Scale-Up of Mouse Embryonic Stem Cell Expansion in Stirred Bioreactors

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The aim of this study was to develop a robust, quality controlled and reproducible large-scale culture system using serum-free (SF) medium to obtain vast numbers of embryonic stem (ES) cells as a starting source for potential applications in tissue regeneration, as well as for drug screening studies. Mouse ES (mES) cells were firstly cultured on microcarriers in spinner flasks to investigate the effect of different parameters such as the agitation rate and the feeding regimen. Cells were successfully expanded at agitation rates up to 60 rpm using the SF medium and no significant differences in terms of growth kinetics or metabolic profiles were found between the two feeding regimens evaluated: 50% medium renewal every 24 h or 25% every 12 h. Overall, cells reached maximum concentrations of (4.2 ± 0.4) and (5.6 ± 0.8) $\times 10^6$ cells/mL at Day 8 for cells fed once or twice per *day; which corresponds to an increase in total cell number of 85 ± 7 and 108 ± 16, respectively. To have a more precise control over culture conditions and to yield a higher number of cells, the scale-up of the spinner flask culture system was successfully accomplished by using a fully controlled stirred tank bioreactor. In this case, the concentration of mES cells cultured on microcarriers increased 85 ± 15-fold over 11 days. Importantly, mES cells expanded under stirred conditions, in both spinner flask and fully controlled stirred tank bioreactor, using SF medium, retained the expression of pluripotency markers such as Oct-4, Nanog, and SSEA-1 and their differentiation potential into cells of the three embryonic germ layers.* © 2011 American Institute of Chemical Engineers *Biotechnol. Prog.*, 27: 1421–1432, 2011
Keywords: mouse embryonic stem cells, expansion, serum-free medium, microcarriers, spinner flask, stirred tank bioreactor

Introduction

Embryonic stem (ES) cells are undifferentiated cells that have the ability to either self-renew, giving rise to two identical pluripotent "daughter" cells, or to differentiate, producing specialized cells. These properties of ES cells make them a very attractive cell source for stem cell-based therapies, for developmental biology studies and also for drug/toxicity-screening studies. Thus, it is not surprising that ES cell research has been rapidly expanding since pluripotent mouse ES (mES) cells were first isolated in 1981[1] followed by the isolation of human ES[2] cells in 1998[3,4] from the inner cell mass of mouse or human blastocysts, respectively. Nonetheless, the successful implementation of stem cell-based technologies will require the ability to generate high numbers of cells with well-defined characteristics. For instance, to treat an adult diabetes patient with insulin-producing cells obtained from ES cells, as many as $13–26 \times 10^9$ pluripotent ES cells might be needed, which requires 6500–13,000 T-flasks (75 cm^2) and takes about 3–4 weeks to produce.[5] For those reasons, it is necessary to implement very efficient scale-up strategies from the commonly used static culture systems (e.g., tissue culture plates), which can still be used for the initial cell expansion, to dynamic culture systems such as spinner flasks and fully controlled bioreactors.

Depending on the application, culture systems must be designed for the expansion of stem cells with uniform properties and/or to promote controlled and reproducible differentiation into selected highly pure mature cell types. Although several alternatives have been developed, stirred tank bioreactors are by far the ones of choice for large-sale production of biopharmaceuticals[6] due to their simplicity, easiness of monitoring and controlling, and operational flexibility, as they may be operated in batch, fed-batch, or continuous mode.[7] In the last years, several methods of expansion and differentiation of ES cells in homogeneous stirred-suspension bioreactors have been reported, including culturing ES cells as aggregates,[8–18] encapsulated[13,19–22] or using microcarriers to support cell adhesion and proliferation.[2,9,13,22–28] Indeed, microcarrier technology remains one of the most effective techniques to immobilize anchorage-dependent cells, as mES cells, under dynamic culture conditions allowing high-density cell growth.[2,23,24] One of the major obstacles to the successful scale-up of stirred bioreactors is the difficulty in satisfying the oxygen demand of large volume, high-density cell cultures, especially when the cultivated cells are the target product. Larger vessels typically features modules for

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forced oxygenation (sparging) since headspace aeration is not sufficient to maintain proper levels of dissolved oxygen (DO).[13] However, the sparging and high agitation rates required to attain the necessary oxygenation of the culture generate hydrodynamic forces that can injure cells and/or affect cell physiology.[7,29] In addition, the potential harmful effects of shear forces are exacerbated under serum-free (SF) conditions, since animal serum, besides its nutritional role, protects the cells from shear stresses.[29] To our best knowledge, no studies targeting the mass production of pluripotent, undifferentiated cells in a high-density, fully controlled stirred tank culture were yet described. The aim of this study was thus to develop a quality controlled and reproducible large-scale microcarrier-based culture system using SF medium to obtain a large number of ES cells for potential applications in tissue regeneration, as well as for drug screening studies.

Materials and Methods

Model ES cell line

The 46C mES cell line[30] was established in the laboratory of Professor Austin Smith at the Wellcome Trust Centre for Stem Cell Research, University of Cambridge, England, UK. 46C mES cells were kept cryopreserved in liquid nitrogen until further use.

mES cell expansion in static conditions before Spinner flask/Bioreactor inoculation

On thawing, 46C mES cells were expanded on gelatinized (1% [v/v] gelatin solution, prepared from 2% gelatin Solution, Sigma) tissue culture plates for, at least, two passages in Knockout Dulbecco's modified Eagle's medium (KDMEM) (GibcoBRL) containing 15% (v/v) Knockout serum replacement (KSR) (GibcoBRL), 1% (v/v) Glutamine 200 mM (GibcoBRL), 1% (v/v) penicillin (50 U/mL)/streptomycin (50 μg/mL) (GibcoBRL), 1% (v/v) non-essential Amino acids 100× (Sigma), 0.1% (v/v) 2-mercaptoethanol 0.1 mM (Sigma) supplemented with 0.1% (v/v) human leukemia inhibitory factor (hLIF) (produced *in house* by 293-HEK EBNA cell line). This expansion medium will be referred as SF medium thereafter. Cells were cultured at 37°C under a 5% CO_2 humidified atmosphere. In each passage, cell number and viability were determined using the Trypan Blue (Gibco) exclusion method in a hemocytometer under an optical microscope (Leica Microsystems).

mES cell expansion under stirred conditions

Microcarriers. The commercially available microcarriers *Cultispher S* (Sigma) were used to support mES cell adhesion and expansion either in spinner flasks or in the stirred tank bioreactor under stirred conditions. These microcarriers are made of cross-linked gelatin with a pore diameter of 10–20 μm, a density of 1.04 and an approximate surface area of 0.4–0.7 m^2/g. mES cell culture on microcarriers was performed following manufacturer's instructions as previously described.[23] 46C mES cells (5×10^4 cells/mL) previously expanded in SF medium for, at least, two passages under static conditions were mixed with 1 mg/mL of microcarriers (±60 cells/bead) previously hydrated, sterilized by autoclaving and equilibrated in pre-warmed (37°C) SF medium.

Spinner Flask Culture. mES cell expansion was performed using a *StemSpan* (StemCell Technologies) spinner flask (30 mL working volume) equipped with an impeller with 90° paddles (normal paddles) and a magnetic stir bar. As spinner flasks do not allow pH and temperature control, they have to be placed inside an incubator at 5% CO_2 in humidified air and 37°C, with aeration taking place in the gas/liquid interface. Before inoculation of the spinner flask, cells, and microcarriers were incubated at 37°C in 1/6 of the final medium volume during 30 min in a conical tube, with gentle agitation every 10 min. Then, fresh pre-warmed (37°C) SF medium was gently added, until half of the final volume, and cell suspension was transferred to the spinner flask. After a 24-h seeding period with intermittent stirring (15 min of stirring at 30–40 rpm, followed by 60 min statically), SF medium was added up to the final volume and the agitation rate set to 40, 60, or 80 rpm. Depending on the feeding regime studied, partial medium renewal was performed everyday by replacing 50% fresh pre-warmed medium every 24 h or 25% every 12 h. The withdrawal/re-feeding of the culture medium was performed immediately after sedimentation of the microcarriers containing the cells.

