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Ex Vivo Expansion of Human Mesenchymal Stem Cells: A More Effective Cell Proliferation Kinetics and Metabolism Under Hypoxia

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The low bone marrow (BM) MSC titers demand a fast ex vivo expansion process to meet the clinically relevant cell dosage. Attending to the low oxygen tension of BM in vivo, we studied the influence of hypoxia on human BM MSC proliferation kinetics and metabolism. Human BM MSC cultured under 2% (hypoxia) and 20% O_2 (normoxia) were characterized in terms of proliferation, cell division kinetics and metabolic patterns. BM MSC cultures under hypoxia displayed an early start of the exponential growth phase, and cell numbers obtained at each time point throughout culture were consistently higher under low O_2, resulting in a higher fold increase after 12 days under hypoxia (40 ± 10 vs. 30 ± 6). Cell labeling with PKH26 allowed us to determine that after 2 days of culture, a significant higher cell number was already actively dividing under 2% compared to 20% O_2 and BM MSC expanded under low oxygen tension displayed consistently higher percentages of cells in the latest generations (generations 4–6) until the 5th day of culture. Cells under low O_2 presented higher specific consumption of nutrients, especially early in culture, but with lower specific production of inhibitory metabolites. Moreover, 2% O_2 favored CFU-F expansion, while maintaining BM MSC characteristic immunophenotype and differentiative potential. Our results demonstrated a more efficient BM MSC expansion at 2% O_2, compared to normoxic conditions, associated to an earlier start of cellular division and supported by an increase in cellular metabolism efficiency towards the maximization of cell yield for application in clinical settings.

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In recent years, human mesenchymal stem cells (MSC) have become one of the most promising candidates for tissue engineering and regenerative medicine applications (Caplan, 2007), mostly due to the differentiative potential (Tae et al., 2006) and immunologic properties (Le Blanc and Ringden, 2007; Jones and McTaggart, 2008) of these multipotent cells. Particularly, in the settings of cellular therapy, MSC have been used to prevent or to treat graft-versus-host disease after hematopoietic stem cell transplantation (Ringden et al., 2006; Kebriaei et al., 2009).

The frequency of MSC is considered to be as low as 0.01% of BM MNC in a newborn, declining with age to 0.001–0.0005% (Caplan, 2007). Though minimal and maximal doses for therapeutic application have not yet been determined, several million BM MSC per kg of patient body weight have been infused (Subbanna, 2007). Therefore, an efficient and Good Manufacturing Practices (GMP)—compliant ex vivo expansion process is required to achieve MSC clinical relevant numbers.

In their BM niche, self-renewal and/or differentiation of MSC are governed by a complex microenvironment signaling that involves cell-to-cell interactions (Ball et al., 2004), soluble factors (proteins or growth factors; Gregory et al., 2003; Choi et al., 2008) and even mechanical forces (Park et al., 2004). The oxygen tension is another important regulator of MSC functions. In fact, Grayson and colleagues showed that, under a 2% O_2 hypoxia, not only the expression of stem cell genes Oct-4 and Rex-1 was up-regulated but also the synthesis of extracellular matrix (ECM) proteins, such as fibronectin, was increased (Grayson et al., 2006, 2007). Moreover, upon induction, levels of differentiation into mesenchymal tissue were increased at lower oxygen tensions (Wang et al., 2005; Grayson et al., 2006, 2007).

Apart from the BM, local hypoxia can also occur in certain pathological conditions, for instance ischemia, infection or tissue injury, where MSC are believed to home and have a regenerative effect through a trophic activity (Caplan, 2007). Concomitantly with local nutrient depletion, the low oxygen level in an injured tissue creates a hostile microenvironment that may jeopardize the efficacy of MSC regenerative role. In fact, some authors demonstrated that the combination of hypoxia and serum deprivation induced MSC apoptosis, although the predominant effect was attributed to the absence of growth factors caused by serum deprivation (Zhu et al., 2006; Potier et al., 2007). Nevertheless, Rosová and co-workers showed that hypoxic preconditioning improved the therapeutic potential of human MSC (Rosova et al., 2008). Thus, in order to develop more efficient protocols of cellular therapy using human MSC, it is crucial to perform more detailed studies of MSC performance on hypoxic microenvironments.

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Recently, there have been an increasing number of studies focusing the influence of hypoxia on MSC differentiation (Wang et al., 2005; Grayson et al., 2006, 2007). However, to our knowledge, no studies are found in the literature concerning the effects of low oxygen tension on cell division kinetics and metabolism for human BM MSC, focusing on the ex vivo expansion of these cells. In this work, we showed that a 2% O_2 hypoxic environment improved BM MSC expansion levels by inducing an early start of the exponential growth phase, with cells starting cell division earlier in the first days of culture. In addition, we observed an increase of cellular metabolism efficiency, associated to the adaptation of BM MSC to the low oxygen tension environment. These results gave important insights on how hypoxia culture favors human BM MSC ex vivo expansion, being advantageous towards the maximization of cell yield in a clinical-scale MSC expansion process.

Methods

Human bone marrow mesenchymal stem cell cultures

Bone marrow (BM) aspirates were obtained from healthy donors after informed consent at Instituto Português de Oncologia Francisco Gentil, Lisboa, Portugal. Low density BM mononuclear cells (MNC) were separated by a Ficoll density gradient (1.077 g/ml) (GE Healthcare, Uppsala, Sweden) and then washed twice in Dulbecco's Modified Essential Medium (DMEM, Gibco, Grand Island, NY) with 10% Fetal Bovine Serum (FBS, MSC qualified, Gibco, Grand Island, NY). BM MNC were then plated at a density of 2×10^5 cells/cm^2 on T-175 flasks (BD Falcon, Franklin Lakes, NJ) in DMEM with 10% FBS, supplemented with streptomycin (0.025 μg/ml), penicillin (0.025 U/ml) (Gibco), at 37°C and 5% CO_2 in a humidified atmosphere. Medium was changed twice a week. BM mesenchymal stem cells (MSC) were isolated based on adherence to plastic, and near cell confluence (70–80%) exhausted medium was removed from the flasks, cells were washed with phosphate buffered saline (PBS, Gibco) and detached from the flask by adding Accutase solution (Sigma, St. Louis, MO) for 7 min at 37°C. Isolated BM MSC expressed their characteristic immunophenotype being CD73, CD90 and CD105 positive and negative for CD31, CD34, CD45, and CD80. Cell number and viability was also determined using the Trypan Blue (Gibco) exclusion method and then cells were replated at an initial density of 3,000 cells/cm^2 in T-175 or T-75 flasks. For these studies, BM MSC from five donors (including both male and female), with ages between 20 and 40 years old, and within passages 3–4, were used.

