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Stem Cell Exosome Production on the SciVario® twin, a Flexible Controller for Your Bioprocess Needs.

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Abstract

Exosomes derived from mesenchymal stem cells (MSC) are a new alternative to restore tissues and organs, avoiding the limitations associated with stem cell therapy. MSC-derived exosomes are involved in MSC's paracrine functions related to cell-to-cell communications and tissue reconstruction, leading to cell self-renewal and differentiation. Despite the benefits of stem cell use, there are limitations, including the large-scale production of cells that mimic physiological conditions. Therefore, increasing the mass production of exosomes in a controlled environment is the necessary next step. In this study, we used a combination of SciVario® twin bioreactor control system and BioBLU® 1c Single-Use Vessels as a model for large scale exosome production in bioreactors. The SciVario twin is a future-proof bioreactor control system developed by Eppendorf. Following the agile development principle, the controller was designed with a maximum working volume of 3.7 L at launch, but the flexibility of the system allows for future hard- and software updates using vessels as large as 40 L.
In this application note, we monitored and analyzed the metabolites derived from the human induced Pluripotent Stem Cells (hiPSC) as well as cell adhesion and morphology, suggesting that we obtained an excellent physiological environment over time. Furthermore, we isolated, purified and quantified (by CD63 ELISA kit) the exosomes produced from MSC.

Introduction

Regenerative medicine is a multidisciplinary field that engineers the structure and function of tissues and organs. Due to their ability to migrate to the site of injury and promote tissue regeneration through paracrine factors (secretome), mesenchymal stem cells have become the most widely used stem cell type for such investigations [1-3]. However, problems associated with inadequate cell localization and low cell survival rate within the target tissue make the MSC less attractive. Recently, paracrine factors have attracted increasing interest due to their potential at overcoming the limitations of MSC. Extracellular Vesicles (EV), including exosomes, are one of the most important paracrine effectors involved in intracellular communication and trafficking [4].

Exosomes are lipid bilayer vesicles with a diameter ranging from 30 to 200 nm, which can be easily confirmed by surface markers such as CD9, CD63, CD81 of tetraspanins [5].

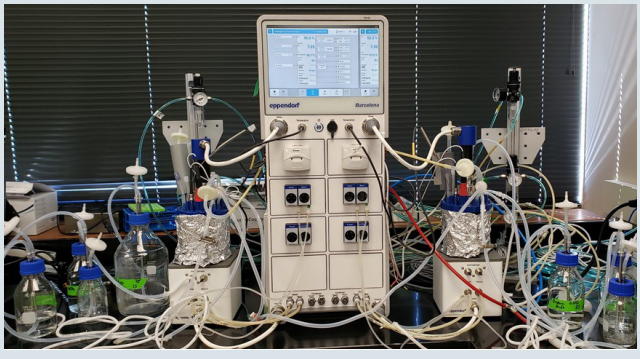

Fig. 1: Human iPSC-derived MSC exosome production in a medium exchange-batch culture using the SciVario twin bioreactor control system and BioBLU 1c Single-Use Vessels.

They offer several advantages compared to cell-based approaches, including exceptional stability and biocompatibility. Moreover, they can be delivered to target tissues easier than whole cells, and can migrate across the blood brain barrier [6]. In addition, since exosomes have the ability to conduct immune modulation, there is much less risk for tumor formation or inhibition of inflammatory cell migration [7, 8]. Moreover, the lack of complex metabolism of exosomes and the influence of the environment on target tissues reduces complications upon use.

In this study, we used BioBLU 1c Single-Use Vessels as bioreactors for human Induced Pluripotent Stem Cells (hiPSC)-derived MSC exosomes production and characterization. We employed the SciVario twin bioreactor control system as a controller device [9] (Figure 1) and collagen-coated microcarriers as cell culture support. We analyzed the cell growth, viability and metabolic activity (levels of glucose, ammonia and lactate in the medium) as well as the exosomes abundance at different times through CD63 Elisa assay.

Material and Methods

SciVario twin

We used the SciVario twin bioreactor control system to perform medium exchange-batch culture using BioBLU 1c Single-Use Vessels equipped with a single pitched-blade impeller. Each bioreactor unit possesses three universal port connectors for pH (port 1) and DO (dissolved oxygen; port 2) sensors, a temperature control block that combines electrical heating and water cooling, agitation control, and a gas module that includes 1 TMFC (Thermal Mass Flow Controller) with an ultra-high turndown ratio of 1:12,000, and 4 solenoid valves for automated 4-gas mixing.