Controlled Stirred Tank Bioreactor Culture. Bioreactor experiments were carried out in a 1.3 L modular benchtop mechanically stirred bioreactor (New Brunswick Bioflo 110) equipped with a three-blade pitched stainless-steel impeller (dimensions: impeller diameter (D_i) 6.5 cm, impeller width (W_i) 6.3 cm, impeller thickness (T_i) 0.1 cm and blades pitched 45° to vertical) and with DO, pH, and temperature (T) probes. Before inoculation of the bioreactor, cells and microcarriers were incubated at 37°C in 1/7 of the final medium volume in a conical tube using a Thermomixer (Eppendorf) for a 6-h period with intermittent stirring (3 s of stirring at 300 rpm, followed by 45 min statically). Then, fresh pre-warmed (37°C) SF medium was gently added until the final volume (700 mL), and cell suspension was transferred to the bioreactor. Based on the results obtained when using spinner flasks, the operational parameters were set to: pH 7.2, DO of 20%, T = 37°C, sparging rate of 100 ccm and an agitation rate of 60 rpm. In our scaling-up strategy (spinner flask to stirred tank), the engineering variable volumetric power input by agitation was kept constant. All controlled parameters were kept constant throughout time in culture with the exception of the sparging rate that was increased to 200 ccm on Day 6. The culture pH was maintained with the addition of 1.0 M of $NaHCO_3$ (Sigma) and CO_2 gas. The DO was monitored by a polarographic oxygen electrode previously calibrated by sparging SF medium with N_2 (DO = 0%) and compressed air (DO = 100%). Aeration was achieved through gentle sparging from the base of the bioreactor with a mixture of N_2, air and CO_2 gas bubbles, and temperature was kept at 37°C by an electric heating jacket. The feeding was performed everyday by replacing 50% of medium and the withdrawal/re-feeding of the culture medium was performed immediately after the quick sedimentation of the microcarriers containing the cells.

Monitoring of cell culture in the spinner flask and stirred tank bioreactor

Cell Counts and Viability. Everyday, duplicate samples of evenly mixed culture were collected from the spinner flask/bioreactor. The beads were washed twice with phosphate buffered saline (PBS) (Sigma) and then 1% trypsin

(GibcoBRL) was added. Cell samples were then incubated for 10–20 min in the thermomixer at 37°C and 1000–1300 rpm. The number of viable and dead cells was determined by using the trypan blue dye exclusion test and counting the cells on a hemocytometer. Supernatant samples were centrifuged at 1500 rpm for 10 min and kept at −20°C for subsequent analysis.

Growth Rates and Doubling Times Calculation. The growth kinetics of 46C mES cells cultured under stirred conditions on microcarriers was also characterized. For the exponential phase (considering the death rate constant negligible), the maximum specific growth rate, μ_{max} (day^{-1}), was calculated as $dX/dt = \mu_{max}X$, where X is the viable cell number for a given t. After calculating μ_{max}, the doubling time, t_d (day), was calculated as $t_d = \ln(2)/\mu_{max}$.

Estimation of Maximum Shear Stress Under Stirred Conditions. Theoretical values of maximum shear stress under stirred conditions, τ_{max}, as result of flow through Kolmogorov eddies,[31] can be determined through Eq. 1:

$$\tau_{max\ .} = 5.33 \cdot \rho \cdot (\varepsilon \cdot \nu)^{1/2}, \quad (1)$$

where ρ denotes fluid density and ν is the kinematic viscosity. The power dissipated per unit mass, ε, is defined by Eq. 2:

$$\varepsilon = \frac{P}{V_L \cdot \rho}, \quad (2)$$

in which V_L is the vessel working volume; the power consumed, P, can be estimated by Eq. 3:

$$P = N_P \cdot N^3 \cdot D_i^5 \cdot \rho, \quad (3)$$

where N_P is the dimensionless power number, N the agitation rate used, and D_i the impeller diameter. N_P can be calculated following Nagata correlations[32] (Eq. 4):

$$N_P = \frac{K_1}{Re} + K_2 \cdot \left[\frac{(10^3 + 1.2 \cdot Re^{0.66})}{10^3 + 3.2 \cdot Re^{0.66}}\right]^{K_4} \quad (4)$$

where:

$$K_1 = 14 + \left(\frac{W_i}{D_t}\right) \cdot \left[670 \cdot \left(\frac{D_i}{D_t} - 0.6\right)^2 + 185\right] \quad (4a)$$

$$K_2 = 10^{K_3} \quad (4b)$$

$$K_3 = 1.3 - 4 \cdot \left(\frac{W_i}{D_t} - 0.5\right)^2 - 1.14 \cdot \left(\frac{D_i}{D_t}\right) \quad (4c)$$

$$K_4 = 1.1 + 4 \cdot \frac{W_i}{D_t} - 2.5 \cdot \left(\frac{D_i}{D_t} - 0.5\right)^2 - 7 \cdot \left(\frac{W_i}{D_t}\right)^4 \quad (4d)$$

where W_i is the impeller width and D_t is the spinner flask diameter. The Reynolds number (Re) can be calculated by Eq. 5:

$$Re = \frac{D_i^2 \cdot N}{\nu}, \quad (5)$$

Vessel dimensions and culture medium properties are depicted in Table 1.

Table 1. Spinner Flask Characteristics and Culture Medium Properties

	Parameter	Value
Spinner flask	Working volume (V_L)	30 mL
	Vessel diameter (D_t)	5 cm
	Impeller width (W_i)	2.4 cm
	Impeller diameter (D_i)	2.7 cm
Culture medium	Density (ρ)	1.005 g/mL*
	Kinematic viscosity (ν)	0.0092 cm^2/s*

*No data was found in the literature concerning our SF medium density or kinematic viscosity. Therefore, these values correspond to average values of other culture media described in the literature.[32,44]

Metabolite Analysis. Glucose, lactate, glutamine, and ammonia concentrations were determined in the supernatant samples collected throughout the experiments by using an automatic analyzer (YSI 7100MBS, Yellow Springs Instruments). The specific metabolic rates ($q_{Met.}$, mol/(day.cell)) were calculated for every time interval using the following equation:

$$q_{Met.} = \frac{\Delta Met.}{\Delta t \cdot \Delta X_v},$$

where ΔMet. is the variation in metabolite concentration during the time period Δt and ΔX_v the average viable cell number obtained during the same time period. The apparent lactate from glucose ($Y'_{Lactate/Glucose}$), and ammonia from glutamine ($Y'_{Ammonia/Glutamine}$) yields were also calculated as the ratio between $q_{Lactate}/q_{Glucose}$ and $q_{Ammonia}/q_{Glutamine}$, respectively.

Cell Viability (Nuclear Integrity). Every 2 days, samples of cell-containing microcarriers were washed with PBS, fixed with 1% (w/v) paraformaldehyde (Sigma) for 20 min at room temperature and washed, once again, with PBS. Then, cells were incubated in the dark with 4',6-diamino-2-phenylindole dilactate (DAPI, 1.5 μg/mL in PBS) for 5 min at room temperature and protected from light, and finally washed with PBS. Stained nuclei (blue) were then visualized under a fluorescence microscope (Leica Microsystems).

Metabolic Activity. Periodically, samples of microcarriers were washed once with PBS and incubated with 40 μL of MTT (3-(4,5-dimethylthiazol-2-yil)-2,5-diphenyltetrazolium bromide, Sigma) (5 mg/mL in PBS) and 400 μL of PBS at 37°C for 45 min. MTT is cleaved by an enzyme of the respiration chain in the mitochondria when cells are metabolically active, generating MTT formazan, a dark blue compound, visible by optical microscopy.