BM MSC expansion under normoxia and hypoxia

BM MSC were expanded in 12-well plates (BD Falcon) at an initial cell density of 1,000 cells/cm^2, using DMEM + 10% FBS, under normoxia (20% O_2) and hypoxia (2% O_2) at 37°C and 5% CO_2. A C-Chamber connected to a Proox Model 21 controller (BioSpherix, Redfield, NY) was used to establish a hypoxic environment.

Proliferative and phenotypic analysis

During time in culture, the ex vivo expansion of the BM MSC was determined by using the Trypan Blue exclusion method for each different culture condition. Fold increase in total cell number was calculated by dividing the number of cells of each day by the number of cells at day 0. Population doublings for both conditions were calculated by dividing the logarithm of the fold increase by the logarithm of 2.

At day 7, cultured cells were also analyzed by flow cytometry (FACSCalibur equipment, Becton Dickinson, San Jose, CA) using a panel of mouse anti-human monoclonal antibodies (PE-conjugated) against: CD73 (Becton Dickinson Immunocytometry Systems, San Jose, CA), CD90 (R&D Systems, Minneapolis, MN), and CD105 (Invitrogen, Carlsbad, CA). Cells were incubated with these monoclonal antibodies for 15 min in the dark at room temperature and then cells were washed in PBS and fixed with 1% paraformaldehyde (Sigma). Isotype controls were also prepared for every experiment. A minimum of 10,000 events was collected for each sample and the CellQuest software (Becton Dickinson) was used for acquisition and analysis.

Modeling cell growth

BM MSC proliferation data was adjusted to a first order kinetic model in order to compute the concentration of viable cells (X_v) over time (t). The balance for viable cells is described in Equation (1)

$$\frac{dX_v}{dt} = \mu X_v \left(\frac{X_{max} - X_v}{X_{max}} \right) - k_d X_v \quad (1)$$

where μ and k_d are the specific growth and death rates, respectively. It was assumed that both μ and k_d were time invariant and that cell differentiation was negligible. The X_{max} term was introduced to take into account the effect of cell confluence. The parameters were determined using a Microsoft Excel worksheet.

Cell division kinetic studies

BM MSC were labeled using PKH26 Red Fluorescent Cell Linker Kit (Sigma) prior to cell culture according to manufacturer's instructions. Cells were then plated in 12-well plates, at an initial cell density of 3,000 cells/cm^2, under 2% and 20% O_2 atmospheres. In the following 6 days, one well of each condition was processed and cells were analyzed by flow cytometry. Since PKH dye binds stably and irreversibly to the lipid layer of cell membrane, each generation of cells is therefore half as fluorescent as the previous one allowing the determination of both the time point at which cultured cells commence cell division and precise quantitation of the number of divisions the cells have undergone at any point in culture. Flow cytometry data were then analyzed using the Proliferation Wizard module of the ModFit software (Becton Dickinson) (da Silva et al., 2009).

Telomere length analysis

The relative size of BM MSC telomeres before and after the expansion was determined using the Telomere PNA Kit/FITC for Flow Cytometry (Dako, Glostrup, Denmark). Briefly, BM MSC samples were mixed with control cells (cell line 1,301, Istituto Nazionale per la Ricerca sul Cancro c/o CBA, Genova, Italy, which is tetraploid, with very long telomeres) and a hybridization solution with or without a Telomere PNA Probe/FITC was added. After a 10 min cycle at 82°C, cell mixtures were kept in the dark at room temperature overnight. Afterwards, samples were washed twice and a DNA Staining Solution was added for, approximately, 6 h. The analyses were then performed by flow cytometry. The relative telomere length (RTL) of BM MSC was calculated relatively to the telomere length of 1,301 cells (control).

Metabolite analysis

At days 0, 3, 5, 7, 10, and 12, supernatants were collected and the concentrations of glucose, lactate, glutamine and ammonia were determined using an automatic analyzer YSI 7100MBS (Yellow Springs Instrument, Yellow Springs, OH). The specific metabolic consumption/production rates (q_{Met}) were calculated for every time interval with the following equation:

$$q_{Met} = \frac{\Delta Met}{\Delta t \overline{X_v}}$$

where ΔMet is the variation of nutrient/metabolite concentration during the time interval Δt and $\overline{X_v}$ is the average viable

cell number for that time period. The apparent yield of lactate from glucose ($Y'_{lactate/glucose}$) was also calculated as the ratio between $q_{lactate}/q_{glucose}$.

Osteogenic differentiation

At cell confluency, osteogenesis was induced using low-glucose DMEM containing 10% FBS, 100 nM dexamethasone, 10 mM β—glycerophophate and 0.05 mM 2-phospho-L-ascorbic acid (all from Sigma). The medium was changed twice a week for 14 days. After induction cells were tested for alkaline phosphatase (ALP) and von Kossa stainings. Briefly, cells were washed in cold PBS and fixed in 10% cold neutral-buffered formalin (Sigma) for 15 min. After fixing, cells were washed and kept in distilled water for 15 min. Cells were incubated with a 0.1 M solution of Tris–HCl (Sigma) containing Naphtol AS MX-PO_4 (0.1 mg/ml) (Sigma) in dimethylformamide (Fischer Scientific, Pittsburgh, PA) and 0.6 mg/ml of Red Violet LB salt (Sigma) for 45 min and washed 4 times with distilled water. Cells were then observed under the microscope (Leica Microsystems, Hamburg, Germany) for ALP staining, as a result of osteogenic commitment. The remaining cells were then stained with silver nitrate (2.5%, w/v) (Sigma) for 30 min at room temperature for von Kossa staining to evaluate the deposits of calcium in the cultures. Cells were washed 3 times in distilled water, set to dry and observed in the microscope.

