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Combined mild hypoxia and bone marrow mesenchymal stem cells improve expansion and *HOXB4* gene expression of human cord blood CD34+ stem cells

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Abstract: Cord blood (CB) is a rich source of hematopoietic stem cells (HSC). It has been used successfully to treat a variety of hematological and non-hematological disorders. Beside the advantages of CB, its main disadvantages are the limited number of stem cells available for transplantation and delayed engraftment. Identifying strategies to enhance expansion and self-renewal of HSCs can improve transplantation efficiency. The aim of this study was to examine different culture conditions on *ex vivo* expansion and homeobox protein Hox-B4 gene (*HOXB4*) expression in cord blood $CD34^+$ stem cells. Human cord blood $CD34^+$ HSC were cultured in serum-free medium supplemented with cytokines with and without a feeder layer in normoxia (21% O_2) and mild hypoxia (5% O_2).At the end of 7 days of culture, the highest number of total nucleated cells (TNC), $CD34^+$ cells, colony forming units (CFUs) and *HOXB4* mRNA were observed in a co-culture of HSC with a bone marrow mesenchymal stem cell(MSC) feeder layer at 5% O_2.We concluded that the combination of bone marrow (BM)-MSC and mild hypoxia (5% O^2) not only improved HSC expansion but also enhanced *HOXB4* gene expression as a self-renewal marker of HSC, and better mimicked the niche microenvironment conditions.

Key words: cord blood; hematopoietic stem cells; $CD34^+$ cells; mesenchymal stem cells; hypoxia; *HOXB4*

Abbreviations: CB – cord blood; HSC – hematopoietic stem cells; HSPC – hematopoietic stem/progenitor cells; *HOXB4* – homeobox protein Hox-B4; TNC –total nucleated cells; CFU – colony forming unit; MSC – mesenchymal stem cells; BM – bone marrow; SCF –stem cell factor; FLT3L – FMS-like tyrosine kinase 3 ligand; TPO –thrombopoietin; IL – interleukin; M-CSF – macrophage-colony stimulating factor; HIF-1 – hypoxia inducible factor-1; SDF1 – stromal cell-derived factor 1; MNC – mononuclear cells; FBS – fetal bovine serum; IMDM – Iscove's modified Dulbecco's medium; DMEM – Dulbecco's modified Eagle's medium; PBS – phosphate-buffered solution; ROS –reactive oxygen species; GEMM – granulocyte/erythroid/macrophage/megakaryocyte, GM – granulocyte/monocyte, G – granulocyte, M – monocyte, E – erythroid (E); BFU – burst forming unit

INTRODUCTION

Stem cells are known for their ability to remain undifferentiated and their capacity to undergo self-renewal, which allows them to proliferate during fetal development and to be maintained throughout adult life [1-4]. There are different sources of HSC. One important source of HSC is CB. Human umbilical CB contains $CD34^+$ hematopoietic stem/progenitor cells (HSPC) with a high proliferative potential [5].

Beside the advantages of CB, including widespread availability and decreased ability to induce immunological reactivity against the patient, CB also has disadvantages, include the low and sometimes limiting number of cells collected in one unit, which can be less than optimal for engraftment in many adults and higher weight children, and the relatively slower engraftment time of neutrophils and platelets [6-8].

There are several ways to overcome these problems. *Ex vivo* expansion of HSPC and co-culture methods with a feeder layer were suggested [9]; however, HSC expansion in culture remains challenging mainly due to our limited knowledge of the factors that drive HSC self-renewal beside expansion. Among the different stimulatory cytokines identified, a cocktail of SCF, FLt3L and TPO are presumed to promote extensive HSC self-renewal [10].

In vivo, HSC are maintained and regulated by a specific microenvironment referred to as a "niche" in the BM. Multiple cellular types, soluble and membrane- bound factors and extracellular matrix components form this niche [11]. MSC in stem cell niches play an essential role in quiescence, proliferation and differentiation of HSC [12].Studies have shown that MSC secrete at the base state IL-6, IL-7, IL-8, IL-11,IL-12, IL-14, IL-15, M-CSF, Flt-3L and SCF [13].

