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Mouse embryonic stem cell expansion in a microcarrier-based stirred culture system

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Abstract

Embryonic stem (ES) cells have the ability to differentiate *in vitro* into a wide variety of cell types with potential applications for tissue regeneration. However, a large number of cells are required, thus strengthening the need to develop large-scale systems using chemically defined media for ES cell production and/or controlled differentiation.

In the present studies, a stirred culture system (i.e. spinner flask) was used to scale-up mouse ES (mES) cell expansion in serum-containing (DMEM/FBS) or serum-free medium, both supplemented with leukemia inhibitory factor (LIF), using either *Cytodex 3* or *Cultispher S* microcarriers. After 8 days, maximal cell densities achieved were (1.9 ± 0.1), (2.6 ± 0.7) and 3.5×10^6 cells/mL for *Cytodex 3* in DMEM/FBS, *Cultispher S* in DMEM/FBS and *Cultispher S* in serum-free cultures, respectively, with fold increases of 38 ± 2, 50 ± 15 and 70. Both microcarriers were suitable to sustain mES cell expansion, though the macroporous *Cultispher S* seemed to be advantageous in providing a more protective environment against shear stress forces, which harmful effects are exacerbated in serum-free conditions. Importantly, mES cells expanded under stirred conditions using serum-free medium retained their pluripotency and the ability to commit to the neural lineage.

Keywords: Mouse embryonic stem cells; Expansion; Neural commitment; Serum-free medium; Microcarriers; Spinner flask

1. Introduction

Embryonic stem (ES) cells can be defined as pluripotent cells having unlimited capacity to replicate themselves through a self-renewal mechanism, with the ability to differentiate into multiple cell types from all three embryonic layers: ectoderm, mesoderm and endoderm (Smith, 2001). Undifferentiated ES cells may potentially provide a starting source material to be used in regenerative medicine or as a basis for biological, pharmacological and toxicological studies *in vitro* as a reliable alternative to animal models (Hook et al., 2005; Passier and Mummery, 2003; Pouton and Haynes, 2005). Therefore, it is important to develop a technology for the scalable and controllable production of undifferentiated ES cells and ES cell-derived cells.

Typically, the mouse has been considered as an animal model of embryonic development in mammals and it has been widely exploited as a model for conducting both *in vitro* and *in vivo* studies, giving an important insight into different aspects of development biology (Keller, 2005). Although mouse ES (mES) are relatively short-lived in the embryo *in vivo*, they can be maintained *in vitro* for long periods of time without any apparent loss of differentiation potential, by culturing them with growth medium containing leukemia inhibitory factor (LIF) with/without a feeder layer of murine embryonic fibroblasts (MEF) (Evans and Kaufman, 1981). In addition, mES cells have shown the ability to differentiate *in vitro* into a wide variety of cell types such as hematopoietic (Dang et al., 2002; Qiu et al., 2005) and neuronal (Lang et al., 2004; Ying et al., 2003b).

Nevertheless, mES cell exploitation has been bewildered by the inclusion of animal serum in the culture medium composition (Wiles and Johansson, 1999; Ying et al., 2003a). Indeed,

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animal serum is poorly characterized and is a potential factor of infectious agent transmission (Ying et al., 2003a). In this context, it has been previously demonstrated that serum-free, chemically defined media can successfully support ES cell proliferation and differentiation (Wiles and Johansson, 1999; Ying et al., 2003a). In particular, a specific complete serum-free medium was developed that maintains ES cell features during prolonged expansion (Ying et al., 2003a). In addition to LIF, this medium is supplemented with bone morphogenic protein 4 (BMP4), whose molecular signals are necessary for suppression of neural differentiation and concomitant self-renewal in the absence of serum (Ying et al., 2003a).

On the other hand, although many studies have addressed mES cell expansion, only few addressed the scale-up of mES cell cultures (Abranches et al., 2007; Bauwens et al., 2005; Cormier et al., 2006; Dang et al., 2004; Fok and Zandstra, 2005). Despite the widespread usage of static culture systems such as T-flasks or tissue culture Petri dishes for expanding ES cells, these have serious limitations especially concerning their non-homogeneous nature which results in concentration gradients (e.g. pH, dissolved oxygen and metabolites) in the culture medium (Hassell et al., 1991; Pörtner et al., 2005). In addition, these culture systems are limited in their productivity by the number of cells that can be supported by a given surface area (Cabrita et al., 2003). Therefore, it turns necessary to consider alternative systems which allow a better homogeneity, a tighter control and/or higher productivity. Simple stirred vessels, the so-called spinner flasks, offer attractive advantages of ready scalability and relative simplicity and homogeneity. However, their use for strictly anchorage dependent cells, like mES cells, is not straightforward. In the last years, several methods of expanding adherent mES cells in homogeneous suspension in spinner flasks have been reported, either in the form of aggregates (Cormier et al., 2006; Fok and Zandstra, 2005), encapsulated (Bauwens et al., 2005; Dang et al., 2004) or using microcarriers as substrate for cell attachment (Abranches et al., 2007; Fok and Zandstra, 2005). Microcarriers are cell-supporting particles which can be made of many different materials (e.g. dextran, polystyrene, gelatin) of various shapes (e.g. spherical). These can be classified as microporous (pore diameter smaller than 1 μm, allowing cell adhesion and growth only on the external surface of the support) or macroporous, allowing cells to use the internal surface of the bead for cell adhesion and proliferation (pore diameter of 10–50 μm).

