Cytotherapy, 2015; 17: 428–442

International Society for Cellular Therapy
ISCT®

Developing a co-culture system for effective megakaryo/thrombopoiesis from umbilical cord blood hematopoietic stem/progenitor cells

JAVAD HATAMI[1], PEDRO Z. ANDRADE[1], ANTÓNIO PEDRO ALVES DE MATOS[2], DUSAN DJOKOVIC[3], CARLA LILAIA[3], FREDERICO CASTELO FERREIRA[1], JOAQUIM M.S. CABRAL[1] & CLÁUDIA L. DA SILVA[1]

[1]Department of Bioengineering and IBB-Institute for Bioengineering and Biosciences, Instituto Superior Técnico, Universidade de Lisboa, Lisboa, Portugal, [2]Centro de Estudos do Ambiente e do Mar (CESAM/FCUL)–Faculdade de Ciências da Universidade de Lisboa and Centro de Investigação Interdisciplinar Egas Moniz (CiiEM), Campus Universitário, Quinta da Granja, Monte de Caparica, Caparica, Portugal, and [3]Department of Obstetrics, Centro Hospitalar Lisboa Ocidental E.P.E., Hospital São Francisco Xavier, Lisboa, Portugal
Andrade is now with Cell2B Advanced Therapeutics SA, Cantanhede, Portugal

Abstract

Background aims. Platelet transfusion can be a life-saving procedure in different medical settings. Thus, there is an increasing demand for platelets, of which shelf-life is only 5 days. The efficient *ex vivo* biomanufacturing of platelets would allow overcoming the shortages of donated platelets. *Methods.* We exploited a two-stage culture protocol aiming to study the effect of different parameters on the megakaryo/thrombopoiesis *ex vivo*. In the expansion stage, human umbilical cord blood (UCB)-derived $CD34^+$-enriched cells were expanded in co-culture with human bone marrow mesenchymal stromal cells (BM-MSCs). The megakaryocytic commitment and platelet generation were studied, considering the impact of exogenous addition of thrombopoietin (TPO) in the expansion stage and a cytokine cocktail (Cyt) including TPO and interleukin-3 in the differentiation stage, with the use of different culture medium formulations, and in the presence/absence of BM-MSCs (direct versus non-direct cell-cell contact). *Results.* Our results suggest that an early megakaryocytic commitment, driven by TPO addition during the expansion stage, further enhanced megakaryopoiesis. Importantly, the results suggest that co-culture with BM-MSCs under serum-free conditions combined with Cyt addition, in the differentiation stage, significantly improved the efficiency yield of megakaryo/thrombopoiesis as well as increasing %CD41, %CD42b and polyploid content; in particular, direct contact of expanded cells with BM-MSCs, in the differentiation stage, enhanced the efficiency yield of megakaryo/thrombopoiesis, despite inhibiting their maturation. *Conclusions.* The present study established an *in vitro* model for the hematopoietic niche that combines different biological factors, namely, the presence of stromal/accessory cells and biochemical cues, which mimics the BM niche and enhances an efficient megakaryo/thrombopoiesis process *ex vivo*.

Key Words: *cell-cell contact, hematopoietic stem/progenitor cells, megakaryocyte, platelet, serum-free medium, umbilical cord blood*

Introduction

Platelet transfusion demand is dramatically increasing because of its essential role in the treatment of cancer, hematological malignancies and marrow failure, including hematopoietic cell transplantation, as well as solid organ transplantation [1]. In the United States only, more than 2 million voluntary-donated platelet units are transfused annually [2]. However, despite persistent platelet shortages, up to 31% of collected platelets are currently wasted because platelets do not tolerate refrigeration, have a shelf-life of 5 to 7 days and should be kept in stringent conditions [1–3]. *Ex vivo* production of platelets from hematopoietic stem/progenitor cells (HSC/HPC), namely $CD34^+$ cells, was previously accomplished with the use of umbilical cord blood (UCB), bone marrow (BM) and mobilized peripheral blood (mPB) cells [3–5]. Recent reports highlighted the potential use of human embryonic stem cells and induced pluripotent stem cells for the clinical-scale production of platelets [6–8]. However, despite significant progress achieved in understanding the mechanisms underlying platelet production, the platelet yield achieved so far has not been sufficient