Stem cell niches are located in regions away from blood vessels. This provides a low O_2 tension environment that enhances stem cell proliferation to the detriment of their differentiation potential [14]. Several studies in murine and human HSC demonstrated that a culture of HSC at 21% O_2 leads to the rapid exhaustion of their stem cell potential. Culture in anoxic conditions (0.1-1% O_2) promotes the maintenance and return of HSC to quiescence in G0 [15]. Cultures at higher O_2 levels (3-5%) maintain cell proliferation [16-18]. The cellular mechanisms by which human hematopoietic progenitors and stem cells respond to hypoxia have not been characterized, but it has been accepted that HIF-1 protein [19] is rapidly degraded under normoxic conditions and is stabilized under low O_2 levels (<5%).

Several molecules have been proposed to control the self-renewal of HSC. Among these molecules, the homeobox family of proteins, *HOXB4* are expressed in most primitive hematopoietic stem cells. A physiological approach suggests that an environment associated with MSC and low O_2 concentration would be the most favorable for *ex vivo* expansion and self-renewal of HSC. In this study, we examined the feasibility of combining serum-free media, BM-MSC and mild hypoxia (5% O2) to establish a novel culture system for the *ex vivo* expansion of HSC and expression of *HOXB4* as a stem cell self-renewal marker.

MATERIALS AND METHODS

CB collection and CD34+ cell purification

CB samples were collected from 4-5 different full-term deliveries from umbilical cord blood after obtaining the parents' informed written consent for each experiment (n=3). CB units were obtained from the Iranian Blood Transfusion Organization. Briefly, up on delivery of the baby the cord was doubly clamped and transected, and blood was collected in special collection bags containing citrate-phosphate dextrose. After collection, the samples were stored at room temperature (15-25°C) and processed within 12 h. Research was carried out according to the guidelines approved by the Ethics Committee at Tarbiat Modares University (reference number 4506).Red cells were precipitated with 6% hydroxyethyl starch (Grifols, Spain). MNC were isolated by Ficoll-Hypaque density gradient centrifugation (Lymphodex, inno-Train Diagnostic, Germany). CB-CD34+ cells were purified from MNC using a MACS $CD34^+$ cell isolation kit following the manufacturer's instructions (Miltenyi Biotec, USA CD34 MicroBead Kit). $CD34^+$ cell purity was evaluated by flow cytometry analysis using an FITC-human CD34 antibody. Nonspecific reactions were excluded using isotype controls.

Feeder layer preparation

BM-MSC were a kind gift from Dr. M. Soleimani (Stem Cells and Tissue Engineering Department, Stem Cell Technology, Tehran, Iran.). Three BM-MSC samples from different healthy donors were cultured in DMEM+10% FBS (Gibco, Grand Island, NY, USA) supplemented with streptomycin (0.025 mg/mL) and penicillin (0.025 U/mL; Gibco BRL), at 37°C and 5% CO_2 in a humidified atmosphere. On reaching 80% confluency, cells were trypsinized and replated at a density of about 10^4 cells/cm^2. Cells were expanded for 2 to 4 passages. Phenotypic characterization and differentiation into osteoblasts and adipocytes were carried out. MSC were characterized by flow cytometric analysis using monoclonal antibodies against CD105, CD73, CD90 and CD45.MSC were positive for CD105, CD73, CD90 and negative for CD45.In order to be used as feeder layers, cells were seeded on 24-well plates (1×10^4 cells/well) and grown until 70-80% confluency.

