Scalable Xeno-Free Expansion of Human Mesenchymal Stromal Cells in a Single-Use, Vertical-Wheel Bioreactor System

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Abstract

Recent advances have paved the way for human derived stem cell based therapeutics; however the development of large-scale and cost-effective manufacturing methods with appropriate quality assurance and quality control to generate cells in compliance with current Good Manufacturing Practices (cGMP) remains a barrier to effective implementation of these treatments. The 2D culture, while suitable for research, proves incredibly cumbersome and expensive at even modest scale. Microcarriers allow for a drastic increase in available surface area for cell growth and coupled with appropriate bioreactors, allow for greatly enhanced harvest capabilities with reduced manipulation steps. The 3D bioreactors have become powerful tools in which biological and/or biochemical processes developed under closely monitored and tightly controlled environmental and operating conditions enable manufacturers to generate improved cell cultures compared to standard 2D monolayer cultures.

In the current study, we describe a process by which human bone marrow-derived mesenchymal stromal cells (hBM-MSC) were successfully expanded in a 3D culture environment using a xeno-free growth media supplemented with human derived platelet lysate. A disposable, vertical-wheel bioreactor system was chosen for its low shear-stress as compared to conventional, horizontally-stirred bioreactors. To determine the appropriate microcarrier system, several commercially available microcarriers were screened in a microwell 2D environment followed by a small scale 100mL disposable, vertical-wheel bioreactor analogue through analyzing the transient glucose consumption, lactate production and harvested cell number. Finally a seed train was completed beginning with a small number of cells plated in a 225cm^2 flask which were transferred to a 500mL vertical-wheel, disposable flask followed by a transfer to a 3L vertical-wheel bioreactor system. Final cell density and growth profile were analyzed, and cells were evaluated for mesenchymal cell surface markers and differentiation ability prior to introduction to the 3D bioreactor environment and at each harvest to determine potency.

Methods

- **2D Microcarrier screening:** hBM-MSCs (RoosterBio Inc, USA) were cultured with 32mg/well of sterilized animal-product-free microcarriers, Plastic, Plastic Plus, Pronectin F, and Star Plus (Pall, USA) in ultralow-attachment 6-well plates (Corning Inc, USA) with 2mL of xeno-free media (RoosterBio Inc, USA) at 57,600 cells/well. Plates were incubated at 37°C, 5% CO_2 for 3 days. 3 wells of each microcarrier were harvested each day by trypsinization, strained through 70µm cell strainers, stained with trypan blue, and counted by hemacytometer.
- **Small Scale 100mL 3D Comparison:** The two best performing microcarriers from the 2D screening experiment were selected for a small scale 3D experiment. hBM-MSCs were cultured with 16g/L of sterilized microcarriers in 100mL vertical wheel flasks at 20 rpm for 9 days with media changes on days 4 and 7. Cells were harvested by removing media, washing with DPBS followed by trypsinization for 7 min at 20 rpm followed by 3 min at 50 rpm. Cells were strained and counted as previously described
- **Seed Train Experiment:** Cryopreserved hBM-MSCs were thawed and expanded in T225 flasks. Cells were harvested, and 10M cells were inoculated into 500mL vertical-wheel flasks with 16g/L of microcarriers. Cells were grown for 6-7 days with a media change on day 4 in 5% CO_2 at 18 rpm, harvested as previously described, and 55-60M cells were transferred to a 3L bioreactor system with 16g/L of Pronectin F microcarriers. Cells were cultured for 11-12 days at 5% CO2 with media changes on days 4 and 7 and xeno-free replenish added on day 9. Cells were then trypsinized for 7 min. at 20 rpm followed by 3 min. at 35 rpm. Microcarriers were strained out, and the cells were counted.
- **Flow Cytommetry and Differentiation Assay:** Cells harvested from 0.5L and 3L were analyzed for presence of cell surface markers CD14, CD19, CD34, CD45, CD73, CD90, CD105, and HLA-DR. Harvested cells were tested for differentiation ability by culturing in adipogenic differentiation medium (Lonza, USA) for 10 days and stained with Oil Red O (Sigma, USA).

