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Combination of Low O_2 Concentration and Mesenchymal Stromal Cells During Culture of Cord Blood CD34$^+$ Cells Improves the Maintenance and Proliferative Capacity of Hematopoietic Stem Cells

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The physiological approach suggests that an environment associating the mesenchymal stromal cells (MSC) and low O_2 concentration would be most favorable for the maintenance of hematopoietic stem cells (HSCs) in course of ex vivo expansion of hematopoietic grafts. To test this hypothesis, we performed a co-culture of cord blood CD34$^+$ cells with or without MSC in presence of cytokines for 10 days at 20%, 5%, and 1.5% O_2 and assessed the impact on total cells, CD34$^+$ cells, committed progenitors (colony-forming cells—CFC) and stem cells activity (pre-CFC and Scid repopulating cells—SRC). Not surprisingly, the expansion of total cells, CD34$^+$ cells, and CFC was higher in co-culture and at 20% O_2 compared to simple culture and low O_2 concentrations, respectively. However, co-culture at low O_2 concentrations provided CD34$^+$ cell and CFC amplification similar to classical culture at 20% O_2. Interestingly, low O_2 concentrations ensured a better pre-CFC and SRC preservation/expansion in co-culture. Indeed, SRC activity in co-culture at 1.5% O_2 was higher than in freshly isolated CD34$^+$ cells. Interleukin-6 production by MSC at physiologically low O_2 concentrations might be one of the factors mediating this effect. Our data demonstrate that association of co-culture and low O_2 concentration not only induces sufficient expansion of committed progenitors (with respect to the classical culture), but also ensures a better maintenance/expansion of hematopoietic stem cells (HSCs), pointing to the oxygenation as a physiological regulatory factor but also as a cell engineering tool.

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Starting from the beginning of the 1990s, a lot of data accumulated showing the importance of low oxygen concentration for the functional maintenance of stem cells. HSC, as a paradigmatic case, was cultured at low O_2 concentrations that were mainly called "hypoxia." This is a confusing term since 1–7% represent physiological oxygen concentrations of the tissues providing HSC environment (Chow et al., 2001; Shima et al., 2009; Guitart et al., 2010; Winkler et al., 2010). These physiologically low O_2 concentrations protect the primitive hematopoietic cells against oxidative stress in vivo (Jang and Sharkis, 2007; Parmar et al., 2007) and ex vivo (Fan et al., 2007, 2008). On the other hand, the reactive oxygen species (ROS) molecules, exhibiting a harmful effect on primitive hematopoietic cells in terms of survival and function, are strongly generated in the culture at 20% O_2 (Ito et al., 2006; Yahata et al., 2011). Most of these articles (Koller et al., 1992a,b; Cipolleschi et al., 1993; Danet et al., 2003; Csete, 2005; Shima et al., 2009; Eliasson et al., 2010), including those of our group (Ivanovic et al., 2004, 2002; Hermitte et al., 2006) refer to experiments performed in cytokine-supplemented liquid cultures. All these data allowed not only a better understanding of self renewal, which is an elementary property of a stem cell, but also a vision concerning the differentiation and the stem cell persistence during development, as a mirror of the evolution: our concept called "oxygen stem cell paradigm" (Ivanovic, 2009). This concept was already employed in the cell engineering enabling new advances in several fields. As illustrative examples, ex vivo expansion of hematopoietic stem and progenitor cells (reviewed in: Ivanovic and Boiron 2009; Ivanovic et al., 2011) and ex vivo red blood cells (RBC) production could be cited (Vlaski et al., 2009).

The liquid culture, however, does not approximate at best in vivo stem cell environment. Some other culture models as the co-culture of hematopoietic cells with stromal cells could

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certainly be considered to better mimic the complexity of in vivo hematopoietic environment—in line with the "stem cell niche" concept (Schofield, 1978). As a matter of fact, the invention of "long-term cultures" (Dexter et al., 1977; Winkler et al., 2010) was based on this approach. Recently, lots of articles were published (Li et al., 2007; Magin et al., 2009; Walenda et al., 2010) evaluating the potential advantages of these co-cultures with respect to liquid cultures in terms of ex vivo expansion of progenitor and stem cells (Yamaguchi et al., 2002; Chivu et al., 2004; McNiece et al., 2004; Wang et al., 2004; Zhang et al., 2004; Robinson et al., 2006; Van Overstraeten-Schlögel et al., 2006; Fei et al., 2007; Huang et al., 2007). The results mainly agreed that the presence of stromal cells provides a better maintenance of stem cell potential. On the contrary, this type of culture is more complex than liquid culture and hence, difficult to be transferred at clinical grade level. Therefore, only one clinical trial using hematopoietic cells produced in co-cultures has been published so far (Robinson et al., 2011).

It is interesting that low oxygen concentrations (in the above-mentioned range) also influence proliferative capacity (Grayson et al., 2007; Carrancio et al., 2008; Lavrentieva et al., 2010), viability (Lavrentieva et al., 2010), motility (Rosová et al., 2008), differentiation potential (Grayson et al., 2006; Fehrer et al., 2007), and proliferation kinetics (Dos Santos et al., 2010) of mesenchymal stem cells (MSC) also enhancing their life span (Jin et al., 2010; Tsai et al., 2011) and modifying their cytokine secretion (Annabi et al., 2003; Potier et al., 2007; Li et al., 2010).

Since there is an obvious interest, both from scientific and technologic viewpoint to optimize ex vivo expansion cultures, we tried to combine the presence of mesenchymal stromal cells with low O_2 concentrations, supposed to be physiological for hematopoietic stem and progenitor cells. In addition, the main factor of cellular response to low O_2 concentration, HIF-1α, is getting stabilized at O_2 concentrations $\leq$5% (Jiang et al., 1996; Jewell et al., 2001; Danet et al., 2003; Kirito et al., 2009). Thus, our hypothesis that the proliferative capacity of HSCs should be better maintained if they were cultured together with mesenchymal stromal cells at appropriately low O_2 concentrations is tested by exposing to 5% and 1.5% O_2 cord (placental) blood (CB) $CD34^+$ cell cultures on the layer of bone MSC.

Materials and Methods

Preparation and culture of cells

Full-term delivery CB samples. Samples were collected (with the mother's informed consent) in sterile bags containing anticoagulant and delivered to the Cell Therapy Unit of the (Etablissement Français du Sang Aquitaine-Limousin, Site de Bordeaux). Only samples not appropriate for banking were used in our experiments.

$CD34^+$ cell purification. Mononuclear cells were isolated on Ficoll (d = 1,077 g/L, Lymphoprep Nyegaard, Oslo, Norway). The $CD34^+$ fraction was isolated with one or two runs of immunomagnetic selection on MiniMACS columns (Miltenyi Biotec, Gladbach, Germany and Miltenyi Biotec GmbH, Paris, France, respectively) according to the manufacturer's instructions. Flow cytometry controls showed >90% pure $CD34^+$ cell populations. $CD34^+$ cells were also selected at the end of primary culture/co-culture (C1) by the same procedure above described to analyze their CD133 expression and ALDH activity.

Preparation of mesenchymal stromal cell (MSC) adherent layers. MSCs were isolated by culture from bone marrow of healthy donors obtained from filters used for allogenic hematopoietic grafts preparation. After RBC lysis in ammonium chloride (8.2 mg/ml; Sigma–Aldrich, St Louis, MO), bone marrow cells were cultured in α-MEM medium (Minimum Essential Medium Alpha Modification) (BioWhittaker, Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS) (HyClone Perbio, Bezons, France), penicillin (100 units/ml), streptomycin (100 μg/ml) (Cambrex BioWhittaker, Walkersville, MD), glutamine (2 mM) (Sigma–Aldrich), and FGF2 (1 ng/ml) (PeproTech, Levallois Perret, France) (referred to as "complete α-MEM"). After 3–4 days at 37°C, 5% CO_2, non-adherent cells were removed and medium was changed. Adherent cells, mainly MSCs, were trypsinized upon reaching confluence, harvested, and cultured at a concentration of $10^3/cm^2$ for 1–3 weeks. MSCs were cryo-preserved or not before usage. For this study, thawed MSCs ($\leq$5th passage) were incubated in 12-well plates (2.5×10^4/ml/well) in complete α-MEM at 37°C, water-saturated atmosphere, 95% air, and 5% CO_2 until monolayer confluence 2 or 3 days later.

