

# Mesenchymal Stem Cell Culture in the New Brunswick™ Galaxy® 170 R CO<sub>2</sub> Incubator Under Hypoxic Conditions

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## Abstract

Common laboratory stem cell cultures involve ambient oxygen conditions in contrast to their native environment where they usually reside in 1 – 6 % O<sub>2</sub>, i.e. under hypoxic conditions. Eppendorf CO<sub>2</sub> incubators have a wide array of options that allow for not only carbon dioxide (CO<sub>2</sub>) and temperature control, but also for oxygen concentration control, which can be used to create a hypoxic internal environment to mimic physiological conditions experienced by stem cells. To demonstrate the low O<sub>2</sub> capability of the New Brunswick Galaxy 170 R CO<sub>2</sub> Incubator, Human Mesenchymal Stem Cells (hMSCs) were cultured under 3 % O<sub>2</sub> conditions. hMSCs were grown on Pall Corporation's commercially available

SoloHill microcarriers in spinner flasks for seven days while their growth and morphology were analyzed. These cells were shown to grow normally under hypoxic conditions and, in some cases, to reach higher cell densities than under normal O<sub>2</sub> (normoxic; ~18 – 21 %) conditions. Following this growth period, the retention of stem cell-like character was confirmed by staining and visualizing several stem cell markers with complimentary antibodies. The differentiation potential, or retention of multipotency, of hMSCs was also confirmed as the cells were able to differentiate into adipocytes and osteocytes at levels comparable to cells grown on flatware under the same conditions.

## Introduction

hMSCs are self-renewing cells that can differentiate into several terminally differentiated cell types. These cells have been isolated from multiple sources such as bone marrow, adipose tissue, and peripheral blood, among other adult tissues [1 – 6]. The interest in these cells lies in their potential to repair damaged tissue and cure disease. Recent research has shown that hMSC growth can be significantly affected by O<sub>2</sub> concentrations. The most common belief is that hMSCs should be cultured in an environment that most closely mimics physiological conditions, which have been shown to be between approximately 1 % and 6 % O<sub>2</sub>

for bone tissue [7 – 8]. Some research has demonstrated that hMSCs cultured at low oxygen, or hypoxic, conditions grow and proliferate to much higher cell densities [9 – 11]. However, there is conflicting research which suggests that hypoxic conditions can negatively impact cell differentiation or reduce cell growth [12 – 15]. In this publication, we demonstrate that low-O<sub>2</sub> cell culture conditions are easily established using the New Brunswick Galaxy 170 R CO<sub>2</sub> Incubator (Figure 1) with 1 – 9 % O<sub>2</sub> control, allowing for the evaluation of hMSC growth on microcarriers in stirred cultures under hypoxic and normoxic conditions.



**Fig. 1:** New Brunswick Galaxy 170 R CO<sub>2</sub> Incubator.

## Material and methods

### Incubator

Cell culture was carried out in a New Brunswick Galaxy 170 R CO<sub>2</sub> Incubator (Figure 1) with high temperature disinfection, 4-door split inner door, and 1 – 19 % O<sub>2</sub> control. First, the New Brunswick Galaxy 170 R was used for culture in hypoxic conditions (3 % O<sub>2</sub>), then for normoxic conditions. Temperature and CO<sub>2</sub> setpoints for both experiments were 37 °C and 5 %, respectively. At normoxic conditions, O<sub>2</sub> control was disabled and the incidental O<sub>2</sub> concentration was 18 – 19 %. Over the course of the culture, neither CO<sub>2</sub> nor O<sub>2</sub> concentrations deviated from the set point by more than 0.2 %, except in instances after the incubator door was opened. Following these instances, gas concentrations returned to their proper set point usually within 10 min. All transient culture parameters, including O<sub>2</sub> concentration, were monitored using the convenient interface provided with the unit.

### Cells and consumable materials

All hMSCs used for this study were isolated from a single human bone marrow donor and were purchased from EMD Millipore® at Passage 1 (EMD Millipore, #SCR108). Cells were cultured in Corning® 125 mL spinner vessels on SoloHill microcarriers. The Bell-ennium™ 5-position stir plate (Bellco Glass®, Inc.) for spinner flasks was housed inside of the New Brunswick Galaxy 170 R. hMSCs were expanded in low glucose DMEM supplemented with 10 % FBS, 2 mM L-glutamine, 50 µg/mL penicillin/streptomycin, and 8 ng/mL bFGF. “Complete” medium refers to this formulation. Table 1 outlines the reagents used in this study.