Results

2D Microcarrier Screening

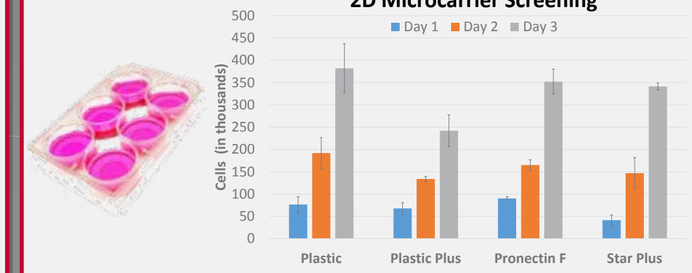


Figure 1: Cells harvested from four microcarrier types cultured in 6-well ultralow attachment plates (Corning) daily for 3 days to determine the two best.

Small Scale 100mL 3D Comparison

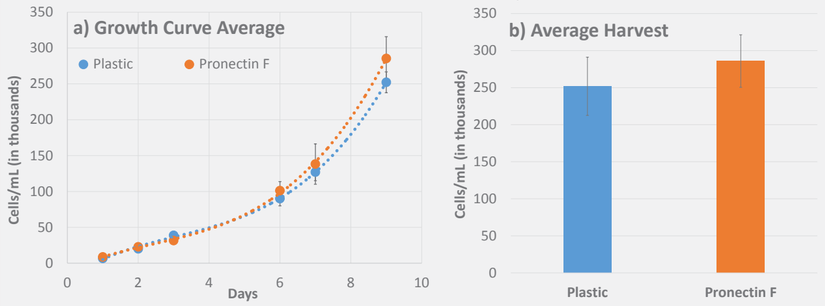
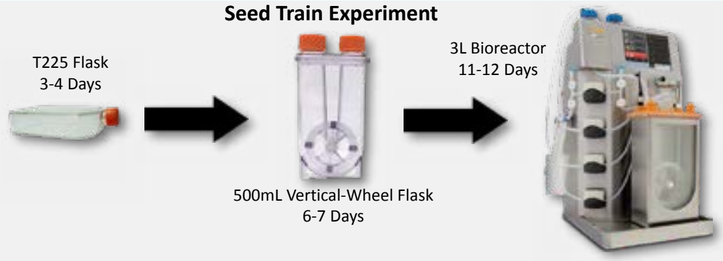


Figure 2: Comparison of best two microcarrier types from 2D screening experiment, Plastic and Pronectin F, in 100mL 3D vertical-wheel spinner flasks a) Growth curve generated from 5 mL samples. b) Average cells/mL harvested over 3 runs. Pronectin F showed the highest capacity for growth and was selected for the seed train experiment to demonstrate scalability.

Seed Train Experiment



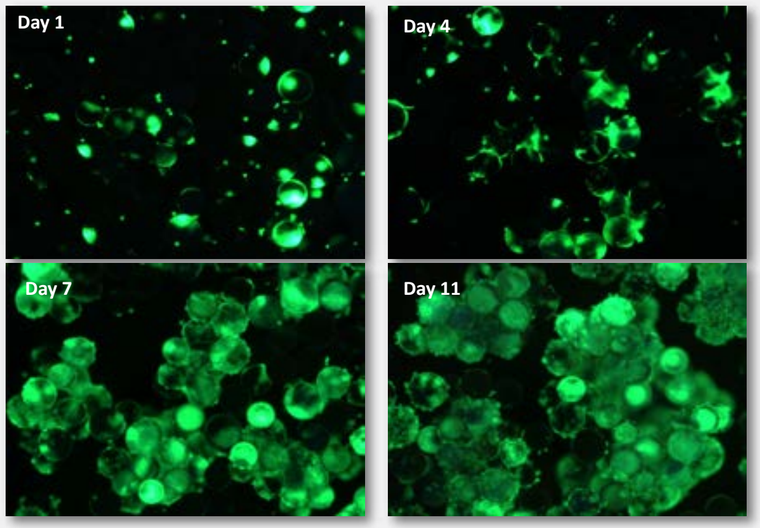


Figure 3: Samples taken from 3L bioreactor, stained with Calcein AM, and imaged using B/G/R fluorescence at 1 day post inoculation and at 3 day intervals until harvest.