In the present studies, the 46C mES cell line (a E14TG2a-derived cell line) was used as a model, which contains a green fluorescent protein (GFP) knock-in at the Sox1 locus (neuroepithelial marker gene) (Ying and Smith, 2003). Ying and collaborators demonstrated that this cell line can be induced to undergo neural commitment in serum-free conditions, where the inductive signals for other cell fates are eliminated (Ying and Smith, 2003; Ying et al., 2003b). Here, we investigated the feasibility of scaling-up 46C mES cell expansion using a microcarrier-based stirred culture system, either in traditional serum-containing medium or serum-free conditions using two different microcarriers, *Cytodex 3* and *Cultispher S*.

2. Materials and methods

2.1. Cell culture

46C mES cells were a gift from the laboratory of Professor Austin Smith at the Institute of Stem Cell Research, University of Edinburgh, Scotland, UK. 46C mES cells were kept cryopreserved in liquid nitrogen until further use.

2.1.1. mES cell expansion in static conditions prior to spinner flask inoculation

Upon thawing, 46C cells were expanded on gelatinized tissue culture plates for two passages, using either Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL) containing 10% fetal bovine serum (FBS) (GibcoBRL), 1% Glutamine 200 mM (GibcoBRL), 1% penicillin (50 U/mL)/streptomycin (50 μg/mL) (GibcoBRL), 1% non-essential Amino acids 100× (Sigma), 0.1% 2-mercaptoethanol 0.1 mM (Sigma) or serum-free ESGRO complete medium (Chemicon). Serum-containing medium (DMEM/FBS) was supplemented with 0.1% LIF (mLIF ESGRO 10^6 U, Chemicon or hLIF produced by 293-EBNA cells), while the ESGRO medium used in serum-free cultures contains LIF in its formulation. The cells were cultured at 37 °C under a 5% CO_2 humidified atmosphere.

In each passage, viable and dead cells were determined by counting in a hemocytometer under an optical microscope using the trypan blue dye exclusion test (GibcoBRL).

2.1.2. mES cell expansion in stirred conditions

mES cell expansion was performed using spinner flasks with microcarriers to support cell adhesion and culture under stirred conditions. Spinner flasks were placed inside an incubator at 5% CO_2 in air and 37 °C, with aeration taking place in the gas/liquid interface.

Two commercially available microcarriers, *Cytodex 3* (GE Healthcare) and *Cultispher S* (Percell Biolytica), designed to be used in stirred systems, were used to support cell expansion in spinner flasks. A summary of the characteristics of the two microcarriers is presented in Table 1. As a preliminary approach, both microcarriers were tested for biocompatibility and mES cell adhesion ability under static conditions in cell culture plates following the manufacturer's instructions.

Two spinner flasks suitable for microcarrier cell culture were tested: *Bellco* (*Bellco* Biotechnology) and *StemSpan* (StemCell Technologies), with working volumes of 80 and 30 mL, respectively. Both spinner flasks are equipped with an impeller with 90° paddles (normal paddles) and a magnetic stir bar.

mES cell culture on both microcarriers was performed following manufacturer's instructions. 46C mES cells (5×10^4 cells/mL) previously expanded for two passages in 60 mm culture plates, were mixed with appropriate concentrations of microcarriers previously hydrated, sterilized by autoclaving and equilibrated in pre-warmed (37 °C) serum-containing medium. The bead concentrations used were 0.5 and 1 mg/mL for *Cytodex 3* and *Cultispher S*, respectively. 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, with gentle agitation every 10 min. Then, fresh pre-warmed (37 °C) medium was gently added, until half of the final volume, and cell suspension was transferred to the spinner flask.

For spinner flask cell inoculation with *Cytodex 3*, after 3 h of intermittent stirring (2 min of stirring at 30–40 rpm followed by 30 min statically), medium was added to 3/4 of the final volume and intermittent stirring was performed during 3 h. Then, the rest of the medium was added and the agitation speed adjusted to 40 rpm (Abranches et al., 2007).

In *Cultispher S* cultures, after a 24-h seeding period with intermittent stirring (15 min of stirring at 30–40 rpm, followed by 60 min statically), medium was added up to the final volume and the speed adjusted to 40 rpm.

In both cases, feeding was performed every 1–2 days by replacing 50% of the medium with fresh pre-warmed medium. The removing/replenishment of the culture medium was performed immediately after the quick sedimentation of the micro carriers containing the cells.

2.2. Monitoring of cell culture in spinner flask

2.2.1. Cell counts and viability

Everyday, duplicate samples of evenly mixed culture were collected from the spinner flask. The beads were washed twice with phosphate buffer saline (PBS) (Sigma) and then trypsin (GibcoBRL) (0.25 and 1% solution for *Cytodex 3* and *Cultispher S* cultures, respectively) was added. Cell samples were then incubated for 10 min in a 37 °C water-bath. Occasional flicking was performed in order to facilitate detachment of cells from the beads and/or gelatin matrix dissolution (*Cultispher S*).

Viable and dead cells were determined by using the trypan blue dye exclusion test on a hemocytometer. Supernatant samples were centrifuged at 1500 rpm for 10 min and kept at −20 °C for subsequent analysis.

2.2.2. Growth rates and doubling times

The growth kinetics of 46C mES cells cultured under stirred conditions on microcarriers was also characterized. Specific growth rates were calculated using the following equation (Melero-Martin et al., 2006):

$$\mu = \frac{1}{X}\frac{\mathrm{d}X}{\mathrm{d}t} \approx \frac{2}{X_2 + X_1}\frac{X_2 - X_1}{t_2 - t_1}$$

where μ (day^{-1}) corresponds to the value of specific growth rate at any given time point, t (days) the culture time and X (cells) the value of viable cell number for a specific t. From this, the doubling time (t_d) was calculated using the following equation, $t_\mathrm{d} = \ln(2)/\mu_\mathrm{max}$.

