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RESEARCH ARTICLE

Dynamic cell–cell interactions between cord blood haematopoietic progenitors and the cellular niche are essential for the expansion of $CD34^+$, $CD34^+CD38^-$ and early lymphoid $CD7^+$ cells

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Abstract

Most clinical applications of haematopoietic stem/progenitor cells (HSCs) would benefit from their *ex vivo* expansion to obtain a therapeutically significant amount of cells from the available donor samples. We studied the impact of cellular interactions between umbilical cord blood (UCB) haematopoietic cells and bone marrow (BM)-derived mesenchymal stem cells (MSCs) on the *ex vivo* expansion and differentiative potential of UCB $CD34^+$-enriched cells. UCB cells were cultured: (a) directly in contact with BM MSC-derived stromal layers (contact); (b) separated by a microporous membrane (non-contact); or (c) without stroma (no stroma). Highly dynamic culture events occurred in HSC-MSC co-cultures, involving cell–cell interactions, which preceded HSC expansion. Throughout the time in culture [18 days], total cell expansion was significantly higher in contact (fold increase of 280 ± 37 at day 18) compared to non-contact (85 ± 25). No significant cell expansion was observed in stroma-free cultures. $CD34^+$ cell expansion was also clearly favoured by direct contact with BM MSCs (35 ± 5- and 7 ± 3-fold increases at day 18 for contact and non-contact, respectively). Moreover, a higher percentage of $CD34^+CD38^-$ cells was consistently maintained during the time in culture under contact (8.1 ± 1.9% at day 18) compared to non-contact (5.7 ± 1.6%). Importantly, direct cell interaction with BM MSCs significantly enhanced the expansion of early lymphoid $CD7^+$ cells, yielding considerably higher (×3–10) progenitor numbers compared to non-contact conditions. These results highlight the importance of dynamic cell–cell interactions between UCB HSCs and BM MSCs, towards the maximization of HSC expansion *ex vivo* to obtain clinically relevant cell numbers for multiple settings, such as BM transplantation or somatic cell gene therapy. **Copyright © 2009 John Wiley & Sons, Ltd.**

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Keywords haematopoietic stem/progenitor cells; bone marrow microenvironment; mesenchymal stem cells; *ex vivo* expansion

1. Introduction

The major obstacle to the widespread use of umbilical cord blood (UCB) in haematopoietic stem cell therapy is the low cell dose available for transplantation (de Lima and Shpall, 2006). The *ex vivo* expansion of UCB-derived haematopoietic stem/progenitor cells (HSCs)

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prior to transplantation could potentially ameliorate inadequate haematopoietic recovery by generating a higher number of cells with haematopoietic reconstitution potential (Shpall *et al.*, 2002). In addition, successful expansion of HSC will broaden the field of application of these cells to include somatic gene therapy, and to the generation of specific mature cell types as adjuncts to bone marrow (BM) transplantation (de Lima and Shpall, 2006).

The major focus in HSC expansion is still the definition of optimal culture conditions concerning haematopoietic growth factor combinations, co-cultures with feeder cells, enrichment procedures, initial cell concentrations used, among other variables, to provide the maximum amplification of the primitive HSC pool (Hofmeister *et al.*, 2007).

In vivo, HSC fate is controlled by the surrounding BM microenvironment or haematopoietic niche, which is characterized by a specific local geometry and cellular arrangement comprising stromal cells, the extracellular matrix (ECM) components and soluble and membrane-bound growth factors/cytokines (Moore and Lemischka, 2006). Contributing to the stromal component of the BM microenvironment are different populations of cells, some, such as macrophages, that are of haematopoietic origin and are derived from HSC, and others that are derived from the so-called mesenchymal stem cells (MSC), such as fibroblasts, smooth muscle cells, adipocytes and osteogenic precursor cells (Yin and Li, 2006). The haematopoietic niche is thought to provide the complex molecular signals determining stem cell self-renewal or differentiation and maturation of functional progeny (Moore and Lemischka, 2006). Therefore, an *ex vivo* model able to mimic the *in vivo* haematopoietic niche requires a complex set-up system that should be able to support stem, progenitor, precursor and mature cells as well as stromal cell elements (da Silva *et al.*, 2005; Goncalves *et al.*, 2006; Zhang *et al.*, 2006).

The interactions of stromal cells with haematopoietic cells are known to be of great importance for the maintenance of the multipotential characteristics of HSCs, although it is not totally yet defined how intrinsic and extrinsic factors regulate stem cell self-renewal, proliferation and differentiation (Bilko *et al.*, 2005; Koller *et al.*, 1999). Indeed, the role of stromal/accessory cells in promoting *ex vivo* expansion/maintenance of human HSCs while preserving the ability of the manipulated cells to engraft in an *in vivo* model has been reported (Ando *et al.*, 2000; Lewis *et al.*, 2001; Shih *et al.*, 1999). Nevertheless, it is still not clear whether interactions between haematopoietic and feeder cells are mediated by constitutively produced soluble factors (Oostendorp *et al.*, 2005) or whether they require direct cell–cell contact (Cheng *et al.*, 2007; Freund *et al.*, 2006; Oritani *et al.*, 1996).

The direct contact of haematopoietic cells with stromal cells has been associated with an improvement in stem/progenitor cell expansion (Cheng *et al.*, 2007) and preservation of stem cell quality during *ex vivo* culture (Breems *et al.*, 1998), as well as an increase in the frequency of gene transfer into primitive cells when using viral supernatants (Nolta *et al.*, 1995). On the other hand, other groups reported that the enhancement effect of stromal cells is due to the soluble factors produced by stroma and that direct cell–cell contact between haematopoietic and stromal cells is not necessary to enhance conservation, proliferation and maturation of primitive progenitors *in vitro*, although it is essential for the regulated production of mature blood cells (Lewis *et al.*, 2001; Verfaillie, 1992). Furthermore, other studies showed that non-contact cultures enhanced *ex vivo* expansion results for UCB (Kawada *et al.*, 1999) and BM HSCs (Oostendorp *et al.*, 2005). However, since distinct co-culture systems have been tested for the expansion of HSC, using different feeder layers with different cytokine combinations, divergent results have been reported and thus the role of stroma in haematopoietic cell co-cultures remains a very controversial issue.