Adipogenic differentiation

The adipogenic differentiation was induced at confluence after culture for 15 days, using DMEM containing 170 nM insulin, 0.5 mM 3-isobutyl-1-methyl-xanthine, 0.2 mM indomethacin (all from Sigma), 1 mM dexamethasone and 10% FBS. Medium was changed every 3 days, and the assessment of differentiation towards an adipocytic phenotype was performed based on the accumulation of lipids, using Oil Red-O stain. Cells were washed with cold PBS and fixed in 2% formaldehyde for 30 min. After fixing, cells were then washed with distilled water and incubated with Oil Red-O solution (Sigma) (0.3% in isopropanol) at room temperature for 1 h. Finally, cells were washed with distilled water and observed under the microscope.

CFU-F assays

Colony forming units-fibroblast (CFU-F) assays were performed at days 0 and 7. Cultured cells were plated on T-25 flasks with a cell density of 10 cells/cm^2 and kept at 37°C, 5% CO_2 in a humidified incubator for 14 days. Cells were then washed once with PBS and incubated with a 0.5% crystal violet (Sigma) solution in methanol (Sigma) for 30 min at room temperature. Finally, stained colonies were rinsed 4 times with PBS and once with distilled water. After drying, colonies with 50 or more cells were counted.

Statistical analysis

Results are presented as mean ± standard error of mean (SEM) of the values obtained for all five donors (performed in duplicate). When appropriate, comparisons between experimental results were determined by the Paired Sampled Wilcoxon Signed Rank Test. A *P*-value less than 0.05 was considered statistically significant.

Results

In this work we evaluated the effect of a 2% O_2 microenvironment on the ex vivo expansion potential of human BM MSC, focusing on the cell division kinetics and metabolism. In order to better qualify and quantify the effect of hypoxia on cell proliferation, cells were plated at a relatively low initial cell density (1,000 cells/cm^2), in order to minimize surface area limitations, and cultured for 12 days.

Expansion of BM MSC under hypoxia resulted in higher cell yields

BM MSC proliferation was evaluated for different five time points in culture (days 3, 5, 7, 10, and 12) for both culture conditions. When cultured under a 2% oxygen tension, the expansion level of BM MSC was higher throughout the entire culture compared to the expansion of cells cultured under normoxia (Fig. 1, $P < 0.05$). In fact, after 12 days of culture, cells expanded under hypoxia reached a fold increase in total cell number of 40 ± 10, which was significantly higher ($P < 0.05$) than the fold increase of 30 ± 6 obtained for cells expanded under normoxic oxygen tension. Cell viability was maintained over 90% for both 2% and 20% O_2 cultures for the entire culture period (data not shown). In order to determine the beneficial effect of hypoxia to maximize cell yield in a clinically relevant time frame culture of 3 weeks, BM MSC from one donor were expanded for three consecutive passages under hypoxic and normoxic microenvironments. On day 7 of each passage, cells were collected by enzymatic treatment, counted and replated at the initial cell density (1,000 cells/cm^2).

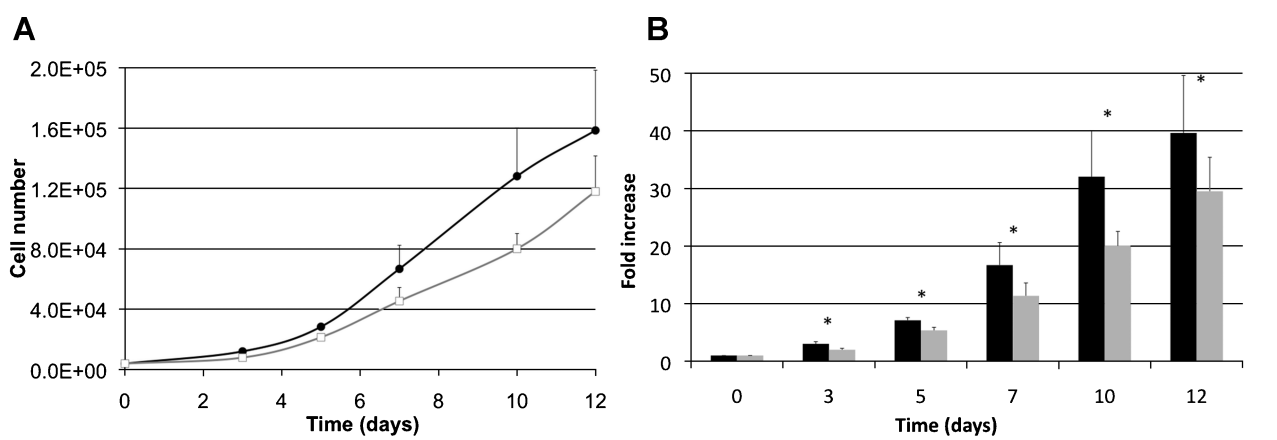

Fig. 1. Ex vivo expansion of human BM MSC under hypoxia (2% O_2) and normoxia (20% O_2). Total cell numbers (A) were determined throughout the culture for both hypoxia (black circles) and normoxia (white squares) and the fold increase values in total cell number (B) obtained for hypoxia (black bars) and normoxia (gray bars) are presented as mean ± SEM (*$P < 0.05$).

TABLE 1. Cumulative fold increase of BM MSC expanded for three passages under hypoxia and normoxia

	Cumulative fold increase (three passages)	RTL (%)	CFU-F fold increase
Hypoxia	360	6.1	19 ± 4
Normoxia	221	9.3	13 ± 3

The relative telomere length (RTL) was also determined for both culture conditions after the expansion. Prior to the cell expansion, the RTL was 12.0% (compared to control 1,301 cell line). The clonogenic potential of BM MSC expanded at 2% and 20% O_2 was also calculated in terms of fold increase.

The cumulative fold increase (product of three consecutive passages fold increase values) was clearly higher for cells expanded at 2% oxygen tension (fold increase of 360) than for cells cultured at 20% oxygen tension (fold increase of 221) (Table 1). Before and after the expansion, the relative telomere length (RTL) of BM MSC was assessed as a qualitatively measurement of cell proliferative potential. On day 0, BM MSC presented an RTL of 12.0% (compared to control cells) and after 3 passages RTL was reduced to 6.1 and 9.3% for cells expanded under hypoxia and normoxia, respectively (Table 1).

