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TECHNOLOGY REVIEW

Billion-Cell Hypoxic Expansion 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 advance the therapeutic treatment of human diseases. Among the various stem cell platforms, mesenchymal stem cells (MSCs) represent one of the most promising options. Currently, there are over 500 clinical trials based on MSCs registered at the NIH's ClinicalTrials.gov website. Although successful expansion of MSCs *in vitro* has been well-established, higher-yield, billion-cell expansion of MSCs remains a bottleneck.

In this study, we successfully demonstrated large-scale culture of human adipose-derived mesenchymal stem cells (AdMSCs) in an industrial, single-use vessel at 3.75 L scale. The vessel offers a precision-controlled environment for the ideal growth of stem cells under simulated hypoxic physiological conditions. Stem cells and their culture media were monitored, analyzed, and controlled, allowing production of AdMSCs in substantial quantities. At the same time, the specific properties of the stem cell were maintained, as evidenced by stem cell marker assays and differentiation assays performed at the conclusion of the run.

Finally, because all steps in the platform were conducted employing single-use consumables, this study demonstrates the fact that the process can be conveniently scaled up to industrial levels of production without having to rely on stainless steel culture facilities.

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. Broadly classified as embryonic, adult, and induced pluripotent stem cells (iPSCs) they can be further characterized by their tissue of origin, including hematopoietic, mammary, intestinal, mesenchymal, endothelial, neural, and hair follicular stem cells. There are numerous peer-reviewed, published articles available based on mesenchymal stem cell (MSC) studies.^[1-3] MEDLINE[®] contains thousands of journal citations and abstracts on the subject from around the world.

Like other adult stem cells, adipose-derived mesenchymal stem cells (AdMSCs) express all of the common stem cell markers and will undergo differentiation into various types of specialized elements under appropriate growth conditions. AdMSCs have a unique advantage over other MSCs since they can be isolated in large quantities from body fat and are resistant to apoptosis.^[2,4-8]

MSCs have important implications in the treatment of myeloablative chemotherapeutic protocols. Hematopoietic stem cell (HSC) transplantation, although a routine treatment after chemotherapy, is not entirely successful in the prevention of post-treatment neutropenia and thrombocytopenia.^[9] In human trials, supplementation of HSCs with autologous stem cells has resulted in positive outcomes. However, this strategy absolutely requires an ample source of MSCs.^[10]

Another important application for MSCs is in the treatment of neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS). In a critical review by Thomsen *et al.*^[11] of current clinical trials using either mesenchymal or neural stem cells to treat ALS patients, the authors wrote that comprehensive pre-clinical trials will be required to establish

the safety and efficacy of the procedure. This line of attack will require a reliable production protocol for clinical-scale quantities of stem cells.

Although MSCs have enormous potential for regenerative medicine, drug screening, and drug discovery, their exploitation has been hindered due to insufficient production quantities required for industrial or cellular therapy applications.^[8] In this report, we scaled-up AdMSC

culture from shaker flasks, a methodology previously developed in our lab^[10], into an industrial-scale, single-use bioreactor. In the bioreactor, cell samples and media are analyzed and closely regulated throughout the expansion process. Moreover, growth parameters (*e.g.*, oxygen, pH, temperature, glucose, glutamine, lactate, ammonia, *etc.*) can be tightly controlled, thus allowing the production of AdMSCs in large quantities.

Materials and Methods

Initial Cell Culture in T-Flasks

AdMSCs were obtained from ATCC[®] at passage 2 and cells were seeded at a density of 5×10^3 cells/cm² into a T-75 cm² flask (USA Scientific[®]) using 15 mL of MSC basal medium (ATCC). The medium was supplemented with ATCC MSC growth kit components at the following concentrations: 2% fetal bovine serum (FBS); 5 ng/mL each: rhFGF-basic (recombinant human fibroblast growth factor)-basic, rhFGF-acidic, and rhEGF (recombinant human epidermal growth factor); and 2.4 mM L-alanyl-L-glutamine.

Single-Use Culture Vessel and Bioreactor Controller

The BioBLU 5c single-use culture vessel (Eppendorf) was used with the CelliGen[®] BLU bioreactor controller (Eppendorf). The vessel has a maximum working volume of 3.75 L and incorporates a pitched blade impeller. This benchtop bioreactor was configured with low-flow thermal mass flow controllers (TMFCs) providing a gas flow range of 0.002–1.0 standard liters per minute (SLPM) and an included gas overlay with a control range of 0.01–5.0 SLPM.

Preparation of Microcarriers

Prior to the start of the experiment, polystyrene and collagen-coated microcarriers (SoloHill[®], Pall[®] Life Sciences) were prepared and sterilized according to the manufacturer's instructions. Cultivation of cells on microcarriers was performed as described previously.^[12]

pH Mixing Study

In order to determine the lowest speed of agitation required for sufficient mixing, the effects of pH were evaluated in a mixing study performed at various speeds including 25, 35, and 55 rpm.^[13] Briefly, a pH sensor (Mettler-Toledo[®]) was calibrated using different standard buffer solutions and placed inside a bioreactor containing 3.75 L of phosphate-buffered saline (PBS; Life Technologies[®]). 18.75 mL of 4 N NaOH (0.5% vessel working volume) was added to the bioreactor to create a temporary pH fluctuation. The pH value was continuously recorded until a steady state was achieved. After each run, the pH value of the bioreactor was brought back to the initial pH using 4 N HCl.