Previous results from our group demonstrated that a serum-free culture system using human BM MSC-derived feeder layers, supplemented with exogenous cytokines, allowed an efficient expansion/maintenance of HSC from BM and UCB (da Silva *et al.*, 2005; Goncalves *et al.*, 2006). In particular, the expansion results for UCB cells were strictly dependent on the presence of the stromal layer (da Silva *et al.*, 2005). Although some previous studies addressed the importance of direct contact between HSC and stromal components (Alakel *et al.*, 2009; Cheng *et al.*, 2007; Freund *et al.*, 2006; Li *et al.*, 2007; Wagner *et al.*, 2007; Zhang *et al.*, 2006), to our best knowledge there are no studies focusing on the dynamics underlying these haematopoietic co-cultures, throughout time in culture, affecting the *ex vivo* expansion and differentiative potential of UCB HSCs. Moreover, few studies perform a direct side-by-side comparison between contact, non-contact and liquid cultures (no stroma) (Cheng *et al.*, 2007; Freund *et al.*, 2006; Li *et al.*, 2007), especially considering the use of a cocktail of cytokines anticipated to exert its effect through stromal and/or accessory cells, as previously reported by our group (da Silva *et al.*, 2005).

Here we studied the effects of direct cellular interactions between UCB cells and MSC-derived feeder layers on the *ex vivo* expansion and differentiative potential of UCB HSCs. We showed that cell–cell interactions with BM MSCs underlie co-culture dynamics, with a significant fraction of progenitors located within the feeder layer early in culture, enhancing the expansion of $CD34^+$ and $CD34^+CD38^-$ cells, as well as early lymphoid $CD7^+$ progenitor cells (Frias *et al.*, 2008) from the UCB. These results are believed to have an important impact in UCB HSC expansion, namely in the design of an efficient clinical-scale expansion system using BM MSC-derived feeder layers.

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2. Materials and methods

2.1. Human donor cell preparation

The umbilical cord blood (UCB) samples were kindly provided by Crioestaminal-Saúde e Tecnologia, S.A. and were collected after maternal donor consent. Low-density UCB mononuclear cells (MNCs) were separated by a Ficoll density gradient (1.077 g/ml; Sigma) and then washed twice in Iscove's modified Dulbecco's medium (IMDM; Gibco). UCB MNCs were kept cryopreserved in liquid nitrogen until further use.

2.2. Human bone marrow mesenchymal stem cell cultures

Heparinized human bone marrow (BM) was obtained from healthy donors after informed consent, according to guidelines from the Office of Human Research Protection at the University of Nevada, Reno, NV, USA. BM MNCs were isolated as previously described for UCB samples. Isolated BM MNCs were magnetically sorted based on Stro-1 positivity, as previously described (Goncalves *et al*., 2006). The MSCs obtained (Stro-1^+, Gly-A^-, $CD45^-$) were then cultured in gelatin-coated (Sigma) T-25 flasks (5 ml) with Mesenchymal Stem Cell Growth Medium (MSCGM; Poietics™, Lonza). After two or three passages, BM MSCs were collected by trypsinization (Trypsin–EDTA, Gibco), frozen and stored in liquid nitrogen until further use. For the establishment of BM MSC-derived feeder layers to support haematopoietic cell expansion, MSCs were thawed at 37 °C in IMDM containing 20% v/v fetal bovine serum (FBS; Sigma). Cell viability was checked using Trypan blue stain 0.4% solution (Gibco) and was always >95%. Feeder layers were obtained after 10 days of culture with MSCGM in gelatin-coated T-25 flasks. At confluence, MSCs were treated with Mitomycin C (Sigma) by removing the culture medium and replacing it with IMDM-FBS (10% v/v) containing 0.5 μg/ml mitomycin C (Loeuillet *et al*., 2001). The cells were incubated at 37 °C under 5% CO_2 humidified air for 2–2.5 h, then washed twice with phosphate-buffered saline (PBS; Gibco) and finally kept in fresh prewarmed MSCGM medium for 24–48 h before establishing co-cultures.

2.3. *Ex vivo* expansion of human $CD34^+$-enriched cells

UCB MNCs were thawed as previously described for BM MSCs. UCB MNCs were then enriched for the $CD34^+$ phenotype, using magnetic cell sorting (MACS; Miltenyi Biotec). The percentage of $CD34^+$ cells upon enrichment was $73 \pm 5\%$ ($n = 14$).

$CD34^+$-enriched cells were cultured (1×10^4 cells/ml) in six-well plates in either the presence or absence of a human BM MSC-derived feeder layer, using Quality Biological serum-free medium (QBSF-60; Quality Biological, Inc.), supplemented with cytokines, at 37 °C and 5% CO_2 humidified air. The cytokine combination used to supplement the culture medium included stem cell factor (SCF; 100 ng/ml), fms-related tyrosine kinase 3 ligand (Flt-3 L; 100 ng/ml), basic fibroblast growth factor (bFGF; 5 ng/ml) (all from Peprotech) and leukaemia inhibitory factor (LIF, 10 U/ml; Chemicon) (da Silva *et al*., 2005). The effect of the presence of MSCs on UCB cells was evaluated in direct contact cultures between the two types of cells (contact) and compared with the sole effects of the soluble released factors by MSCs (non-contact). In this case, both types of cells were separated by a microporous membrane of 0.4 μm pore diameter (BD Falcon™).

Periodically, cultures were half-fed, with half of the cultures being harvested and used for analysis and the same volume being replaced with fresh medium. Co-culture periods were limited to <3 weeks in order to prevent stromal cell detachment.

