

Research article

**MESENCHYMAL STEM CELLS PROMOTE A PRIMITIVE PHENOTYPE CD34+c-kit+ IN HUMAN CORD BLOOD-DERIVED HEMATOPOIETIC STEM CELLS DURING *ex vivo* EXPANSION**

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**Abstract:** The purpose of this study was to evaluate the influence of bone marrow-mesenchymal stem cells (BM-MSc) and exogenously added cytokines on the proliferation, primitive cell subpopulation maintenance (including the c-kit+ marker) and clonogenic capacity of hematopoietic stem cells (HSC). BM-MSc were collected from volunteer donors, isolated and characterized. Umbilical cord blood (UCB) samples were collected from healthy full-term deliveries. UCB-CD34+ cells were cultured in the presence or absence of BM-MSc and/or cytokines for 3 and 7 days. CD34+ cell proliferation was evaluated using the CFSE method and cell phenotype was determined by CD34, c-kit, CD33, CD38, HLA-DR, cyCD22 and cyCD3 detection. Cell clonogenic ability was also assessed. Exogenously added SCF, TPO and FLT3L increased

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Abbreviations used: BFU-E – burst-forming unit erythroids; BM – bone marrow; BM-MSc – bone marrow mesenchymal stem cells; CFSE – carboxyfluorescein diacetate succinimidyl ester; CFU-E – colony-forming unit erythroids; CFU-GEMM – colony-forming unit–granulocyte, erythrocyte, monocyte, megakaryocyte; CFU-GM – colony-forming unit–granulocyte, monocyte, macrophage; Flt-3L – FMS-like tyrosine kinase 3 ligand; FRS – frozen supernatant; FS – fresh supernatant; HC – hematopoietic cytokines; HM – hematopoietic mesenchymal; HMC – hematopoietic-mesenchymal cytokines; HSC – hematopoietic stem cells; LTC-IC – long-term culture-initiating cell; MACS – magnetic-activated cell sorting; MFI – medium fluorescence intensity; MSC – mesenchymal stem cells; SCF – stem cell factor; TPO – thrombopoietin; UCB – umbilical cord blood; UCB-CD34+ – umbilical cord blood–hematopoietic stem cell

CD34<sup>+</sup> cell proliferation in the presence or absence of BM-MSC, but with concomitant cell differentiation. Without any added cytokines, BM-MSC are able to increase the percentage of primitive progenitors as evaluated by c-kit expression and CFU-GEMM increase. Interestingly, this latter effect was dependent on both cell-cell interactions and secreted factors. A 7-day co-culture period will be optimal for obtaining an increased primitive HSC level. Including c-kit as a marker for primitive phenotype evaluation has shown the relevance of BM-MSC and their secreted factors on UCB-HSC stemness function. This effect could be dissociated from that of the addition of exogenous cytokines, which induced cellular differentiation instead.

**Key words:** Hematopoietic stem cell, Mesenchymal stem cell, Bone marrow stroma, CD34<sup>+</sup>c-kit<sup>+</sup> subpopulation, Cytokines, Progenitor cell, Umbilical cord blood

## INTRODUCTION

Experimental evidence from the last two decades has established that hematopoietic stem cells (HSC) from umbilical cord blood (UCB) can be used as a cell source for treatments for hematopoietic system-associated diseases [1]. However, the low numbers of hematopoietic progenitors present in UCB remains a drawback for their clinical application and further biological studies. Therefore, researchers have designed various strategies and cytokine cocktails for *in vitro* HSC expansion to ensure an adequate number of cells for transplantation [2, 3]. In particular, cytokines that are presumed to promote extensive cell self-renewal and to limit apoptosis levels have been used, such as stem cell factor (SCF), FMS-like tyrosine kinase 3 ligand (Flt-3L) and thrombopoietin (TPO) [4, 5].

UCB-HSC expansion protocols have led to improved results in clinical trials, with reduced neutrophil and platelet count time recovery in patients who received transplanted cells [6]. However, other studies have also revealed changes in HSC biological features after *in vitro* cytokine-driven HSC expansion. In fact, *in vitro*-expanded hematopoietic progenitors have reduced engraftment capacity [7], altered VLA-4 and VLA-5 expression [8], increased Fas ligand (CD95) expression [9], downregulation of the anti-apoptotic protein Bcl-2 [10] and increased caspase activation [11].

It is likely that the observed changes in HSC after *in vitro* expansion are attributable to the lack of a suitable microenvironment that maintains their biological properties, as per Schofield's "niche hypothesis" [12]. Several studies have supported this concept and have extended it to show that the HSC niche in the bone marrow (BM) consists of several cell types, including fibroblasts, pericytes, endothelial cells, osteoblasts and mesenchymal stem cells (MSC) along with extracellular matrix glycoproteins [13, 14]. In particular, BM-MSC are important regulators of the hematopoietic microenvironment because they have the capacity to differentiate into specialized stem cells. They also

contribute to provide suitable support for hematopoiesis in this milieu by synthesizing a complex network of cytokines, adhesion molecules and extracellular matrix proteins, regulating HSC survival, proliferation, growth and differentiation [15, 16]. BM-MSCs have thus been included in HSC expansion strategies and niche studies for maintaining HSC biological characteristics *ex vivo*. There are two important issues to consider: if BM-MSCs-secreted cytokines are sufficient to maintain HSC characteristics or if exogenous cytokines are needed to induce cell expansion and undifferentiated phenotype maintenance. The effect of BM-MSCs on HSC proliferation [17] and maintenance has been studied in populations that are considered primitive due to their CD34<sup>+</sup>CD38<sup>-</sup> phenotype or slight modifications thereof [16, 18-20]. Flores-Guzman *et al.* showed that BM-MSCs play a key role in primitive cell phenotype maintenance in CD34<sup>+</sup>CD38<sup>-</sup>Lin<sup>-</sup> populations. Others have demonstrated that BM-MSCs support HSC expansion or self-renewal in CD133<sup>+</sup>, CD34<sup>+</sup>CD38<sup>-</sup>CD133<sup>+</sup> or CD34<sup>+</sup>CD38<sup>-</sup> cells [6, 18, 19, 21]. It is believed that CD34<sup>+</sup>CD38<sup>-</sup> is the most primitive phenotype since it is associated with human long-term culture-initiating cell (LTC-IC) production and contains SCID-human-repopulating and NOD-SCID-repopulating cells [22-24]. However, other studies have shown that the CD34<sup>+</sup>CD38<sup>+</sup> population is also capable of repopulating immunodeficient mice [25-27]. Therefore, the presence or absence of CD38 is not enough to define HSC as primitive. In clinical diagnosis, it is not enough to consider a patient sample as having a high content of primitive cells by detecting only CD34 or CD38 antigens [28]. Therefore, it is important to evaluate other antigens or their combinations to establish the HSC primitive phenotype and stemness function.