The homogeneity (H) of pH mixing was calculated and plotted against elapsed time using the following equation:

$$H(t) = \frac{\text{pH}(t) - \text{pH}(i)}{\text{pH}(f) - \text{pH}(i)} \times 100$$

H(t) = homogeneity at time, t
 pH(t) = pH value at time, t
 pH(f) = final pH value under the complete homogenized condition
 pH(i) = initial pH value upon trace (NaOH) addition

Optimization and Cultivation of AdMSCs in Single-Use Vessels

Two independent, large-scale experiments were performed in single-use vessels using two different types of microcarriers.

For the first experiment, polystyrene microcarriers containing AdMSCs were harvested from single-use polycarbonate flask (Corning[®]) cultures after 15 days of growth and seeded directly into the BioBLU 5c single-use vessel containing 1.5 L of AdMSC complete growth medium with microcarriers at a concentration of 15 g/L. The day after inoculation, another 1.5 L of AdMSC complete growth medium was added to the vessel with microcarriers at a concentration of 45 g/L to reach the final concentration of microcarriers (30 g/L). The agitation speed was set at 25 rpm and the temperature, 37 °C. The bioreactor pH was maintained at 7.0 by the controller using the automatic addition of CO₂ gas and 7.5% NaHCO₃ solution. During the experiment, the dissolved oxygen (DO) level was set to 10% and the controller was set to 4-gas mode to automatically maintain the DO setpoint by delivering four gas choices (air, CO₂, N₂, and O₂) through the overlay (vessel head space) instead of the sparger. The overlay gas flow was maintained at 0.1 SLPM during the first ten days of the experiment. After ten days, the overlay gas flow was increased to 0.3 SLPM. A 25% medium exchange was performed at day 5 and an additional 0.75 L AdMSC complete medium was added at day 11 to reach the maximum working volume of the vessel (3.75 L). Furthermore, a 50%

medium exchange was performed at day 14.

For the second experiment, collagen-coated microcarriers containing AdMSCs were harvested from single-use polycarbonate flask cultures after 15 days of growth and seeded directly into the BioBLU 5c single-use vessel (**Figure 1**) containing 3.5 L AdMSC complete medium with collagen-coated microcarriers at a concentration of 17 g/L. The initial agitation speed was again set to 25 rpm. After one hour of incubation, the cell culture volume was adjusted to a total working volume of 3.75 L. The working volume included targeted final concentration levels of: 0.25 L serum-containing medium (total FBS 4%), and growth supplements (10 ng/mL each of rhFGF-basic, rhFGF-acidic, and rhEGF; and 2.4 mM L-alanyl-L-glutamine). The bioreactor control parameters were the same as the first bioreactor run except that the agitation speed was increased to 35 rpm after six days of cell culture. In addition, the overlay gas flow was increased to 0.3 SLPM and N₂ 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 containing 0.1% **Pluronic® F-68 surfactant** (Life Technologies). On day 15, 0.5 g/L of glucose was added to the vessel to sustain cell growth without additional media exchange.

Cell Counting and Metabolite Measurement

Cells on microcarrier beads were counted by the **NucleoCounter® NC-100™** (ChemoMetec® A/S) according to the manufacturer's protocol. The supernatants collected during cell counting were used for metabolite measurement with the automated **Cedex® Bio Analyzer** (Roche®). In addition to the NucleoCounter, a **Vi-CELL™ XR** (Beckman Coulter®) was also used to count the cells that were collected from the T-75 cm² flasks used in the initial cell culture. However, the Vi-CELL was not used for counting cells from the microcarrier cultures due to the risk of jamming the Vi-CELL's needle sipper with microcarriers.

Stem Cell Surface Marker Assays

To assess the quality of AdMSCs after expansion, and to confirm that the stem cell markers were retained during cultivation in the vessel, CD44, CD90, and CD105-specific fluorescent immunoassays were performed using the following procedure: Cells on the microcarrier beads were fixed with 4% paraformaldehyde for 30 minutes followed by three washes with ATCC's **Dulbecco's PBS** (DPBS), Ca²⁺ and Mg²⁺ free, and subsequently blocked with 5% FBS at room temperature for one hour. Immunostaining was performed using **BioLegend® FITC-conjugated anti-human CD44 antibody solution** containing the nuclear stain 4', 6-diamidino-2-phenylindole (**DAPI**; Life

Technologies) for one hour at room temperature. For immunostaining of the CD90 and CD105 markers, cells were fixed and blocked using the same protocol as described above. The cells were incubated with **mouse anti-human CD90 and CD105 antibodies** (Abcam®) for one hour and washed five times with room-temperature DPBS for five minutes each. The cells were further incubated with **Alexa Fluor® 546 and 594 anti-mouse secondary antibodies** (Life Technologies) and DAPI solutions at room temperature for one hour. The cells were washed five times with room-temperature DPBS for five minutes each and visualized under an **EVOS® FL LED-based fluorescence microscope** (Life Technologies).