2.4. Proliferative and phenotypic analysis

During the time in culture, for each different culture condition at each time point x, the number of viable cells was calculated using the Trypan blue exclusion method; the number of cells obtained was then multiplied by a 2^n factor, n being the number of medium renewals performed, in order to account for the half-feeding procedure before day x, as previously described (Goncalves *et al*., 2006). Fold increase in total cell number was calculated by dividing the number of cells at each day by the number of cells at day 0. The cells were also analysed by flow cytometry (FACScalibur, Becton Dickinson), using a panel of monoclonal antibodies (FITC-, or PE-conjugated) against: CD34 and CD38 for stem/progenitor cells; CD14, CD15 and CD33 (myeloid lineage); and CD7 (marker for early lymphopoiesis) (Becton Dickinson). Cultured cells were incubated with these monoclonal antibodies for 15 min in the dark at room temperature. Then the cells were washed in PBS and fixed with 1% paraformaldehyde (Sigma). Appropriate isotype controls were also prepared for every experiment to exclude the possibility of non-specific binding of antibodies to Fc receptors. A minimum of 10 000 events was collected for each sample.

2.5. Analysis of haematopoietic cell distribution

Concerning haematopoietic cellular distribution, every 2 days the suspension and adherent fractions of one well (of a six-well plate) were harvested and analysed independently for cell proliferation and phenotype. The fraction of cells in suspension was obtained by harvesting the culture supernatant after flushing, and this was combined with the cells released after rinsing the adherent layer with PBS. Adherent cells were obtained by treatment with trypsin–EDTA after washing with PBS.

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Haematopoietic cellular distribution between suspension and the adherent fraction was determined simply by dividing the number of suspension/adherent cells by the number of total cells (suspension + adherent). The number of cells (#) expressing each phenotype in suspension or adherent fractions was calculated by multiplying the percentages determined by flow cytometry by the total number of cells in suspension or adherent cell fraction (equation 1):

$$\#_{CDX^+CDY^+_{suspension}} = \frac{\%CDX^+CDY^+}{100} \times \#_{suspension\ cells} \quad (1)$$

$$\#_{CDX^+CDY^+_{adherent}} = \frac{\%CDX^+CDY^+}{100} \times \#_{adherent\ cells}$$

The distribution or frequency of each cell type between suspension and the adherent fraction represents the ratio of the number of cells in each fraction and the number of total cells in both fractions (equation 2):

$$\%CDX^+CDY^+{}_{suspension} = \frac{\#_{CDX^+CDY^+_{suspension}}}{\#_{CDX^+CDY^+_{suspension}} + \#_{CDX^+CDY^+_{adherent}}}$$

$$\%CDX^+CDY^+{}_{adherent} = \frac{\#_{CDX^+CDY^+_{adherent}}}{\#_{CDX^+CDY^+_{suspension}} + \#_{CDX^+CDY^+_{adherent}}} \quad (2)$$

2.6. Scanning electron microscopy analysis

Co-cultures of haematopoietic cells in direct contact with the BM MSCs were fixed with 2.5% glutaraldehyde (Sigma), dehydrated with ethanol (Merck) (70%, 90% and absolute), washed with hexamethyldisilazane (Sigma) for 15 min and gold-coated prior to observation. A scanning electron microscope (SEM) Hitachi S-2400, with an accelerating voltage set to 15 kV, was used for visualization.

2.7. Clonogenic assays

Both fresh and expanded UCB CD34$^+$-enriched cells were characterized in terms of clonogenic potential. The clonogenic assays were performed in triplicate by plating 1000 (day 0) or 10 000 cells (days 10 and 18) in MethoCult GF H4434 (Stem Cell Technologies). The cultures were maintained at 37 °C and 5% CO_2 humidified air. After 14 days, the colonies were counted and categorized according to standard criteria (Almeida-Porada *et al*., 2000). Colony-forming unit–granulocyte macrophage (CFU–GM), colony-forming unit–granulocyte, erythroid, macrophage, megakaryocyte (CFU-Mix) and burst-forming unit–erythroid (BFU–E) progenitors were identified. CFU numbers were calculated by dividing the number of colonies present at days 10 and 18 by the number of cells plated, and this value was then multiplied by the total number of cells in culture for the day of harvest.

2.8. Statistical analysis

Results are presented as mean ± standard error of mean (SEM). Comparisons between experimental results were determined by Mann–Whitney U-test for two independent samples, when appropriate. $p < 0.05$ was considered statistically significant.

3. Results

UCB CD34$^+$-enriched cells were cultured directly over a confluent BM MSC feeder layer (contact) that allowed the establishment of direct cell–cell interactions between the two types of cells. Indeed, scanning electron micrographs of co-cultures (at day 6) demonstrate the occurrence of physical interactions between the two types of cells (Figure 1).

The effects of the soluble factors released by the MSC feeder layer on haematopoietic cells were evaluated by culturing CD34$^+$-enriched cells on top of a microporous membrane (non-contact). This membrane was placed 1 mm above the feeder layer, which prevented any type of physical interaction, not permitting direct cell–cell communication between UCB HSCs and BM MSCs, only allowing the trafficking of soluble factors. Importantly, the presence of the membrane, by itself, did not display any cytotoxic effects on the UCB HSCs (data not shown). The proof of concept of the importance of the presence of feeder cells in this culture system was demonstrated by including a stroma-free experimental condition (no stroma).

