Microcarrier expansion of mouse embryonic stem cell-derived neural stem cells in stirred bioreactors

Carlos A. V. Rodrigues,[1,2] *Maria Margarida Diogo,*[1,2] *Cláudia Lobato da Silva,*[1,2] *and Joaquim M. S. Cabral*[1,2]*

[1]*Department of Bioengineering, Instituto Superior Técnico, Technical University of Lisbon, Lisboa, Portugal*

[2]*IBB-Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Technical University of Lisbon, Lisboa, Portugal*

Abstract.

Neural stem cells (NSCs) are self-renewing multipotent cells, able to differentiate into the phenotypes present in the central nervous system. Applications of NSCs may include toxicology, fundamental research, or cell therapies. The culture of floating cell clusters, called "neurospheres," is widely used for the propagation of NSC populations *in vitro* but shows several limitations, which may be circumvented by expansion under adherent conditions. In particular, the derivation of distinct populations of NSCs from embryonic stem cells capable of long-term culture under adherent conditions without losing differentiation potential was recently described. However, the expansion of these cells in agitated bioreactors has not been addressed until now and was the aim of this study. Selected microcarriers were tested under dynamic conditions in spinner flasks. Superior performance was observed with polystyrene beads coated with a recombinant peptide containing the Arg–Gly–Asp (RGD) motif (Pronectin F). After optimization of the culture, a 35-fold increase in cell number was achieved after 6 days. High cellular viability and multipotency were maintained throughout the culture. The study presented here may be the basis for the development of larger scale bioprocesses for expansion of these and other populations of adherent NSCs, either from mouse or human origin.

1. Introduction

Neural stem cells (NSCs) are able to self-renew and generate neurons, astrocytes, and oligodendrocytes throughout the nervous system development and are responsible for the limited regeneration capacity of the adult brain [1]. First identified in the mouse brain by Reynolds and Weiss [2], NSCs brought new hope for the field of regenerative medicine, especially by providing novel potential approaches for the treatment of devastating neurodegenerative diseases such as Parkinson's disease, multiple sclerosis, Huntington's disease, or amyotrophic lateral sclerosis [3].

NSCs can be expanded *in vitro*, in the presence of growth factors, and typically are cultured as floating spherical aggregates known as neurospheres. For the generation of neurosphere cultures, mouse or rat neural tissue is harvested from embryos at E10.5–E18.5 or from the adult subventricular zone [1]. Although this culture system was the basis of important findings in terms of biological and molecular properties of NSCs, it also faces some limitations [1],[4]. For instance, heterogeneity occurs within and between neurospheres, stem cells maintained within neurospheres have an uncertain relationship to central nervous system precursors *in vivo* [5], there are oxygen and nutrient diffusion limitations and waste accumulation in the cluster center, and cells expanded as neurospheres differentiate much more readily into astrocytes than into neurons *in vivo* [6],[7].

The expansion of NSCs under adherent conditions would circumvent most of these limitations. As a particularly relevant model of NSCs that can be cultured under adherent conditions, NS cell lines that can be expanded clonally in monolayer, by symmetrical self-renewal, with maintenance of their neuronal differentiation potential for over 100 passages were derived for the first time a few years ago from embryonic stem (ES) cells. They have been termed NS cells to highlight the similar experimental attributes when compared with ES cells [4]. These NS cells are obtained after in *vitro* neuralization of ES cells and, most importantly, by exposing the resulting SOX1-positive cells to epidermal growth factor (EGF) and fibroblast growth

factor-2 (FGF-2). Under these culture conditions, a cell population with similar characteristics to radial glia, SOX1-negative, nestin-positive, and SOX2-positive is obtained.

The huge potential of NS cells makes them appealing for diverse applications. Some possibilities include drug testing, research in developmental and stem cell biology, or *in vivo* therapeutic settings such as gene therapy, drug delivery, or transplantation. For these applications, a high number of cells or high cell concentrations are likely to be required. However, the traditional static culture systems, such as tissue culture flasks or multiwell plates, are not a good solution if this is the final purpose. Indeed, the stationary nature of the culture leads to gradients in the culture medium in terms of pH, dissolved O_2, nutrient and metabolite concentration. Furthermore, static culture systems are labor intensive, expensive, and do not allow the tight control of culture parameters such as dissolved oxygen and pH. To generate a very high number of cells, the culture also needs to be scaled-up, bringing additional complications. The reduced surface area/volume ratio, for instance, in T-flasks, implies the use of multiple culture vessels or flasks with multiple trays, with the subsequent increase in incubator occupancy, handling time (for feeding, culture monitoring, and harvesting of cells), risk of contamination, and also an increased price.

A more efficient high-density culture of NSCs is possible if bioreactors are used. Extensive work has been done both with mouse (mNSC, [8–14]) and human (hNSC, [6],[15],[16]) cells toward the development of successful protocols for the expansion of NSCs as neurospheres in stirred bioreactors.

Inoculation and culture conditions of mNSC in bioreactors were optimized [8] including pH, osmolarity, and initial cell density. In order to minimize cell death (necrosis) in the center of the neurospheres, by oxygen or nutrient starvation due to diffusion limitations, it was shown that the diameter of NSC aggregates can be controlled below critical levels through manipulation of the agitation rate [12]. Efficient protocols were developed as well for the extended culture of mNSCs by successive passaging the cells over 35 days [9]. These protocols were developed for 125–250 mL spinner flasks and the mass transfer, shear stress, and hydrodynamic guidelines obtained from these studies directed the scale-up to large-scale (500 mL) computer-controlled reactors [17].

The same group also presented successful protocols for serum-free generation of clinically relevant quantities of human telencephalon-derived neural precursor cells (hNPC) in 500-mL computer-controlled suspension bioreactors [15]. hNPC harvested from different levels of the neuraxis were also expanded in bioreactors and transplanted into rodent models of Parkinson's disease and the influence of precursor cell origin in cell survival, differentiation, and migration *in vivo* were evaluated. The results obtained confirmed that hNPC can be expanded extensively in standardized conditions in suspension bioreactors; although upon transplantation, the expanded cells are able to survive and migrate in different patterns, depending on the region of origin, differentiation occurred predominantly into astrocytes instead of neurons. Other authors also reported preferential astroglial differentiation of neurosphere-expanded cells upon transplantation [7]. More recently, neuronal differentiation was observed after transplantation of NSCs expanded

as neurospheres [18] but with lower efficiency when compared with primary NSCs.

In this study, an alternative approach was used for *in vitro* expansion of NSCs. The previously described adherent ES cell-derived NS cells [4] were cultured on particles, known as microcarriers, in small-scale stirred bioreactors (spinner flasks). Microcarrier systems were introduced by Van Wezel in 1967, in a publication where this author used DEAE-Sephadex A50, a positively charged ion exchanger, for the growth of human fibroblast-like cells[19],[20]. This culture system was already extensively used for the expansion of anchorage-dependent mammalian cells such as hepatocytes [21] and cells for production of growth factors, vaccines, or antibodies[22–24]. A successful microcarrier culture, in bioreactors, was also described for ES cells, both from mouse[25–27] and human[28–32] origin as well as for mesenchymal stem cells[33–36]. Microcarriers are small spherical particles (100–300 μm in size) that provide a surface on which cells are ideally cultured in a monolayer and thus are equally exposed to the bulk medium, facilitating nutrient supply and metabolic by-product elimination. These cultures are characterized by high surface-to-volume ratio, accommodating higher cell densities compared with those in static cultures, and the available surface for cell growth can be easily adjusted by changing the amount of particles. Microcarriers can be composed of different materials, including plastic, cellulose, dextran, or gelatin. According to the current regulatory issues, pointing toward the removal of animal products from pharmaceutical- or clinical-grade manufacturing processes, in this work, only microcarriers in compliance with this condition were evaluated and cultures were performed in serum-free media.