Cell culture in T-flasks and multilayers flasks

We purchased the iPSC-derived MSC from American Type Culture Collection (ATCC®, ACS-7010™) and we established the culture by seeding 10,000 cells/cm2 per T-75 flask (Eppendorf, 0030711122) using either 15 mL of DMEM/F12 medium (Thermo Fisher Scientific® ,11320033) supplemented with 10 % fetal bovine serum (FBS) (Thermo Fisher Scientific, 16000044) and 1 % antibiotic-antimycotic (100X) (Sigma Aldrich, A5955) or mesenchymal stem cell basal medium (ATCC, PCS-500-030™) (ATCC medium) supplemented with 7 % FBS, (Thermo Fisher Scientific, 16000044), 125 pg/mL rhFGF basic, 15 ng/mL rh IGF-1, 2.4 mM L-alanyl-L-glutamine (Mesenchymal Stem Cell Growth Kit (ATCC PCS-500-041™) and 1 % antibiotic-antimycotic (100X) (complete ATCC medium).

We incubated the flasks at 37 °C and 5 % CO_2 in a CellXpert® C170i Incubator (Eppendorf, 6731011045). When the cultures had reached approximately 80 % to 90 % confluence, we made a second passage as follow: For each flask, we aspirated the spent medium, rinsed the cell layer with 5 mL of Dulbecco's phosphate buffered saline (DPBS) (Thermo Fisher Scientific, 14190144) to remove residual traces of serum and aspirated the DPBS. Then, we added 3 mL of pre-warmed trypsin-EDTA solution (0.25 %) (Thermo Fisher Scientific, 25200056) and incubated flasks at 37 °C and 5 % CO_2 for 5 minutes. When most cells were detached, we added the double volume of the ATCC complete medium to each flask. We collected and counted the cells using a Vi-CELL XR cell viability analyzer (Beckman Coulter®). Finally, we centrifuged the iPSC-derived MSCs at 1500 rpm (Centrifuge 5430R, Eppendorf, 022620601) for 5 minutes (we aspirated the neutralized dissociation solution from the cell pellet), resuspended in DMEM/F12 medium or ATCC complete medium and seeded at a density of 10,000 cells/cm² in T-175 flasks (Eppendorf, 0030712129).

We performed a third passage (required to achieve the appropriate inoculation cell density) following the same cell subculture procedure described above, but seeding the cells in multilayer flasks, a HYPERFlask® M cell culture vessel (Corning®, 09-761-22) with the same cell culture density.

Microcarriers preparation

We used collagen coated cross-linked polystyrene microcarriers (Pall Corporation, C-221-020) as support matrix. We conducted the sterilization process according the manufacturer's instructions [10] but with a few modifications. Briefly, we transferred 17 g of collagen coated microcarriers (125 – 212 µm of size range and 360 cm²/g of superficial area) into a 250 mL glass bottle along with 100 mL of DPBS. Then, we autoclaved the glass bottle at 121°C for 30 minutes. After the sterilization process, we carefully aspirated the autoclaved PBS, added 100 mL of medium to the microcarriers and incubated for 24 hours.

Sensor calibration

Prior to the preparation of the BioBLU 1c Single-Use Vessels, we connected the ISM® gel-filled pH sensors to the SciVario twin bioreactor control system that was automatically detected by the software of the controller. We performed the calibration process according to the operation's manual using buffer solutions of pH 7 and pH 4 as "zero" and "span" respectively. Then, we disconnected the pH sensors and sterilized them in an autoclavable pouch.

BioBLU 1c Single-Use Vessel preparation and process parameters

We outfitted each BioBLU 1c with magnetic drive and one

pitched-blade impeller with a pH sensor and a dip tube (Eppendorf, M1287-908402) along with a compression probe adapter (Eppendorf, M1287-503001), both inserted in a spare PG 13.5 port under aseptic conditions in the BioSafety Cabinet following sterilization. In addition, we fitted a polarographic DO sensor (Mettler Toledo®) in the headplate, an exhaust condenser, a sampling dip tube, a 4-gas mixing line connected to the gas sparge port and an extra N_2 gas line connected to an overlay gas port and 3 liquid addition ports. There is one additional port for the inoculation and medium addition, one for the addition of the base and another for the addition of 0.1 % antifoam (Pluronic®-F68 surfactant, Life Technologies®, 24040-032). We then placed the assembled vessels in their respective temperature control blocks to maintain constant temperature. Finally, we introduced the DMEM/F12 or ATCC complete medium into each vessel and conditioned the vessels for at least 24 hours under the parameters and setpoints listed in Table 1.

Table 1: Process parameters and setpoints of the first and second experiments.