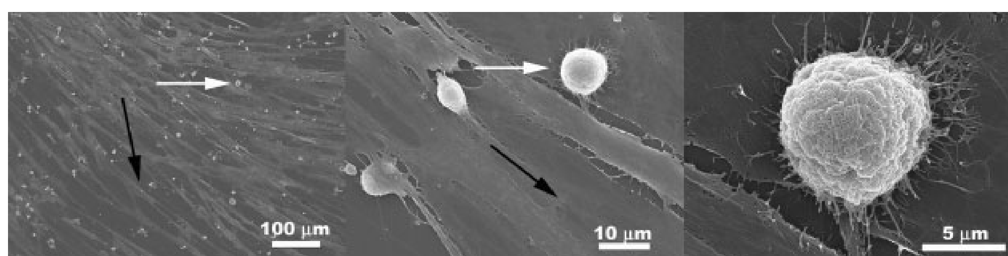

Figure 1. Scanning electron micrographs of UCB CD34$^+$-enriched cells (white arrows) cultured directly over a BM MSC feeder layer (black arrows). Day 6 co-cultures are presented using different amplifications, showing well-established cell–cell contact interactions between haematopoietic cells and MSCs

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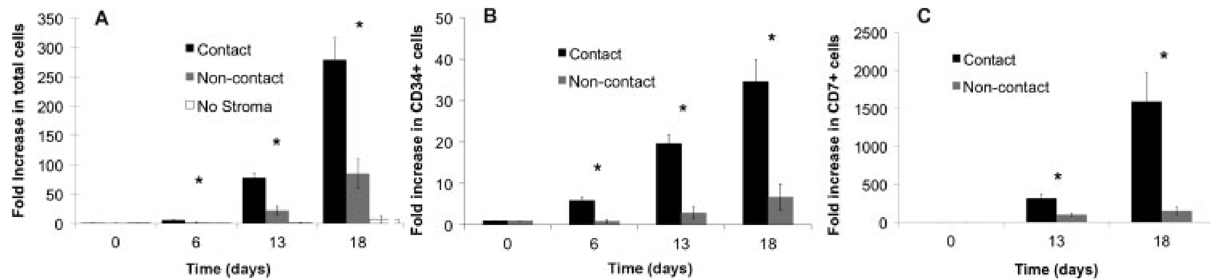

Figure 2. ***Ex vivo*** **culture of UCB CD34^{+}-enriched cells under different culture conditions. UCB cells were cultured either directly over BM MSC-derived feeder layers (contact; black bars), separated from each other by a 0.4 μm microporous membrane (non-contact; grey bars) or in the absence of stroma (no stroma; white bars) for 18 days. During the time in culture, total haematopoietic cell numbers were determined and the fold increase in total cells (A) ($n = 6$) was calculated for the three conditions tested; immunophenotypic analyses for CD34 expression were performed and fold increase values in CD34^{+} cells ($n = 4$) were determined (B); CD7^{+} expression was determined by flow cytometry and fold increase in CD7^{+} cells calculated ($n \geq 2$) (C). No immunophenotypic analyses were performed for the no stroma condition, due to the limited cell numbers obtained, throughout the time in culture (*$p < 0.05$)**

3.1. Cell–cell interactions with a BM MSC feeder layer enhanced the *ex vivo* expansion of UCB stem/progenitor cells

UCB CD34^{+}-enriched cells were cultured for 18 days in a cytokine-supplemented serum-free medium system, using the different configurations previously described. Total haematopoietic cell expansion (starting population 1×10^4 cells/ml) was assessed at days 6, 13 and 18, for contact, non-contact and no stroma conditions. The levels of expansion in total cell numbers were consistently higher when haematopoietic cells were cultured directly over the feeder layer compared to the other conditions (non-contact and no stroma), reaching a fold increase of 280 ± 37 after 18 days in culture (Figure 2A) ($n \geq 6$), corresponding to an average of 2.8×10^6 cells/ml. In non-contact cultures, total haematopoietic cell expansion was significantly lower ($p < 0.05$), reaching a 85 ± 25-fold increase at day 18, while no significant cell expansion was observed in stroma-free cultures (no stroma) throughout the time in culture (Figure 2A). Cell viability was >90% for contact and non-contact configurations during culture, while consistently lower for the no stroma condition, reaching values <60% on day 13 (data not shown).

In order to assess the more primitive stem/progenitor haematopoietic cell content during time in culture, immunophenotypic analyses were performed using anti-CD34 and anti-CD38 antibodies. The expansion of CD34^{+} cells (average initial content 73 ± 5%, corresponding to 7.3×10^3 cells/ml) was also significantly improved by direct interactions with BM MSCs when compared to the non-contact condition ($n \geq 4$, $p < 0.05$; Figure 2B). The maximal expansion values were attained after 18 days for both conditions, when fold increase values of 35 ± 5 and 7 ± 3 were obtained for the contact and non-contact configurations, respectively. No immunophenotypic analyses were performed for the no stroma condition due to the limited cell numbers obtained for all time points.

As commonly observed for the UCB CD34^{+}-enriched cells (Mayani and Lansdorp, 1998), the initial content of CD34^{+}CD38^{-} was considerably low (3.0 ± 1.2%), although upon culture initiation these values tended to increase, especially within the first 2 weeks, in the presence of BM MSCs (Figure 3). For instance, at day 6 the percentage of CD34^{+}CD38^{-} cells increased to 15.8 ± 2.3% and 8.6 ± 3.4% for the contact and non-contact configurations, respectively (Table 1). Overall, the maintenance of these primitive cells in our culture system was favoured under contact conditions, with a consistently higher percentage of CD34^{+}CD38^{-} cells obtained when the cells were cultivated in direct contact with BM MSCs, at different time points. More importantly, the cell expansion obtained when direct cell interactions occurred between the UCB and the feeder cells yielded considerably higher primitive progenitor cell numbers after 18 days (2.3×10^5 and 4.9×10^4 cells/ml, for the contact and non-contact conditions, respectively) ($p < 0.05$).

It is notable that when UCB CD34^{+}-enriched cells were cultivated in MSC-conditioned medium, on top of the ECM remaining upon harvest of the confluent MSC feeder layer, the levels of expansion of both total and CD34^{+} cells obtained were in between the results for the non-contact and no stroma conditions (data not shown).

The clonogenic potential of the expanded cells was also evaluated to assess the impact of the cellular interactions with BM MSCs on the maintenance/expansion of haematopoietic progenitor cells (Figure 4). On day 0, before starting the *ex vivo* culture, the clonogenic potential of the enriched population as CFU-GM, BFU-E and CFU-Mix colonies was $(3.6 \pm 1.0) \times 10^3$, $(1.0 \pm 0.5) \times 10^3$ and $(3.0 \pm 1.0) \times 10^3$ per 1000 cells, respectively.