The CD117 or c-kit, characterized by Yarden *et al.* in 1987 [29], is expressed in hematopoietic cells and also in several non-hematopoietic tissues and tumors [30]. Together with CD33 and HLA-DR, it has been used for leukemia diagnosis. The c-kit antigen is specifically expressed in BM in the progenitor compartment. Upon differentiation, its expression is downregulated, other than in the mast cells [28, 29]. Up to 70% of CD34<sup>+</sup> BM progenitor cells co-express c-kit [30, 31] and the CD34<sup>+</sup>c-kit<sup>+</sup> subpopulation contains the cells with the highest clonogenic activity (CFU-GM, CFU-mix and BFU-E) [32, 33]. Interestingly, human clinical studies have shown that the primitive hematopoietic progenitor cells, including the burst-forming unit megakaryocyte, the high proliferative potential colony-forming cell and the long-term BM culture-initiating cell, are contained within the CD34<sup>+</sup>c-kit<sup>+</sup>HLA-DR subpopulation, indicating that human stem cells are c-kit positive [34]. It has also been shown that changes in c-kit and HLA-DR antigen expression on the CD34<sup>+</sup> cell population can be indicative of HSC primitive progenitor characteristics or commitment to the monocytic lineage [28, 35].

Consequently, short-term (3- and 7-day) UCB-HSC cultures were established to study the effect of BM-MSCs on secreted or exogenously added cytokines over HSC proliferation, myeloid or lymphoid primitive-associated progenitor

phenotypes and clonogenic capacity. Our results showed that in the absence of exogenously added cytokines, BM-MSC induce slight CD34+ proliferation, promoting primitive progenitor maintenance with higher multi-lineage potential and increased CD34 and c-kit expression.

## **MATERIALS AND METHODS**

### **Mesenchymal stem cell isolation from bone marrow**

BM aspirates were collected in a sterile tube containing ethylene diaminetetraacetic acid anticoagulant (EDTA) from the iliac crest of healthy donors aged 19-65 after they had signed an informed consent form and approval had been given by the ethical committee of Hospital Universitario San Ignacio. Mononuclear cells were isolated by Ficoll density gradient centrifugation (Histopaque d = 1.077 g/cm<sup>3</sup>, Sigma-Aldrich, USA) and cells were plated at a density of 10<sup>6</sup> cells/cm<sup>2</sup> in IMDM Glutamax-I (GIBCO, Invitrogen) supplemented with 1% sodium pyruvate (GIBCO, Invitrogen), 1% MEM non-essential amino acid solution 100X (Sigma-Aldrich, USA) and 10% fetal calf serum (FCS). After obtaining 90% cell confluence, BM-MSC were detached by treatment with 0.25% Trypsin and 1 mM EDTA (GIBCO, Invitrogen) and replated at about 1.5 x 10<sup>5</sup> cells/ml for all experiments. MSC were used for the various experiments in passages 3-5.

### **Mesenchymal stem cell immunophenotyping**

After the 3<sup>rd</sup> passage, adherent cells were trypsinized and labeled with combinations of monoclonal antibodies (CD105/CD73/CD45/CD34; CD44/CD90/CD45/CD13; and HLA-I/HLA-DR/CD45/CD34) in four color stainings per group using fluorescein isothiocyanate, phycoerythrinperidinin chlorophyll protein, phycoerythrinperidinin Cy5.5 and allophycocyanin. Data were acquired using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). CellQUEST PRO software, FlowJo and Paint-a-Gate (Becton Dickinson Biosciences) were used for data analysis.

### **Mesenchymal stem cell *in vitro* differentiation**

After the 3<sup>rd</sup> passage, the osteogenic, adipogenic and chondrogenic differentiation capacities were qualitatively determined using specific staining and optical microscopy examination. MSC were replated at 2x10<sup>4</sup> cells/1.5 cm<sup>2</sup> and cultured to reach 90% confluence in IMDM with culture medium to induce osteogenic [36, 37], adipogenic [36, 38] and chondrogenic differentiation [36, 39].

### **CD34+ cell isolation from human umbilical cord blood**

UCB samples from normal full-term deliveries were collected after obtaining informed consent in accordance with the guidelines approved by the ethics committees of the Hospital Materno-Infantil and Hospital Universitario San Ignacio. Mononuclear cells were isolated by Ficoll density gradient centrifugation (Histopaque d = 1.077g/cm<sup>3</sup>, Sigma-Aldrich, USA) and CD34+

cells were enriched using a previously described magnetic-activated cell-sorting (MACS) CD34 isolation kit (MiltenyiBiotec, Auburn, CA, USA) [40]. Cell purity was evaluated by flow cytometry using allophycocyanin anti-CD34 (Clone 8G12, BD Biosciences). The sample purity was above 87%. Cell viability was determined using trypan blue dye. The results were greater than 95% in all trials.

#### **CD34+ cell divisions by carboxyfluorescein diacetate succinimidyl ester labeling**

After isolation, UCB-CD34+ cells were labeled for 7 min at room temperature and 3 min at 37°C with 5  $\mu$ M carboxyfluorescein diacetate succinimidyl ester (CFSE; CellTrace CFSE Cell Proliferation Kit, Invitrogen) in 0.1% BSA-supplemented PBS. CFSE-labeled cells were suspended in RPMI (Invitrogen Corporation, Carlsbad, CA, USA) with 10% FCS for 5 min in ice and washed three times with PBS for 7 min at 400 x g and 20°C. CFSE labeling was confirmed using fluorescence microscopy. A fraction of the CD34 cells were FCS deprived for 48 h (synchronized cells) to establish non-proliferating cells (the highest CFSE mean fluorescence intensity).

#### **Culturing umbilical cord blood-CD34+CFSE+ cells with bone marrow-mesenchymal stem cells and/or cytokines**

Third passage confluent and characterized BM-MSCs were used as the UCB-CD34+ feeder layer. The CD34+CFSE+ cells were cultured under three experimental conditions.