	First Experiment	Second Experiment
Parameters	**Setpoints**	
Starting volume	700 mL	
Ending volume	1 L	
Initial agitation	80 rpm (0.2 tip speed)	
Temperature	37 °C	
Inoculation density	3 x 10^4 cell/mL	10.4 x 10^4 cells/ mL
Cell culture medium	DMEM/F12 medium	ATCC complete medium
DO Setpoint	40% (P=0.1; I=0.001)	
pH Setpoint	7.2 (deadband = 0.1), cascade to CO_2 (acid) cascade to 0.45 M sodium bicarbonate (base)	7.6 (deadband = 0.1), cascade to CO_2 (acid) cascade to 0.45 M sodium bicarbonate (base)
Overlay N_2 gas flow	0.20 SLPM	0.25 SLPM
Gassing range	0.1 SLPH-30 SLPH	
Gassing cascade	Set O_2 % at 30 % controller output to 21 % and at 100 % controller output to 21 %. Set flow at 0 % controller outputto 0.5 SLPH, and at 100 % controller output to 30 SLPH.	

Cascade control of DO

We establish the following DO cascade to control the culture with air only, without Oxygen suplementation:
"Set O_2 % at 30 % controller output to 21 % and at 100 % controller output to 21 %. Set flow at 0 % controller output to 0.50 SLPH and at 100 % controller output to 30 SLPH."

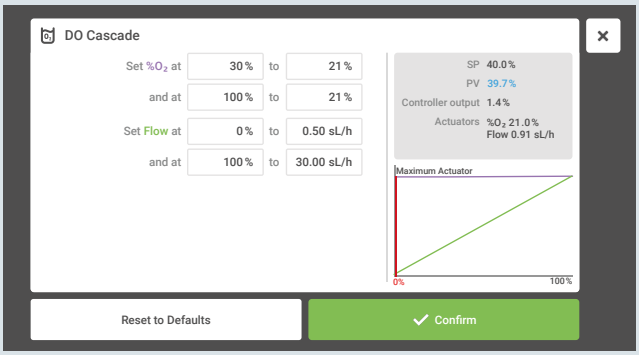

Fig. 2: Visualization of the DO cascade parameters used in our experiment.

hiPSC-derived MSCs culture on BioBLU 1c Single-Use Vessels

We harvested the cells from multilayer flasks (see section "**Cell culture in T-flasks and multilayers flaks**") and seeded at a density described in Table 1 into the glass bottles containing collagen coated microcarriers (see section "**Microcarriers preparation**") and 300 mL of DMEM/F12 or ATCC complete medium. We kept the bottles in a CellXpert C170i incubator for 2 hours at 37 °C and 5 % CO_2 under static conditions. Following incubation, the 300 mL of cells on microcarriers were then transferred to a BioBLU 1c bioreactor, already filled with 700 mL medium, reaching the working volume of 1 L and maintaining the microcarriers concentration at 17 g/L. Furthermore, we set the overlay N_2 gas flow between 0.2 and 0.25 SLPM to maintain the DO level at 40 %.

Medium exchange

As we previously described in the "**BioBLU 1c Single-Use Vessel preparation and process parameters**" section, we inserted a dip tube along with a compression probe adapter in a spare PG 13.5 port allowing for medium exchange without disturbing the cell culture. Briefly, after day 5 of cell culture, we performed a medium exchanged every two days until day 9 and then on daily basis as required. We stopped the agitation and gases flow for 5 minutes, allowing the microcarriers to settle at the bottom of the BioBLU 1c and exchanged 10 % of medium, using the dip tube to remove the cell culture medium from the surface and using the feeding port to add fresh medium. Moreover, in the second experiment, additional glucose solution with a concentration of 1 g/L was added on day 11 of the culture to the fresh medium to increase glucose levels in the cell culture with subsequent medium exchanges.