Correspondence: **Frederico Castelo Ferreira**, PhD, Department of Bioengineering and IBB-Institute for Bioengineering and Biosciences, Instituto Superior Tecnico, Universidade de Lisboa, Av. Rovisco Pais, Nr. 1, 1049-001, Lisboa, Portugal. E-mail: frederico.ferreira@ist.utl.pt

(Received 17 November 2014; accepted 23 December 2014)

ISSN 1465-3249
http://dx.doi.org/10.1016/j.jcyt.2014.12.010

for adult transfusion ($3.0-6.0 \times 10^{11}$ platelets/adult) [2,3,9].

Megakaryocytes (Mks) are large cells that reside in the BM niche and derive from lineage commitment of HSC/HPC *in vivo*. Platelets are enucleated fragments of Mks that are formed and released into the vascular sinusoids by terminal differentiation of mature Mks [10]. This process, thrombopoiesis, involves endomitosis, with cytoplasmic enlargement, significantly increasing in size and DNA contents (polyploidy) of Mks [4]. The final step of thrombopoiesis includes pro-platelet formation from the cytoplasm of Mks by a network of specialized membranes, the so-called demarcation membrane system (Dm); nascent platelets are then released from pro-platelet fragments [4]. Thrombopoietin (TPO) is a key regulator of megakaryo/thrombopoiesis involved in early Mk development, proliferation and maturation, as well as supporting survival and proliferation of primitive cells/progenitors [11]. However, it was shown that other cytokines such as interleukin (IL)-3, IL-6 and IL-11 are needed, in combination with TPO, for Mk development and maturation [12].

The interactions between HSC/HPC and mesenchymal stromal cells (MSC) or/and MSC-derived cells, residing in the BM niche, can take place through cell-cell cross-talk or by produced soluble factors. Such interactions have a pronounced impact on HSC/HPC self-renewal, proliferation and differentiation [13–15]. The significant role of BM stromal/accessory cells on promoting the proliferation/maintenance of HSC/HPC, while preserving its engraftment ability, has been previously reported using an *in vivo* model [16]. The results of our group demonstrated successful expansion of UCB-derived $CD34^+$ cells in co-culture with BM-MSC with the use of serum-free medium supplemented with growth factors *ex vivo* [17–19]. In particular, direct contact with a BM-MSC feeder layer boosted proliferation of UCB-derived $CD34^+$ cells [13]. The BM niche is a complex system that involves a 3D structure in which different factors, such as the extracellular matrix components, cell-cell interactions and extrinsic cues provided by accessory/stromal cells, modulate Mk development and maturation [20,21]. It was also shown that signaling molecules secreted by stromal cells, as well as cell-cell interactions in the BM niche, are elementary modulators of the megakaryo/thrombopoiesis process [15,22]. For example, besides providing physical support for HSC/HPC, BM-MSC secrete cytokines, such as granulocyte-macrophage colony-stimulating factor, IL-6 and stem cell factor (SCF), which regulate megakaryopoiesis [15,23,24].

