Expansion of Human Induced Pluripotent Stem Cells on Dissolvable Microcarriers Under Dynamic Culture Conditions

Sara Filipa Violante Vieira

Master's student in Biotechnology at Instituto Superior Técnico, Universidade Técnica de Lisboa, Av. Rovisco Pais, 1, 1049-001, Lisbon, Portugal

ABSTRACT: Human induced pluripotent stem cells (hiPSCs) have been regarded as very promising for applications in the biomedical field, due to their abilities to self-renew and differentiate into all cell types of the human body. hiPSC expansion in vitro to large numbers is already possible through xeno-free chemically defined large scale processes, with microcarrier culture systems amongst the most popular. Microcarriers pose disadvantages regarding cell harvesting after expansion: cells are left on the surface of microcarriers or lost during filtration – a process yet to be optimised, particularly when considering large-scale production. Corning Inc. has developed microcarriers based on a digestible pectin matrix, which can be dissolved with pectinase after hiPSC expansion, preventing one of the problems of cell recovery and making hiPSC production more economically viable. Here, hiPSCs expansion was tested under static and dynamic conditions using dissolvable microcarriers (DM) and compared with the results obtained using polystyrene microcarriers (PSM). Adhesion was very similar on both types of microcarriers using similar coatings. Expansion achieved higher cell numbers on a shorter time frame using DM – $4.61\pm0.07x10^7$ cells on Matrigel-coated DM using E8 culture medium, and $3.80\pm0.73x10^7$ cells on Synthemax®II DM using mTeSR™1 culture medium, in 5 days of culture. Cell recovery efficiency was similar using both protocols of cell harvesting – 50.8±7.8% with protease and pectinase incubation, and 51.1±9.4% with protease incubation and filtration. Preparation of DM for use was also easier and less time consuming. hiPSC expansion on DM proved to be a very promising alternative to replace PSM.

KEYWORDS: human induced pluripotent stem cells; microcarriers; dissolvable microcarriers; expansion; cell harvesting; spinner-flask.

INTRODUCTION

The use of human embryonic stem cells (hESCs) for clinical, pharmacological and developmental research purposes has always raised ethical issues, due to the origin of the cells which are isolated from human embryos [1, 2]. This is one of the main reasons why the successful reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) [3, 4] was a major breakthrough and has put human iPSCs (hiPSCs) at the forefront of potential regenerative medicine therapies; hiPSCs could be used as an unlimited source of cell lines for pharmaceutical applications, in automated, high-throughput methods for synthesizing and screening libraries of biomaterials, and also as a way to monitor local microenvironments of soluble factors, such as small molecules, siRNAs and other signalling molecules [2].

In order for these applications to become a reality, it is imperative to expand hiPSCs to clinically relevant numbers *in vitro*. Human pluripotent stem cells (hPSCs) are adherent cells and have traditionally been expanded on feeder layers and coating matrices, as two-dimensional adherent colonies [5]; the major limitation of the two-dimensional culture systems is the surface area available for cells to adhere and proliferate, and the impossibility to successfully scale-up the system. Optimisation of hPSC expansion *in vitro* led culture systems away from small static cultures– such as multi-well plates – to larger scale three-dimensional (3D) dynamic culture systems.

Within 3D culture system options, hPSC culture on microcarriers may be the solution for a scalable way to control cell aggregation in suspension [10]. Microcarriers act as the surface for the growth of cells that are dependent of adhesion, enhancing the available surface area per volume for cell growth, allowing for a reduction in the consumption of culture medium and growth factors [2]. The development of microcarriers as a support for anchorage-dependent cell culture began in 1967, with the studies of van Wezel using Dextran particles [6]. Since then, many different types of microcarriers were developed, varying from porous and non-porous, with or without functional coating, made from plastics (polystyrene, polyethylene, polyester and polypropylene), glass, acrylamide, silica, cellulose, dextran, collagen (gelatine) and glycosaminoglycans. The existing data suggests that most of the commercially available microcarriers, used in mammalian cell culture, are not suitable for long term expansion of undifferentiated hPSCs, without any surface modification [7, 8], which demonstrates the relevance of using appropriate biological or synthetic substrates specifically developed for hiPSCs. In recent years, hPSC expansion on microcarriers has made great improvements and there are currently many reports on hPSC expansion yields using different types of microcarriers with diverse coatings, and the cell numbers obtained seems to be ever increasing, which shows that optimisation of the culture system is on the right track [9]. Microcarrier culture optimisation has to take into consideration parameters such as surface charge, microcarrier diameter, microcarrier density, and seeding density of the cultured cells [10], in order to promote efficient cell adhesion and a robust cell proliferation without differentiation, but also allow efficient and technically simple cell harvesting [9].

Nevertheless, cell expansion on microcarriers still poses some disadvantages. Because hPSCs expand as multilayers on the microcarrier surface, large microcarrier aggregates are formed [9], making the process of cell harvesting – which has yet to be fully characterised and monitored – rather difficult and not as efficient desired. A variable amount of cells is left still attached to the surface of the microcarriers and some cells are lost during the filtration step used to separate the detached cells from the microcarriers; the use of proteases to detach the cells from the surface of the microcarriers often leads to cleavage of cell surface proteins, which can in turn lead to dysregulation of the cell functions [11] and also affect the efficiency of cell sorting techniques that are based on the identification of surface proteins. Optimisation of the cell recovery step could improve the cell recovery yields Furthermore, due to the formation of these microcarrier agglomerates, quantification

of cells attached to the microcarriers becomes very challenging.

In order to prevent the loss of these cells, and in turn increase the yield of the cell expansion, it is possible to use microcarriers based on a completely digestible matrix, which eliminate the need to perform separation protocols, by dissolving the microcarriers altogether [12, 13]. This type of microcarriers have been described for many decades but were never fully developed and although they had been tested with animal cells [12], hiPSC expansion had not been tried before.

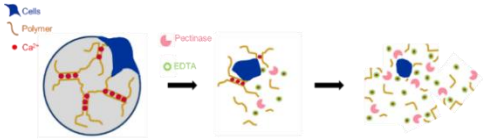

***Figure 1. Digestion of** DM **(Corning®).** The new ionically cross-linked polysaccharide microcarriers can be digested after hPSC expansion using a harvesting solution made up of pectinase and EDTA, which fully dissociates the matrix that comprises the microcarriers, leaving cells in suspension. From Corning Inc.*

Corning Inc.'s dissolvable microcarriers (DM) are made up of an ionically cross-linked polysaccharide – pectic acid or polygalacturonic acid (PGA), which is a gelatinous water soluble acid – and can be fully digested using a harvesting pectinase/EDTA solution. These microcarriers can be functionalised with cell adhesion promoting peptides, preferably by coating with a synthetic polymer bearing adhesion peptides, which enable bio-specific adhesion of the cultured cells. Cell recovery from these microcarriers can be achieved without using a protease, but rather pectinase and a chelating agent – in this case, EDTA – the pectinase will dissolve the PGA microcarrier and EDTA will help dissociate cells, resulting in a cell suspension which can then be further processed (**Figure 1**). Still, this process has yet to be optimised; since the cells covering the surface of the microcarrier do not allow for the harvesting solution to reach the microcarrier and thus dissolve it, the use of a protease is still necessary [13].

In this work, these DM are tested for hiPSC expansion, and the results of both expansion and havesting are compared with those obtained on polystyrene microcarriers (PSM).

MATERIALS AND METHODS

Maintenance of human induced pluripotent stem cells: hiPSC cell line F002.1.13, derived from healthy donor (46, XX) fibroblasts through retroviral transduction of pluripotency genes OCT4, SOX2, C-MYC and KLF4, was cultured on Matrigel™ (Corning®)-coated 6-well tissue culture plates (Falcon®) using 1.5mL/well of either Essential 8™ (Life Technologies™) or mTeSR™1 (STEMCELL Technologies™) culture media; culture media was changed daily. Cells were kept at 37 °C, 5% CO_2 and 20% O_2 inside a CO_2 incubator (Memmert). Cells were and passaged onto new plates at 80% confluence: cells were washed twice with 1 mL/well of ethylenediaminetetraacetic acid (EDTA) dissociation solution (0.5 mM EDTA (Invitrogen®) and 1.8 g/L sodium chloride (Sigma-Aldrich®) in phosphate-buffered saline (PBS) solution (Gibco)), and left for 5 min in 1 mL/well of EDTA. EDTA was removed, cells were flushed up to 5 times with 1 mL/well of culture medium and collected in a tube. The total cell suspension recovered was homogenised and plated onto a new multi-well plate, previously coated with Matrigel™ and homogenised on the plate to assure an even distribution of the cells. Passaged cells were kept in a CO_2 incubator.

