Scalable culture of human induced pluripotent cells on microcarriers under xeno-free conditions using single-use vertical-wheel™ bioreactors

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Abstract

BACKGROUND: Human induced pluripotent stem cells (hiPSC) are expected to become powerful tools for disease modelling, for the discovery and testing of new drugs and, ultimately, for regenerative therapies. The success of these applications depends on the development of scalable bioprocesses capable of generating large numbers of hiPSC and derivatives.

RESULTS: In this work, the novel vertical-wheel single-use bioreactors were used for the first time for the expansion of hiPSC under xeno-free conditions. Cultures were performed on microcarriers in two different scales of vessels (100 and 500 mL with 80 and 300 mL working volumes, respectively), leading to maximum cell densities up to $1.21 \pm 0.02 \times 10^6$ cells mL $^{-1}$ and volumetric productivities of $2.01 \pm 0.04 \times 10^5$ cells mL $^{-1}$ day $^{-1}$. The pluripotency as well as a normal karyotype were maintained after cell expansion. Consistency of the processes was confirmed with a different hiPSC line, which is an important aspect for a personalized medicine approach.

CONCLUSION: The results here described demonstrate the feasibility of scalable production of hiPSC in a microcarrier-based system using vertical-wheel bioreactors. The protocols developed in this study provide a Good Manufacturing Practices (GMP)-compliant system for hiPSC manufacturing which may be an important step towards the successful implementation of hiPSC-based products.

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Keywords: induced pluripotent stem cells; vertical-wheel bioreactors; microcarriers; xeno-free

INTRODUCTION

Human pluripotent stem cells (hPSC), including induced pluripotent stem cells (hiPSC) and embryonic stem cells (hESC), are defined by the capacity for long-term proliferation (self-renewal) and the potential to differentiate into all cells from the three embryonic germ layers. Cellular reprogramming methods allow the generation of hiPSC from adult somatic cells, a discovery that is changing the fields of regenerative medicine and drug discovery. In fact, clinical translation of hiPSC will enable innovative strategies to repair tissues affected by disease or injury, without the ethical and immune compatibility issues raised by hESC. Moreover, hiPSC can be used to generate personalized disease models in vitro, which constitutes an unparalleled strategy for the discovery of new therapeutic drugs. The success of these hiPSC-based clinical and commercial applications requires the development of scalable cell manufacturing processes able to generate relevant cell numbers while maintaining safety and cell functionality. Stirred bioreactors have been widely used to culture mammalian cells in suspension for industrial production of biopharmaceutical products (e.g. vaccines, recombinant proteins or monoclonal antibodies) and have been seen also as a platform for large-scale culture of stem cells. Cultivation of adherent cells in bioreactors is typically performed using microcarriers (MC), which are small particles ($d = 100–300 \mu m$) that are suspended in the vessel and provide higher culture surface area per reactor volume compared to the planar platform. The stirred environment in the bioreactor is crucial to provide homogeneous concentrations of...
nutrients and growth factors in the vessel but also to maintain the MC in homogeneous suspension. However, the hydrodynamic shear stress generated by the impeller may cause damage to the cells grown on the surface of MC and potential detachment from the MC.8

Culture of hPSC on MC has been successfully performed using a variety of strategies (Fig. 1(A)). Initial reports focused on testing different MC types to demonstrate their application for hPSC culture on static conditions.9 Culture of hPSC in stirred bioreactors has also been described in the literature using MC coated with Matrigel, an undefined commercial mixture of animal-origin extracellular matrix (ECM) proteins.10,11 Despite the significant improvement on these approaches, animal product-free (xeno-free) culture conditions, which are important to avoid immunogenic responses, pathogen contamination, batch-to-batch variability and to comply with current Good Manufacturing Practices (cGMP) regulations, are not widely used yet in this field. MC have been engineered with synthetic vitronectin-derived peptides covalently linked to the surface and were used, in combination with TeSR2 medium, for xeno-free expansion of hiPSC.12 This system enabled cell densities up to 1.6 × 10^6 cells mL^-1, in spinner-flasks. However, although the results are encouraging, the preparation of these MC can be a time-consuming process. Ready-to-use MC coated with Synthemax, a synthetic surface containing also vitronectin-based peptides, were later introduced by Corning and were used, in combination with mTeSR1 culture medium, to expand hiPSC and hESC in spinner-flasks13,14 up to cell densities of 2.1 × 10^6 cells mL^-1.13 Lam et al. reported an alternative culture system, where plastic microcarriers coated with recombinant human laminin-521 were used to culture hESC and hiPSC with mTeSR1 medium, leading to cell densities up to 3.6 and 1.9 × 10^6 cells mL^-1, respectively.15 More recently, a completely xeno-free system for hiPSC expansion was reported, using polystyrene MC coated with recombinant human vitronectin and Essential 8 (E8) as the culture medium. With this system, a 3.5-fold increase in cell number was obtained after culturing hiPSC in spinner flasks for 10 days.16