- i)  $10^5$  CD34+CFSE+ cells were plated in direct contact with confluent BM-MSCs feeder cells in 24-well plates in standard medium (RPMI with vitamins) in the presence of 50 ng/ml stem cell factor (SCF), FMS-like tyrosine kinase 3 ligand (Flt-3L) and thrombopoietin (TPO). These were the HMC culture conditions.
- ii)  $10^5$  CD34+CFSE+ cells were plated in direct contact with confluent BM-MSCs feeder cells in 24-well plates in standard medium (RPMI with vitamins) without cytokines. These were the HM culture conditions.
- iii)  $10^5$  CD34+CFSE+ cells were plated in the presence of SCF, Flt-3L and TPO without BM-MSCs. These were the HC culture conditions. Cultures were maintained in a humidified atmosphere at 37°C for 3 and 7 days.

#### **Umbilical cord blood-CD34+ immunophenotype evaluation after culture**

All the cells were harvested by trypsin treatment after 3 and 7 days of co-culture, washed in PBS and stained with monoclonal antibodies in the four color stainings. The antigens were detected by flow cytometry according to the availability of fluorochromes (CD34/c-kit/CD33 or CD38/HLA-DR and CD34/cyCD22/cyCD3/HLA-DR). Reliable discrimination between BM-MSCs and UCB-CD34+ cells was possible using their different forward scatter and side scatter signals and the high fluorescence of the CFSE-labeled hematopoietic cells (detected in FL1). Dead cells were excluded during acquisition (gate: intermediate forward scatter and low side scatter) and result analysis (FlowJo and

Paint-A-Gate software to exclude the remaining dead cells; Supplementary Fig. 1 at <http://dx.doi.org/10.2478/s11658-012-0036-1>).

### **Umbilical cord blood-CD34<sup>+</sup> cell clonogenic capacity**

After co-culture for 3 and 7 days, the UCB-CD34<sup>+</sup> cells were separated by negative selection using an antibody against BM-MSC (CD105 Microbeads, 120-000-316 MACS, MiltenyiBiotec). Cultivated cells ( $0.5-1 \times 10^3/1.1$  ml) were then cultured for 15 days in HSC-CFU complete with EPO medium (130-091-278, MiltenyiBiotec). Hematopoietic colonies were quantified with inverted microscopy and morphologically characterized with cytospin and Wright's staining, using the following colony criteria ([www.stemcell.com](http://www.stemcell.com)):

- i) Colony-forming unit–granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM) – granulocytes, erythrocytes and monocytes having at least 500 cells;
- ii) Burst-forming unit erythroids (BFU-E) and colony-forming unit–erythroids (CFU-E) – erythroblasts respectively having at least 200 and 10-200 cells;
- iii) Colony-forming unit–granulocyte macrophage (CFU-GM) – granulocytes and monocytes having at least 40 cells.

In some experiments, UCB-CD34<sup>+</sup> cells were cultured with BM-MSC supernatants obtained from fresh cultures (fresh supernatant, FS) or from supernatants frozen for 3 days at  $-70^{\circ}\text{C}$  (frozen supernatant, FRS). After 3 days, UCB-CD34 cells were cultured in HSC-CFU complete with EPO medium and colonies were counted as described previously.

### **Statistical analysis**

The data were analyzed with Friedman's test or Kruskal Wallis method using SPSS v16.0 and GraphPad Prism 5.0 for graphics. Results were considered statistically significant when  $p < 0.05$ .

## **RESULTS**

### **BM-MSC phenotype and multi-lineage differentiation**

Following the recommendations of the International Society of Cellular Therapy, BM-MSC were morphologically (Fig. 1A-B) and immunophenotypically (Fig. 2) characterized. BM-MSC were negative for CD34 and CD45 and positive for CD105, CD90, CD73, CD13 and CD44, as previously described by other authors [41, 42]. Supplementary Table 1 at <http://dx.doi.org/10.2478/s11658-012-0036-1> shows the percentage of positive cells for MSC antigens. Differentiation potential was evidenced in osteogenic, adipogenic and chondrogenic media (Fig. 1C-E), confirming MSC identity. All of the experiments were reproduced with samples from different patients to avoid variations between samples.

### **UCB-CD34<sup>+</sup> cell proliferation in BM-MSC co-cultures**

The BM repopulating efficiency of UCB-CD34<sup>+</sup> cells in a transplant setting depends in part on the number of transplanted cells [1]. Short-term UCB-CD34<sup>+</sup> co-cultures were performed under different conditions with or without BM-MSC

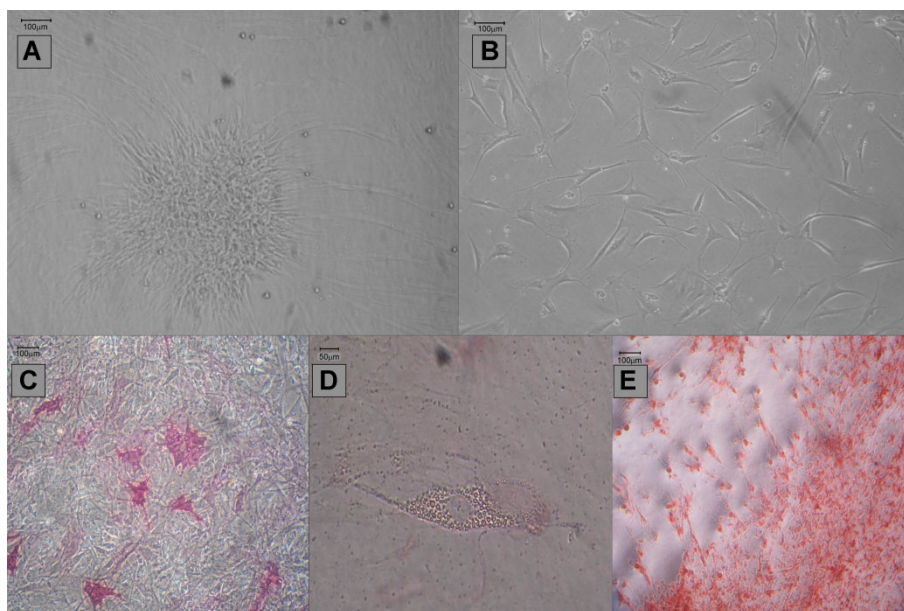


Fig. 1. Determining BM-MSc morphology and differentiation potential. A – BM-MSc showed a colony with fibroblast-like morphology after 15 days' culture (Olympus CK2, magnification x10. Scale bar: 100  $\mu$ m). B – BM-MSc showed a fibroblast-like morphology after 15 days' culture (Olympus CK2, magnification 5x. Scale bar: 100  $\mu$ m). C – BM-MSc showed osteogenic differentiation with increased ALP expression (alkaline phosphatase stain, Olympus CK2, magnification x10. Scale bar: 100  $\mu$ m). D – Adipogenic differentiation showed a lipid-rich vacuole (Oil Red O stain, Olympus CK2, magnification x20. Scale bar: 50  $\mu$ m). E – For chondrocyte differentiation, sulphated proteoglycan accumulations and extensive matrix were evident (Safranin O stain, Olympus Ck2, magnification x5. Scale bar: 100  $\mu$ m). Photographs were taken with a Sony Cybershot DSC-W7 digital camera and colors corrected after acquisition with Adobe Photoshop and BioImagenXD software.