In terms of CFU-GM progenitors, by day 10, $(2.4 \pm 1.3) \times 10^4$ were obtained for the contact configuration, whereas $(5.2 \pm 1.2) \times 10^3$ were achieved for the non-contact condition (Figure 4A). Additionally, CFU-Mix numbers increased after the first week of culture, reaching values of $(2.2 \pm 1.2) \times 10^4$ and $(4.2 \pm 0.2) \times 10^3$ by day 10, for the contact and non-contact, respectively (Figure 4B). As expected, the clonogenic potential for BFU-E progenitors was lost upon culture initiation for both culture conditions (da Silva *et al*., 2005). Due to the reduced cell numbers obtained

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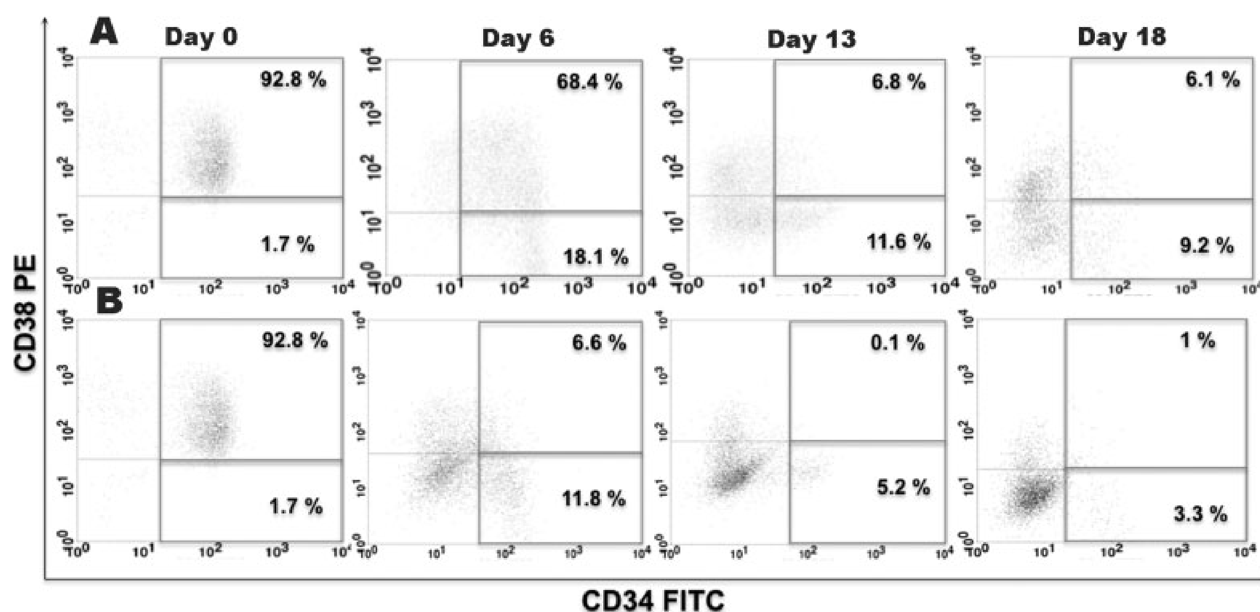

Figure 3. Flow cytometry analyses of UCB CD34$^+$-enriched cells expanded in the presence of a BM MSC-derived feeder layer. Dot-plots display a representative experiment of UCB cells highlighting the expression of CD34$^+$CD38$^-$ cells at days 0, 6, 13 and 18 for the contact (A) and non-contact (B) configurations

Table 1. Phenotypic analyses of the expanded UCB cells in terms of progenitor cell content and differentiative potential

Time (days)	%CD34$^+$ CD38$^-$		%CD7$^+$		%CD14$^+$		%CD15$^+$		%CD33$^+$	
	Contact	*Non-contact*	*Contact*	*Non-contact*	*Contact*	*Non-contact*	*Contact*	*Non-contact*	*Contact*	*Non-contact*
0	3.0 ± 1.2		10.3 ± 3.3		18.2 ± 5.1		30.0 ± 5.1		73.1 ± 5.5	
6	15.8 ± 2.3	8.6 ± 3.4	n.d.		n.d.		n.d.		n.d.	
13	10.2 ± 2.4	6.6 ± 2.2	37.7 ± 6.7*	6.2 ± 0.3*	28.9 ± 3.8	25.8 ± 4.7	49.1 ± 5.3	56.9 ± 8.7	89.7 ± 2.6	83.0 ± 7.8
18	8.1 ± 1.9	5.7 ± 1.6	40.0 ± 7.9	22.3 ± 4.8	29.0 ± 2.0	35.5 ± 6.6	44.6 ± 3.7	48.1 ± 1.7	75.0 ± 7.9	64.1 ± 12.2

UCB CD34$^+$-enriched cells were cultivated either directly over a BM MSC-derived feeder layer (contact) or separated using a microporous membrane (non-contact) for 18 days ($n = 4$). Non-adherent cells were harvested periodically and analysed by flow cytometry (*$p < 0.05$).