In order to assess the differentiative potential of BM MSC after the expansion under a 2% oxygen tension, cells were subsequently cultured in osteogenic and adipogenic differentiation media. The positive staining for ALP and von Kossa (osteogenesis) and the formation of lipid vacuoles stained with Oil Red O (adipogenesis) were observed for cells expanded both under hypoxia or normoxia, with no detectible differences among them (Fig. 2).

Immunophenotypic assays were also performed by flow cytometry after cell expansion to verify if BM MSC maintained their characteristic immunophenotype (Dominici et al., 2006). Flow cytometry analyses showed that more than 95% of cells expanded under a low oxygen tension expressed CD73, CD90 and CD105, as well as cells expanded under the atmospheric oxygen tension (Fig. 2).

In addition, the clonogenic potential of BM MSC was assessed after 7 days of expansion (Table 1). A hypoxic microenvironment led to a 19 ± 4-fold increase in CFU-F progenitors by day 7, whereas a 13 ± 3-fold increase was obtained for cells expanded under 20% O_2.

BM MSC start cellular division earlier under hypoxia

In order to evaluate if the different oxygen tensions studied would have an effect on BM MSC specific growth and death rates, we used a first order kinetic model (Eq. 1) to determine those values for hypoxic and normoxic culture conditions. Regarding the specific growth rate (μ), cells expanded under different oxygen tensions displayed similar values (0.49 ± 0.04 and 0.45 ± 0.06 day^{-1} for hypoxia and normoxia, respectively). Also for the specific death rate (k_d), no significant differences were found for cells expanded under low (0.03 ± 0.02 day^{-1}) and normal (0.06 ± 0.04 day^{-1}) oxygen tension. We also determined the population doublings throughout time in culture for both culture conditions. Cells cultured under hypoxia displayed consistently higher cumulative population doublings throughout time in culture ($P < 0.05$), reaching a maximum of 5.2 ± 0.3 versus 4.8 ± 0.3 for normoxia at day 12.

To find potential differences for the influence of oxygen tension of BM MSC proliferation among different cultures, especially in the lag phase preceding proliferation, we also performed cell division kinetics studies for the first 6 days of culture. Cells were labeled with PKH26, processed daily by flow cytometry and respective data were analyzed using the Proliferation Wizard of the ModFit Software, where the percentage of cells for each generation was determined (Fig. 3). After 24 h, BM MSC cultured under both conditions remained undivided (as Parent generation cells); active cell division must then have started upon this period and at day 2, BM MSC cultured under hypoxia presented a significant lower percentage of undivided cells when compared to normoxia expanded cells (22.4 ± 3.1% and 30.0 ± 2.6%, respectively, $P < 0.05$). Accordingly, at this time point, the percentage of hypoxia cells on generation 3 (26.4 ± 2.7%) was significantly higher than for normoxia cells (17.1 ± 4.6%). In the following days (days 3, 4, and 5), this earlier start of cellular division was also observed as BM MSC expanded under low oxygen tension displayed consistently higher percentages of cells in the latest generations (generations 4–6), although cell distribution profile

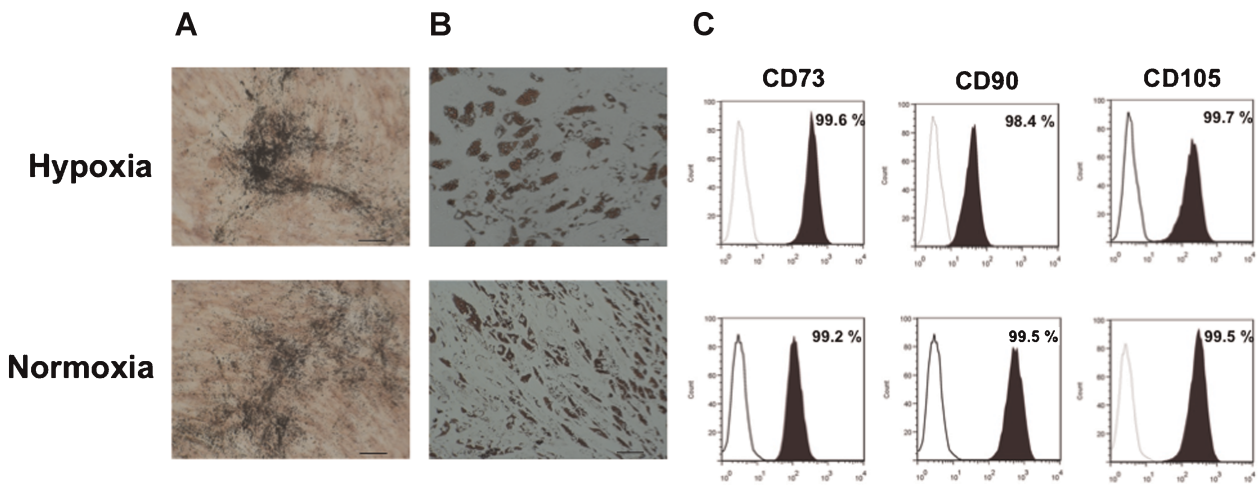

Fig. 2. Differentiative potential and immunophenotype of human BM MSC expanded under different O_2 tensions. After the expansion, cells were cultured in either osteogenic or adipogenic inductive media for 14 days. A: Osteogenic differentiation was detected by alkaline phosphatase (osteogenic progenitors in pink) and Von Kossa stainings (calcium deposits in black). B: Adipogenic differentiation was confirmed by Oil Red O staining showing lipid vacuoles (in red) in cells. C: BM MSC characteristic immunophenotype was also not altered by the expansion under a low oxygen tension.