Coculture with a feeder layer

One$\times10^4$ isolated $CD34^+$cells were co-cultured with 1×10^4/well BM-MSC in 24-well plates in Stemline II serum free media (Sigma, Germany) supplemented with/without the combination of human recombinant

cytokines: Flt3-L (50 ng/mL, ORF Genetics, Iceland), SCF(50 ng/mL, ORF Genetics, Iceland) and TPO (50 ng/mL, Peprotech, Rocky Hill, NJ, USA). In this study, the cells harvested at 2-4 passages were used as BM-MSC. All experiments were cultured at 37°C, 5% CO_2 in 5% O_2 (two-gas incubator) and 21% O_2 for 7 days. The culture conditions for the present study were classified into six groups as follows: (i) cytokine group: $CD34^+$ cells cultured in serum-free medium in the presence of cytokines in 21% O_2; (ii) feeder group: $CD34^+$ cells cultured in the serum-free medium directly on the confluent MSC feeder layer without cytokines in 21% O_2; (iii) feeder+cytokine group: $CD34^+$ cells cultured in the serum-free medium on a confluent MSC feeder layer and in the presence of cytokines in 21% O_2; (iv) cytokine group: $CD34^+$ cells cultured in serum-free medium in the presence of cytokines in 5% O_2; (v) feeder group: $CD34^+$ cells cultured in serum-free medium directly on the confluent MSC feeder layer without cytokines in 5% O_2; (vi) feeder+cytokine group: $CD34^+$ cells cultured in serum-free medium on a confluent MSC feeder layer and in the presence of cytokines in 5% O2.HSC were additionally cultured in Stemline II serum-free medium without any cytokines and the MSC layer as the control group. After 7 days, the co-cultures were harvested, and viable cell counts were taken using trypan blue dye exclusion. The total number of nucleated cells was counted.

Flow cytometric analysis

Expanded $CD34^+$ cells from each culture condition were collected and resuspended in PBS. After labeling the cell suspension with monoclonal antibodies (CD34-FITC, BD Pharmingen™, USA), the cells were incubated for 30-45 min on ice. The expansion-fold of $CD34^+$ cells was calculated by comparing the TNC×$CD34^+$ percentage obtained after 7 days with the TNC×$CD34^+$ percentage before culture initiation. Data analysis was performed using FlowJo analytical software (Tree Star, Inc., Ashland, OR).

Colony-forming cell (CFC) assay

One×10^3freshly isolated $CD34^+$ or expanded cells in 6 different culture conditions were harvested on day 7 and cultured on 12-well plates in cytokine-supplemented methylcellulose media (MethoCult H4434 classic with cytokine, Stem Cell Technologies, Canada) with 2% FBS in IMDM as indicated in the manufacturer's user instructions to enumerate the CFC. CFU colonies consisting of 50 cells were counted after 14 days of incubation at 37°C under humidified conditions in 5% CO_2. The experiments were performed in duplicate in three independent experiments. The total CFU fold change was calculated by dividing the number of colonies obtained per 10^3cultured cells by the number of the colonies obtained at day 0, and multiplying by the fold increase in total number of cells.

RNA extraction and cDNA synthesis

Cell RNA was extracted using TRIzol (Sigma, Germany). Total RNAs were reverse transcribed using the cDNA kit (Primescript RT reagent kit perfect real time, Takara) according to the manufacturer's instructions. Each cDNA sample was run in duplicate for target (*HOXB4*) or *HPRT* endogenous control.

Real-time PCR

Quantitative reverse transcriptase (q-PCR) was performed using 1μL total cDNA in 10μL reaction volume with 0.3 μM of each primer and 5 μL of real-time master mix (Amplicon, Denmark).Thermal cycling was initiated at 95°C for 15 min followed by 40 cycles of PCR (95°C, 20 s). The annealing temperature was 61°C. Quantification of qRT-PCR signals was performed using the delta-delta Ct method which calculates the relative changes in gene expression of the target gene normalized to the *HPRT* endogenous control. The values obtained were represented as the relative quantity of mRNA level variations. The sequence of genesisas follows: *HOXB4*: forward: 5′-TGCAAAGAGCCCGTCGT-3′; reverse: 5′-GGCGTAATTGGGGTTTACCG-3′.*HPRT*: forward: 5′-CCTGGCGTCGTGATTAGTG-3′ and reverse: 5′-TCAGTCCTGTCCATAATTAGTCC-3′

Statistical analysis

All data were obtained from three independent experiments. Data are represented as the mean values±standard deviation. Significant differences

were assessed by either the Student t-test or ANOVA. Comparison of the data between normoxia and hypoxia was done using an independent t-test. One-way ANOVA was used to calculate the significance within groups. Statistics were calculated with using Graph Pad Prism 6 software. The value <0.05 were considered statistically significant.