Isolation of cDNA and Polymerase Chain Reaction (PCR) Amplification of Stem Cell Markers

Total RNA was isolated from the AdMSCs grown in the T-75 cm² flasks and on the microcarrier beads using **TRIzol® reagent** (Life Technologies). cDNA was synthesized using the high-capacity **cDNA Reverse Transcription Kit** (Life Technologies) in a **Mastercycler® pro** thermocycler (Eppendorf). The primer sequences and PCR conditions used for the CD45, CD105, and beta actin genes were described previously.¹⁴ The Oct3/4 and Sox2 genes were amplified using **primer pair kits** from R&D Systems®. The human CD44 gene was amplified using forward 5' AGAAGAAAGCCAGTGCCTCT 3' and reverse 5' GGGAGGTGTTGGATGTGAGG 3' primers, which were designed using the **BLAST®** program with **Entrez gene: 960 human** as a template. All the primers were validated by aligning with respective gene sequences using the BLAST program.

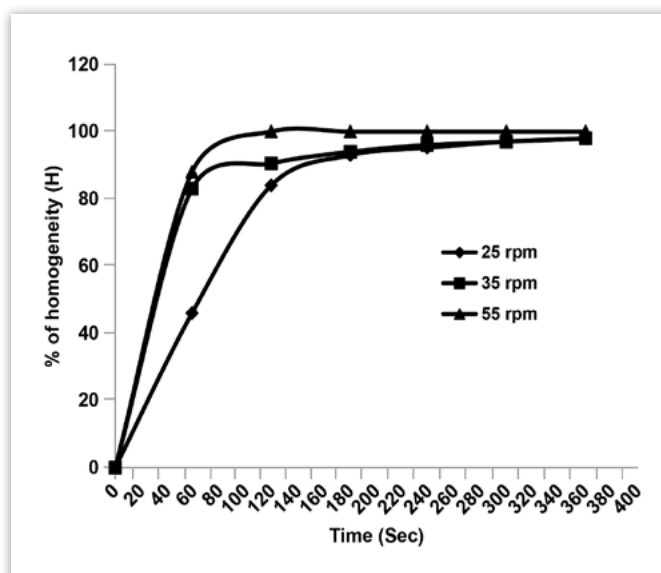


FIGURE 1. Homogeneity curves during the pH-based mixing study at various rpms in a BioBLU 5c single-use vessel.

Stem Cell Differentiation Assays

AdMSCs were harvested from the bioreactor into [50 mL conical tubes](#) (USA Scientific). Once the microcarrier beads settled to the bottom of the tube, the supernatants were removed and cells were washed with DPBS. Afterward, the microcarrier beads were treated with 5 mL of pre-warmed [trypsin-EDTA solution](#) (ATCC) at 37 °C for ten minutes. During incubation, the tubes were occasionally vortexed for two seconds and then neutralized by adding an equal volume of [trypsin-neutralizing solution](#) (ATCC). Microcarrier beads were allowed to settle to the bottom of the tube and the supernatants were collected as soon as possible.

Microcarrier beads were washed 2–3 times with DPBS

and the supernatant was combined into a 50 mL tube. Following washing, AdMSCs were collected from the bottom of the tube by centrifugation at 120×g for five minutes and resuspended into 5 mL of MSC complete medium. Cells were seeded at a density of 1.8×10^4 cells/cm² into [6-well plates](#) (USA Scientific). Differentiations were induced with [Adipocyte](#) (ATCC) and [Osteocyte](#) (ATCC) Differentiation Toolkits. Following manufacturer's instructions, differentiated adipocytes were identified by [oil red O staining](#) (ScienCell™) and osteocytes were identified with [alizarin red S staining](#) (ScienCell). Both were visualized using an [Olympus® CK40 inverted microscope](#) equipped with an [Infinity2 CCD camera](#) (Lumenera®).

Results and Discussion

Optimization of Bioprocess Parameters Using Polystyrene Microcarriers

From the mixing study, it was found that 100% homogeneity was achieved in two minutes with agitation at 55 rpm; whereas 90.4% and 84.0% homogeneity were achieved by 35 and 25 rpm agitation, respectively (**Figure 1**). MSCs are very sensitive to shear force damage, so gentle agitation at a lower rpm is preferred whenever possible. Since a significant amount ($\geq 84.0\%$) of homogeneity was achieved in the bioreactor at 25 or 35 rpm within two minutes, the bioreactor agitation speed was maintained between 25 and 35 rpm during the entire experiment. AdMSCs were initially expanded under shake flask culture conditions using single-use polycarbonate flasks. Microcarriers containing AdMSCs were collected from these flasks and used to inoculate the BioBLU 5c single-use vessel with an initial cell density of 5×10^3 cells/mL. For the first experiment, 30 g/L of microcarrier was used in order to explore the maximum microcarrier

concentration for AdMSCs cultured under a controlled environment. Although AdMSCs quickly expanded in the bioreactor within 24 hours of inoculation, there was a four-day lag phase in cell growth following the addition of a high concentration of microcarriers. This might have been due to collisions between microcarriers and shear forces resulting from their ultra-high density. The initial culture also showed that the DO level could not be maintained at the 10% setpoint. Thus, the overlay gas flow was increased to 0.3 SLPM after ten days of cell growth. However, the 0.3 SLPM overlay gas flow was still not enough to bring the DO down to the 10% setpoint. Direct gas sparging was not used in this experiment, but was subsequently used in later experiments. The actual DO fluctuated around 20% throughout the bioreactor run. After the 50% medium exchange on day 14, cell growth increased and reached its maximum density of 3.9×10^4 cells/mL by day 18. The final density was ~7-fold higher than the initial cell density (**Figure 2**).