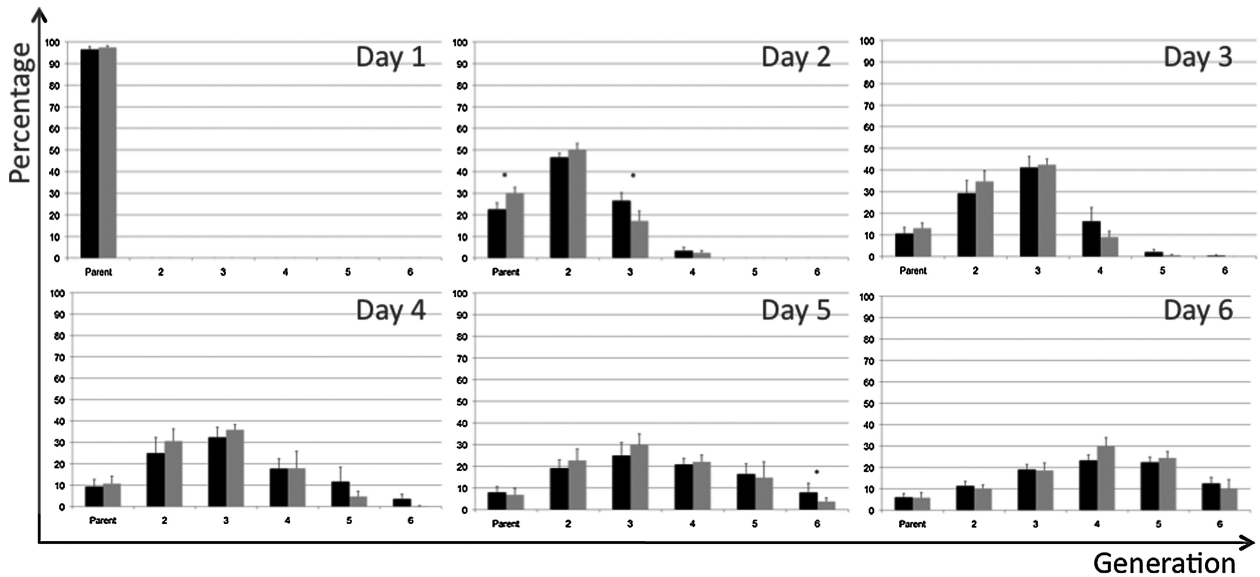

Fig. 3. Cell division kinetics of human BM MSC under hypoxia and normoxia. Cells were labeled with PKH26 and cultured for 6 days. Every day, cells from both hypoxia (black bars) and normoxia (gray bars) cultures were analyzed by flow cytometry and the percentage of cells from each generation was determined using the Proliferation Wizard of the ModFit Software. Values are represented as mean ± SEM (*$P < 0.05$).

among generations 2–6 was similar for both culture conditions. Indeed, at day 5, a significantly higher percentage of cells expanded under low O_2 already reached generation 6 ($7.8 \pm 4.3\%$), compared to normoxic conditions ($3.7 \pm 1.7\%$) ($P < 0.05$). Later in culture, no significant differences were found between cell distributions among the different generations, for both culture conditions.

Human BM MSC metabolism is more efficient under low oxygen tension

The analysis of cell nutrient consumption and metabolite production allows a better understanding of how different culture conditions affect cellular metabolism. Cell culture supernatants were collected, in different time points, and concentrations of nutrients (glucose and glutamine) and metabolites (lactate and ammonia) were determined using the metabolite analyzer. Then, the average specific consumption rates of glucose and glutamine and average specific production rates of lactate and ammonia were calculated for different culture time intervals (Fig. 4 and 7).

In the beginning of the culture, until day 7, BM MSC expanded under a 2% oxygen tension showed consistently higher values of specific consumption rate of glucose (Fig. 4A), ranging between 14 and 17 pmol day^{-1} $cell^{-1}$, when compared to normoxia (12–14 pmol day^{-1} $cell^{-1}$). Nevertheless, as cultures reached days 7–12, values of glucose specific consumption rate became similar for both conditions, with

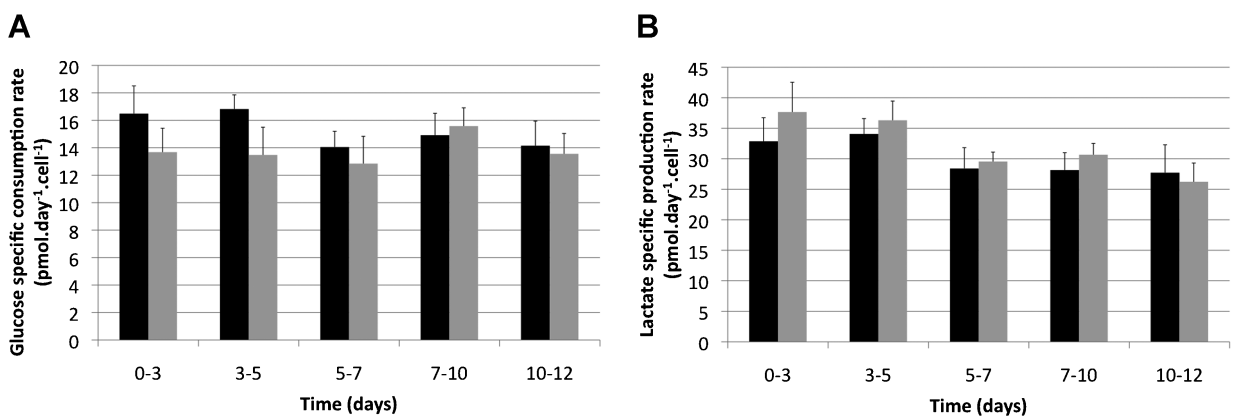

Fig. 4. Glucose specific consumption rate and lactate specific production rate of human BM MSC expanded under different O_2 tensions. Throughout time in culture, concentrations of glucose and lactate were monitored and specific consumption/production rates of glucose (A)/ lactate (B) were determined for cells cultured under hypoxia (black bars) and normoxia (gray bars) for different time intervals. Values are represented as mean ± SEM.

minor differences of 0.6 pmol day^{-1} cell^{-1} between them. Thus, the average value of glucose specific consumption rate (referred to the entire culture period) was approximately the same for hypoxia (15 ± 1 pmol day^{-1} cell^{-1}) and normoxia (14 ± 1 pmol day^{-1} cell^{-1}). The glucose concentration reached minimum values only by day 12 of culture (1.0 ± 0.4 and 2.1 ± 0.5 mM for hypoxia and normoxia, respectively).

In terms of lactate specific production rate, BM MSC presented higher values under normoxic culture conditions until day 10 (Fig. 4B). Moreover, for both oxygen tensions, the specific production rate of lactate presented higher values in the first days of expansion (days 0–5), with average values of 30 ± 3 and 32 ± 5 pmol day^{-1} cell^{-1} for hypoxia and normoxia, respectively. Importantly, lactate concentration never reached inhibitory values (over 35 mM; Schop et al., 2009) throughout time in culture for both studied conditions as shown in Figure 5 (maximum values of 10.3 and 8.2 mM of lactate for hypoxia and normoxia, respectively).