or cytokines (HMC, HC and HM conditions) to explore UCB-CD34<sup>+</sup> proliferation and cytokine contribution to growth (see the Materials and Methods section). After 3 and 7 days, UCB-CD34<sup>+</sup> cells cultured in the presence of recombinant cytokines (SCF, Flt-3L, TPO) with or without BM-MSc (HMC and HC conditions) significantly increased the total UCB-CD34<sup>+</sup> numbers and the percentage of cells with several cell divisions (Fig. 3B, C, F and G) compared to arrested cells (Fig. 3A) or HM setting (Fig. 3D-E). The percentage of cells with only one cell division (P1) was higher under HM conditions than under HMC or HC conditions in the two periods studied. After 3 days under HM conditions, there were 95% cells in P1 compared to 70% and 60% for HMC and HC, respectively ( $p < 0.05$ ; Fig. 3H). A similar situation was observed after 7 days, with CD34<sup>+</sup> cells undergoing 3 to 4 cell cycles under HMC and HC conditions, while most cells (about 85%) under HM conditions were still in the first round of cell division. BM-MSc do not induce changes in the CD34<sup>+</sup> cell proliferation (Fig. 3D, E) and the addition of exogenous cytokines is crucial to increase cell numbers.

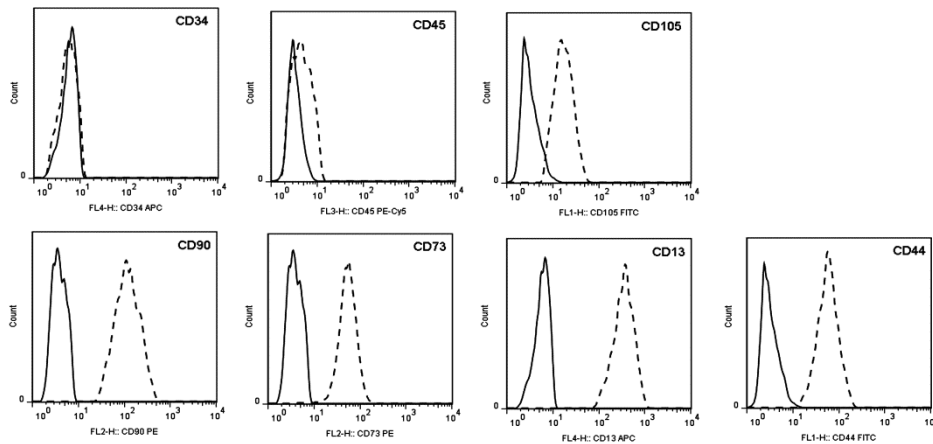


Fig. 2. BM-MSc immunophenotype characterization. These histograms represent antigen expression as signaled (FlowJo software). The figure shows data from one representative experiment (dashed line: antigen expression; solid line: auto-fluorescence control).

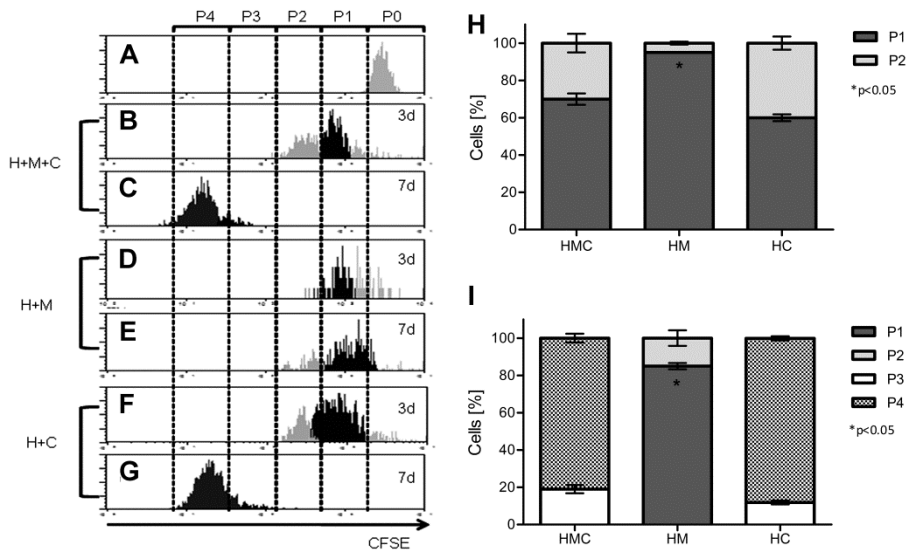


Fig. 3. UCB-CD34<sup>+</sup> cell proliferation in the presence or absence of cytokines or BM-MSc. CD34<sup>+</sup>CFSE<sup>+</sup> cell proliferation was evaluated after 3 and 7 days under three different experimental conditions. A – CD34<sup>+</sup>CFSE<sup>+</sup> cells were synchronized by serum starvation as a control. B,C – HMC conditions: hematopoietic and mesenchymal cells and cytokines. D,E – HM conditions: hematopoietic cells and mesenchymal cells only. F,G – HC conditions: hematopoietic cells and cytokines. H – The percentage of cells with one (P1) or two (P2) cell divisions after 3 days is indicated ( $p < 0.05$ ). I – The percentage of cells with more ( $P_n$ ) cell divisions after 7 days is indicated ( $p < 0.05$ ).



### Extended (7-day) BM-MSC and UCB-CD34+ co-culturing maintained primitive progenitor percentage

Although the number of CD34+ cells from UCB is critical when considering clinical applications, maintaining an undifferentiated phenotype is also of great relevance. Several studies have shown that cell proliferation is closely related to the state of cell differentiation [43-45]. UCB-CD34+ cells were plated under each culture condition and the expression of the antigens CD34, c-kit, CD33, CD38, cyCD22, cyCD3 and HLA-DR was analyzed to assess their differentiation state. After 3 days, no significant differences ( $p > 0.05$ ) in the percentages of CD34+CD38-, CD34+CD38+ or CD34+CD38+c-kit+ cells were observed under the different conditions. After 7 days, the HM conditions had a higher percentage of CD34+CD38- cells (6.5% vs. around 1% in the other two conditions,  $p < 0.05$ ) and CD34+CD38+c-kit+ cells (92.4% vs. 47% in HMC and 23% in HC,  $p < 0.05$ ; Fig. 4). This increase was mainly due to a reduction in a much more differentiated population expressing the myeloid marker CD33 (CD34+CD38+CD33+). Although the CD34+CD38- subpopulation was found within c-kit+ cells (Fig. 5D), it is unclear whether the CD34+CD38-c-kit+ subpopulation has the same functional capabilities as the “classic” primitive CD34+CD38- phenotype.