Material	Company	Product Number
Corning® 125 mL spinner vessels	Corning®	10-203B
Collagen Coated Microcarriers	SoloHill®	C102-1521
Plastic Microcarriers	SoloHill®	P102-1521
Pronectin® F-Coated Microcarriers	SoloHill®	PF102-1521
Plastic Plus Microcarriers	SoloHill®	PP102-1521
Low Glucose DMEM	Life Technologies®	11054
Fetal Bovine Serum (FBS)	Thermo Scientific®	SH30071.03
L-Glutamine	ATCC®	30-2300™
basic Fibroblast Growth Factor (bFGF)	EMD Millipore®	GF003
Canted neck cell culture T-flasks	Corning®	430825, 430639, 430641
Dulbecco's Phosphate Buffered Saline (DPBS)	Thermo Scientific®	SH30028.03
TrypLE™	Life Technologies®	12563-029
Pluronic® F68	Life Technologies®	24040-32
Trypan Blue	MP Biomedicals®	1691049
100 µm cell strainer	Thermo Fisher Scientific®	22-363-549

**Table 1:** Consumables and reagents used in this study

### Cell expansion on flatware

To prepare enough cells for use in this study, cells were initially expanded for two passages in T-flasks. Flasks were seeded at 3,000 cells/cm<sup>2</sup> and cultured for ~7 days until the flasks were ~100 % confluent. 50 % medium exchanges were performed on days 3 and 5. To subculture cells, the medium was decanted and the cells were rinsed once with DPBS. The DPBS was immediately decanted and 1 – 3 mL of TrypLE + 0.3 % Pluronic was added, depending on T-flask size. Pluronic acid is believed to have membrane stabilizing properties, protecting cell viability during this step. Flasks were incubated at 37 °C until the cells detached (5 – 10 min). The cells were resuspended in complete medium and then centrifuged at 300 x g for 5 min to pellet the cells. Medium and TrypLE were removed. Cells were resuspended in 10 mL complete medium without bFGF (volume depends on T-flask size and number) and counted using trypan blue stain and a hemocytometer.

### Cell growth in spinner vessels

Spinners were operated at a microcarrier concentration of 5 cm<sup>2</sup>/mL and a volume of 75 mL. Cells subcultured on flatware (as described previously) were used to seed the spinners at a density of 5,000 cells/cm<sup>2</sup>. Spinner cultures were performed as described in previous microcarrier protocols [16]. Briefly, for Collagen Coated, Plastic, Pronectin F, and Plastic Plus microcarriers, cells were seeded in medium (acclimated to culture conditions for 20 min prior to seeding) and supplemented with 0.05 % FBS in the absence of bFGF until > 80 % of the cells attached (3 – 4 h). Serum and bFGF were then added to the culture to supplement the medium to the target of 10 % FBS and 8 ng/mL bFGF. Microcarrier cultures were agitated at 40 rpm. 50 % medium exchanges were performed on days 3 and 5. Glucose supplementation to 100 mg/dL was performed on days 4 and 6. Cell counts to quantify growth were performed on days 3, 5, 6, and 7.

### Cell counting and harvesting from spinner vessels

Cell counts were performed routinely during the culture to monitor the progress of cell growth. In addition, on day 7, the spinners were harvested to determine final cell recoveries. The same protocol was used in both cases, with volumes adjusted appropriately. First, the microcarriers were allowed to settle, then the medium was removed and replaced with DPBS. TrypLE containing 0.3 % Pluronic was added. The mixture was gently pipetted once or twice to thoroughly mix and then incubated at 37 °C for 5 – 10 min (with occasional gentle rocking by hand). Cells and microcarriers were then pipetted to mechanically dissociate the cell clumps into a single-cell suspension. For standard cell counting, the resulting cell slurry was then counted via hemocytometer and trypan blue staining. Following the count, sampled microcarriers were dried and weighed so that the cell count could be adjusted to account for the sampled microcarrier surface area. For cell harvesting, the cell slurry was passed through a 100 µm mesh cell strainer to remove the microcarriers. The cell pool was centrifuged, decanted, and resuspended in medium containing 0.05 % FBS for cell counting.

