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Scalable Expansion of Human induced Pluripotent Stem Cells (hiPSC) under Xeno-free Conditions

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Abstract Human induced pluripotent stem cells (hiPSCs) represent a very promising tool in the biomedical research field by being capable of making multiple copies of themselves for long periods in culture and generating cells from the three embryonic germ layers upon differentiation. Thus and to fully realize their potential for disease modelling, drug screening and cell therapy, it is mandatory the conception of chemically defined culture systems for large-scale production of hiPSCs, under good manufacturing practices (GMP), free of xenogeneic components and without batch-to-batch variability. In this work, an optimized xeno- and serum-free culture system is proposed for the maintenance of hiPSCs using a microcarrier-based system in spinner-flasks. A multifactorial design was used to identify the operation conditions that maximize the expansion index of hiPSCs in culture. Cells were seeded at 55000 cells/cm^2 in vitronectin-coated beads, after an EDTA treatment with ROCK inhibitor, and cultured with xeno-free E8 medium. An agitation speed of 44 rpm was used for a proper homogenization of the culture. Under this optimized protocol, an expansion index of 4.4 ± 0.7 was achieved after 10-13 days of culture. Cells were further characterized in terms of pluripotency by intracellular immunostaining for markers Oct4 and Nanog, and flow cytometry for Oct4, Nanog and Sox2 markers. 92 ± 2% of the cells were found to be positive for Oct4, 97 ± 1% for Sox2 and 98 ± 1% for Nanog. The proposed culture system thus provides an efficient and cost effective platform for the expansion of hiPSCs for numerous applications, from basic biological research to clinical development.

Keywords: human induced pluripotent stem cells (hiPSCs), vitronectin, E8 medium, microcarriers, spinner-flask.

Introduction

Human stem cells are unspecialized cells with a remarkable ability for self-renewal and differentiation into functional and specific tissue cells under defined and controlled conditions. Owing to their properties, they have been identify as a potential source and starting point for the development of cell-based therapies for regenerative medicine and gene therapy, as well as the main instrument for drug screening and disease modelling (1) (2). According to their differentiation potential, stem cells can be classified into totipotent, pluripotent or multipotent stem cells (3).

A single human pluripotent stem cell (hPSC) is capable of giving rise to all cells derived from the three embryonic germ layers, known as mesoderm, endoderm and ectoderm, hence all cells of the human body. Human embryonic stem cells (hESCs) are derived from the inner cell mass of the blastocyst, an early stage of the human embryo, and they were first isolated in 1998 by James Thompson and his co-workers at University of Wisconsin (4). Despite their enormous potential due to their pluripotency, long-term self-renewal capacity and relatively easy isolation and maintenance in culture, there are some ethical concerns regarding the sacrifice of human embryos for hESCs generation that may limit and compromise their use in research studies and cell-based therapies (5).

In 2007, a significant breakthrough in human stem cell research field was established when Takahashi and Yamanaka surprised the scientific community by showing that enforced expression of four key transcription factors, Oct4, Sox2, Klf4 and c-Myc, in human somatic cells was enough to reprogram them to a pluripotency-like state (6). These so called human induced pluripotent stem cells (hiPSCs) were similar to human embryonic stem cells in terms of morphology, proliferation, feeder dependence, surface antigens, gene expression profile, telomerase activity, and were also able to differentiate *in vitro* into all cell types of the three embryonic germ layers. Therefore, hiPSCs can potentially overcome the ethical concerns associated with hESCs, since their generation does not involve the manipulation and destruction of human embryos, and potentially circumvent immune rejection in patients when an autologous transplantation of hiPSC-derived cells is performed (7).

In the last few years, significant progress has been made towards the development of scalable and robust culture systems for hPSCs expansion that ensure the maintenance of their pluripotency and self-renewal properties. In particular, and to fully realize their potential for disease modelling, drug screening and clinical application, it is mandatory the conception of chemically defined culture systems for the production of increased cell quantities, under good manufacturing practices (GMP), free of contaminants and without batch-to-batch variability. The early culture conditions for hPSCs were established by effectively following the developed methods for mouse embryonic stem cells, particularly using feeder-cell layers. Over time, these cultures were replaced by feeder-free systems, using for example biological substrates, coupled with serum-based or serum-free media (8) (9) (10).

