination of additional animal-derived components, no special pre-treatment of the microcarrier beads was carried out, such as coating with Matrigel™ or MEF, as recently proposed by Nie et al. (22).

Although cells in the spinners underwent a long lag phase, cell growth between 120 and 340 h followed an exponential pattern (data fitting with R = 0.989). However, the doubling time in the exponential phase was 75 h, as compared to 25 h in the static plates coated with MEFs and this could be an indication that cells need to be further adapted to feeder-free culture conditions on microcarriers for some passages. Nevertheless, 101 million cells were obtained, on average, within 340 h in each spinner. The spinner flasks used are relatively small (6.5 cm in diameter and 14.5 cm in height) and cells can be cultivated in them with no manipulation (except for the daily partial medium exchange), indicating that the proposed stirred culture technique is a promising method to scale-up hES cell production.

As shown in Figure 1, in terms of cells per growth surface, cells in the spinners grew to a maximum concentration of 0.21 million cells/cm^2 (Figure 1B), which is 30% more than the maximum concentration obtained in static plates, 0.16 million cells/cm^2 (Figure 1A). However, the most significant difference was observed in terms of cells per volume of medium. In the spinner, a maximum concen-

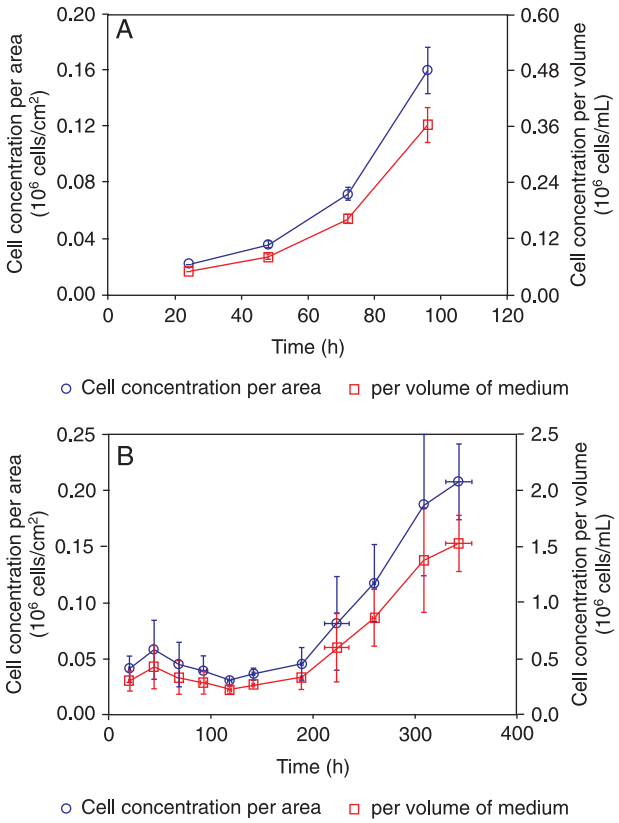

Figure 1. Kinetics of human embryonic stem cell growth cultured in: *A*, 6-well plates; *B*, spinner flasks. Data are reported both in terms of cells obtained per surface area available for cell attachment and in terms of cell concentration per volume of medium in the culture vessel. Data are reported as means ± SE for experiments in duplicate.

tration of 1.5 x 10^6 cells/mL was achieved compared to 0.36 x 10^6 cells/mL in the static plates.

As stated earlier, daily medium exchanges (partial exchanges in spinners and complete exchanges in plates) were carried out. In view of this, the cell yield per total volume of medium spent was calculated and resulted in 240,000 cells/mL in spinners versus 110,000 cells/mL in static culture. This over 2-fold higher cell yield obtained in the stirred system significantly decreased the costs associated with culture medium and with expensive supplements, such as FGF-2.

To characterize the pluripotency of hES cells grown in stirred microcarrier cultures, we evaluated the presence of the pluripotency markers Oct-4, SSEA-4 and TRA 1-60 by immunocytochemistry and confirmed their presence by flow cytometry of the same markers plus SOX-2 (Figure 2). The procedures were done before inoculating the hES cells into the spinner bioreactors during stirred microcarrier cultivation, and after spinner culture in the cells that were recovered from microcarriers and re-plated onto inactivated MEFs (Figure 2).