RESULTS

Total nucleated cell expansion

To assess whether various culture conditions could accelerate cell expansion (Fig. 1), we analyzed the count increase of cells after culture as defined by the conditions in different experimental groups. The trypan blue exclusion test demonstrated >85% viable cells both at 21% and 5% O_2 in different culture conditions. The initial cell count was 1×10^4, and after 7 days culture in normoxia, the mean TNC count was 75600±4900 in the cytokine culture without the MSC feeder layer, 41540±2660 in the coculture system without cytokines and 112000±3361 in the co-culture system with cytokines. In hypoxic cultures, the mean TNC count was 89000±6151 in the cytokine culture without the MSC feeder layer, 48700±3161 in the co-culture system without cytokines, and 144080±8518 in the co-culture system with cytokines. In all co-cultures, TNC increased significantly compared to the feeder-free culture conditions (n=3, P<0.05). In all of the groups in hypoxic conditions compared to normoxic conditions, TNC increased significantly (P<0.05) (Fig. 2).

CD34⁺ cell expansion

As CD34⁺ is often used as a surrogate marker for HSC, the enumeration of CD34⁺ cells has been used to quantify the progenitor and stem cell contents.CD34⁺ cell purity, evaluated by flow cytometry analysis using an FITC-human CD34⁺ antibody, was >90% in all cases on the first day of purification. Fig. 3 shows the fold change of CD34⁺ cells in different culture conditions after 7 days. The mean fold change of CD34⁺ cells was 1.96±0.15 in the cytokine culture without the MSC feeder layer, 1.04±0.08 in the co-culture system without cytokines, and 2.98±0.2 in the co-culture system with cytokines in 21% O_2. In the hypoxic culture,

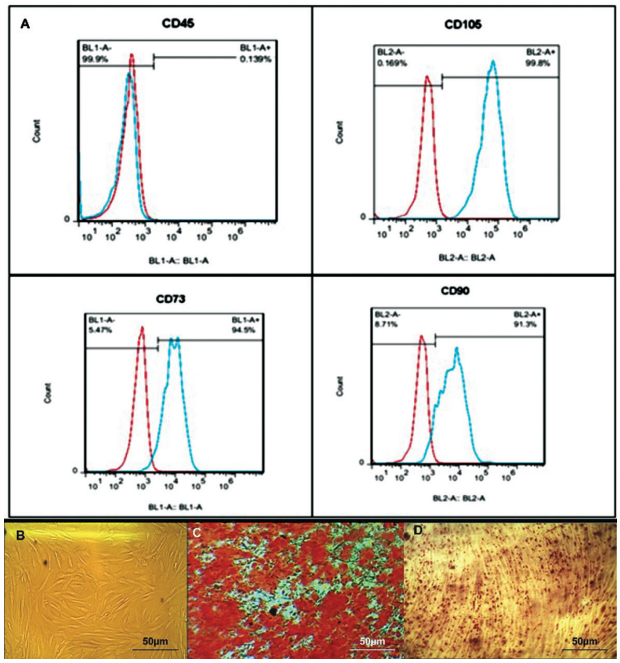

Fig. 1. Characterization of human BM-MSC *in vitro*. **A** – flow cytometry showing that BM-MSC were positive for the markers CD105,CD73 and CD90, and were negative for the leukocyte common antigen CD45. **B** – BM-MSCs exhibiting a spindle-like morphology under light field microscopy. Osteogenic(**C**) and adipogenic (**D**) differentiation was detected by Alizarin Red S staining and by oil red O staining, respectively, to confirm the multipotency of BM-MSCs. Scale bar: 50µm.

the mean fold change in CD34⁺ cells was 2.45±0.15 in the cytokine culture without the MSC feeder layer, 1.32±0.15 in the co-culture system without cytokines, and 4.91±0.23 in the co-culture system with cytokines. The mean fold change in CD34⁺ cells on day 7 in the co-culture system with cytokines was higher than in the cytokine culture without the MSC feeder layer and the co-culture system without cytokines (N=3, P<0.05). Our results showed that both the co-culture and the hypoxic environment had a significant effect on the final CD34⁺ cell output, and that the collective impact of these two factors was remarkable.

