Characterization of the growth modulatory activities of osteoblast conditioned media on cord blood progenitor cells

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Abstract Engraftment outcomes are strongly correlated with the numbers of hematopoietic stem and progenitor cells (HSPC) infused. Expansion of umbilical cord blood (CB) HSPC has gained much interest lately since infusion of expanded HSPC can accelerate engraftment and improve clinical outcomes. Many novel protocols based on different expansion strategies of HSPC and their downstream derivatives are under development. Herein, we describe the production and properties of serum-free medium (SFM) conditioned with mesenchymal stromal cells derived-osteoblasts (OCM) for the expansion of umbilical CB cells and progenitors. After optimization of the conditioning length, we show that OCM increased the production of human CB total nucleated cells and CD34⁺ cells by 1.8-fold and 1.5-fold over standard SFM, respectively. Production of immature CD34⁺ subpopulations enriched in hematopoietic stem cells was also improved with a shorter conditioning period. Moreover, we show that the growth modulatory activities of OCM on progenitor expansion are

regulated by both soluble factors and non-soluble cellular elements. Finally, the growth and differentiation modulatory activities of OCM were fully retained after high dose-ionizing irradiation and highly stable when OCM is stored frozen. In summary, our results suggest that OCM efficiently mimics some of the natural regulatory activities of osteoblasts on HSPC and highlight the marked expansion potentials of SFM conditioned with osteoblasts.

Keywords Cord blood · Osteoblasts · Conditioned medium · Hematopoietic stem and progenitor cells · Ex vivo expansion

Abbreviations

BM	Bone marrow
CB	Umbilical cord blood
CM	Conditioned medium
ECM	Extracellular matrix
FL	FLT-3 ligand
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
MSC	Mesenchymal stromal cells
M-OST	MSC-derived osteoblasts
MCM	MSC conditioned media
OCM	Osteoblast conditioned medium
IL	Interleukin
Lin	Lineage
SCF	Stem cell factor
SFM	Serum free medium

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TPO Thrombopoietin

Introduction

The qualities and quantities of HSPC are key players in successful transplant for the treatment of hematological diseases. Compared to bone marrow (BM) and mobilized peripheral blood, umbilical CB has the highest counts of lineage (Lin) negative CD34⁺CD38[−] cells but is limited to a one time donation and lower absolute HSPC counts (Arber et al. 2011). Notably, higher cell dose in CB transplantation promote early engraftment and prevents graft failure.

Different strategies are considered to improve engraftment of CB HSPC to resolve the slow engraftment. One strategy has been focused on the ex vivo expansion of HSPC prior to transplantation which has now been tested in several clinical trials (Pineault and Abu-Khader 2015; Norkin et al. 2013; Delaney et al. 2005; Cairo et al. 2016; de Lima et al. 2012). Indeed, decreased graft failure and accelerated neutrophil recovery have been reported in patients undergoing double CB transplantation with an expanded and unmanipulated units, each unit providing short- and long-term engraftment, respectively (de Lima et al. 2012; Delaney et al. 2010; Horwitz et al. 2014).

The majority of HSPC protocols are liquid SFM-based suspension culture supplemented with high concentration of cytokines. Also gaining much interest is the use of co-culture with stromal components of the hematopoietic microenvironment such as MSC (reviewed in Pineault and Abu-Khader 2015).

Hematopoiesis is normally modulated by a combination of functionally divergent molecules and physiological regulators that include growth factors, extracellular matrix (ECM) proteins, MSC and osteoblasts (Taichman and Emerson 1998). Perivascular MSC, vascular endothelial cells and osteoblasts have all been linked to regulation of HSC and downstream progenitors (Schepers et al. 2015). For instance, the number of HSPC was shown to correlate with osteoblast numbers in some transgenic mice models (Calvi et al. 2003; Visnjic et al. 2004). Moreover, Chitteti et al. (2010) also showed that expanded murine Sca-1⁺Lin^{neg}c-Kit⁺ HSPC have better engraftment activity when the expansion occur in a primary murine osteoblasts coculture system. It was also reported that mature osteoblasts derived from an immortalized HM3-