2.2.3. Viability (nuclear integrity)

Every 2 days, samples of cell-containing beads were washed with PBS, fixed with 1% paraformaldehyde (Sigma) for 20 min at room temperature and washed, once again, with PBS. Then, the 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, washed with PBS and stained nuclei (blue) were visualized under a fluorescence microscope.

2.2.4. 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 3 h or 45 min for *Cytodex 3* and *Cultispher S*. MTT is cleaved by an enzyme in the respiration chain in the mitochondria if the cell is metabolically active, generating MTT formazan, a dark blue compound, visible by microscopy.

2.2.5. 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, the cells were washed and kept in distilled water for another 15 min. Following the washing step, the cells were incubated with a 1:1 solution of distilled water/Tris–HCl (Sigma) containing Naphthol AS MX-PO_4 (0.1 mg/mL, Sigma) and Red Violet LB salt (0.6 mg/mL, Sigma) for 45 min and washed three times in distilled water. Finally, cells were kept in distilled water and observed with an optical microscope.

2.2.6. 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 ($q_\mathrm{Met.}$, mol/(day cell)) were calculated for every time interval using the following equation: $q_\mathrm{Met.} = \Delta\mathrm{Met.}/(\Delta t\, \Delta X_\mathrm{v})$, where ΔMet. is the variation in metabolite concentration during the time period Δt and ΔX_v the average viable cell number during the same time period. The apparent lactate from glucose ($Y'_{\mathrm{lactate/glucose}}$), and ammonia from glutamine ($Y'_{\mathrm{ammonia/glutamine}}$) yields were also calculated as the ratio between $q_\mathrm{lactate}/q_\mathrm{glucose}$ and $q_\mathrm{ammonia}/q_\mathrm{glutamine}$.

2.3. Neural commitment of 46C mES cells expanded under serum-free conditions

After cell expansion in the spinner flask, the cells were tested for their neural commitment potential. After the trypsinization step for *Cultispher S* gelatin matrix dissolution, the cells were firstly expanded during 24 h under static conditions in gelatinized tissue-culture grade plates at high cell concentration (1×10^5 cells/cm^2). Then, cells were collected after trypsinization, resuspended in serum-free RHB-A medium (Stem Cell Sciences) and 10^4 cells/cm^2 were re-plated in 1 mL in gelatinized 12-well tissue-culture plates. Cells were cultured for 6 days and the medium was replaced every 2 days by fresh pre-warmed (37 °C) RHB-A medium.

2.4. Flow cytometric quantification of neural conversion

As previously mentioned, the use of 46C mES cells allows the quantification of neural progenitors by flow cytometry based

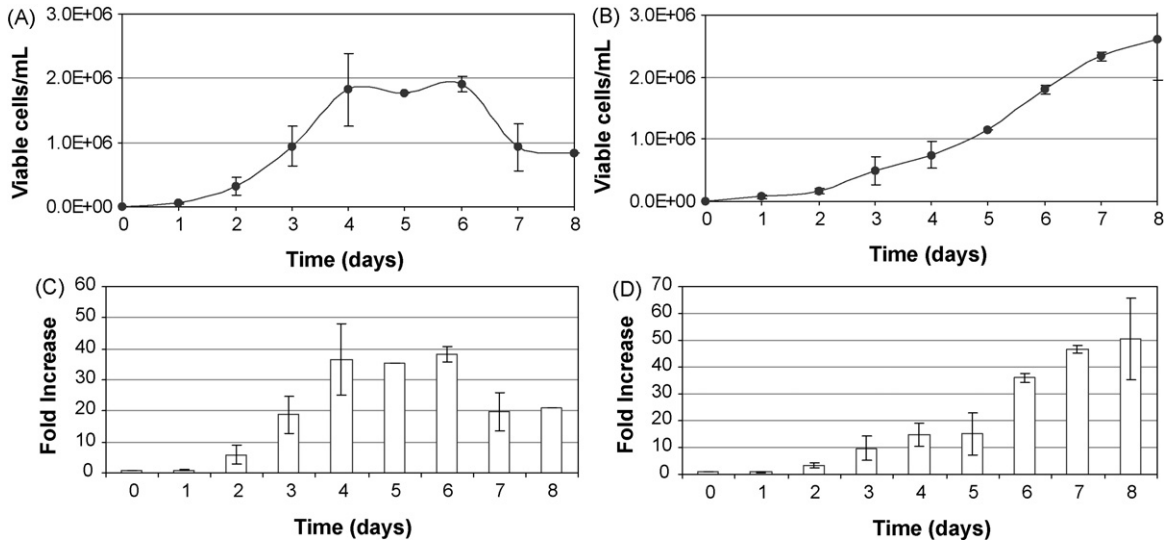

Fig. 1. 46C mES cell culture on microcarriers in a spinner flask culture system using serum-containing medium. Growth curves in terms of viable cells per milliliter (A and B) and cell expansion in terms of fold increase in total cell number (C and D) of 46C mES cells cultured on *Cytodex 3* and *Cultispher S*, respectively, are represented. Cells were inoculated at 5×10^4 cells/mL on 0.5 or 1 mg of microcarriers/mL in DMEM/FBS supplemented with LIF. Error bars represent standard deviation of two independent experiments.

on GFP expression (Ying and Smith, 2003). To this end, after culture in differentiation medium (Section 2.3), the cells were firstly trypsinized, washed in serum-containing medium, centrifuged at 1000 rpm for 2 min, resuspended in PBS containing 4% FBS and counted. The cells were then analyzed using a flow cytometer (FACSCalibur, Becton Dickinson) and the CellQuest software (Ying and Smith, 2003). Undifferentiated 46C ES cells were used as negative control.