The culture system described here may provide a process to expand other NSC populations, besides NS cells, that grow preferably as adherent monolayer [1]. These cells could be useful for the applications previously described either by themselves or as a complement of cells grown as neurospheres. Furthermore, and importantly, this culture system and this cell population may have a different outcome in terms of neuronal differentiation after transplantation, both in terms of quantity and neuronal subtypes obtained.

2. Materials and methods

2.1. NS cell expansion in static conditions

The NS cell line CGR8-NS, derived from the mouse ES cell line CGR8 and provided by the laboratory of Professor Austin Smith, Wellcome Trust Centre for Stem Cell Research, Cambridge, United Kingdom, was used as the cell model. CGR8-NS cells used in this work were always at passages P35–P40 and were kept preserved at $-80°C$ or liquid nitrogen until further use.

CGR8-NS cell culture was performed as described before [4],[37]. Briefly, upon thawing, CGR8-NS cells were expanded under static conditions on uncoated tissue culture plates or T-flasks (Falcon, BD Biosciences, San Jose, CA, USA) for one passage, using serum-free culture medium RHB-A (Stem Cells Inc., Palo Alto, CA, USA) containing 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco, Invitrogen Corporation, Carlsbad, CA, USA), and supplemented with 15 ng/mL of FGF-2 and

EFG (Peprotech, London, UK). The cells were cultured at 37°C under a 5% CO_2 humidified atmosphere.

Cells were passaged using Accutase (Sigma, St. Louis, MO, USA). In each passage, viable and dead cells were determined by counting in a hemocytometer under an optical microscope (Olympus, Hamburg, Germany) using trypan blue dye (Gibco BRL) exclusion test. Viabilities were always above 90%.

2.2. NS cell expansion on microcarriers under static conditions

To determine the most suitable microcarrier type for CGR8-NS cell culture, different commercially available animal-free products were tested under static conditions. In particular, Plastic, Plastic Plus, Glass, Hillex® II, and Pronectin® F microcarriers (all from Solohill Engineering, Inc., Ann Arbor, MI, USA) were used. Before inoculation, the microcarriers were suspended in phosphate-buffered saline (PBS) (Gibco BRL), sterilized by autoclaving, and incubated (at 37°C and 5% CO_2) at least for 1 H in culture medium supplemented with the growth factors.

An equivalent area of 15 cm² was used for each microcarrier type. CGR8-NS cells (10^4 cells/cm² of microcarrier area), previously thawed and expanded in static conditions until 80%–90% of confluence, were mixed with the microcarriers and cultured in 24-well ultra-low attachment plates (Corning Inc., Corning, NY, USA). Cell attachment was examined visually after 3 days. Attachment was also verified after 4′,6-diamino-2-phenylindole dilactate (DAPI) staining. Briefly, a sample of the microcarriers, after 3 days of culture, was washed twice with PBS, fixed with 2% paraformaldehyde (PFA, Sigma) for 15 Min at room temperature, and washed again twice with PBS. The samples were incubated with a solution of 1.5 µg/mL DAPI (Sigma) in PBS, for 5 Min at room temperature, and protected from light. After the incubation time, the samples were washed three times with PBS and blue-stained nuclei were visualized under a fluorescence optical microscope (DMI 3000B; Leica, Wetzlar, Germany) and digital images were taken with a digital camera (DXM 1200 F; Nikon, Tokyo, Japan).

2.3. Spinner flask operation and cell counts

Cell culture under dynamic conditions was performed in StemSpan spinner flasks (StemCell Technologies, Vancouver, Canada), suitable for microcarrier culture, with working volumes of 30 mL. Spinner flasks are equipped with an impeller with paddles and a magnetic stir bar and were placed inside a CO_2 incubator at 37°C over a magnetic stirring plate (2 mag, Germany).

Unless otherwise specified, the microcarrier concentration used was 20 mg/mL, an intermediate value in the range recommended by the manufacturer (5–40 g/L). This corresponds to an equivalent area of approximately 216 cm². CGR8-NS cells were thawed and expanded in static conditions until 80%–90% of confluence, and detached and dissociated to a single-cell suspension with Accutase (Sigma). Sterilized microcarriers were incubated for at least 1 H at 37°C in the CO_2 incubator prior to mixing with 2 × 10^6 cells, in 15 mL (half of the final volume) of medium. After 24 H, the culture volume was adjusted to 30 mL.

During the first 24 H after inoculation, two different intermittent stirring schemes were tested: scheme A, stirring on (50 rpm) for 2 Min followed by 60 Min off; scheme B, continuous stirring for up to 18 H at 50 rpm followed by 6 H of intermittent stirring (3 Min on, 30 Min off).

Unless otherwise noted, feeding was performed on a daily basis by replacing 50% of the medium with fresh prewarmed medium. The total volume of medium (30 mL) was supplemented every 24 H with 15 ng/mL of FGF-2 and EFG to prevent growth factor depletion due to thermal degradation [38].

Duplicate samples of evenly mixed culture were collected from the spinner flasks every 24 H. The microcarriers were washed twice using PBS before incubation with Accutase (Sigma) to detach the cells from the beads. Samples were incubated in a heated mixer (Thermomixer Comfort, Eppendorf, Germany) for 7 Min at 37°C, using 650 rpm of mixing speed to facilitate detachment of cells from the microcarriers. Accutase (Sigma) was diluted with fresh culture medium and the samples were centrifuged at 208g for 3 Min. After resuspending the cells in fresh culture medium, viable and dead cells were determined by counting in a hemocytometer under an optical microscope using the trypan blue dye exclusion test. Fold increase (FI) in cell number was calculated as the ratio between the cell number determined at the end of the culture and the number of cells initially inoculated. To calculate the specific growth rate (µ), ln X, where X is the number of cells, was plotted against time (t) in the period corresponding to the exponential growth phase. According to Eq. (1)

$$\ln X = \ln X_0 + \mu t \qquad (1)$$

where X_0 is the number of cells in the initial point, the specific growth rate is the slope of a linear trend line adjusted to the plot. Doubling time was calculated as indicated in Eq. (2)

$$\text{Doubling time} = \frac{\ln 2}{\mu} \qquad (2)$$

2.4. Analysis of metabolites and nutrients in culture medium

In order to determine the concentration of culture medium components, the microcarriers were allowed to sediment and then samples (1.5 mL) of the medium were collected, centrifuged at 468g for 10 Min, and kept at −20°C for subsequent analysis. After medium changes, and after at least 10 Min of stirring to ensure homogenization, a sample was also collected. Glucose, lactate, and glutamine concentrations were determined in the supernatant of the samples collected throughout the experiments by using an automatic analyzer (YSI 7100MBS; Yellow Springs Instruments, Yellow Springs, OH, USA). Specific lactate production rate (q_{Lac}) was calculated according to Eq. (3)

$$q_{Lac} = \frac{\Delta Lac}{\overline{X} \Delta t} \qquad (3)$$

where ΔLac is the lactate produced during the time period Δt and $\overline{X}$ is the average number of cells during that period. The average specific lactate production rate was calculated as the average of the q_{Lac} values obtained for all days.