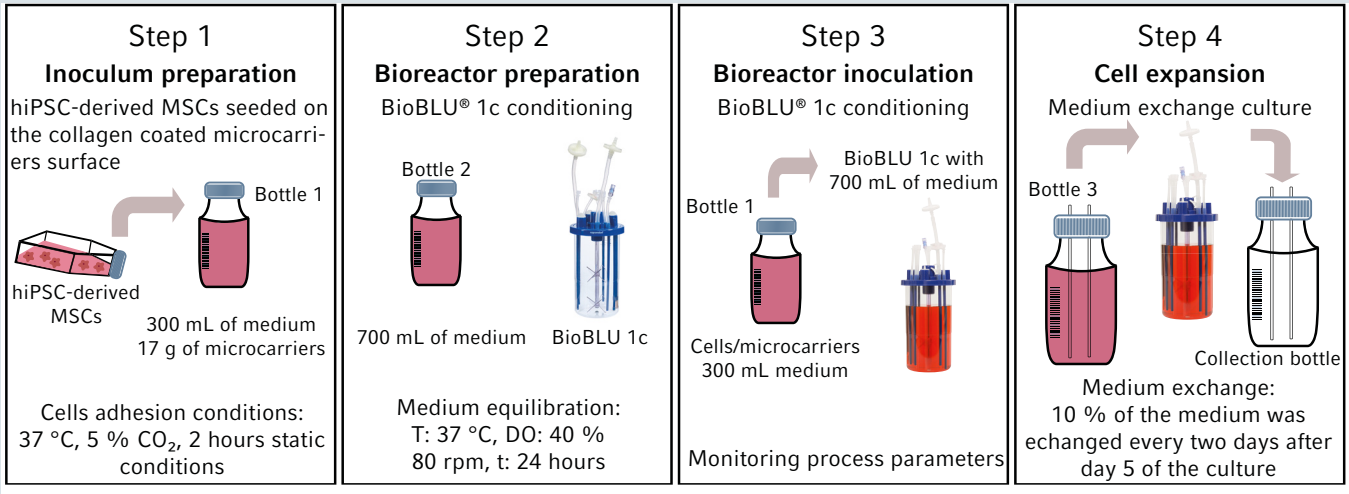

Fig 3: Process flow diagram of the experimental setup

Exosome collection assay

After day 5 of culture, we collected 50 mL of iPCS-derived MSC/microcarriers/medium using a Labtainer Pro BioProcess Container (BPC) bag with line sets (Thermo Fisher Scientific, SH30658.11). We transferred the 50 mL into 50 mL conical tube (Eppendorf, 0030122186) allowing the iPCS-derived MSC/microcarriers system to settle to the bottom of the tube. We removed the supernatant and added additional 50 mL of medium with the same composition (dependent on the experiment) but replacing the regular FBS by 2 % exosome-depleted FBS (ED-FBS) (Fisher Scientific, A2720801) and transferring the contents into a 250 mL shake flask (Schott Duran®). Finally, we incubated the iPSC-derived MSC/microcarriers system for 48 hours at 37 °C, 5 % CO_2 and 50 rpm using the New Brunswick™ S41i CO_2 incubator shaker. We repeated the collection process at days 8, 11, and 14 of culture.

Isolation and purification of human iPSC-derived MSC exosomes

At 48 hours after each collection day, we removed the supernatant and performed the isolation and purification of exosomes according to the ExoQuick-TC PLUS (System Bioscience, EQPL10TC-1) protocol with some modifications. Briefly, we centrifuged the supernatant at 3000 x g (Centrifuge 5430R for 15 minutes and filtered using a 0.22 µm filter to remove cells and cell debris). Then, we filtered the supernatant using the Amicon® Ultra-15 Centrifugal system (Millipore Sigma, UFC910024), with molecular weight cut-off (MWCO) of 100,000, to concentrate the exosome fraction. We transferred the supernatant to a sterile tube, added ExoQuick-TC Exosome Precipitation Solution in a ratio of 5:1 and incubated at 4 °C overnight. After the incubation period, we centrifuged the ExoQuick-TC/supernatant mixture at 1500 × g for 30 minutes, bringing the exosomes pellet to the bottom of the tube. Then, we carefully aspirated the supernatant, centrifuged the pellet again at 1500 × g for 5 minutes to remove any residue of ExoQuick-TC solution and resuspended the exosomes in 250 µL of resuspension buffer.

Finally, we washed the microsphere beads three times with resuspension buffer and added 250 µL of the exosome sample to the washed microspheres. The microspheres beads are customized to reduce protein carryover from serum/plasma and tissue culture. We collected the supernatant containing the purified exosomes after mixing the exosomes and microspheres at room temperature on an inverting shaker for 15 minutes and centrifuged the mixture for 5 minutes at 8500 rpm (Centrifuge MiniSpin® Plus, Eppendorf, 022620207).

Cell viability and metabolic activity

We collected a sample every day in the course of the first experiment or every two days in the second experiment from the bioreactors to determine the cell viability, cellular density, and metabolites concentration (glucose, ammonia (NH_3), and lactate). This was accomplished by connecting a sterile 5 ml syringe to the Luer Lock sample port. Then, we discarded 3 mL of dead volume and collected again 3 mL (using a new 5 mL sterile syringe) as a workable sample for analysis. We transferred the viable samples to a 15 mL Eppendorf conical tube, allowing the microcarriers to settle in the tube. Before aspirating the medium, we used 1 mL to measure the metabolite levels employing a Cedex® Bio Analyzer (Roche). Then, we washed the microcarriers with 1 mL of DPBS and incubated at 37 °C for 5 minutes with 300 µL of