The novel vertical-wheel bioreactors have an agitation mechanism provided by a large vertical impeller and a U-shaped bottom, promoting mixing and particle suspension with reduced power input and agitation speeds,17 which may be beneficial for hiPSC culture. The vertical-wheel bioreactors can be scaled-up, maintaining consistent mixing efficiency, from lab-scale vessels (0.1–0.5L) to larger production units (up to 80L).17 Moreover, these bioreactors are single-use vessels, allowing reducing operation time between batches as well as the risk of contamination, which can be important if operation under cGMP-compatible mal system; Thermo Fisher Scientific, USA), from now on referred to as ‘TeSR2’, were used in this work. The hiPSC were routinely cultured on 6-well culture plates coated with Matrigel (1:100; Corning, USA), using the E8 culture medium (Thermo Fisher Scientific), in a humidified 5% CO2 incubator, at 37°C. Culture medium was refreshed daily and cells were routinely passaged, when colonies reached 80% confluence, at a split ratio of 1:4, using EDTA.19 Briefly, cells were washed with EDTA (0.5 mmol L^-1 in Dulbecco’s phosphate-buffered saline, DPBS) and incubated for 5 min, again with the EDTA solution. After this incubation, the EDTA was removed and the cells were rinsed and collected by pipetting with culture medium. Cultures for bioreactor inoculation did not exceed four passages in monolayer culture.

**Microcarrier preparation**

SoloHill plastic microcarriers (Pall, USA), with 360 cm^2 g^-1 of superficial area, were used for hiPSC culture in the bioreactors. Microcarriers were sterilized for 1 h with Ethanol 70% at room temperature and washed three times with sterile DPBS. Coating of microcarriers with vitronectin (VTN-N; ThermoFisher Scientific) was performed at 0.5 μg VTN-N per cm² of microcarrier area, in DPBS, for 2 h at room temperature in a rocking platform.16 Prior to cell inoculation, microcarriers were incubated at 37°C, for 30 min, in culture medium.

**hiPSC culture on vertical-wheel bioreactors**

PBS MINI vertical-wheel bioreactors (PBS Biotech, USA), in particular the PBS 0.1 and PBS 0.5, were operated at a working volume in which the impeller wheel is completely covered by medium (80 and 300 mL respectively). A microcarrier concentration of 20 g L^-1 was used (PBS 0.1: 576 cm² per reactor; PBS 0.5: 2160 cm² per reactor). Cells from 80% confluent 6-well plates were harvested with EDTA, as small clumps, counted with a hemocytometer, using the trypan blue dye exclusion test and incubated for 30–60 min with the MC on ultra low attachment culture plates (Corning) at an initial density of 5 or 2.5 × 10^4 cells cm⁻². Cells were then transferred to the bioreactors and culture medium was added until reaching the respective working volume. Medium was supplemented with the ROCK inhibitor Y-27632 (10 μmol L⁻¹; Stem Cell Technologies, Canada) for the first 24 h after inoculation. Feeding was performed on a daily basis by replacing 80% of volume with pre-warmed medium. The culture was continuously stirred at a reduced speed in the first 48 h and the just suspended speed (NJS) afterwards (Table 1). To qualitatively assess cell viability, cells were incubated with Calcein AM (2 μmol L⁻¹; Thermo Fisher Scientific) for 15 min and observed by fluorescence microscopy.

Two samples (700 μL) of the culture were collected daily from the bioreactors. In order to detach the cells, microcarriers were incubated with Accutase (Sigma, USA) at 37°C for 10 min after which the reaction was stopped by dilution with culture medium. Mechanical dissociation was performed by pipetting up and down, to obtain a single-cell suspension and the mixture was then filtered through a 100 μm cell strainer (BD Biosciences, USA) to remove the microcarriers. Cells were centrifuged at 210 g for 5 min and
Figure 1. Experimental design of hiPSC expansion in vertical-wheel bioreactors. (A) Evolution of the culture of hPSC on MC. The culture conditions selected for this study, in terms of culture medium and MC coating are highlighted in black boxes. Abbreviations: KO-SR – Knock-Out™ Serum Replacement. (B) Diagram of the bioprocess here developed for xeno-free human induced pluripotent stem cell expansion in vertical-wheel bioreactors using microcarriers.