B10 MSC line can induce better expansion of multipotent progenitors (MPP), as determined in a long-term culture initiating cell (LTC-IC) assay (Mishima et al. 2010). Both growth factor secretions and cell–cell contact have been reported to be needed for the growth-modulatory activates of MSC on HSPC (Flores-Guzman et al. 2013; Peled et al. 2004; Amsellem et al. 2003). Moreover, coculture of HSPC in MSC contact-based cocultures has been shown to improve human chimerism after transplantation (Ferreira et al. 2012; Goncalves et al. 2006; Walenda et al. 2011).

Our group has previously shown that ex vivo expansion of CB CD34⁺ cell can be promoted by coculture without contact with irradiated MSC or MSC-derived osteoblasts (M-OST) (Celebi et al. 2011). Just recently, we showed in the context of conditioned medium (CM) that M-OST had superior potentials in enhancing the growth and expansion of CB CD34⁺ cells than MSC. Culture of CB cells in M-OST CM (OCM) and to a lesser extent MSC CM also resulted in increased frequency of cells positive for the cell-surface expression of C–X–C chemokine receptor type 4 (CXCR4) implicated in the HSPC homing to the BM (Peled et al. 1999; Kollet et al. 2001; Peled et al. 2000). Importantly, OCM was shown to modulate the engraftment properties of expanded HSPC. Indeed, human platelet levels were significantly greater in mice transplanted with HSPC expanded in OCM compared to control SFM or MSC CM (Dumont et al. 2014).

The OCM HSPC expansion protocol is based on the differentiation of MSC into immature M-OST after a 6 day osteogenic differentiation period induced in standard osteogenic medium (Birmingham et al. 2012). Non-filtered centrifuged supernatant is then frozen without cryoprotectant until use (Dumont et al. 2014). Herein, we sought to better understand and characterise the growth promoting activity of OCM on CB CD34⁺ cells which remains poorly understood at this time. First, we optimized the length of condition for the production of OCM. Then, we investigated the impact of short and long length of conditioning on the production of CB cells enriched in MPP- and HSC-like cells and downstream committed lineages. We also characterized the functional contribution of soluble and non-soluble fractions of OCM to its growth promoting activities and platelet engraftment modulating properties. Finally, we showed that the growth promoting activities of OCM are retained after irradiation and after prolonged cryostorage.

Materials and methods

Isolation and culture of human MSC and production of M-OST

The research project described herein was approved beforehand by the Canadian Blood Services research ethical board (protocol #2014.011). Human BM MSCs were established from purchased fresh BM mononuclear cells (Lonza, Walkersville, MD, USA) as previously described (Dumont et al. 2014). A total of six plastic-adherent MSC lines were generated from independent samples and used between passages 2–8. Cytometry analyses confirmed that MSC were negative for CD14, CD19, CD33, CD45 and CD144 but expressed CD73, CD90, CD105 and CD146. In vitro assays confirmed their capacity to undergo osteoblast, adipocyte and chondrocyte differentiation (data not shown). Osteogenic differentiation was induced by culturing MSC cultures (~90 % confluency) with osteogenic medium composed of α -minimal essential medium supplemented with 10 % FBS, 10^{-7} M dexamethasone (Sigma, St-Louis, MO, USA), 0.2 mM ascorbic acid (Sigma), and 10 mM glycerol 2-phosphate (Sigma) for 6 days with complete medium replacement done every 2 days.

Production of OCM

Plates containing M-OST were rinsed twice with phosphate-buffered saline (PBS) and then incubated with standard SFM (15 mL for T-75 flasks) consisting of Iscove's modified Dulbecco medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 20 % BIT serum substitute (bovine serum albumin, insulin, and transferrin solution, Stem Cell Technologies, Vancouver, BC, Canada), 40 μ g/ml of low-density lipoproteins (Stem Cell Technologies) and 5×10^{-5} M 2-mercaptoethanol (Sigma). For OCM conditioned for 6 days, 1 volume of fresh SFM was added to cultures for a final volume of 30 mL for T-75 flask. At the time point indicated, supernatants were collected, centrifuged ($329 \times g/5$ min) and supernatants were aliquoted and stored at -80 °C before use. When indicated, OCM was filtered using low protein binding 0.2 μ m filters (GVS Filter Technology Inc., Indianapolis, IN, USA). X-ray irradiated (15 G) OCM was also used when indicated. OCM was prepared from individual MSC line and tested individually with a minimum of two MSC donors tested for each experiment.