Alkaline Phosphatase Staining. Samples of beads containing mES cells were washed with PBS and fixed in 10% cold neutral-buffered formalin (Sigma) for 15 min. After fixing, cells were washed and kept in distilled water for another 15 min. Following the washing step, cells were incubated with a pre-thawed aliquot of Fast Violet Solution containing 4% (v/v) Naphthol AS-MX Phosphate Alkaline Solution 0.25% (Sigma) for 45 min and washed three times in distilled water. Finally, cells were kept in distilled water and observed with an optical microscope.

Flow Cytometry Analysis of Pluripotency Markers. Intracellular markers Oct-4 and Nanog. The percentage of Oct-4 and Nanog-expressing cells was evaluated by flow cytometry after intracellular staining. After culture, cells were collected and fixed in 2% (w/v) paraformaldehyde solution in PBS. Cells were then permeabilized with 1% (w/v) saponin

Table 2. Properties of the Oligonucleotide Primers Used in RT-PCR

Primer	Size (bp)	Annealing Temperature (°C)	Sequence
Oct-4	82	54.5	(Fw) 5′-GCCTTGCAGCTCAGCCTTAA-3′ (Re) 5′-CTCATTGTTGTCGGCTTCCTC-3′
Nanog	109	51.5	(Fw) 5′-ATGCCTGCAGTTTTTCATCC-3′ (Re) 5′-GAGGCAGGTCTTCAGAGGAA-3′
GAPDH	151	54.5	(Fw) 5′-GCACAGTCAAGGCCGAGAAT-3′ (Re) 5′-GCCTTCTCCATGGTGGTCGTCAA-3′
EOMES	179	59.5	(Fw) 5′-CCACTGGATGAGGCAGGAGATTTCC-3′ (Re) 5′-AGTCTTGGAAGGTTCATTCAAGTCC-3′
FGF-5	115	59	(Fw) 5′-GGCAGAAGTAGCGCGACGTT-3′ (Re) 5′-GCCCCGAAGGGCTCCACTG-3′
AFP	108	60.5	(Fw) 5′-CCAGAACCTGCCGAGAGTTGC-3′ (Re) 5′-GGCTGGGGCATACATGAAGGGG-3′

(Sigma) in PBS, followed by incubation in blocking solution (3% [v/v] normal goat serum [NGS, Sigma] in PBS) for 15 min. The primary antibody (mouse monoclonal anti-Oct-3/4 (Chemicon), 1:30 (v/v) dilution in blocking solution, or rabbit polyclonal anti-Nanog (Santa Cruz Biotechnology), 1:500 (v/v) dilution in blocking solution) was added to the samples, and then cells were incubated for 2 h at room temperature. After washing thoroughly, the secondary antibody (Alexa Fluor 488 conjugated goat-anti mouse or goat-anti rabbit IgG, (Molecular Probes), 1:1000 dilution in blocking solution) was added to the cells that were then incubated for 45 min at room temperature. For each marker, cells incubated with the proper secondary antibody were used as negative control. After washing, the acquisition and analysis of the samples were performed in a FACSCalibur flow cytometer (Becton Dickinson [BD] Biosciences) and using the CellQuest software (BD Biosciences). Cell debris and dead cells were excluded from the analysis based on electronic gates using forward scatter (cell size) and side scatter (cell complexity) criteria. A minimum of 10,000 events was collected for each sample.

Extracellular marker SSEA-1. The percentage of SSEA-1-expressing cells was evaluated by flow cytometry after expansion. Cells were collected and resuspended in PBS. The antibody was then added to the cell suspension (mouse monoclonal FITC-conjugated anti-CD15 [BD], 1:10 dilution in PBS), followed by incubation for 15 min at room temperature in the dark. Isotype controls were used in every experiment to exclude non-specific binding of the antibody. After washing, the samples were analyzed by flow cytometry as previously described.

Induction of EB-based ES cell differentiation.

To assess the multilineage differentiation potential of ES cells after spinner flask/bioreactor cultures, expanded cells were used to generate embryoid bodies (EBs). Briefly, 4×10^5 cells collected from the spinner flask/bioreactor cultures were separated from the microcarriers, diluted in 10 mL of differentiation medium (i.e., SF expansion medium without LIF), and plated on common 100 mm bacterial petridishes. Medium was changed every other day. At Day 10, the EBs were dissociated by incubating with 1% trypsin, centrifuged at 1200 rpm, and the pellet was kept at −80°C for RNA isolation.

RNA isolation

Cells were harvested for RNA isolation at the beginning and at the end of the spinner flask/bioreactor cultures and from EBs, as previously indicated. Total RNA was collected using the High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. RNA was quantified by UV spectrophotometry (Nanodrop) at 260 nm. Complementary DNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche) with anchored-oligo$^{10}_{18}$ primers and 1 μg of RNA. The integrity of this cDNA was verified using control primers for porphobilinogen deaminase (PBGD), according to manufacturer's instructions.

Quantitative RT-PCR Analysis. The expression levels of two ES cell pluripotency markers (Oct4 and Nanog) and of three early ES cell differentiation genes (AFP [Endoderm], FGF5 [Ectoderm] and EOMES [Mesoderm]) characteristic of each one of the three embryonic germ layers were determined by Real Time PCR (RT-PCR). For this purpose, a two-step PCR run was performed in a Light-cycler (Roche) using a SYBR green PCR master mix (Roche, Switzerland), containing one LightCycler FastStart DNA Master Plus SYBR green, 4 mM $MgCl_2$, 0.5 μM of each primer (Table 1) and 2 μL of template resulting from the cDNA synthesis reaction in 20 μL of final volume. This two step program consisted in an initial denaturation step at 95°C followed by 45 rounds of cycling between 10 s at 95°C, 10 s at the respective annealing temperature (Table 1) and 10 s at 72°C. Expression was normalized to the metabolic housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The specificity of the reactions was confirmed by using gel electrophoresis (i.e., presence of a single band). Control assays containing no templates were also performed.

Statistical Analysis. Results are presented as mean ± standard error of mean.[33] When appropriate, comparisons between experimental results were determined by Mann-Whitney test for independent samples. A P-value less than 0.05 was considered statistically significant.

Results and discussion

Optimization of mES cell expansion in spinner flasks

Effect of the Agitation Rate on mES Cell Growth and Metabolism. All cells are sensitive to hydrodynamic forces and animal cells are particularly fragile because they lack a protective cell wall and are larger than microbial cells. Cells in most bioreactor culture systems are subjected to fluid mechanical shear stress through the process of agitation. Thus, for the design of rational scale-up strategies, an understanding of the effect of agitation on cellular viability and metabolism is required. To study the effect of agitation rate, suspension cultures of mES cells on microcarriers were performed at the agitation rates of 40 (used in our previous studies[2,23]), 60 and 80 rpm. These correspond to calculated shear stress values of 1.2, 2.0, and 3.0 dyn/cm^2, respectively

(Eqs. 1–5). Cells were cultured for 8 days in a spinner flask culture system with SF medium, using the manufacturer's recommended density of microcarriers (1 mg/mL). The comparison of the growth curves of mES cells under the different agitation rates, which is presented in Figure 1, suggests that cells were more efficiently expanded under 40 and 60 rpm as compared with 80 rpm. Throughout the experiments, for 40 and 60 rpm agitation rates, cell viabilities were always above 90%, and the fraction of live and/or dead cells found in suspension was always lower than 5%. On the other hand, at 80 rpm, the fraction of cells (live and dead) in suspension at Day 2 was ±70%, suggesting that, at this agitation rate, the cells that were loosely attached to the microcarriers after the initial adhesion step (first 24 h) were probably dislodged. At 40 and 60 rpm, the growth curves display an exponential phase leading to maximal cell concentrations of (2.8 ± 0.3) and (4.2 ± 0.4) × 10^6 cells/mL at Day 8 for 40 and 60 rpm, respectively. The concentrations correspond to 56 ± 6 and 85 ± 7 fold increase in total cell number, respectively. No significant differences were observed in terms of cell number until Day 5 for the two agitation rates tested, although becoming significantly different in the following days ($P < 0.05$). The higher final cell number achieved at 60 rpm could be associated to the lower number of microcarrier aggregates observed, with a lower size, as a consequence of the higher shear stress observed at this agitation rate (2.0 vs. 1.2 dyn/cm^2). This might have facilitated the mass transport compared with the 40 rpm condition, where larger and numerous and large microcarrier clusters were observed (Figures 2A–D). Indeed, as already described for mES cells cultured as aggregates, clusters with lower diameters demonstrated to be advantageous as they probably minimize mass transfer limitations of nutrients and oxygen to cells within the aggregates, thereby allowing greater ES cell expansion.[8] It should be noticed that in our studies with spinner flasks we decided not to continue cultures beyond Day 8 since the intense microcarrier aggregation (6–8 microcarriers clustered) observed on this time point, for the two agitation rates studied, turned the cell culture analysis very difficult and the estimation of cell numbers inaccurate (Figures 2D,H). One possible strategy to minimize the occurrence of microcarrier aggregation throughout time in culture would be to vary the agitation scheme, using lower agitation