Cultures of hES cells grown on static plates have been

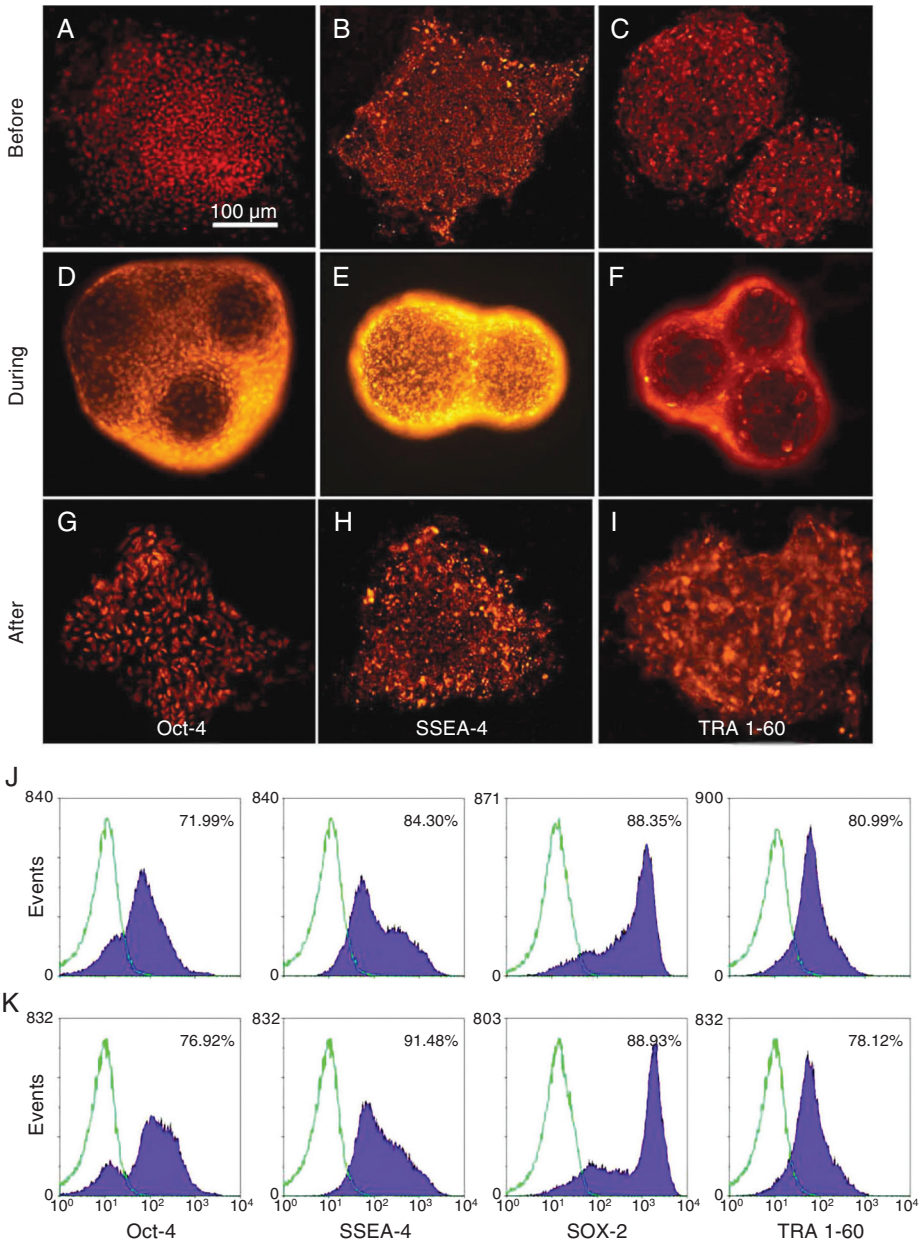

Figure 2. Pluripotency analysis: *A-I*, immunocytochemistry of H9 human embryonic stem cells (P33) for Oct-4, SSEA-4 and TRA 1-60 before, during and after cell growth in spinners. Cells sampled from spinners were replated onto inactivated mouse embryonic fibroblasts and cultured for 2 weeks before the immunocytochemical analyses (scale bar: 100 µm). *J-K*, FACS results of human embryonic stem cells before (J) and after (K) cell culture in spinners, for each of the four markers analyzed (Oct-4, SSEA-4, SOX-2, and TRA 1-60).

shown to maintain a stable karyotype even after long-term culturing (26). Likewise, in the present study, using TrypLE™ to detach cells from the microcarriers (21,27), hES cells maintained a stable karyotype after 2 weeks of cultivation in the stirred microcarrier system (Figure 3).

Cultures of human EBs form cystic structures characterized by cavitations and fluid accumulation (28) followed by spontaneous differentiation into multiple cell types of three germ layers (29-31). To evaluate whether cultivation of hES cells in stirred microcarrier systems maintain these characteristics, EBs formed either from hES cells grown on static plates or re-plated onto MEFs after spinner culture were compared. Spinner-cultured hES cells were able to aggregate as EBs with diameter and morphology similar to those of EBs derived from naive cells grown on static cultures (Figure 4). Immunocytochemical and FACS analysis showed that, as expected, spinner-derived hES cells