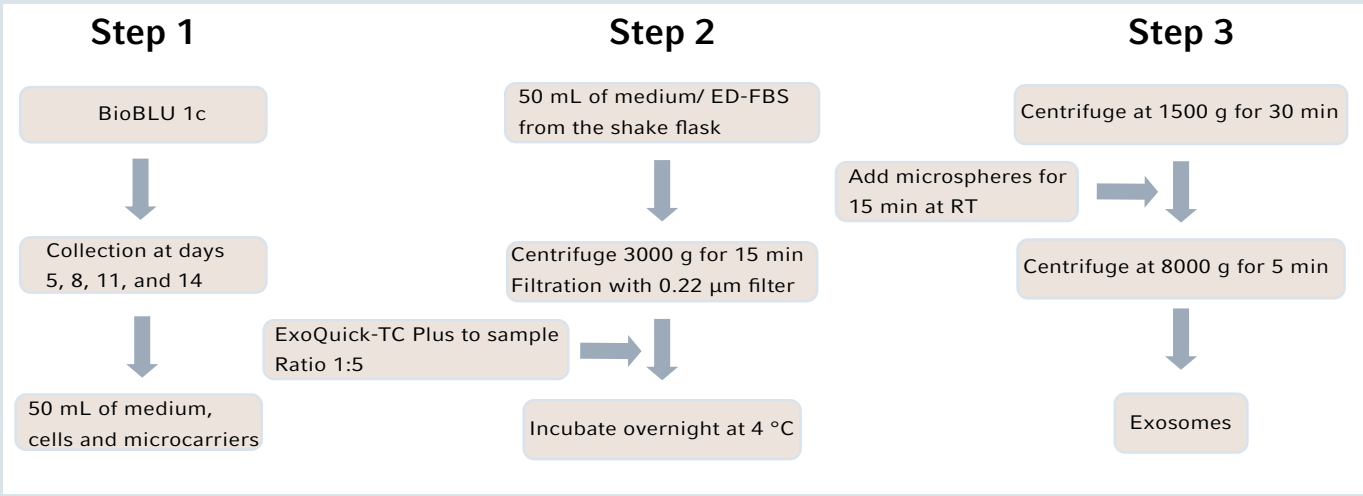

Fig 4: Isolation and purification of iPSC-derived MSC exosomes.

trypsin (0.25%).

To inactivate the trypsin solution, we added 700 µL of fresh medium and gently pipetted the sample up and down, creating a cell suspension. We filtered the cells and microcarriers through a 40-micron strainer into a 50 mL Eppendorf conical tube. Finally, we measured the cell viability and density using a Vi-Cell XR Viability Analyzer.

Quantification of human iPSC-derived MSC exosomes

Following the exosomes enrichment by the ExoQuick-TC PLUS protocol, we quantified tetraspanin-containing exosomes through ExoELISA-ULTRA CD63 Kit (System Bioscience, EXEL-ULTRA-CD63-1). We immobilized the exosomes on the surface of the CD63 ExoELISA microtiter plate. Then, we finished the assay according to the manufacture's protocol instructions.

iPSC-derived MSC morphology

To study the human iPSC-derived MSC morphology during the cell expansion process in the BioBLU 1c Single-Use Vessel, we followed the protocol described below:
We collected 500 µL of cells/microcarriers/medium at days 5 and 9 of culture and transferred them to a 1.5 mL Eppendorf microtube, allowing the microcarriers to settle to the bottom. Then, we removed most of the supernatant and used the cells and microcarriers to facilitate visualization of the bright-field images, using an EVOS® FL fluorescence microscope (Life Technology).

Results and Discussion

To demonstrate the ability of human iPSC-derived MSC to secrete exosomes, we performed two medium exchanges using the BioBLU 1c Single-Use Vessels and SciVario twin bioreactor control system. After the initial expansion of the cells in T-flasks culture conditions, we analyzed their stemnnes capacity by flow cytometry noting that they displayed the typical phenotype of MSCs (see Figure 5). Specifically, cells were positive for CD90 and CD29 (typical MSC markers) and negative for hematopoietic markers such as CD34 and CD11b.

Next, we established the optimal cell culture conditions. We used 17 g/L microcarrier concentration to provide cell support and employing DMEM/F12 as the cell culture medium. The inoculum was ready after the cell expansion and the initial cell attachment process provided by the microcarrier in the CellXpert C170i incubator (at 37 °C and 5 % CO_2 for 2 hours under static conditions). We then inoculated the BioBLU 1c Single-Use Vessel at an initial cell density of 5 x 10^3 cells/cm^2 (3 x 10^4 cell/mL) under a controlled environment. In addition, we added the anti-foaming agent Pluronic-F68 surfactant (0.1 %) to the medium.