2.5. Neuronal and astrocyte differentiation of NS cells expanded under dynamic conditions

For astrocyte differentiation, NS cells expanded during 9 days in spinner flasks, were plated (density: 10^4 cells/cm²) in RHB-A medium supplemented with 1% fetal bovine serum (Gibco-BRL), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were incubated for 12 days at 37°C under a 5% CO_2 humidified atmosphere with 50% medium change every 2–3 days. For neuronal differentiation, NS cells expanded during 9 days in spinner flasks, were plated (density: 1–1.5×10^5 cells/cm²) on uncoated well plates using RHB-A medium supplemented with 1% B27 (Invitrogen), 0.5% N_2 Supplement (Invitrogen), and 10 ng/mL FGF-2. After 3 days at 37°C, cells were gently dissociated with Accutase (Sigma), and were replated (density: 5–7.5×10^4 cells/cm²) on polyornithine/laminin (both from Sigma) coated well plates in a medium composed of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM)/F12 and Neurobasal medium (Invitrogen) containing 0.5% N_2 supplement, 1% B27, 20 ng/mL brain-derived neurotrophic factor (BDNF, Invitrogen), and 10 ng/mL FGF-2. After 3 days, the culture medium was shifted to a 1:1 mixture of DMEM/F12 and Neurobasal medium containing 0.5% N_2 supplement, 1% B27, 30 ng/mL BDNF, and 6.7 ng/mL FGF-2. After 3 days more, medium composition was changed to one with the same composition but having a reduced FGF-2 concentration (5 ng/mL). All media for neuronal differentiation were supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. At day 12 of the neuronal differentiation protocol, neuronal differentiation efficiency was assessed by immunocytochemistry and flow cytometry.

2.6. Intracellular staining for flow cytometry

Cells were analyzed for the expression of intracellular proteins by flow cytometry after intracellular staining. Briefly, a minimum of 1×10^6 cells were washed with PBS and fixed in 2% PFA for at least 20 Min. Cells were then washed with 1% normal goat serum (NGS, Sigma), resuspended in 3% NGS and permeabilized with 0.5% saponin solution (Sigma) during 15 Min at room temperature. Cells were incubated with 3% NGS solution for 15 Min at room temperature and then with the indicated antibody dilution for 1–2 H at room temperature. The following primary antibodies were used: anti-Ki-67-FITC (dilution 1:37; Invitrogen), anti-Nestin mouse monoclonal antibody (dilution 1:100; Millipore, Billerica, MA, USA), anti-Sox2 (dilution 1:50; R&D systems, Minneapolis, MN, USA), anti-glial fibrillary acidic protein (dilution 1:100; GFAP, Millipore), anti-βIII-Tubulin (dilution 1:500; Tuj1, Covance, Princeton, NJ, USA). After washing with 1% NGS, cells were incubated with the appropriate secondary antibodies, all conjugated with Alexa Fluor 488 (dilution 1:500; Invitrogen), for 1 H at room temperature. Finally, the cells were washed with 1% NGS and resuspended in PBS, prior to analysis. For the negative control sample, the same protocol was followed but during the incubation period with the primary antibody, the cells were incubated with 1% NGS but without any antibody. All flow cytometry analyses were performed in a FACS calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and a minimum of 10,000 events was collected for each sample. The CellQuest software (Becton Dickinson) was used for all acquisition/analyses.

2.7. Immunocytochemistry

Cells (either on tissue culture plates or attached to microcarriers) were fixed in cold 4% PFA for 10 Min at room temperature. After washing twice in PBS, the cells were permeabilized with 0.1% Triton X-100 (Sigma) in PBS with 10% NGS for 1 H at room temperature. Primary antibodies were incubated overnight at 4°C in PBS with 0.1% Triton X-100 and 5% NGS. The primary antibodies used and their dilutions were the same as indicated above. At the end of the incubation with primary antibody, cells were washed three times in PBS. Appropriate secondary antibodies, all conjugated with Alexa Fluor 488 (dilution 1:500; Invitrogen), were incubated with the cells for 1 H at room temperature in the dark. Finally, cells were washed with PBS, and the nuclei were counterstained with DAPI (1.5 µg/mL in PBS) for 5 Min at room temperature. After the incubation time, the samples were washed two times with PBS and the stained cells were visualized under a fluorescence microscope.

3. Results

3.1. Microcarrier screening under static conditions

The first step toward the development of a bioreactor system for the adherent culture of mouse NS cells was a testing of different commercially available microcarriers under static conditions in order to find the one most suitable for cell attachment. CGR8-NS cells can be cultured, in static conditions, on gelatin-coated tissue culture flasks [39]. However, envisaging a xeno-free process, the presence of gelatin is not desirable and thus previous studies were conducted in uncoated flasks [37]. Following the same principle, different microcarriers free from animal products were used. In particular, SoloHill's Plastic, Plastic Plus, Glass, Hillex II, and Pronectin F microcarriers were tested. As regular culture flasks are made of polystyrene, these particles, with a solid polystyrene core, would be an obvious starting point, with different coatings to enhance cell adhesion under dynamic conditions.

After 3 days of culture, the efficiency of cell adhesion and survival in the different microcarriers was assessed by visual inspection under the microscope, both in bright field and with DAPI staining (Table 1, Fig. 1). Because of the poor performance of the Plastic and Hillex II microcarriers for CGR8-NS cell attachment, demonstrated with this experiment, these were not considered for further studies. The Glass, Plastic Plus, and Pronectin F microcarriers were thus selected for preliminary experiments in spinner flasks.

3.2. Mouse NS cell culture in spinner flasks

For dynamic culture of mNS cells, spinner flasks with a working volume of 30 mL were used. On the basis of previous reports of spinner flask culture of stem cells [26],[30],[31],[40], for the first experiment, an intermittent stirring regime was used in the initial 24 H of culture (see Section 2.3). In this first day of culture,
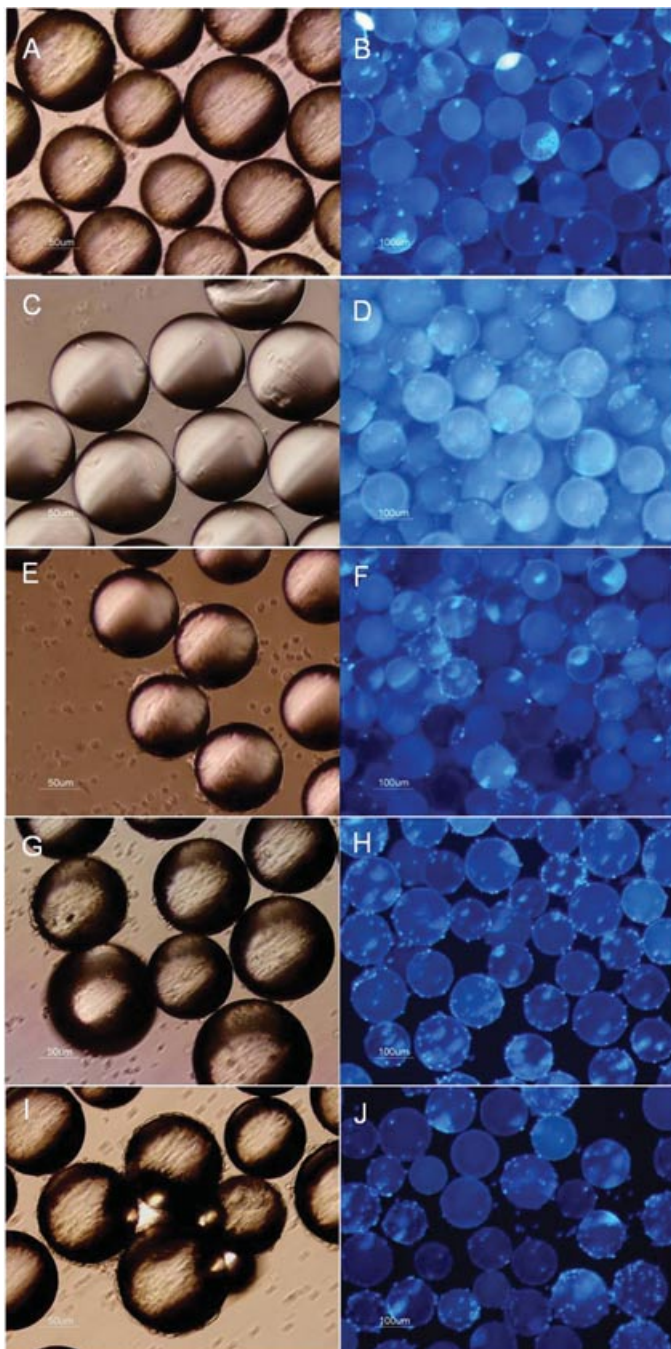
Fig. 1. Screening of microcarriers in static conditions. Bright field (left column) and DAPI staining (right column) are shown. Microcarriers tested include Plastic (A and B), Hillex II (C and D), Glass (E and F), Plastic Plus (G and H), and Pronectin F (I and J) all from SoloHill.

