Large-scale Production of Human Mesenchymal Stem Cells in BioBLU® 5c Single-use Vessels

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Abstract

Stem cell-based regenerative medicine has great potential to revolutionize human disease treatments. Among the various stem cell platforms, mesenchymal stem cells (MSCs) represent one of the highest potentials as evidenced by clinical trial activities. Currently, there are over 400 clinical trials based on MSCs registered at clinicaltrials.gov. Although successful expansion of MSCs in vitro has been well established, the large clinical-scale production of MSCs remains a bottleneck, potentially limiting the immediate clinical applications should some of the ongoing trials receive FDA approval. In this study, we demonstrate the success of large clinical-scale culture of human adipose-derived mesenchymal stem cells (AdMSCs) in an industrial single-use vessel at 3.75 liter (L) scale (working volume). The vessel offers a precision controlled environment for the ideal growth of stem cells under simulated physiological conditions. Stem cells and culture media were monitored, analyzed, and controlled, thus allowing us to produce AdMSCs in large clinical-scale quantities while maintaining healthy stem cell properties as evidenced by stem cell marker assays and differentiation assays performed at the end of the culture. Furthermore, with clinical relevance in mind, every cell culture step from T-flask to shake flask to bioreactor vessel was conducted strictly using single-use consumables.

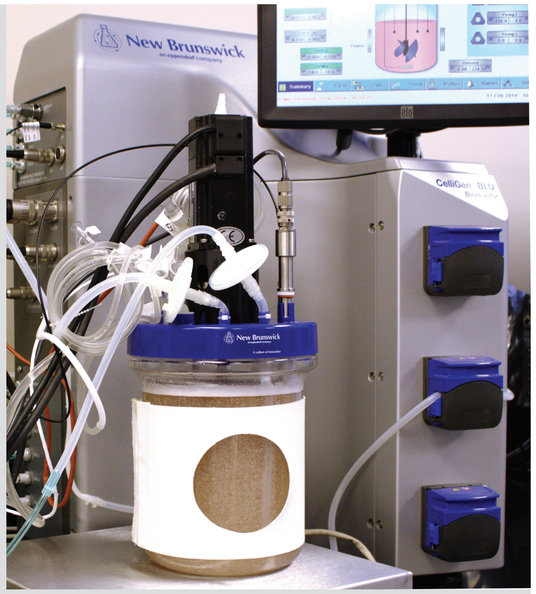
Introduction

Stem cells are undifferentiated cells which have the capability of self-renewal and the potential to differentiate into a variety of cell types, thus performing a critical role in tissue repair and regeneration. Stem cells can be broadly classified as: embryonic, adult, and induced pluripotent stem cells (iPSCs). Adult stem cells can be further characterized by their tissue of origin, such as: hematopoietic, mammary, intestinal, mesenchymal, endothelial, neural, and hair follicle stem cells. According to recent market reports, MSCs are the most studied stem cells [1].

Like other adult stem cells, adipose-derived mesenchymal stem cells (AdMSCs) express all of the common stem cell markers and can be differentiated into various types of specialized cells under appropriate growth conditions. AdMSCs have a unique advantage over other MSCs, since they can be isolated in large quantities from fat tissue and are resistant to apoptosis.

Although MSCs have enormous potential for regenerative medicine, drug screening, and drug discovery, their applications are limited by the quantities required for industrial or clinical applications. Here in this study, we scaled-up AdMSC culture from shake flasks, a method previously developed in our lab, into a BioBLU 5c (Eppendorf) single-use vessel. In the vessel, cell samples, and medium can be analyzed throughout the expansion process and the growth process can be tightly controlled (e.g., oxygen, pH, temperature, glucose, glutamine, lactate, ammonia), thus allowing us to produce AdMSCs in large clinical-scale quantities.

Materials and Methods



Cell culture & cultivation of cells on microcarriers
Expansion of AdMSCs (ATCC®, PCS-500-011™) in a T-75 cm^2 (USA Scientific®, CC7682-4175) flask and cultivation of AdMSCs on microcarrier in shake flask culture were performed as described in previous study [2].

pH mixing study
In order to determine the lowest speed of agitation required for sufficient mixing, a pH-based mixing study was performed at various speeds such as: 25, 35, and 55 rpm according to Xing, Kenty, Li, and Lee [3].

Cell counting
Cells were counted by NucleoCounter® NC-100™ (ChemoMetec® A/S) and Vi-CELL™ XR (Beckman Coulter®).

Metabolite measurement
The supernatants collected during cell counting were used for metabolite measurement using Cedex® Bio Analyzer (Roche®).

Stem cell surface marker immunoassay and stem cell differentiation assays
Both were performed as described in previous study [2].