After the initial cell expansion, the cell count was erratic, especially after each collection day (Figure 6). We attributed this behavior to the low inoculation density and the use of DMEM/F12 cell culture medium whose composition may be suboptimal for the iPSC-derived MSC expansion. Additionally, the DO level is known to be an important factor that can affect the growth and performance of the MSC. Although some studies reported that hypoxic conditions enhance the therapeutic potential of MSC and its secretome, others indicated that no matter under what conditions the experiment is established (hypoxia or normoxia), the collected secretome showed similar effects [11]. In this study, the O_2 demand is expected to be low due to the low inoculation cell

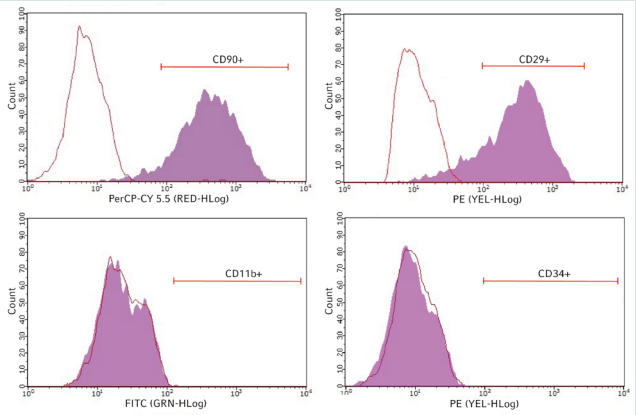

Fig. 5: Immunophenotyping of human iPSC-derived MSC at passage 3. We immunophenotyped the human iPSC-derived MSC for CD90, CD29, CD34, and CD11b (filled histogram). We used unstained cells as control (empty histogram).

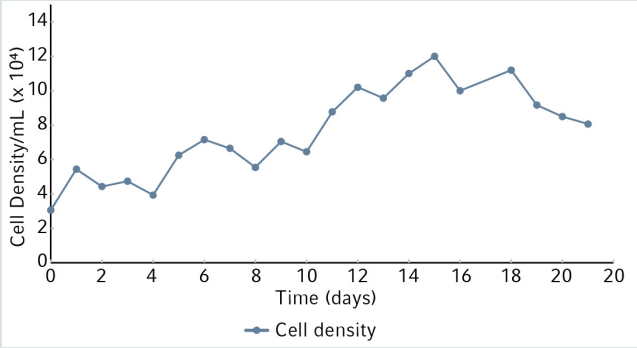

Fig. 6: iPSCs-derived MSCs cell growth trending BioBLU 1c Single-Use Vessel with DMEM/F12 medium.

density used, which is why we established a cascade with air flow only, without oxygen supplementation (21%) (Figure 2) throughout the experiment to reach the desired setpoint. We also added an N_2 overlay to balance the system using an external flow meter (Omega Engineering, FMA-1608A) directly connected to the nitrogen gas outlet, bypassing the controller.

Overall, cell growth increased 4-fold more than the initial cell density on day 15 of culture, but the final density was low.

We performed a second experiment to increase the iPSCs-derived MSCs inoculation density and the exosomes production yield from each sample. To achieve that, we changed the cell culture conditions of the iPSC-derived MSC relative to the first experiment. First, we performed the cell expansion and the initial cell attachment to the microcarriers in ATCC complete medium. Then, we increased the inoculation cell density to 17 x 10^3 cells/cm^2 (10.4 x 10^4 cell/mL), maintaining the microcarrier concentration at 17 g/L. We kept the rest of the conditions the same as for the first experiment, with the exception that the overlay gas flow was increased to 0.25 SLPM to provide a better balance to the minimum air flow. Additionally, we performed a medium exchange on a daily basis starting from day 5 of the culture. We observed an initial lag phase 24 hours after the inoculation followed by a steady increase of cell growth between days 1 and 9 of culture.

We then determined the consumption of glucose and production of lactate and NH_3 while maintaining the concentration of lactate and NH_3 below 1.2 g/L and 1.2 mmol/L respectively during the whole run. The glucose level was significantly lower in the ATCC complete medium than in the DMEM/F12, necessitating the addition of 1 g/L of glucose.