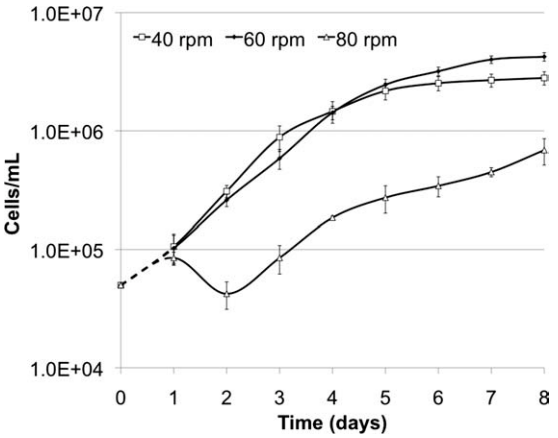

Figure 1. Effect of the agitation rate on 46C mES cell growth on *Cultispher S* microcarriers in a spinner flask culture system using SF medium.

Growth curves in terms of viable cell number per mL of 46C mES cells cultured on *Cultispher S* microcarriers under 40 (□, $n = 5$), 60 rpm (•, $n = 6$) and 80 rpm (△, $n = 2$) are displayed. Cells were inoculated at 5 × 10^4 cells/mL on 1 mg of microcarriers per mL and 50% of culture medium was changed everyday. Values are represented as mean ± SEM (*$P < 0.05$).

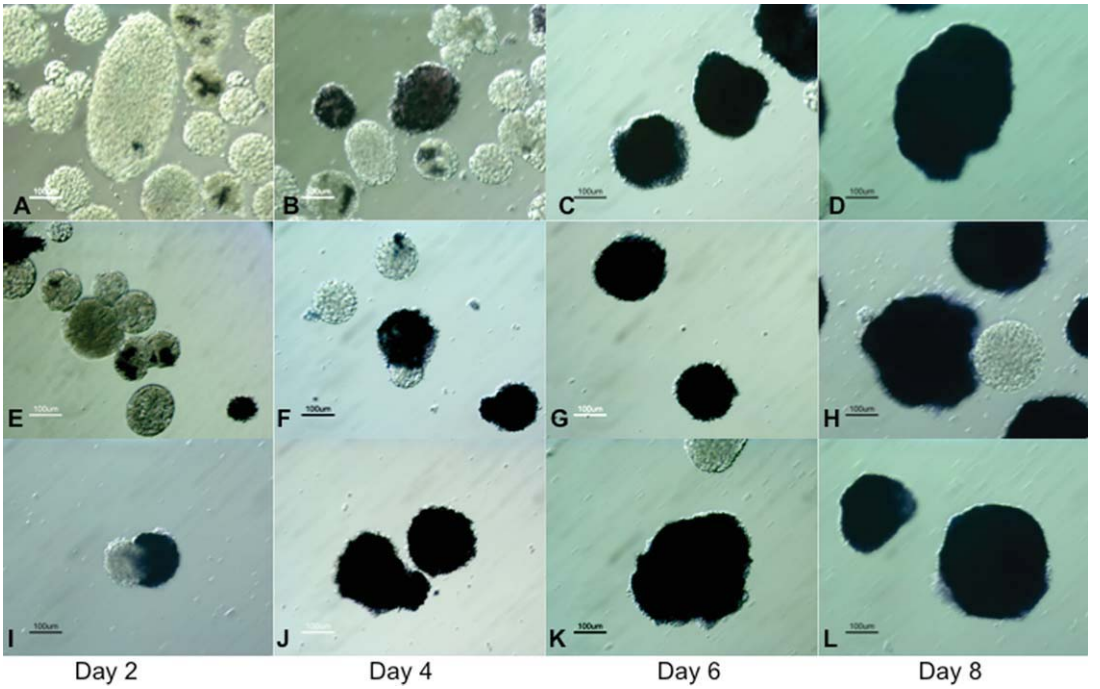

Figure 2. Micrographs of 46C mES cells cultured on *Cultispher S* microcarriers under stirred culture conditions.

Cells were visualized at Day 2, Day 4, Day 6, and Day 8 after MTT staining (100× amplification) for spinner flask (A–H) and bioreactors (I–L) cultures, respectively. The fraction of cells-containing beads after the adhesion step (first 24 h) ranged 50–60%; at the end of the culture this fraction increased to 80%.

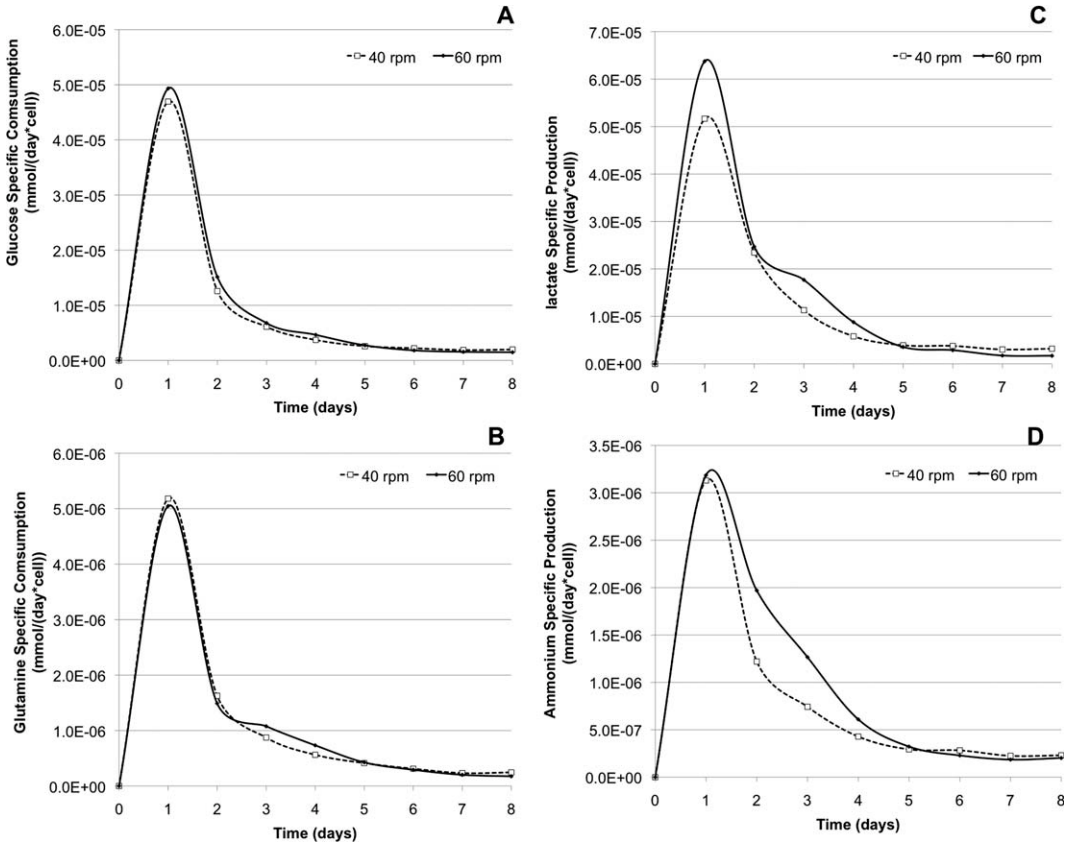

Figure 3. Metabolic quotient profiles (A–D) and metabolite concentration profiles (E–H) during 46C mES cell culture on *Cultispher S* microcarriers in SF medium obtained under agitation rates of 40 (dashed line, $n = 5$) and 60 (solid line, $n = 6$) rpm.