In the co-culture systems without cytokines, TNC and CD34⁺ cell numbers increased 5- and 1.5-fold, respectively, and cell viability remained 90% after 7 days. In both hypoxia and normoxia cultures, the TNC count and CD34+ fold change were higher in the cytokine groups when compared to the feeder groups (Figs. 2 and 3).

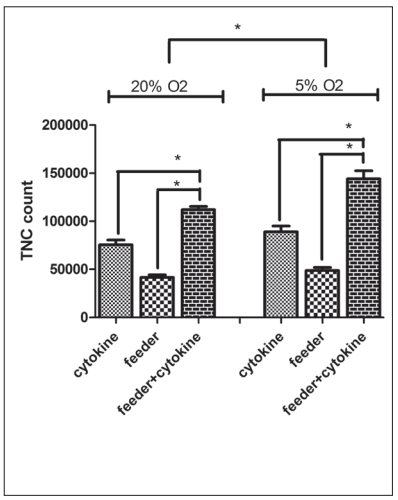

Fig. 2. Total nucleated cell count in different culture conditions. One×10^4 CD34$^+$ cells/well cultured in Stemline II serum-free media supplemented with the combination of Flt3-L(50 ng/mL), SCF (50 ng/mL) and TPO (50 ng/mL) in 5% O_2 and 21% O2, for 7 days with/without the bone marrow MSC feeder layer. After 7 days, the TNC was counted. Data represents the mean±SD from three independent experiments. Error bars represent SD, significant values are *p<0.05 (two-tailed Student's t test).

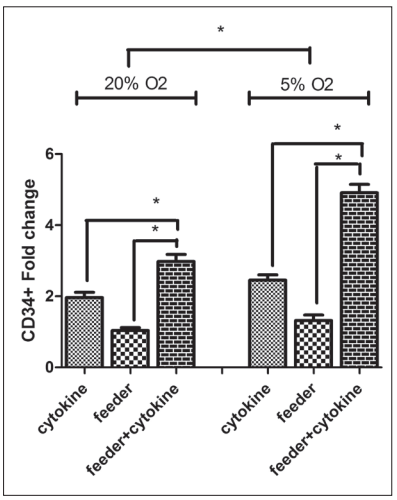

Fig. 3. CD34$^+$ cell fold change in different culture conditions. One×10^5 expanded CD34$^+$cells from different culture conditions evaluated by labeling the cells with antibody conjugated to FITC by flow cytometry. The fold-expansion of CD34$^+$ cells was calculated by comparing the TNC×CD34$^+$% obtained after 7 days with the TNC×CD34$^+$% before culture initiation. Data represent the means±SD from three independent experiments. Error bars represent SD, significant values are*p<0.05 (two-tailed Student's t test).

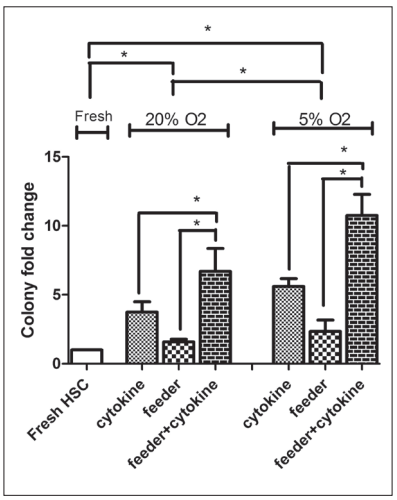

Fig. 4. Colony fold change in different culture conditions. Results of statistical analysis of methylcellulose-based clonogenic assay performed on CB-CD34$^+$ cells in different culture conditions plated after 14 days. The experiments were performed in duplicate in three independent experiments. Error bars represent SD, significant values are *p<0.05 (two-tailed Student's t test).