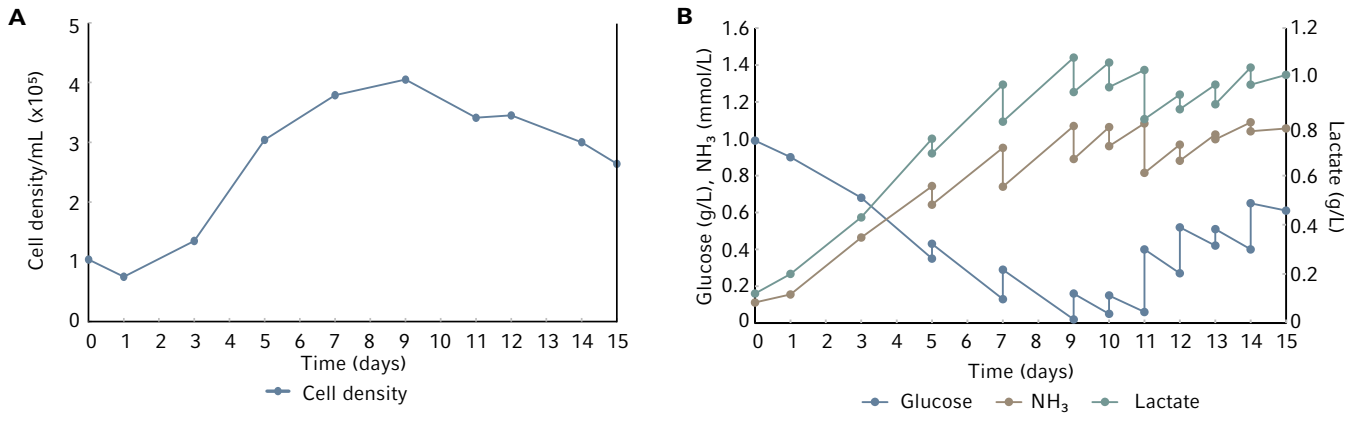

Fig. 7: iPSCs-derived MSCs growth profile in BioBLU 1c Single-Use Vessel with ATCC complete medium. A: iPSCs-derived MSC density and viability. B: Metabolic profile.

The culture reached the stationary phase around day 9, followed by a subsequent decrease in cell density until day 15 (Figure 7). At day 9, the iPSC-derived MCS reached a maximum cell density of 4.1 x 10^5 cell/mL. However, we observed substantial microcarrier aggregation later in the course of the run. Therefore we based the cell count in the later stages on only the floating microcarriers, thus the true average of the cell expansion in the vessel is undoubtedly higher.

To isolate and characterize the iPSC-derived MSC exosomes, we used a labtainer bag with line sets connected to the BioBLU 1c. At day 5, 8, 11 and 14, we collected the exosomes from the ATCC complete culture medium containing ED-FBS and purified them using the ExoQuick-TC PLUS kit. As shown in Figure 8A, exosomes are highly enriched in proteins in which tetraspanins (membrane proteins: CD63, CD9, CD81, CD82) play a key role in cell invasion, penetration and fusion events. We used a direct Enzyme-Linked ImmunoSorbent Assay (ELISA), specifically the ExoELISA-ULTRA CD63 Kit to quantify the exosome abundance. We incubated the exosomes with a primary anti-CD63 antibody that binds the tetraspanin protein CD63 on the exosomal surface. The results show that the number of iPSC-derived MSC exosomes constantly increases from day 5 (2.6 x 10^{10}) to day16 (8.6 x 10^{10}, Figure 8B). In addition, we found a direct correlation between cell density and secreted exosomes up to day 9 while the decrease in cell density in the bioreactor did not influence the exosome secretion after the stationary state.

To study the three-dimensional cell morphology on collagen coated microcarriers, we collected samples at days 5 and 9 of culture and visualized them through bright-field images (Figures 9A and B).

Taking into account the limitations of using bright-field systems for live cell imaging, we evaluated the morphology of human iPSC-derived MSC, demostrating that at the early stage of the cell growth profile, a few cells attached on the microcarriers surface (Figure 9A) and progressively formed intermicrocarriers cellular bridges and aggregates (Figure 9B). It is well known that the 3D cellular environment allows the cells to mimic the in vivo cellular behavior, involving cell-cell and cell-extracellular matrix (ECM) interactions, and promoting cell signaling and proliferation. It has recently been reported that although the mechanisms of exosome biogenesis, as well as the trafficking and release of vesicles are still not well understood, findings suggest that the exosome secretion and molecular cargoes can be altered by the cell microenvironment [12].