Glucose (A) and glutamine (B) specific consumption rates; lactate (C) and ammonia (D) specific production rates as well as glucose (E), glutamine (F), lactate (G), and ammonium (H) concentrations during time in culture are represented. Fifty percentage of culture medium was changed everyday.

rates (40–60 rpm) in the beginning of the culture and increase it later in culture (e.g., 80 rpm).

Specific growth rates (μ_{max}) were also calculated for the two different agitation rates according to "Materials and Methods" and were 1.0 ± 0.3 and 1.0 ± 0.2 day^{-1}, respectively, for 40 and 60 rpm. Consequently, the doubling time was similar for both agitation rates, 0.7 day, consistent with the almost nonexistent differences found between cell expansion at 40 and 60 rpm until Day 5.

To characterize the cell metabolic patterns, the consumption rates and production rates per viable cell (i.e., metabolic quotients) were calculated. Glucose and glutamine consumption rates, as well as lactate and ammonia production rates during time in culture are represented in Figure 3. Overall, the specific consumption/production rates are higher during the first 2 days of culture consistent with the initiation of the exponential growth phase; then these values decreased, becoming roughly constant. These observations suggest that the medium replacement schedule performed (50% every day) was efficient at supplying nutrients (glucose and glutamine), while removing the waste products that can inhibit cell growth (lactate and ammonia). Indeed, during time in culture, glucose and glutamine concentrations did not decrease beyond 4.7 ± 2.2 and 0.5 ± 0.1 mM, respectively, which are above the respective K_S values reported in the literature for BHK[34] and hybridoma[34,35] cultures. Concerning lactate concentrations, in both cases, the values were always under 20 mM which is reported to inhibit cell proliferation for other mammalian cells.[36,37] Ammonia, a by-product of glutamine metabolism, has also a harmful effect to cells, being an order of magnitude more toxic than lactate, especially if it does not evaporate from cell culture medium at an appreciable rate. Inhibitory effects to cell growth by ammonia accumulation have been reported for concentrations above 4 mM for mammalian cells.[37] However, in our experiments, ammonia levels never reached concentrations higher than 3.6 mM.

The overall apparent lactate from glucose ($Y'_{Lact./Gluc.}$), and ammonia from glutamine ($Y'_{Ammo./Glut.}$) yields were also calculated. The $Y'_{Lact./Gluc.}$ provides an estimate of the fraction of glucose converted to lactate via glycolysis[38] and its theoretical maximum yield is 2 because no more than two molecules of lactate can be obtained from a single molecule of glucose. Though $Y'_{Lact./Gluc}$ is only an apparent yield because lactate can be produced via metabolism of glutamine (also present in the culture medium) and it may be also converted into others compounds such as alanine, it provides a measure of metabolic efficiency of a specific culture. No significant differences were found in terms of overall $Y'_{Lact./Gluc.}$values between 40 and 60 rpm cultures (1.6 ± 0.1 and 1.6 ± 0.2 $mol_{Lac.}/mol_{Gluc}$, respectively). On the other hand, $Y'_{Ammo./Glut.}$ provides an estimate of the fraction of glutamine converted to ammonia, a major inhibitory waste product in mammalian cell cultures, which is released by chemical decomposition of glutamine and by metabolic deamination of glutamine to glutamate and by the conversion of glutamate to α-ketoglutarate. In this study, the calculated $Y'_{Ammo./Glut.}$ was 0.8 ± 0.1 and 1.0 ± 0.1 $mol_{Ammo.}/mol_{Glut.}$,

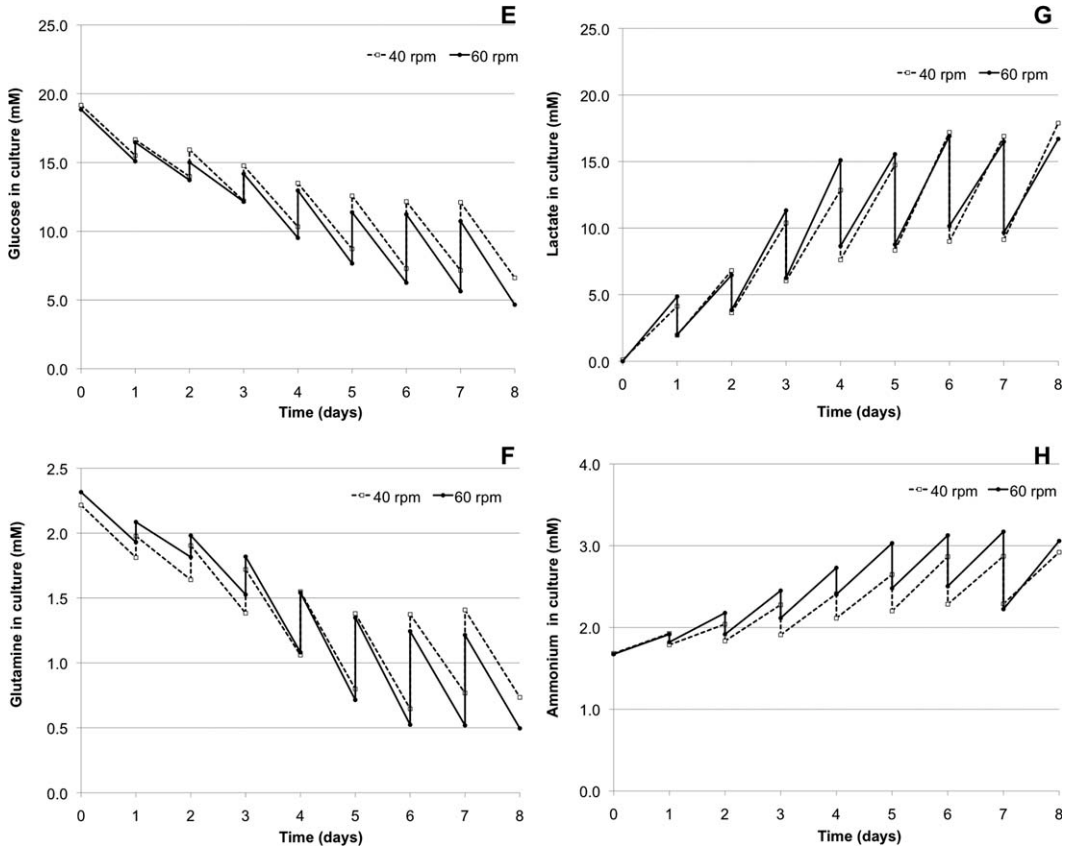

Figure 3. Continued

for 40 and 60 rpm respectively, which are in agreement with the values reported in the literature for other mammalian cells such as BHK and CHO cells.[39,40]

Effect of Feeding Regimen on mES Cell Growth and Metabolism. The first experiments in spinner flasks were performed using a feeding strategy of 50% medium change every day according to our previous results.[23] It can be hypothesized that a more programmed feeding protocol would potentially result in fewer cell growth fluctuations. Indeed, the protocol for culture medium replenishment might affect cell growth: if this is performed too early in culture or a large portion of medium is replaced at a time, a dilution of important autocrine factors can occur, while accumulation of toxic metabolic by-products takes place when medium is replaced later in culture. For that purpose, every 12 h, 25% of the medium was changed. Figure 4 represents the growth curves of cells fed once (50% medium change) or twice per day (25% medium change). The agitation rate used for these experiments was 60 rpm since the results presented in the previous section indicated an improvement in cell expansion performance.