Viable cells were counted using a hemocytometer. Cell viability was always above 90%. For cell replating, after the end of the culture in the bioreactors, cells on microcarriers were incubated with Y-27632 for 1 h before harvesting and afterwards the protocol described above was used for cell recovery. The replating density was $5 \times 10^4$ cells cm$^{-2}$.

Cell adhesion to the microcarriers was calculated as the ratio $X_{day1}/X_i$ where $X_{day1}$ is the number of viable cells attached to the microcarriers at day 1 and $X_i$ is the initial number of cells (at day 0). The maximum fold increase in cell number was calculated as the ratio $X_{max}/X_i$, where $X_{max}$ is the maximum cell number achieved during the culture. Results are presented as mean ± SEM.

Glucose and lactate analysis

Glucose, glutamine and lactate concentrations from cell-free supernatants were analyzed using an YSI 2700 Select™ Biochemistry Analyzer (YSI Incorporated Life Sciences, USA). The yield of lactate from glucose, $Y_{Lac/Glc}$, was calculated for each day as $\Delta Lac/\Delta Glc$, where $\Delta Lac$ is the production of lactate during that day and $\Delta Glc$ is the consumption of glucose during the same period.

Flow cytometry

At the end of the culture in vertical-wheel bioreactors, cells were harvested and analyzed for the expression of pluripotency markers by flow cytometry. For surface marker staining, antibodies for SSEA4 (1:10; PE-conjugated, Miltenyi Biotec, Germany) and TRA-1-60 (1:10; PE-conjugated, Miltenyi Biotec) were used. Approximately $2 \times 10^5$ cells were resuspended in 100 $\mu$L of FACS buffer (3% (v/v) fetal bovine serum (FBS, Thermo Fisher Scientific) in DPBS) with the primary antibody, and incubated for 15 min at room temperature in the dark. Cells were washed with DPBS and resuspended in 300 $\mu$L of DPBS prior to flow cytometry analysis (FACSCalibur; Becton Dickinson, USA). For negative...
controls, cells were incubated with the appropriate isotype controls (1:10, Miltenyi Biotec). For intracellular staining, the protocol used is described elsewhere. Antibodies for OCT4 (1:300; Millipore, USA) and SOX2 (1:200; R&D Systems, USA) were used and goat anti-mouse IgG- AlexaFluor 488 (1:500; ThermoFisher Scientific) was used as secondary antibody. For the negative controls, cells were incubated only with secondary antibody in 3% (v/v) normal goat serum (NGS; Sigma) in DPBS. The CellQuest software (Becton Dickinson) was used for all acquisition/analyses. A minimum of 10,000 events were collected for each sample.

**Immunocytochemistry**

Cells, either on tissue culture plates or on microcarriers, were analyzed using a previously described protocol. Cells were analyzed for the expression of the intracellular pluripotency markers using primary antibodies for OCT4 (1:150; Millipore) and SOX2 (1:200; R&D Systems) and using the secondary antibody goat anti-mouse IgG- AlexaFluor 546 (1:500; ThermoFisher Scientific). For the surface markers of pluripotency, antibodies for SSEA4 (1:100; Stemgent, USA) and TRA-1-60 (1:100; Stemgent, USA) were used and with the secondary antibodies goat anti-mouse IgG- AlexaFluor 546 (1:500) and goat anti-mouse IgM- AlexaFluor 546 (1:500), respectively, were used (both ThermoFisher Scientific). For surface antigens, cells were incubated with bovine serum albumin (BSA, 3% (v/v) diluted in DPBS), without Triton-X. Differentiated cells were tested for the expression of alpha smooth muscle actin (α-SMA; 1:1000; Dako, Denmark), neuron-specific class III β-Tubulin (TUJ1; 1:4000; Covance) and SOX17 (1:1000; R&D Systems). Neural progenitor cells were immunostained for NESTIN (1:200; R&D Systems), PAX6 (1:400; Covance), ZO-1 (1:250, Thermo Fisher Scientific) and SOX2 (1:200, R&D Systems). Cells were examined under a fluorescence microscope (Leica DMI3000B/Nikon Digital Camera Dxm1200F) or a confocal microscope (Zeiss LSM 710).