3. Results

3.1. 46C mES cell expansion on microcarriers in stirred culture conditions using serum-containing medium

Herein, envisaging the need to scale-up mES cell expansion under serum-free conditions, we firstly compared the ability of two microcarriers—*Cytodex 3* and *Cutispher S* to support 46C mES cell expansion in a spinner flask using serum-containing medium.

46C mES cells were cultured for 8 days in a spinner flask culture system with DMEM/FBS supplemented with LIF. Throughout the experiments, for both microcarriers, the cell viabilities were always above 90%, and the fraction of cells found in suspension was always less than 5% (data not shown). Viable cell densities obtained (A and B), as well as the fold increase in total cell number achieved (C and D) are represented in Fig. 1 for *Cytodex 3* and *Cultispher S* culture systems, respectively.

It is possible to conclude that both microcarriers were able to support mES cell expansion. In both cases, the growth curve comprises a 24 h lag phase followed by an exponential phase leading to maximal cell concentrations of (1.9 ± 0.1) and $(2.6 \pm 0.7) \times 10^6$ cells/mL at days 6 and 8 for *Cytodex 3* and *Cultispher S* cultures, respectively; these correspond to fold increase values of 38 ± 2 (Fig. 1C) and 50 ± 15 (Fig. 1D). Although under the present culture conditions no significant differences can be found in terms of the proliferative supporting ability of the two microcarriers tested, it seems that the "start up" of the culture was faster for the mES cells expanded on *Cytodex 3* (i.e. shorter lag phase) since the fold increase values were higher in the early beginning.

On the other hand, the *Cytodex 3* culture reached the stationary (plateau) phase by day 4, while in *Cultispher S* experiments, the cell growth seemed to slow down only beyond day 7. By day 6, a decrease in cell density was observed in *Cytodex 3* cul-

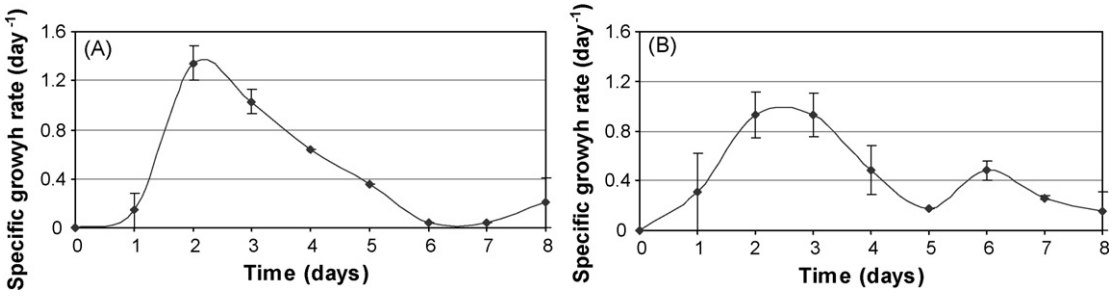

Fig. 2. Specific growth rate profile of 46C mES cells cultured on microcarriers in a spinner flask using serum-containing medium. Throughout time in culture, specific growth rates were calculated for cells cultured on *Cytodex 3* (A) and *Cultispher S* (B). Error bars represent standard deviation of two independent experiments.

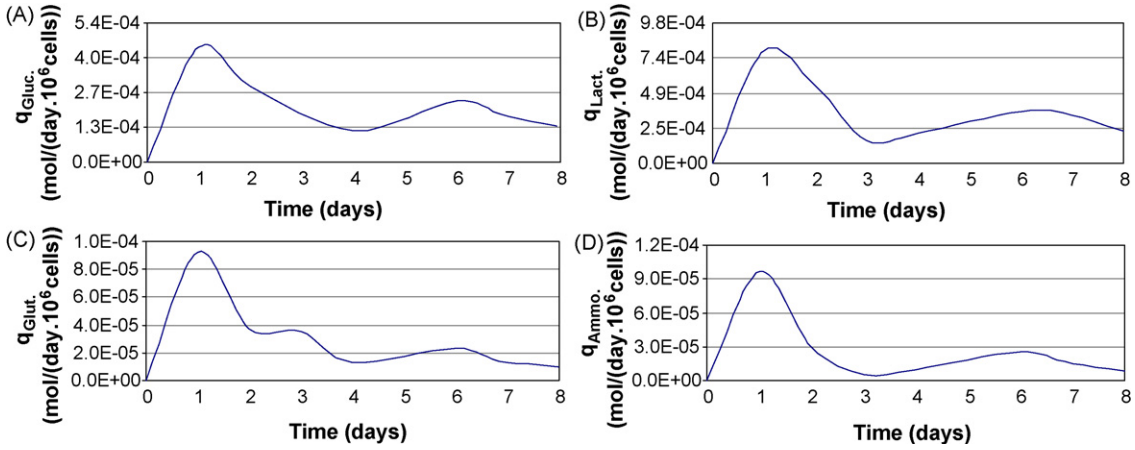

Fig. 3. Metabolic quotient profiles during 46C mES cell culture on *Cytodex 3* microcarriers in serum-containing medium. Glucose (A) and glutamine (B) specific consumption rates, as well as lactate (C) and ammonia (D) specific production rates during time in culture are represented. Values displayed represent the average of two independent experiments.

tures, but no significant increase in cell death was observed. In fact, it should be mentioned that cell monitoring during *Cytodex 3* cultures required, after trypsinization, a filtration step in order to separate the dextran beads from the cells. However, this step was extremely inefficient in the last days of the culture, since the beads became "clumped", obstructing the filter surface, turning difficult the recovery of the cells from the beads.