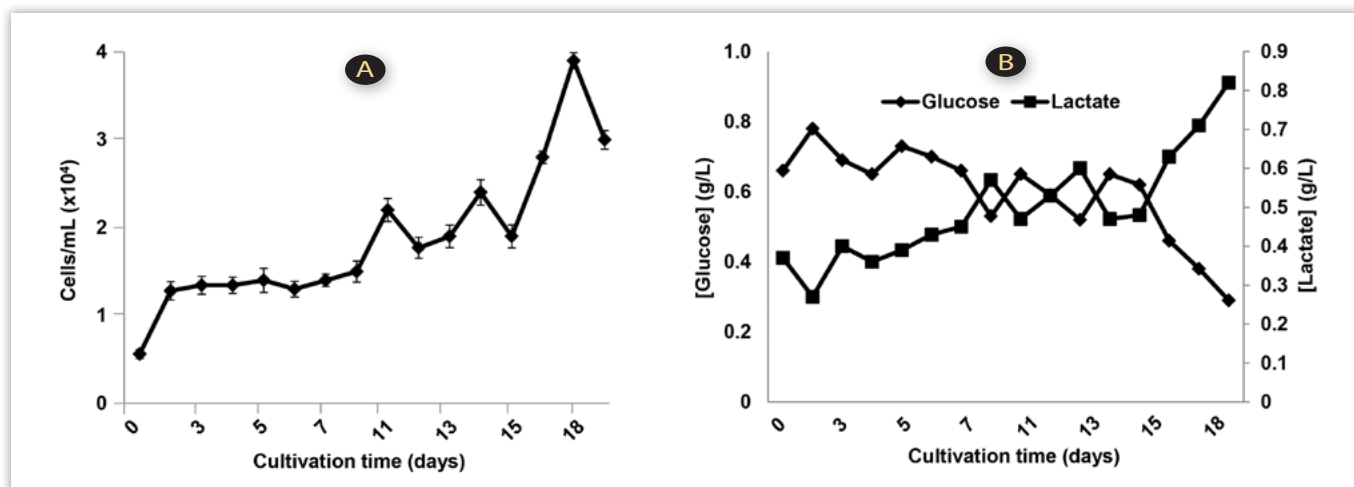


FIGURE 2. Growth profile of AdMSCs in a BioBLU 5c single-use vessel with polystyrene microcarrier beads. (A) cell density; and (B) glucose and lactate concentrations over time.

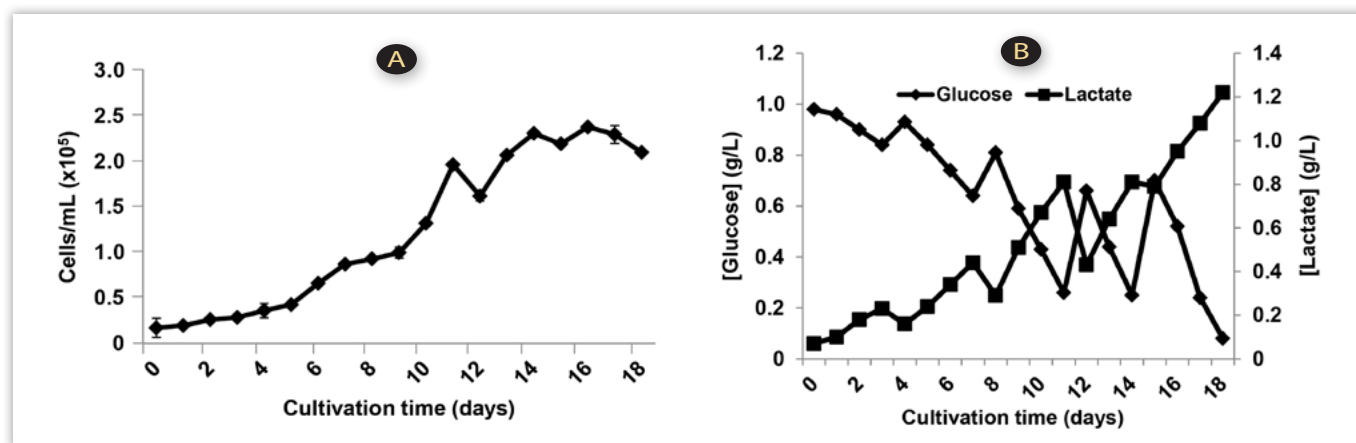


FIGURE 3. Growth profile of AdMSCs in a BioBLU 5c single-use vessel with collagen-coated microcarriers. (A) cell density; (B) glucose and lactate concentrations over time: 50% medium exchange was performed every four days and 0.5 g/L glucose was added at day 15.