Co-culture of MSCs and $CD34^+$ cells. Purified $CD34^+$ cells were seeded at 2×10^4/ml/well with or without MSCs monolayers in 12-well plates (Nunc, Roskilde, Denmark) and cultured for 10 days in Stem Alpha A serum free medium (Stem Alpha SA, St. Genis l'Argentière, France) supplemented with human cytokines [stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF) (Amgen-Roche, Neupogen, Neuilly sur seine, France, 100 ng/ml each), thrombopoietin (TPO) (PeproTech; 20 ng/ml), and interleukin (IL)-3 (recombinant human—rHu; Pepro Tech, London, England; 0.5 ng/ml)].

The cultures were incubated at 37°C either at 5% CO_2 and 20% (Incubator Igo 150 Cell Life, Jouan, St. Herblain, France), 5%, or 1.5% O_2 (Proox Culture Chamber with O_2 and CO_2 regulators BioSpherix, Ltd, Redfield, NY) in a water-saturated atmosphere. At Day-10, non-adherent cells were carefully harvested and washed, the viable cells enumerated (trypan blue exclusion test) and processed for further analyses as described below.

Flow cytometry analysis

Membrane antigens expression. After 10 days of culture with or without MSCs at different O_2 concentrations, non-adherent cells were washed in Basal Iscove medium (Biochrom AG, Berlin, Germany) and suspended in phosphate buffer saline, labeled (20 min in dark) with anti-CD33 and -CD13 (myeloid markers), -CD19 (lymphoid marker), -CD41 and -CD61 (megakaryocytic markers), and -CD34 monoclonal antibodies in order to observe lineage differentiation. The anti-CD45 (pan-hematopoietic marker) was used to exclude the non-hematopoietic cells (MSC cells) from analysis. For analysis of undifferentiated cells, anti-CD133 (Hess et al., 2006; Isidori et al., 2007) was used in combination with CD34 expression and ALDH activity (see below). All these antibodies were conjugated either with FITC, PE, PercP, or APC fluorochromes as mentioned in Table 1. After labeling, cells were washed and re-suspended and their phenotype was analyzed by flow cytometry (FACSCalibur, Becton Dickenson, San Jose, CA). Fluorochrome-conjugated isotype antibodies were used to determine the level of non-specific binding.

Aldehyde dehydrogenase (ALDH) activity. The high activity of ALDH is known as a marker of freshly isolated primitive hematopoietic progenitors and stem cells (Hess et al., 2004, 2006).

ALDH labeling was performed using Aldefluor reagent (StemCell Technologies, Grenoble, France) according to the manufacturer's instructions. Briefly, activated Aldefluor substrate (1 μl) was added to 0.1×10^6 $CD34^+$ cells (purified at Day-0 and after culture (Day-10)) suspended in 100 μl Aldefluor assay buffer and incubated for 30–60 min at 37°C to allow the conversion of Aldefluor substrate, a green fluorescent product retained within the cell (Storms et al., 1999). As negative control, an aliquot of Aldefluor-stained cells was incubated with 1 μl of a specific ALDH inhibitor (DEAB). Cells were co-stained with human CD34-PE and CD133-APC antibodies.

Colony-forming cell (CFC) assay. To detect the committed hematopoietic progenitors, freshly isolated $CD34^+$ or cultured cells harvested at Day-10 were seeded in a cytokine-supplemented methylcellulose medium Stem Alpha ID (Stem Alpha SA) in a Petri dish or 24-well plates (Nunc), respectively, at concentration of 250 cells/ml or volume of 0.5/250 μl, respectively (Ivanovic et al., 2004). After 14 days of incubation at 37°C in water-saturated atmosphere, 20%

TABLE 1. Summary of the monoclonal antibodies used for cell labeling

BD Pharmingen, Pont-de-Claix, France	CD33 PE, CD41 PE, CD90 FITC, CD19 PE
BD Bioscience, San Jose, CA	CD34 PE, CD13 PE, CD61 FITC, CD45 PerCep, CD33 APC, CD45 FITC
Miltenyi Biotec, Gladbach, Germany	CD133 APC

O_2 and 5% CO_2, the CFCs [colony-forming units-granulocyte macrophage (CFU-GM), burst-forming units-erythroid (BFU-E), and colony-forming units-Mix (CFU-Mix)] (>50 cells) were enumerated using an inverted microscope.

Pre-CFCs (primitive progenitors) assay

The production of committed progenitors during a long-term secondary culture (C2) reflects the presence and quantity of a primitive population situated between progenitors and stem cells (pre-CFC) in C1 (Ivanovic et al., 2000, 2002; Kovacevic-Filipovic et al., 2007). C1 cells (total Day-10 progeny of 20,000 $CD34^+$ cells plated at Day-0 in 1-ml cultures) were washed and resuspended in 1 ml of medium. The C2 was carried out with 200 μl of C1 cell suspension (in each condition) and the volume was adjusted to 1 ml of cytokine-supplemented (IL-3, SCF, G-CSF, and TPO, as C1 culture concentrations) serum-free medium (Stem Alpha A), and cultured for 8 weeks at 20% O_2 with a weekly demi-depopulation and addition of fresh medium that was taken into account for normalization of total CFC contents in C2. Every week of C2, the cells were plated in methylcellulose to detect committed progenitors as mentioned above.

Scid-repopulating cells (SRC) assay

Stem cell activity was evaluated by in vivo assay—transplantation of human cells to immunodeficient (NOG/Scid) mice. After 10 days, 1,000 cells sorted on Day-0 (and stored at −80°C), or their progeny obtained under different C1 conditions were injected to 6- to 10-week-old NOG/Scid mice conditioned by intraperitoneal injections of Busulfan 25 mg/kg (Busilvex®, Pierre Fabre, Boulogne, France) at D2 and D1 (according to the previously established protocol) (Robert-Richard et al., 2006) (central animal-keeping facility of University of Bordeaux 2). Positive control (mice injected with 15,000–20,000 $CD34^+$ purified at D0) and negative control (non-injected mice) were included. After 8 weeks, the animals were euthanized, their femora were isolated and the BM was flushed with 1 ml of RPMI 1640 complemented with human albumin (0.4%). Cells were washed [phosphate-buffered saline, EDTA 5 mM, human albumin (0.4%)] and incubated with a FITC-coupled anti-human CD45, PE-coupled anti-human CD19, and APC-coupled anti-human CD33 antibodies for 20 min at 4°C in the dark.

Washed cells were analyzed on a FACSCalibur (Becton Dickinson) to detect and quantify human chimerism. To avoid false-positive results due to control isotype, we used non-engrafted mice as controls.

To detect the progenitors (CFC) of human origin in murine bone marrow, 15 and 30 μl of femoral cell suspension (see above) were seeded per 250 μl of methylcellulose (in duplicate) (Stem Alpha-1; Stem Alpha SA) supplemented with 10% human plasma, 25 ng/ml (rHu IL-3) (PeproTech, London), 25 ng/ml rHu GM-CSF) (R&D Systems Europe, Lille, France), 50 ng/ml rHu SCF (Amgen-Roche, Neupogen), and 3 U/ml rHu erythropoietin (EPO) (Tebu-Bio, Le Perray, France) (Robert-Richard et al., 2006).

After 14 days of incubation, the human CFCs (>50 cells) were enumerated using an inverted microscope.