Cultivation of AdMSCs in BioBLU 5c single-use vessel
Collagen coated microcarriers containing AdMSCs were harvested from shake flask cultures after 15 days of growth and seeded directly into the BioBLU 5c single-use vessel containing 3.5 L AdMSC complete medium with collagen coated microcarriers at a concentration of 17 g/L. The initial agitation speed was set to 25 rpm. The temperature was set at 37 °C. The pH of the bioreactor was maintained at 7.0 by the controller using automatic addition of CO_2 gas and 7.5 % sodium bicarbonate ($NaHCO_3$) solution. After 1 h of incubation, the cell culture volume was adjusted to total 3.75 L with 0.25 L of serum-containing medium to reach a final FBS concentration of 4 % and the targeted level of final concentration of growth supplements (10 ng/mL final concentration of rh FGF basic, rh FGF acidic, and rh EGF, and 2.4 mM final concentration of L-alanyl-L-glutamine). The agitation speed was increased to 35 rpm after 6 days of cell culture. In addition, the overlay gas flow was increased to 0.3 SLPM and N_2 gas was introduced at 0.01 SLPM through the macrosparger to maintain the DO level at 15 %. A 50 % medium exchange was performed at days 4, 8, and 12 with AdMSC complete medium.

Isolation of cDNA and PCR amplification of stem cells markers
Total RNA was isolated from the AdMSCs grown on the microcarrier beads and T-75 cm^2 flasks using TRIzol® reagent (Life Technologies®, 15596-018). cDNA was synthesized using the High-capacity cDNA Reverse Transcription Kit (Life Technologies, 4374966) in a Mastercycler® pro thermocycler (Eppendorf). The primer sequences and PCR conditions used for the CD45, CD105, and beta actin genes were described previously [4]. The Oct3/4 and Sox2 genes were amplified using primer pair kits from R&D Systems® (RDP-321 and RDP-323). The Human CD44 gene was amplified using forward 5´ AGAAGAAAGCCAGTGCGTCT 3´ and reverse 5´ GGGAGGTGTTGGATGTGAGG 3´ primers, which were designed using the BLAST program with Entrez Gene: 960 human as a template.

Results

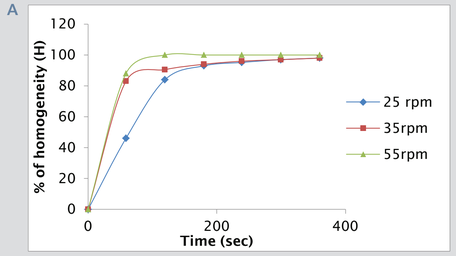


Figure 1:
A: Homogeneity curves during the pH-based mixing study at various rpm in a BioBLU 5c single-use vessel

Growth profile of AdMSCs in BioBLU 5c single-use vessel with collagen coated microcarriers:
B: Cell density
C: Glucose and Lactate concentrations over time: 50 % medium exchanged was performed every 4 days and 0.5 g/L glucose was added at day 15

Differentiation assays for AdMSCs expanded in BioBLU 5c vessel

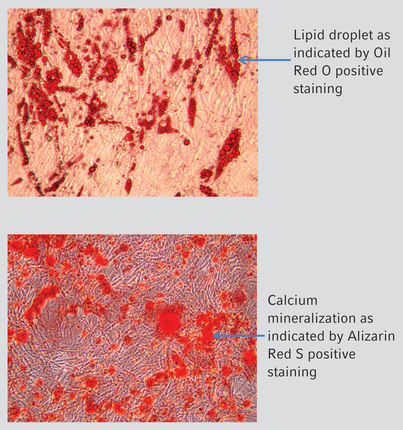


Figure 4: Differentiation assays for AdMSCs expanded on microcarriers in bioreactor
A: Adipogenic differentiation formed lipid droplets as indicated by Oil Red O positive staining
B: Osteogenic differentiation caused calcium mineralization of extracellular matrix as indicated by Alizarin Red S positive staining