Table 1

Qualitative assessment of the attachment efficiency of mNS cells to microcarriers under static conditions

Microcarrier type	Material	Attachment
Plastic	Cross-linked polystyrene with no surface charge	Poor
Hillex II	Modified polystyrene with surface charge	Poor
Glass	Cross-linked polystyrene with only incidental surface charge coated with high silica glass	Medium
Plastic Plus	Cross-linked polystyrene with cationic surface charge	Medium
Pronectin F	Cross-linked polystyrene with surface charge coated with recombinant RGD-containing protein	Good

nutrient shortage or metabolite accumulation. The culture was kept for 9 days, until growth started slowing down (Fig. 2).

These three microcarriers exhibit different surface properties. In the case of glass microcarriers, the beads have no surface charge. In opposition, the Plastic Plus beads have a positively charged surface that enhances cell adhesion to the beads through electrostatic interactions, whereas the Pronectin F spheres are coated with an adhesion-promoting recombinant peptide, containing the RGD cell attachment motif. These differences led to distinct behaviors within these microcarriers under dynamic culture conditions and because the Pronectin F microcarriers provided the best results in terms of cell expansion, these beads were selected for further optimization of the system.

The first 24 H are of critical importance for the success of the culture because a homogeneous inoculation of the microcarriers will lead to an efficient use of the available growth area. Thus, additional effort was made to improve this step. Following recent indications from the microcarrier manufacturer, a different inoculation scheme was employed. The novelty in this new scheme is the use of continuous agitation in the first 24 H, instead of intermittent agitation. The purpose of this procedure is to avoid cell aggregation, as single cells are more likely to attach to the microcarriers than cell clumps, which will not attach at all or, at best, will attach as clumps. Intermittent stirring was used, 18 H after the beginning of the culture, for cell spreading on the microcarriers. This protocol was compared with the "traditional" one (Fig. 3) and better results in terms of cell expansion were achieved. Although in the initial days both schemes lead to comparable cell numbers, in the end of the culture period, higher FIs in cell number were possible with the new scheme, which was thus adopted for further studies.

As mentioned above, half the volume of culture medium was replaced every day with fresh medium. It was important, however, to determine whether this was the optimal procedure in terms of cell growth. For that purpose, the output in terms of cell expansion was compared between daily feeding of the culture and replacing half the volume of medium only every 2 days (Fig. 4A). In both situations, the growth factors EGF and FGF-2

the microcarriers were suspended in only half the final volume (15 mL) to increase the frequency of cell–bead interactions, improving cell attachment, as well as to concentrate the autocrine factors released by the cells. A stirring speed of 60 rpm was used in these first exploratory experiments as well as a feeding regime where half of the medium is changed every day to avoid
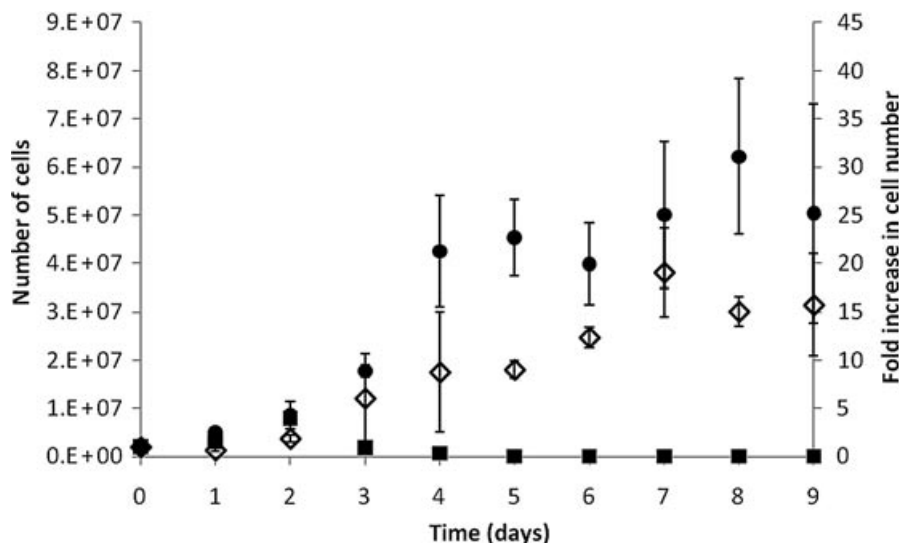
Fig. 2. Growth curve of mES cell-derived NS cells cultured in spinner flasks with different microcarriers (Glass, white diamonds; Pronectin F, black circles; Plastic Plus, black squares). The results shown are the average of two independent experiments. Error bars indicate standard error of the mean (SEM).
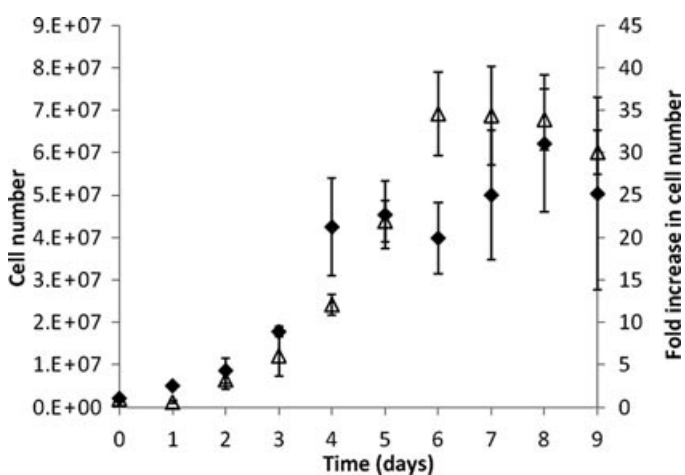

Fig. 3. Growth curve of mES cell-derived NS cells cultured in spinner flasks with different inoculation strategies. (A) Intermittent stirring (black diamonds) and (B) continuous stirring for 18 H followed by intermittent stirring for 6 H (white triangles). See Section 2.3 for more details. The results shown are the average of two independent experiments. Error bars indicate SEM.

were added in a daily basis since at least FGF-2 is known to have a reduced thermal stability (up to an 80% drop in concentration after 24 H) at 37°C [38].