Microcarrier preparation: Polystyrene microcarriers (PSM, Solo Hill®) and high concentration Synthemax®II microcarriers (polystyrene-based microcarriers from Corning®) (surface area of 360 cm^2/gram) were sterilised by weighing the microcarriers in a 15mL-tube and incubating them in ethanol 70% for 1 h under agitation. After incubation the ethanol was removed and the microcarriers were washed three times with sterile Phosphate-buffered saline (PBS, Gibco), agitated and left to settle completely before removing the PBS solution, and were stored at room temperature. Dissolvable microcarriers (surface area of 5000 cm^2/gram) were prepared according to manufacturer's instructions: according to the necessary surface area, in a laminar hood, the appropriate amount of microcarriers was weighted in a sterile glass bottle. Microcarriers were hydrated by adding 150 mL of sterile water (Corning®) per gram of microcarriers, swirling the suspension to ensure homogeneous hydration, and left to hydrate for 1 h. Before use, microcarriers should be allowed to settle in order to remove the water used for the hydration and replace it with culture medium. All manipulations should be performed using glass vials. Uncoated microcarriers were coated before inoculation by incubating the necessary amount of microcarriers in 200 µL of Matrigel™ and 6 mL of medium, for 1.5 h at room temperature under agitation.

hiPSC culture in 3D dynamic culture system: Spinner-flask's inner surface is siliconized using SIGMACOTE® (SigmaAldrich) according to manufacturer's instructions, after which the spinner-flask is sterilised in an autoclave. Before inoculation, 10 mL of culture medium are added to the spinner-flask to remove any water droplets that were left behind, this culture medium is discarded. Spinner-flasks are inoculated using cells previously expanded on monolayer culture, recovered using EDTA and counted, in order to know the amount of cell suspension to be used. Spinner-flasks are inoculated using half the final volume that will be used for cell expansion – 15 mL for inoculation and 30 mL of final volume. This volume includes the cell suspension needed, the previously prepared microcarrier suspension, and the necessary culture media (supplemented with 0.5% PenSrep and 10 µM Rho associated protein kinase inhibitor (ROCKi) Y-27632 (STEMCELL Technologies) to make up the total volume. After 24 h of culture time, microcarriers are left to settle to the bottom of the vessel, and exhausted media is removed until only 20% of the volume (6 mL) that will be used for expansion is left. To obtain the total final volume, 24 mL of fresh culture media (supplemented with PenStrep) are added. For the remaining culture time, 80% of exhausted media is replaced with the same volume of fresh culture media (supplemented with PenStrep). Every 24 h, two 500 µL samples are obtained from the spinner flask; for this, agitation is stopped and the samples are recovered immediately, using a 2 mL pipette. The samples are processed using the cell quantification protocols.

Alamar Blue calibration: A calibration curve was established by measuring the fluorescence of pre-determined cell numbers – 0 cells, 20,000, 40,000, 60,000 80,000 and 100,000 cells – and plotting the results in four different conditions: cells on Matrigel™-coated plates, on Matrigel™-coated polystyrene microcarriers and on Matrigel™-coated dissolvable microcarriers using Essential 8 culture medium, and cells on Synthemax®II dissolvable microcarriers on mTeSR™1 culture medium, using ultra-low attachment plates. Triplicates were prepared for each condition and for each number of cells desired, after inoculation with the cells all plates were incubated for 2 h at 37 ºC; after this first incubation, 25 µL of Alamar Blue (Invitrogen®) were added to each well and all plates were incubated for 1 h at 37 ºC. After the second incubation, 380 µL of the culture medium were recovered from each well, without carrying any microcarriers, and centrifuged in Eppendorf vials for 3 min at 1000 rpm; on a 96-well plate, duplicates of each condition were added using 170 µL in each well and the plate was analysed on a multimode microplate reader (Infinite® 200 Pro, Tecan) to measure the fluorescence intensity, which was monitored at 560 nm excitation wavelength and 590 nm emission wavelength; optimal gain is measured from one of the wells containing 100,000 cells.

Cell quantification: Indirect quantification – For 500 µL samples: in each well, 80% of the medium was removed and the same volume of fresh culture medium was added; 25 µL of Alamar Blue were added to each well and the plates were incubated for 1 h at 37ºC. At the same time, two wells containing only culture medium,

without cells, were incubated with 25 µL of Alamar Blue. After incubation, 350 µL of the culture medium were recovered from each well, without carrying any microcarriers, and centrifuged in Eppendorf vials for 3 min at 1000 rpm; the supernatant was used to obtain dilutions – in the same culture medium used for the culture medium change – in order for the measurements to fit in the calibration curves established. On a 96-well plate, duplicates of each dilution were added using 170 µL in each well, and the plate was analysed on a multimode microplate reader to measure the fluorescence intensity. **Direct quantification –** After the indirect cell quantification procedure, cells were harvested. For cells cultured on PSM: culture medium is removed from each well which is then washed with 500 µL of PBS. 300 µL of Accutase (Sigma Aldrich) are added and the plate is incubated for 5 min at 37 ºC; each well is flushed using a micropipette in order to detach cells from each other and from the microcarriers and 500 µL of washing medium (Knock-out DMEM (KO-DMEM, Gibco) culture medium, supplemented with Knockout-Serum Replacement (KO-SR, Gibco), MEM-non essential aa, L-glutamine (Gibco) and Penicillin-Streptomycin (PenStrep, Gibco)) are added to each well to stop Accutase reaction; all contents of the wells are recovered and the suspension containing polystyrene microcarriers is sifted through a strainer with a 100 µm mesh into a 50-mL tube. For cells culture on DM: culture medium is removed from each well which is then washed with 500 µL of PBS; 300 µL of Accutase and 200 µL of pectinase/EDTA solution (50 U/mL pectinase (Sigma-Aldrich) and 5 mM EDTA (Life Technologies) pH 8 in PBS) are added and the plate is incubated for 5 min at 37ºC; each well is flushed using a micropipette in order to detach cells from each other and 500 µL of washing medium are added to each well to stop Accutase reaction; after recovery, all contents of the wells are transferred into 15 mL-tubes. The contents of the tubes were centrifuged for 5 min at 1000 rpm. After centrifugation, supernatant was discarded and each tube grinded against the grid of the chamber in order to loosen the pellet, which was then resuspended in 730 µL of washing medium; from the suspensions obtained, 10 µL were diluted in a 1:2 proportion with Trypan Blue, 10 µL of the final solution were added to Neubauer chambers and the cells were counted under the microscope.

Cell recovery: The enzymes needed were used according to the volumes suggested by the manufacturer on the protocol for cell expansion on dissolvable microcarriers, which states the following: add 250mL of protease per gram of microcarriers, in a concentration appropriate for the cell type; add pectinase and EDTA directly to the protease solution, ensuring a final pectinase concentration of 100 U/mL and EDTA concentration of 10 mM. Before recovery cells were incubated for 1 h in washing medium supplemented with ROCKi. **Protocol A – Cell recovery using protease treatment followed by filtration:** cells and microcarriers were allowed to settle to the bottom of the tube, washing medium was removed, the cells were washed using PBS solution, left to settle once more, and the PBS removed. The appropriate volume of protease was added to the cells/microcarriers mixture, transferred back to the spinner flask, and incubated at 37ºC, under 35 rpm agitation; after 10 min, cells were flushed using a micropipette to help loosen the cells from the microcarriers, and incubated for 10 more minutes. At the end of the incubation time, washing medium was added to the contents of the spinner-flask (twice the volume of protease used), the total volume was filtered through a 100µm mesh strainer into a new 50mL-tube, and centrifuged for 10min at 1500 rpm. The supernatant was discarded, cells were resuspended using either washing medium or culture medium supplemented with ROCKi (if cells were to be re-plated), and a sample was used to quantify the amount of cells recovered, in order to calculate the efficiency of the process. **Protocol B – Cell recovery using protease and harvesting solution:** This protocol was only performed when working with dissolvable microcarriers. Cells and microcarriers were allowed to settle to the bottom of the tube, washing medium was removed, the cells were washed using PBS solution, left to settle once more, and the PBS removed. The appropriate volume of protease was added to the cells/microcarriers suspension, transferred back to the spinner-flask, and incubated for 10 min at 37ºC, under 35 rpm agitation; after the first incubation, cells were flushed using a micropipette to help loosen the cells from the microcarriers, the appropriate volume of harvesting solution (1.3 mL of pectinase and 1 mL od EDTA to 97.7 mL of Dulbecco's Phosphate-Buffered Saline (dPBS, Gibco), obtaining a final solution with 50 U/mL pectinase and 5 mM EDTA pH8 in dPBS) was added to the contents of the spinner flask and the whole volume was incubated for 10 min. At the end of the incubation time, washing medium was added to the contents of the spinner-flask (twice the volume of enzyme/protease used), the total volume transferred into a new 50mL-tube, and centrifuged for 10 min at 1500 rpm. The supernatant was discarded, cells were resuspended using either washing medium or culture medium supplemented with ROCKi (if cells were to be re-plated), and a sample was used to quantify the amount of cells recovered, in order to calculate the efficiency of the process.