The present work aims to further extend our previous study [25] by investigating the influence of several culture parameters on the effective megakaryo/thrombopoiesis process in the two-stage cultivation protocol with the use of UCB-derived $CD34^+$-enriched progenitors. Therefore, we used our previously established cytokines cocktail [25] containing only TPO and IL-3, which was demonstrated to be a simple and effective protocol for megakaryocytic differentiation of UCB cells. Overall, TPO is used as a main driver of megakaryo/thrombopoiesis and IL-3 as a proliferative signal. The impact of TPO inclusion in the expansion cocktail was assessed, first in terms of expansion profile and then in terms of effects on megakaryocytic differentiation of UCB $CD34^+$-enriched cells. In the second (differentiation) stage, combination and interaction of biological factors and biochemical cues with HSC/HPC, which recapitulate the BM niche, were evaluated aiming to identify an efficient megakaryo/thrombopoiesis process *ex vivo*. The three parameters assessed at this stage, alone or in combination with each other, included (i) exogenous addition of cytokines (TPO and IL-3; 100 and 10 ng/mL, respectively); (ii) co-culture of expanded HSC/HPC with a BM-MSC feeder layer (in direct or non-direct cell-cell contact), aiming to study the effect of the feeder layer on megakaryo/thrombopoiesis either by direct cell-cell contact interaction or caused by the secreted trophic factors and/or in combination with cytokines addition; and (iii) serum-containing versus serum-free culture medium. In the current study, two culture medium formulations were used in the differentiation stage. A schematic representation of different parameters explored in the current study is presented in Figure 1.

Methods

UCB samples

UCB samples were obtained from healthy donors after maternal donor-informed consent at Hospital São Francisco Xavier, Centro Hospitalar Lisboa Ocidental, E.P.E., Portugal. Low-density mononuclear cells (MNC) were separated by means of a Ficoll density gradient (1.077 g/mL; GE Healthcare) according to the manufacturer's protocol. UCB MNC were either cryopreserved for further use or processed directly.

Human BM-MSC cultures

BM-MSC were isolated from healthy donors after informed consent was obtained as described elsewhere [17,18] and kept cryopreserved in liquid/vapor nitrogen. To establish the feeder layers, BM-MSC were thawed and cultured (3.0×10^3

cells/cm^2) in Dulbecco's modified essential medium (Gibco) containing 10% fetal bovine serum (FBS) (MSC-qualified; Gibco), supplemented with streptomycin (0.025 mg/mL, Gibco) and penicillin (0.025 U/mL, Gibco) at 37°C and 5% CO_2 in a humidified atmosphere with medium changed twice per week. At near full confluence (80–90%), BM-MSC were harvested from the flask by use of Accutase (Sigma) [25]. BM-MSC were re-seeded at 3.0×10^3 cells/cm^2 in T25 flasks or six-well plates (BD) and cultured until confluence and then were treated with mitomycin C (Sigma) (0.5 μg/mL in Iscove's modified Dulbecco's media (IMDM) + 10% FBS) to prevent feeder layer overgrowth [13,19]. BM-MSC (passages 3 to 6) were then washed twice with phosphate-buffered saline (PBS; Gibco) for 5 min and used as a feeder layer within the next 12 to 24 h.

Ex vivo expansion of UCB CD34$^+$-enriched cells (stage I)

When cryopreserved, UCB MNC were thawed at 37°C with the use of IMDM (Gibco) supplemented with 20% FBS and 50 μg/mL deoxyribonuclease (DNAse; Sigma) and were then centrifuged (1250 rpm, 7 min) and washed with PBS [25]. UCB MNC (either thawed or fresh) were enriched for CD34 expression by magnetic activated cell sorting (MACS; Miltenyi Biotech) according to the manufacturer's instructions. CD34$^+$ cell expansion was carried out by use of a pool of the UCB CD34$^+$-enriched cells (at least from two different UCB units) in co-culture with BM-MSC (direct cell-cell contact). Two cytokine cocktails, referred to as Z9 and T0, were used. Z9 contains SCF (60 ng/mL), Flt-3 (55 ng/mL), TPO (50 ng/mL) and basic fibroblast growth factor (b-FGF) (5 ng/mL) (all from Peprotech) [19]. T0 contains SCF (60 ng/mL), Flt-3 (75 ng/mL) and b-FGF (5 ng/mL). In the current study, UCB CD34$^+$-enriched cells were seeded to a T25 flask (BD) with the use of QBSF-60 (Quality Biological Inc) as an expansion culture medium at the density of 3.0×10^4 cells/mL, over a mitomycin C–treated BM-MSC feeder layer.