**RT-PCR**

RNA was isolated using PureLink® RNA Mini Kit (Life Technologies). cDNA was synthesized using 1 μg of total RNA and the High Capacity cDNA Reverse Transcription kit (Life Technologies). Reactions were run in triplicate using Applied Biosystems' Taqman™ Gene Expression Assays for NANOG, OCT4, BRACHYURY, SOX17 and SOX1 (Thermo Fisher Scientific) on the StepOne Real-Time PCR System (Thermo Fisher Scientific). The analysis was performed using the ΔΔCt method and values were normalized against the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

### Table 1. Agitation speeds used for the hiPSC culture in the vertical-wheel bioreactors

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**In vitro hiPSC differentiation potential**

hiPSC differentiation potential was evaluated in vitro with the embryoid body (EB) formation assay. Cells from the bioreactors were harvested and replated as single-cells, as described. At 80% confluence, cells were passaged with EDTA to a 6-well ultra-low-attachment plate (Corning) in EB medium (DMEM with 20% (v/v) FBS, 1% (v/v) MEM-non essential amino acids, 1 mmol L⁻¹ sodium pyruvate, 0.1 mmol L⁻¹ β-mercaptoethanol and 1% (v/v) Penicillin/Streptomycin, all from Thermo Fisher Scientific) supplemented with ROCK inhibitor for the first 24 h. Medium was changed every 2 days for the next 4 weeks. EBs were then dissociated with Trypsin 0.025% (Thermo Fisher Scientific) and cells inoculated in a 24-well plate coated with 15 μg mL⁻¹ poly-ornithine (Sigma) and 20 μg mL⁻¹ laminin (Sigma). Medium was changed every 2 days for 1 week. Finally, cells were stained with anti-SOX17, TUJ1 and α-SMA antibodies.

**Neural induction by dual-SMAD inhibition**

Confluent microcarriers harvested from the vertical-wheel bioreactors were placed in 24-well low-attachment plate and cultured with N2B27 medium, a 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM)/F12 and Neurobasal medium supplemented with 1 × N2 and 1 × B27, respectively (all from Thermo Fisher Scientific), supplemented with 10 μmol L⁻¹ of SB431542 (Sigma) and 100 mmol L⁻¹ of LDN193189 (Stemgent). Complete medium was refreshed daily for 12 days. At the end of the differentiation protocol, cells attached to microcarriers were stained for NESTIN and PAX6. For neural induction in 2D, hiPSC cultured in the bioreactors were recovered from the microcarriers (as described) and re-plated on Matrigel-coated 6-well plates, with the medium described above. This medium was replaced daily for 12 days and at day 12 cells were stained for NESTIN and PAX6. Cells were passaged using EDTA and cultured for four additional days after which (day 16) the cells were stained with ZO-1 and SOX2.

**Karyotype analysis**

Confluent microcarriers were harvested from the bioreactor and cells were replated in 2D, with Y-27632 (10 μmol L⁻¹). After 24 h, when cells are actively dividing, cells were arrested in metaphase by incubation with colcemid (Thermo Fisher Scientific) for 4 h at 37 °C. Cytogenetic analysis was performed by Genomed SA (Portugal).

**RESULTS AND DISCUSSION**

**Selection and optimization of hiPSC culture conditions in vertical-wheel bioreactors**

The expansion of hiPSC in bioreactors using microcarriers has been developed and optimized by various research groups worldwide. Different microcarriers and protein coatings have been tested in combination with various culture media formulations, envisaging the establishment of effective and robust methods for hPSC expansion. Although reagents containing animal-origin materials, including mTeSR1 or Matrigel, have been widely used, more recently the field is adopting completely defined and xeno-free solutions. In particular, the culture of hiPSC in spinner-flasks using microcarriers coated with recombinant human vitronectin (rhVTN-N) and E8, a completely xeno-free culture medium which formulation is available, was optimized with a Design of Experiments approach. It is relevant to note that the change from more complex media formulations (e.g. mTeSR1 or StemPro) to E8 formulations approach. It is relevant to note that the change from more complex media formulations (e.g. mTeSR1 or StemPro) to E8...
Expansion of human induced pluripotent stem cells in vertical-wheel bioreactors

Figure 2. hiPSC culture in Vertical-Wheel bioreactors with a seeding density of 5 × 10⁴ cells cm⁻² (50 k) or 2.5 × 10⁴ cells cm⁻² (25 k). (A-D) Representative image of microcarriers harvested from the bioreactor at day 1 and day 6 and analyzed by light microscopy (A, B respectively) or after incubation with calcein AM, by fluorescence microscopy (C, D respectively). (E) Growth curves of hiPSC cultured in the PBS 0.1 bioreactor with the two seeding densities. (F-H) Concentration profiles of glucose (F), glutamine (G) and lactate (H) during the culture in the PBS 0.1. Results are presented as mean ± SEM of three independent bioreactor runs (25 k) or two independent runs (50 k). Scale bars = 100 μm.