Immunocytochemistry: Surface markers – culture medium was removed from all the wells which were then washed three times with 500 µL of PBS and incubated with 300 µL of primary antibody solution – TRA-1-60 (Stemgent, 1:135) and SSEA4 (Stemgent, 1:135) in washing medium – for 30 min at 37ºC. Afterwards, each well was washed three times with 500 µL of PBS and incubated with 300 µL of secondary antibody solution – Alexa 488 or 546 Goat anti-mouse IgM (Invitrogen, 1:500) for TRA-1-60 and Goat anti-mouse IgG (Invitrogen, 1:500) for SSEA4 – for 30-45 min, at 37ºC in the dark. The wells were washed two times with 500 µL of PBS and 500 µL of PBS were added to each well. **Intracellular markers –** culture medium was removed and each well washed with PBS; 4% Paraformaldehyde (PFA, Sigma Aldrich) in PBS solution was added and incubated for 10-15 min at room temperature. After incubation, each well was washed with PBS and incubated with 300 µL of Blocking solution (10% Normal Goat Serum (NGS, Sigma Aldrich), 0.1% Triton-X (Sigma Aldrich), in PBS) for 1 h at room temperature. Blocking solution was removed and 300 µL of primary antibody solution was added to each well – NANOG (Merck Millipore, 1:5000) and OCT4 (Merck Millipore, 1:150) in Staining buffer (5% NGS, 0.1% Triton-X, in PBS) – and incubated at 4ºC, overnight. Each well was washed three times using 500 µL of PBS and incubated using 300 µL of the secondary antibody solution – Alexa 546 or 488 goat anti-rabbit IgG (1:500) in staining buffer – for 1h at room temperature, in the dark. Each well was once again washed three times with 500 µL of PBS and incubated with DAPI solution for 3 min at room temperature, in the dark. Each well was washed two times with 500 µL of PBS to remove DAPI crystals and 500 µL of PBS were added to each well.

Flow cytometry: **Surface markers –** Cells kept in 2% PFA solution are centrifuged for 3 min at 1000 rpm and each pellet is resupended in 100 µL of FACS buffer (4% foetal bovine serum (FBS; Sigma Aldrich) in PBS) for each analysis that is to be performed. 100 µL of cell suspension is transferred to a FACS vial, 10 µL of phycoerythrin (PE) conjugated antibody solution – SSEA4 (Miltenyi Biotec), TRA-1-60 (Miltenyi Biotec) and SSEA1 (Miltenyi Biotec) – are added to each vial and incubated for 15 min at room temperature, in the dark. After incubation, 2 mL of PBS solution are added to each vial, which is then centrifuged for 3 min at 1000 rpm; supernatant is discarded and 2 mL of PBS are added to each vial, which are again centrifuged and the supernatant discarded. 300 µL of PBS are used to resuspend the pellet and the cell suspension obtained is then analysed in a FACScalibur (Becton Dickinson) flow cytometer, using CellQuest™ software (Becton Dickinson) for data acquisition. **Intracellular markers –** The Eppendorf vials to be used in this procedure are coated with 400 µL of BSA solution for 15 min at room temperature. Meanwhile, samples kept in 2% PFA solution are centrifuged for 5 min at 1000 rpm, washed twice with 5 mL of 3% NGS solution, and centrifuged for 5 min at 1000 rpm, each time. The supernatant is discarded and cells are resuspended in 500 µL of 3% NGS solution for each analysis to be performed with that same sample. BSA solution is removed from the Eppendorf vials, and 500 µL of cell suspension is added to each one. Vials are centrifuged for 3 min at 1000 rpm, supernatant is removed, and the pellet is resuspended in 150 µL of 3% NGS solution and 150 µL of saponin saponin (Sigma Aldrich) and incubated for 15 min at room temperature. After incubation the vials are centrifuged for 3 min at 1000 rpm, the supernatant is removed, and the pellet is resuspended in 300 µL of NGS solution and incubated for 15 min at room temperature. After incubation the vials are centrifuged for 3 min at 1000 rpm. The supernatant is removed, the pellet is resuspended in the appropriate primary antibody solutions – Anti-OCT4 (mouse IgG, Merck Millipore) antibody is used in a 1:300

proportion – and incubated for 1.5 h in the dark. After incubation vials are centrifuged for 3 min at 1000 rpm, pellet is washed twice with 1% NGS solution, and centrifuged for 3 min at 1000 rpm, each time. The supernatant is removed, all samples are resuspended in 300 µL of secondary antibody solution – Alexa 488 anti-mouse IgG antibody (Invitrogen) in a 1:300 proportion –, and incubated for 45 min in the dark. After incubation, vials are centrifuged for 3 min at 1000 rpm, pellet is washed twice with 1% NGS solution, and centrifuged for 3 min at 1000 rpm, each time. Supernatant is removed, the pellet is resuspended in 500 µL of PBS solution, cell suspension is transferred to FACS vials and analysed in a FACScalibur flow cytometer, using CellQuest™ software for data acquisition.

Embryoid body formation: Cells were recovered as if for a normal passage using EDTA, counted, and plated on ultra-low attachment 6-well plates, at a density of $1x10^6$ cells per well, using 2 mL of expansion culture medium (either E8™ or mTeSR™1) supplemented with PenStrep and ROCKi. After 24 h, 80% of the culture medium was replaced with fresh medium not supplemented with ROCKi. After 2 days of cell expansion, 80% of spent culture medium was replaced with EB differentiation medium (Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with Foetal Bovine Serum (FBS, Sigma Aldrich), Minimum essential medium (MEM)-non essential amino acids (aa, Gibco), Sodium pyruvate (Gibco), □-Mercaptoethanol (Sigma Aldrich), and Penicillin-Streptomycin (PenStrep, Gibco)), every other day for 28 days. In order to re-plate the cells from the EBs, the necessary wells in a 24-well plate were coated with poly-ornithine (200 µL per well) for 1 h at 37ºC, followed by an incubation with laminin solution (1 µL of laminin stock solution per well on 200 µL per well of PBS solution), for 2 h at 37ºC. On the 29th day of differentiation, aggregates were dissociated. Differentiation medium was removed, aggregates were washed twice using 2 mL of PBS solution per well, and incubated with 1 mL of Trypsin per well for 5 min at 37ºC; after incubation, 1 mL of EB differentiation medium was added to each well, cells were flushed using a micropipette, cell suspension was transferred to a 15mL-tube and centrifuged. After centrifugation, the supernatant was discarded. Cells were resuspended in 2 mL of EB differentiation medium, and 500 µL of cell suspension were re-plated in each well of a previously coated 24-well plate. On the 7th day after re-plating, cells were fixed using a PFA 4% solution, and immunocytochemistry analysis was performed using antibodies to detect OCT4 (1:150), SOX17 (R&D Systems, 1:1 000), TUJ1 (Covance, 1:20 000) and α-SMA (Dako, 1: 1000), and Alexa 546 Goat anti-mouse IgG (1:500) as secondary antibody.

Data analysis: The values obtained from all the cell quantifications were used to calculate: difference (in percentage) in cell numbers 24h after inoculation, through direct and indirect cell quantification methods, relatively to the number of cells initially inoculated; Fold increase, which corresponds to the ratio between the highest cell numbers achieved and the number of cells after 24h, both obtained through direct cell quantifications; Seeding yield which corresponds to the ratio between the highest cell numbers achieved and the number of cells inoculated, through values obtained through direct cell quantifications. Standard deviation values are always presented and were calculated from the measurements obtained.