From Figure 4, it is possible to conclude that there were no significant differences between growth curves of cells fed once or twice per day. Maximal cell concentrations of (4.2 $\pm$ 0.4) and (5.6 $\pm$ 0.8) $\times 10^6$ cells/mL at Day 8 were attained for cells fed once or twice per day; these correspond to fold increase values in total cell number of 85 $\pm$ 7 and 108 $\pm$ 16, respectively. Changes in the feeding strategy did not significantly influence the specific growth rate, which was kept almost constant (i.e., 1.0 $\pm$ 0.1 and 1.0 $\pm$ 0.2 day^{-1}, for 25% and 50% feeding regimen, respectively). The metabolic profiles of glucose, glutamine, lactate, and ammonia are represented in Figure 5. For the 25% medium change, glucose and glutamine levels never dropped below 9.5 $\pm$ 0.7 and 0.7 $\pm$ 0.1 mM, respectively, whereas lower concentrations of 4.7 $\pm$ 0.2 and 0.5 $\pm$ 0.1 were attained for glucose and glutamine respectively, with the 50% medium change regimen. On the other hand, the 50% medium change schedule originated a 23% increase in maximum lactate concentration value compared with the highest concentration of lactate observed for the 25% medium change. Nevertheless, despite the concentration of nutrients and by-products was steadier in the case of 25% medium change, it did not represent an improvement in terms of the total number of cells. Overall, it is reasonable to conclude that the daily 50% medium exchange was sufficient for supplying the necessary nutrients, as well as for removing the toxic by-products of metabolism.

Stirred tank bioreactor culture

To develop an efficient, reproducible, and cost-effective process for ES cell production for use in multiple settings, the spinner flask microcarrier culture system was scaled-up to a fully controlled stirred tank bioreactor which allows a more precise control over culture conditions to yield a greater number of cells while maintaining their pluripotency. Thus, mES cells were expanded in a stirred tank with a working volume of 700 mL during 11 days. The scale-up strategy was implemented taking into consideration the feeding regimen and cell inoculation procedure already optimized using spinner flasks (Optimization of mES cell expansion in spinner flasks section). In addition, the set-points of operational parameters such as T, pH, DO, and

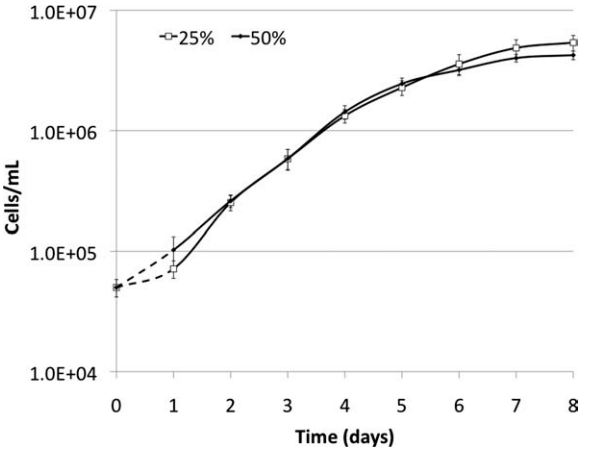

Figure 4. Effect of feeding regimen on 46C mES cell growth on *Cultispher S* microcarriers in a spinner flask culture system using SF medium.

Growth curves in terms of viable cell densities per mL. 46C mES cells were fed once per day with 50% medium change every 24 h (solid line, $n = 6$) or twice per day with 25% medium change every 12 h (dashed line, $n = 3$). Cells were inoculated at 5×10^4 cells/mL on 1 mg of microcarriers per mL of culture medium and agitation rate was set to 60 rpm. Values are represented as mean ± SEM.

agitation rate were optimized in an integrated mode. Based on previous results using spinner flasks, the operational parameters were set to: pH 7.2, DO = 20%, $T = 37°C$ and agitation rate = 60 rpm. The growth curve of mES cells expanded in the bioreactor is represented in Figure 6. Overall, the growth curve followed the expected pattern, similar to the growth curves of mES cells in spinner flasks, displaying a exponential phase leading to a maximum cell concentration of $(4.3 \pm 0.5) \times 10^6$ cells/mL, which represents a 85 ± 15-fold increase in total cell number (Figure 6).

These results show that the growth kinetics of mES cells cultivated in the large-scale bioreactor is comparable with the one observed in the spinner flasks (Figure 1). Nevertheless, although the maximum value of fold-increase in total cell number obtained was similar in both systems (85 ± 15-fold vs. 85 ± 7-fold for the spinner flask at 60 rpm (Figure 1)), this was achieved later in culture in the bioreactor (11th vs. 8th day). These differences might be due to one or a combination of the following factors, especially related with the hydrodynamic shear fields generated inside the bioreactor. First, there was an increase in the shear stress experienced by the cells in the bioreactor due to the modification of the impeller design and the addition of measuring probes. On the other hand, ES cells cultured in the bioreactor experience an additional hydrodynamic shear stress caused by the sparging with gas bubbles, required to achieve adequate oxygen delivery in large volumes, high-cell-density bioreactors. Notably, and to the best of our knowledge, all prior studies that focused on ES cell expansion for the purpose of mass production of undifferentiated cells in bioreactors used surface aeration to assure oxygen supply. Sparging was used in

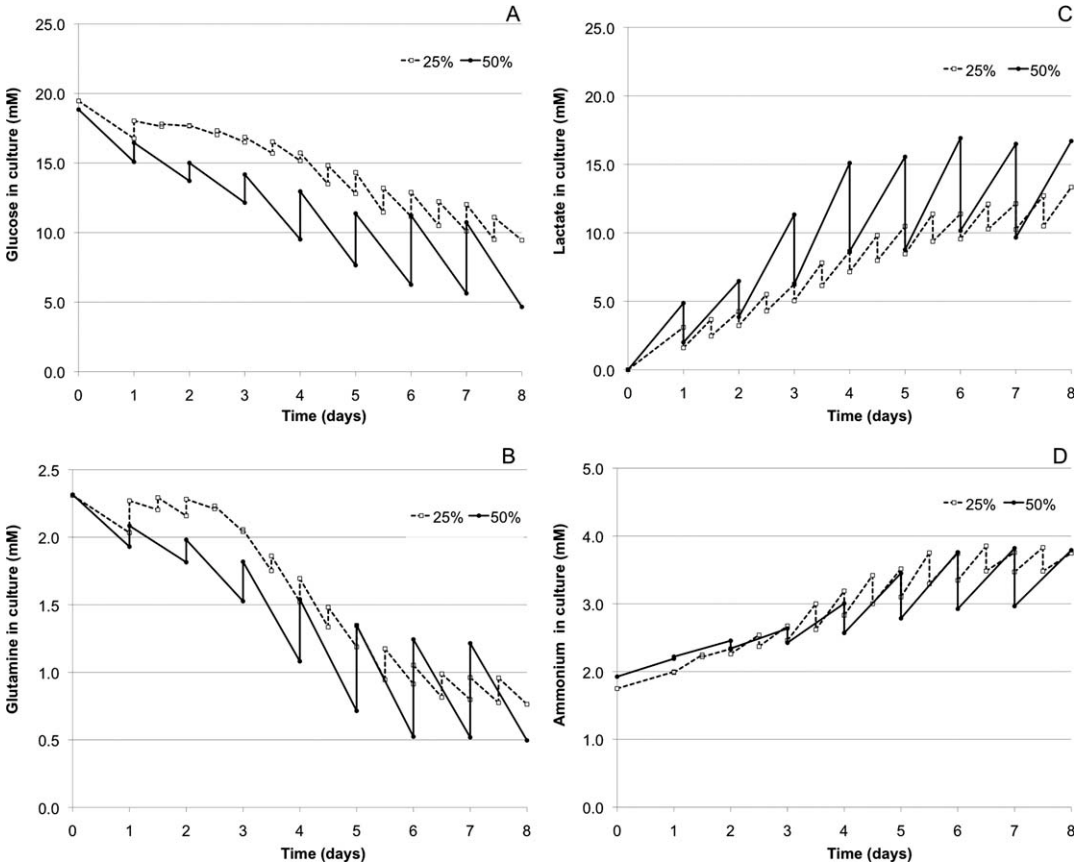

Figure 5. Metabolite concentration profiles during 46C mES cell culture on *Cultispher S* microcarriers in a spinner flask culture system using SF medium.