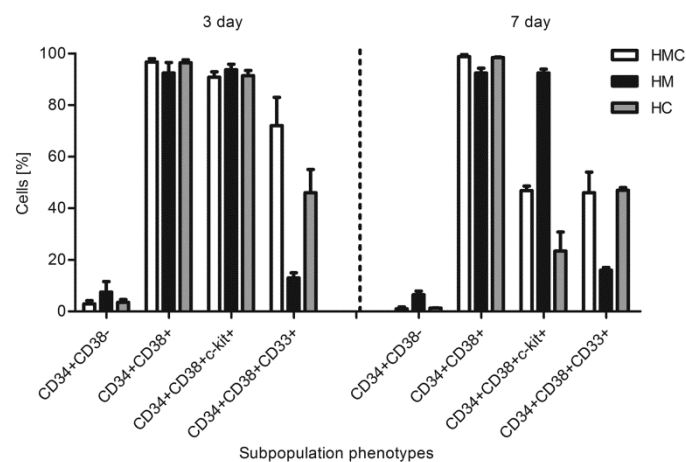


Fig. 4. UCB-CD34+ subpopulations obtained after 3 and 7 days' culture. After 3 days, no significant differences were found between the percentage of CD34+CD38-, CD34+CD38+ and CD34+CD38+ c-kit+ cells ( $p > 0.05$ ). After 7 days, a higher percentage of primitive progenitors (CD34+CD38+ c-kit+) was obtained under the HM conditions ( $p < 0.05$ ). The CD34+CD38+CD33+ population was higher under the culture conditions with cytokines (HMC and HC). The figure shows the mean values for the percentage of cells and the standard deviation.

Interestingly, the CD34 and c-kit mean fluorescence intensity (MFI) was different under the three different conditions. After 3 days, the CD34+ MFI was lower ( $p < 0.05$ ) under the HM conditions than under the conditions with cytokines (HMC and HC; Supplementary Table 2). After 7 days, the situation

reversed, with the HM conditions having the highest CD34+ MFI. A very similar situation was found with the c-kit MFI, with cells expressing a higher c-kit level under HM conditions after 7 days ( $p < 0.05$ ; Fig. 6, Supplementary Table 2). No changes were detected in the HLA-DR antigen, and lymphoid antigens were not detected in the co-culture systems used here after 3 or 7 days (data not shown).

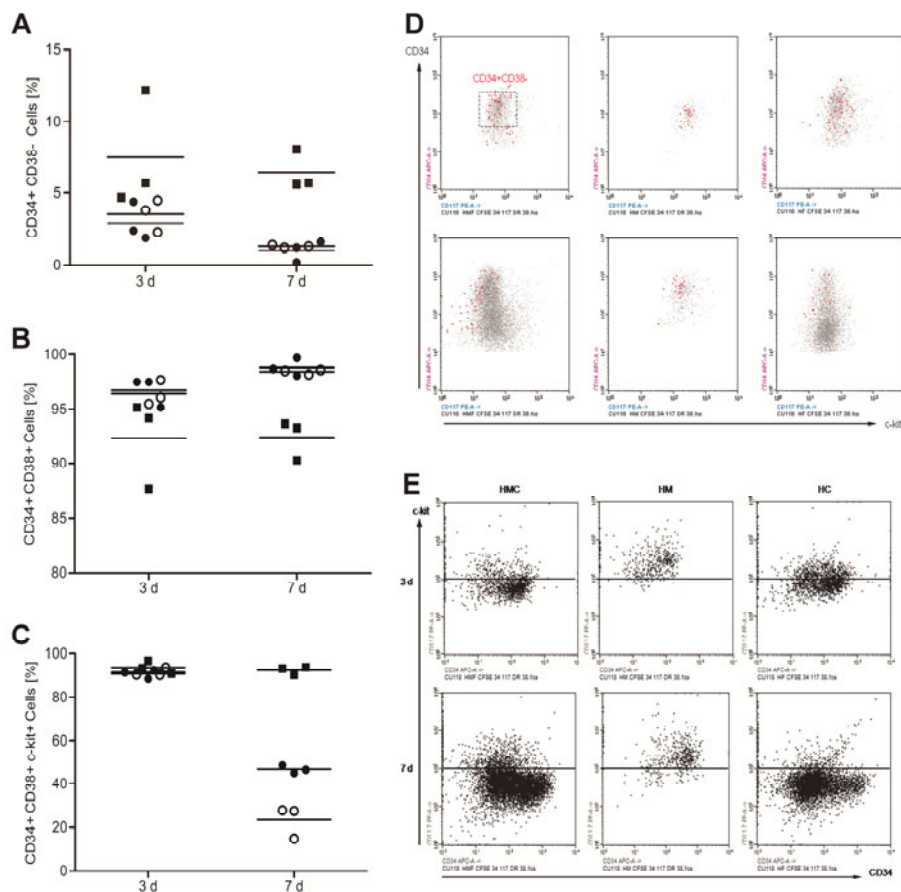


Fig. 5. Percentage of primitive progenitors under the three different conditions. A – Under the HM (■) conditions, there was a higher percentage of CD34+CD38- cells after 7 days compared to the results under the HMC (●) and HC (○) conditions. All of these experiments were done in triplicate. By contrast, (B) the same condition (HM) yielded the lowest percentage of committed (CD34+CD38+) cells. C – Under the HM conditions, 91.6% of CD34+ cells co-expressed c-kit and CD38 after 7 days. D – Red dot plots show CD34+CD38- that co-expressed c-kit under culture conditions. E – After 7 days, the HM conditions maintained the highest percentage of cells co-expressing CD34 and c-kit. An arbitrary division was performed on  $10^2$  units of the dot plots to clarify differences in antigen expression.