Colony-forming assay

The colony-forming unit ability of CB-CD34$^+$ cells was investigated by performing a clonogenic assay in methylcellulose-based cultures (14 days) under different culture conditions that expanded for 7 days, and with fresh CD34$^+$ cells. The results showed that the expanded cells in all culture conditions possessed the ability to produce clonogenic progenitor cells: CFU-GEMM,-GM, -G, -M, -E and -BFU-E, similar to fresh CD34$^+$ cells. The total fold increase in clonogenic potential was calculated by dividing the number of colonies obtained per 10^3 cultured cells under the different culture conditions by the number of the colonies obtained at day 0 and multiplying by the fold increase in total number of cells.

The mean CFU fold change of different culture conditions compared to fresh CD34$^+$cells was 3.7±0.7 in the cytokine culture without the MSC feeder layer,1.6±0.2 in the co-culture system without cytokines, and 6.7±1.6 in the co-culture system with cytokines in normoxia. In 5% O_2, the mean CFU fold changes were as follows: 5.6±0.5 in the cytokine culture without the MSC feeder layer, 2.3±0.8 in the co-culture system without cytokines, and 10.8±1.5 in the co-culture system with cytokines.

Cultures in the presence of the feeder layer and cytokines were capable of maintaining a higher clonogenic potential (P<0.05). There was higher colony fold change in hypoxia compared to normoxia (P<0.05). The data are shown in Fig. 4 and the morphology of the colonies is presented in Fig. 5.

HOXB4 gene expression

The expression of *HOXB4* in fresh CD34$^+$ cells and expanded cells after 7 days of growth in all culture conditions was evaluated by RT-PCR. The mean fold change ratio of *HOXB4* mRNA expression in the expanded cells to fresh CD34$^+$cells in normoxia was 0.5±0.1 in the cytokine culture without the MSC feeder layer, 0.8±0.1 in the co-culture system without

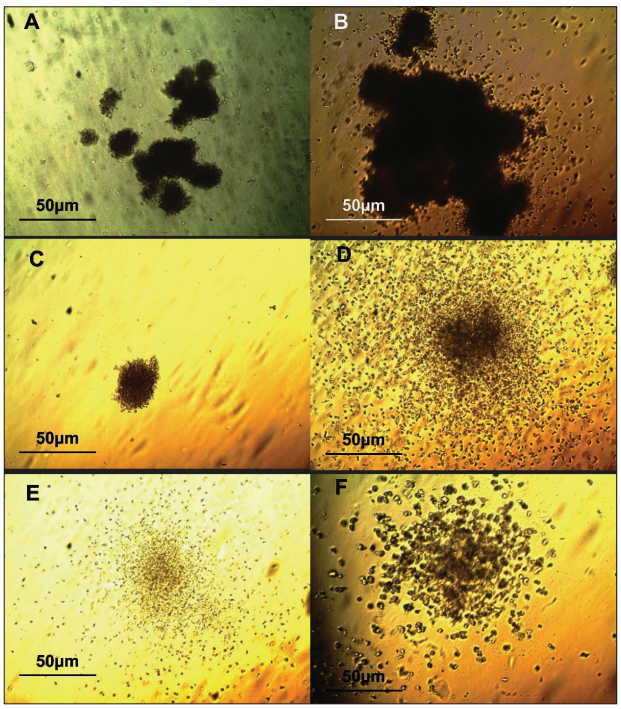

Fig. 5. Morphology of colonies cultured 14 days in MethoCult H4434 with cytokine. **A** – BFU-E, **B** – CFU-GEMM, **C**– CFU-E, **D** – CFU-GM, **E** – CFU-G, **F** – CFU-M (scale bars in A-F:50µm).