This work aims to establish an optimized and scalable culture system for the expansion of hiPSCs, under xeno-free and chemically defined conditions, using human vitronectin-coated microcarriers in spinner-flasks supplied with serum- and xeno-free Essential 8™ medium. For this purpose, the influence of three culture parameters will be evaluated, namely cell dissociation method, initial hiPSCs density and agitation speed. Generated cells will be further characterized concerning their pluripotency through intracellular immunostaining and flow cytometry.

Material and Methods

Cell Line

The hiPSC line used in all studies, Gibco® Episomal hiPSC line, was gently provided by Life Technologies for the purpose of this study. The Gibco® Episomal hiPSC line is a zero-footprint, viral-integration-free human iPSC line, adapted to feeder-free and serum-free culture conditions (11).

Culture on Feeder-free Layers

Human induced pluripotent stem cells were thawed and allowed to expand in daily supplemented Essential 8™ medium (E8, 0.5% Penicillin-Streptomycin (P/S)) on culture plates pre-coated with Matrigel™ (BD Biosciences) or human vitronectin (VTN-N, Life Technologies). Cells were passaged as small clumps with sterile EDTA 0.5 mM when ≈ 85% confluency was reached, as described elsewhere (12). Plates were placed in a humidified incubator at 37ºC, 5% CO_2.

Microcarrier-based Culture System

<u>Beads Preparation</u>

Prior to coating, xeno-free plastic microcarriers (SoloHill®) were sterilized with ethanol 70% during 1h, 10 rpm, at room temperature. After washing with sterile PBS, beads were left to incubate with coating solution of VTN-N (diluted in PBS at a proportion of 1:100 (v/v), 1% P/S) or Geltrex® (GT, Life Technologies) (diluted in DMEM-F12 at a proportion of 1:60 (v/v), 1% P/S), during 2h, 20 rpm, at room temperature. After removing the coating solution, beads were incubated at 37ºC with E8 medium (0.5% P/S) for at least 30 min. Before culture, medium was replaced with a defined volume of E8 medium (0.5% P/S) with or without ROCK inhibitor (Y-27632; StemGent®, 10 µM).

<u>EDTA Inoculation of hiPSCs</u>

Cells were harvested from 6-well plates according to the aforementioned protocol for human iPSCs passaging with EDTA. A defined volume of E8 medium (0.5% P/S) with or without ROCK inhibitor (10 µM) was used to flush cells from wells.

- *Static Conditions*: cells were inoculated in an ultra-low attachment 24-well plate (beads surface area: 3 cm^2/well; beads density: 360 cm^2/g). Cell suspensions with concentrations of 30000, 50000 and 70000 cells/cm^2 were prepared in E8 medium (0.5% P/S, 10 µM of ROCK inhibitor) and added to the beads as 250 µL/well. Pre-coated beads were already suspended in E8 medium (0.5% P/S, 10 µM of ROCK inhibitor) and placed as 250 µL/well. A total culture volume of 500 µL/well was used for inoculation. The plate was carefully placed in humidified incubator at 37ºC, 5% CO_2. E8 medium without ROCK inhibitor (ROCKi) was daily replaced (500 µL/well).

- *Dynamic conditions*: for inoculation in a StemSpan™ Spinner Flask (StemCell™ Technologies) a cell suspension of 55000 cells/cm^2 was prepared in E8 medium (0.5% P/S, 10 µM of ROCK inhibitor) and added to VTN-N pre-coated beads (beads concentration: 20 g/L) suspended in E8 medium (0.5% P/S, 10 µM of ROCK inhibitor). A total volume of 25 mL was used for cell inoculation, at Day 0, and cells were incubated with ROCK inhibitor for 24 h in a humidified incubator at 37ºC, 5% CO_2, without mechanical agitation. Intermittent agitation (stirring at 40-50 rpm during 3 min, every 2h) was used to promote cell adaptation to shear stress at Day 1. From Day 2, inclusive, continuous agitation (44 rpm) was applied for mixing. Daily medium change was performed by replacing 80% of the

medium with fresh E8 medium without ROCK inhibitor (working volume of 50 mL).