may represent a reduction of 30–60% in the cost of the materials for hiPSC expansion. In the present study (Fig. 1(B)), we used these xeno-free culture conditions to demonstrate the use of the novel vertical-wheel bioreactors for hiPSC culture. As an initial approach, a seeding density of 5 × 10⁴ cells cm⁻² of MC area was used (≈40 cells per MC) to inoculate the PBS 0.1, a vertical-wheel bioreactor with a working volume of 80 mL. A reduced agitation speed (10–14 rpm) was used during the first 48 h, to facilitate cell adhesion, while still promoting suspension of the MC. Under these conditions, at day 1, 53 ± 1% of the seeded cells adhered to the microcarriers. After 48 h, the agitation speed was adjusted to the ‘just suspended speed’, Nₚₛ, which is the minimum speed that allows complete suspension of the microcarriers. As observed in previous studies in this field, cells did not grow on the microcarriers as a monolayer but instead formed multi-layer cell-microcarrier aggregates (Fig. 2 (A-B)) with a visible increase in viable cell number throughout culture (Fig. 2 (C-D)). Fig. 2(E) shows the growth kinetics of the bioreactor culture. Cells enter the exponential phase of growth after day 1 and at day 6 growth stops and the culture reaches a plateau. A maximum of 1.0 ± 0.1 × 10⁸ cells were obtained at day 6, which corresponds to a 3.5 ± 0.3-fold increase in cell number. A lower seeding density of 2.5 × 10⁴ cells cm⁻² was also tested (≈20 cells per MC), envisaging an improvement in the overall fold expansion in the reactor (Fig. 2(E)). With this condition, 59 ± 7% of the seeded cells adhered to the microcarriers and an identical maximum cell number (average of 9.7 ± 0.2 × 10⁷ cells, with individual runs reaching 1.0 × 10⁸ cells) was obtained at day 6, resulting in a 6.7 ± 0.1-fold increase in cell number, almost twice that obtained in the initial condition tested. The maximum specific growth rate obtained was 0.63 ± 0.05 day⁻¹, which corresponds to a doubling time of 1.1 ± 0.1 days. The concentration of glucose, glutamine and lactate in the cell culture supernatant was measured, in order to analyze the availability of nutrients/accumulation of waste products throughout the culture time (Fig. 2(F-H)). We did not observe exhaustion of nutrients at any point in the culture, as glucose and glutamine never dropped below 40% of the respective initial value and the accumulation of lactate was never above 18 mmol L⁻¹, which according to the literature, is under the inhibitory concentration for hiPSC growth (> 20 mmol L⁻¹).

The vertical-wheel bioreactors have an innovative mixing mechanism, which combines characteristics of radial and axial flow impellers, the radial component being in a vertical plane and the axial component in the horizontal plane. This mixing mechanism is generated by a large vertical wheel, located inside the vessel, which rotates around a horizontal axis. These characteristics provide mixing and suspension of particles inside the bioreactors with low agitation speeds and were here tested for hiPSC xeno-free culture on MC. The culture conditions used in the present study (culture medium, MC type and coating) are the same as described in a previous publication from our group reporting hiPSC expansion...
Process robustness and scalability

To evaluate the robustness of the bioprocess, a second hiPSC line (Gibco) was cultured in the PBS 0.1 bioreactor under the same experimental conditions. As shown in Fig. 3(A), the exponential phase of growth starts at day 1 and a maximum cell number of $8.8 \pm 2.1 \times 10^7$ cells mL$^{-1}$ was obtained after 5 days, which corresponds to a fold increase of $6.1 \pm 0.2$. As shown in Table 2, the results obtained with both cell lines are comparable, which indicates that, despite the differences observed due to the inherent variability of the cell lines, the process is robust. The possibility to use this culture system with different hiPSC lines and to have a predictable behavior in terms of cell expansion is particularly important for the generation of cells for personalized drug screenings or autologous cell therapies, for instance in a precision medicine context.