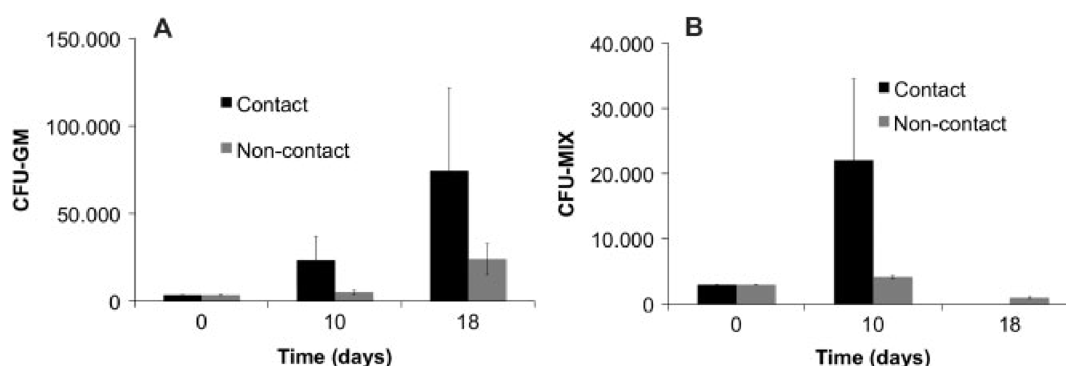

Figure 4. Clonogenic potential of UCB CD34$^+$ enriched cells cultivated in contact and non-contact configurations. The number of colony-forming units (CFU) for UCB cells cultured under direct contact with BM MSC-derived feeder layer (black bars) or separated from the feeder layer by a microporous membrane (non-contact; grey bars) are shown. CFU-granulocyte macrophage (CFU-GM) (A) and CFU-MIX (B) were determined throughout the time in culture, using standard criteria ($n = 2$)

in no stroma cultures, no clonogenic assays were performed for this experimental condition, although data reported by our group demonstrated that, in the absence of stroma, the maintenance of the clonogenic potential of haematopoietic progenitors is dramatically impaired (da Silva *et al*., 2005). At the end of the culture, on day 18, the number of CFU-GM was $(7.4 \pm 4.2) \times 10^4$ and $(2.4 \pm 0.8) \times 10^4$ for contact and non-contact, respectively, corresponding to a fold increase in clonogenic potential of 21 for contact and 6.8 for non-contact (Figure 4B). Overall, the clonogenic potential of the cells expanded in the contact condition with BM MSCs was consistently higher compared to the non-contact condition; nonetheless, after 18 days, no CFU-Mix

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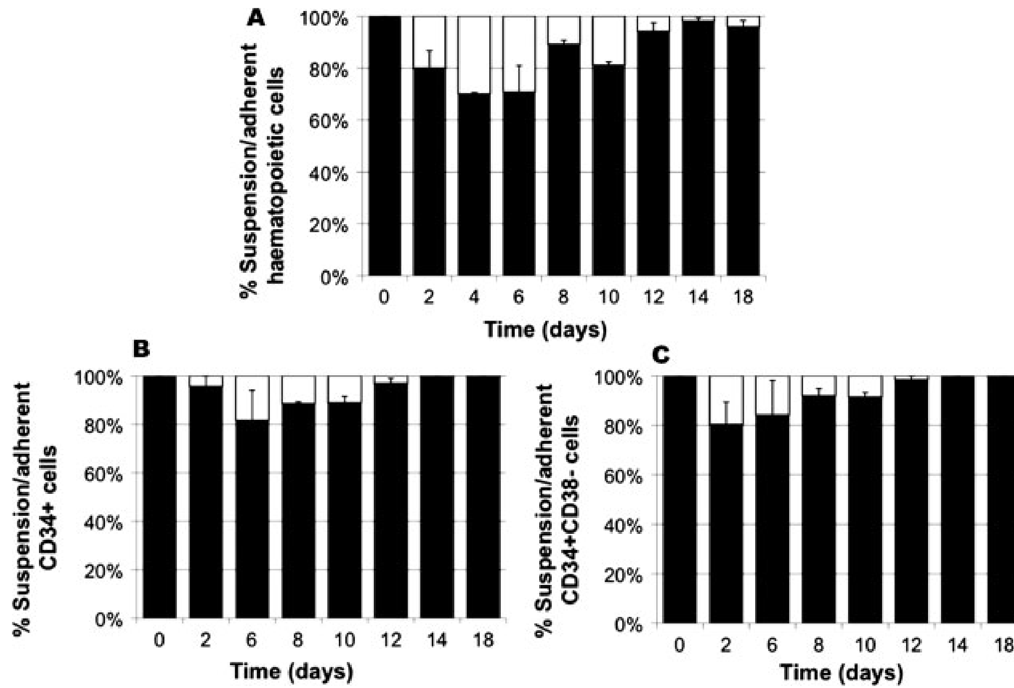

Figure 5. Distribution of haematopoietic cells between suspension and adherent cell fractions in UCB HSC-BM MSC co-cultures. Percentages of total (A), CD34$^+$ (B) and CD34$^+$CD38$^-$ (C) cells in suspension (black bars) and adherent to the feeder layer (white bars) ($n = 3$) are presented

colonies were detected for the contact condition, while non-contact cells originated $(1.0 \pm 0.1) \times 10^3$ colonies (Figure 4A).

3.2. Dynamic cellular distribution within suspension/adherent fractions occurs in UCB HSC–BM MSC co-cultures

We also investigated the location of haematopoietic cells, either in suspension or within the adherent cell fraction during time in culture. The fraction of haematopoietic cells adherent to the feeder layer reached its maximum value at days 4–6 (30%) (Figure 5A). In what concerns the more primitive cells, the maximal ratio of CD34$^+$ cells adherent to the stromal layer occurred by day 6 (19 ± 12% of total CD34$^+$ cells were adherent) (Figure 5B), which was maintained roughly constant until day 10; then most of the CD34$^+$ progenitors were almost exclusively found in the suspension fraction. A similar pattern was observed for the CD34$^+$CD38$^-$ phenotype, although the maximal proportion of these progenitors adherent to the feeder layer was found early in culture, at day 2 (20 ± 8%) (Figures 5C).

3.3. Cell–cell interactions with a BM MSC feeder layer influences the differentiative potential of UCB expanded cells

An *ex vivo* culture system for the expansion of haematopoietic cells should be able not only to support the proliferation of the more primitive stem/progenitor cells but also to generate more committed progenitor cells with short-term reconstitution ability, which can potentially accelerate haematopoietic recovery upon HSC transplantation (de Lima and Shpall, 2006; Shpall *et al*., 2002).