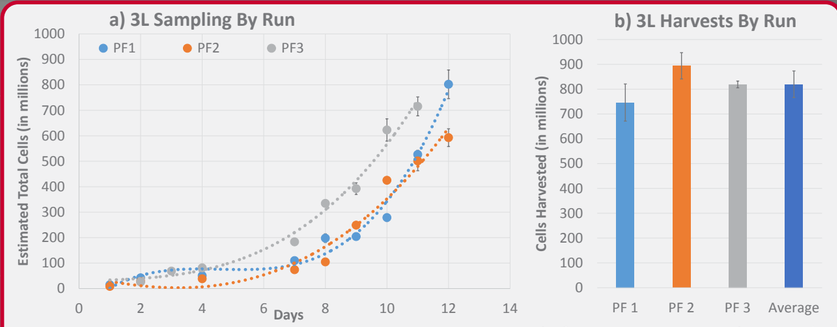


Figure 4: 3 experiments using Pronectin F microcarriers to scale from a T225 to 3L volume. a) Total cells estimated from 5mL samples collected from 3L bioreactor. b) Cells harvested from 3L bioreactors. Harvests of the 3L bioreactor showed an average of 820 ± 50 M cells collected after 11-12 days of culture time. Slower culture times were observed for cells harvested from the same donor on the same day, but from different lots illustrating potential variation in performance not only between donors, but lots as well.

Flow Cytommetry and Differentiation Assay

	0.5L	3L
CD105	63 ± 30%	44 ± 16%
CD73	99.1 ± 0.5%	99.5 ± 0.2%
CD90	99.7 ± 0.3%	99.9 ± 0.1%
CD45	0.7 ± 0.3%	2.4 ± 0.6%

	0.5L	3L
CD34	13 ± 4%	9 ± 4%
CD14	3.1 ± 0.4%	8 ± 3%
CD19	1.3 ± 0.3%	1.6 ± 0.5%
HLA-DR	25 ± 6%	23 ± 9%

Table 1: cell-surface marker comparison of cells harvested after 0.5L and 3L expansion.

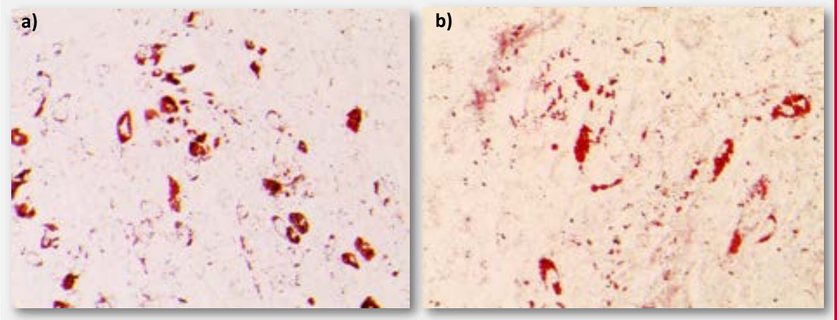


Figure 5: Adipogenic differentiation of cells harvested from a) 0.5L and b) 3L at 20x. Both cells harvested from the 0.5L and the 3L bioreactor showed lipid droplets stained in red by Oil Red O after 10 days in differentiation medium.

Conclusions

- Small-scale 2D microcarrier screening experiments showed that, for the selected hBM-MSCs donor, Plastic and Pronectin F microcarriers allowed for the greatest capacity for cell growth.
- 3D culture of cells on Plastic and Pronectin F microcarriers in 100 mL vertical-wheel flasks suggested that Pronectin F would be the most viable candidate for demonstrating scalability in a seed train experiment.
- A seed train was completed by expanding a few million cells in a T225, transferring them to a 500mL vertical wheel vessel with Pronectin F microcarriers for a 6 fold expansion, then transferring them once more to a 3L vertical wheel bioreactor system for an additional 13 fold expansion. Overall, an 82 fold expansion was completed after transfer to 3D in two vessels.
- Flow cytommetry showed that cells harvested from 0.5L and 3L vessels were similar in cell surface marker expression, and adipogenic differentiation assays revealed that both cell populations were able to generate lipid droplets.