RESULTS AND DISCUSSION

ALAMAR BLUE CALIBRATION FOR INDIRECT CELL QUANTIFICATION: The first step towards getting information about the efficiency of cell recovery from microcarriers was to establish a way to quantify the cells attached to the microcarriers' surface before the harvesting protocol; this way it would be possible to compare the cell numbers obtained before and after recovering the cells from the microcarriers and determine the amount of cells that were being lost. Alamar Blue can be used to correlate the number of cells in culture with the intensity of the fluorescence measured. To establish the calibration curves six cell densities were used, three independent wells were inoculated for each cell density, and each well's fluorescence was measured twice. The equations for the calibration curves were obtained through linear regression of the average fluorescence measured in each condition, by using the fluorescence intensity values with the corrections to exclude the culture media interference, and the expressions obtained were: $y=0.3295x+507.08$ ($R^2=0.9987$) for Matrigel™-coated PSM , $y=0.3344x+834.08$ ($R^2=0.9951$) on Matrigel™-coated DM, and on Matrigel™-coated plates $y=0.3631x+152.73$ ($R^2=0.9971$), using E8™ culture medium, and $y=0.2401x+206.01$ ($R^2=0.9955$) for Synthemax®II-coated DM using mTeSR™1 culture medium. Where x stands for the number of cells, and y stands for the fluorescence intensity measured. The use of Alamar blue for indirect cell quantifications proved to work only to the point before cells started to form large aggregates; beyond that, the cell numbers obtained with this technique were always below the values obtained through direct quantification of the samples recovered.

HIPSC EXPANSION ON MICROCARRIERS UNDER STATIC CONDITIONS: DM were developed all cell types but tested for Mesenchymal Stem Cell (MSC) culture [13] and had never been tried for hiPSC expansion before, therefore, it was important to begin testing by analysing hiPSC adhesion to the surface of the DM, monitoring cell expansion and distribution over the available surface area, and analysing cells after being cultured on DM.

hiPSCs were inoculated on six different combinations of microcarriers – polystyrene or dissolvable –, surfaces – Matrigel™ or Synthemax®II –, and culture media – Essential 8™ or mTeSR™1. Cells were cultured for 4 days (cells cultured on E8™ medium) or 5 days (cells cultured on mTeSR™1), and cell expansion was monitored everyday through direct and indirect cell quantifications; the cell numbers obtained were used to determine: number of cells 24 h after inoculation (to analyse cell adhesion to the microcarriers), fold increase, and seeding yield.

Table 1. hiPSC expansion in static three-dimensional culture. *Values obtained for: difference in hiPSCs numbers 24h after inoculation of the microcarriers, determined through indirect (Alamar Blue) and direct cell quantification methods, and culture time at which highest hiPSC expansion was achieved, on polystyrene (PSM) and dissolvable (DM) microcarriers, with Matrigel™ and Synthemax®II surfaces, for cultures using Essential 8™ and mTeSR™1 culture media. All values were calculated from duplicates.*

Static 3D culture – hiPSC expansion on microcarriers			
Culture system	**After 24h (%)**		**Highest expansion**
	Alamar Blue	**Direct Counts**	
PSM+Mat+E8	98.5±12.6	32.2±1.1	Day 3
DM+ Mat+E8	128.6±12.8	48.3±17.2	Day 3
PSM+Mat+mTeSR1	92.5±20.4	41.7±8.9	Day 4
DM+ Mat+mTeSR1	112.9±33.5	61.1±5.0	Day 5
PSM+SII+ mTeSR1	48.3±2.1	29.7±1.4	Day 5
DM+SII+mTeSR1	75.3±24.0	48.1±0.3	Day 5

The first experiments were performed using E8™ culture medium; when comparing PSM and DM with the same Matrigel™ coating, cell quantification 24h after inoculation showed higher cell numbers on DM, both through direct and indirect cell quantifications – 48.3 ± 17.2% and 128.6 ± 12.8%, respectively – than when compared to the cells on

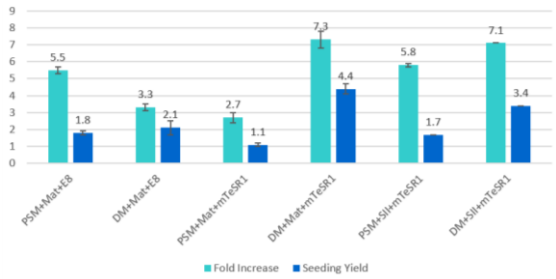

Figure 2. hiPSC expansion in static three-dimensional culture. *Values obtained for: fold increase (calculated from the highest values obtained from direct cell quantifications), and seeding yield (calculated from the highest values obtained from direct cell quantifications) considering only the cells that were recovered, on polystyrene (PSM) and dissolvable (DM) microcarriers, with Matrigel™(Mat) and Synthemax®II (SII) surfaces, for cultures using Essential 8™ (E8) and mTeSR™1 culture media. All values were calculated from duplicates.*

PSM – 32.2 ± 1.1% and 98.5 ± 12.6%, respectively –, suggesting that DM allow for a better adhesion of hiPSCs to their surface; the same was observed when replacing E8™ medium with mTeSR™1, with direct and indirect cell quantifications 24 after inoculation showing 61.1 ± 5.0% and 112.9 ± 33.5% for the DM, and 41.7 ± 8.9% and 92.5 ± 20.4% for PSM. This difference was also observed when inoculating cells on microcarriers with Synthemax®II surface and culturing them on mTeSR™1 medium, where direct and indirect cell quantifications 24 h after inoculation were 48.1 ± 0.3% and 75.3 ± 24.0% for DM, and 29.7 ± 1.4% and 48.3 ± 2.1% on PSM, these two conditions having the lowest cell adhesion of all six (**Table 1**).

On the other hand, regarding cell expansion efficiency (**Figure 2**), the use of mTeSR™1 proved to have better results for hiPSC expansion, when compared to E8™ culture medium, except when culturing hiPSCs on Matrigel™-coated PSM – only achieving a fold increase of 2.7 ± 0.3 and a seeding yield of 1.1 ± 0.1, after 4 days of culture; regarding these two parameters, the best hiPSC expansion was achieved using Matrigel™-coated DM on mTeSR™1 culture medium – achieving a fold increase of 7.3 ± 0.5 and a seeding yield of 4.4 ± 0.3, after 5 days of culture –, closely followed by a combination of Synthemax®II DM on mTeSR™1 culture medium – achieving a fold increase of 7.1 ± 0.0 and a seeding yield of 3.4 ± 0.0, after 5 days of culture –, being the difference in seeding yield due to the better adhesion observed for the first system, when compared to the second. Experiments using a combination of Synthemax®II DM in E8™ culture medium were also performed and proved unsucseful as cells staarted to expand as aggregates instead of adhering to the surface of the microcarriers.

Daily visual monitoring (through optical microscopy, results not shown) was used to keep track of cell distribution throughout the available surface area. It was possible to understand that microcarrier aggregates start to appear as early as 24 h after inoculation; these aggregates become larger throughout culture time and may be a great source of concern regarding the diffusion of nutrients and gases, especially if hiPSCs were to be cultured for longer periods of time. The formation of aggregates was aggravated by the use of Matrigel™ coating, due to its consistency being very similar to that of gelatine which naturally promotes aggregation, even of empty microcarriers; in comparison, the use of Synthemax®II surface helps reduce aggregation but does not completely prevent it. The composition of the microcarriers does not appear to have any impact on aggregation. The overall distribution of cells throughout the available microcarriers shows that some microcarriers were completely covered in cells while others were left with no cells. Characterisation of the cells after expansion was performed by immunohistochemistry to detect pluripotency markers SSEA4 and OCT4 on cells attached to the microcarriers at the final day of culture; the results were positive for every condition tested (results not shown) which suggested that cells were maintaining their characteristics during expansion.

The global analysis of all the results obtained indicates that microcarrier culture systems, under static conditions, are able to support hiPSC expansion, as long as there is surface area available for cell adhesion; also, regarding the DM under analysis, it is safe to state that they are very promising for hiPSC expansion, showing better results than those obtained on PSM, under the culture conditions used in these experiments.

HIPSC EXPANSION ON MICROCARRIERS UNDER DYNAMIC CONDITIONS

ADHESION PROTOCOLS: The first experiment performed under dynamic culture conditions combined DM with Matrigel™ coating and E8™ culture medium; indirect cell quantifications after 24 h showed 100% adhesion of the cells seeded, and cell proliferation during that time frame – with 115.5% ± 2.0% cells comparing to the number of cells used for initial seeding. In order to try and optimise cell adhesion, three different protocols were used, which differed in terms of agitation regimes during the first 48h: A1 – 24 h static, 24 h intermittent agitation – 3 min at 25 rpm every 2 h –, continuous agitation at 25 rpm until end of culture; A2 – 2 h static, intermittent agitation – 3 min at 25 rpm every 2 h – until 24 h, continuous agitation at 25 rpm until end of culture; A3 – 24 h static, continuous agitation at 25 rpm until end of culture. All protocols showed complete adhesion (100%) of the cells used for seeding, however, protocol A2 showed higher expansion within the first 24h when compared with the other two; on the other hand, when considering the average cell numbers, neither protocol A2 nor A3 achieved cell numbers as high as those obtained using protocol A1 (**Figure 3**).