Optimization of Bioprocess Parameters Using Collagen-Coated Microcarriers

Since the maximum expected AdMSC density was not achieved from the first bioreactor experiment using polystyrene microcarriers, a second experiment was performed using collagen-coated microcarriers. Recent studies have shown that collagen-coated microcarriers may support higher MSC density in single-use vessels.^{115,161} In the second experiment, microcarriers containing AdMSCs were collected from shake flasks and inoculated into the bioreactor to a final density of 1.75×10^4 cells/mL. Medium exchanges were performed every four days during the experiment. The DO was set to a more controllable 15% and maintained using the addition of N₂ through the overlay. Beginning on day 6, additional N₂ gas was introduced through the sparger at 0.01 SLPM. Since 100% DO was calibrated using 100% air, 15% DO represents ~3% O₂ in the medium, which is within the targeted hypoxic physiological conditions (2–5% O₂). Furthermore, the agitation speed of the bioreactor was increased to 35 rpm to support the complete suspension of AdMSC-containing microcarriers in the BioBLU vessel. Pluronic-F68 surfactant (0.1%) was also introduced into the medium to reduce foaming, a result of N₂ sparging. Pluronic-F68 is also known to protect cell membranes and reduce the shear force during cell culture agitation.¹¹⁶¹ Cell growth steadily increased in the bioreactor from day 6, which was accompanied by an increase in glucose consumption and lactic acid production. Although cells were still metabolically active at day 15, as seen from continued glucose consumption and lactic acid production, the addition of 0.5 g/L glucose at day 15 did not result in a significant increase in cell growth (Figure 3), which indicated that the AdMSCs had reached a stationary state. This might have been due to cell growth being limited by either space for propagation or exhaustion of certain essential nutrients other than glucose. After 16 days of cell culture, AdMSCs in

the vessel reached a maximum density of $\sim 2.4 \times 10^5$ cells/mL, about 14-fold higher than the initial seeding density.

Corrections of NucleoCounter Cell Measurement

Since the Vi-CELL could not be used for cell counting in the presence of microcarriers, the NucleoCounter NC-100 was used to conduct daily cell counts throughout the bioreactor run. However, the NC-100 appeared to have a smaller dynamic range than the Vi-CELL, thus giving inaccurate readings at the higher cell densities. In order to provide more precise cell counts, a comparative study was performed between the NC-100 and the Vi-CELL. For this purpose, AdMSCs were collected from T-75 cm² flasks and counted with both the Vi-CELL and the NC-100 counter after a 3-fold dilution. The cell count results indicated that, at high cell concentrations, the NC-100 undercounted the cells significantly, as compared to the Vi-CELL (an industry standard). In the high cell concentration range, Vi-CELL reported on average, 1.8-fold higher than the NC-100 from the same sample (Figure 4).

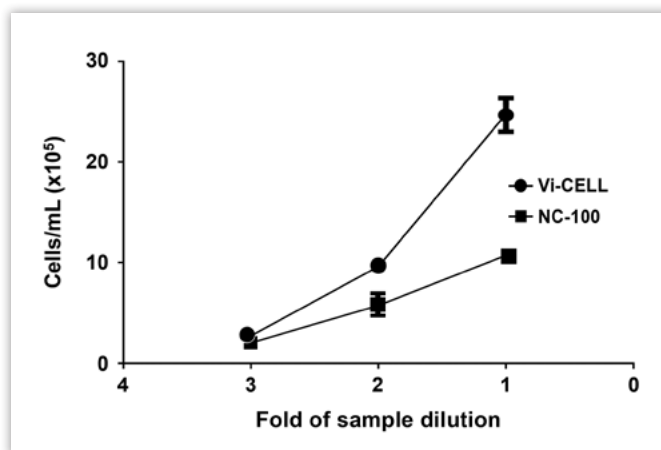
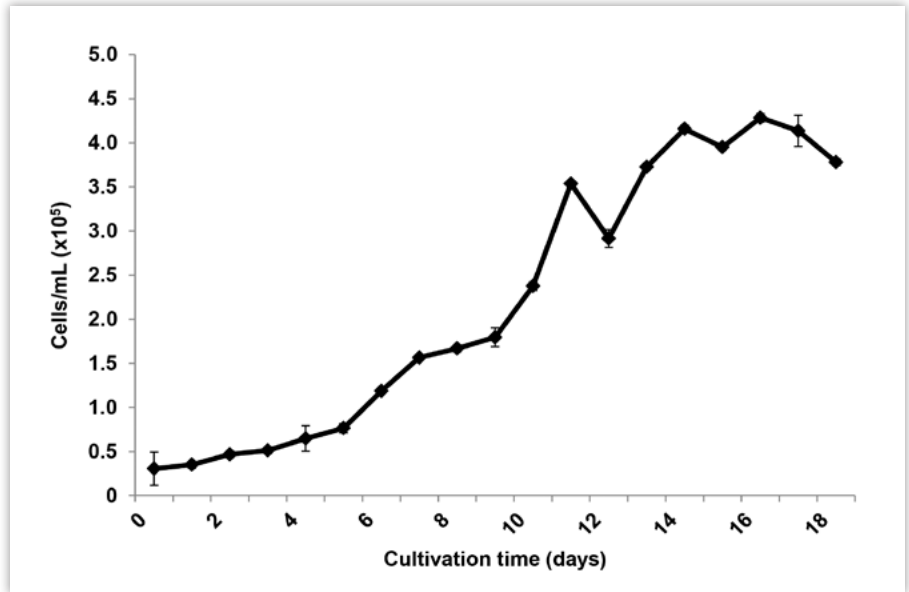


FIGURE 4. Comparison between Vi-CELL and NC-100 for measuring cell counts from the same AdMSC samples.