Glucose (a), glutamine (b), lactate (c), and ammonium (d) concentrations during time in culture are represented for cells fed once per day (50% medium change every 24 h; solid line) or twice per day (25% medium change every 25%; dashed line). Cells were inoculated at 5×10^4 cells/mL on 1 mg of microcarriers per mL of culture medium.

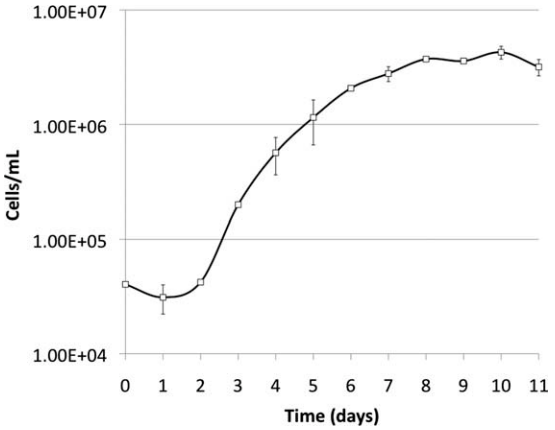

Figure 6. 46C mES cell culture on *Cultispher S* microcarriers in a fully controlled stirred tank bioreactor culture system in SF medium.

Growth curve in terms of number of viable cells per mL. Cells were inoculated at 5×10^4 cells/mL on 1 mg of microcarriers per mL of culture medium in a spinner flask. After 24 h, the cells were transferred to the bioreactor and agitation rate set to 60 rpm. Values are represented as mean ± SEM ($n = 2$).

the present studies since surface aeration would be ineffective for oxygen delivery in our high-density ES cell cultures (up to 3.0×10^9 cells). Nevertheless, sparging might cause cell damage because cells attach to the gas bubbles and are subjected to high shear stress during bubble rupture.[41] Also, sparging of high-protein-content media produces extensive foam that decreases the reactor capacity. The use of protectants such as Pluronic-F68 and antifoams, commonly used in the biopharmaceutical industry to overcome issues of foaming and mechanical stress on cells when higher air sparging rates are employed,[41–43] could be a good solution for stem cell technology applications. Although this additional shear might not have affected cell viability (always above 90%), it can be the responsible for the delay observed in cell growth kinetics, especially by affecting the initial cell adhesion to the microcarriers. Indeed, the initial cell distribution on microcarriers is known to affect overall growth kinetics of microcarrier cultures. In particular, the microcarriers that were not occupied by any cells initially will certainly remain barren during the entire culture period. In this context, different initial stirring regimens were employed during the microcarrier cell seeding step in the spinner flask and in the stirred bioreactor. In the former, after an initial inoculation step in a small portion of the culture medium in a conical tube, there was an initial 24 h period *in situ,* with an intermittent agitation regimen (15 min ON at 30–40 rpm plus 60 min OFF) in half of the final volume. In the latter, after the initial inoculation step in a reduced volume outside the bioreactor, with an intermittent agitation regimen during only 6 h, cell suspension with the microcarriers was then transferred to the bioreactor, in the final volume of SF medium (700 mL), and agitated continuously at 60 rpm thereafter. The different inoculation procedures potentially originated a different initial cell distribution after inoculation, which is known to affect the maximum cell concentration attainable in the culture. Specifically for the stirred tank bioreactor, the "harsher" culture conditions might have affected the initial adhesion step of the cells to the microcarriers thereby delaying the cell growth kinetics. Accordingly, the maximum specific growth rate attained in the bioreactor was 0.9 ± 0.1 day^{-1} (vs. 1.0 ± 0.2 day^{-1} for the spinner flask at 60 rpm), which corresponds to a doubling time of 0.8 ± 0.1 day (compared with 0.7 ± 0.2 day^{-1} for spinner flask). The specific consumption rates of glucose and glutamine, as well as the specific production rates of lactate and ammonium per day obtained in the bioreactor are represented in Figure 7. The mean values of $Y'_{Lact./Gluc.}$ and $Y'_{Ammo./Glut.}$ were 1.7 ± 0.3 $mol_{Gluc.}/mol_{Lact.}$ and 1.5 ± 0.2 $mol_{Ammo.}/mol_{Glut.}$, respectively. Although $Y'_{Lact./Gluc.}$ is comparable with the one calculated for the expansion of mES cells in spinner flasks, $Y'_{Ammo./Glut.}$ in the stirred bioreactor is higher suggesting that the glutamine metabolic pathways might have changed among the two culture systems studied. A comparison of growth and metabolic parameters of cells expanded in the spinner flask and bioreactor is presented in Table 3.

Qualitative Evaluation of Cell Adhesion*/Viability (DAPI Staining),* ***Metabolic Activity (MTT Staining) and Pluripotency (ALP Staining) of 46C mES Cells Cultured on Microcarriers in Spinner Flask/Stirred Tank Bioreactor Culture Systems****.* To evaluate cell adhesion, viability, metabolic activity, and pluripotency of mES cells immobilized on the microcarriers during time in culture, DAPI, MTT (Figures 2I–L), and ALP stainings, respectively, were performed every 2 days for the different culture conditions tested (spinner flask and stirred tank bioreactor). In both systems, a gradual increase in microcarrier occupancy by mES cells was observed throughout time in the culture, which remained viable, metabolically active and pluripotent.

Evaluation of Pluripotency of 46C mES Cells Cultured on Microcarriers in Spinner Flask/Stirred Tank Bioreactor Culture Systems by Flow Cytometry. When developing a mES cells production system, it is crucial to ensure that the large quantities of cells generated remain undifferentiated and exhibit pluripotency features. The analysis of three pluripotency markers, Oct-4, SSEA-1, and Nanog was performed by flow cytometry. These markers have been found to be expressed in undifferentiated mouse ES cells and are downregulated on differentiation. In this study, culture samples were taken at Day 0 (control cells) and at the end of the spinner flask/bioreactor cultures.

The data in Table 4 indicates that after culture under stirred conditions, using the two different systems (the spinner flask and fully controlled stirred tank bioreactor), >90% of the cells maintained the expression of the characteristic pluripotency markers Oct4, Nanog and SSEA-1.