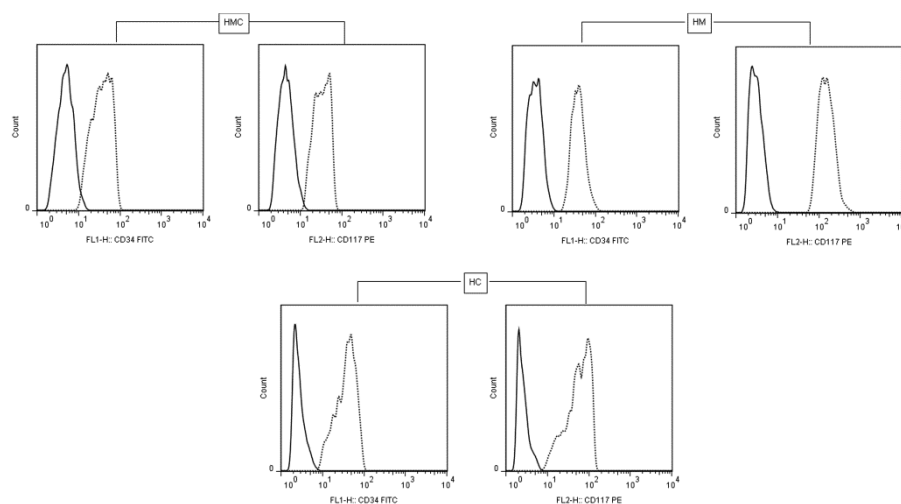


Fig. 6. Mean fluorescence intensity (MFI) histograms for CD34 and c-kit antigens after 7 days. The MFI for CD34 and c-kit (CD117) antigens was significantly higher after 7 days under the HM conditions ( $p < 0.05$ ) than under the HMC or HC conditions. The figure shows data from one representative experiment (dotted line: antigen expression; solid line: auto-fluorescence control).

Without exogenous cytokines, BM-MSc influences HSC primitive phenotype maintenance, as may be observed after 3 days of co-culture (increase in CD34+CD38- and a decrease in CD34+CD38+CD33+). After 7 days, additional differences in the subpopulations were observed, with an important increase in the CD34+CD38-c-kit+ and CD34+CD38+c-kit+ populations. Under these conditions, the CD34 and c-kit MFI are higher (Fig. 6). In terms of HSC (CD34+) primitive phenotype (CD38-, CD38+c-kit+ or CD33-) maintenance, it is clear that BM-MSc exerts a supportive effect that is recognizable after 7 days of co-culture.

### **BM-MSc without exogenous cytokines increased UCB-CD34+ clonogenic capacity**

The differentiation potential of UCB-CD34+ cells into hematopoietic lineages was also evaluated. It was found that UCB-CD34+ cells isolated from the HM conditions showed a higher absolute count and percentage of CFU-GEMM than the other culture conditions. After 3 days, the HM conditions yielded 18 colonies of CFU-GEMM per  $10^3$  CD34+ cells (60%) compared to 5 colonies (14%) and 7 colonies (20%) under HMC and HC conditions, respectively (Fig. 7A, B). Likewise, after 7 days under the HM conditions, an increased number of CFU-GEMM colonies was observed compared to the situation with the other two conditions (15 vs. 1 and 2 per  $10^3$  CD34+ cells; Fig. 7C, D). Cytospins were performed on all of the colonies and it was found that CFU-GEMM colonies were indeed enriched in cells having immature morphology with basophilic and agranular cytoplasm and the presence of a low condensed nucleus having 2 or 3 nucleoli (Fig. 8A, B), whereas the more committed colonies consisted of cells

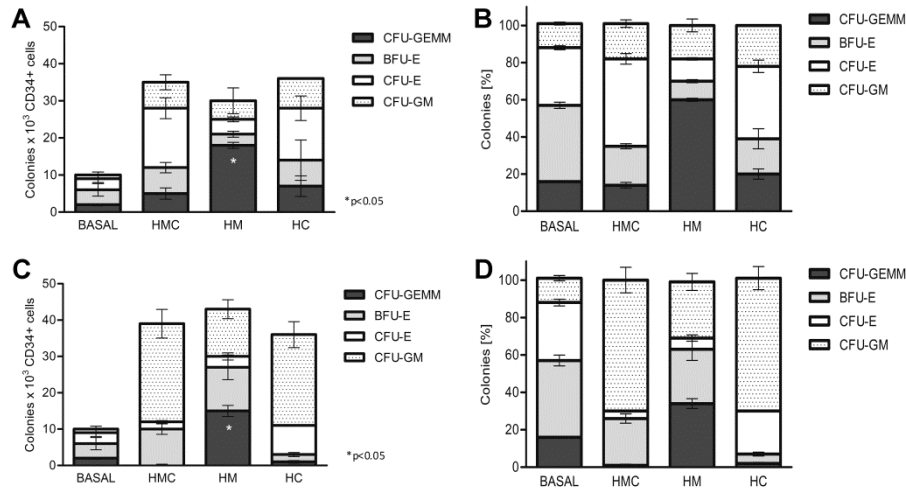


Fig. 7. UCB-HSC clonogenic capacity after co-culture with BM-MSC. A, B – An increase in the absolute count (colonies per 10<sup>3</sup> CD34<sup>+</sup> cells) and percentage of primitive progenitor colonies (CFU-GEMM) was detected under the HM conditions after 3 days compared to the basal or HMC and HC conditions ( $p < 0.05$ ). C, D – The absolute count and percentage of CFU-GEMM remained higher under the HM conditions after 7 days ( $p < 0.05$ ). The basal condition corresponds to freshly isolated CD34<sup>+</sup> cells. The figure shows the mean values for the absolute count and percentage of colonies  $\pm$  standard deviation.

with the expected morphology. Erythroid progenitor colonies (BFU-E, CFU-E) had cells with an acidophilic cytoplasm and nucleus with condensed chromatin (Fig. 8C-F). Granulocytic colonies (CFU-G) had abundant granules in their cytoplasm and monocytic colonies (CFU-M) had a semi-lobed nucleus with basophilic cytoplasm (Fig. 8G-I). Again, BM-MSC (HM conditions) are able to induce not only higher percentages of CFU-GEMM but also a higher number of colonies compared to other culture conditions.

UCB-CD34<sup>+</sup> clonogenic assays were performed after BM-MSC co-culture or after adding fresh (FS) or frozen (FRS) BM-MSC culture supernatants. It was revealed that cells in direct contact (DC) under the HM conditions had a higher absolute count (18 colonies per 10<sup>3</sup> CD34<sup>+</sup> cells, 60%) of CFU-GEMM colonies compared to hematopoietic cells in culture with supernatants (FS or FRS;  $p < 0.05$ ; Fig. 9A). However, it should be noted that 10 colonies (28%) of CFU-GEMM were observed when UCB-CD34<sup>+</sup> were cultured with FS, suggesting that BM-MSC-secreted cytokines may also promote colony growth (Fig. 9A, B). No CFU-GEMM colonies were observed when determining UCB-CD34<sup>+</sup> clonogenic capacity after culturing with FRS; only colonies with committed lineages were detected (Fig. 9A-E). These results demonstrate that cell contact in short-term culture UCB-CD34<sup>+</sup> with BM-MSC can play an important role in the maintenance of primitive hematopoietic populations retaining their clonogenic ability.