The scalability of the process is also a critical aspect, foreseeing the necessity to generate hiPSC at a relatively large scale for many hiPSC applications. To evaluate the scalability of our process we tested a vertical-wheel bioreactor with a higher working volume, the PBS 0.5. This bioreactor was operated at a working volume of 300 mL with seeding density of $2.5 \times 10^4$ cells cm$^{-2}$, condition that led to the highest fold expansion in the PBS 0.1. The ‘just suspended speed’ (in rpm) was lower (Table 1) than established for the PBS 0.1, since the diameter of the impeller in the PBS 0.5 is higher. In fact, the diameter of the PBS 0.5 impeller is 6.7 cm, which at a speed of 20 rpm corresponds to an impeller linear tip speed of $421$ cm min$^{-1}$ (vs $351$ cm min$^{-1}$, at 26 rpm, in the PBS 0.1, $D = 4.3$ cm). Figure 3(B-C) shows the growth curve under these conditions, where a maximum of $2.6 \pm 0.5 \times 10^8$ cells (4.8 $\pm$ 0.2-fold increase in cell number) was obtained after 6 days of culture. The specific growth rate observed was $0.65 \pm 0.06$ day$^{-1}$, which is comparable with the value obtained with the PBS 0.1. The concentration profiles of glucose, lactate and glutamine (Fig. 3(D-F)) are also similar and without limitations of nutrients or inhibitory concentrations of lactate, indicating a comparable performance of the culture at this higher scale. However, the expansion factor and, consequently, the maximum cell density ($8.55 \pm 1.51 \times 10^7$ cells mL$^{-1}$) were lower, which suggests that

Figure 3. Process robustness and scalability. (A) Growth curve of the Gibco hiPSC line (vs. TC Lab) in PBS 0.1 vertical-wheel bioreactors. (B-C) Growth curves of the TC Lab hiPSC line in the PBS 0.5 and PBS 0.1 bioreactors in total cell numbers (B) and cell density (C). (D-F) Concentration profiles of glucose (D), lactate (E) and glutamine (F) in the PBS 0.5 and PBS 0.1 bioreactors. All results are presented as mean ± SEM of two independent bioreactor runs.
the operational conditions may be further optimized. Nevertheless, this culture volume is within the highest reported yet for xeno-free expansion of hiPSC on microcarriers, as well as the absolute cell number obtained (>2 x 10^9). The predicted cell doses for regenerative medicine applications are between 5 x 10^8 (macular degeneration) and 10^9 – 10^10 (myocardial or liver regeneration) hPSC-derived cells/patient, which is still 1 – 2 orders of magnitude above the values reported here. The vertical-wheel system may thus be a promising platform for the production of hPSC-derived cells as it is available in higher scales, up to 80 L controlled bioreactors.

Characterization of cells expanded in vertical-wheel bioreactor

To demonstrate that the culture in vertical-wheel vessels did not affect cell quality, we tested if the expression of pluripotency markers, energy metabolism, normal karyotype and differentiation potential (pluripotency) of the hiPSC was maintained after culture in the PBS 0.5 vertical-wheel bioreactor. The expression of pluripotency markers such as OCT4, SOX2, TRA-1-60 and SSEA4 on cells cultured on the microcarriers was assessed by immunocytochemistry. As shown in Fig. 4(A), expression of the pluripotency markers was detected with the expected nuclear (OCT4 and SOX2) or cell surface (TRA-1-60 and SSEA4) localization. Cells were also recovered from the microcarriers and replated in 2D Matrigel-coated culture plates. The typical colony morphology was observed and the cells expressed the same pluripotency markers (Fig. 4(B)). Flow cytometry analysis of the cells harvested from the bioreactors at the end of culture was performed (Fig. 4(C)) and the results showed that >90% of the cells maintained the expression of pluripotency markers, which therefore was not affected by the culture in the vertical-wheel bioreactor. Table 3 shows the flow cytometry results for the other conditions tested in this study, which also confirm that this bioreactor configuration did not have an impact on pluripotency marker expression. The expression of OCT4 and NANOG, as well as early markers of differentiation into ectoderm (SOX1), mesoderm (BRACHYURY) and endoderm (SOX17), was investigated by quantitative RT-PCR. As shown in Fig. 4(D), after 8 days in the vertical-wheel bioreactor there was no downregulation of pluripotency genes or upregulation of differentiation genes in relation to the cells cultured in 2D and used as inoculum. The consumption of glucose and production of lactate allows calculation of the yield of lactate from glucose (Y_{Lac/Glc}), which gives an indication of the metabolic activity of the cells. Notably, for all the conditions tested (Fig. 4(E)) the Y_{Lac/Glc} was around 2, a value that is typically observed when cells rely on glycolysis for energy metabolism. hPSC are reported to use glycolysis even in the presence of oxygen, a process known as the ‘Warburg effect’, which is also supported by the present data. A recent study describes a shift from glycolysis to oxidative phosphorylation in later stages of hiPSC culture, in microcarrier-free cultures, which is not evident in our cultures. However, the different culture medium used in that study (mTeSR1) and the presence of microcarriers in our system may lead to differences in cell metabolism. The maintenance of genomic integrity under dynamic culture conditions is critical to the success of a bioprocess for stem cell production. The karyotype of the expanded cells was analyzed and, as shown in Fig. 4(F), no abnormalities were detected.