When considering fold increase and seeding yield, protocol A1 showed the best results from all three experiments compared – with a fold increase of 5.8 ± 1.7 at day 5 when compared to the number of cells present after 24 h, and a seeding yield of 3.2 ± 1.2 at day 5 when compared to the

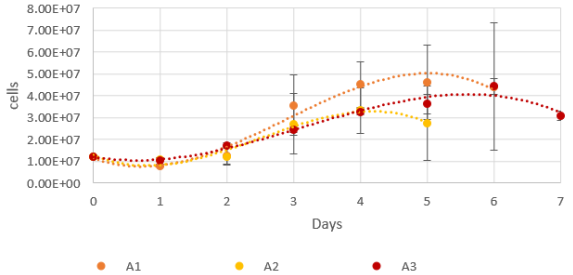

Figure 3. Adhesion protocols of hiPSCs on Matrigel™-coated dissolvable microcarriers in Essential 8™ culture medium. *The graphic represents the number of cells present in the culture vessel at each day of culture time, regarding each adhesion protocol tested using hiPSCs on Matrigel™-coated dissolvable microcarriers in E8™ culture medium. Each curve represents a polynomial regression of the values obtained for hiPSC expansion using different types of agitation during the first 48h of culture. All values were calculated from duplicate samples collected during each experiment.*

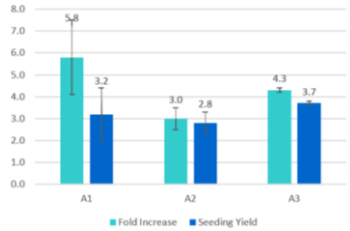

Figure 4. Adhesion protocols of hiPSCs on Matrigel™-coated dissolvable microcarriers. *Values obtained for: fold increase (calculated from the highest values obtained from direct cell quantifications), and seeding yield (calculated from the highest values obtained from direct cell quantifications) considering only the cells that were recovered, on Matrigel™-coated dissolvable microcarriers, using Essential 8™ and culture medium. All values were calculated from duplicates.*

initial cell numbers used for seeding, which was only bested by the seeding yield of protocol A3 with a value of 3.7 ± 0.1 at day 6 (**Figure 4**). Cell distribution over the surface area available for expansion was monitored daily through microscopic visualisation; distribution seemed more even when using adhesion protocol A2, while more surface area seemed to remain available when using adhesion protocol A1. Regarding microcarrier aggregation, by the end of culture time, large microcarrier aggregates were visible in all three experiments, however, when using adhesion protocols A1 and A3, the aggregates obtained appeared to be larger than those formed when using protocol A2; these conclusions are only as representative as the samples recovered for microscopy and cell quantification. Considering all the results obtained, which showed that protocol A1 allowed to achieve higher cell numbers and obtain the highest fold increase, protocol A1 was kept as the protocol of choice for the rest of the experiments performed, keeping in mind that agitation could still be improved to prevent formation of large microcarrier aggregates and allow for a better cell distribution.

HIPSC EXPANSION: The vessels used for hiPSC culture were spinner-flasks which are specifically designed for suspension cell culture. The spinner-flask chosen had a horizontal impeller for agitation and a maximum working volume of 50 mL – experiments were performed with a total volume of 30 mL. The optimal microcarrier density for hiPSC expansion on PSM within spinner flasks is recommended by the manufacturer as 20 g of microcarriers per litre of culture media; in a working volume of 30 mL, this translates to 216 cm^2 of surface area available for cell growth – this was the surface area considered for all experiments performed. The optimal number of cells for inoculation of microcarriers had been established as 55,000cells/cm2 [14], which for the surface area available was rounded up to $1.2x10^7$ cells.

In terms of cell adhesion (**Table 2**), with the exception of hiPSC expansion on Synthemax®II DM using mTeSR™1 – cell quantifications after 24 h were between 49.0 ± 2.3% and 52.7 ± 5.6% with direct quantifications, and between 48.3 ± 0.8% and 59.6 ± 5.0% with indirect cell quantifications, relating to the numbers of cells used for inoculation of the spinner-flasks –, cells used for inoculation adhere completely to the microcarriers available, and begin expansion within the first 24 h of culture. These results are in accordance to those obtained in static experiments, where adhesion to Matrigel™-coated microcarriers was also higher than adhesion to Synthemax®II microcarriers. Adhesion to Matrigel™-coated DM in mTeSR™1 appears to be the highest – 187.5 ± 0.0% and 228.1% through direct and indirect cell quantifications, respectively – however, due to the gelatinous nature of Matrigel™, microcarriers in this systems formed very large aggregates within the first 24h of culture, making it virtually impossible to retrieve homogenous and representative samples for hiPSC quantifications, meaning that the values obtained after 24 h were highly overestimated and should not be taken into account without considering all this.

Table 2. hiPSC expansion in dynamic three-dimensional culture*. Values obtained for: difference in hiPSCs numbers 24h after inoculation of the microcarriers, determined through indirect (Alamar Blue) and direct cell quantification methods, fold increase (calculated from the highest values obtained from direct cell quantifications), and seeding yield (calculated from the highest values obtained from direct cell quantifications) considering only the cells that were recovered, on polystyrene (PSM) and dissolvable (DM) microcarriers, with Matrigel™ and Synthemax®II surfaces, for cultures using Essential 8™ and mTeSR™1 culture media. All values were calculated from duplicates.*

Dynamic 3D Culture (30mL) – hiPSC expansion			
Culture system	After 24h (%)		Maximum cells (culture time)
	Alamar Blue	Direct quantification	
DM+Mat+E8	115.5 ± 2.0	66.5 ± 4.8	4.61 ± 0.17 x10^7 (Day 5)
PSM+Mat+E8	117.6 ± 33.5	55.3 ± 3.6	5.68 ± 0.78 x10^7 (Day 9)
DM+Mat+mTeSR1	228.1	187.5 ± 0.0	3.22 ± 0.06 x10^7 (Day 5)
PSM+SII+ mTeSR1	132.9 ± 39.1	88.6 ± 1.9	4.68 ± 0.07 x10^7 (Day 10)
DM+ SII+ mTeSR1	48.3 ± 0.8	49.0 ± 2.3	3.80 ± 0.73 x10^7 (Day 5)
	59.6 ± 5.0	52.7 ± 5.6	3.26 ± 0.27 x10^7 (Day 6)

Regarding hiPSC expansion (**Figure 5**), in order to establish a base for comparison, hiPSCs were expanded on PSM with Matrigel coating on E8™ culture medium (PSM+Mat+E8) and on PSM with Synthemax®II surface on mTeSR™1 medium (PSM+SII+mTeSR1), systems that had been previously tested in the laboratory. hiPSC expansion in each of these systems achieved 5.68 ± 0.78x10^7 cells after 9 days of culture time, and 4.68 ± 0.07 x10^7 cells after 10 days of culture time, respectively. Even though none of the experiments performed on DM achieved a number of cells as high as any of the systems using PSM – which were used

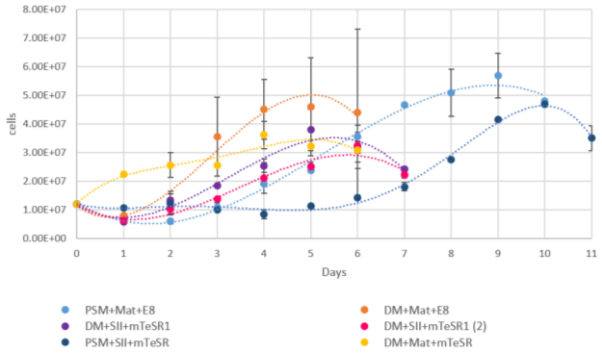

Figure 5. hiPSCS expansion in 3D dynamic culture conditions in spinner-flasks (V=30mL). *The graphic represents the number of cells present in the culture vessel at each day of culture time, regarding each condition used for the experiments; the curves used as basis for comparison of expansion are represented in light blue (PSM+Mat+E8) and dark blue (PSM+SII+mTeSR1), the remaining curves show hiPSC expansion on dissolvable microcarriers, in different culture conditions: in orange DM+Mat+E8, in purple and pink DM+SII+mTeSR1, and in yellow DM+Mat+mTeSR1. The dotted curves represent polynomial regressions of the values obtained All values were calculated from duplicates. Key: PSM – polystyrene microcarriers; DM – dissolvable microcarriers; Mat – Matrigel™; SII – Synthemax®II; E8 – Essential 8™ culture medium; mTeSR1 – mTeSR™1 culture medium.*

as a basis for comparison –, the results obtained are still very promising. Although hiPSC expansion using PSM+Mat+E8 and PSM+SII+mTeSR1 allowed to achieve higher cell numbers – 5.68 ± 0.78x10^7 cell in 9 days and 4.68 ± 0.07x10^7 cells in 10 days, respectively – hiPSC expansion on Matrigel™-coated DM using E8™ culture medium (DM+Mat+E8) allowed for a faster expansion of the cells, achieving highest cell numbers – 4.61 ± 1.71x10^7 cells – after 5 days of culture. The higher efficiency of this system is even more evident when considering that at 5 days of culture time, hiPSC expansion on PSM+Mat+E8 had achieved 2.38 ± 0.07x10^7 cells, and on PSM+SII+mTeSR1 only 1.15 ± 0.04x10^7 cells. Looking at hiPSC expansion on Synthemax®II DM (which would be the most interesting when considering establishing a xeno-free culture system) using mTeSR™1 culture medium (DM+SII+mTeSR1), which achieved their highest of 3.80 ± 0.72x10^7 cells (purple) and 3.26 ± 0.27x10^7 cells (pink) between days 5 and 6, respectively, the results obtained, while not as high as those obtained with expansion on DM+Mat+E8, are still higher than the number of cells obtained through expansion on PSM within that culture time.