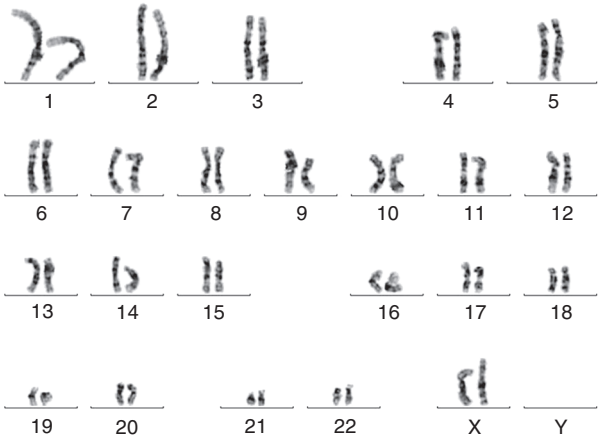

Figure 3. Karyotype analysis of cells after culture in the microcarrier-bioreactor system. Chromosomes were stained with Giemsa and show a normal karyotype.

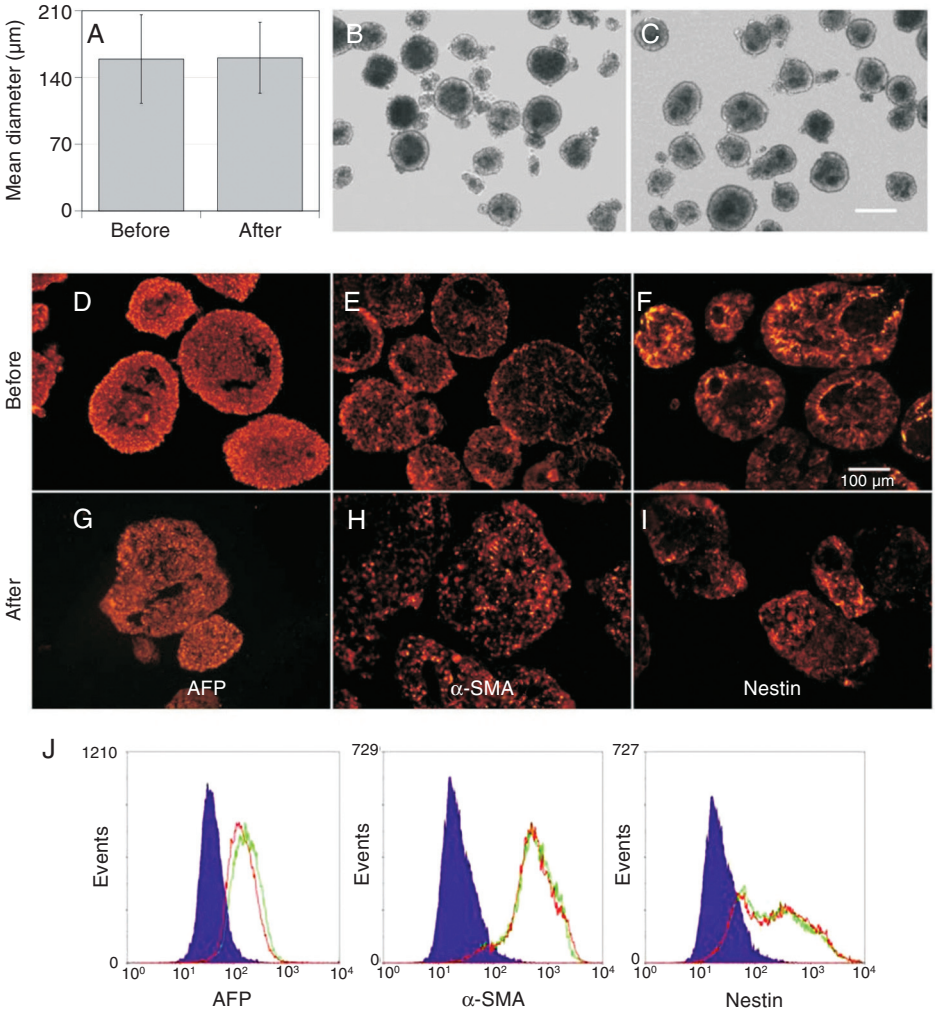

Figure 4. Spontaneous differentiation through embryoid body (EB) formation: *A*, average size of EBs; *B*, micrograph of EBs formed from human embryonic stem (hES) cells before spinner propagation; *C*, micrograph of EBs formed from hES cells after spinner propagation (scale bar: 200 µm for panels B and C). *D-I*, Immunocytochemistry for AFP (endoderm), α-SMA (mesoderm) and nestin (ectoderm) in EBs formed after 7 days in traditional static culture on inactivated mouse embryonic fibroblasts (MEFs) and after growth in spinner and then re-plating onto inactivated MEFs (scale bar: 100 µm for panels D-I). *J*, FACS results of EB samples for markers of the three germ layers: AFP (endoderm), α-SMA (mesoderm) and nestin (ectoderm), before (red line) and after (green line) cell growth in the spinner system. AFP = alphafetoprotein; α-SMA = α-smooth muscle actin.

form EBs with the three germ layers (Figure 4). As previously reported (28), endodermal markers were expressed in both the interior and exterior layers, with ectodermal labeling being peripheral and adjacent to the endoderm layer.