CD34⁺-enriched cell cultures and progenitor assays

Fifteen human umbilical CB units were obtained from the Canadian Blood Services' Cord Blood for Research Program after obtaining institutional review board approval and written informed consent. MNC were isolated with Ficoll-Paque PLUS (GE Health Care Inc., Baie D'Urfe, QC, Canada) gradient and CB CD34⁺ cells were enriched from pooled CB MNCs (2–4 donors) with EasySepTM Human Cord Blood CD34 Positive Selection Kit (StemCell Technologies) following manufacturer's instruction (final CD34⁺ purity ≥ 95 %).

All CB CD34⁺ cultures used SFM or non-diluted OCM supplemented with the megakaryocyte progenitor cytokine cocktail (OMPC) composed of 10 ng/mL Stem Cell Factor (SCF), 35 ng/mL thrombopoietin (TPO) and 11 ng/mL FLT-3 ligand (FL), optimized for the expansion of megakaryocyte and myeloid progenitors (Pineault et al. 2011). All cytokines were purchased at Peprotech (Rocky Hill, NJ, USA). CB CD34⁺ cultures were initiated at a starting density of 7500–12,000 cells/mL in 250 μ L of medium, with one volume of fresh media added at day-4 and cultures analyzed at day-6. CB cultures were done in 24-well tissue culture polystyrene plates (Falcon from Corning, Tewksbury, MA, USA). All in vitro culture assays were done in duplicate and incubated in humidified atmosphere (5 % CO₂) at 37 °C. Viable nucleated cells were enumerated manually with trypan blue exclusion (Invitrogen, Burlington, ON, Canada) or by flow cytometry. Results were repeated in at least two biological experiments that included the use of different donor-derived MSC lines and independent donor-derived CB CD34⁺ cell preparations. Colony forming cells (CFC) were measured using MethoCult H4434 (StemCell Technologies). Colonies were counted after 14 days using Olympus CKX41 inverted microscope (Olympus Corporation, Japan).

Flow cytometry analysis

CB cells were phenotyped by flow cytometry using a FACS-Attune (Thermo Fisher Scientific Inc.). At least 10,000 events were acquired for each sample, with dead cell and debris gated out by the forward and side-scatter and/or Sytox (Invitrogen). The antibodies used included anti-CD235a-phycoerythrine (PE), anti-CD41a (GPIIb)-fluoresceine isothiocyanate (FITC),

anti-CD14- allophycocyanine (APC), anti-CD34-PE, anti-CD45-APC, anti-CD45RA-APC, anti-CD38-FITC, anti-CD90-PerCP-Cy7, anti-CD73-PE, anti-CD184-APC (CXCR4), anti-CD63-PE and anti-CD105-FITC antibodies. All antibodies purchased at Becton–Dickinson Pharmingen (Mississauga, Canada). AlexaFluor-488 Annexin V and Sytox were used for viability analysis of expanded CB cells incubated without cytokines for 2–4 days following the manufacturer's instruction (Invitrogen).

Western blots analysis

OCM without BIT serum substitute was prepared as described above. OCM and FCM were mixed with an equal volume of 2× sample buffer (60 mM Tris–HCl pH 6.8, 2 % (w/v) SDS, 10 % (v/v) glycerol, 0.01 % (v/v) bromophenol blue, and 5 % (v/v) beta-mercaptoethanol) then heated for 5 min at 95 °C. A total of twenty µL of FCM or OCM were loaded per well on 8 % SDS-PAGE gels, then transferred to Immobilon P-PVDF (Millipore, Montreal, PQ, Canada). Blots were incubated with polyclonal anti-fibronectin (Millipore) then washed using PBS and 0.1 % Tween 20. Membranes were then incubated with secondary anti-rabbit antibodies conjugated to alkaline phosphatase (AP) (Bio-RAD, Hercules, CA, USA) and detection was performed to visualize the proteins using AttoPhos® AP fluorescent substrate system (Promega, Fitchburg, WI, USA). AP signal was detected using AlphaImager™ 2200 detection system (Thermo Fisher Scientific Inc.).