Considering the differences between the number of hiPSCs used to inoculate the spinners, and the number of cells achieved by the end of culture, hiPSC expansion achieved higher fold increase and seeding yield when cells were cultured on PSM+Mat+E8 (8.6 ± 1.2 and 4.7 ± 0.6, respectively). hiPSC expansion on DM+Mat+E8 achieved 5.8 ± 1.7 and 3.2 ± 1.2, fold increase and seeding yield, respectively and expansion on DM+SII+mTeSR1 reached 6.5 ± 1.2 and 3.2 ± 0.6, on the first experiment, and 5.2 ± 0.4 and 2.7 ± 0.2 on the second experiment, for those same parameters. Cell expansion on DM+Mat+mTeSR1 proved to be the least productive (**Figure 6**). It is important to keep in mind that the conditions – cell density and microcarrier surface area – used in these experiments, had been optimised for expansion on PSM, therefore, hiPSC expansion on DM still has room for optimisation, and could achieve even better results.

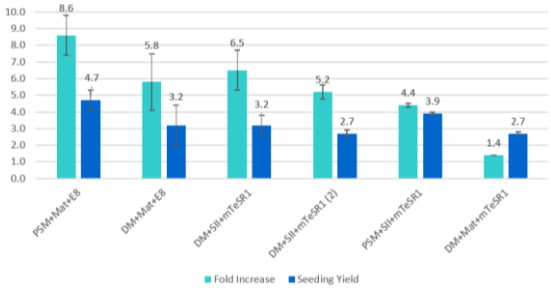

***Figure 6. hiPC expansion in dynamic three-dimensional culture**. Values obtained for: fold increase (calculated from the highest values obtained from direct cell quantifications), and seeding yield (calculated from the highest values obtained from direct cell quantifications) considering only the cells that were recovered, on polystyrene (PSM) and dissolvable (DM) microcarriers, with Matrigel™(Mat) and Synthemax®II (SII) surfaces, for cultures using Essential 8™ (E8) and mTeSR™1 culture media. All values were calculated from duplicates.*

Regarding cell distribution over the microcarriers' surface area (**Figure 7**), it is possible to observe that 24h after inoculation of the microcarriers: on DM cells start to expand between the microcarriers, which begins formation of small microcarrier aggregates; on PSM cell distribution appears to be more homogeneous. At the final day of culture, it was still possible to observe some empty microcarriers in all situations but mostly large aggregates completely covered in hiPSCs – aggregates were larger on cultures using DM

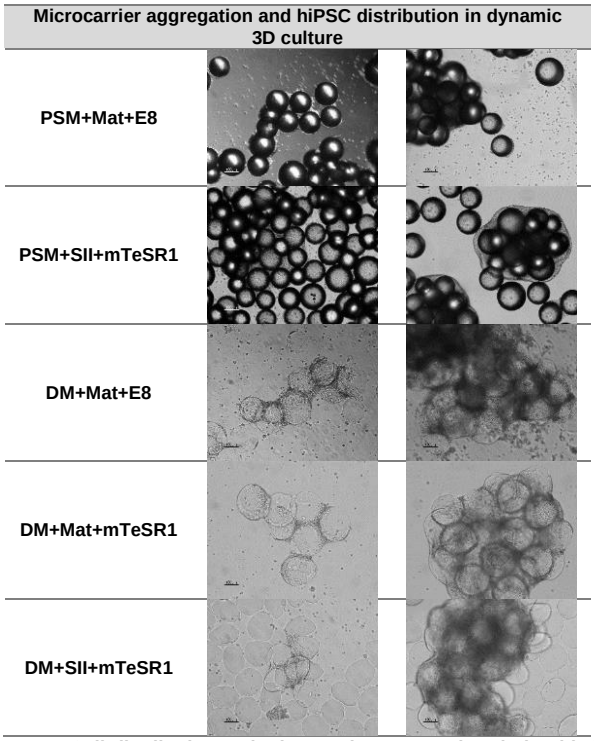

***Figure 7. Cell distribution and microcarrier aggregation during hiPSC expansion.** The images show the distribution of hiPSCs over the available microcarrier surface and aggregation 24h after inoculation (left) and at the final day of culture time (right). Bar scale: 100µm.*

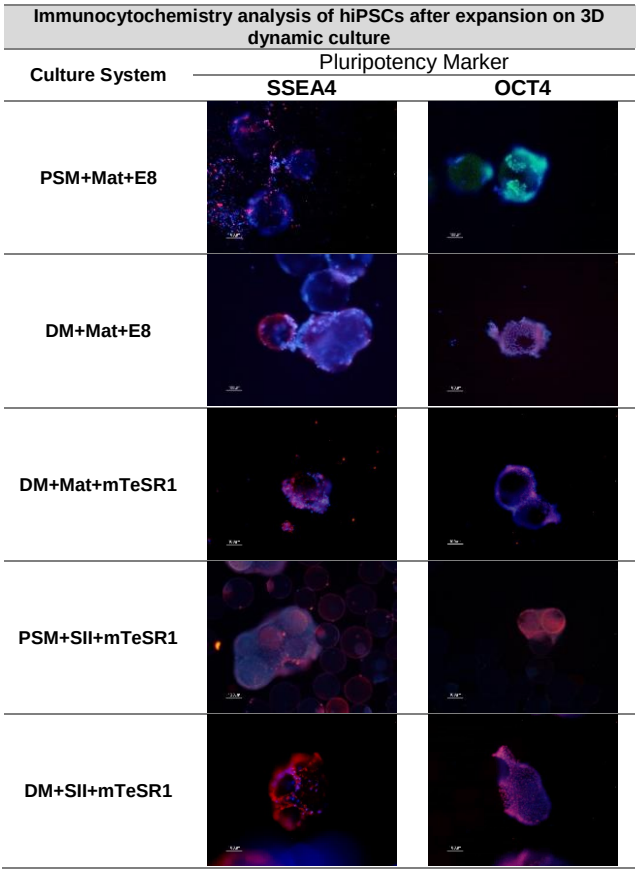

***Figure. 8 Immunocytochemistry analysis of hiPSCs after expansion on 3D dynamic culture.** Immunocytochemistry results obtained after merging immunofluorescence imaging (red and green) of pluripotency markers SSEA4 and OCT4 and DAPI counterstaining imaging (blue). Images were obtained from cell/microcarrier samples obtained on the final day of expansion in 3D dynamic culture. Bar scale: 100µm.*

than on those using PSM. Once again, these conclusions were obtained through visual interpretation alone and are only as representative as the samples recovered for microscopy and cell quantification. Overall, by allowing to achieve higher cell numbers within a shorter time frame, hiPSC expansion on DM seem to be the most cost effective choice, making DM a very promising product, as long as hiPSCs were conserving all their characteristics. Following each experiment, cells attached to the surface of the microcarriers were analysed using immunocytochemistry, using known pluripotency markers, namely OCT4, SSEA4 (**Figure. 8**), and TRA-1-60 (results not shown), as well as the early differentiation marker SSEA1 (results not shown). The immunostaining analysis of the cells with DAPI counterstaining after culture on polystyrene and dissolvable microcarriers, showed that, in all cases, 90-100% of the cells showed a positive result regarding the pluripotency markers analysed and an even distribution of those throughout all the cells, while at the same time, the analysis of SSEA1 marker was completely negative, which is a good indication that hiPSCs do maintain their phenotype and pluripotency characteristics when cultured under these conditions and that no cells had started to differentiate.