Cytokine production

Quantitative studies of cytokine production during C1 (primary culture/co-culture) and the culture of MSC alone (at 20% and 5% O_2) were carried out by the multiplexed bead immunoassay combined technique. The supernatants were collected and stored at −20 and −80°C. Multiplex analysis was performed using the kits purchased from (Biosources- Invitrogen, Cergy Pontoise, France) [human IL-1β (interleukine-1β), human IL-10 (interleukine 10), human IL-6 (interleukine 6), and human GM-CSF] according to the manufacturer's protocol. To generate a standard curve, serial dilutions of appropriate standards provided by the manufacturer were prepared and used. The limits of detection for growth factors were as follows: IL-1β (<15 pg/ml), IL-10 (<5 pg/ml), IL-6 (<3 pg/ml), GM-CSF (<15 pg/ml). The fluorescent signature was detected by Luminex 100 Analyzer.

Statistical analysis

The *t*-test was used to evaluate the significance of difference between the experimental conditions. In the experiences with mice, Mann–Whitney test was used.

Results

Impact of oxygen concentration on amplification of total cells, $CD34^+$ cells, and clonogenic progenitors (CFC) after 10 days of C1

In culture conditions used here, the decrease in O_2 concentration (from 20%, 5%, and 1.5%) provokes a relative decrease in fold expansion of total cells both in culture and co-culture. In both cases, at 1.5% O_2, this decrease was close to half the value found at 20%. There is a trend to enhance the fold expansion in co-culture comparing to culture, but it was not statistically significant (Fig. 1A). This enhancement in co-culture is, however, evident for $CD34^+$ cells: two to three times higher fold expansion was evidenced in co-culture with respect to the cultures (comparing the same O_2 concentrations). Again, the decrease of fold expansion was paralleled by decrease in O_2 concentration becoming statistically significant at 1.5% O_2 versus 20% O_2 in co-culture (Fig. 1B). When the fold expansion of committed progenitors (CFU-GM, BFU-E, and CFU mix) was analyzed, the situation was similar to the one of $CD34^+$ cells (Fig. 1C). This fold expansion at 1.5% and 5% O_2 was lower than at 20% O_2 after 10 days in co-cultures. However, co-culture at low O_2 (1.5% and 5%) assured similar and even higher fold expansion than classical culture at 20% O_2.

Effect of low O_2 concentrations and co-culture on membrane markers expression and ALDH activity

We did not find a significant difference in terms of the expression of myeloid markers (CD33 and CD13) or megakaryocytic markers (CD41 and CD61) in our conditions. The CD19 (lymphoid marker) was not detectable.

However, the decrease in O_2 concentration results in decrease in percentage of $CD34^+$ cells expressing CD133. This decrease is statistically significant in 1.5% of O_2 (Fig. 2A). Moreover, both 5% and 1.5% O_2 concentrations resulted in a marked decrease (one half) of $CD34^+$ cells expressing $ALDH^+$ activity (Fig. 2B).

Low O_2 concentrations better maintain primitive progenitors (pre-CFC) in co-culture

Committed progenitors production in secondary liquid cultures initiated with the cells recovered from primary cultures or co-cultures demonstrated that:

(a) This sub-population of primitive progenitors and stem cells was not better preserved at low O_2 concentration with respect to 20% O_2 in a simple culture since the small observed differences were not statistically significant (Fig. 3A).
(b) Production of committed progenitors by the cells issued from co-culture at 1.5% and 5% is significantly higher with respect to 20%. This difference is more pronounced at week-2 of secondary culture (threefold higher at 1.5% and 5% with respect to 20% of O_2) (Fig. 3B, inset).

Low O_2 concentrations increase Scid repopulating cells (SRC) activity in co-culture

When 1,000 Day-0 $CD34^+$ cells or their Day-10 progeny were injected to NOG/Scid mice, human cells were detected in some

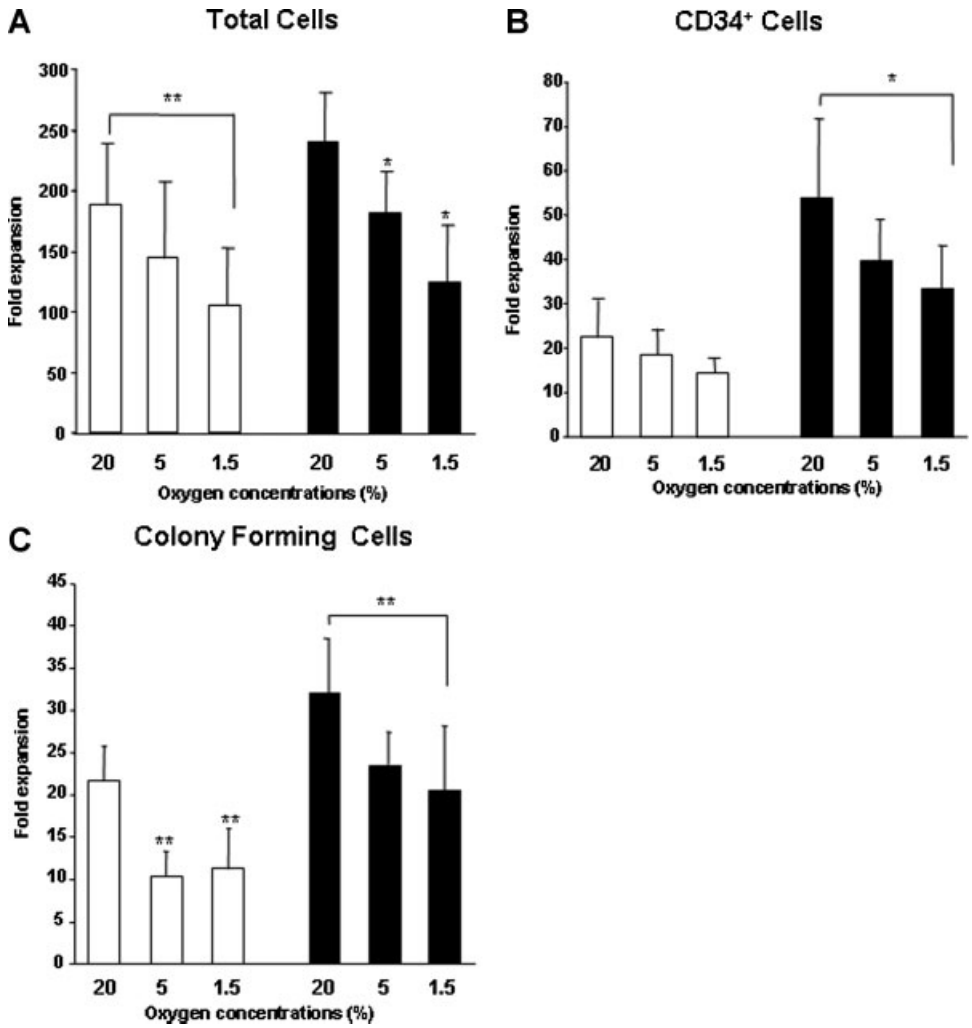

Fig. 1. Impact of oxygen concentrations and MSC on hematopoietic cells amplification in a Day-10 culture. Purified CD34$^+$ cells were cultured without (white bars, simple cultures) or with (black bars, co-cultures) mesenchymal stromal cells. A: Total cells, (B) CD34$^+$ cells, (C) colony forming cells (CFC). Results are expressed as mean ± SE; n = 8–9; $^*P < 0.05$; $^{**}P < 0.01$.

mice but were not in others 7–8 weeks later, demonstrating that the dose of 1,000 cells falls within the linear portion of a sigmoid dose/response (transplanted/cell engraftment) curve, that is, this cell number represents a limiting dilution dose. Based on human CD45 chimerism in NOG/Scid mice (SRC-$_{CD}$), it is evident that the co-culture better preserves this sub-population of stem cells than simple culture (Fig. 4A). In addition, this effect is enhanced with decrease in O_2 concentration (Fig. 4A). Although the proportion of positive mice (the human CD45$^+$ cells detected in their bone marrow) increases in parallel with a decrease in O_2 concentration, even in simple culture, this increase reaches its peak in co-culture, starting from 5% O_2 (Fig. 4B). So, at 1.5% and 5% O_2, the co-cultures seem to maintain a full SRC-$_{CD}$ Day-0 number. When analyzing the percentage of CD45$^+$ cells per femur of engrafted mice, this low O_2 concentration-dependent maintenance of stem cells became even more pronounced. The most pronounced effect, however, remains a combination of co-culture with 1.5% O_2 (almost six times higher values compared to Day-0 cells) (Fig. 4C). Having in mind that the frequency of SRC$_{CD}$ at Day-0 and at Day-10 in co-cultures at 5% and 1.5% O_2 seems to be similar (as shown in Fig. 4B), this result suggests that an appropriately low O_2 concentration, associated with the presence of stromal cells enhanced the individual proliferative capacity of SRC. An analysis of human CD19 and CD33$^+$ cells in bone marrow of the same mice revealed that low O_2 concentrations combined with co-culture maintained the differentiation balance between lymphoid and myeloid line similar to that of Day-0 cells (Fig. 4D).