The differentiation potential of the expanded cells was evaluated by the EB formation assay (Fig. 5(A)). After 5 weeks of differentiation, cells from mesoderm, ectoderm and endoderm germ layers, were detected, demonstrating that pluripotency was maintained. Finally, directed differentiation into neural precursors was performed using the dual-SMAD inhibition method. Neural induction was performed (Fig. 5(B–E)) either on cells replated in 2D (Fig. 5(B, E)) and directly using confluent microcarriers harvested from the bioreactor and transferred to culture plates and cultured in static conditions (Fig. 5(C, D)). The results show that in both cases NESTIN^+ PAX6^+ progenitors were obtained as well as neural rosettes with polarized expression of ZO-1 (Fig. 5(E)). Altogether, these data demonstrate that expansion of hiPSC on microcarriers, using vertical-wheel bioreactors, under xeno-free conditions does not affect cell quality.

Xeno-free expansion of hiPSC in vertical-wheel bioreactors: a viable alternative

Table 4 summarizes the culture conditions and results obtained in recent studies of MC-based hPSC expansion, in terms of maximum cell densities, time in culture and volumetric productivity. Although higher productivities are reported in the literature, our xeno-free system only requires a microcarrier coating with recombinant vitronectin by passive adsorption, without the need for more complex chemical conjugation steps and does not require the use of more expensive coating proteins (e.g. laminin-521). Furthermore, our results suggest that the performance obtained can be repeated in higher-scale vertical-wheel systems. Our process constitutes thus an easy to implement and economically feasible alternative for GMP-compatible production of hiPSC.

It is important to note that MC-free systems for hiPSC expansion have also been reported, in which cells are cultured as floating aggregates. This approach facilitates the downstream processing of the culture, avoiding a filtration step to remove the MC. Nevertheless, we believe that both culture methods can be interesting, depending on the context. In the case of MC culture, during the first 24 h the MC provide a vitronectin-coated surface where cells adhere and spread. Afterwards, we observed the formation of cell/MC aggregates (Fig. 2A,B) in which the MC work primarily not as a surface for cell attachment but as an ‘aggregation nucleus’. At this stage of the culture, the presence of the MC within the cell
Figure 4. Characterization of hiPSC cultured in the Vertical-Wheel bioreactor. (A) Immunocytochemistry analysis of hiPSC attached to MC for pluripotency markers (OCT4, SOX2, SSEA4 and TRA-1-60) after 8 days of culture, by confocal microscopy. Scale bar = 100 μm, except for TRA-1-60 (50 μm). (B) Cells replated in 2D culture plates after recovery from the MC. Immunocytochemistry for pluripotency markers is shown. Scale bar = 100 μm. (C) Flow cytometry analysis for pluripotency markers of cells harvested from the bioreactor. Representative histograms are shown, with the negative control outlined in green and the stained population in purple. The percentage (average ± SEM) of positive cells is indicated (n = 2). (D) Quantitative RT-PCR analysis of the cells harvested from the bioreactor. Fold change is relative to cells cultured in 2D culture plates (n = 2). (E) Yield of lactate from glucose, obtained in the different conditions studied (n = 2 for all conditions, except for PBS 0.1 25 k; n = 3). (F) Karyotype of the cells cultured in Vertical-Wheel bioreactor for 8 days.

Table 3. Flow cytometry results (% of positive cells) in the different conditions tested

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<tbody>
<tr>
<td>PBS 0.1  (5 x 10⁴ cells cm⁻²)</td>
<td>88 ± 3</td>
<td>92 ± 1</td>
<td>91 ± 4</td>
<td>98.3 ± 0.1</td>
</tr>
<tr>
<td>PBS 0.1  (2.5 x 10⁴ cells cm⁻²)</td>
<td>97 ± 1</td>
<td>98 ± 1</td>
<td>81 ± 6</td>
<td>93 ± 5</td>
</tr>
<tr>
<td>PBS 0.1  (Gibco cell line)</td>
<td>95 ± 2</td>
<td>97.4 ± 0.2</td>
<td>91 ± 2</td>
<td>98.1 ± 0.4</td>
</tr>
<tr>
<td>PBS 0.5</td>
<td>95 ± 4</td>
<td>95 ± 3</td>
<td>91 ± 6</td>
<td>97 ± 2</td>
</tr>
</tbody>
</table>

aggregates limits the thickness of the layer of cells, which may facilitate the exposure of the cells to the culture medium avoiding the formation of a necrotic/differentiated aggregate core. Moreover, the use of engineered microcarriers with specific mechanical properties or biochemical functionalization may constitute an interesting strategy to improve the expansion factor or, considering the possible integration of a directed differentiation step after expansion, the differentiation efficiency.