FIGURE 5. Corrected cell growth profile of AdMSCs in a BioBLU 5c single-use bioreactor with collagen-coated microcarriers.

A corrected bioreactor cell growth profile was provided, based on NC-100 to Vi-CELL correlation, using the averaged correction factor of 1.8-fold (**Figure 5**). The peak cell density reached $\sim 4.3 \times 10^5$ cells/mL in the BioBLU 5c single-use vessel's 3.75 L maximum working volume, resulting in a total cell number yield of ~ 1.6 billion cells (1.6×10^9) on day 16.



Immunostaining of Stem Cell Markers

To confirm that AdMSCs retained their stem cell properties during expansion in the bioreactor, immunostaining of stem cell surface markers was performed. MSCs express various cell surface markers such as CD73, CD90, CD105, and CD44.^[3-5, 7] Microcarrier beads containing AdMSCs were characterized based on surface marker expression using CD44, CD90, and CD105-specific antibodies followed by fluorescence imaging. The results revealed that AdMSCs retained stem cell surface markers during the experiment (**Figure 6**).

PCR Analysis of AdMSCs

In addition to immunostaining, PCR was also performed to monitor gene expression of additional stem cell markers. This approach revealed

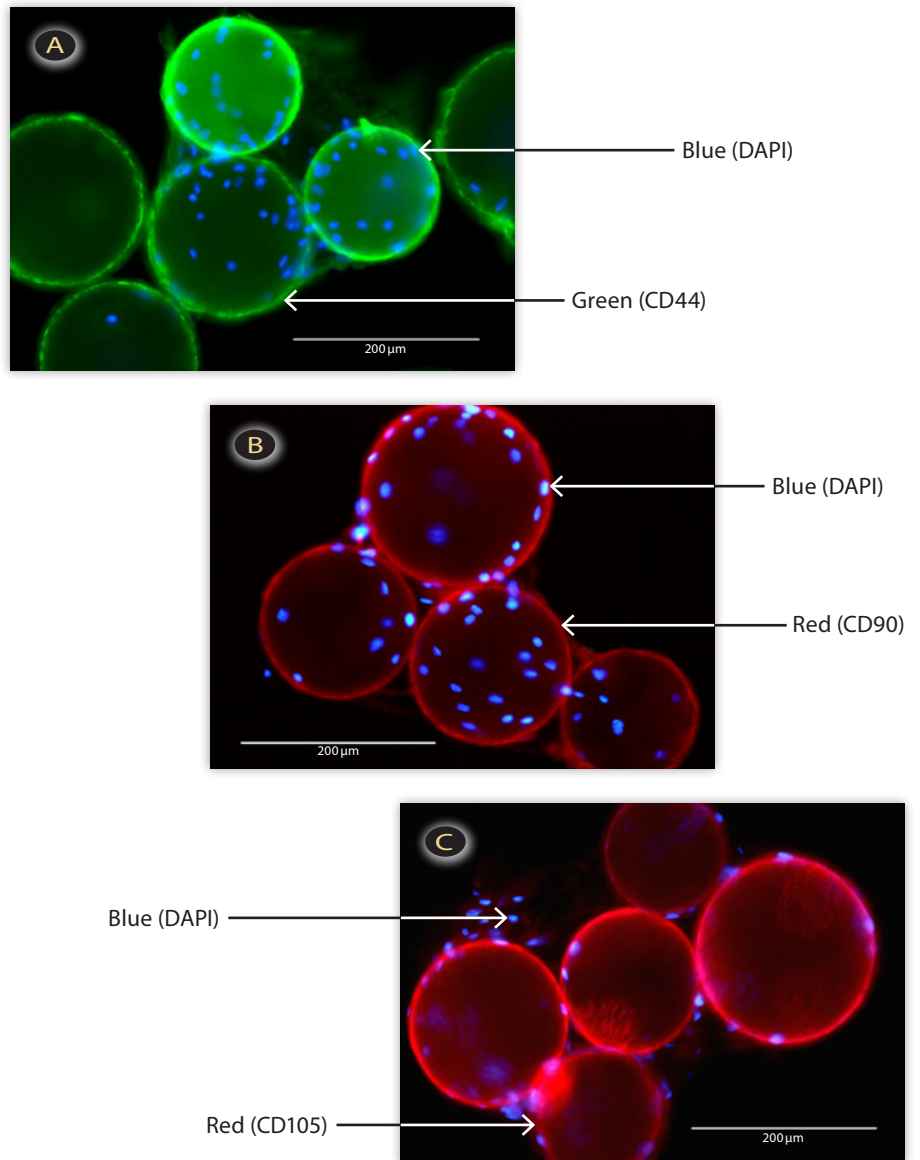


FIGURE 6. Stem cell marker identification immunoassays for AdMSCs expanded on collagen-coated microcarriers in bioreactor. (A) AdMSCs on microcarrier beads are positive for CD44 stem cell marker, as indicated in green by fluorescence imaging; (B) AdMSCs on microcarrier beads are positive for CD90 stem cell marker, as indicated in red by fluorescence imaging; and (C) AdMSCs on microcarrier beads are positive for CD105 stem cell marker, as indicated in red by fluorescence imaging.

that AdMSCs collected towards the end of the bioreactor culture were positive for CD44, CD90, CD105, Oct3/4, and Sox2 gene expression, whereas they were negative for CD45 gene expression. The post-bioreactor stem cell marker gene expression was compared to cells cultured in T-75 cm² flasks. From the comparison, it was observed that AdMSCs collected from the T-75 cm² flasks (used in the initial culture) and the BioBLU 5c expansion bioreactor had the same stem cell marker gene expression pattern (**Figure 7**).