Statistical analysis

Statistical analyses were performed using GraphPad InStat version 3.00 (La Jolla, CA, USA). Multigroup comparisons were done by paired ANOVA while two groups comparison were done with paired student *t* test as indicated. *P* values smaller than 0.05 were considered significant.

Results

Impact of the length of conditioning time on the growth promoting activity of OCM

In the original study reporting that OCM promoted the expansion of CB CD34⁺ cells and myeloid clonogenic

progenitors, OCM was produced by conditioning SFM for 6 days. Herein, we sought to determine whether an optimal length of conditioning time existed for the expansion of CB cells and CD34⁺ cells. In this study, CB CD34⁺ cells were cultured for a total of 6 days in SFM or in OCM after which the cells were harvested for analyses. All cultures were supplemented with the same cytokine cocktail composed of SCF, FL and TPO (Pineault et al. 2011).

Preliminary data showed that conditioning for 6 hours raised the expansion of TNC and CD34⁺ cells by 40 and 25 % compared to SFM, whereas expansion induced with 6 days of conditioning was 200 % greater (data not shown). Therefore we focused our attention to the length of conditioning between 1 and 3 days. Cellular phenotyping by flow cytometry with markers for different cell subsets was used to monitor the growth and differentiation of CD34⁺ cells in the cultures (Fig. 1a). Expansion of TNC and CD34⁺ cells was strongly promoted by all OCMs tested (Fig. 1b, c) though 3 days of conditioning consistently induced the lowest expansions likely due likely to nutrients depletion of OCM since fresh SFM media was added at day 4 during conditioning. The frequency of CB cells that retained expression of CD34, a marker commonly used to estimate the graft content in HSPC, tended to be decreased in OCM cultures independently of the conditioning length though the differences were not significant (Fig. 1d). However, the net production of CD34⁺ cells in OCM cultures tended to be superior versus control (Fig. 1c).

Next, we investigated the impact of the conditioning length on the commitment and differentiation of CB CD34⁺ cells. The frequency and net production of CD14⁺ myelomonocytic cells were significantly greater in all OCM cultures (Fig. 1a, e). Conversely, the frequency and net output of CD41⁺ megakaryocytes tended to be reduced in OCM cultures independently of the conditioning length (Fig. 1a, f). No significant differences were observed for the frequency and expansion of CD235⁺ erythroblasts (data not shown).

Shorter conditioning period favors greater production of immature CB hematopoietic cells

The previous result suggested that a short-conditioning period of 1 day of SFM with M-OST provided similar proliferative promoting activity on UCB TNC

Fig. 1 Impact of the length of conditioning on the growth modulatory activities of OCM on CB CD34⁺ cells. **a** Representative flow cytometry profile showing the frequencies of CD34⁺, CD14⁺, and CD41⁺ expressing cells in SFM control (CTL) (dashed line) and OCM (solid line) cultures (unstained sample shown in grey). **b**, **c** TNC and CD34⁺ cell production per seeded cells after 6 days of culture in SFM (CTL) medium and in OCM conditioned for 1, 2, 3 and 6 days (1–6d). **d** Frequency of CD34⁺

cells in CTL and OCM cultures. **e**, **f** Production of CD14⁺ monocytes and CD41⁺ megakaryocytes per seeded cells after 6 days of culture in SFM and different OCM cultures. Mean $\pm$ SEM of five independent experiments presented. Significant differences were determined by paired ANOVA test; * $P < 0.05$ versus CTL, ** $P < 0.01$ versus CTL, *** $P < 0.001$ versus CTL

and CD34⁺ cells than SFM conditioned for 6 days. To further investigate potential differences arising from different conditioning length, we compared the capacity of SFM conditioned for 1 and 6 days with M-OST to support the production of CB cells enriched in HSPC subpopulations (Fig. 2a).