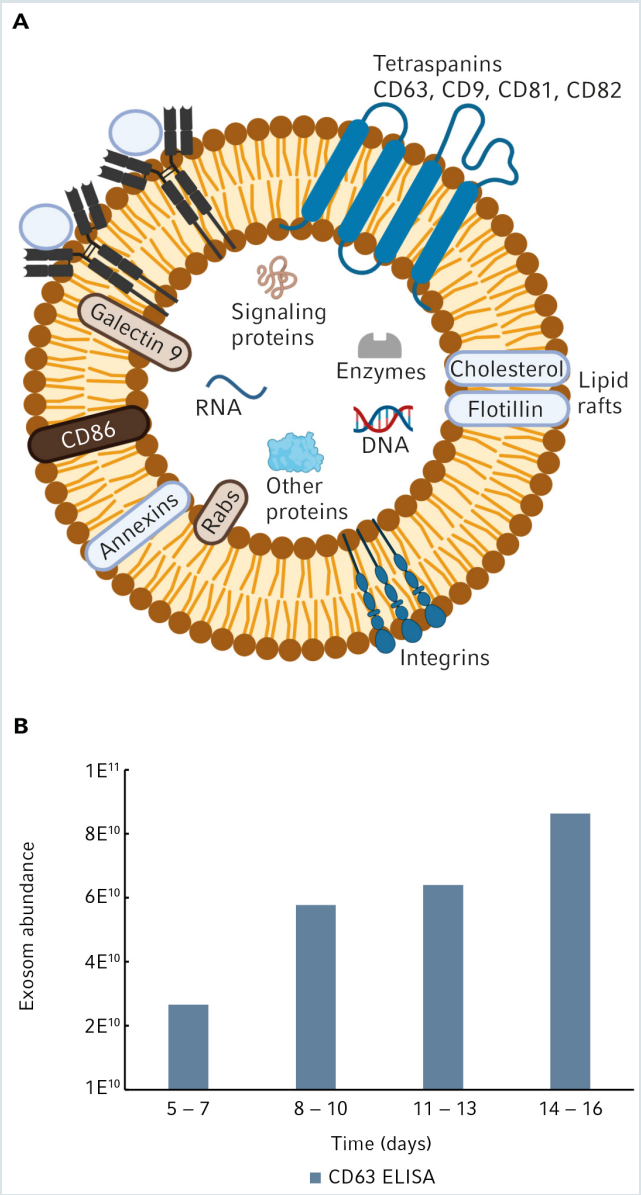

Fig. 8: A) Schematic representation of an exosome composition. (Created with BioRender.com) **B)** Exosome abundance secreted by iPSC-derived MSC on each collection period.

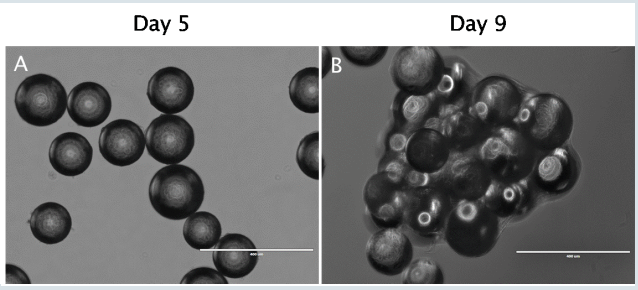

Fig. 9: Bright-field (A and B) images (10x magnification) of human iPSC-derived MSC on collagen coated microcarriers at days 5 and 9 of culture.

Conclusions

We have established the feasibility of producing MSC-derived exosomes using the SciVario twin bioreactor control system and BioBLU 1c Single-Use Vessels. Future updates of the SciVario twin will allow users to run bioreactors as large as 40 L. The design of the control system allows the precise manipulation of the cell culture environment, leading to rapid adhesion and proliferation of human iPSC-derived MSC to the microcarrier's surface. We showed that by increasing the cell inoculation density, larger amounts of MSC exosomes were produced over time. These SciVario twin experiments are preliminary studies and have not yet been optimized to ascertain the maximum exosome production levels. However, our observations can serve as a guideline for further improvements in MSC-derived exosomes isolation, purification, and scale-up protocols.

Literature

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Ordering information

Description	Order no. international
SciVario® twin Fermenter/Bioreactor Control System, base unit, 100 – 240 V/50/60 Hz, for 2 vessels	7600100001
BioBLU® 1c Single-Use Vessel, cell culture, open pipe, 2 pitched-blade impellers, optical pH, sterile, 4 pieces	1386110500
Centrifuge MiniSpin® plus, non-refrigerated, with Rotor F-45-12-11, 120 V/50 – 60 Hz (US)	022620207
Centrifuge 5430 R, keypad, refrigerated, with Rotor FA-45-30-11 incl. rotor lid, 120 V/50 – 60 Hz (US)	022620601
Incubator CellXpert® C170i, inner door with 4 door segments, handle right side, 1 – 20 % oxygen control, 100 – 127 V/50 – 60 Hz (JP/US)	6731011045
Recirculation tube (from 3L decanter kit (M1287-119102))	M1287-908402
Adaptor, compression fitting, for Pg 13.5 port, (from 3L decanter kit (M1287-119102))	M1287-503001
Eppendorf Conical Tubes, sterile, pyrogen-, DNase-, RNase-, human and bacterial DNA-free, colorless, 50 mL	0030122178
Eppendorf Conical Tubes, sterile, pyrogen-, DNase-, RNase-, human and bacterial DNA-free, colorless, 15 mL	0030122151

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