Assessment of AdMSC Differentiated Functions

To further confirm that the AdMSCs cultured in the bioreactor retained their differentiation capacity, adipocyte and osteocyte differentiation assays were performed. AdMSCs were collected from the microcarrier beads and seeded into 6-well plates containing either adipocyte or osteocyte differentiation media. In the osteocyte differentiation medium, the cells transformed into long polygonal-shaped osteocytes and produced calcium

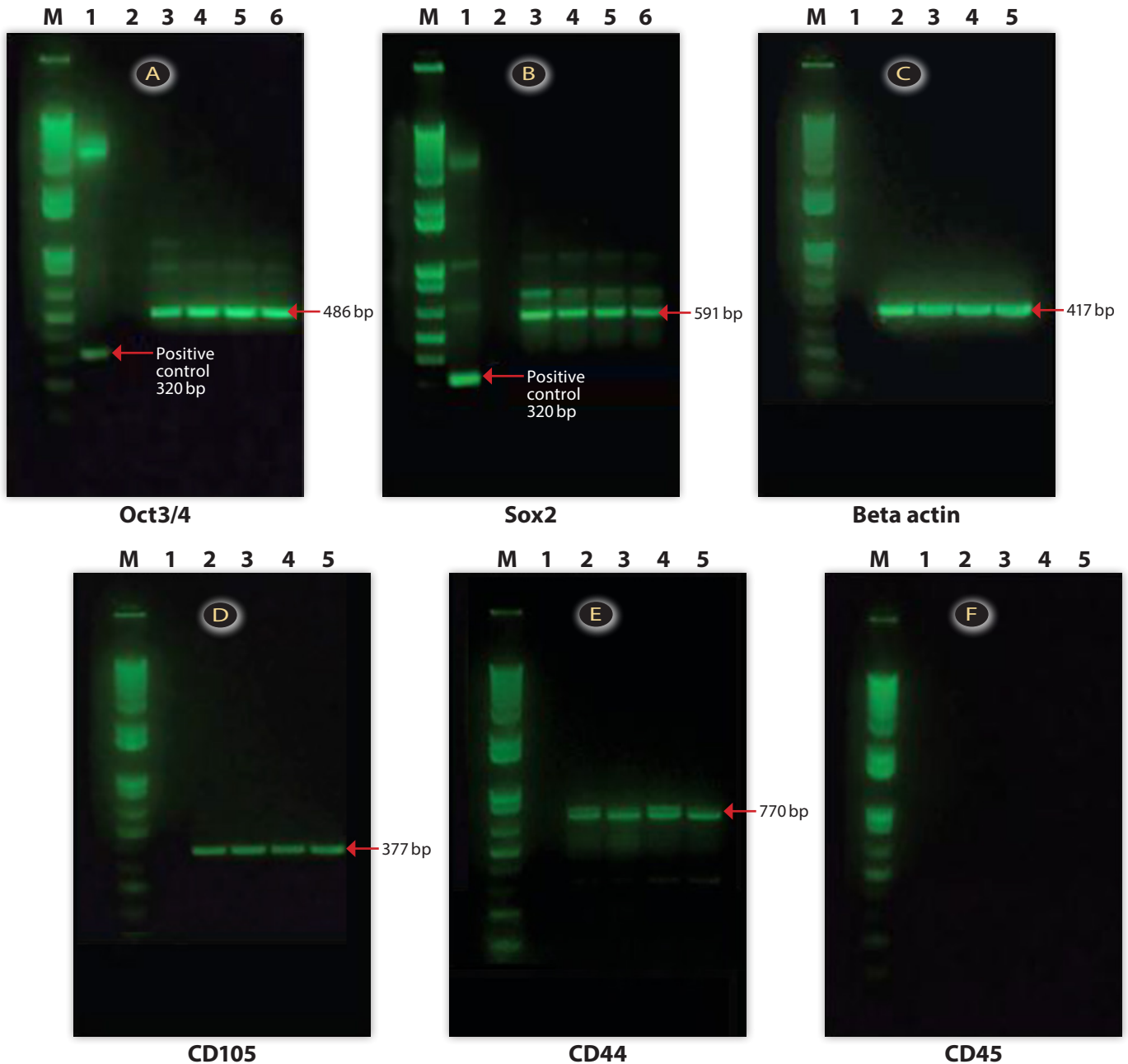


FIGURE 7. PCR analysis of multipotency markers in AdMSCs cultured in T-flasks and in BioBLU single-use vessels. For gels (A) and (B), **M**: DNA ladder; **Lane 1**: positive control synthetic DNA sequence for Oct3/4 or Sox2; **Lane 2**: PCR negative control; **Lane 3**: sample at 0.20 million cells/mL; **Lane 4**: sample at 0.24 million cells/mL; **Lane 5**: sample from T-75 cm² flask at passage 4; and **Lane 6**: sample from T-75 cm² flask at passage 5. For gels (C), (D), (E), and (F), **M**: DNA ladder; **Lane 1**: PCR negative control; **Lane 2**: sample at 0.20 million cells/mL; **Lane 3**: sample at 0.24 million cells/mL; **Lane 4**: sample from T-75 cm² flask at passage 4; **Lane 5**: sample from T-75 cm² flask at passage 5.