If the analysis was based on human progenitors (CFC), generated during 7–8 weeks after injection of cells to NOG/Scid mice, it is obvious that the simple cultures maintained better this stem cell sub-population (SRC-$_{CFC}$) activity at lower O_2 concentrations (5% and 1.5%) (Fig. 5A, inset). The co-culture by itself has a positive effect on the SRC-$_{CFC}$ maintenance, even at 20%. In general, co-culture ensures maintenance of proliferative capacity of SRC-$_{CFC}$ at a level similar to Day-0 (Fig. 5A). However, if the differentiation ratio (CFU-GM and BFU-E) was analyzed, only co-culture at 1.5% O_2 maintained the Day-0 ratio while in all other conditions of culture or co-cultures, this ratio shifted in favor of CFU-GM. This result suggests that a low O_2 concentration is necessary to maintain not only the proliferative capacity of stem cells, but also their differentiation potential.

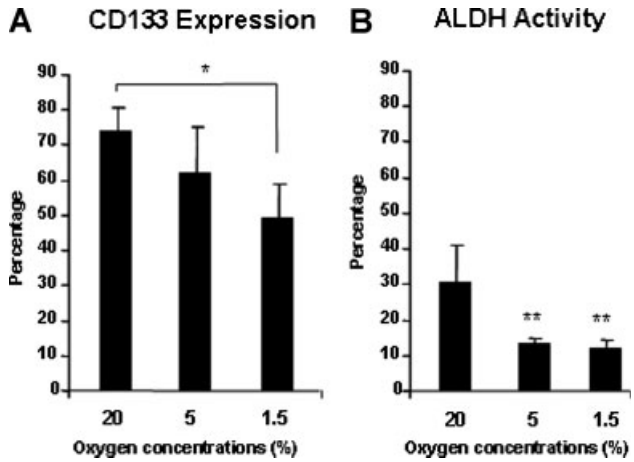

Fig. 2. Analysis of CD34$^+$ cells for CD133 expression and ALDH activity. At the end of co-culture, CD34$^+$ cells were purified and analyzed by flow cytometry for (A) Expression of CD133, (B) ALDH activity. Results are expressed as mean ± SE; n = 7; $^*P < 0.05$; $^{**}P < 0.01$.

Impact of O_2 concentration on cytokine secretion

Production of IL-6 is higher in co-culture at 5% with respect to 20% O_2 until Day-5, but at Day-7 this trend is not statistically significant (Fig. 6A). This "stagnation" in IL-6 production was not detected in simple MSC culture: it increases exponentially, tripling at Day-7 with respect to Day-5 (Fig. 6B). We have not noted a secretion of IL-6 in simple CD34$^+$ culture. These data implying the consumption of IL-6 by hematopoietic cells amplified in co-culture. The IL-1beta, IL-10, and GM-CSF were not detected in our conditions.

Discussion

The most interesting result of this study is that association of low O_2 concentration (1.5% O_2) and co-culture was the condition able not only to maintain the frequency and full differentiation capacity of stem cells (SRC), but also to enhance their proliferative capacity. The last point is suggested by more than five times higher human chimerism in engrafted murine bone marrow, in situation when SRC frequency did not change with respect to Day-0 (the same proportion of "positive" mice).

Being heavily stimulated by cytokines (very high concentrations with respect to the physiological ones) the hematopoietic stem and progenitor cells in ex vivo expansion cultures represent rather a paradigm of regenerating hematopoiesis than of steady state one (Ivanovic and Boiron, 2009). It is evident that in these circumstances a high differentiation pressure on stem cells exists (analogous to the model of Loeffler et al., 1989). The above-mentioned results confirm the major role of micro-environment (mimicked by stromal cells and low O_2 concentrations in this case) for stem cell maintenance. On the basis of the analysis of the results concerning co-cultures presented here, it is evident that the better maintenance of stem cells is paralleled with the lower numerical expansion of committed progenitors: the lowest number of CFC in co-culture was found at 1.5% of O_2 where the best preservation of SRC functional capacity was detected. It should be stressed, however, that this number of committed progenitors corresponds to their maximal expansion in simple

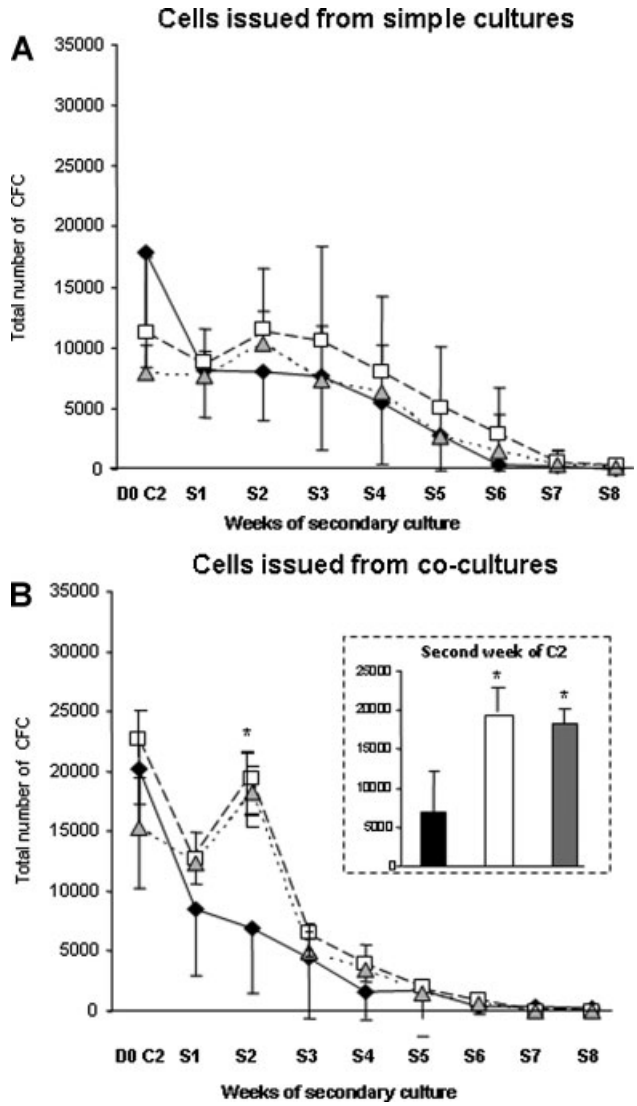

Fig. 3. Impact of low O_2 concentrations and MSC on pre-CFC activity. The curves represent generation of CFC during 8 weeks of secondary cultures (C2) initiated with the cellular products obtained after 10 days of primary culture/co-culture (C1) (20,000 CD34$^+$ cells at Day-0 (D0)). A: Time course of CFC in C2 initiated with the cells issued from simple cultures. B: Time course of CFC in C2 initiated with the cells issued from co-cultures. Inset: zoom on situation in C2 after 2 weeks. Black lozenges/bars: 20% O_2; white squares/bars: 5% O_2; gray triangles/bars: 1.5% O_2. Results are expressed as mean + SE; n = 5–9; $^*P < 0.05$.