Single-cell Inoculation

Prior to enzymatic dissociation, cells were incubated with 10 µM ROCK inhibitor for 1 h at 37ºC. After washing with sterile PBS, cells were left to incubate with Accutase (Sigma®) for 5-10 min at 37ºC, until colonies break into single cells. Cells were then flushed with washing medium (87,4 % KO-DMEM, 10 % KO-SR, 1 % MEM-non essential aa, 1 mM L-Glutamine, 0.1 mM β–Mercaptoethanol and 1 % P/S) and centrifuged at 1000 rpm for 5 min. For inoculation in 24-well plate, a cell suspension of 50000 cells/cm^2 was prepared with E8 medium (0.5% P/S, 10 µM of ROCK inhibitor) and added to beads as 250 µL/well. Pre-coated beads were already suspended in E8 medium (0.5% P/S, 10 µM of ROCK inhibitor) and placed as 250 µL/well. A total culture volume of 500 µL/well was used for inoculation. The plate was carefully placed in humidified incubator at 37ºC, 5% CO_2. E8 medium without ROCK inhibitor was daily replaced (500 µL/well).

Characterization of hiPSCs

Immunofluorescence

- *Surface Marker Staining*: the analysed surface markers were Tra-1-60, Tra-1-81 and SSEA-4. Medium from wells was removed, replaced by washing medium containing the primary antibody and incubated for 30 min at 37ºC. Cells were then washed three times with 1 mL of washing media to remove any excess of primary antibody. Medium containing the secondary antibody was placed and left to incubate during 30 min, at 37ºC. Finally, cells were once more washed three times, with washing media, and examined using a fluorescence microscope.

- *Intracellular Staining*: Medium from wells was replaced with 4% paraformaldehyde (PFA; Gibco®) for cell fixation, for 10 min at room temperature. Cells were then washed twice with PBS (Phosphate Buffered Saline) and incubated for 30-60 min, at room temperature, with Blocking Solution (10% NGS, 0.1% Triton in PBS), in order to reduce background staining. After blocking solution removal, cells were incubated with the primary antibody in staining solution (5% NGS, 0.1% Triton in PBS), overnight at 4ºC. After three washes with PBS, cells were incubated with secondary antibody diluted in staining solution for 1 h, in the dark, at room temperature. Later on, cells were washed once with PBS to remove the excess of secondary antibody and incubated with diluted 4',6-diamidino-2-phenylindole (DAPI, 1:10000 in PBS), during 2 min at room temperature, for nuclear-staining. Finally, the cells were washed twice with PBS to remove any remaining DAPI crystals and left with PBS for further examination under a fluorescence microscope.

For the analysis of cells anchored on beads (Oct4 and Nanog), the immunostaining procedure was slightly different. A sample from the spinner-flask was transferred to a 15-mL tube and culture medium was replaced with 4% PFA. After cell fixation, PFA was removed and 1 mL of blocking solution was added. The total volume was equally distributed in two wells, of a 12-well plate, and cells were incubated for 1h at room temperature. Later, blocking solution was replaced with 400 µL/well of a primary antibody solution and left overnight at 4ºC. After incubation with primary antibody, cells were washed three times with PBS (1 mL/ well) and incubated with secondary antibody solution) for 1h, in the dark. Cells were then washed three times with PBS and 500 µL/well of DAPI solution was added. Finally, two washes with PBS were performed and cells were left with PBS for further examination under a fluorescence microscope.

Flow Cytometry

For intracellular staining, cells were fixed with 2% PFA, centrifuged at 1250 rpm, for 5 min, and washed twice with 1% NGS, centrifuging each time at 1250 rpm, for 5 min. Cells were then resuspended in 3% of NGS and equally distributed in Eppendorf tubes, previously coated with 1% Bovine Serum Albumin (BSA, Invitrogen™). After centrifuging at 1000 g, for 3 min, the supernatant was discarded and the pellet resuspended in a 1:1 solution of 3% NGS and 1% Saponin (Sigma®). Cells were left to incubate for 15 min, at room temperature, for membrane permeabilization. Following another centrifugation, cells were washed with 3% NGS, incubated at room temperature, for 15 min, and centrifuged once again. The obtained pellet was resuspended in primary antibody solution in 3% NGS and incubated in the dark, for 1h30 min, at room temperature. After another centrifugation step, cells were washed twice with 1% NGS, to remove any remaining primary antibody solution, centrifuging each time at 1000 g for 3 min. The pellet was incubated for 45 min, in the dark, with the secondary antibody diluted in 3% NGS. At last, and after washing twice with 1% NGS, cells were resuspended in PBS and transferred to FACS tubes for further analysis in the flow cytometer (Becton Dickinson®).