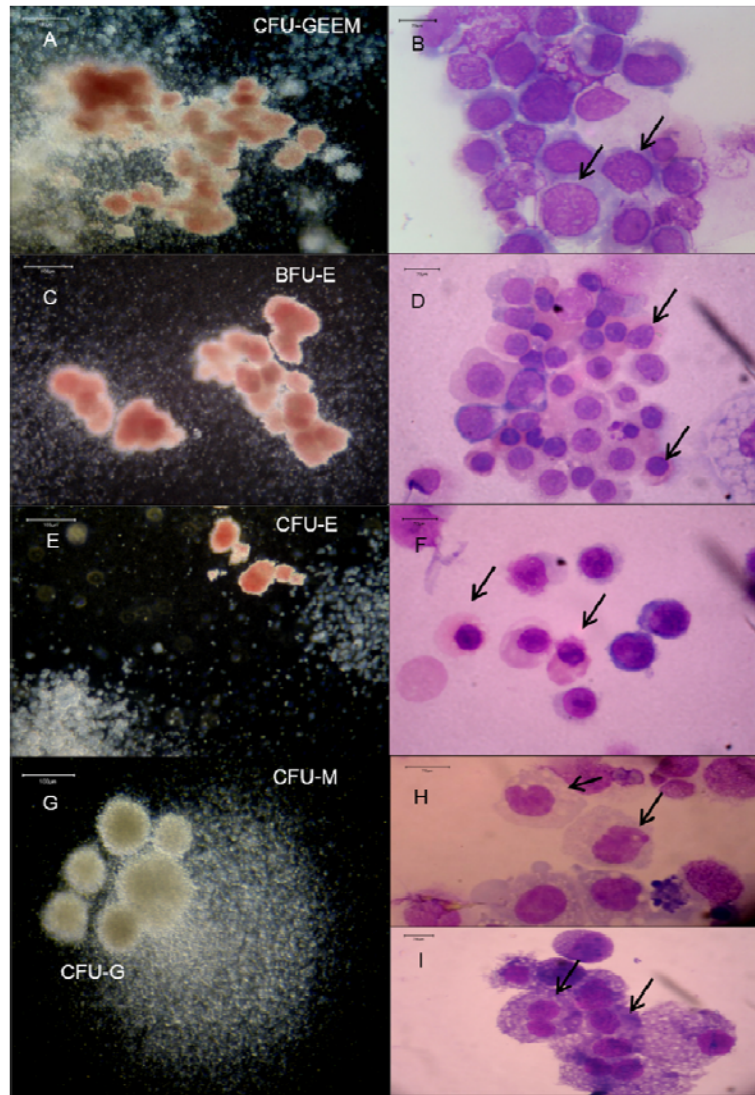


Fig. 8. Morphological evaluation of the colonies. Colonies were processed by cytopsin. A, B – CFU-GEEM showed blasts with open chromatin, some nucleoli and agranular cytoplasm (Hematoxylin-eosin stain, Olympus CH30, magnification x100). C-F – The correlation between the other colonies and their morphology: for example, erythroid colonies showed orthochromic and polychromatic normoblasts (Hematoxylin-eosin stain, Olympus CH30, magnification x100). G, H – CFU-M show pro-monocytes and mature monocytes and G-I – CFU-G show cells with granular cytoplasm (Hematoxylin-eosin stain, Olympus CH30, magnification x100). Scale bar A, C, E, G: 100  $\mu$ m. Scale bar B, D, F, H, I: 70  $\mu$ m.

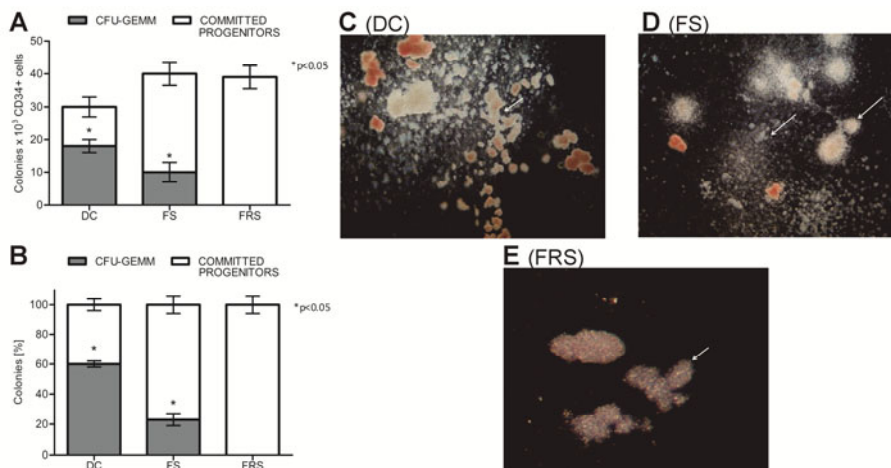


Fig. 9. The effect of MSC-HSC contact on UCB-HSC clonogenic capacity. A, B – A higher absolute count and percentage of primitive progenitor colonies (CFU-GEMM) was found in direct contact (DC) compared to conditions with fresh (FS) or frozen (FRS) BM-MSC supernatant. Nevertheless, the latter conditions yielded no primitive progenitor colonies. C-E – Photographs of the colonies obtained under each of the culture conditions with BM-MSC supernatant.

## DISCUSSION

Different strategies have been designed for UCB-HSC *ex vivo* expansion. Several studies have shown that some cytokines can increase CD34<sup>+</sup> cell number several-fold [46-52]. Clinical trials using pre-expanded UCB-CD34<sup>+</sup> cells have shown that this is a safe and efficient procedure and that it does not increase the percentage of patients who develop graft-versus-host disease (GVHD) [53]. However, this procedure is expensive and the most commonly used cytokine concentrations (in the ng/ml range) do not correspond to the concentrations detected *in vivo* in the BM microenvironment (in the pg/ml range), which could alter the “quality” of the primitive progenitors responsible for long-term BM reconstitution [54]. The use of mesenchymal stem cells has also led to optimizing UCB-CD34<sup>+</sup> cell expansion [54]. More recently, it has been shown that co-culturing UCB-HSC and mesenchymal cells (in the absence of cytokines) has stimulated the expansion of more primitive hematopoietic progenitors (CD34<sup>+</sup>CD38<sup>-</sup> or CD133<sup>+</sup>CD38<sup>-</sup>) [18].