From the growth curves obtained, it is clear that the daily feeding led to better results in terms of expansion. As the growth factor supply was identical, the different results could be related with shortage or exhaustion of nutrients, such as glucose or glutamine, or excessive accumulation of toxic metabolites, such as lactate. Therefore, analysis of the concentration profiles of

these compounds during the culture could provide clues for explaining the results (Figure 4B–4D).

As glucose consumption is relatively low throughout the 9 days of culture, exhaustion of this nutrient never occurs. In terms of glutamine, and in contrast to what happens with glucose, the initial concentration is rather low. However, even though consumption is observed, this nutrient is never completely exhausted. The lactate profile reveals an increasing concentration of this metabolite as the cell number increases. The maximum concentration achieved was 13.5 mM and the average specific lactate production rate was $6.41 \times 10^{-6} \pm 4.64 \times 10^{-7}$ mmol/(cell day) for the daily half-fed culture and $5.22 \times 10^{-6} \pm 9.06 \times 10^{-7}$ mmol/(cell day) for the culture half fed every 2 days. Given that the best results were obtained with daily half feeding, this practice was adopted for further experiments.

The effect of stirring speed over the culture performance was also studied (Fig. 5) by comparing the growth curves obtained by stirring the culture at 40, 60, and 80 rpm. Although these three agitation rates led to expansion of mNS cells, better results in terms of cell expansion were observed with 60 rpm, which was considered the optimal speed.

Finally, a different concentration of microcarriers was tested. In all the previous studies, a concentration of 20 g/L of microcarriers was used, corresponding to an area of 216 cm². Having in mind hypothetical positive effects of an increased initial cell density (e.g., improved attachment efficiency), the same number of cells (2×10^6) was inoculated in a bead suspension with a lower concentration. The lower value in the manufacturer-recommended range, 5 g/L, was then compared with 20 g/L (Fig. 6A). The results show that a lower increase in cell number was observed when a concentration of 5 g/L of microcarriers was used. This may be explained by plotting cell density throughout the culture time (Fig. 6B). Indeed, both cultures seem to reach a "plateau" around 3–4×10^5 cells/cm², which probably correspond to the point
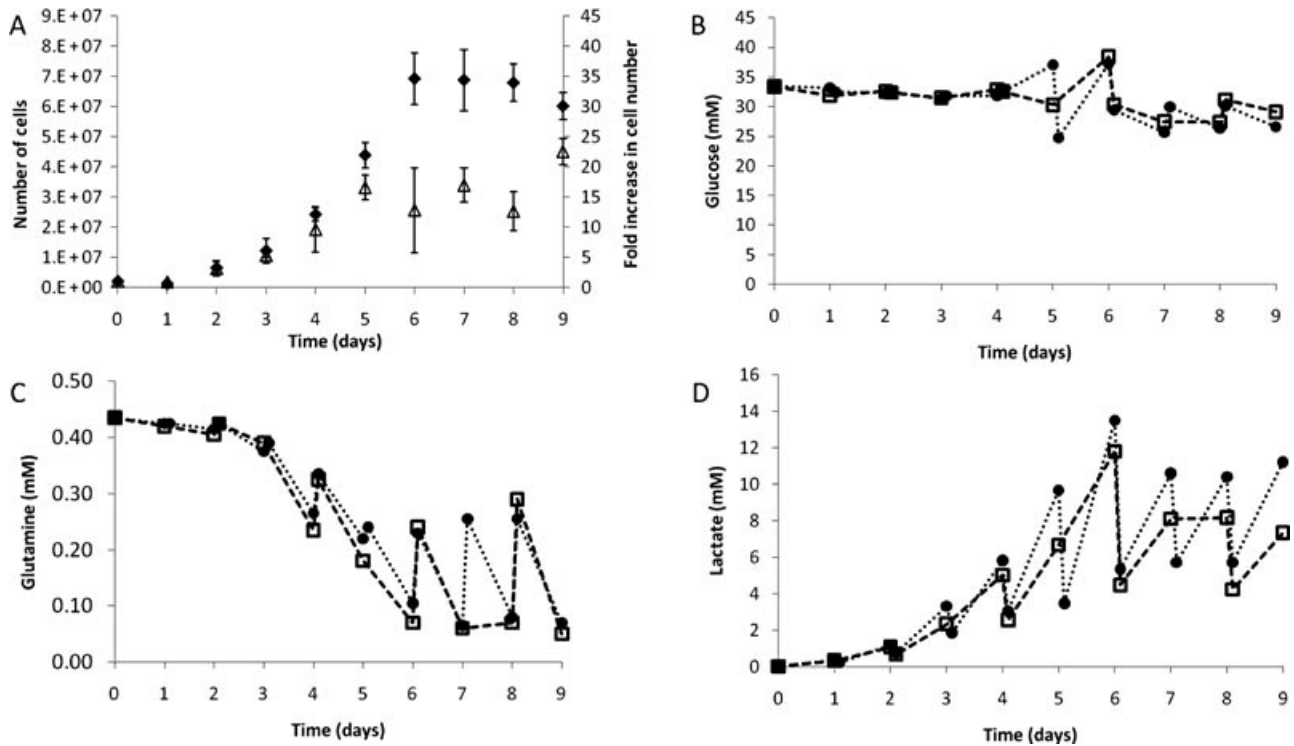
Fig. 4. Effect of feeding regime on the expansion of mES cell-derived NS cells in spinner flasks. (A) Growth curve of cells with different feeding schemes; half-medium exchange every day (black diamonds) and every 2 days (white triangles). The results shown are the average of two independent experiments. Error bars indicate SEM. Representative data of concentration profiles of glucose (B), glutamine (C), and lactate (D) over time, for half-medium exchange every day (black circles) and every 2 days (white squares).
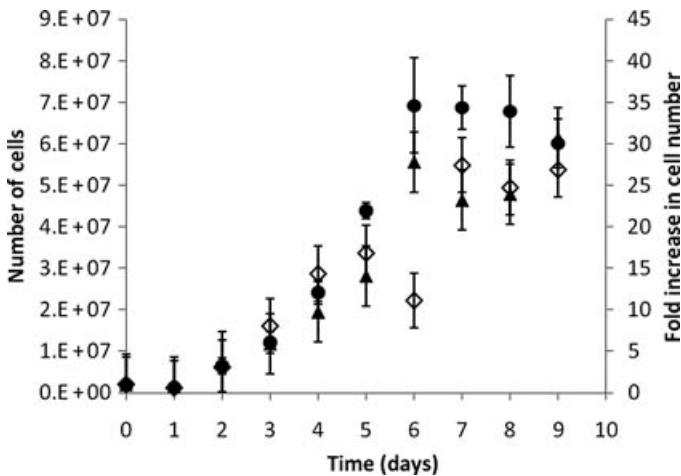

Fig. 5. Growth curve of mES cell-derived NS cells cultured in spinner flasks with different stirring speeds: 40 (white diamonds), 60 (black circles), and 80 (black triangles) rpm. The results shown are the average of two independent experiments. Error bars indicate SEM.

Table 2

Specific growth rate and doubling times of CGR8-NS cells cultured on microcarriers and in tissue culture plates

	Spinner flask	Static 2D culture[a]
Specific growth rate (day^{-1})	0.76 ± 0.02	1.2 ± 0.02
Doubling Time (H)	22 ± 0.69	13.9 ± 0.3

[a]Taken from ref. 37.

condition, the growth rate and doubling time were calculated (Table 2).