Specific growth rates were also calculated according to Section 2 and are represented in Fig. 2. In both cases, the specific growth rate curve followed the expected pattern for mammalian cell culture in general: after an initial increase reaching the maximum specific growth rate by days 2–3 (1.3 ± 0.1 and $1.0 \pm 0.2\,\text{day}^{-1}$ for *Cytodex 3* and *Cultispher S*, respectively) this rate decreases, either due to depletion of essential nutrients or accumulation of inhibitory metabolites. Concerning the doubling times (t_d), the fact of $t_{d(Cytodex\ 3)} < t_{d(Cultispher\ S)}$ (0.53 day versus 0.69 day) is consistent with the observation that cells needed more time to adhere and to start proliferate in *Cultispher S* cultures.

3.2. 46C mES cell metabolism during microcarrier cell culture in stirred culture conditions using serum-containing medium

The best way to characterize cell metabolism patterns is in terms of consumption rates and production rates *per* viable cell (i.e. metabolic quotients). Glucose and glutamine consumption rates, as well as lactate and ammonia production rates during the time in culture are represented in Figs. 3 and 4 for *Cytodex 3* and *Cultispher S* experiments, respectively. Overall, the specific consumption/production rates are higher during the first 2 days of culture consistent with the initial higher cell growth; then, after the adaptation of the cells to the culture environment, these values decrease becoming roughly constant.

It was also possible to conclude that, in general, the medium replacement was efficient at supplying nutrients (glucose and glutamine). Indeed, during time in culture, the glucose and glutamine concentrations did not decrease beyond 5 and 0.6 mM, respectively, never being completely depleted from culture medium.

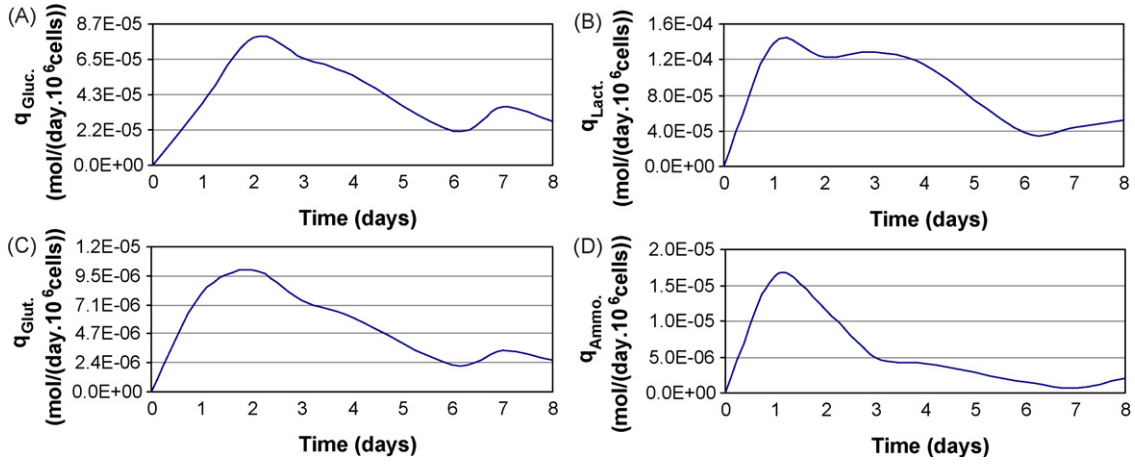

Fig. 4. Metabolic quotient profiles during 46C mES cell culture on *Cultispher S* microcarriers in serum-containing medium. Glucose (A) and glutamine (B) specific consumption rates, as well as lactate (C) and ammonia (D) specific production rates during time in culture are represented. Values displayed represent the average of two independent experiments.

Concerning the lactate concentrations, in both cases, the values were above 20 mM after the day 3. Although no specific data was found in the literature concerning inhibitory lactate concentrations for mES cell proliferation, it has been reported that hematopoietic cell proliferation, as well as for other mammalian cells, is inhibited above 20 mM (Ozturk et al., 1992; Patel et al., 2000). For *Cytodex 3* culture, the stationary phase was reached by day 4, which might suggest that the concentration of lactate above 20 mM observed could be inhibitory for the mES cell growth as well. However, for *Cultispher S* cultures, although lactate concentrations also exceeded this value, an extended exponential growth phase was observed compared to *Cytodex 3* experiments. Therefore, from these results it is not possible to assure that 20 mM lactate concentration is inhibitory for mES cell growth.

Ammonia, a by-product of glutamine metabolism, has also a harmful effect to cells, being an order of magnitude more toxic than lactate. Here, for all cultures ammonia levels never reached concentrations higher than 3 mM. Inhibitory effects to cell growth by ammonia accumulation have been reported for concentrations above 4 mM for mammalian cells (Ozturk et al., 1992).

The overall apparent lactate from glucose ($Y'_{\text{lact./gluc.}}$), and ammonia from glutamine ($Y'_{\text{ammo./glut.}}$) yields were also calculated. The $Y'_{\text{lact./gluc.}}$ provides an estimate of the fraction of glucose converted to lactate via glycolysis (Santos et al., 2005). The overall value of $Y'_{\text{lact./gluc.}}$ was 1.4 and 1.7 $\text{mol}_{\text{lac.}}/\text{mol}_{\text{gluc.}}$, for *Cytodex 3* and *Cultispher S* cultures, respectively. Although these values were similar for both microcarrier culture systems, the fact of $Y'_{\text{lact./gluc.}}$ was slightly higher for *Cultispher S*, might suggest the occurrence of oxygen limitation due to the higher cell numbers achieved during mES cell culture with this microcarrier.

On the other hand, the calculated $Y'_{\text{ammo./glut.}}$ was 0.7 and 0.6 $\text{mol}_{\text{ammo.}}/\text{mol}_{\text{glut.}}$, which is similar to the values reported in the literature for hybridoma, BHK and CHO cells (Cruz et al., 2000; Lao and Toth, 1997; Zhou et al., 1997). For instances, as long as the glutamine concentration is above 0.3 mM (Cruz et al., 2000), $Y'_{\text{ammo./glut.}}$ has been shown to be constant in hybridoma cell culture.