The discrepancies found in the consumption of glucose and production of lactate, between cells expanded under different oxygen tensions, were also reflected on the values of the apparent yield of lactate from glucose ($Y'_{lactate/glucose}$) throughout the culture (Fig. 6). Under hypoxia, BM MSC presented consistently lower values of $Y'_{lactate/glucose}$, particularly for the first week of culture. In the following days, similar values of $Y'_{lactate/glucose}$ were obtained for both culture conditions. Consequently, the global average value of $Y'_{lactate/glucose}$ was considerably lower for cells expanded under hypoxia, 2.0 ± 0.1, when compared to a 2.4 ± 0.5 value for normoxia-expanded cells.

Contrarily to the consumption of glucose, that showed small variations throughout all culture time, the specific consumption rate of glutamine (Fig. 7A), a source of carbon and nitrogen, was significantly higher ($P < 0.05$) for the first 3 days of culture under both conditions (48 ± 7 and 31 ± 12 pmol day^{-1} cell^{-1} for hypoxia and normoxia, respectively) compared to the following time period (days 3–5: 19 ± 2 and 13 ± 5 pmol day^{-1} cell^{-1} for hypoxia and normoxia, respectively). Moreover, after day 5, the consumption of glutamine by BM MSC was clearly reduced, independently of the oxygen tension, and resulted in a average specific consumption rate of glutamine between days 5 and 12 of 5 ± 2 and 4 ± 1 pmol day^{-1} cell^{-1} for hypoxia and normoxia, respectively. Throughout time in culture, the concentration of glutamine was constantly higher than 2 mM both for hypoxic and normoxic cultures.

As expected, the specific production rate of ammonia (Fig. 7B), a by-product of glutamine metabolism, showed a corresponding variation trend to glutamine consumption, with higher values in the beginning of the culture and decreasing with culture time. However, under a 2% oxygen tension, the specific production rate of ammonia was consistently lower throughout time in culture. In fact, BM MSC expanded in hypoxia presented a 50% average reduction on ammonia specific production rate. Furthermore, it was also important to verify if ammonia concentration reached cell growth inhibitory values throughout time in culture (Fig. 5). Under 20% oxygen tension, the ammonia concentration consecutively exceeded the referenced ammonia inhibitory concentration of 2.4 mM (Schop et al., 2009), while in hypoxia cultures the concentration of ammonia never reached values higher than 2.3 mM.

Discussion

The ex vivo expansion of BM MSC for clinical applications is, presently, a very time consuming and expensive cell culture process. Moreover, the time frame to achieve clinical relevant cell numbers is normally within 3–5 weeks, which may be a considerably long period of time in the settings of cellular therapy, such as the treatment of acute graft-versus-host disease. The optimization of the expansion process should then consider all these aspects and focus on the maximization of cell yield, while reducing cell culture time, which will, as a result, reduce total process costs (which include equipment/facilities/specialized human resources costs as well as culture-associated costs) (Kirouac and Zandstra, 2008).

In an attempt to mimic the bone microenvironment hypoxia, where the oxygen tension is described to vary between 1% and 6% (Chow et al., 2001), we cultured BM MSC under 2% O_2 and compared cell proliferative potential, as well as cell division kinetics and metabolic patterns with BM MSC expanded under normoxia (20%). It should be noticed that preliminary results in our lab demonstrated no significant differences in terms of cell growth between BM MSC expanded under 5% O_2 compared to normoxic levels (20%) (unpublished results).

BM MSC expanded under hypoxia continually presented significantly higher levels of expansion throughout time in

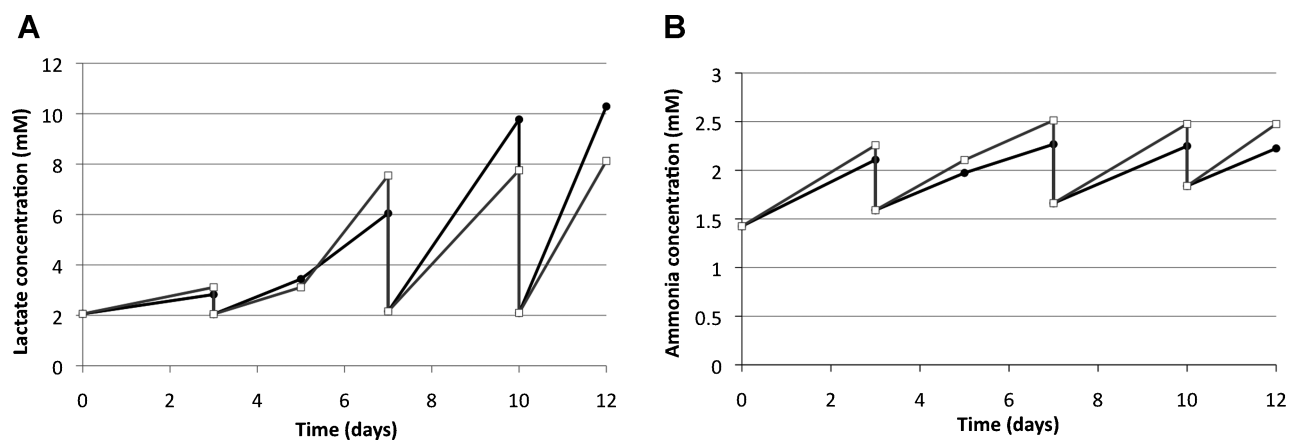

Fig. 5. Concentration profiles of lactate and ammonia throughout time in culture for hypoxia and normoxia cultures. For BM MSC cultures under hypoxia (black circles) and normoxia (white squares), the concentration of lactate (A) and ammonia (B) was monitored in order to verify if BM MSC growth inhibitory values were reached. Values are represented as mean of five experiments.