The capacity of the OCMs to support CB TNC outputs was similar (Fig. 2b). However, significant differences were observed in regards to the production of cell subpopulations normally enriched in stem and progenitor cells. Indeed, net production of CD34⁺ (1.2-fold vs. day 6 OCM), CD34⁺CD38[−] (2.7-fold) and CD34⁺CD38[−]CD45RA[−] (1.8-fold) cells were significantly greater with OCM conditioned for just 1 day (Fig. 2c–e). Similar results were observed for the yield of CD34⁺CD38[−]CD45RA[−]CD90[−] MPP-like cells (Fig. 2f) (Majeti et al. 2007; Notta et al. 2011). Finally, production of CB cells with phenotype similar to that of HSC (i.e. HSC-like cells) was increased twofold in 1-day OCM versus 6-days OCM

(Fig. 2g). Taken together, these results suggest that a shorter conditioning length improves the ex vivo production of CB cells with phenotypes associated with stem and progenitor cells. The remainder of this study was pursued with SFM medium conditioned for 1 day.

The growth promoting activity of OCM is mediated by soluble and non-soluble factors

We previously reported that 0.2 μ m filtration reduced the capacity of OCM to support expansion of TNC (Dumont et al. 2014). However, the impact of filtration on the growth and differentiation modulatory activities of OCM was not addressed further. Herein, we observed again a significant reduction in the capacity of OCM to support TNC expansion following 0.2 μ m filtration designed to capture large cellular elements (Fig. 3a). Moreover, filtration significantly reduced the yields of CD34⁺ as well as that of CD34⁺CD45RA[−]

Fig. 2 A shorter length of medium conditioning is associated with improved production of immature CB cells. **a** Representative flow cytometry analysis of CB cultures for HSPC-enriched subpopulations. **b–g** Production of TNC and HSPC-enriched cell subpopulations after 6 days of culture in OCM conditioned for 1 or 6 days (mean $\pm$ SEM, $n = 3$). Significant differences were determined by two-tailed paired T-test, * $P < 0.05$, ** $P < 0.01$

and CD34⁺CD38⁻CD45RA⁻ cells (Fig. 3a, b). Of note, FCM also promoted CB cell growth though the difference versus SFM was not quite significant ($P = 0.08$ for TNC, Fig. 3a). On the other hand, filtration did not have a profound effect on the differentiation modulatory activity of OCM on CB

CD34⁺ cells. Indeed, the frequencies of lineage committed cells were similar between OCM and FCM cultures (Fig. 3c). Moreover, the positive effect of OCM on the expression of CxCR4 on CB cells was retained in FCM cultures (data not shown). We also addressed the impact of filtration on the capacity of

Fig. 3 Soluble and non-soluble cellular elements mediate the growth and platelet engraftment modulatory activities of OCM on CB CD34⁺ cells. **a, b** Production of TNC, CD34⁺, CD34⁺CD45RA⁻ and CD34⁺CD38⁻CD45RA⁻ per seeded cells after 6 days of culture in SFM (CTL), filtered OCM (FCM), unfiltered OCM (CM) and in SFM supplemented with OCM retentate (AB-CM) cultures. **c** Frequencies of CD14⁺, CD41⁺ and CD235a⁺ cells in CTL, FCM, CM and AB-CM cultures. **d** CFC produced per seeded cells after 6 days of culture

in SFM medium (CTL), FCM and CM cultures. Granulocyte/macrophage progenitors (GM), burst-forming unit erythroid progenitors (BFU-E), and multi-potential progenitors (GEMM). Mean $\pm$ SEM of three independent experiments are shown for (a)–(d). Significant differences determined by paired ANOVA test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **e** Detection of fibronectin in OCM, FCM and SFM by western blot. Representative results are presented ($n = 2$), the integrated density value (IDV) of each band is indicated below

OCM to support the production of myeloid progenitors detected by the colony-forming cell (CFC) assay. The highest output was seen in OCM cultures (Fig. 3d). Indeed, the yield of myeloid CFC was significantly reduced by filtration though it was slightly greater than that seen in control (Fig. 3d). The same trend was seen for all myeloid progenitor subtypes (i.e. CFU-GM, BFU-E, and GEMM).