3.3. CGR8-NS cells remain multipotent after spinner flask expansion

Optimal parameters for spinner flask expansion of CGR8-NS cells were determined. However, it is of crucial importance to verify whether cell culture on the microcarriers as well as the dynamic environment provided by agitation affect the multipotency of the cells. For that purpose, after 9 days of culture, the expression of neural stem/progenitor markers Nestin and Sox2 was assessed by immunocytochemistry, directly on the microcarriers (Figs. 7A–7F). Cells were also harvested from the

where the microcarriers are confluent and thus there is no more area available for cells to grow. We can thus conclude that with 20 g/L, it is possible to achieve a higher cell number due to the larger microcarrier surface area available. For this
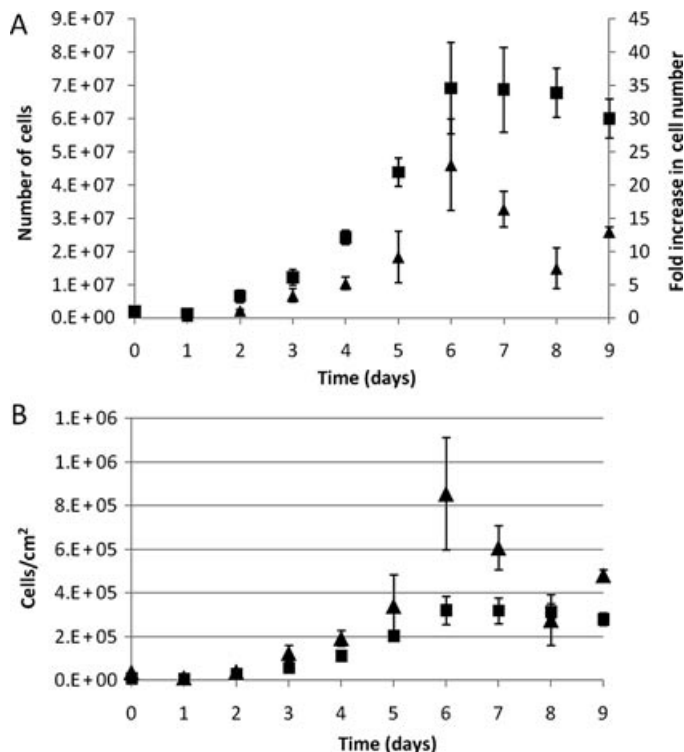
Fig. 6. Effect of different microcarrier concentrations on spinner flask expansion of mES cell-derived NS cells. Growth curve of cells cultured with different microcarrier concentrations (A) and cell density (cells/cm²) over time (B). Concentrations used were 5 g/L (black triangles) and 20 g/L (black squares). Error bars indicate SEM.

microcarriers and analyzed for the expression of these markers by flow cytometry, after intracellular staining (Figs. 7G and 7H). The markers were expressed by more than 90% of the cells and the staining directly performed on the microcarriers showed the expected morphology. The expression of the proliferation marker Ki-67 [41], which is present in proliferative cells (in cell cycle) and is absent in quiescent cells, was also assessed (Fig. 7I). More than 90% of the cells expressed this marker, indicating that spinner flask-cultivated cells are actively proliferating.

Although the expression of these markers is an important point, it was essential to verify if the cells retained the capability to differentiate into neuronal and glial cells and therefore CGR8-NS cells were expanded for 9 days in the spinner flasks, with the optimized parameters, and then differentiated under static conditions into neurons and astrocytes, by following the described protocols [4],[42]. After 12 days of differentiation, cells with characteristic astrocyte morphology were observed (Fig. 8A), and successful differentiation was confirmed by the presence of immunoreactive cells for GFAP (Figs. 8B–8D). Flow cytometry analysis confirms the existence of GFAP positive cells (Fig. 8E). In terms of neuronal differentiation, also after 12 days of differentiation under appropriate conditions, cells acquired neuronal morphology (Fig. 8F) and became immunoreactive for βIII-Tubulin (Figs. 8G–8I). Also, this result was confirmed with flow cytometry (Fig. 8J).

4. Discussion

This study presents a stirred bioreactor system for the suspension culture of mouse ES cell-derived NS cells, attached to microcarriers. The first step adopted here was a screening of different animal component-free microcarriers. A first selection step was performed, in static conditions, in order to determine which microcarriers were capable of supporting cell attachment and growth. The selected microcarriers consisted of cross-linked polystyrene beads coated either with a surface without charge (Glass), with a cationic surface, which facilitates cell attachment (Plastic Plus), or with a recombinant protein polymer (Pronectin F). These substrates were tested for spinner flask culture and the best results were obtained with Pronectin F. This result may be explained with the conclusions obtained by Forestell et al. [43]. These authors hypothesized that, in a first step, cells have to overcome the van der Waals forces separating the cells from the microcarriers, probably by interactions based on hydrophobic surface properties, followed by an attachment and spreading step, mediated by attachment proteins. In the present case, the Plastic Plus microcarriers may have had a good performance in the initial adhesion step, due to their positively charged surface, but were inappropriate for the spreading, whereas Pronectin F, by mimicking the presence of extracellular matrix proteins, may have led to better overall results. In fact, the protein polymer present in the Pronectin F beads, with a molecular weight of 72 kDa, has been engineered to include 13 copies of the cell attachment epitope of human fibronectin, Arg–Gly–Asp (RGD) interspersed between structural peptide segments, corresponding to the crystalline region of silk [44]. Some studies even mention an impaired production of extracellular matrix when cells grow on charged surfaces [45], which may explain the poor performance of the Plastic Plus. Although the mechanism by which the glass microcarriers promote cell adhesion is not clear, these particles were able to sustain cell growth under dynamic conditions almost as well as the Pronectin F carriers. The silica surface, being free of electrical charge, may lead to a slower attachment and growth than observed with Pronectin F but maybe without, for instance, the detrimental effect on extracellular matrix production that may have occurred with Plastic Plus. Moreover, the shear forces generated in spinner flask cultures may further emphasize the differences in attachment characteristics of the different types of microcarriers [46]. For these reasons, Pronectin F carriers were used in the subsequent optimization studies.