Ex vivo differentiation of expanded CD34$^+$-enriched cells (stage II)

After 7 days of expansion, expanded cells were cultured with the use of differentiation culture medium, IMDM supplemented with 10% FBS or QBSF-60, at a density of 2.0×10^5 cells/mL. Both culture conditions were supplemented with streptomycin (0.025 mg/mL) and penicillin (0.025 U/mL). When indicated, expanded cells were co-cultured in direct and non-direct contact with BM-MSC in combination with/without cytokine addition (IL-3 and TPO; 10 ng/mL and 100 ng/mL, respectively; both from Peprotech). For co-cultures with no cell-cell contact, six-well plates (BD) with an insert of 0.4-μm pore diameter (BD) were used to avoid direct contact with the feeder layer [13]. BM-MSC were previously inactivated with mitomycin C through the use of the same protocol described before. At days 3, 5 and 7 of the culture, half of the volume of the exhausted medium was replaced by addition of fresh medium.

Proliferative and phenotypic analysis

Cell numbers and viability were determined by use of the trypan blue (Gibco) dye exclusion method. The proliferation extent of CD34$^+$ cells was assessed as fold increase in CD34$^+$ cell number (FI-CD34$^+$) and calculated according to Equation 1.

$$\text{FI} - \text{CD34}^+ = \frac{\text{number of CD34}^+ \text{ cells (at the end of expansion stage)}}{\text{number of CD34}^+ \text{ cells (seeded at day 0)}} \quad \text{Eq. 1}$$

Phenotypic analysis was performed by use of flow cytometry (FACSCalibur equipment, BD), as previously described [25]. Briefly, cells were labeled with a panel of monoclonal antibodies (fluorescein isothiocyanate–conjugated or phycoerythrin-conjugated) including anti-CD34 (for early HSC/HPC), CD33 and CD14 (both for myeloid lineage), CD41 and CD42b (both for megakaryocytic lineage) and CD7 (myeloid and early lymphocytic lineage) (all from BioLegend) [17]. The degree of megakaryocytic commitment was quantified by calculation of the efficiency yield

$$\text{EY} = \frac{\text{number of CD41}^+ \text{ cells (at the end of differentiation stage)}}{\text{number of CD34}^+ \text{ cells (seeded at day 0)}} \quad \text{Eq. 2}$$

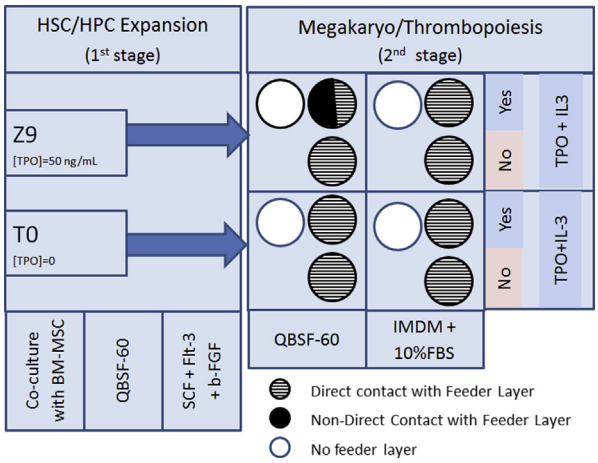

Figure 1. Schematic representation of the two-stage cultivation protocol (HSC/HPC expansion, megakaryo/thrombopoiesis) and the combination of the different parameters exploited in the current study.

(EY) according to Equation 2. $CD34^+$ and $CD41^+$ cells were considered in this study as HSC/HPC and megakaryocytic cells, respectively.