culture at 20% O_2. Thus, co-culture combined with appropriately low O_2 concentration (1.5%) could provide a tool to get a big number of committed progenitors, in parallel with complete preservation or even enhancing of stem cells functional capacities. These data stress the paramount importance of respecting physiological conditions in ex vivo cell engineering. In this regard, it was demonstrated that "hyperoxia" (20%) favors a high production of ROS inducing important negative effects in different cell types (Ito et al., 2006; Fan et al., 2007, 2008; Kubo et al., 2008; Carrera et al., 2010; Yahata et al., 2011). In line with the SRC data are the results concerning pre-CFC. Again, only the low O_2 concentration conditions combined with co-culture were able to preserve a

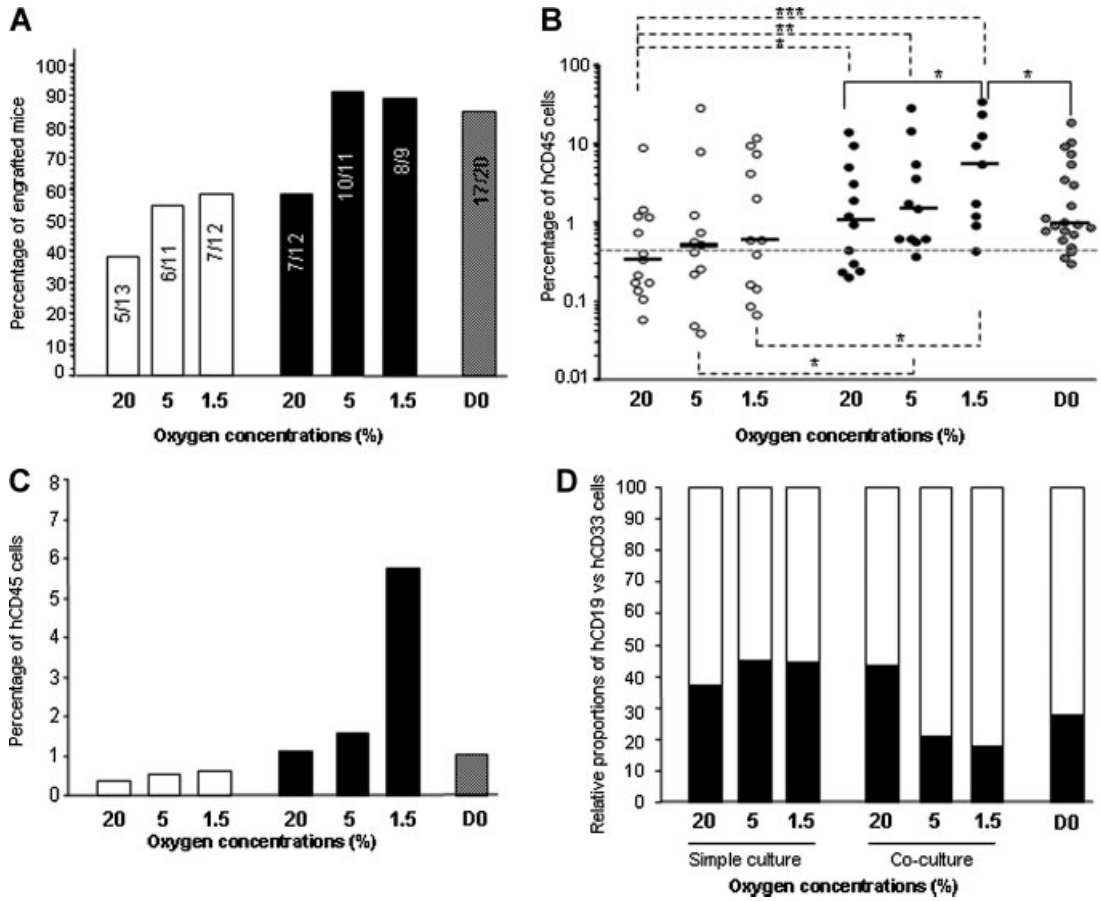

Fig. 4. Impact of low O_2 concentrations and MSC on SRC activity ($SRC\text{-}_{CD}$). The activity of Scid Repopulating Cells was detected on the basis of human $CD45^+$ chimerism in bone marrow of NOG/Scid mice ($SRC\text{-}_{CD}$) 7 and 8 weeks after injections with 1,000 steady state cord blood cells (D0) or their progeny after 10 days of cultures without (simple cultures) or with (co-cultures) MSC under 20%, 5%, or 1.5% O_2. A: Frequency of positive mice for human engraftment (human $CD45^+$ chimerism >0.5%). The cultures, co-cultures and Day-0 point (D0) are represented by white, black, and gray bars, respectively. The number (N) of positive per number of analyzed mice is presented inside bars for each condition separately. Note that the percentage of engraftment with respect to Day-0 was reduced in cultures but fully maintained in co-cultures at low O2 concentrations (5% and 1.5%) ($P < 0.05$). B: Individual mice bone marrow labeling with human $CD45^+$ antibody; white, black and gray circles represent cultures, co-cultures, and D0, respectively. C: Median human CD45 chimerism in positive mice. D: Relative proportion of B lymphoid ($CD19^+$, white bars) and myeloid ($CD33^+$, black bars) cells within human $CD45^+$ cells. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. The number of "positive" mice (N) for each corresponding condition in (C) and (D) is stated inside the bars in (A) and individually visible in (B). Cumulative results from four independent experiments presented.

high capacity of repopulation of secondary culture with committed progenitors (pre-CFC activity). However, unlike SRC_{CD}, where 1.5% O_2 concentration was most effective, both 5% and 1.5% were effective for pre-CFC. This underlines functional difference between two stem cell populations, a schedule completely in line with all the data related to the heterogeneity of stem and progenitor cells sub-populations with respect to their demands for O_2 published so far (Cipolleschi et al., 1993; Simsek et al., 2010, reviewed in Parmar et al., 2007; Ivanovic, 2009).

In our conditions, $CD34^+/CD133^+$ cells, as well as the degree of $ALDH^+$ activity in $CD34^+$ cell population behaved with respect to O_2 concentrations similarly as committed progenitor's number: decreasing in parallel with O_2 concentrations. Even in steady state, most of $CD34^+/CD133^+$ cells and $CD34^+/ALDH^+$ cells are, in fact, the committed progenitors. So, this finding is perfectly coherent, having in mind a low frequency of stem cells in these phenotypically/metabolically characterized populations. The relative enhancing of stem cells (SRC and pre-CFC) frequency at low O_2 concentrations could not be evidenced in overwhelming progenitor population, having opposite numerical trend.

Although several studies concerning low O_2 concentration (5%) for co-cultures of hematopoietic and stromal cells were published (Koller et al., 1992b; Song et al., 2009; Zhambalova et al., 2009), our study is first performed in serum-free conditions, comparing 1.5% and 5% O_2 and using in vivo approach (SRC) for stem cell detection. These studies could not be directly compared due to differences in conditions: Zimbalova et al. studied the culture of mononuclear cells (MNC) on stromal cells without adding cytokines, Koller et al. did not employ SRC assay, Song et al. employed the osteoblasts as stromal cells . . . Nevertheless, the common feature in all studies, a better maintenance of primitive cells in co-culture at low O_2 concentrations, is confirmed here with the "gold standard" essay for human stem cell detection—in vivo SRC assay. Furthermore, our study introduces the notion of enhancing of proliferative activity of SRC co-cultured with stromal cells.