Results and Discussion

Maintenance of hiPSCs on 2D Surface

In order to assess the efficiency of vitronectin (VTN-N) as a xeno-free and chemically defined substrate for the feeder-free expansion of human pluripotent stem cells, hiPSCs were initially cultured and expanded in E8 medium as a monolayer in tissue culture plates coated with VTN-N. After expansion under these conditions for 3 passages, hiPSCs presented a typical morphology characterized by flat and densely packed colonies with defined borders and round shape (13).

Cells were routinely passaged as small clumps using EDTA when approx. 85% confluency was reached, typically every 3-4 days. At each cell passage, cell counting was performed by trypan blue staining and the expansion index determined according to the average fold increase per passage (Fi), given by the Equation 1. Cells cultured on VTN-N-coated plates in E8 medium present an expansion index of 4.3 ± 0.4.

$$Fi = \frac{N_i}{N_0} = \frac{Total\ number\ of\ generated\ cells}{Number\ of\ plated\ cells} \qquad \textbf{Equation 1}$$

Cultured cells were characterized in terms of pluripotency by intracellular and surface antigens immunostaining for the expression of the pluripotency markers Oct4, Sox2 and Nanog, and Tra-1-60, Tra-1-81 and SSEA4, respectively. The results for the intracellular staining were merged with DAPI nuclei counterstaining (Figure 1).

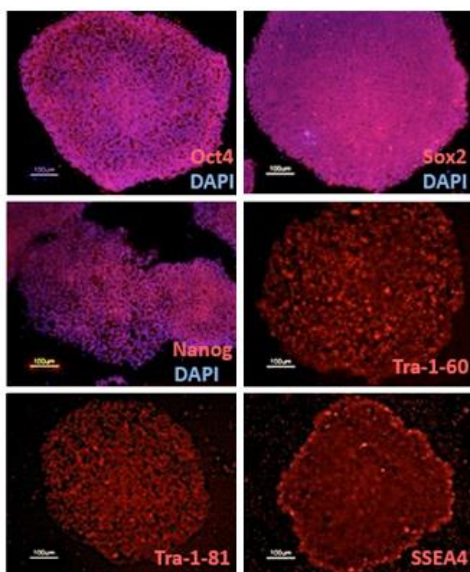

Figure 1 Immunostaining of hiPSCs colonies cultured on VTN-N in E8 medium. Colonies were stained for intracellular pluripotency markers Oct4, Sox2 and Nanog and nuclei counterstained with DAPI; and for surface pluripotency markers Tra-1-60, Tra-1-81 and SSEA4 (scale bars – 100 µm).

Flow cytometry was additionally performed to quantify the Oct4, Sox2 and Nanog intracellular markers. Results indicate that 95 ± 1% of the analysed cells were positive for Oct4, 92 ± 1% for Sox2 and 89 ± 3% for Nanog. These results confirm the efficiency of VTN-N and E8 medium combination as a robust xeno- and serum-free culture system for the maintenance of human induced pluripotent stem cells.

Expansion of hiPSCs in Microcarrier-based Culture

As previously mentioned, this work aims to develop a scalable system for the expansion of hiPSCs under xeno-free and chemically defined conditions using a microcarrier-based culture in spinner-flasks. Therefore, preliminary studies were initially performed under static conditions to evaluate once more the efficiency of human vitronectin and to identify the optimal conditions for cell seeding on VTN-N coated microcarriers, namely dissociation method and initial cell density.

Static Conditions

To evaluate the optimal dissociation method for inoculation on xeno-free compact microcarriers, 50000 cells/cm^2 were seeded separately to vitronectin (VTN-N) and Geltrex® (GT) pre-coated beads, in an ultra-low attachment 24-well plate. Cells were inoculated as small clumps using EDTA treatment, with E8 medium, with or without ROCK inhibitor (ROCKi), and as single-cells (SC) using enzymatic dissociation with Accutase and ROCKi. Geltrex® was deliberately used as a control in order to compare and assess the efficiency of VTN-N in promoting and maintaining hiPSCs expansion for being a multi-component, undefined and xenogeneic substrate, in opposition to vitronectin.

At day 5, cells were harvested and counted and the expansion index for each condition was determined. According to the results (Figure 2), when cells were inoculated in E8 medium with EDTA, EDTA with ROCKi and as single-cells with ROCKi present an expansion index of 0.76, 4.43 and 3.50, on VTN-N coated beads, and 0.97, 4.30 and 4.80, on GT coated beads, respectively.