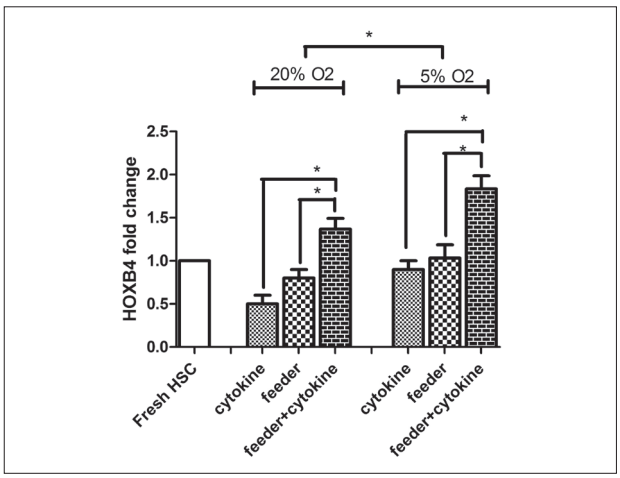

Fig. 6. The mean fold change ratio of *HOXB4* mRNA expression in different culture conditions by RT-PCR (n=3; significant values are *$p<0.05$). Data are normalized to *HPRT* expression levels.

cytokines, and 1.36±0.12 in the co-culture system with cytokines. In hypoxic culture, the mean fold change ratio was 0.9±0.1 in the cytokine culture without the MSC feeder layer, 1.03±0.15 in the co-culture system without cytokines, and 1.83±0.15 in the co-culture system with cytokines.

We observed that in cytokine groups, either in normoxia or hypoxia, *HOXB4* expression decreased rapidly, but in the feeder groups without cytokines, *HOXB4* expression was better maintained. The highest *HOXB4* levels were detected in the feeder+cytokine groups. The results showed that *HOXB4* gene expression was sensitive to the oxygen level and the presence of the MSC feeder layer (Fig. 6) (N=3, P<0.05).

DISCUSSION

The major focus in HSC expansion is still the definition of optimal culture conditions as regards hematopoietic growth factor combinations, co-cultures with feeder cells and other stem cell niche requirements. Our experiments were carried out in serum-free culture medium because in clinical use, serum-free culture medium has been proposed to avoid some concerns, such as the risk of infection by viruses or prions, variation between individual batches [20], the presence of inhibitors or stimulating factors of hematopoiesis, and finally, the use of serum free-media provides biochemically defined culture conditions [21].

The best results in HSC expansion have been obtained by researchers using early-acting factors, such as SCF, FLT3L and TPO. Although the application of cytokine-supplemented media results in the fast and effective expansion of hematopoietic cells, rapid expansion is accompanied by differentiation, resulting in depletion of the stem cell pool [22].

Interaction between stem cells and their niches can modulate HSC functions *in vitro* [23].A more natural stem cell proliferation was achieved by culturing the stem cells on a layer of MSC feeder cells [24,25]. We showed that a serum-free culture system using human BM MSC-derived feeder layers supplemented with a cytokine cocktail of SCF, TPO and FLT3L, allowed for a higher expansion of $CD34^+$ HSC from CB.

The mean fold changes of TNC and $CD34^+$ cells in the cytokine culture with MSC were higher than in the cytokine culture without MSC. Consistent with our results, co-culture with MSC maintained HSC with a primitive immune phenotype ($CD34^+CD38^-$ or $CD133^+CD38^-$) [26], and *ex vivo* contact with stromal components of the hematopoietic microenvironment preserved HSC [27,28].

In the co-culture system without cytokines, TNC and CD34+cell numbers were increased up to 5- and 1.5-fold, respectively, but cell viability was more than 90%.MSC as a feeder layer without the addition of any cytokines maintained HSC expansion at a low but constant level for 7 days. In keeping with our results, Amirizadeh et al. [29] also showed that the proliferation capacity of HSC in recombinant cytokine culture with a MSC feeder layer was higher than in a cytokine culture without MSC and in a co-culture system without cytokines. It seems that BM-MSC secreted cytokines and growth or survival signals that were transferred to the HSC via adhesive molecules through the modulation of cytokine- and growth factor-dependent signals.