It has also been reported by Kropp et al. that perfusion of culture medium, in aggregate-based culture using instrumented stirred bioreactors, enabled cell densities up to 2.85 x 10⁶ cells mL⁻¹. In the present study, perfusion was not performed since the implementation of this culture mode in the miniaturized bioreactors used is not straightforward. However, it is predicted that perfusion of culture medium could be implemented in scaled-up versions of the vertical-wheel bioreactors, improving significantly the culture outcome by reducing the oscillations in the culture microenvironment (e.g. Fig. 2 (F-H)). Moreover, although lactate concentrations did not reach the reported inhibitory levels (>20 mmol L⁻¹), we observed pH values as low as 6.5 in the culture medium supernatant (result not shown), which are likely to negatively affect the culture and could be avoided with continuous renewal of the medium.

CONCLUSION

One of the challenges for the widespread use of hiPSC either in the development/testing of novel drugs or in cell-replacement therapies is the generation of functional cells in high quantities in a short time. The use of bioreactors has been seen as the most
rational solution for the design of standardized and reproducible bioprocesses for cell production. In fact, the possibility to culture cells in a homogeneous environment, which can be monitored and controlled, using a single vessel with an appropriate scale, provides a safer and viable alternative to the conventional planar platforms (e.g. T-flasks). The work here presented describes a GMP-compliant culture system, which uses the novel vertical-wheel bioreactors together with xeno-free reagents, leading to an almost 7-fold expansion of hiPSC in 6 days, maintaining the pluripotency of the cells, their multilineage differentiation potential and a normal karyotype. The upscaling of the process will depend on the desired application. For instance, an autologous therapy or a personalized drug screening will typically require a scale-out strategy (in a lower volume) while an allogeneic product will require scale-up

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**Table 4.** Microcarrier-based culture systems for the expansion of hiPSC in defined conditions

<table>
<thead>
<tr>
<th>Reference</th>
<th>Cell type</th>
<th>Xeno-free conditions</th>
<th>Bioreactor type / volume</th>
<th>Maximum cell density (cell mL⁻¹)</th>
<th>Days in culture</th>
<th>Volumetric productivity (cells mL⁻¹ day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS 0.1 (this study)</td>
<td>hiPSC</td>
<td>Yes</td>
<td>vertical-wheel (80 mL)</td>
<td>1.21 ± 0.02 × 10⁶</td>
<td>6</td>
<td>2.01 ± 0.04 × 10⁵</td>
</tr>
<tr>
<td>PBS 0.5 (this study)</td>
<td>hiPSC</td>
<td>Yes</td>
<td>vertical-wheel (300 mL)</td>
<td>8.55 ± 1.51 × 10⁵</td>
<td>6</td>
<td>1.43 ± 0.25 × 10⁵</td>
</tr>
<tr>
<td>Badenes et al., 2016</td>
<td>hiPSC</td>
<td>Yes</td>
<td>Spinner Flask (50 mL)</td>
<td>1.6 × 10⁶</td>
<td>10</td>
<td>1.6 × 10⁵</td>
</tr>
<tr>
<td>Fan et al., 2012</td>
<td>hiPSC</td>
<td>Yes</td>
<td>Spinner Flask (50 mL)</td>
<td>1.6 × 10⁶</td>
<td>6</td>
<td>2.6 × 10⁵</td>
</tr>
<tr>
<td>Badenes et al., 2014</td>
<td>hiPSC</td>
<td>No</td>
<td>Spinner Flask (15 mL)</td>
<td>7.3 × 10⁵</td>
<td>6</td>
<td>1.2 × 10⁵</td>
</tr>
<tr>
<td>Lam et al., 2015</td>
<td>hiPSC</td>
<td>No</td>
<td>Spinner Flask (50 mL)</td>
<td>1.9 × 10⁶</td>
<td>7</td>
<td>2.7 × 10⁵</td>
</tr>
<tr>
<td>Silva et al., 2013</td>
<td>hESC</td>
<td>No</td>
<td>Spinner Flask (60 mL)</td>
<td>2.1 × 10⁶</td>
<td>10</td>
<td>2.1 × 10⁵</td>
</tr>
</tbody>
</table>
to larger volume reactors. The results here obtained suggest the applicability of this system in both settings, which makes it a versatile candidate for future hiPSC-based bioprocesses.

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REFERENCES