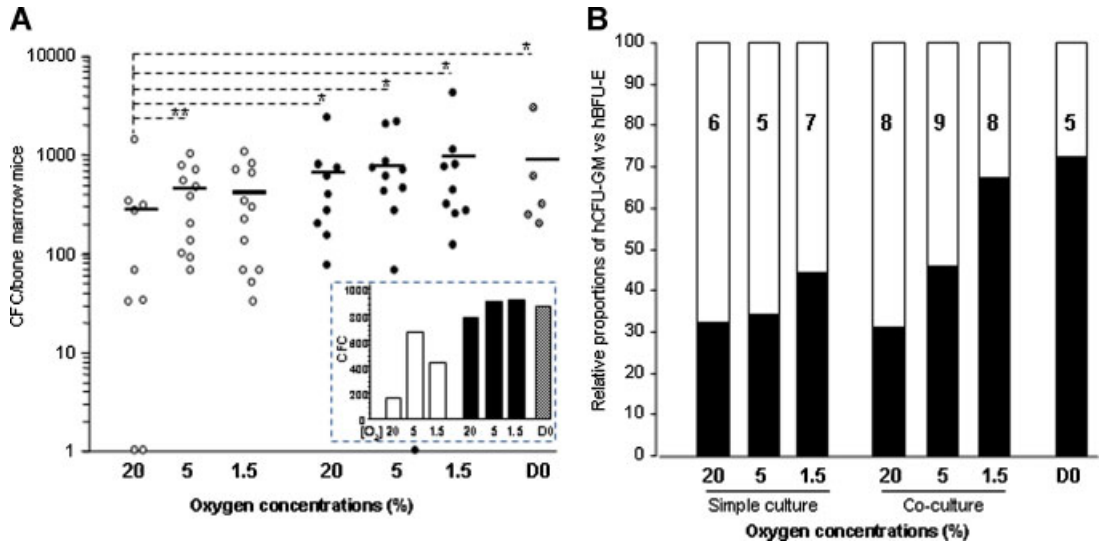

Fig. 5. Impact of oxygen concentrations and MSC on SRC activity (SRC-$_{CFC}$). The mice were sacrificed 7–8 weeks after injection of 1,000 steady state cord blood cells (D0) or their Day-10 (D10) cultures/co-culture progeny, and their bone marrows were harvested and analyzed for human CFC content. A: Individual mice human CFC content per femur. Inset: median CFC content per femur (only mice positive for human CD45 antigen were considered—the number of mice analyzed is given in corresponding bars of plot B). The cultures, co-cultures and D0 are represented by white, black, and gray circles/bars, respectively. B: Relative proportion of granulo-monocytic (white bars) and erythroid progenitors (black bars) of human origin in murine bone marrow. Only mice positive for human CD45 antigen were considered; the number of mice (n) is given in the bars. *$P < 0.05$; **$P < 0.01$. Cumulative results from four independent experiments presented.

The detailed examination of experiments done with IL-3 imply that its pro-differentiation effect on stem cells is related (i) to the presence of serum in medium and (ii) to a high dose employed (Ivanovic, 2004). So, IL-3 in a low dose in a serum-free culture showed some synergistic benefic effect with low O_2 concentration on stem cell maintenance and, simultaneously, on expansion of committed progenitors (Ivanovic et al., 2004). So, the serum-free condition employed here enabled us to add a low dose of IL-3 to cytokine cocktail.

Positive effect of low O_2 concentration associated to MSC on HSC maintenance could result from numerous factors whose production is affected by O_2 concentration (HGF, VEGF, IL1, THF, IL-10, etc.) (Annabi et al., 2003; Potier et al., 2007; Li et al., 2010; Tamama et al., 2011). The list of genes coding cytokines, growth factor and their receptors, as well as other molecules exhibiting the "HIF-1-binding sequence"—hypoxia response element (HRE), is long (reviewed in Semenza, 2007). Our results suggest that at least in part, this effect could be operated by IL-6 (Ulich et al., 1991; Rodriguez et al., 2004) whose production by MSC is stimulated at low O_2 concentration. This viewpoint is in line with recent literature data, demonstrating a decrease of expansion of hematopoietic cells cultivated with bone marrow MNC supernatants treated with an IL-6 inhibitor. This effect was only observed with the supernatant from low O_2 concentration culture (Li et al., 2011). Similarly, the positive effects on committed progenitors in co-cultures at low O_2 concentration upon IL-17 stimulation seem to be associated with an increased IL-6 secretion (Krstic et al., 2009). Furthermore, this scenario is supported by data obtained with murine bone marrow cells: the exogenous IL-6 synergizes with the low O_2 concentration (1%) to functionally maintain the pre-CFCs sub-population, an effect undetectable at 20% O_2 (Kovacevic-Filipovic et al., 2007).

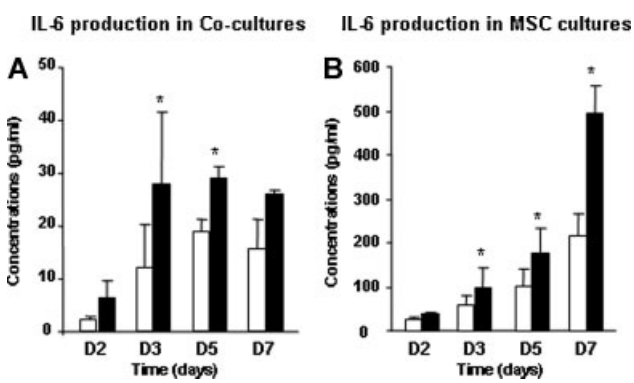

Fig. 6. Time-course of IL-6 production in co-cultures and MSC cultures. A: Co-cultures of MSC with hematopoietic CD34$^+$ cells. B: MSC cultures. White bars = 20% O_2; Black bars = 5% O_2; n = 4–8; *$P < 0.05$.

In conclusion, the data presented in this article firmly support the thesis that low O_2 concentration is a physiological regulator of hematopoiesis. In fact, it would be better to say that the data obtained in cultures at low O_2 concentrations approximate better the physiological reality and therefore, some data obtained at 20% O_2 might be rather considered as an artifact than as a "standard" control (Ivanovic, 2009).

In addition, from a biological viewpoint (excluding safety and technology considerations), a co-culture with mesenchymal stromal cells seems to be an interesting start-point for development of clinical grade ex vivo expansion procedure.

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Literature Cited

Annabi B, Lee YT, Turcotte S, Naud E, Desrosiers RR, Champagne M, Eliopoulos N, Galipeau J, Béliveau R. 2003. Hypoxia promotes murine bone-marrow-derived stromale cell migration and tube formation. Stem Cells 21:337–347.

Carrancio S, López-Holgado N, Sánchez-Guijo FM, Villarón E, Barbado V, Tabera S, Díez-Campelo M, Blanco J, San Miguel JF, Del Cañizo MC. 2008. Optimization of mesenchymal stem cell expansion procedures by cell separation and culture conditions modification. Exp Hematol 36:1014–1021.

Carrera S, de Verdier PJ, Khan Z, Zhao B, Mahale A, Bowman KJ, Zainol M, Jones GD, Lee SW, Aaronson SA, Macip S. 2010. Protection of cells in physiological oxygen tensions against DNA damage-induced apoptosis. J Biol Chem 285:13658–13665.

Chivu M, Diaconu CC, Bleotu C, Alexiu I, Brasoveanu L, Cernescu C. 2004. The comparison of different protocols for expansion of umbilical-cord blood hematopoietic stem cells. J Cell Mol Med 8:223–231.

Chow DC, Wenning LA, Miller WM, Papoutsakis ET. 2001. Modeling pO(2) distributions in the bone marrow hematopoietic compartment. II. Modified Kroghian models. Biophys J 81:685–696.

Cipolleschi MG, Dello Sbarba P, Olivotto M. 1993. The role of hypoxia in the maintenance of hematopoietic stem cells. Blood 82:2031–2037.

Csete M. 2005. Oxygen in the cultivation of stem Cells. Ann NY Acad Sci 1049:1–8.

Danet GH, Pan Y, Luongo JL, Bonnet DA, Simon MC. 2003. Expansion of human SCID-repopulating cells under hypoxic conditions. J Clin Invest 112:126–135.

Dexter TM, Allen TD, Lajtha LG. 1977. Conditions controlling the proliferation of haemopoietic stem cells in vitro. J Cell Physiol 91:335–344.

Dos Santos F, Andrade PZ, Boura JS, Abecasis MM, da Silva CL, Cabral JM. 2010. Ex vivo expansion of human mesenchymal stem cells: A more effective cell proliferation kinetics and metabolism under hypoxia. J Cell Physiol 223:27–35.