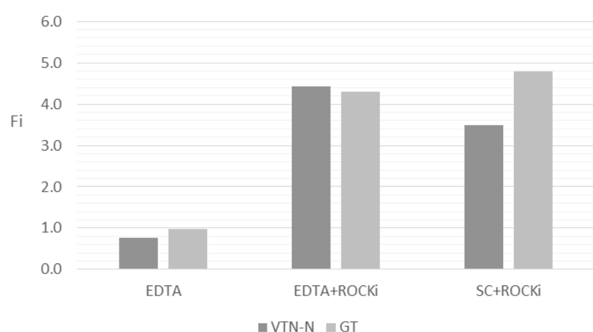

Figure 2 Expansion index of human iPSCs culture in E8 medium on VTN-N and GT coated beads for each inoculation condition tested: seeding as small cell clumps with and without ROCKi and as single-cell with ROCKi (n=1).

As the results suggest, EDTA treatment with ROCK inhibitor is the most promising and efficient inoculation protocol as it presents the higher average expansion index for cell seeding on VTN-N coated beads. To be noted that the use of ROCK inhibitor has been reported as not required when cultivating hiPSCs as a monolayer culture after an EDTA treatment (12). Nevertheless, the achieved results demonstrate that its use is crucial and mandatory for cell inoculation with EDTA on microcarriers, independently of beads coating, at least with the particular hiPSC line used in this work.

This might be related to the difficulty of cells to establish an immediate interaction with the substrate, and thus re-establishing cell-ECM interactions which are crucial for cell survival, when inoculated on the surface of 3D structures, as the case of compact beads. The use of ROCK inhibitor increases the survival time of cells and, consequently, the adherence efficiency to beads. In fact, cell-ECM interaction is more easily restored in a 2D monolayer culture since gravity promotes sedimentation of cells on the top of coated culture plates.

By analysing Figure 2, it is still possible to note that results are relatively similar for VTN-N and GT, being the only observed variation related to inoculation as single-cells with ROCK inhibitor. Nevertheless, this observation does not invalidate EDTA treatment with ROCKi as the most efficient method.

For the assessment of the optimal initial cell density for cell inoculation with EDTA and ROCKi on VTN-N coated beads, three cell densities were tested, namely 30000, 50000 and 70000 cells/cm^2. Once more, at day 5 cells were harvested and counted and the expansion index for each condition was determined. When cells were inoculated in E8 medium on VTN-N coated beads they presented an expansion index of 3.5 ± 0.3, 4.4 ± 0.5 and 2.2 ± 0.2, after seeding of 30000, 50000 and 70000 cells/cm^2, respectively. By looking at the results (Figure 3), is clearly evident that inoculation with 50000 cells/cm^2 is preferable to the other assessed options.

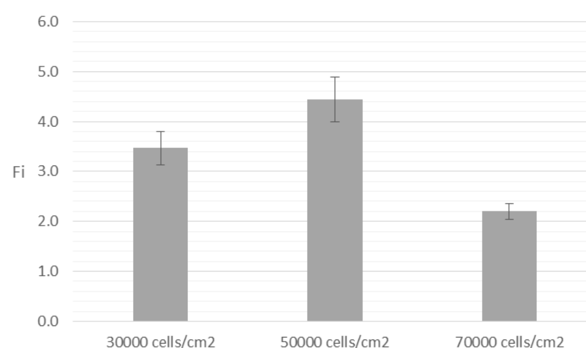

Figure 3 Expansion index of human iPSCs culture in E8 medium after cell inoculation with EDTA and ROCKi of 30000, 50000 and 70000 cells/cm^2 on VTN-N coated beads (n=3).

Therefore, and taking into account the results from the assays performed under static conditions, it is expected that the system resulting from hiPSCs seeded with close to 50000 cells/cm^2 in VTN-N coated microcarriers, after an EDTA treatment with ROCK inhibitor, is the most suitable for spinner-flask expansion and maintenance of hiPSCs.

Dynamic Conditions

A Face-Centered Composite Design (FC-CD) was performed to identify the optimal operating condition (OOC) for the expansion of hiPSCs on VTN-N coated beads in a spinner-flask system supplied with E8 medium, after EDTA inoculation with ROCK inhibitor. In this factorial design, two parameters were used as input, namely initial cell density (30000, 50000 or 70000 cells/cm^2) and agitation speed (30, 50 or 70 rpm), to maximize fold increase (output). Therefore, nine different conditions were tested resulting from the combination in pairs of these two parameters. The optimal condition given by the model was 55000 cells/cm^2 and 44 rpm, as initial cell density and agitation speed, respectively, with a predicted fold increase of 3.5. Interestingly, the optimal cell density given by the model is similar to the one predicted in assays under static conditions.