3.3. Evaluation of cell adhesion/viability, metabolic activity and pluripotency of 46C mES cells cultured on microcarriers

MTT and DAPI stainings were performed every 2 days during time in culture for both microcarriers. It was observed a gradual increase in microcarrier occupancy by mES cells. In addition, cells remained viable and metabolically active in culture. Fig. 5 indicates a successful cell adhesion of mES cells to both microcarriers after 2 days of culture (1); at day 4, the number of viable cells in the microcarriers had significantly increased and some microcarrier aggregation was observed (2). At days 6 and 8 (Fig. 5(3) and (4), respectively), a high percentage of beads is totally covered by mES cells, and the intense microcarrier aggregation becomes more evident. In addition, it was possible to observe that cells grew preferentially around the external surface of the macroporous *Cultispher S*; however, by changing the microscope amplification and focusing plans, different colonized plans of the 3D particles are visualized, suggesting that cells are also found in the interior of the gelatin matrix. Importantly, cell cultured for 8 days, stained positively for alkaline phosphatase, indicating that a high percentage of cells remained pluripotent, in their undifferentiated state (data not shown).

3.4. 46C mES cell expansion on Cultispher S microcarriers in stirred culture conditions: serum-containing versus serum-free conditions

Results in the previous sections indicated the feasibility of using macroporous gelatin *Cultispher S* microcarriers to support 46C mES cell expansion using a serum-containing medium. Here, we performed a side-by-side comparison of 46C mES cell culture on *Cultispher S* using either serum-containing or serum-

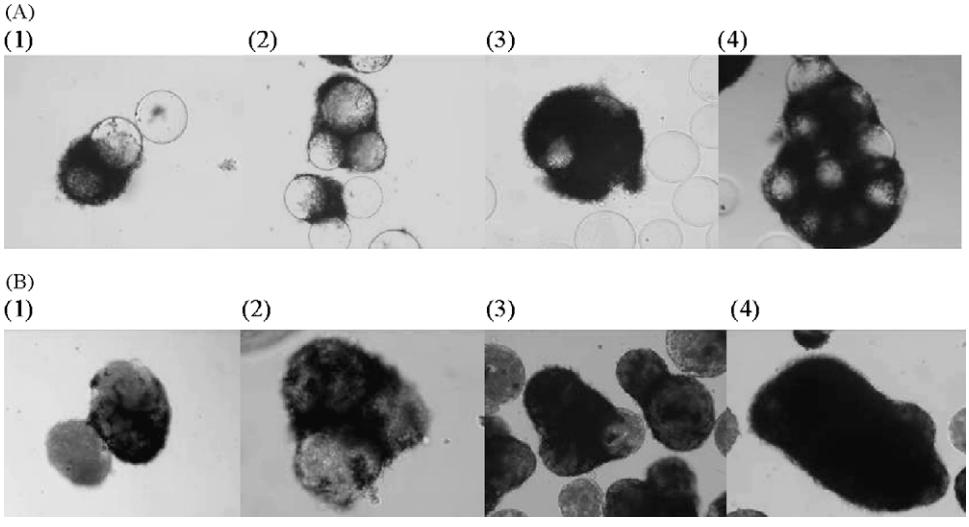

Fig. 5. Optical microscope photographs of 46C mES cells cultured on microcarriers under stirred culture conditions. Cells were visualized at Day 2 (1), Day 4 (2), Day 6 (3) and Day 8 (4) after MTT staining (100× amplification) for *Cytodex 3* (A) and *Cultispher S* (B) cultures, respectively.

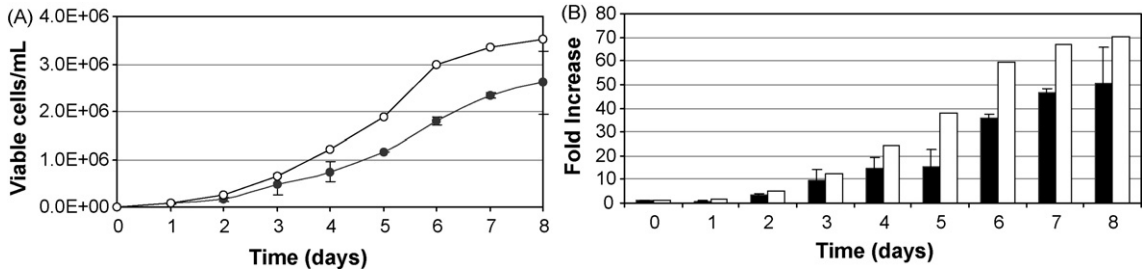

Fig. 6. 46C mES cell expansion on *Cultispher S* microcarriers in a spinner flask. Growth curve in terms of viable cells per milliliter (A) and cell expansion in terms of fold increase in total cell number (B) of 46C mES cells cultured on *Cultispher S*, respectively, are represented. Cells were inoculated at 5×10^4 cells/mL on 1 $mg_{Cultispher\ S}$/mL in serum-containing (in black) or serum-free medium (in white), both with LIF. Error bars represent standard deviation of two independent experiments for serum-containing experiments; one experiment is represented for serum-free conditions.

free medium. Viable cell densities obtained are represented in Fig. 6A, as well as the fold increase in total cell number achieved throughout time in culture—Fig. 6B.

It is possible to conclude that *Cultispher S* microcarriers were also suitable supports for mES cell expansion in serum-free conditions. After a 24 h, cells started to divide with a growth pattern similar to that obtained for cell culture in serum-containing medium, reaching a maximum cell concentration of 3.5×10^6 cells/mL, which represents a 70-fold increase (Fig. 6A and B respectively, in white). However, before cell inoculation, it was found necessary to incubate the beads alone overnight in serum-containing medium in order to enhance cell adhesion.