Eliasson P, Rehn M, Hammar P, Larsson P, Sirenko O, Flippin LA, Cammenga J, Jönsson JI. 2010. Hypoxia mediates low cell-cycle activity and increases the proportion of long-term-reconstituting hematopoietic stem cells during in vitro culture. Exp Hematol 38:301.e2–310.e2.

Fan J, Cai H, Tan WS. 2007. Role of the plasma membrane ROS-generating NADPH oxidase in CD34+ progenitor cells preservation by hypoxia. J Biotechnol 130:455–462.

Fan J, Cai H, Yang S, Yan L, Tan W. 2008. Comparison between the effects of normoxia and hypoxia on antioxidant enzymes and glutathione redox state in ex vivo culture of CD34 (+) cells. Comp Biochem Physiol B Biochem Mol Biol 151:153–158.

Fehrer C, Brunauer R, Laschober G, Unterluggauer H, Reitinger S, Kloss F, Gülly C, Gassner R, Lepperdinger G. 2007. Reduced oxygen tension attenuates differentiation capacity of human mesenchymal stem cells and prolongs their lifespan. Aging Cell 6:745–757.

Fei XM, Wu YJ, Chang Z, Miao KR, Tang YH, Zhou XY, Wang LX, Pan QQ, Wang CY. 2007. Co-culture of cord blood CD34 (+) cells with human BM mesenchymal stromal cells enhances short-term engraftment of cord blood cells in NOD/SCID mice. Cytotherapy 9:338–347.

Grayson WL, Zhao F, Izadpanah R, Bunnell B, Ma T. 2006. Effects of hypoxia on human mesenchymal stem cell expansion and plasticity in 3D constructs. J Cell Physiol 207:331–339.

Grayson WL, Zhao F, Bunnell B, Ma T. 2007. Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells. Biochem Biophys Res Commun 358:948–953.

Guitart AV, Hammoud M, Dello Sbarba P, Ivanovic Z, Praloran V. 2010. Slow-cycling/quiescence balance of hematopoietic stem cells is related to physiological gradient of oxygen. Exp Hematol 38:847–851.

Hermitte F, Brunet de la Grange P, Belloc F, Praloran V, Ivanovic Z. 2006. Very low O_2 concentration (0.1%) favors G0 return of dividing $CD34^+$ cells. Stem Cells 24:65–73.

Hess DA, Meyerrose TE, Wirthlin L, Craft TP, Herrbrich PE, Creer MH, Nolta JA. 2004. Functional characterization of highly purified human hematopoietic repopulating cells isolated according to aldehyde dehydrogenase activity. Blood 104:1648–1655.

Hess DA, Wirthlin L, Craft TP, Herrbrich PE, Hohm SA, Lahey R, Eades WC, Creer MH, Nolta JA. 2006. Selection based on CD133 and high aldehyde dehydrogenase activity isolates long-term reconstituting human hematopoietic stem cells. Blood 107:2162–2169.

Huang GP, Pan ZJ, Jia BB, Zheng Q, Xie CG, Gu JH, McNiece IK, Wang JF. 2007. Ex vivo expansion and transplantation of hematopoietic stem/progenitor cells supported by mesenchymal stem cells from human umbilical cord blood. Cell Transplant 16:579–585.

Isidori A, Motta MR, Tani M, Terragna C, Zinzani P, Curti A, Rizzi S, Taioli S, Giudice V, D'Addio A, Gugliotta G, Conte R, Baccarani M, Lemoli RM. 2007. Positive selection and transplantation of autologous highly purified CD133 (+) stem cells in resistant/relapsed chronic lymphocytic leukemia patients results in rapid hematopoietic reconstitution without an adequate leukemic cell purging. Biol Blood Marrow Transplant 13:1224–1232.

Ito K, Hirao A, Arai F, Takubo K, Matsuoka S, Miyamoto K, Ohmura M, Naka K, Hosokawa K, Ikeda Y, Suda T. 2006. Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. Nat Med 12:446–451.

Ivanovic Z. 2004. Interleukin-3 and ex vivo maintenance of hematopoietic stem cells: Facts and controversies. Eur Cytokine Netw 15:6–13.

Ivanovic Z. 2009. Hypoxia or in situ normoxia: The stem cell paradigm. J Cell Physiol 219:271–275.

Ivanovic Z, Boiron JM. 2009. Ex vivo expansion of hematopoietic stem cells: Concept and clinical benefit. Transfus Clin Biol 16:489–500.

Ivanovic Z, Dello Sbarba P, Trimoreau F, Faucher JL, Praloran V. 2000. Primitive human HPCs are better maintained and expanded in vitro at 1 percent oxygen than at 20 percent. Transfusion 40:1482–1488.

Ivanovic Z, Belloc F, Faucher JL, Cipolleschi MG, Praloran V, Dello Sbarba P. 2002. Hypoxia maintains and interleukin-3 reduces the pre-colony-forming cell potential of dividing CD34 (+) murine bone marrow cells. Exp Hematol 30:67–73.

Ivanovic Z, Hermitte F, Brunet de la Grange P, Dazey B, Belloc F, Lacombe F, Vezon G, Praloran V. 2004. Simultaneous maintenance of human cord blood SCID-repopulating cells and expansion of committed progenitors at low O_2 concentration (3%). Stem Cells 22:716–724.

Ivanovic Z, Duchez P, Chevaleyre J, Vlaski M, Lafarge X, Dazey B, Robert-Richard E, Mazurier F, Boiron JM. 2011. Clinical-scale cultures of cord blood CD34+ cells to amplify committed progenitors and maintain stem cell activity. Cell Transplant 20: (in press). DOI: 10.3727/096368910X552853

Jang YY, Sharkis SJ. 2007. A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. Blood 110:3056–3063.

Jewell UR, Kvietikova I, Scheid A, Bauer C, Wenger RH, Gassmann M. 2001. Induction of HIF-1alpha in response to hypoxia is instantaneous. FASEB J 15:1312–1314.

Jiang BH, Semenza GL, Bauer C, Marti HH. 1996. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. Am J Physiol 271:C1172–C1180.

Jin Y, Kato T, Furu M, Nasu A, Kajita Y, Mitsui H, Ueda M, Aoyama T, Nakayama T, Nakamura T, Toguchida J. 2010. Mesenchymal stem cells cultured under hypoxia escape from senescence via down-regulation of p16 and extracellular signal regulated kinase. Biochem Biophys Res Commun 391:1471–1476.

Kirito K, Hu Y, Komatsu N. 2009. HIF-1 prevents the overproduction of mitochondrial ROS after cytokine stimulation through induction of PDK-1. Cell Cycle 8:2844–2849.

Koller MR, Bender JG, Miller WM, Papoutsakis ET. 1992a. Reduced oxygen tension increases hematopoiesis in long-term culture of human stem and progenitor cells from cord blood and bone marrow. Exp Hematol 20:264–270.

Koller MR, Bender JG, Papoutsakis ET, Miller WM. 1992b. Effects of synergistic cytokine combinations, low oxygen, and irradiated stroma on the expansion of human cord blood progenitors. Blood 80:403–411.

Kovacevic-Filipovic M, Petakov M, Hermitte F, Debeissat C, Krstic A, Jovcic G, Bugarski D, Lafarge X, Milenkovic P, Praloran V, Ivanovic Z. 2007. Interleukin-6 (IL-6) and low O (2) concentration (1%) synergize to improve the maintenance of hematopoietic stem cells (pre-CFC). J Cell Physiol 212:68–75.

Krstic A, Vlaski M, Hammoud M, Chevaleyre J, Duchez P, Jovcic G, Bugarski D, Milenkovic P, Bourin P, Boiron JM, Praloran V, Ivanovic Z. 2009. Low O2 concentrations enhance the positive effect of IL-17 on the maintenance of erythroid progenitors during co-culture of CD34+ and mesenchymal stem cells. Eur Cytokine Netw 20:10–16.

Kubo M, Li TS, Suzuki R, Shirasawa B, Morikage N, Ohshima M, Qin SL, Hamano K. 2008. Hypoxic preconditioning increases survival and angiogenic potency of peripheral blood mononuclear cells via oxidative stress resistance. Am J Physiol Heart Circ Physiol 294:H590–H595.