Next, we set to investigate whether the retentate (i.e. fraction trapped in filters) of OCM could

modulate the growth of CB cells. This was tested by adding it to SFM. The retentate tended to increase the yields of TNC, CD34⁺ and CD34⁺CD38⁻CD45RA⁻ cells produced in cultures but not to the same magnitude as seen in OCM cultures (Fig. 3a, b). Moreover, the differentiation modulatory activities of the retentate resembled that of the SFM and not that of OCM (Fig. 3c).

Osteoblasts are known to secrete a large quantity of ECM proteins that can form large complexes (Knothe

Fig. 4 Impact of non-cryoprotected M-OSTs and irradiation on the growth promoting activities of OCM. **a** Viability analysis of thawed M-OST frozen without cryoprotectant ($n = 4$). **b–d** Production of TNC, CD34⁺ and CD34⁺CD45RA⁻ cells per seeded cells in SFM (CTL), unfiltered OCM (CM), filtered OCM (FCM) and FCM supplemented with non-cryoprotected thawed M-OST (2500–40,000 cells/mL). Mean ± SEM of five

independent experiments. Significant differences were determined by paired ANOVA test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **e** Production of TNC, CD34⁺, CD34⁺CD45RA⁻, CD14⁺, CD41⁺ and CD235⁺ cells per seeded cells in OCM and irradiated OCM (IR-OCM). Mean ± SEM of two independent experiments

Tate et al. 2004; Palumbo 1986). Therefore we addressed whether the loss of activity associated with filtration correlated or not with a loss in the presence of fibronectin, a protein known to have proliferative and differentiation regulatory activities on hematopoietic cells. Fibronectin was readily detected in OCM and its concentration was reduced by 4.4 ± 1.7 fold (Fig. 3e) in FCM (range 3.2–5.5, $n = 2$). Altogether, these results demonstrate that the growth and differentiation modulatory activities of OCM are carried out by a combination of soluble and non-soluble elements.

The modulatory activities of OCM are not dependent on remaining M-OSTs

We sought to determine whether large cellular-based elements in OCM are partially responsible for the

growth promoting activity on CB cells. We hypothesised that M-OST may be retained in OCM since it is not filtrated and that these may promote the growth of CB cells. Only low concentration of cells with forward and size scattering properties similar to M-OST was measured in OCM (172 ± 279 per mL, mean ± SD, $n = 3$, range 0–494). Viability analysis carried out on M-OSTs frozen in condition similar to OCM revealed that the majority would either be apoptotic or necrotic (Fig. 4a). Consistent with this, such M-OSTs were unable to grow back when placed in osteogenic cultures (data not shown). We tested whether such non-viable M-OSTs could impact the growth and differentiation of CB CD34⁺ cells. The addition of thawed non-cryoprotected M-OSTs to filtered OCM slightly increased expansion of TNC and CD34⁺ cells in a dose dependent fashion, but the difference versus

FCM was only significant when greater than 10,000 M-OSTs per mL were added (Fig. 4b, c). Taken together, these results suggest that trace numbers of M-OSTs in OCM are not key players responsible for the growth promoting activity of OCM.

The growth promoting activities of OCM are retained after irradiation and long-term storage at -80°C

Irradiation is useful mean to inactivate potential infectious agents in biological solutions. We tested whether the principal activities of OCM would be retained following high dose-ionizing irradiation (X-ray, 15 Gy). The ex vivo expansion of CB TNC and CD34^{+} cells was unaffected by irradiation (Fig. 4d). Moreover, the production of $\text{CD34}^{+}\text{CD45RA}^{-}$ and differentiated cells were unperturbed in irradiated OCM (Fig. 4d).