The specific growth rate profile was also determined for serum-free conditions (Fig. 7). No differences were found concerning the specific growth rate time evolution for both medium conditions and the doubling time of mES cells cultured on macroporous microcarriers was similar for both media (0.69 day versus 0.63 day, for serum-containing and serum-free cultures, respectively).

The metabolic patterns of 46C mES cells cultured on *Cultispher S* were similar to those obtained in the presence of serum (data not shown). However, in serum-free conditions the lactate concentrations were lower (<20 mM) than the observed with DMEM/FBS medium.

As for serum-containing conditions, mES cells expanded for 8 days in serum-free conditions using *Cultispher S* maintained their nuclear integrity and metabolic activity (by DAPI and MTT staining, respectively). In addition, cells retained their pluripotency as it was attested by ALP staining (Fig. 8A).

In addition, since the absence of serum exacerbates the harmful effects of shear stress, we wondered if the agitation could affect detrimentally cell viability. A flow cytometric analysis of the expanded cell population in serum-free conditions allowed us to conclude that only 8% of the cells were apoptotic, whereas only 2% were necrotic (data not shown).

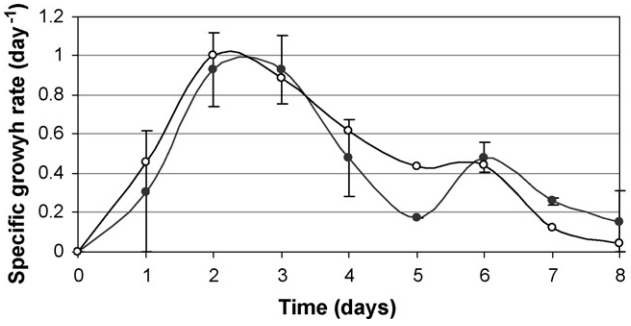

Fig. 7. Specific growth rate curve of 46C mES cells cultured on *Cultispher S* in a spinner flask culture system. Cells were inoculated at 5×10^4 cells/mL on 1 mg/mL of *Cultispher S*, in serum-containing medium (in black) or serum-free medium (in white). Error bars represent standard deviation of two independent experiments for serum-containing experiments; one experiment is represented for serum-free conditions.

3.5. Neural commitment of 46C mES cells expanded on Cultispher S microcarriers in stirred culture conditions using serum-free medium

After mES cell expansion in the spinner flask on *Cultispher S* microcarriers under serum-free conditions, the cells were tested for their neural commitment potential, according to Section 2. The neural commitment protocol was performed during 6 days, since results previously obtained in our laboratory showed that the maximum percentage of Sox1-GFP$^+$ neural progenitor cells is obtained at day 6 (Diogo et al., 2007). The percentage of Sox1-GFP$^+$ cells determined by flow cytometry was 90% (Fig. 8B and C) which indicates that even after expansion in stirred conditions for 8 days, under serum-free conditions, most of the cells kept the potential for neural commitment.

4. Discussion

Concerning the development of novel stem cell-based therapies, ES cells have been considered as a potential cell source since they can be maintained in culture as undifferentiated cells.

In the present studies, two different microcarrier-based culture systems under stirred conditions were evaluated as alternatives to the traditional monolayer mES cell culture, for the scaling-up of mES cell expansion, envisaging a production-scale system. Both microcarriers tested (*Cytodex 3* and *Cultispher S*) provided a suitable matrix for the expansion of these cells under stirred conditions. Concerning mES cell expansion using *Cytodex 3* microcarriers with serum-containing medium in the spinner flask, a maximal 38 ± 2-fold increase was achieved. The maximum specific growth rate was 1.3 day^{-1}, which corresponds to a doubling time of 0.53 days. This value of specific growth rate is in agreement with other results from our labo-

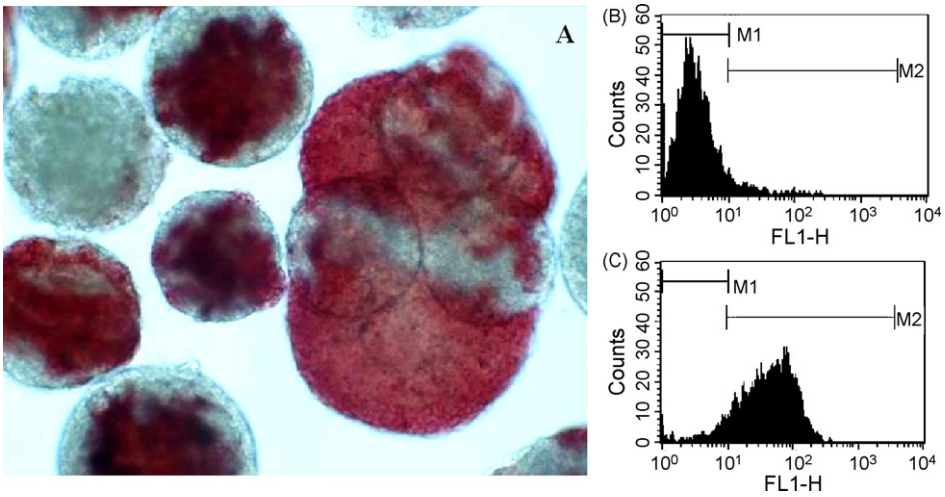

Fig. 8. Evaluation of pluripotency and neural commitment potential of 46C ES cells cultured on *Cultispher S* microcarriers under stirred culture conditions in serum-free medium. Pluripotency was evaluated at day 8 of the spinner flask expansion by alkaline phosphatase staining (optical microscope photograph at 200× amplification) (A). The percentage of neural progenitors was determined by flow cytometry using undifferentiated 46C ES as the negative control (B) and after culturing 46C ES cells during 6 days in the presence of RHB-A medium (C).

ratory by Abranches et al. who reported a maximum specific growth rate of 1.2 day^{-1} for the expansion of S25 mES cells on *Cytodex 3*, using the same culture conditions (Abranches et al., 2007). On the other hand, Fok and Zandstra (2005) have also reported a doubling time of 0.5–0.70 day for the expansion of mES cells on *Cytodex 3* microcarriers.