### Stem cell marker visualization and assessment of attachment to microcarriers

To visualize the expression of several stem cell markers on hMSCs, samples were transferred from the spinners into 15 mL tubes. Once the microcarriers settled, the medium was removed and the cells/microcarriers were carefully washed with DPBS for 5 min at room temperature. When the cells and microcarriers settled, the DPBS was removed and cells and microcarriers were fixed in 4 % paraformaldehyde for 10 min at room temperature. The paraformaldehyde was

then removed, and the cells were washed in DPBS and stored at 4 °C until use. Non-specific binding was blocked by incubation with 5 % FBS in DPBS for 1 h at room temperature. Samples were washed in 500 µL DPBS three times for 5 min at room temperature. Samples were then incubated in 500 µL of the dye/antibody solutions. Dyes and antibodies used were: DAPI (Life Technologies, D3571), phalloidin-FITC (Life Technologies, A12379), FITC anti-human CD44 (BioLegend®, 338803), APC anti-human CD90 (BioLegend, 328113), and Alexa Fluor® 647 anti-human Stro-1 (BioLegend, 340103). All antibodies were used at 1:1000 except for anti-Stro-1 which was used at 1:500.

To assess the distribution of cell attachment across the microcarrier population, a 2 mL sample was taken from each spinner approximately 5 – 6 h after seeding and processed exactly as outlined here. To visualize cell attachment, 500 µL of sample in DPBS was transferred to a single well of a 24-well plate and incubated with 0.5 µL of DAPI stain. Cells were visualized under fluorescent light, and the number of cells per microcarrier was tabulated for a population of > 50 microcarriers.

### Adipogenesis and osteogenesis differentiation

To determine differentiation potential of the hMSCs expanded on microcarriers, cells harvested from spinners were frozen, then thawed, and seeded on 24-well plates at 6,000 cells/cm<sup>2</sup>. Growth/expansion medium was removed, and 1 mL of either Osteogenesis Induction Medium (EMD Millipore, SCR028) or Adipogenesis Induction Medium (EMD Millipore, SCR020) was added. Induction and maintenance media were changed according to EMD Millipore protocol (as recommended by supplier). Osteocyte differentiation was determined by Alazarin Red S staining and adipocyte differentiation was determined by Oil Red O staining (protocols supplied with EMD Millipore kits).

## Results and discussion

### Attachment

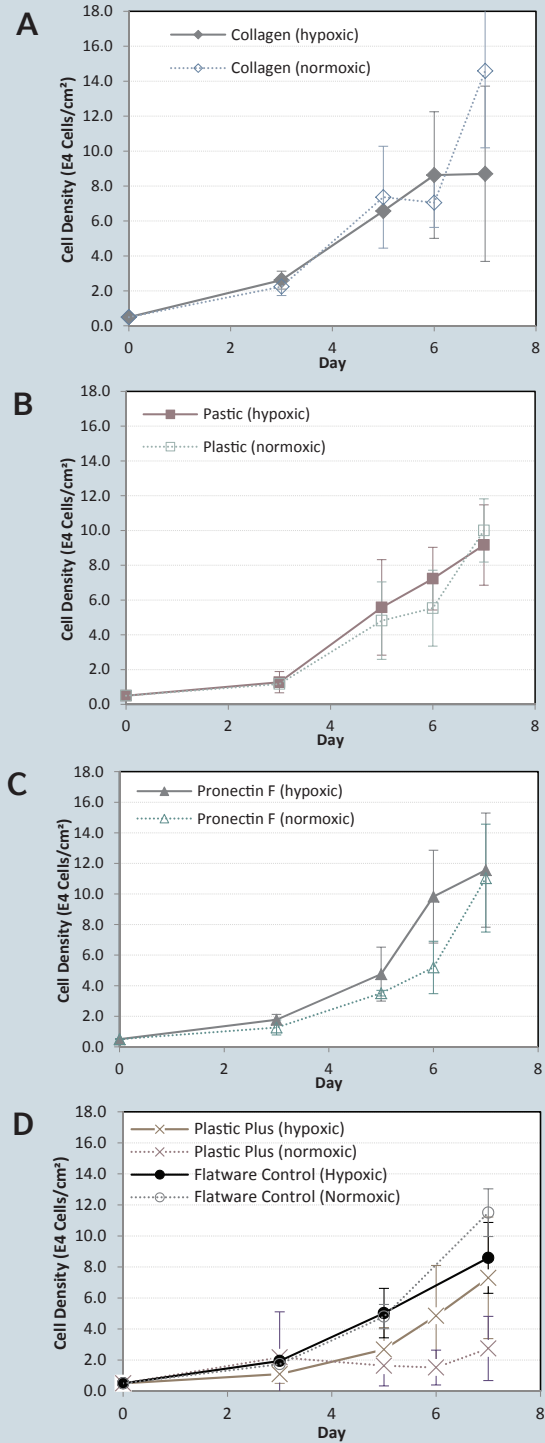
hMSCs were grown in the New Brunswick Galaxy 170 R for 7 days under normoxic and hypoxic conditions. The attachment distribution was assessed 5 to 6 h after seeding. At the current cell seeding density (5,000 cells/cm<sup>2</sup>), each microcarrier on average should contain about 4 cells per bead. An even distribution of cells across the microcarrier population leads to the most effective utilization of the available surface area and usually correlates with the highest cell densities if the surface is favorable for growth. Unused microcarriers (microcarrier with no cells bound) can be