Lavrentieva A, Majore I, Kasper C, Hass R. 2010. Effects of hypoxic culture conditions on umbilical cord-derived human mesenchymal stem cells. Cell Commun Signal 16:18.

Li N, Feugier P, Serrurrier B, Latger-Cannard V, Lesesve JF, Stoltz JF, Eljaafari A. 2007. Human mesenchymal stem cells improve ex vivo expansion of adult human $CD34^+$ peripheral blood progenitor cells and decrease their allostimulatory capacity. Exp Hematol 35:507–515.

Li Z, Wei H, Deng L, Cong X, Chen X. 2010. Expression and secretion of interleukin-1β, tumour necrosis factor-α and interleukin-10 by hypoxia- and serum-deprivation-stimulated mesenchymal stem cells. FEBS J 277:3688–3698.

Li P, Huang J, Tian HJ, Huang QY, Jiang CH, Gao YQ. 2011. Regulation of bone marrow hematopoietic stem cell is involved in high-altitude erythrocytosis. Exp Hematol 39:37–46.

Loeffler M, Pantel K, Wulff H, Wichmann HE. 1989. A mathematical model of erythropoiesis in mice and rats. Part 1: Structure of the model. Cell Tissue Kinet 22:13–30.

Magin AS, Körfer NR, Partenheimer H, Lange C, Zander A, Noll T. 2009. Primary cells as feeder cells for coculture expansion of human hematopoietic stem cells from umbilical cord blood—A comparative study. Stem Cells Dev 18:173–186.

McNiece I, Harrington J, Turney J, Kellner J, Shpall EJ. 2004. Ex vivo expansion of cord blood mononuclear cells on mesenchymal stem cells. Cytotherapy 4:311–317.

Parmar K, Mauch P, Vergilio JA, Sackstein R, Down JD. 2007. Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. Proc Natl Acad Sci USA 104:5431–5436.

Potier E, Ferreira E, Andriamanalijaona R, Pujol JP, Oudina K, Logeart-Avramoglou D, Petite H. 2007. Hypoxia affects mesenchymal stromal cell osteogenic differentiation and angiogenic factor expression. Bone 40:1078–1087.

Robert-Richard E, Ged C, Ortet J, Santarelli X, Lamrissi-Garcia I, de Verneuil H, Mazurier F. 2006. Human cell engraftment after busulfan or irradiation conditioning of NOD/SCID mice. Haematologica 91:1384.

Robinson SN, Ng J, Niu T, Yang H, McMannis JD, Karandish S, Kaur I, Fu P, Del Angel M, Messinger R, Flagge F, de Lima M, Decker W, Xing D, Champlin R, Shpall EJ. 2006. Superior ex vivo cord blood expansion following co-culture with bone marrow-derived mesenchymal stem cells. Bone Marrow Transplant 4:359–3566.

Robinson SN, Simmons PJ, Yang H, Alousi AM, Marcos de Lima J, Shpall EJ. 2011. Mesenchymal stem cells in ex vivo cord blood expansion. Best Pract Res Clin Haematol 24:83–92.

Rodriguez Mdel C, Bernad A, Aracil M. 2004. Interleukin-6 deficiency affects bone marrow stromal precursors, resulting in defective hematopoietic support. Blood 103:3349–3354.

Rosová I, Dao M, Capoccia B, Link D, Nolta JA. 2008. Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. Stem Cells 26:2173–2182.

Schofield R. 1978. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells 4:7–25.

Semenza GL. 2007. Oxygen-dependent regulation of mitochondrial respiration by hypoxia-inducible factor1. Biochem J 405:1–9.

Shima H, Takubo K, Iwasaki H, Yoshihara H, Gomei Y, Hosokawa K, Arai F, Takahashi T, Suda T. 2009. Reconstitution activity of hypoxic cultured human cord blood CD34-positive cells in NOG mice. Biochem Biophys Res Commun 378:467–472.

Simsek T, Kocabas F, Zheng J, Deberardinis RJ, Mahmoud AI, Olson EN, Schneider JW, Zhang CC, Sadek HA. 2010. The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. Cell Stem Cell 7:380–390.

Song K, Zhao G, Liu T, Zhang L, Ma X, Liu J, Cui Z. 2009. Effective expansion of umbilical cord blood hematopoietic stem/progenitor cells by regulation of microencapsulated osteoblasts under hypoxic condition. Biotechnol Lett 31:923–928.

Storms RW, Trujillo AP, Springer JB, Shah L, Colvin OM, Ludeman SM, Smith C. 1999. Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. Proc Natl Acad Sci USA 96:9118–9123.

Tamama K, Kawasaki H, Kerpedjieva SS, Guan J, Ganju RK, Sen CK. 2011. Differential roles of hypoxia inducible factor subunits in multipotential stromal cells under hypoxic condition. J Cell Biochem 112:804–817.

Tsai CC, Chen YJ, Yew TL, Chen LL, Wang JY, Chiu CH, Hung SC. 2011. Hypoxia inhibits senescence and maintains mesenchymal stem cell properties through down-regulation of E2A-p21 by HIF-TWIST. Blood 117:459–469.

Ulich TR, del Castillo J, Yin SM, Egrie JC. 1991. The erythropoietic effects of interleukin 6 and erythropoietin in vivo. Exp Hematol 19:29–34.

Van Overstraeten-Schlögel N, Beguin Y, Gothot A. 2006. Role of stromal-derived factor-1 in the hematopoietic-supporting activity of human mesenchymal stem cells. Eur J Haematol 76:488–493.

Vlaski M, Lafarge X, Chevaleyre J, Duchez P, Boiron JM, Ivanovic Z. 2009. Low oxygen concentration as a general physiologic regulator of erythropoiesis beyond the EPO-related

downstream tuning and a tool for the optimization of red blood cell production ex vivo. Exp Hematol 37:573–578.

Walenda T, Bork S, Horn P, Wein F, Saffrich R, Diehlmann A, Eckstein V, Ho AD, Wagner W. 2010. Co-culture with mesenchymal stromal cells increases proliferation and maintenance of haematopoietic progenitor cells. J Cell Mol Med 14:337–350.

Wang JF, Wang LJ, Wu YF, Xiang Y, Xie CG, Jia BB, Harrington J, McNiece IK. 2004. Mesenchymal stem/progenitor cells in human umbilical cord blood as support for ex vivo expansion of CD34 (+) hematopoietic stem cells and for chondrogenic differentiation. Haematologica 89:837–844.

Winkler IG, Barbier V, Wadley R, Zannettino AC, Williams S, Lévesque JP. 2010. Positioning of bone marrow hematopoietic and stromal cells relative to blood flow in vivo: Serially reconstituting hematopoietic stem cells reside in distinct nonperfused niches. Blood 116:375–385.

Yahata T, Takanashi T, Muguruma Y, Ibrahim AA, Matsuzawa H, Uno T, Sheng Y, Onizuka M, Ito M, Kato S, Ando K. 2011. Accumulation of oxidative DNA damage restricts the self-renewal capacity of human hematopoietic stem cells. Blood 118: [Epub ahead of print]. DOI 10.1182/blood-2011-01-330050

Yamaguchi M, Hirayama F, Wakamoto S, Fujihara M, Murahashi H, Sato N, Ikebuchi K, Sawada K, Koike T, Kuwabara M, Azuma H, Ikeda H. 2002. Bone marrow stromal cells prepared using AB serum and bFGF for hematopoietic stem cells expansion. Transfusion 42:921–927.

Zhambalova AP, Darevskaya AN, Kabaeva NV, Romanov YA, Buravkova LB. 2009. Specific interaction of cultured human mesenchymal and hemopoietic stem cells under conditions of reduced oxygen content. Bull Exp Biol Med 147:525–530.

Zhang Y, Li C, Jiang X, Zhang S, Wu Y, Liu B, Tang P, Mao N. 2004. Human placenta-derived mesenchymal progenitor cells support culture expansion of long-term culture- initiating cells from cord blood CD34^{+} cells. Exp Hematol 32:657–664.