Several publications support the fundamental role of low O_2 concentrations in the regulation of stem cell homeostasis (similar to those in bone marrow). It is generally believed that the hypoxic niche protects stem cells from oxidative stress, and thus, the HSC present in such niches contain low *in vivo* ROS levels [30].

The relationship between the cycle/quiescence balance for HSC and physiological O_2 tension (<5%) in their environment has been reported both *in vitro* and *in vivo* [31]; for these reasons we choose 5% O_2 tension.

A higher TNC count and $CD34^+$ fold change in mild hypoxia (5% O_2 tension) demonstrated that mild hypoxia stimulated the expansion of HSC. Recently, it was reported that cultivation of human $CD34^+$, 38^- HSC in hypoxic conditions prior to transplantations maintains and to some extent improves their engraftment to immune-compromised mice [32,33]. Our results showed that expanded cells in all culture conditions possessed the ability to produce clonogenic progenitor cells similar to fresh $CD34^+$ cells. The colony-forming potential depended on the number of HSC, and our results showed that the number of CFC in HSC cultured on the MSC feeder layer in the presence of cytokines at 5% O_2 was higher than other culture conditions. We choose *HOXB4* gene expression as a self-renewal marker of HSC because *HOXB4* is probably one of the most important regulators of HSC self-renewal [34]. It is expressed in the stem cell fraction of the bone marrow and subsequently downregulated during differentiation in humans [35] and mice [36]. Several studies showed that *HOXB4* induces *ex vivo* expansion of HSC and *in vivo HOXB4* overexpression accelerated stem cell regeneration and led to a marked competitive repopulating advantage [37-39].Our results showed that despite the higher expansion of HSC in the cytokine group, *HOXB4* expression was lower in this group when compared to the feeder group and the feeder+cytokine group. Several studies have reported that cytokines such as GM-CSF, IL-3, SCF and TPO increased murine and human HSC proliferation with a rapid increase in the level of ROS in cells [40].It seems that MSC co-culture attenuated the side effects of ROS and better-preserved HSC self-renewal. We showed higher HSC expansion and expression of *HOXB4* in hypoxia (5% O_2 tension). Our study is in accordance with previous studies that reported that hypoxia is beneficial in maintaining the self-renewal properties of HSC [41,42].

CONCLUSIONS

Harvested $CD34^+$ cells were significantly increased when HSC were co-cultured on MSC with combinations of FLT3-L, TPO and SCF, when compared to a cocktail of cytokines or feeder alone. In the culture conditions used here, the decrease in O_2 concentration provoked a relative increase in the expansion of total cells both in culture and co-culture. The positive effect of low O_2 concentration associated with MSC on HSC maintenance was the result of numerous factors such as cytokines, growth factors and their receptors, as well as other molecules exhibiting the HIF-1-binding sequence, whose production was affected by O_2 concentration. The results of this experiment support the conclusion that the co-culture of CB-$CD34^+$ and BM-MSC under mild hypoxia(5% O_2) not only improved expansion of TNC, $CD34^+$cells and CFU-C, but also the level of *HOXB4* gene expression as a self-renewal marker of HSC increase, as compared to freshly isolated $CD34^+$ cells. We speculated that hypoxia induced expression of HIF-1 and maintained elevated levels of downstream genes such as *HOXB4*.We found that several environmental factors, such as concentrations and combinations of cytokines, stromal interactions and other stem cell niche conditions (such as O_2 tension) were required to modulate the quiescence/proliferation balances and self-renewal capacity of hematopoietic stem cells. These findings are expected to con-

tribute to the development of more efficient culture systems for *ex vivo* expansion of CB HSC for cellular therapy that resemble the *in vivo* microenvironment in which these cells reside.

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Author contributions: FM performed the experiments, analyzed the data and wrote the manuscript.SA and AA contributed to the experimental design and manuscript editing. All authors reviewed and approved the final manuscript.

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