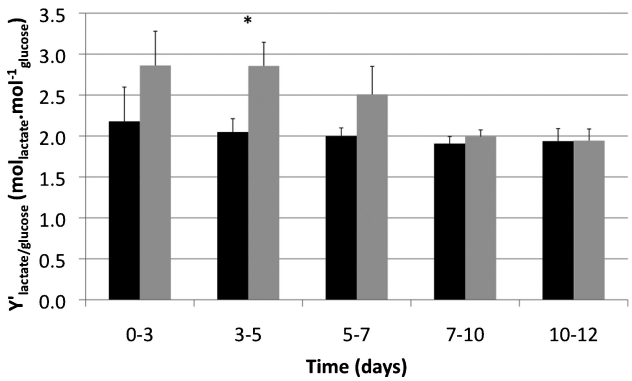

Fig. 6. Apparent yield of lactate from glucose ($Y'_{lactate/glucose}$) determined for BM MSC expanded under hypoxia and normoxia throughout time in culture. $Y'_{lactate/glucose}$ was calculated by dividing the specific production rate of lactate by the specific consumption rate of glucose for cells cultured under hypoxia (black bars) and normoxia (gray bars), for different five time intervals, during 12 days of culture. Values are represented as mean ± SEM (*$P < 0.05$).

culture, reaching a higher fold increase in total cell number compared to cells cultured under normoxia. In order to also study the effect of hypoxia on a clinical-scale BM MSC expansion process, we cultured cells for consecutive three passages of 7 days each, and verified that cell yield was considerably higher at the low oxygen tension. It confirmed that the potential reduction of culture time associated with the differences observed in cell proliferation might be relevant to accelerate significantly the clinical cell expansion process. In fact, Grayson and collaborators had also verified that 2% O_2 atmosphere improved human BM MSC expansion over seven passages (Grayson et al., 2007), even though cells displayed a longer lag phase when cultured in 3D constructs compared to monolayer cultures (Grayson et al., 2006). The beneficial effects on cell proliferation observed under hypoxic conditions might be associated to an improvement in cell adhesion and ECM formation patterns (Grayson et al., 2007).

As expected, after three passages, BM MSC expanded under hypoxia presented a larger shortening of telomere length, which was certainly related with the higher proliferation observed at this oxygen tension (Bonab et al., 2006; Kim et al., 2009). Although the reduction of telomere length is normally associated with cell senescence (Baxter et al., 2004), the lack of studies correlating in vivo activity of BM MSC and telomere size, does not allow to determine if the therapeutic performance (in both cellular therapy and tissue engineering settings; Caplan, 2007) of those cells would be affected.

Specific cell growth rate is a global measurement of proliferation performance (in a temporal basis) of a cell culture population providing a quantitative parameter for cell growth during the exponential phase. Since we found no differences in the specific growth rate values, but differences in terms of final cell yield and cumulative population doublings were observed, we performed cell division kinetic studies in order to get a deeper knowledge of the cell growth curve profile, namely concerning potential differences observed in the initial lag phase. In fact, the analysis of cell growth curves showed that, under a 2% oxygen tension, BM MSC entered the cell cycle earlier, with a shorter lag phase, which consequently resulted in higher cell expansion after 12 days of culture.

By using PKH cell tracking it was possible to conclude that BM MSC cultured under the different oxygen tensions remained undivided after 24 h and active cell division only started upon this time point. The delay of the first mitosis observed during the first 24 h might be due the time needed to complete cell adhesion to the tissue culture flask and/or the "trauma" of cells upon enzymatic treatment during cell passaging and cannot be straightly attributed to true quiescent state of the initial cell population. For both culture conditions, BM MSC only started to divide between day 1 and 2, with cells expanded under hypoxia presenting an earlier start of cell division, consistent with the observation of a shorter lag phase for cells cultured under low O_2. To our best knowledge this is the first report of a different cell division kinetics for BM MSC cultured under hypoxia, by using a PKH cell tracking approach. Although reports in the literature (including this present work) described the beneficial effect of hypoxia in BM MSC expansion as assessed by common proliferation studies, here we were able to detect subtle differences on cell division entry for cells cultured under the different conditions. Our results thus demonstrate the ability of BM MSC cultured under hypoxia to enter cell division earlier in culture. These results contrast with the observations of Grayson et al. (2007) who described no

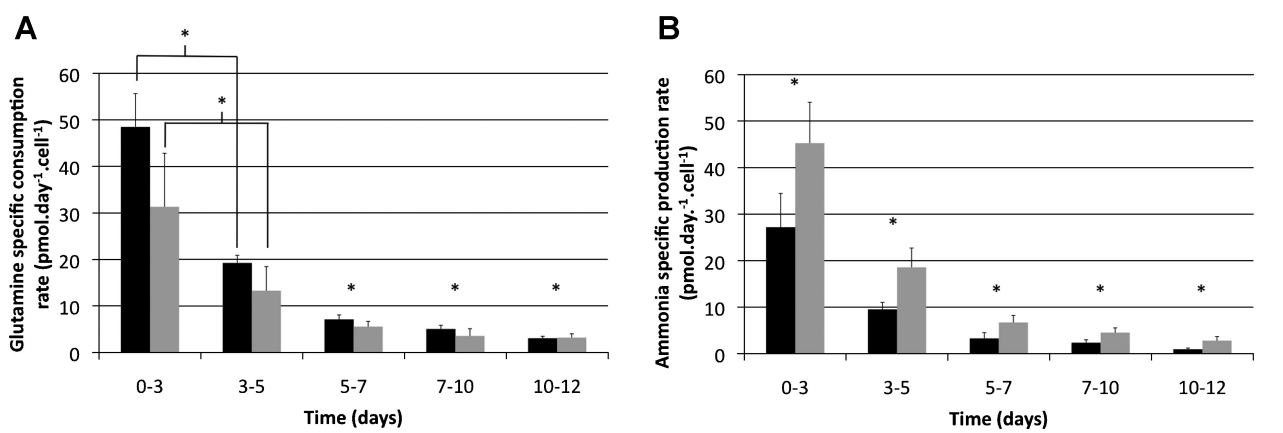

Fig. 7. Glutamine specific consumption rate and ammonia specific production rate of human BM MSC expanded under different O_2 tensions Throughout time in culture, concentrations of glutamine and ammonia were monitored and specific consumption/production rates of glutamine (A) and ammonia (B) were determined for cells cultured under hypoxia (black bars) and normoxia (gray bars). Values are represented as mean ± SEM (*$P < 0.05$).

significant differences in the number of cells, within 5 days of culture, under the two conditions (2% and 20% O_2). This discrepancy might be due to differences in experimental culture conditions such as the cell passage (P3-P4 cells were used in our studies vs. P2-P7 in Grayson et al., 2007 or donors age (Stenderup et al., 2003)).