This study aimed to evaluate the effect of BM-MSC on some biological characteristics of UCB-HSC, in the absence of high levels (ng/ml) of exogenous added cytokines, in a model much closer (only concerning *in situ*-produced cytokines) to what might be occurring *in vivo*. This is in agreement with several cytokine-free UCB-CD34<sup>+</sup> expansion protocols that have been shown to be efficient [55, 56]. BM-MSC-secreted cytokines should have had synergistic effects with the added cytokines. Nevertheless, this study has shown that UCB-

CD34<sup>+</sup> cells expanded with exogenous cytokines in the presence (HMC) or absence (HC) of BM-MSCs proliferate to the same extent (Fig. 3). This was an unexpected result, since it had previously been shown that BM-MSCs express SCF, TPO, Flt-3L, leukemia inhibition factor (LIF), IL-11 and IL-6 at the mRNA level [21]. Additionally, it is important to note that the paracrine effect of MSC could be dependent on the donor [57] and the culture conditions (e.g., type of culture medium) [58].

An extra supply of growth factors (SCF, Flt-3L and TPO) is definitely required for UCB-CD34<sup>+</sup> expansion. Andrade *et al.* showed that UCB-CD34<sup>+</sup> cells and BM-MSCs co-cultured using the same cytokines tested here (SCF, Flt-3L and TPO) resulted in a 17-fold CD34<sup>+</sup> cell expansion [46], thereby partly agreeing with our results. Nevertheless in this study, cell differentiation was not as deeply evaluated as in other studies where the *in vitro* increase in UCB-HSC number was not accompanied by an evaluation of commitment status using additional antigens other than CD34 and CD38 [46].

HSC primitive phenotype characterization in humans is essentially based on CD34 (presence) and CD38 (absence) marker assessment, but many cells subsist within the CD38<sup>+</sup> population with repopulating capability [59-61]. This shows that there are probably many more CD34<sup>+</sup> cell subpopulations that have not been completely characterized to date, suggesting the need for other markers when evaluating primitive or progenitor status. Appealingly, different markers are used in BM clinical evaluation to define differentiation status. Recent results have shown a significant association between HSC c-kit expression and perivascular niches. Butler *et al.* showed that in cytokine-free co-cultures, endothelial cells stimulate the expansion of the CD34<sup>-</sup>, Flt3<sup>-</sup>, c-kit<sup>+</sup>, Lin<sup>-</sup> and Sca-1<sup>+</sup> populations, retaining a higher repopulation capacity of BM in *in vivo* assays [62]. Other authors have shown that SCF deletion (c-kit ligand) in endothelial and perivascular stem cells or leptin receptor (+) cells dramatically affect the number of HSC [63]. Also, the c-kit<sup>+</sup> population may further regulate its microenvironment by inducing osteoblast formation from MSC [64]. The latter may suggest a reciprocal positive effect of c-kit-bearing cells on niche maintenance and vice versa.

We took advantage of this concept to consider the c-kit antigen as an alternative marker for *in vitro* primitive progenitor cell evaluation. Our results showed that the HM conditions promoted the maintenance of the CD34<sup>+</sup>c-kit<sup>+</sup> population (92.4%) with a reduction in the more differentiated CD34<sup>+</sup>CD33<sup>+</sup> (16%) population (Fig. 4.). We also showed higher CD34 and c-kit MFI under HM conditions after 7 days' co-culture when compared with both cytokine conditions (HMC and HC; Fig. 6, Supplementary Table 2). This finding is very interesting since this population (CD34<sup>+</sup>c-kit<sup>+</sup>) has an important role in normal [65] and pathological hematopoiesis [66], acute myocardial infarction [67] and vascular remodeling [68, 69].

We have detected an intermediate HLA-DR expression without significant changes between the cell populations and culture conditions analyzed (data not

shown). Lymphoid antigens were not detected in the three systems assayed here, probably because of the culture period assayed. In fact, Gardner *et al.* have shown that lymphoid progenitor generation from CD34<sup>+</sup> cells may require longer cultivation periods [70] and other studies have shown that the number of lymphoid progenitors in UCB is very low (0.02%) [71, 72].

Several reports have shown that BM-MSC increase CD34<sup>+</sup> cell proliferation and short-term engraftment in recipient NOD/SCID mice [73] but the influence of BM-MSC on UCB-CD34<sup>+</sup> multi-lineage capacity has not been shown by clonogenic assays. In particular, clonogenic assays are widely used as a test for assessing HSC *in vitro* differentiation capacity. Here we have observed (Fig. 7 and Fig. 8) that UCB-CD34<sup>+</sup>c-kit<sup>+</sup> developed a greater number of CFU-GEMM under the HM conditions compared to the two culture conditions with cytokines. Interestingly, the absolute CFU-GEMM count increased in the HM condition by more than 3 times compared to the basal count. It is likely that contact with BM-MSC could regulate HSC self-renewal. This might have indicated that the CD34<sup>+</sup>c-kit<sup>+</sup> phenotype corresponded to primitive progenitors and that they were regulated, at least partly, by direct contact with BM-MSC, as shown in our assays with BM-MSC supernatants (Fig. 9).

Taking all these results into consideration, one could argue that increased UCB-HSC proliferation may be more dependent on paracrine factors (cytokines), while increased CD34<sup>+</sup>c-kit<sup>+</sup> cells and CFU-GEMM may be promoted by direct contact with BM-MSC. Once physical association between MSC and HSC has occurred, sufficient time must elapse for the various signals [74, 75] to be able to modulate HSC cell fate, retaining their original characteristics but allowing some proliferation to increase cell number.

A co-culture system that increases the CD34<sup>+</sup>c-kit<sup>+</sup> cell subpopulation, improving UCB-HSC “quality”, would be also very useful for HSC stored for long periods on CB banks. These findings may optimize the use of CD34<sup>+</sup>c-kit<sup>+</sup> cell subpopulation for patients with congenital or acquired aplasias, leukemia patients undergoing myeloablative therapy or patients with diseases not associated with the hematopoietic system, such as myocardial infarction or vascular disease.

**Authorship and disclosures.** Viviana M. Rodriguez-Pardo and Jean Paul Vernot designed the study. Viviana M. Rodriguez-Pardo performed the experiments. Viviana M. Rodriguez-Pardo and Jean Paul Vernot analyzed the data and wrote the paper. The authors reported no potential conflicts of interest.

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