To evaluate the impact of the cellular interactions with BM MSC feeder cells on the myeloid differentiative potential of UCB-expanded cells, we analysed the expression of CD14, CD15 and CD33 antigens by flow cytometry throughout the culture for both the contact and non-contact configurations. As previously demonstrated, using our culture system, the differentiative potential of CD34$^+$-enriched cells is primarily shifted towards the myeloid lineage, as observed by the presence of CD14$^+$, CD15$^+$ and CD33$^+$ cell populations (Table 1) (da Silva *et al*., 2005; Goncalves *et al*., 2006). Overall, no significant differences were found between contact and non-contact cultures concerning the expression of these myeloid markers, suggesting that these are independent of direct cellular interactions with feeder cells.

Although no mature lymphoid cells (CD3$^+$ and CD19$^+$) were detected upon the first days of the culture, as previously reported (da Silva *et al*., 2005), we were able to maintain a CD7$^+$ population, with early lymphocytic potential (Frias *et al*., 2008). CD7$^+$ progenitor cell content increased upon culture initiation (expression on day 0, 10.3 ± 3.3%), reaching a significantly higher ($p < 0.05$) expression in contact cultures (37.7 ± 6.7%) compared to non-contact cultures (6.2 ± 0.3%) after 13 days (Table 1). Moreover, this CD7$^+$ cell population was much more efficiently expanded in contact cultures, reaching a fold increase of 315 ± 56 and 1588 ± 381 at days 13 and 18, respectively ($p < 0.05$ compared to

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non-contact) (Figure 2C). From these $CD7^+$ cells, nearly 5% were $CD7^+CD34^+$ at day 0 and this percentage was roughly maintained within the first week of culture under contact conditions, decreasing thereafter; concomitantly, there was an increase of the fraction of $CD7^+CD34^-$ cells and, after 2 weeks of culture, nearly all $CD7^+$ cells did not express CD34 (data not shown).

4. Discussion

We have previously reported that the expansion/maintenance of HSCs from both BM and UCB is highly favoured in the presence of a BM MSC-derived feeder layer of human origin in a cytokine-supplemented serum-free culture system (da Silva *et al.*, 2005; Goncalves *et al.*, 2006). Importantly, transplantation into pre-immune fetal sheep demonstrated that the expanded cells retained their engraftment capability (Goncalves *et al.*, 2006). Interestingly, UCB HSCs in particular could not be expanded in the absence of the feeder layer, using a cytokine cocktail which was anticipated to exert their effect through stromal or accessory cells (da Silva *et al.*, 2005).

Although the successful expansion of UCB HSCs in stroma-free culture systems (supplemented with cytokines and other stroma-derived molecules) has recently been described (Zhang *et al.*, 2008), an MSC-derived stroma layer has the ability to better replicate the haematopoietic niche microenvironment and, consequently, might not require high amounts of exogenously added cytokines. Indeed, human MSCs have been suggested and recognized as a suitable model for studying the interaction mechanisms of haematopoietic progenitors, recreating the supportive cellular milieu of the niche (de Lima and Shpall, 2006; Wagner *et al.*, 2007).

However, the haematopoietic supportive capacity of MSC-derived stroma remains not fully understood, particularly the role of direct cell–cell interactions with HSCs. In this work, we focused on interactions between haematopoietic and BM MSC feeder cells, concerning the existence of contact-dependent mechanisms involved in the expansion/maintenance of UCB HSCs, as well as affecting their differentiative potential throughout the time in culture. Upon 6 days of co-culture in the contact condition, cell–cell interactions between haematopoietic and feeder cells were clearly visible, as assessed by scanning electron microscopy.

Our results indicate that $CD34^+$ expansion is clearly favoured by direct cellular interactions with BM MSCs, rather than dependent on soluble factors only. No cell expansion was achieved in stroma-free conditions, in agreement with our previously published results (da Silva *et al.*, 2005). Similar results were obtained by other authors, reinforcing the importance of direct contact with stroma for the expansion of UCB haematopoietic stem/progenitor cells (Alakel *et al.*, 2009; Cheng *et al.*, 2007; Freund *et al.*, 2006; Li *et al.*, 2007; Wagner *et al.*, 2007; Zhang *et al.*, 2006). However, few studies have performed a direct side-by-side comparison between contact, non-contact and liquid (no stroma) cultures (Cheng *et al.*, 2007; Freund *et al.*, 2006; Li *et al.*, 2007), especially using UCB HSCs in co-culture with BM MSCs. In addition, no studies have been found focusing the time course of these haematopoietic co-cultures, while assessing both *ex vivo* expansion and differentiative potential of UCB HSCs. On the other hand, in none of these studies a purified Stro-1^+ BM MSC population was used as a feeder layer (Goncalves *et al.*, 2006), supplemented with a cocktail of cytokines known to exert its effect through stromal and/or accessory cell elements (da Silva *et al.*, 2005).

Regardless of the direct cellular interactions or non-contact with the feeder layer, our culture system was also able to support the expansion of the more primitive haematopoietic progenitors, the $CD34^+CD38^-$ cells. In the early stage of the culture, it was possible to observe a significant increase of $CD34^+CD38^-$ cell content, followed by a steady decrease thereafter. Accordingly, we recently demonstrated that UCB $CD34^+$-enriched cells cultivated on a BM MSC feeder layer downregulate CD38 expression early in culture, even in the absence of significant cell division (da Silva *et al.*, 2009). When combined with the levels of total cell expansion, the contact condition yielded a higher expansion in terms of $CD34^+CD38^-$ cells with reported repopulating activity (McKenzie *et al.*, 2007).

Moreover, the results obtained from distribution studies of haematopoietic cells revealed highly dynamic culture events occurring during co-culture of haematopoietic cells and feeder layers. The frequency of adherent haematopoietic cells reached a maximum of 30% at days 4–6, which corresponds to the starting time point of effective expansion of haematopoietic cells. This suggests that early contact events are required for subsequent expansion during the time in culture. Also for $CD34^+$ and $CD34^+CD38^-$ cells, the maximum frequency within the adherent layer was found at days 6 and 2, respectively, which decreased steadily until day 14. Concomitantly, a significant increase in $CD34^+$ and $CD34^+CD38^-$ cell numbers was observed in suspension throughout the time in culture. In fact, it was previously reported that CD34-expressing cells migrate rapidly toward stroma cells and that the multiplication of haematopoietic progenitors correlated with a reduced CD34-mediated cell adhesion (Gordon, 2000).