deposits in the extracellular matrix. On the other hand, when cells were treated with adipocyte differentiation medium, cells became oval-shaped and accumulated lipid droplets. After 21 days of culture, the plates were

stained with oil red O or alizarin red S staining solutions. Microscopic observation revealed that the AdMSCs from the bioreactor were successfully differentiated either into adipocytes or osteocytes (**Figure 8**).

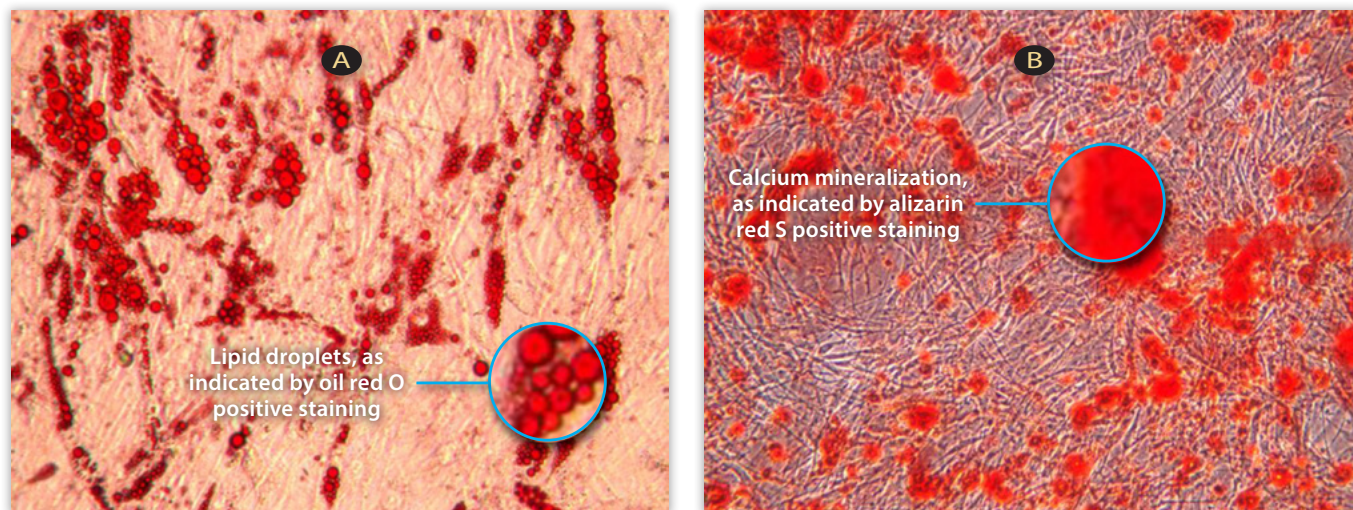


FIGURE 8. Differentiation assays for AdMSCs expanded on microcarriers in bioreactor. (A) adipogenic differentiation formed lipid droplets, as indicated by oil red O positive staining; and (B) osteogenic differentiation caused calcium mineralization of extracellular matrix, as indicated by alizarin red S positive staining.

Conclusions

The lack of reliable technology for large-scale production of mesenchymal stem cells had been a major drawback to their evaluation in clinical studies. Nienow *et al.*^[17] have addressed this challenge through the use of microcarriers. These researchers were able to achieve high levels of expression for several mesenchymal markers. Using this approach, they were able to grow the cells to numbers as high as 1.75×10^8 total cells; however, it is still far below the billion-cell scale.

It was estimated that the average human would require approximately one billion cells per treatment dose for certain cell therapies.^[18] Our study demonstrates the feasibility of employing single-use bioreactors for the large-scale production of MSCs. The smaller BioBLU 5c single-use vessel used in this study has a maximum working volume of 3.75 L

and is capable of producing over one billion (1.62×10^9) MSCs. The larger BioBLU 50c, with a maximum working volume of 40 L, can be used to produce much greater quantities of MSCs using the same CelliGen BLU controller. In addition, the single-use vessel is equipped with a large, pitched-blade impeller that provides stem cells with ample mixing under low rpm conditions to avoid potential shear force damages. In this study, we have also shown that AdMSCs cultured in single-use vessels retained their differentiation and multipotency properties, as evidenced by immunostaining, PCR, and differentiation assays. Our study results validated the general applicability of single-use bioreactor technology for process optimization and large-scale production of stem cells in numbers appropriate for the cellular therapy market.

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COVER IMAGE: Immunostaining of induced pluripotent stem cells (iPSC). Red, SSEA-4 pluripotency marker; Green, TRA-1-66 pluripotency marker; Blue, Hoechst nuclear stain. — *Courtesy of Ma Sha, PhD, R&D Lab, Eppendorf, Inc.*