Animal serum has been typically included in ES culture media compositions. However, taking into account the technical disadvantages of using serum (Mukhopadhyay et al., 2004; Wiles and Johansson, 1999), it was envisaged to perform the scale-up of mES cell expansion in a chemically defined serum-free medium (Ying et al., 2003a). In addition, there are increasing concerns about animal suffering inflicted during serum collection that add an ethical imperative to avoid the use of serum wherever possible. However, serum-free conditions potentially exacerbate the harmful effect of shear stress on cultured cells (McDowell and Papoutsakis, 1998). In this context, the possibility of using an alternative type of microcarriers (*Cultispher S*) to support mES cell expansion in a spinner flask system was tested. The macroporous nature of *Cultispher S* (contrarily to the microporosity of *Cytodex 3*) would potentially provide a more protective environment to the cells, favoring cell expansion (Ng et al., 1996). In addition, cells are potentially less dependent of cell adhesion factors, since the concentration of those factors are locally higher inside the pores, allowing the culture of cells under lower serum concentrations (Xiao et al., 1999).

A maximal fold increase of 50 ± 15 was obtained when 46C mES cells were cultured in serum-containing medium using *Cultispher S* microcarriers, having a maximal specific growth rate of 1 day^{-1}. Concerning the doubling time, this was lower for *Cytodex 3* compared to *Cultispher S* cultures, which is consistent with the growth curve: it was possible to observe that the "start up" of the culture was faster for the mES cells expanded on *Cytodex 3*. This behavior might be due to the fact that cells need more time to adhere and to start their proliferation on the *Cultispher S*. However, growth curves of both microcarrier cultures indicate that the stationary phase of growth was clearly reached in *Cytodex 3* experiments (from day 4), whereas with *Cultispher S* (specially under serum-free medium) it suggests that proliferation did not cease. The differences of cell growth curve patterns observed between *Cytodex 3* and *Cultispher S* cultures in serum-containing medium are most likely due to surface area limitation in *Cytodex 3* cultures and/or the more protective environment provided by *Cultispher S* microcarriers to withstand the shear stress forces within the culture system. For instances, by using a microcarrier concentration of 0.5 mg/ml in *Cytodex 3* cultures (Abranches et al., 2007), the surface area might be limited in comparison to *Cultispher S* culture where a 1 mg/mL microcarrier concentration was used. Indeed, though some beads were empty even after 8 days in *Cytodex 3* cultures, the ones occupied by the cells were truly "crowded" and the bead-to-bead cell transfer is not likely to occur.

In serum-free conditions using *Cultispher S* in the spinner flask, a maximal fold increase of 70 is obtained after 8 days of culture. The higher fold increases observed in serum-free cultures might be, at least in part, due to the more specific action of certain compounds of the medium such as BMP-4, which together with LIF, allow mES self-renewal (Ying et al., 2003a). In addition, the use of macroporous particles is potentially advantageous by protecting cells from shear forces under stirred conditions.

In what accounts to the metabolic characterization of mES cell expansion in the spinner flask culture system, no specific data was found in the literature concerning inhibitory concentrations of lactate and ammonia, neither limiting concentrations of glucose of glutamine for this particular culture system. However, waste accumulation might have been one cause for the slowing down of the cell growth at the end of the cultures, especially in serum-containing cultures.

Results of metabolic analysis also suggest that the higher glutamine consumption is observed upon culture starting, after 1–2 days in culture, indicating a key role for this amino acid. In fact, it

was observed that $Y'_{lact./gluc.}$ was higher than the theoretical value of 2 at the first day of culture, suggesting the utilization of glutamine as the preferential carbon source. Glucose and glutamine concentrations were never less than 5 and 0.6 mM, respectively, staying within the accepted physiological rate for mammalian cells in general (Ozturk et al., 1997).

The ultimate goal of our studies was to confirm that 46C mES cells expanded for 8 days under stirred conditions using microcarriers retained their pluripotency and neural commitment potential. Alkaline phosphatase staining confirmed the maintenance of pluripotency by the 46C mES cells expanded under serum-free conditions using *Cultispher S*. In addition, those cells were able to undergo neural commitment using an appropriate culture medium and, after 6 days of culture, 90% of cells were Sox1-GFP positive. This value is comparable, though slightly higher, to the results obtained for 46C mES neural commitment in static culture conditions (<80%) (Ying and Smith, 2003).

In conclusion, our results show the feasibility of using a microcarrier-based spinner flask culture system to scale-up mES cell expansion. Importantly, we describe a successful strategy for the expansion of mES cells on macroporous microcarriers, under stirred conditions using serum-free medium, while retaining the pluripotency and neural commitment potential of the expanded cells. Although spinner flasks are regarded as bioreactors at laboratory scale, the high cell densities achieved in culture, especially when using serum-free conditions, demonstrate the potential of this particular culture system for use in scale-up strategies. The efficient, reproducible and cost-effective production of ES cells for use in multiple setting will require a bioreactor system which allow a more precise control over culture conditions to yield a greater number of cells (for example, by monitoring and continuously adjusting of oxygen content or pH).

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