HIPSC RECOVERY: After being cultured within the spinner-flasks, hiPSCs were recovered using different harvesting protocols in order to compare the efficiency of each one and perform characterisation of the cells after recovery. In order to be able to establish a direct comparison with polystyrene microcarriers, cell recovery was conducted using two different harvesting protocols: A – Using Accutase and a 100µm mesh cell strainer; B – Using Accutase and pectinase/EDTA harvesting solution. The goal was to understand whether or not cell loss during harvesting from the microcarriers could be reduced and to determine if the use of the harvesting solution would have any impact on cell viability after cell recovery.

In terms of recovery efficiency, the results obtained (**Table 3**) are still inconclusive since the cell harvesting protocol is yet to be optimised and these are only preliminary results, but the recovery efficiencies from both protocols were almost identical. The average cell recovery obtained using harvesting protocol A (protease + strainer) is 51.1 ± 9.4%. The lowest recovery obtained with this protocol (18.1%) was attained using dissolvable microcarriers – this low efficiency may be due to prolonged exposure to the protease but was at first attributed to dissolvable microcarriers being able to pass through the strainer and contaminate the cell suspension; however, dissolvable microcarriers are moderately rigid, and have a narrow size distribution (200-300 µm), which should prevent any microcarriers from passing through a 100 µm mesh strainer, and therefore,

***Table 3. hiPSC recovery efficiency after expansion on microcarriers**. Values obtained for cell recovery efficiency after expansion of hiPSCs under 3D dynamic culture conditions, using different combinations of polystyrene (PSM) and dissolvable (DM) microcarriers, Matrigel™ (Mat) and Synthemax®II (SII) surfaces, and Essential 8™ (E8) and mTeSR™1 culture media, and corresponding viability of the cells recovered, comparing two different harvesting protocols: A - use of protease and a 100µm mesh cell strainer; B – use of protease and pectinase/EDTA harvesting solution.*

Cell recovery efficiency after expansion		
hiPSC expansion conditions	Cell recovery Efficiency % (cell viability %)	
	Harvesting Protocol	
	A	B
PSM+Mat+E8	56.0	-
PSM+SII+mTeSR1	41.4 (78.9)	-
DM+Mat+mTeSR1	18.1 (89.8)	42.2 (93.0)
DM+SII+mTeSR1	42.9	61.0
DM+SII+mTeSR1 (2)	64.0 (90.9)	49.3 (97.8)

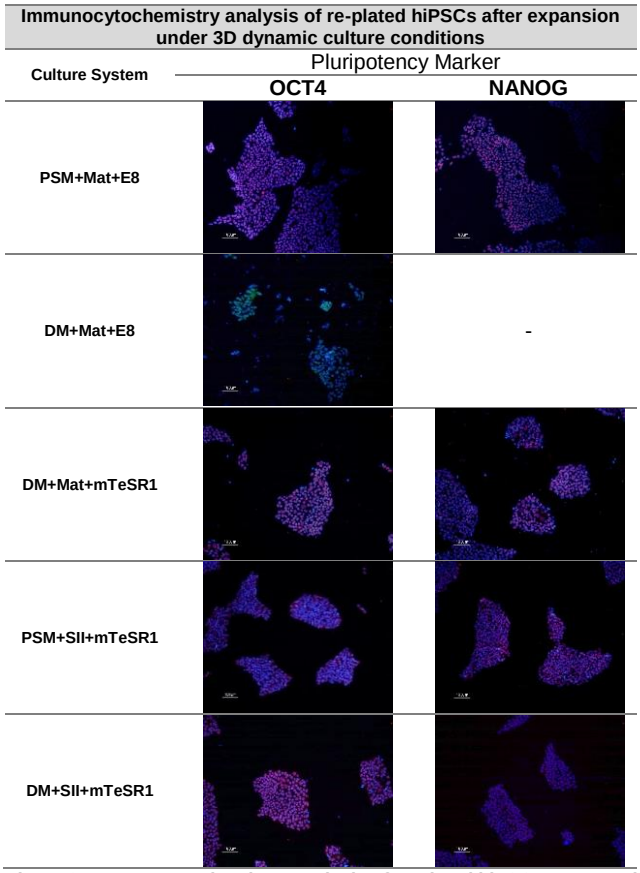

***Figure 9. Immunocytochemistry analysis of re-plated hiPSCs recovered after expansion under 3D dynamic culture conditions.** Merging of immunofluorescence imaging (red or green) of intracellular pluripotency markers OCT4 and NANOG, with DAPI counterstaining imaging (blue), on days 2-3 after re-plating on Matrigel-coated multi-well plates. Bar scale: 100µm.*

should not interfere with cell recovery. When using protocol B (protease + harvesting solution) the average cell recovery is 50.8 ± 7.8%. Regarding cell viability, the use of harvesting protocol A has a viability average of 86.5 ± 5.4%, while protocol B shows, on average, a cell viability of 95.4 ± 2.4%, slightly higher than the first one. These experiments are only the preliminary testing regarding cell recovery from dissolvable microcarriers, and recovery protocol B has yet to be optimised. The ultimate goal would be to be able to recover cells without the use of a protease. As it is, protocol B already allows for easier cell recovery, with the benefit of avoiding the risk of microcarriers debris on cell suspension (broken microcarriers that get through the filters). Scale-up of this protocol would also be easier to perform, and more cost effective, as there would be no need for a large-scale filtration step.

Following the application of the recovery protocols, cells were re-plated onto Matrigel™-coated multi-well plates in order to understand if they retained their ability to expand as colonies and to characterise those cells through immunocytochemistry assays to establish whether cells still expressed pluripotency surface and intracellular markers. Re-plating of the cells allowed to confirm that hiPSCs did not lose their characteristic colony morphology after being expanded in 3D dynamic culture conditions, and were able to expand normally as 2D monolayers. When colonies reached the appropriate size, immunocytochemistry analysis was performed in order to characterise the cells obtained and make sure cells were expressing pluripotency

markers and not showing signs of differentiation. The immunocytochemistry analysis of the colonies obtained after re-plating the cells showed that all cells maintained normal expression of pluripotency markers OCT4 and NANOG (**Figure 9**), as well as SSEA4 and TRA-1-60 (results not shown), and there was no expression of early differentiation markers (results not shown), which allows the conclusion that neither of the harvesting protocols used affect hiPSCs in any of the characteristics analysed.

FLOW CYTOMETRY: For the characterisation of cell populations used to inoculate the microcarriers, and the resulting populations after expansion under stirred culture conditions, samples of both cell populations were analysed through flow cytometry. This analysis was performed in order to quantify cells expressing pluripotency markers and the alterations in that expression after expansion; the goal was to determine the percentage of cells expressing pluripotency markers and verify if those numbers were maintained at the end of culture. According to the values obtained (**Table 4**), in hiPSCs cultured on dissolvable microcarriers, expression of pluripotency marker OCT4 decreased only when cells were expanded on Matrigel™-coated dissolvable microcarriers on Essential 8™ culture medium (DM+Mat+E8) (2.8% less expression of OCT4 after 5 days of culture), which may be attributed to the transition from a 2D static culture system to a 3D dynamic culture system. Expression of SSEA4 and TRA-1-60 never decreased when compared to the expression of those same pluripotency markers on the respective hiPSC population used to inoculate the spinner-flask, with expression values increasing 0.5 to 5.5%. In all cases, the differences observed are not very significant – therefore it is possible to consider that no alterations in expression of pluripotency markers occurred. Regarding cells expanded on polystyrene microcarriers, OCT4 and TRA-1-60 expression dropped 9.9 and 17.4% respectively when cells were cultured on PSM+Mat+E8, which could be due to the prolonged time of cell culture, however, no significant alterations were obtained on hiPSCs cultured on PSM+SII+mTeSR1 which could indicate that prolonged culture time on its own is not a decisive factor, and only affects cells cultured on a specific substrate (Matrigel™) or with a specific culture medium (E8™). Overall, hiPSC expansion on dissolvable microcarriers seems to have no negative effect on pluripotency markers expression.