an early indication of an inefficient attachment that may not reach its maximum cell density. The results differed slightly depending on the oxygen condition. Under hypoxic conditions, less than 15 % of the microcarrier population was without cells after 5 – 6 h of culture. At normoxic conditions, this number was slightly higher at ~20 % for some microcarrier types; however, there was much higher variability among the replicates for this condition. Attachment did not seem to be affected by the microcarrier type evaluated.

**Cell growth in the New Brunswick™ Galaxy® 170 R**

Cell densities of cultures were quantified by using the described cell counting method to generate growth curves (Figure 2). Results obtained were generally consistent with past findings [17] in which cell densities reached 6 – 10 x 10<sup>4</sup> cells/cm<sup>2</sup>, with some exceptions. Most notably, cells grew to much higher densities on flatware in this experiment, as previous flatware densities only reached about 4 x 10<sup>4</sup> cells/cm<sup>2</sup>. This difference may be due to the 40 % higher densities that were used to seed flatware in this study. In addition, growth on Collagen Coated microcarriers under normoxic conditions was higher than the previously reported range, while growth on Plastic Plus microcarriers under normoxic conditions was lower than the historical range. However, given the large variance among the different spinner replicates in this study (indicated by the error bars in Figure 2) and the variation in seeding densities tested historically, these differences may not be significant. All other conditions tested were comparable to the historical range.

The dependency of cell densities on O<sub>2</sub> condition (as indicated by daily cell counts) was variable and depended on the microcarrier type tested. The nature of the growth curve for the normoxic conditions differed from the hypoxic conditions in that normoxic growth lagged behind hypoxic growth earlier in the culture, but then accelerated later in the culture. In the case of Collagen Coated microcarriers, cell densities were similar through day 6, but growth appeared to accelerate under normoxic conditions and the resulting cell density was higher on day 7. The same trend was observed on the flatware controls. For Pronectin F microcarriers, cell densities under hypoxic conditions were higher through day 6, but normoxic growth appeared to accelerate, and cell densities were equivalent on day 7. These trends are difficult to conclude with certainty, as there was a large amount of variation between the replicates, either due to sampling or biological variability. A similar trend was observed on Plastic Plus microcarriers, but cell densities remained lower in normoxic conditions throughout the duration of the culture. On Plastic microcarriers, there were no observed differences in cell densities.