Next, we addressed the stability of OCM major proliferative modulatory activities of OCM on CB CD34^{+} cells. To limit as much as possible the loss of biological activity inherent to long-term storage, OCM prepared from independent MSC donors were dispensed in single use aliquots that were stored at -80°C . In general, the proliferative and differentiation modulatory activities of OCM were found to be very stable at -80°C . This was evident by the significant increase in cell growth measured in cultures established with OCM conserved for few weeks and up to 1 year (Fig. 5a), and by the relative stability of this effect (Fig. 5b). Indeed, when results from two lines were analysed together, no significant loss of TNC expansion was observed between successive time points tested (Fig. 5b). Moreover, the capacity of OCM to promote expansion of CD34^{+} and production of $\text{CD34}^{+}\text{CD45RA}^{-}$ cells were also largely retained over this storage period (Fig. 5c–f). Likewise, the strong pro-monocytic activity of OCM was retained over the yearlong storage period tested (data not shown).

Discussion

Co-transplantation of a CB unit with ex vivo expanded progenitors has shown to improve engraftment and accelerate neutrophil recovery in CB transplant

(Pineault and Abu-Khader 2015; Cairo et al. 2016). However, platelets recovery remains a major issue given its extended delays. Interestingly, we previously showed that CB CD34^{+} cells expanded in OCM improved platelet engraftment compared to cells expanded in standard SFM (Dumont et al. 2014). However, the mean of action and active components of OCM responsible for this and other properties remained unresolved. Herein, we optimized the conditioning length and showed that soluble and non-soluble elements are together responsible for the increased growth of CB CD34^{+} cells and progenitors, and that OCM can be stably stored and even irradiated before use.

First, we showed that a short conditioning period of 1 day was sufficient to expand CB TNC and CD34^{+} cells up to more than 75- and 18-fold in just 6 days of culture, respectively. Such level of expansion in only 6 days of culture compares advantageously with other protocol previously reported with SFM (Rossmann et al. 2001; Ueda et al. 2000; Araki et al. 2006) although protocols with longer expansion periods are as expected associated with greater overall level of cell expansion (Pineault and Abu-Khader 2015). Moreover, phenotypic analyses showed that the productions of immature CB subpopulations such as $\text{CD34}^{+}\text{CD38}^{-}\text{CD45RA}^{-}$, $\text{CD34}^{+}\text{CD38}^{-}\text{CD45RA}^{-}\text{CD90}^{-}$ MPP-like and $\text{CD34}^{+}\text{CD38}^{-}\text{CD45RA}^{-}\text{CD90}^{-}$ HSC-like cells were significantly improved with the shorter 1 day conditioning than the original longer 6 days. Such finding suggest that longer conditioning period may result in the accumulation of factors such as MIP-1 β , IL-8 and NAP-2 present in OCM (Dumont et al. 2014) that promote the differentiation over the maintenance of immature cells such as MPP and HSC (Csaszar et al. 2012). Besides, longer conditioning is associated with metabolic wastes buildup (e.g. lactic acid) and depletion of cell nutrients such as vitamins, amino acids and other components required to sustain mammalian cell growth (Duval et al. 1992). However, the putative increase in production of HSC- and MPP-like cells associated with shorter conditioning would need to be properly confirmed in transplant experiments.

Also revealed herein is the fact that OCM contains physically divergent elements that together are responsible for the hematopoietic enhancing activity of OCM. On one hand, OCM contains large cell-based elements lost by $0.2\text{ }\mu\text{M}$ filtration that collaborate with soluble

Fig. 5 Impact of storage length at -80°C on the growth modulatory activities of OCM on CB CD34⁺ cells. **a** Expansion of TNC per seeded cells in control (white) or in OCM cultures (light and dark) as a function of storage time (x-axis) at -80°C . OCM prepared from two independent donors are presented (mean $\pm$ SD of duplicate cultures). **b** Expansion of TNC in OCM cultures normalized to SFM control as a function of storage time (mean $\pm$ SD, n = 2). **c** Expansion of CD34⁺ cells per seeded cells (see **a** for details). **d** Normalized expansion of