Table 4. Flow cytometry analysis of hiPSCs obtained after expansion under three-dimensional dynamic culture conditions. *Percentage of cells expressing the pluripotency markers; cells were analysed at day 0 (inoculation) of each experiment, and on the final day of each expansion in order to quantify the cells expressing surface – TRA-1-60 and SSEA4 – and intracellular – OCT4 – pluripotency markers. DM – dissolvable microcarriers; PSM – polystyrene microcarriers; Mat – Matrigel™; SII – Synthemax®II; E8 – Essential 8™; mTeSR1 – mTeSR™1; A1 – adhesion protocol 1; A2 – adhesion protocol 2; A3 – adhesion protocol 3.*

hiPSC characterisation after expansion on 3D dynamic culture - Flow cytometry analysis				
Culture Conditions (Adhesion Protocol)	**Day**	**% of cells expressing the pluripotency marker**		
		OCT4	**TRA-1-60**	**SSEA4**
DM+Mat+E8 (A2)	0	97.7	96.2	97.9
	5	94.9	96.7	99.7
DM+Mat+E8 (A3)	0	95.5	90.5	-
	7	96.6	96.0	-
DM+SII+mTeSR1 (A1)	0	95.5	-	99.0
	7	98.4	-	91.8
DM+Mat+mTeSR1 (A1)	0	94.8	-	95.3
	6	98.5	-	99.7
PSM+Mat+E8 (A1)	0	98.3	92.5	-
	10	88.4	75.1	-
PSM+SII+mTeSR1 (A1)	0	99.5	-	96.6
	11	99.0	-	89.8

EMBRYOID BODY FORMATION: In order to verify if cells obtained after expansion on dissolvable microcarriers under dynamic culture conditions retained their ability to differentiate into cell types derived from the three germ layers of embryogenesis, some of the cells recovered after expansion were used for embryoid body formation assays. Two independent assays were performed, using cells cultured on dissolvable microcarriers with either Matrigel™ or Synthemax®II surfaces. On both experiments, the cells obtained at the end of the assay showed positive results for markers of cell types from each of the three germ layers (**Figure 10**) as well as the morphology expected, and negative results for the pluripotency marker tested (OCT4, results not shown), showing that even after being subjected to different stimuli during expansion under 3D dynamic culture conditions, hiPSCs maintain their ability to differentiate into cell types from the three germ layers of embryonic development, which is one of their two main characteristics.

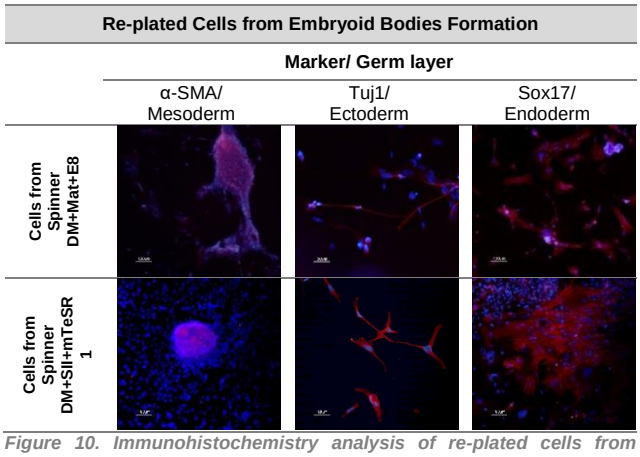

Figure 10. Immunohistochemistry analysis of re-plated cells from Embryoid Bodies differentiation*. Merging of immunofluorescence imaging (red) of differentiation markers SOX17, TUJ1, and α-SMA and DAPI counterstaining imaging (blue) Analysis performed 7 days re-plating of the cells from embryoid body differentiation on laminin-coated multi-well plates.*

CONCLUSIONS AND FUTURE WORK

hiPSC culture *in vitro* is challenging process, but many progresses have been achieved in recent years. In the development of new supports for hiPSC expansion is it very important to take into account the need to comply with GMP, the development of chemically-defined and xeno-free systems – which in this case include both the microcarriers and surface coating –, the possibility of scaling up the system, the downstream processing, and ultimately, the cost effectiveness of the whole process. Regarding hiPSC adhesion and expansion on dissolvable microcarriers in 3D culture systems, overall, adhesion to dissolvable microcarriers was comparable to adhesion to polystyrene microcarriers when using Matrigel™ coating, and although adhesion was lower when using Synthemax®II dissolvable microcarriers, the fold increase obtained at the end of the expansion was higher when compared to Synthemax®II polystyrene microcarriers. hiPSC expansion allowed to achieve higher cell numbers – 4.61 ± 0.17x10^7 cells on Matrigel™-coated dissolvable microcarriers using E8™ culture medium, and 3.80 ± 0.73x10^7 cells on Synthemax®II dissolvable microcarriers using mTeSR™1 culture medium – within a shorter time frame (5 days), than the cell numbers obtained after expansion on polystyrene microcarriers, which took 9 days to achieve maximum yield; meaning hiPSC expansion on dissolvable microcarriers is a more cost

effective choice when directly compared to polystyrene microcarriers, and making dissolvable microcarriers a very promising product for hiPSC expansion. Concerning cell recovery, efficiency and cell viability results obtained using two different cell harvesting protocols were very similar – both on harvesting from polystyrene microcarriers using a protease and a strainer (51.1 ± 9.4% cell recovery with 86.5 ± 5.4% cell viability), and on recovery from dissolvable microcarriers using a pectinase/EDTA harvesting solution (50.8 ± 7.8% cell recovery with 95.4 ± 2.4%,); Nevertheless, cell recovery protocol from dissolvable microcarriers is simpler, easier, and requires less manipulation of the cells, requiring only one additional reagent that can be used together with the protease used for cell harvesting from polystyrene microcarriers.

Since both cell recovery efficiency and cell viability after recovery are very similar, and there is no alteration of the characteristics of the cells after re-plating, the choice between polystyrene and dissolvable microcarriers has to be made taking into account not only hiPSC expansion, but also microcarrier preparation and manipulation, and the differences between the harvesting protocols. Due to their composition, dissolvable microcarriers adhere very easily to polystyrene surfaces, which means that all manipulations should be performed using glass materials and failing to do so will result in extensive losses of microcarriers during manipulation, especially in the presence of culture media. Also, due to their transparent appearance, dissolvable microcarriers are very difficult to identify in culture media when they are not covered in cells – which could be easily overcome by adding some colour to the microcarriers. Nevertheless, because packaged dissolvable microcarriers are completely sterilised through γ-radiation and, in some cases, their surface is functionalised with small peptides during production (Synthemax®II dissolvable microcarriers), preparation time for these microcarriers requires only that they are fully hydrated before being used, which widely shortens preparation time and, therefore, the time needed to begin an experiment. Moreover, regardless of cell recovery efficiency, the recovery protocol itself – using a combination of a protease and a pectinase/EDTA harvesting solution – requires less mechanical manipulation of the cells which makes it a less time consuming and more practical approach to cell recovery than the traditional protocol of protease incubation and filtration step, especially when considering large scale production. Furthermore, the translucent appearance that makes it so challenging to work with dissolvable microcarriers is also one of their best characteristics; because these microcarriers are completely transparent they are much easier to observe through microscopic analysis, allowing for better visualisation of the cells on the surface of the microcarriers. Taking all this into consideration, although it may present some challenges, working with dissolvable microcarriers allows a faster and easier preparation of the microcarriers, a simpler and faster recovery of the cells after expansion, and, in general, a better monitoring of cell growth throughout expansion.

The experiments described throughout this report represent only the initial studies of hiPSC expansion on dissolvable microcarriers. The next steps should include further optimisation of hiPSC expansion under dynamic conditions using laboratory scale spinner flasks, and further testing and optimisation of cell recovery after said expansion. All experiments should be replicated for this cell line and confirmed using a different hiPSC line to properly validate all the results obtained so far. In addition to proper validation it would be important to perform a complete characterisation of the cells recovered after expansion on dissolvable microcarriers to complement the characterisation that was described here; the full characterisation panel should include: immunostaining for detection of pluripotency markers, alkaline phosphatase staining, flow cytometry analysis, RT-PCR, analysis of Embryoid Bodies formation and spontaneous differentiation into cell types from the three germ layers, karyotype analysis, and, ultimately, the formation of teratomas on immunocompromised mice. Another very important step will be a parallel cost analysis in order to compare the cost of hiPSC *in vitro* production using polystyrene microcarriers anda harvesting protocol that includes the use of a protease and a strainer, with the production of the same amount of cells on dissolvable microcarriers using a recovery protocol that requires a protease and the appropriate harvesting solution. If hiPSC expansion on dissolvable microcarriers and respective recovery of those cells proves to be successful, the final outcome from these experiments will be the establishment of a new protocol for hiPSC expansion under chemically-defined and xeno-free conditions, and a downstream cell recovery process which does not require the filtration step to separate cells from the microcarriers, but rather relies on the dissolution of the microcarriers to recover the cells without damage or loss. Further optimisation of cell recovery would ideally lead to a protease-free recovery protocol, but at this time, this has yet to be accomplished. Ultimately, the protocol established ought to comply with all GMP guidelines and should be an important step towards an integrated process of hPSC expansion – with or without controlled differentiation of the cells after expansion – and downstream processing within a closed system.

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