There is considerable interest in the use of agitated systems to culture embryonic stem cells. However, no study was able to successfully scale-up undifferentiated hES cells using a defined microcarrier matrix. Although some groups working with murine embryonic stem cells have reached a higher final cell concentration in stirred cultures than that achieved with our microcarrier-based culture system (12,32), two important facts should be taken into account: i) murine embryonic stem cells have a faster cell cycle, which ultimately results in a higher proliferation rate when compared to human cells (33), and ii) the well-established culture conditions of murine embryonic stem cells provide a good basis for their adaptation to other culture types. It is relatively easy to make murine embryonic stem cells adhere to beads in suspension, whereas hES cell culture conditions are not robust, and any changes in these conditions may result in them ultimately losing their pluripotentiality (9,20,34).

Phillips et al. (21) showed that hES cells are able to grow attached to microcarriers in a stationary environment and presented data of a proof-of-principle experiment in stirred spinner flasks. In that experiment, using a culture medium containing 100 ng/mL FGF-2, they achieved a cell concentration of 1.4 x 10^5 cells/mL. In the present study, we obtained an 11-fold higher final cell concentration (Table 1) using a 13-fold lower FGF-2 concentration, with a consequent significant cost reduction for cell production.

Recently, Nie et al. (22) were able to culture hES cells in stirred microcarrier systems as well. However, their protocol required additional pre-treatment of the beads by coating with Matrigel™ or murine embryonic fibroblasts, raising the cost and introducing additional animal-derived compounds into the system.

As stated earlier, our stirred microcarrier system was able to yield 240,000 cells/mL of medium used, while in static culture plates the yield was only 110,000 cells/mL of medium used. As discussed by Abranches et al. (12), the improvement in cell growth achieved in stirred microcarrier cultures is most likely due to better oxygenation, nutrient availability and metabolite mass transport, as well as limited microenvironmental toxicity.

Table 1. Overview of studies using microcarriers for the expansion of human embryonic stem cells and comparison with data of the present study.

	Oh and Choo (20)	Phillips et al. (21)[a]	Phillips et al. (21)	Nie et al. (22)	This study	This study
Scale-up methodology	Suspension culture attached to feeders on microcarriers (Cytodex™ 3)	Static plates with microcarriers (Hillex-II)	Spinner flasks with microcarriers (Hillex-II)	Spinner flasks with microcarriers (Cytodex™ 3 coated with Matrigel™ or MEFs)	Static plates	Spinner flasks with microcarriers (Cytodex™ 3)
Inoculum concentration (cells/mL)	Not informed	3.3 x 10^5	0.6 x 10^5	1-2 x 10^5	0.5 x 10^5	2-2.5 x 10^5
Final cell concentration (cells/mL)	Unsuccessful human embryonic stem cell growth attempt	10 x 10^5	1.4 x 10^5	11.2 x 10^5	3.6 x 10^5	15.3 x 10^5
Fold increase related to the inoculum	–	3	2.2	7.5	7.2	6.8
FGF-2 concentration (ng/mL)	–	100	100	4	8	8
Total medium needed to achieve 1 x 10^8 cells	–	810 mL	1600 mL	360 mL	936 mL	420 mL
Total vessels needed to achieve 1 x 10^8 cells	–	5 plates	10 spinner flasks	1 to 2 spinner flasks	12 plates	1 spinner flask
Costs based only on FGF-2 to achieve 1 x 10^8 cells[b]	–	$1113	$2200	$20	$108	$47

FGF-2 = fibroblast growth factor-2; MEFs = mouse embryonic fibroblasts. [a]Under the best conditions determined in the study (21). [b]The cost given for FGF-2 was the market price in Brazil for the imported product used in this study.

Stem cell therapies require large amounts of cells (35,36) and several protocols have been proposed in recent years for the expansion of adult stem cell, (8,10,12, 13,19,32,37-40). The use of hES cells in cell therapy implies that a considerable number of cells are available for cell differentiation and subsequent transplantation. This poses the need for scaling-up the methods currently used for culturing these cells. The culture method proposed here was not only able to yield a high cell concentration and an advantageous yield of cells per volume of culture medium, but, most importantly, it was able to support the proliferation of hES cells in a pluripotent state.

Acknowledgments

We thank Ismael Gomes and Maria R.L.S. Pinheiros for technical assistance and Marcia Triunfol for manuscript revision and editing. We also thank Dr. Michael McConnell (Salk Institute) for helpful discussions.

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