Most of the protocols described for stem cell culture in microcarriers use an intermittent stirring scheme for inoculation [26],[30],[31],[40]. In this work, initially, this scheme was also used and was compared with a different inoculation paradigm, where continuous stirring is used during the attachment period to avoid cell aggregation and to promote a more homogenous cell distribution. Higher cell numbers were observed when the latter scheme was used, confirming the importance of a good initial distribution of the inoculum among the microcarriers for the development of the culture, as a better occupation of the available area is possible. The choice of the ideal inoculation scheme is, however, probably dependent on the individual characteristics of each cell line, such as the attachment speed or the
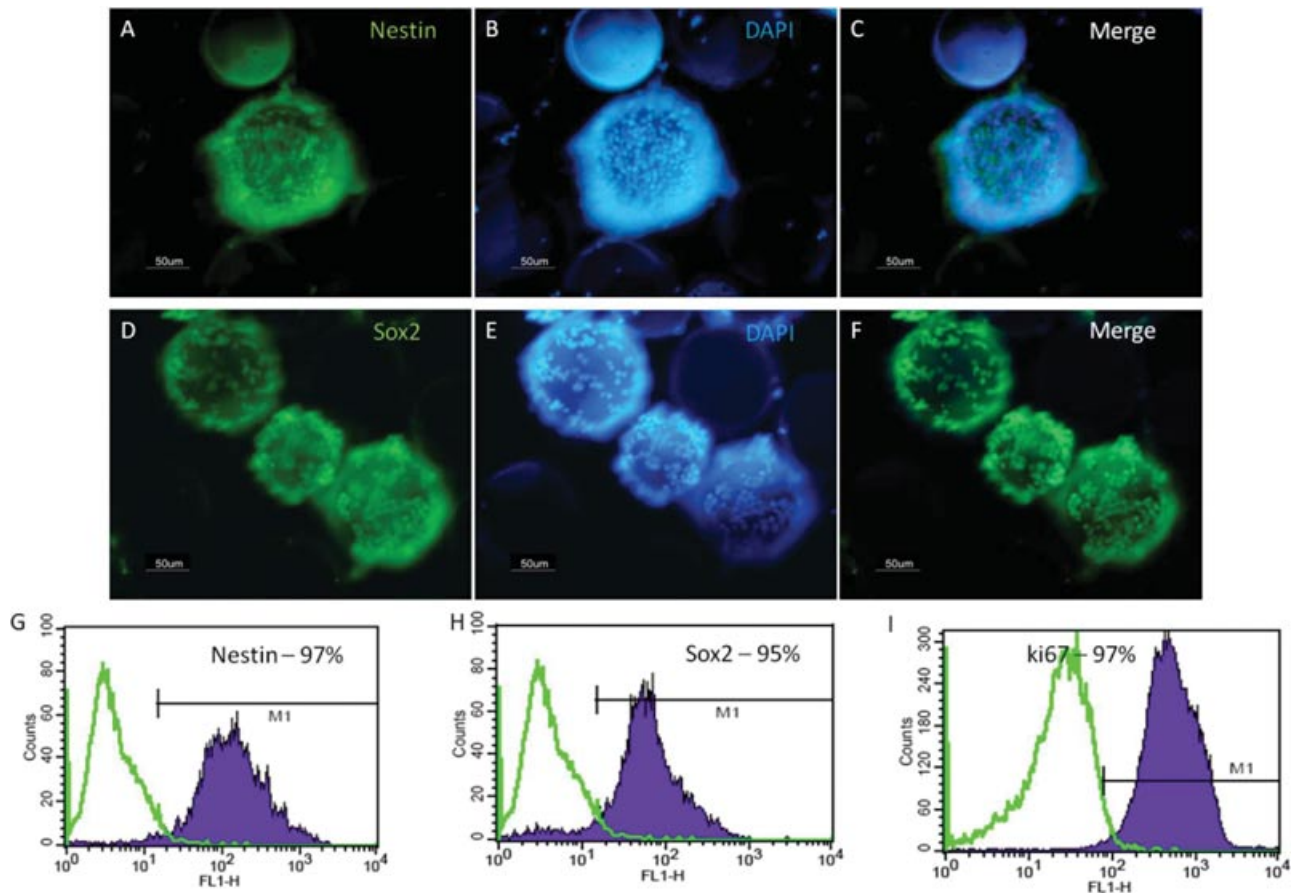
Fig. 7. Expression of neural stem/progenitor cell markers and a cell division marker in mES cell-derived NS cells cultured in spinner flasks for 9 days. Immunostaining for Nestin (A–C), Sox2 (D–F), and flow cytometry for Nestin (G), Sox2 (H), and ki-67 (I).
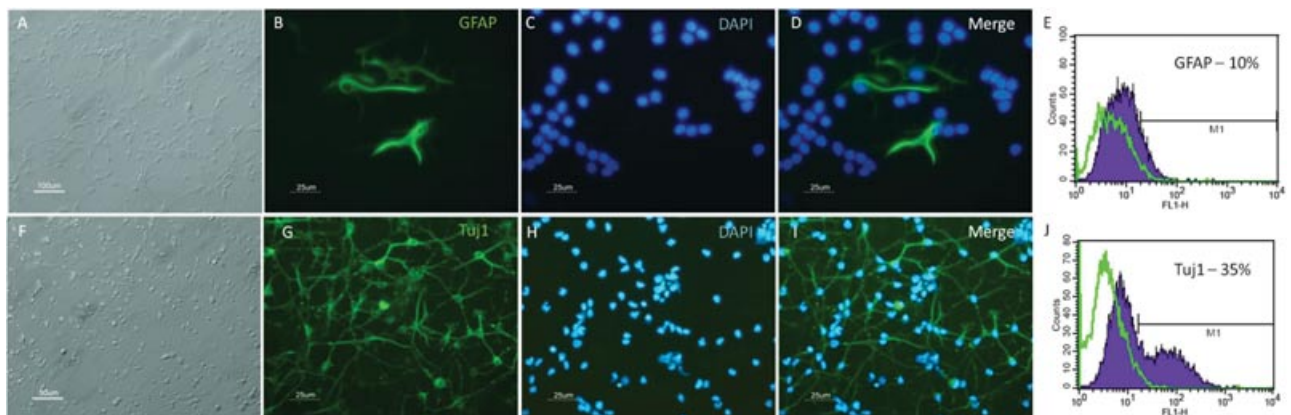

Fig. 8. Differentiation of mES cell-derived NS cells cultured in spinner flasks for 9 days. Bright-field images of cells differentiated into astrocytes for 12 days (A) and expression of the astrocyte marker GFAP, detected by immunostaining (B–D) and flow cytometry (E). Bright-field images of cells differentiated into neurons for 12 days (F) and expression of the neuronal marker Tuj1, detected by immunostaining (G–I) and flow cytometry (J).

aggregation tendency, and is also dependent on the microcarrier coating; thus, it is likely that for different cell types and for different microcarriers, different strategies have to be adopted.

Besides these inoculation procedures, the feeding scheme of the culture is a crucial point for the efficiency of the bioprocess. The information obtained from the analysis of the glucose, glutamine, and lactate concentration profiles throughout the culture period indicates that the difference in cell expansion observed with the different feeding regimes was probably not due to glucose or glutamine exhaustion or excessive lactate accumulation. However, some important points emerge from the analysis of the concentration profiles. The first important note is that the initial concentration of glucose ($\approx$33.5 mM or 6.0 g/L) in RHB-A medium is higher than, for instance, in DMEM high glucose (4.5 g/L). As only very low amounts of glucose are consumed, lower levels of this nutrient (such as 4.5 g/L or lower) could be used in order to reduce lactate production. In contrast, the initial value of glutamine concentration ($\approx$0.45 mM) is lower than the 2–4 mM, usually seen in culture media for mammalian cells [35],[47]. As the culture medium is a proprietary formulation and therefore its composition is not known, even in terms of basal components, this observation suggests that instead of containing "free" glutamine, the RHB-A medium may be supplemented with GlutaMAX (which our method is unable to detect), limiting the interpretation of the glutamine profile determined. Concerning lactate, in accordance with previous results [37], the concentration increases with an increasing number of cells. The specific lactate production rate of mES cell-derived NS cells determined here, in dynamic microcarrier culture, is also comparable to the value determined in static two-dimensional (2D) culture ($\approx$3 $\times$ 10^{-6} [37]). The maximum concentration obtained ($\approx$14 mM) is below the values reported to be inhibitory for the proliferation of other stem cell populations (16–35 mM for mesenchymal stem cells [48]).

The differences observed in cell expansion with the different feeding regimes are not explained by these results and may thus be due to another medium component, such as proteins present in these complex medium formulations or other amino acids besides glutamine, whose concentration was not determined and whose role should be crucial under dynamic conditions. Indeed, under static conditions, medium change may be performed every 2 days without any detrimental effect toward cell expansion. This kind of study was, however, out of the scope of this work and may be the subject for future optimization studies.