CD34⁺ cells (see **b** for details). **e** Expansion of CD34⁺CD45RA⁻ cells per seeded cells (see **a** for details). Results for this response were not available at 3.5 months-time point. **f** Normalized expansion of CD34⁺CD45RA⁻ cells (see **b** for details). Significant differences in **a**, **c**, **e** were determined by unpaired ANOVA, * $P < 0.05$ versus CTL, ** $P < 0.01$ versus CTL, *** $P < 0.001$ versus CTL, \$ $P < 0.05$ between OCM# 1 versus OCM# 2

factors in promoting the growth of most CB cells including clonogenic progenitors and immature subpopulations. Osteoblast and M-OSTs secrete a wide array of matrix materials and ECM proteins such as collagen I, fibronectin, osteocalcin and osteopontin (Kulterer et al. 2007; Neve et al. 2011). ECM proteins have been implicated in the regulation of homing, survival, differentiation and proliferation of hematopoietic cells (Yokota et al. 1998; Voermans et al. 1999;

Sagar et al. 2006). Furthermore, growth factors and cytokines have a natural affinity for ECM proteins (Taipale and Keski-Oja 1997) and such interaction has been reported to potentiate the mitogenic and functional properties of several growth factors (Clark 2008). We previously showed that ECM proteins have common and distinct regulatory activities on CB CD34⁺ cell growth and differentiation and that a combination of ECM proteins (collagen I and IV, fibronectin and

laminin) increased the net production of CB CD34⁺ in SFM (Celebi et al. 2011). Since ECM proteins can form large complexes, we hypothesise that those could be lost to filtration, and that such combination could be contributing to the growth promoting activity of M-OST retentate. In support of this hypothesis, fibronectin was significantly depleted in FCM versus OCM. Further investigations are required to extend this hypothesis and to narrow down ECM elements playing a role in the activities of OCM.

We also explored the possibility that M-OSTs at low concentration are retained in OCM. However, over 99 % are non-viable since no cryoprotectant agents were used. Nonetheless, it is possible that M-OST or related debris can present cell surface ligands [e.g. Notch ligands (Chitteti et al. 2010)] to CB CD34⁺ cells that can activate signaling pathways leading up to cell proliferation (Ducas et al. 2009). However, doses of thawed non-cryoprotected M-OST at least 20-fold greater were required to provide a significant growth benefit which suggests that M-OSTs retained in OCM are not a major contributor.

While filtration reduced cell growth of most subpopulations, it did not have a profound effect on the differentiation modulatory activity of OCM on CB CD34⁺ cells, monocytic and erythroid lineages as well as on the expression of CXCR4 demonstrating that soluble factors released by M-OST were largely responsible for these effects. Moreover, cell growth in FCM cultures was halfway that observed between the control and OCM cultures suggesting that secreted growth factors are also partially responsible for the increase in expansion observed with OCM. We reported in our previous study that OCM contains many growth factors including interleukins, colony-stimulating factors (i.e. GM-CSF, GM-CSF, and M-CSF), RANTES, insulin-like growth factor binding protein 2 and multiple chemokines (Dumont et al. 2014). We confirmed by Q-PCR the expression of 4 colony-stimulating factors (GM-CSF, G-CSF, M-CSF and IL-6) in M-OSTs (data not shown). The presence of these in factors that can synergize with exogenous SCF partially explained the increased expansion of CD14⁺ myeloid cells and CFC myeloid progenitors in OCM cultures (Pineault et al. 2011; Metcalf 2008; Broudy 1997). It is also possible that OCM contains growth nutrients that have been partially metabolized by M-OST (Ishii and Mann 2014).

In conclusion, the OCM represents a hybrid culture system between contact and noncontact coculture systems for the ex vivo expansion of HSPC with a stromal component of the hematopoietic microenvironment. OCM provide an interesting alternative to coculture system as it can easily be used in any HSPC culture vessels including bioreactors. Moreover, OCM is highly stable and can even be irradiated to reduce any safety concerns. Future work will be required to characterize the larger cellular elements and soluble factors that support the increase in the expansion of CD34⁺ cells.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to disclose.

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