**Figure 2:** Cell densities for hMSC cultures in the New Brunswick Galaxy 170 R incubator under normoxic and hypoxic conditions on various microcarriers. Cell densities were obtained via cell counts. Data points represent the average of n = 3 replicates. Error bars indicate +/- one standard deviation. For easier visualization, data were broken down into 4 graphs:

- A: Collagen Coated microcarriers
- B: Plastic microcarriers
- C: Pronectin F microcarriers
- D: Plastic Plus microcarriers and flatware controls

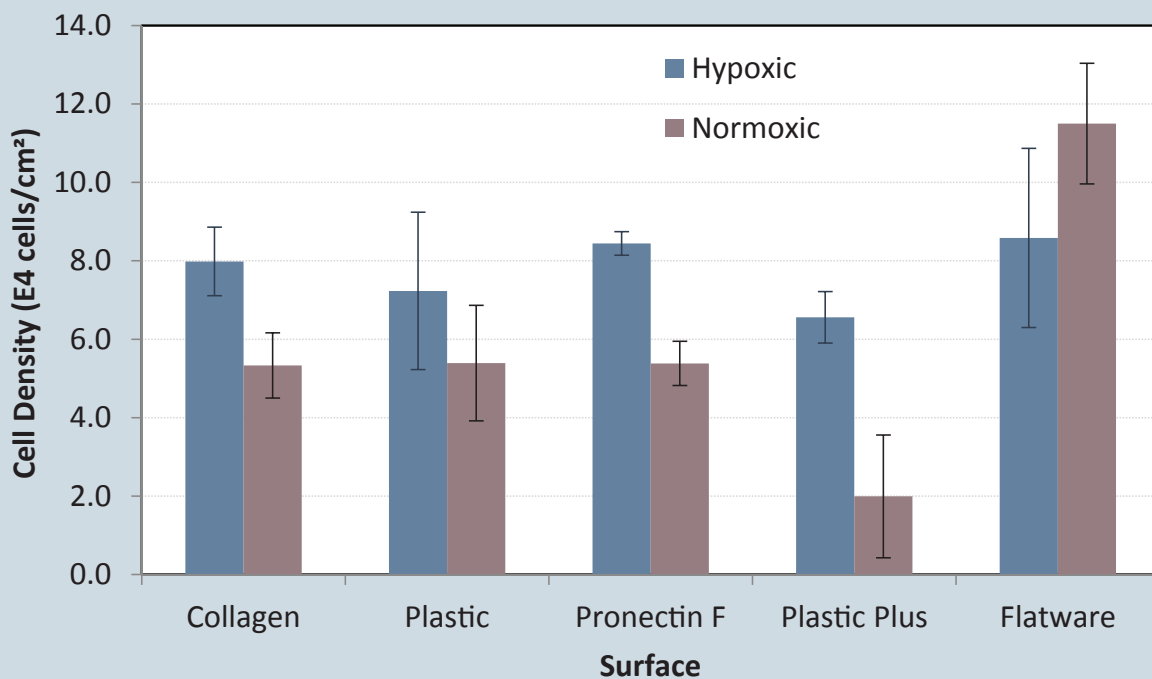
Cell densities were also dependent on the type of microcarrier tested; while the variability between the replicates made it difficult to make a statistical distinction between growth on Collagen Coated, Plastic, and Pronectin F microcarriers, cells did display more favorable growth on these microcarriers in comparison to Plastic Plus.

**Cell harvest**

As observed previously [17], hMSCs tend to form bridges between microcarriers late in the culture. This network of bridges leads to microcarrier aggregates that vary in size, which can contribute to variability in daily sampling. The exact mechanism for microcarrier aggregate formation is an area of ongoing investigation, as well as how the average density of cells in these clumps compares to the density of cells on single suspension microcarriers. Therefore, spinners were harvested on day 7, and the resulting cell pool was counted to generate a harvest cell density (Figure 3) as a potential means to gain a more accurate understanding of the true cell density over the entire microcarrier population. This approach also provides insight into how many cells can be harvested and further expanded.

Results from this harvest method suggested that hypoxic conditions were slightly more favorable for generating high cell densities on microcarriers in comparison to normoxic

conditions. This result is supported by the data suggesting attachment under hypoxic conditions was, on average, better than normoxic conditions, as better attachment kinetics often lay the foundation for higher cell densities later in the culture. Daily cell density measurements were also generally similar or higher under hypoxic conditions through day 6 of the culture, suggesting that the apparent accelerated normoxic growth on day 7 might be a sampling or assay artifact. Alternately, the harvest method used for these spinners could be preferentially favorable to cells grown under hypoxic conditions, as hypoxic conditions may be improving cell robustness and making cells more resistant to the stresses of harvesting. Regardless of interpretation, these data suggest that there is some advantage to growing hMSCs on microcarriers under hypoxic conditions. In the case of flatware controls, the data do not support the contention that hypoxic conditions are beneficial for growth. However, the procedure used to harvest flatware was slightly different than the procedure to harvest microcarriers, namely cells do not experience the same magnitude of shear forces. Although cell densities appear higher for normoxic conditions, this difference is not statistically significant and therefore may not represent a true departure from the overall trend.