Therefore, two spinner flasks were performed to validate the optimal operating condition (Figure 4-A). Cell counting was performed daily, in order to monitor cell growth, and cells were maintained in culture until death phase was reached. The resulting growth curves are presented in Figure 4-B.

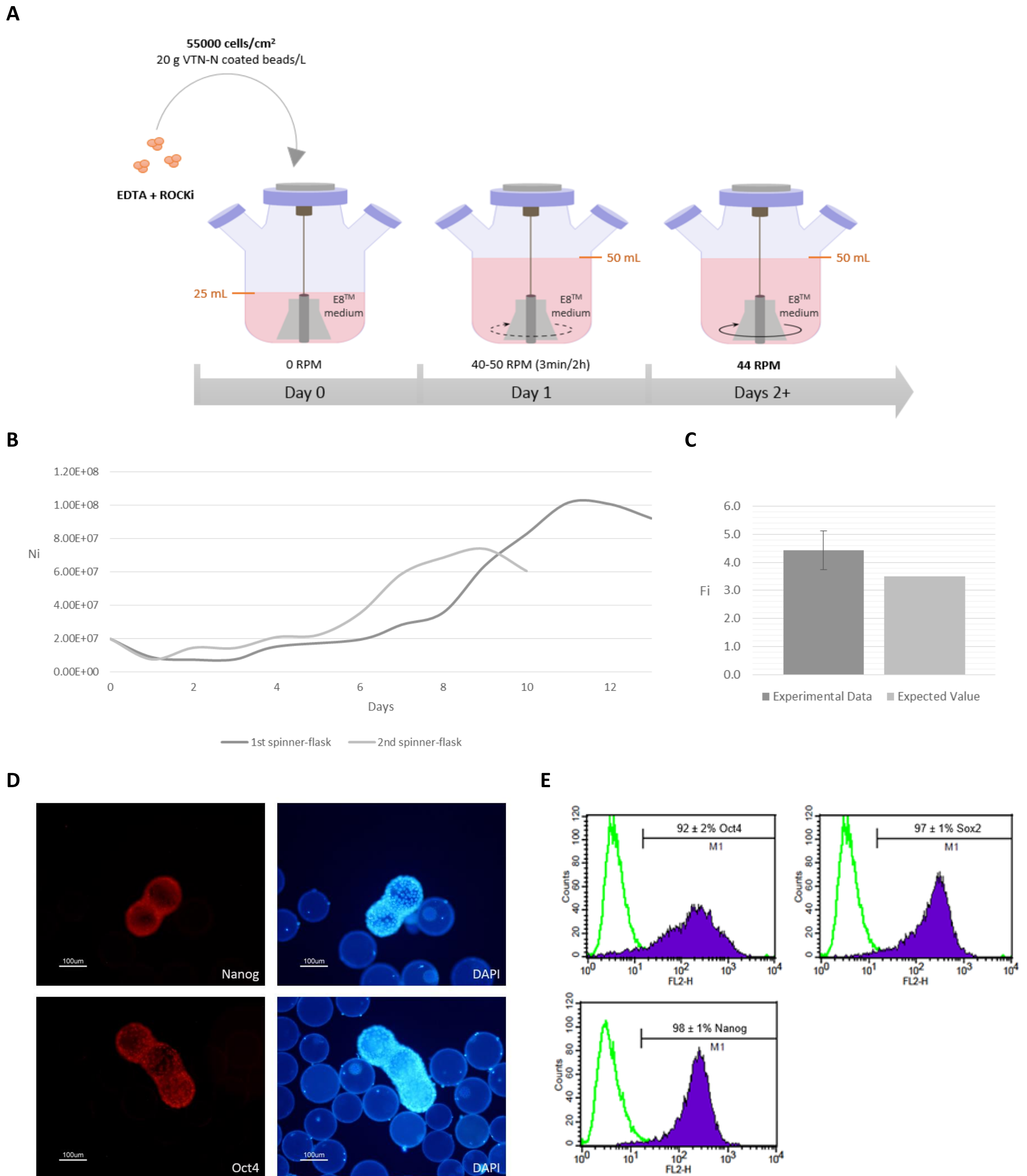

Figure 4 Validation of the optimal operational condition for the expansion of hiPSCs on VTN-N coated beads in a spinner-flask system, supplied with E8 medium (A). Growth curves of hiPSCs cultured in a spinner-flask system at optimal operating conditions determined by multifactorial design (n=1) (B). Expansion indexes of hiPSCs: experimental (n=2) and expected values (C). Immunostaining of hiPSCs cultured on VTN-N coated beads: cells were stained for pluripotency markers Nanog and Oct4, and nuclei counterstained with DAPI (scale bar – 100 µm) (D). Flow cytometry analysis of intracellular pluripotency markers Oct4, Sox2 and Nanog: area under green curve represents the negative control and purple area corresponds to the analysed samples. The percentage of analysed cells expressing each pluripotency marker is shown above bars (E).

According to these results, the rate of cell adhesion to VTN-N coated microcarriers is 41 ± 1%. As observed in both growth curves, stationary phase takes more than 7 days to be reached. Cells when cultured in a spinner-flask system at optimal operating condition present an expansion index of 4.4 ± 0.7, which is 27% higher than the one predicted by the model (Figure 4-C).

Despite the observed difference between expansion indexes, it cannot be unequivocally concluded that the model was not efficient in predicting fold increase value for OOC, since only two validations were performed, thus not being statistically significant. Nevertheless, it can be clearly stated that the model allowed finding an operation condition which maximizes expansion index of hiPSCs in culture.

Prior to harvesting, hiPSCs were characterized in terms of pluripotency by intracellular immunostaining for the expression of the pluripotency markers Nanog and Oct4. DAPI nuclei counterstaining was performed, as well. Results confirm that the majority of the cells attached to the microcarriers express both pluripotency markers (Figure 4-D). Flow cytometry was performed after cell harvesting to quantify the Oct4, Sox2 and Nanog intracellular pluripotency markers. Results indicate that 92 ± 2% of the analysed cells were positive for Oct4, 97 ± 1% for Sox2 and 98 ± 1% for Nanog (Figure 4-E).

Conclusions and Future Work