Transmission electron microscopy analysis of Mks and platelets

The internal structure of Mks and platelets was observed by use of transmission electron microscopy (TEM). UCB culture-derived cells and PB-derived platelets were fixed in a solution containing 2.5 % glutaraldehyde (Sigma) in 0.1 mol/L sodium cacodylate buffer (pH 7.4; Sigma) for 1 h at room temperature (22–25°C). After rinsing with cacodylate buffer, cells were post-fixed with a 1% osmium tetroxide (Sigma) in 0.1 mol/L cacodylate buffer for 1 h at room temperature. Cells were then fixed with uranyl acetate (0.5%; Sigma) in citrate-acetate acid buffer (pH 5–6) and dehydrated by gradually increasing ethanol (Sigma) concentration (70%, 95% and 100% in distilled water). Finally, cell populations were embedded in Epon (Momentive Specialty Chemicals, Inc.), cut and Mks and platelets ultrastructures were observed with the use of a transmission electron microscope (Hitachi 8100, Hitachi Instruments Inc).

Analysis of DNA ploidy

Mk ploidy was determined by means of a double-staining technique with flow cytometry and the use of the CellQuest Pro software (BD) by choosing $CD41^+$ events as a reference gate [25,26]. Briefly, culture-derived cells were incubated 15 min with anti-CD41 antibody (Biolegend) and then were fixed by 70% cold ethanol (4°C). Cells were re-suspended in a staining solution containing propidium iodide (50 µg/mL; Sigma), sodium citrate (4 mol/L; Sigma), RNase A (0.1 mg/mL; Sigma) and Triton X-100 (0.1%; Sigma) in pH 7.8 for 1 h before flow cytometry analysis was performed.

Preparation of fresh UCB, PB and UCB culture-derived platelets

For UCB culture-derived platelets, culture-derived cells were collected at the end of differentiation stage and centrifuged (750 rpm, 10 min) to remove larger, nucleated cells. Supernatants, which display a lighter platelet fraction, were collected and centrifuged again (3000 rpm, 5 min). The obtained pellets were re-suspended in washing solution that contained sodium citrate (200 mmol/L; Sigma) and

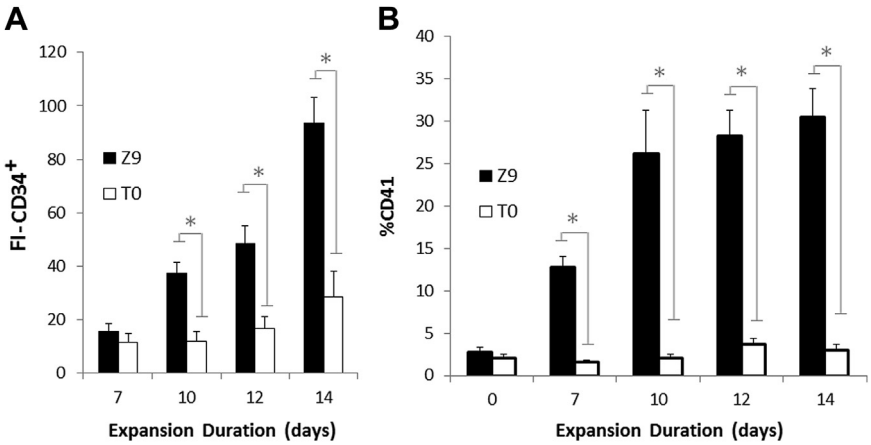

Figure 2. (A) Proliferation extent of $CD34^+$ cells, assessed as fold increase in the number of $CD34^+$ cells (FI-$CD34^+$) during 14 days of the expansion stage. (B) Phenotypic characterization of UCB $CD34^+$-enriched cells during 14 days of expansion in terms of %CD41 for cell population co-cultured with human BM-MSC in cytokine cocktails Z9 (black bars) and T0 (white bars). Results presented as average ± SEM. *$P < 0.05$, $n \geq 4$.

chloride sodium (2 mmol/L, Sigma) in PBS and distributed equally into two 15-mL Falcon tubes (BD). For non-activated platelet analysis, the content of one of the tubes was fixed in 2 mL of paraformaldehyde (2% in PBS; Sigma) and kept at room temperature. The content of the other tube was used for the platelet activation assay; 2 mL of human thrombin (2 U/L; Sigma) was added to the tube and kept inside a humidified incubator (37°C) for 10 min. Afterward, 5 mL of washing solution was added to each tube and centrifuged (3000 rpm, 5 min) and was then re-suspended in PBS and labeled with anti-CD41, anti-CD42b, anti-CD62p and anti-CD63 antibodies (Biolegend). Appropriate (fluorescein isothiocyanate—conjugated or phycoerythrin-conjugated) isotype controls were used for each condition. Labeled cells were kept in a dark condition for 15 min (room temperature) and were then washed with PBS, fixed with 500 μL paraformaldehyde (2% in PBS) and analyzed by means of flow cytometry.