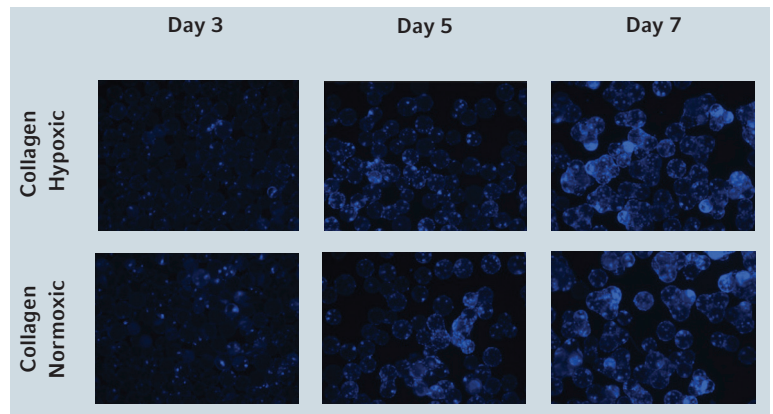
**Figure 3:** Harvested day 7 cell densities for hMSC cultures at hypoxic vs. normoxic O<sub>2</sub> concentrations in the New Brunswick Galaxy 170 R incubator grown on microcarriers and flatware. Cell densities were obtained via cell counts. Data points represent the average of n = 3 replicates. Error bars indicate +/- one standard deviation.

**Cell morphology, identity and differentiation potential**

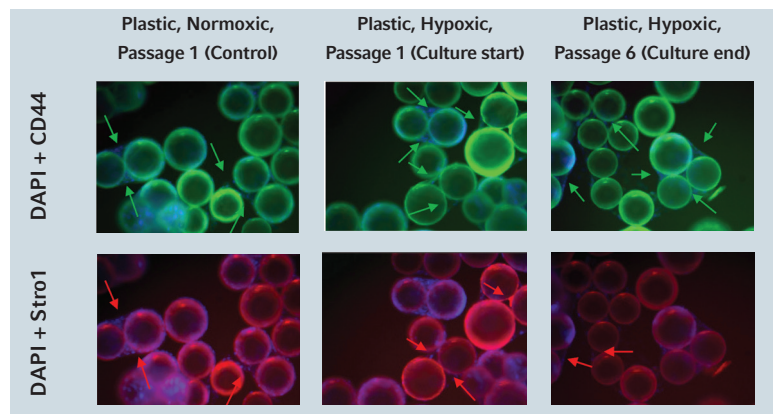
On days 3, 5, and 7 of the culture, samples were obtained and stained with DAPI to help visualize the three-dimensional confluency and morphology of cells on microcarriers. A comparison of cells cultured in hypoxic vs. normoxic conditions on Collagen Coated microcarriers (Figure 4) revealed no differences in confluency levels during culture. All cells were of proper morphology, indicating good health and viability of the culture.

Day 7 samples were obtained prior to harvest and stained to verify the retention of stem cell markers as an indication of stem cell identity. Additionally, cells were serially passaged on plastic microcarriers at hypoxic conditions for a total of 6 passages, and samples from passage 6 were compared to cells obtained from passage 1 (Figure 5). Both samples stained positive for CD44, CD90 (not shown), and Stro1, indicating retention of stem cell identity. Results are representative for all microcarrier types after passage 1 at both hypoxic and normoxic conditions (data not shown), but serial passaging was performed only on plastic.