BM MSC expansion under a hypoxic microenvironment also favored the proliferation of the more primitive cells, with clonogenic ability (CFU-F), in agreement to what have been reported by Grayson and collaborators (Grayson et al., 2006). In view of the fact that BM MSC population is quite heterogeneous in culture (Colter et al., 2001), the selective survival of the more primitive cells over their progeny may be especially beneficial in the settings of cellular therapy. Additionally, no differences were observed in immunophenotype and multilineage differentiative potential for cells expanded at 2% or 20% oxygen tension.

Regarding cellular metabolism, there was a clear adaptation of BM MSC to the low oxygen atmosphere. Cells cultured at 2% O_2 presented a higher specific consumption rate of glucose during the first week of culture, corresponding to the beginning of the exponential growth phase. This higher consumption of glucose under hypoxia can be explained by the higher requirement of carbon for the faster cell proliferation induced by the low oxygen tension. Schop et al. (2009) obtained lower values for the specific consumption rate of glucose for the cell exponential growth phase (9 pmol day^{-1} $cell^{-1}$ vs. 14 pmol day^{-1} $cell^{-1}$ in our study, under normoxia), while Higuera and collaborators determined values of an order of magnitude lower (Higuera et al., 2009). However, as previously stated, BM MSC proliferation and, consequently, cellular metabolism are known to be affected by culture conditions (Sotiropoulou et al., 2006), as well as cell passage number and donors age. These reasons may indeed be the explanation for the discrepancy shown between our work's metabolic rates and those reported by other authors.

Concerning the production of lactate in the same time period, it was considerably lower for cells expanded under hypoxia, resulting in noticeably lower values of $Y'_{lactate/glucose}$. In fact, the $Y'_{lactate/glucose}$ obtained when BM MSC were cultured at a normal oxygen level (higher than 2 until day 7) indicates that glutamine was also metabolized as a carbon source into lactate.

Nevertheless, the average $Y'_{lactate/glucose}$ was superior or equal to the theoretical values of 2 for both conditions showing that, even at a normal oxygen tension (20%), BM MSC metabolism is not primarily based on mitochondrial oxidative phosphorylation (Fig. 8), as it had also been reported by other authors (Grayson et al., 2006; Higuera et al., 2009; Schop et al., 2009). In fact, Vander Heiden and collaborators reported that proliferating cells, such as cancer or stem cells, rely on aerobic glycolysis to generate energy (Warburg effect). Although aerobic glycolysis is a less efficient metabolic pathway in terms of ATP production, this is counterbalanced by the non-limiting availability of glucose provided by the culture medium, and therefore, the production of ATP is not impaired for these cells throughout culture time. Moreover, it has been proposed that the metabolism of all proliferating cells (including cancer cells) is adapted to facilitate the uptake and incorporation of nutrients into biomass (e.g., amino acids, nucleotides) (Vander Heiden et al., 2009).

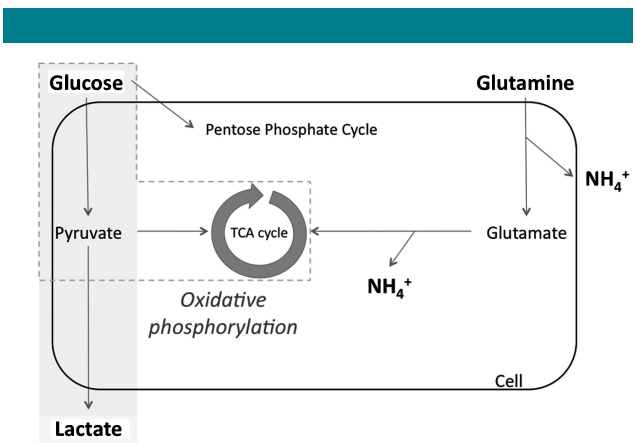

Fig. 8. Simplified version of the main metabolic pathways of animal cells. Proliferating cells, such as stem cells, rely primarily on aerobic glycolysis (shaded in gray) to generate energy as an alternative to the oxidative phosphorylation (dashed line).

Furthermore, the specific consumption rate of glutamine was higher for hypoxic cultures throughout the entire culture, even though its tendency was similar for both conditions: glutamine was consumed at a higher rate until day 5, and afterwards its consumption was drastically reduced to significantly lower values. Indeed, glutamine is known to have an important role to start cell proliferation, namely as supplier of carbon in the form of mitochondrial oxaloacetate (vital to maintain citrate production in the tricarboxylic acid cycle) and nitrogen for other amino acids synthesis (Vander Heiden et al., 2009). Even though it had been suggested by other authors that glutamine was not an important energy source for human MSC (Schop et al., 2009), our results demostrate an effective consumption of this amino acid, especially within the first days of culture, in agreement to what have been described for other animal/stem cells (Neermann and Wagner, 1996; Follmar et al., 2006). Growth limitation by glucose starvation was not likely to occur before day 12 since glucose concentration values were above the non-limiting concentration range (0.13–1 mM) (Ozturk et al., 1997; Gódia and Cairó, 2006; Acosta et al., 2007). Glutamine was also kept above non-limiting concentrations (within a range of 0.09–0.15 mM for different animal cell lines) throughout the entire culture (Gódia and Cairó, 2006).

Interestingly, the specific production rate of ammonia was significantly reduced under hypoxia throughout the entire culture. It may be related to a more efficient cell internal mechanism to balance ammonia accumulation due to the rapid glutamine consumption: the ammonium ion released in the conversion of glutamate in α-ketoglutarate is transferred to oxaloacetate to form aspartate or to pyruvate to form alanine (Gódia and Cairó, 2006). Consequently, the ammonia concentration in hypoxic cultures never reached inhibitory values, while the higher accumulation of ammonia in the culture medium of normoxic cultures might have contributed for the low cell expansion levels observed.

In conclusion, our results clearly showed that the expansion of human BM MSC under a low oxygen tension microenvironment significantly improved cell proliferation, by accelerating cell division kinetics, while increasing metabolism efficiency. In addition, expanded BM MSC maintained their clonogenic ability, immunophenotypic profile and differentiative potential. Importantly, hypoxia culture can be rapidly and easily implemented into the clinical-scale expansion process in order to maximize BM MSC yield and, at the same time, reduce culture time while maintaining cell product quality.

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