Fresh UCB and PB-derived platelets

After the Ficoll density-gradient centrifugation of fresh UCB cells, the upper supernatant layer, which contained the platelet-rich plasma, was collected. PB-derived platelets were kindly donated by Instituto Português do Sangue (Lisboa, Portugal). Both fresh UCB and PB-derived platelets were processed similarly to UCB culture-derived platelets, except for the first centrifugation step (750 rpm, 10 min), which was not performed. These platelets were used for platelet functional assessment in the current study.

Statistical analysis

Results are presented as a mean ± standard error of mean (SEM). Statistical analysis was performed by use of IBM SPSS statistics (v. 20) with the use of Mann-Whitney U analysis. Differences in results were considered statistically significant at a value of $P < 0.05$.

Results

Effect of TPO addition on the expansion profile of UCB CD34$^+$-enriched cells (stage I)

In the present study, UCB CD34$^+$-enriched cell expansion was based on a previously established protocol. In this protocol [19], a mathematical model was established, with the use of a factorial design approach, targeting the optimization of cytokines concentrations to maximize UCB CD34$^+$-enriched cell expansion in co-culture with BM-MSC. The optimized cocktail Z9 that was obtained contained SCF (60 ng/mL), Flt-3 (55 ng/mL), TPO (50 ng/mL) and b-FGF (5 ng/mL). To study the effect of TPO addition on the expansion profile of UCB CD34$^+$-enriched cells, we used the above-mentioned modeling approach, imposing the limitation that the concentration of TPO should be zero, and therefore a TPO-free cytokine cocktail, referred to as T0, was identified. T0 contains SCF (60 ng/mL), Flt-3 (75 ng/mL) and b-FGF (5 ng/mL). The percentage of CD34$^+$ cells in the starting population submitted to the Z9 and T0 expansion protocols was similar at values of 81% ± 4.0% and 83% ± 4.9%, respectively, without having significant differences ($n \geq 4$, $P > 0.05$). Similarly, the percentage of CD41$^+$ cells in the starting population submitted to the Z9 and T0 was similar, at values of 2.8 ± 0.60 and 2.0 ± 0.50, respectively, without having significant differences ($n \geq 4$, $P > 0.05$).

The predicted values of FI-CD34$^+$, for Z9 and T0 condition, with the use of the mathematical model, were 21 ± 3.5 and 13 ± 3.5, respectively, after 7 days of expansion. These values are in agreement with the FI-CD34$^+$ obtained experimentally, 16 ± 3.0 (using Z9) and 12 ± 3.4 (using T0), for CD34$^+$ cells expanded in co-culture with BM-MSC after 7 days of expansion. However, considering 14 days of expansion, the values of FI-CD34$^+$ for the Z9 condition were significantly higher compared with the T0 condition ($P < 0.05$ for days 10, 12 and 14 of expansion) (Figure 2A). Percentage of CD34 decreased over the expansion stage, with no significant differences observed between Z9 and T0 (data not shown) [19]. There was also no significant difference between Z9 and T0 in terms of differentiative potential, namely, %CD33, %CD14 and %CD7 during 14 days of expansion (Table I). Nevertheless, phenotypic analysis revealed that the cell population cultured in Z9 condition had significantly higher %CD41 and %CD42b, compared with the T0 condition, which suggests a superior early megakaryocytic commitment during the Z9 expansion condition (Figure 2B; Table I).