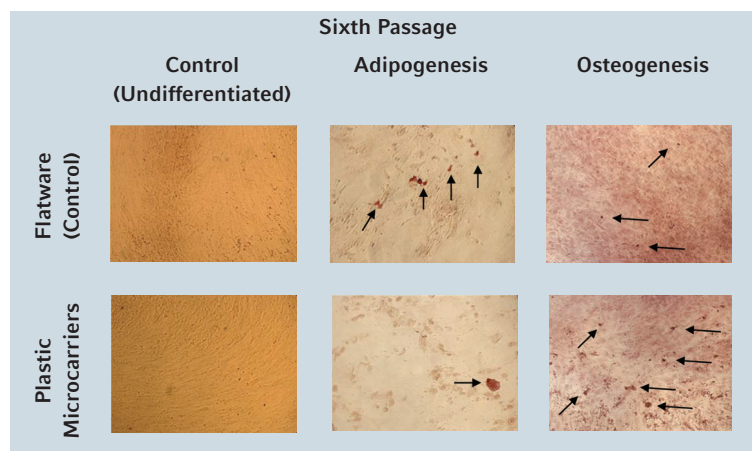
Finally, cells serially passaged 6 times on animal-derived component-free plastic microcarriers under hypoxic conditions were successfully differentiated into adipocytes and osteocytes following both the first and sixth passage (data not shown, Figure 6). Adipogenesis was indicated by the presence of red-stained lipid droplets and osteogenesis was indicated by a calcification layer and the presence of red-stained calcium phosphate deposits.



**Figure 4:** 3D-stacked Fluorescent DAPI stained images (4 X magnification) of hMSCs on Collagen Coated microcarriers at 3, 5, and 7 days. The similarities in cell morphology between hypoxic and normoxic conditions are representative of other microcarrier types.



**Figure 5:** hMSCs expanded in the New Brunswick Galaxy 170 R were incubated with anti-CD44 (green), anti-Stro1 (red) and DAPI (blue) at 10 X magnification. Exposure times were adjusted for maximum contrast. High background resulted from lengthy exposure times due to low expression levels. Populations of CD44 expressing cells are indicated by green arrows. Populations of Stro1 expressing cells are indicated by red arrows. Results are representative of all microcarrier types tested.



**Figure 6:** Cells serially passaged six times on plastic microcarriers in the New Brunswick Galaxy 170 R incubator differentiate into adipocytes and osteocytes. Bright field images were taken with a Nikon® Ti65 at 20 X (adipocytes) and 10 X (osteocytes). Arrows indicate lipid droplets and calcium phosphate deposits. Sixth passage undifferentiated samples were stained with hematoxylin solution.

## Conclusion

hMSCs grown in the New Brunswick Galaxy 170 R CO<sub>2</sub> Incubator on microcarriers under hypoxic conditions display normal growth as compared to those grown at normoxic O<sub>2</sub> concentrations. The data presented here suggest that more cells may be harvested from microcarriers grown under hypoxic conditions, indicating that hypoxic conditions favor a slightly better growth rate. Large differences in growth rates between hypoxia and normoxia may not be expected in this experiment, since the cell line was already adapted to normoxic conditions when the cells were harvested from the patient and removed from their native hypoxic environment. Recent reviews indicate that significant changes may happen at the gene expression level during adaptation to culture conditions, where O<sub>2</sub> level plays an important role by regulating hypoxia-inducible factor-1 (HIF-1)-mediated expression of different genes [18].

The ease of setup and the tight O<sub>2</sub> concentration control displayed by the New Brunswick Galaxy 170 R provided

the ideal conditions for this experiment. This study is a demonstration of the low oxygen feature of an advanced incubator and it could be used to successfully conduct stem cell studies under physiological conditions. As recently pointed out by Dr. Elaine Fuchs (Rockefeller University) during her keynote speech at the 2013 annual meeting of the International Society for Stem Cell Research (ISSCR), many *in vitro* cell culture techniques may produce questionable results since the culture environment is greatly deviated from native physiological conditions. Dr. Fuchs's group created a mouse embryo-based "biological incubator" in which to study the effects of gene knockout and stem cell transplantation under native physiological conditions instead of in a flask [19,20]. Such principle and practice is enchanting but may not be feasible for universal adaptation by all cell culture labs. However, it is certainly feasible to employ an incubator with precision O<sub>2</sub> control to conduct cell culture research under physiological oxygen conditions.

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