Stem Cell marker Immunoassays of AdMSCs expanded in BioBLU 5c vessel

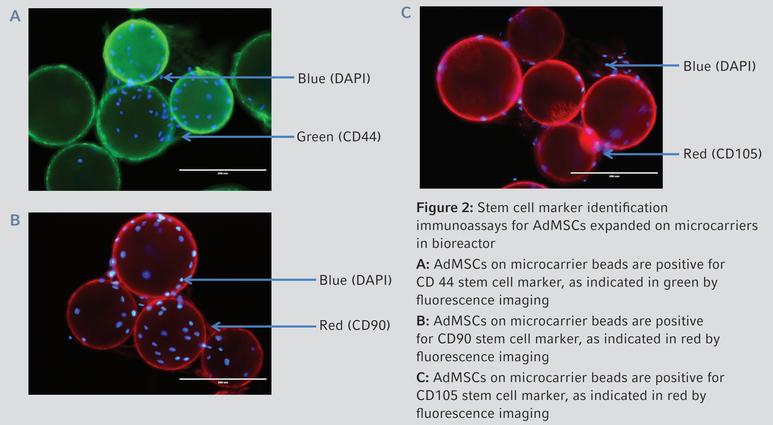


Figure 2: Stem cell marker identification immunoassays for AdMSCs expanded on microcarriers in bioreactor
A: AdMSCs on microcarrier beads are positive for CD 44 stem cell marker, as indicated in green by fluorescence imaging
B: AdMSCs on microcarrier beads are positive for CD90 stem cell marker, as indicated in red by fluorescence imaging
C: AdMSCs on microcarrier beads are positive for CD105 stem cell marker, as indicated in red by fluorescence imaging

PCR based gene expression analysis of stem cell multipotency markers

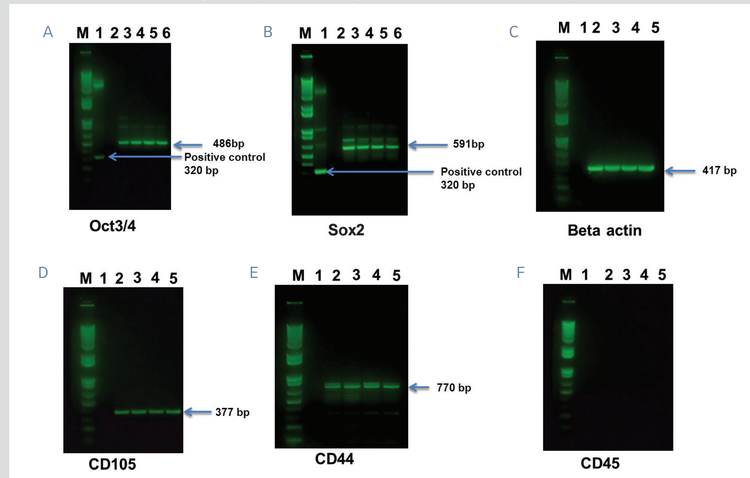


Figure 3: For gel A & B: M: DNA ladder; Lane 1: Positive control synthetic DNA sequence for Oct3/4 or Sox2; Lane 2: PCR negative control; Lane 3: Sample at 0.2 million cells/mL; Lane 4: Sample at 0.24 million cells/mL; Lane 5: Sample from T-75 cm^2 flask at passage 4; Lane 6: Sample from T-75 cm^2 flask at passage 5
For gel C, D, E, & F: M: DNA ladder; Lane 1: PCR negative control; Lane 2: Sample at 0.2 million cells/mL; Lane 3: Sample at 0.24 million cells/mL; Lane 4: Sample from T-75 cm^2 flask at passage 4; Lane 5: Sample from T-75 cm^2 flask at passage 5

Conclusion

> Our study clearly demonstrated the feasibility of using BioBLU 5c single-use vessels for the production of clinical dose-scale numbers of MSCs. The BioBLU 5c single-use vessel has a maximum working volume of 3.75 L, capable of producing clinical dose-scale numbers of MSCs in a single run.
> In this study, we have also shown that AdMSCs cultured in BioBLU 5c single-use vessels retained their differentiation and multipotency properties as evident by immunostaining, PCR, and differentiation assays.
> BioBLU 5c is equipped with a pitched-blade impeller which allows stem cells to be cultured under low rpm conditions to avoid shear force damages. In addition, BioBLU vessels are produced from USP Class VI and animal component-free materials, and do not contain any toxic leachables/extractables, such as bis(2,4-di-tert-butylphenyl)phosphate (bDtBPP).
> The above studies validated the general applicability of the New Brunswick™ CelliGen® BLU benchtop bioreactor and BioBLU single-use vessels for large-scale process optimization and production of stem cells in numbers appropriate for clinical applications in regenerative medicine.

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[2] Siddiquee K, Sha M. Microcarrier-Based Expansion of Adipose-Derived Mesenchymal Stem Cells in Shake Flasks. *Bioprocessing Journal* 2013; 12(4):32-8.
[3] Xing Z, Kenty BM, Li ZJ, Lee SS. Scale-up analysis for a CHO cell culture process in large-scale bioreactors. *Biotechnology and Bioengineering* 2009; 103(4):733-46.
[4] Potdar P, Prasannan P. Differentiation of Human Dermal Mesenchymal Stem Cells into Cardiomyocytes by Treatment with 5-Azacytidine: Concept for Regenerative Therapy in Myocardial Infarction. *ISRN Stem Cells* 2013; 2013:Article ID 687282.