Our observations on the beneficial effects of cell–cell interactions with the BM niche on HSCs expansion might be associated with a particular signalling mechanism or combination of multiple intercellular mechanisms, already established or still unknown. Some cell signalling mechanisms involving Notch, Wnt, bone morphogenetic proteins, osteopontin and the homeobox gene *HoxB4* are already known to be involved in HSCs proliferation and maintenance (Haylock and Nilsson, 2006; Hofmeister *et al.*, 2007; Kim *et al.*, 2009; Sadlon *et al.*, 2004) and may explain our observations. For instance, Notch

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ligands present in the membrane surface of bone marrow stromal cells (Jones *et al.*, 1998) have been described as promoters of UCB HSC expansion (Suzuki *et al.*, 2006). In fact, Delta-1 is actually in phase I clinical trials for cord blood expansion (Bernstein *et al.*, 2008). Other possible interactions between integrin receptors present in haematopoietic cell membranes may be also involved, such as for $\alpha 4$ and $\alpha 5$ (Foguenne *et al.*, 2009). Nevertheless, preliminary results in our laboratory demonstrated that the sole ECM produced by MSCs, in the presence of conditioned medium, is not sufficient to support the efficient expansion/differentiation of HSCs. This clearly indicates that in our culture conditions the full haematopoietic supportive capacity of MSCs requires the occurrence of dynamic cell–cell interactions with the haematopoietic cells.

Concerning the clonogenic potential of the expanded cells, the numbers of CFU-GM progenitors in contact cultures were consistently higher compared to non-contact cells during the culture period. On the other hand, a higher number of primitive CFU-Mix progenitors was obtained for contact cells at day 10, compared to non-contact condition. After 18 days, no CFU-Mix colonies were found in the clonogenic assays for the contact condition, which can be a consequence of the commitment of these early progenitors with the myeloid lineage, giving rise to cells with CFU-GM potential.

In fact, as previously demonstrated by our group, the differentiative potential of $CD34^+$-enriched cells cultivated in this serum-free stromal-based culture system was primarily shifted towards the myeloid lineage (da Silva *et al.*, 2005; Goncalves *et al.*, 2006). Herein, no significant differences were observed in the content of $CD14^+$, $CD15^+$ and $CD33^+$ cells in contact and non-contact cultures, suggesting that soluble factors produced by the BM MSC feeder layer have a predominant role on the haematopoietic cell myeloid differentiation process. Nevertheless, the importance of direct contact with MSCs on the myeloid differentiation process should not be overlooked, since Li and co-workers described that MSCs inhibited $CD34^+$ cell commitment into myeloid dendritic cells via cell–cell contact mechanisms (Li *et al.*, 2008), and Hidalgo *et al.* (2002) demonstrated a complex role of CD44-mediated haematopoietic progenitor cell adhesion in myelopoieisis.

Although the differentiative potential of expanded cells was shifted to the myeloid lineage, with no mature lymphoid cells ($CD3^+$ and $CD19^+$) detected upon the first days of the culture, our culture system was also able to expand a population $CD7^+$ with early lymphocytic potential, as previously described by us (da Silva *et al.*, 2005), which promotes the maturation of the immune cellular component of the graft. In addition, also human adipose-derived stromal cells demonstrated to support the expansion of $CD7^+$ cells from UCB, although with limited capacity to expand $CD34^+$ cells (Kilroy *et al.*, 2007). This suggests that our BM MSC-derived feeder layer supports both the expansion and differentiation potential of HSCs, whereas adipose-derived MSCs support complete differentiation only (Corre *et al.*, 2004). Previous results from our laboratory demonstrated that the expanded UCB $CD7^+$ population can efficiently originate dendritic and natural killer cells, after *in vitro* stimulation (Frias *et al.*, 2008). Importantly, in the present studies we demonstrated that expansion of $CD7^+$ cells was strongly contact-dependent, reaching significantly higher levels of expansion ($p < 0.05$) compared to non-contact. Under contact conditions our culture system reproducibly drove the *ex vivo* expansion of UCB HSCs, generating, at earlier time points, a known lympho-progenitor population $CD7^+CD34^+$ (Hao *et al.*, 2001), while a significant number of more mature $CD7^+CD34^-$ cells arise later in culture, capable of differentiating into NK and dendritic cells *in vitro* (Frias *et al.*, 2008). Our results thus demonstrate that, contrarily to myeloid phenotypes, the expansion/maintenance of a $CD7^+$ population, with lymphocytic potential, depends on cell–cell interactions between haematopoietic cells and MSCs, rather than soluble factors only. Accordingly, the critical requirement of BM stromal cells for cell differentiation has been reported for the lymphoid lineage (Katayama and Frenette, 2003).

In conclusion, direct cell–cell interactions with BM MSC feeder layers are crucial for the efficient expansion of $CD34^+$, $CD34^+CD38^-$ and $CD7^+$ cells from the UCB, while maintaining their clonogenic ability and potential for the myeloid ($CD33^+$, $CD15^+$, $CD14^+$) haematopoietic lineage. These results are expected to have an important impact in the design of an efficient clinical-scale expansion system allowing the co-culture of UCB HSC with BM MSC-derived feeder layers.

The co-culture of UCB HSC with a BM MSC feeder layer of human origin, non-transformed, may better preserve and expand a component of the more primitive, long-term repopulating cells, while also producing increased numbers of more lineage-committed, shorter-term repopulating cells which could potentially ameliorate inadequate haematopoietic recovery upon HSC transplantation (da Silva *et al.*, 2005; de Lima and Shpall, 2006).

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