The effect of agitation rate was also evaluated. Agitation rate has a role in important culture parameters: the shear forces experienced by the cells, the homogeneity of the culture environment, and the oxygen mass transfer from the headspace to the liquid medium. Additionally, in the case of microcarrier culture, stirring influences the homogeneity of microcarrier occupation (mainly in the beginning of the culture) and avoids excessive aggregation of the beads in later culture times. In the present work, an intermediate agitation speed of 60 rpm was found to be optimal. In fact, a slower stirring rate (40 rpm) may be insufficient to keep the microcarriers in suspension; thus, the particles may accumulate, in particular under the stirring shaft, leading to an undesired heterogeneity in terms of access

Table 3

Available area, initial cell density, and initial cells/bead ratio for both microcarrier concentrations tested

	5 g/L	20 g/L
Available area (cm²)	54	216
Initial cell density (cells/cm²)	3.7×10^4	9.3×10^3
Initial cells/bead ratio (cells/bead)	29	7.2

to the culture medium nutrients and oxygen and suboptimal usage of the available culture surface area. A higher stirring speed (60 rpm) may also contribute to a more efficient oxygen mass transfer by surface aeration. This effect, however, would be more pronounced if larger culture volumes were used. Increasing stirring to 80 rpm resulted in a lower expansion ratio, probably because of excessive shear, leading to dislodging of the cells from the microcarriers and eventually cell death. Nevertheless, at 80 rpm, this effect is not dramatic. Thus, as some aggregation of the microcarriers was observed, mainly in the last days of culture, a future improvement in the process may consist of starting the culture with 60 rpm and increasing the speed (at least to 80 rpm) in the late exponential phase.

The final study performed, in terms of culture optimization, consisted of the determination of the effect of different microcarrier concentrations in culture development. This parameter is obviously related to the available surface area for the cells to grow; in the present study, because the initial number of cells was the same, changing microcarrier concentration means also changing the initial cells/area and cells/bead ratio (Table 3). In static 2D culture, for mNS cells, an optimal initial cell density of 1 $\times$ 10^4 was determined [37], which is remarkably close to the calculated cell density for 20 g/L of microcarriers (Table 3), considered here the best condition for microcarrier culture of those cells. The results show also that cell growth in both microcarrier concentrations tested achieves a plateau when cell density is around 3–4 $\times$ 10^5 cells/cm². This may correspond to the density at which the microcarriers are confluent and is in good agreement with the estimated maximum cell density possible to achieve in static 2D cultures for these cells [37], which was approximately 2.6 $\times$ 10^5 cells/cm². Increasing even more the quantity of microcarrier leads to very concentrated slurries, which are difficult to manipulate and which can harm the cells because of bead–bead or bead-to-cell collisions. Furthermore, such a procedure would decrease the initial cell density, which in static 2D culture leads to lower FIs in cell number [37].

With the optimized process, a cell concentration of 2 $\times$ 10^6 cells/mL could be achieved. However, apart from this optimization of the bioprocess, a fundamental step is the validation of the expanded cells. The fact that the cells cultured on the beads, after 9 days, still express the neural stem/progenitor cell markers Nestin and Sox2 as well as the proliferation marker Ki-67 and are able to differentiate into neuronal and glial fates, confirms that the culture on microcarriers, on the Pronectin F substrate, and the dynamic culture conditions (especially the agitation-induced shear) do not affect the multipotency of mouse mNS cells.

The growth rate of the mNS cells, with the optimized conditions, was 0.76 day^{-1} (Table 2), slightly lower than observed in static 2D culture [37]. This is probably caused by the shear environment in the spinner flasks and suggests that maybe alternative bioreactor configurations, without mechanical agitation, can be used to improve this bioprocess.

A final reflection can be made regarding the advantage of this process over conventional tissue culture flask culture, in terms of incubator space saving. To plate 2×10^6 cells, at a density of 1×10^4 cells/cm², an area of 200 cm² is required, which corresponds approximately to 3 T75 flasks, each occupying approximately a volume of 250 mL. Considering a 10-FI in cell number (as described in [37] for cells cultured under 20% O_2) after 3 days, when cells should be ready for passaging, 30 flasks would be required, occupying a volume of 7.5 L. After 3 more days, assuming the same FI, 300 flasks would be required, occupying a volume of 75 L (almost the volume of a CO_2 incubator), leading theoretically to a 1,000-FI in cell number. The bioprocess presented here allows achieving approximately a 35-FI in cell number after 6 days. The incubator space occupied by a spinner flask is about 235 mL. Although lower expansion is obtained in relation to 2D culture, the $\approx 7 \times 10^7$ cells obtained after 6 days in a single spinner flask run could be passaged to a larger-scale bioreactor. Simple calculations indicate that a working volume of approximately 1 L would be enough to inoculate the cells; assuming a similar performance as observed in small scale, a final 1,225-FI in cell number would be attained. We can thus conclude that even though more time may be required to achieve the same number of cells, a microcarrier-based culture could be much simpler to perform (manipulating two bioreactors instead of 333 T-flasks). Regarding the process economy, there is still room for improvement. First, a better understanding of the limiting components of the culture medium would allow us to feed to the culture only the required elements and not the complete medium (with components that are not exhausted, such as glucose) and not deplete the culture of important autocrine factors. Likewise, concerning growth factors, which are expensive (and essential) components of the process, studies could be made to determine the effective concentration of EGF and FGF-2 over time. This would allow the supplementation of the medium only with the minimum amount necessary to maintain the appropriate concentration, minimizing the costs.

Although this work was developed with mouse cells, human populations of NSCs exist with similar characteristics to the mouse ES cell-derived NS cell line used here. For instance, human NS cells derived from the fetal brain, and capable of growing as a monolayer, were described [49] with very similar characteristics to this cell population. A population of human ES cell-derived NSCs was also described [50], which although corresponding to a different developmental stage [1] are also cultured adherently and show long-term self-renewal and differentiation potential into neural cells. The methods developed here may find application in the expansion of these cell populations.

The process described here constitutes an efficient way to obtain large amounts of NS cells for diverse applications such as stem cell research, toxicological studies, or ultimately for cell transplantation. A long road is however still ahead before cell therapies for neurodegenerative diseases can become a reality. For instance, the performance of NS cells expanded as a monolayer on microcarriers in stirred bioreactors upon transplantation in animals has to be studied and compared with what was obtained when using neurosphere-based expansion [6]. Additionally, it is estimated that, to treat a Parkinson's disease patient, about 10^9 human NSCs would be required [17]. Although the study presented here was performed in small-scale spinner flasks, scale-up to higher volume reactors would be essential and, in principle, possible. With the cells used in the present study, a cell density of $\approx 2 \times 10^6$ cells/mL can be obtained, which means that a single bioreactor with a volume of 500 mL could provide a therapeutically relevant number of cells. Moreover, engineering strategies can be used to develop microcarriers made of new materials, capable of creating biomimetic microenvironments to enhance even more cell expansion or direct cell differentiation toward specific phenotypes. These strategies could include presentation of mechanical cues (*e.g.*, optimal material architecture and mechanical properties), incorporation of ligands inspired by the natural extracellular matrix (similar to Pronectin F), or growth factors, and so forth [51],[52].

Now that some clinical trials with NSCs are already being started [53], efficient bioreactor protocols could become a powerful tool for the implementation of successful therapies in the future.

5. Conclusions

The present work describes, for the first time, a bioreactor system for the adherent expansion of mouse ES cell-derived NS cells. The optimized system consisted of using Pronectin F microcarriers in a concentration of 20 g/L, a stirring speed of 60 rpm, and replacing half the volume of culture medium every day. An almost 35-FI in cell number after 6 days was achieved, with no observable loss of multipotency, in terms of expression of neural stem/progenitor cell markers and neuronal and astrocyte differentiation potential. This work is a proof of principle for the feasibility of microcarrier culture of mNS cells in a stirred suspension bioreactor.

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