In this work, a scalable culture system was established for the expansion and maintenance of hiPSCs. Xeno- and serum-free Essential 8^{TM} medium was used as a culture medium for daily supply of all crucial nutrients and growth factors and xeno-free microcarriers coated with human recombinant vitronectin (VTN-N) were used to provide a feeder-free adherent culture for hiPSCs expansion in spinner-flasks. This system is the first to combine the use of a microcarrier-based culture with E8 medium.

Taking into account the results presented above, it can be clearly stated the efficiency of the optimized spinner-flask culture system for expansion and maintenance of hiPSCs. Under this optimized spinner-flask culture system, cells after EDTA inoculation with ROCKi presented an adhesion efficiency of 41 ± 1% and an expansion index of 4.4 ± 0.7, 27% higher than the one predicted by the model. The expression of pluripotency markers was confirmed by intracellular immunostaining and flow cytometry. In fact, 92 ± 2% of the cells were positive for Oct4, 97 ± 1% for Sox2 and 98 ± 1% for Nanog.

In sum, the established culture system allows an efficient scale-up bioprocess for the expansion of hiPSCs under fully xeno-free and chemically defined conditions. The simplicity of Essential 8^{TM} medium dramatically reduces cost and lot-to-lot variability thus representing a practical alternative to other serum-free cultures. Furthermore, the use of EDTA treatment with ROCK inhibitor as cell dissociation method reduces time consumption and variability between batches.

Future approaches will consist in testing at first the system robustness through the use of other feeder-free hiPSC lines at optimal operational condition. Furthermore, and when transposing and up-scaling the proposed culture system from spinner-flasks to stirred-suspension bioreactors, there are other parameters that can be optimized and adjusted in order to maximize fold increase, such as osmolarity, dissolved oxygen tension and hydrodynamic shear stress.

Additionally, it would also be interesting and useful to optimize cell yield by increasing the rate of cell adhesion to microcarriers. The proposed culture system allows an adhesion rate of 41 ± 1%, which is actually common for inoculation of hiPSCs on microcarriers. However, and particularly when comparing to other human stem cells, this is still a sub-optimal value. Moreover, the development of a bioprocess combining expansion and differentiation of hiPSCs with purification step would also be desirable and valuable.

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