Evaluation of Pluripotency and Multilineage Differentiation Potential of 46C mES Cells Cultured on Microcarriers in Spinner Flask/Stirred Tank Bioreactor Culture Systems by Real Time PCR (RT-PCR). One of the most important features of ES cells is their ability to differentiate into cells of all three embryonic germ layers. Thus, the expression levels of two ES cell pluripotency markers (Oct4 and Nanog) and of three early ES cell differentiation genes (AFP [Endoderm], FGF5 [Ectoderm]. and EOMES [Mesoderm]) characteristic of each one of the three embryonic germ layers were determined through RT-PCR (Figure 8), as well as the GAPDH, which is a housekeeping gene. Cells collected at Day 0 of cell expansion were used as control and compared with cells collected in the last day of the spinner flask/bioreactor cultures (Day 8 for spinner flask cultures and Day 11 for bioreactor cultures) and to cells collected after 10 days of EB culture. Overall, the pluripotency genes expression was maintained during the stirred cultures with cells keeping their undifferentiated state when

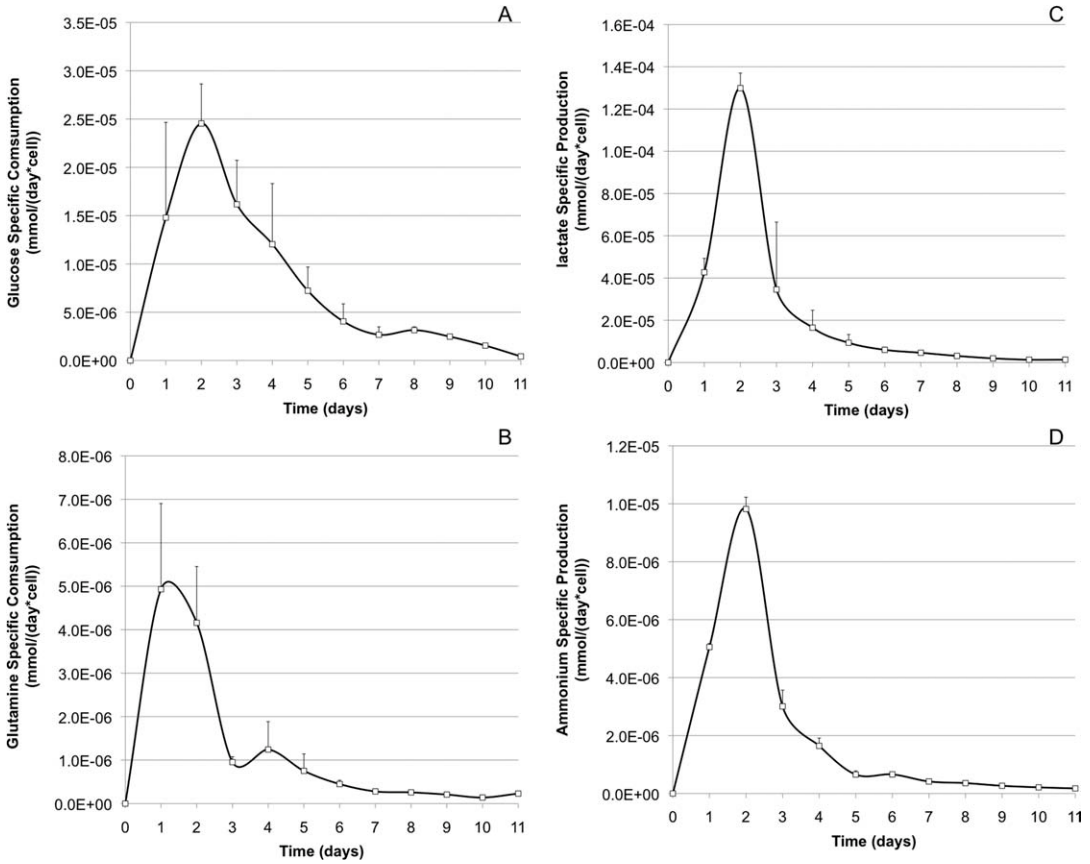

Figure 7. Metabolic quotient profiles during 46C mES cell culture on *Cultispher S* microcarriers in SF medium in a fully controlled large-scale bioreactor.

Glucose (a) and glutamine (b) specific consumption rates, as well as lactate (c) and ammonia (d) specific production rates during time in culture are represented. Values displayed represent the average of two independent experiments.

Table 3. Comparison of Growth and Metabolic Parameters of Cells Expanded in Stirred Culture Systems

Parameter	Spinner Flask	Bioreactor
Working volume (mL)	30	700
Culture time (days)	8	11
Agitation rate (rpm)	60	60
Feeding scheme	50% Renewal everyday	50% Renewal everyday
Maximum cell concentration (cells/mL)	$(4.2 \pm 0.4) \times 10^6$	$(4.3 \pm 0.5) \times 10^6$
Maximum fold increase in total cell number	85 ± 7	85 ± 15
Maximum specific growth rate (day^{-1})	1.0 ± 0.2	0.9 ± 0.1
Doubling time (day)	0.7 ± 0.2	0.8 ± 0.1
$Y'_{Lact./Gluc.}$ ($mol_{Gluc.}/mol_{Lact.}$)	1.6 ± 0.2	1.7 ± 0.3
$Y'_{Ammo./Glut.}$ ($mol_{Ammo.}/mol_{Glut.}$)	1.0 ± 0.1	1.5 ± 0.2

Table 4. Flow Cytometry Analysis of mES Cell Characteristic Pluripotency Markers (Oct4, Nanog and SSEA-1) Obtained at Day 0 and After 8 or 11 Days of Culture Under Stirred Conditions

		Day 8 - Spinner Flask (%)			
		40 rpm	60 rpm	60 rpm	
Pluripotency Marker	Day 0 (%)	50% Feed	50% Feed	25% Feed	Day 11 Bioreactor (%)
Oct-4	97 ± 2	98 ± 1	97 ± 2	98 ± 2	96 ± 2
Nanog	96 ± 2	97 ± 2	96 ± 2	95 ± 2	98 ± 1
SSEA-1	94 ± 5	93 ± 6	92 ± 5	92 ± 6	97 ± 2

proliferating on the microcarriers (expansion conditions) and diminished after 10 days of EBs culture (differentiation conditions). On the other hand, the expression of the three early differentiation genes increased in the EBs culture, meaning that ES cells maintained their differentiation potential into cells of the three embryonic germ layers after culture for 8

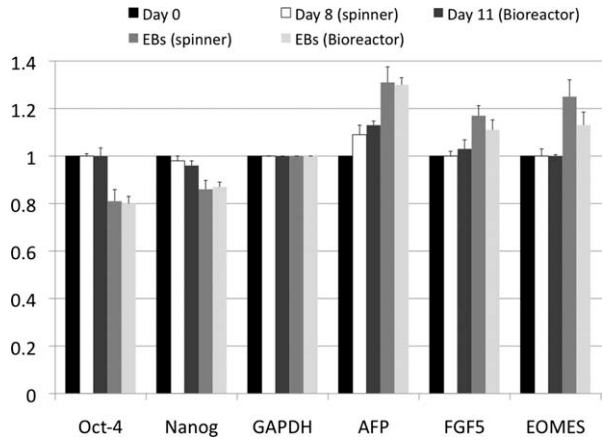

Figure 8. RT-PCR analysis for pluripotency (Oct4 and Nanog) and early differentiation markers (AFP, FGF5, and EOMES) expression on the beginning (Day 0) and at the end of spinner flask/stirred tank bioreactor cultures (Day 8 for spinner flask and Day 11 for bioreactor) and after 10 days of EBs cultures (started with cells expanded in the spinner flask-EBs [spinner] or in the bioreactor-EBs [bioreactor]).

Values represent expression levels normalized to GAPDH of $n = 2$–3 independent experiments standardized to control values at Day 0.

or 11 days under stirred conditions in the spinner flask and stirred tank bioreactor, respectively.

Conclusions

In this study, we developed a quality controlled and reproducible scalable culture system for the expansion, under SF conditions, of the 46C mES cells, which were used as a model cell line. The results demonstrated the feasibility of successfully scaling-up mES cell expansion to a fully controlled stirred tank bioreactor culture system (700 mL volume) reaching cell densities as high as $(4.3 \pm 0.5) \times 10^6$ cells/mL ($[3.0 \pm 0.5] \times 10^9$ ES cells) after 11 days, corresponding to a fold increase in total cell number of 85 ± 15 and a doubling time of <1 day.

The controlled bioprocess developed herein is potentially adaptable to other cell types, including human ES cells and induced pluripotent stem cells, thus representing a promising starting point for the development of novel technologies for the production of differentiated derivatives from human pluripotent cells.

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Notation

DAPI = 4',6-diamino-2-phenylindole dilactate
DO = dissolved oxygen
ES = embryonic stem
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
KDMEM = knockout Dulbecco's modified Eagle's medium
KSR = knockout serum replacement
LIF = leukemia inhibitory factor
NGS = normal goat serum
PBGD = porphobilinogen deaminase
PBS = phosphate buffered saline
SF = serum-free

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