Impact of co-culture with BM-MSC, addition of cytokines and medium formulation on megakaryocytic differentiation of UCB CD34$^+$-enriched cells in the differentiation stage (stage II)

UCB CD34$^+$-enriched cells expanded in co-culture with BM-MSC, in Z9 or T0 cytokine cocktails, were seeded to the differentiation stage for additional 7 days. The two-stage cultivation protocol to generate megakaryocytic cells was recently established in our lab with the use of IMDM supplemented with FBS 10% (IMDM+10% FBS), TPO

Table I. Phenotypic characterization of expansion of UCB CD34$^+$-enriched cells including %CD33, %CD14, %CD7 and %CD42b during 14 days of expansion for cell population co-cultured with human BM-MSC using cytokine cocktails Z9 and T0.

	%CD33		%CD14		%CD7		%CD42b	
Expansion duration (days)	Z9	T0	Z9	T0	Z9	T0	Z9	T0
0	44 ± 10	40 ± 12	6.5 ± 1.3	7.0 ± 3.5	8.2 ± 1.6	8.5 ± 3.2	2.5 ± 1.1	2.0 ± 0.9
7	96 ± 1.0	80 ± 8.5	17 ± 5.5	16 ± 4.2	37 ± 7.1	32 ± 5.3	7.5 ± 2.5*	0.3 ± 0.2*
10	90 ± 1.5	76 ± 3.9	24 ± 4.9	14 ± 3.2	40 ± 7.6	39 ± 6.0	7.6 ± 2.0*	0.1 ± 0.1*
12	84 ± 3.1	78 ± 6.1	28 ± 4.0	19 ± 3.5	35 ± 10	39 ± 4.6	7.2 ± 1.1*	0*
14	86 ± 3.5	91 ± 2.5	27 ± 4.3	17 ± 0.0	47 ± 7.1	44 ± 0.0	8.4 ± 1.5*	0*

Results are presented as average ± SEM.
*$P < 0.05$ for each day, $n \geq 4$.

(100 ng/ml) and IL-3 (10 ng/mL) [25]. Herein, QBSF-60, a commercially available, serum-free formulation, was tested against IMDM+10% FBS. Although several studies addressed the role of stromal cells, including BM-MSC, on the proliferation profile of HSC/HPC, few studies were focused on the role of accessory/stromal cells, particularly BM-MSC, on the megakaryocytic differentiation of HSC/HPC populations. We investigated the effect of the presence of a BM-MSC feeder layer (Feeder) in the differentiation stage and the use of the cytokine cocktail containing TPO (100 ng/mL) and IL-3 (10 ng/mL) (referred to as Cyt) on the megakaryocytic differentiation of expanded cells. These two conditions, Feeder and Cyt, were used alone or in combination with each other.

CD34$^+$-enriched cells expanded in the Z9 condition had significantly higher EY and %CD41 compared with cells expanded in the T0 condition ($P < 0.05$) with the same differentiation condition (Figure 3). The EY and %CD41 obtained for conditions that combine a BM-MSC feeder layer with cytokine addition (Feeder+Cyt) were higher (Figure 3) than the values obtained for conditions that used the BM-MSC feeder layer only (no cytokines) or cytokines alone (no BM-MSC feeder layer) in the differentiation stage (ie, EY: Feeder+Cyt > Feeder>Cyt; %CD41: Feeder+Cyt>Cyt>Feeder; $P < 0.05$), regardless the differentiation culture medium used (QBSF-60 or IMDM+10% FBS). Namely, for UCB CD34$^+$-enriched cells expanded in the Z9 condition and then differentiated with the use of QBSF-60, EY values obtained were 202 ± 34 (Feeder+Cyt), 75 ± 4.9 (Feeder) and 57 ± 7.8 (Cyt); %CD41 values obtained were 58 ± 4.9 (Feeder+Cyt), 20 ± 5.4 (Feeder) and 45 ± 4.2 (Cyt). EY and %CD41 were significantly higher for expanded CD34$^+$-enriched cells when those were differentiated toward Mk lineage with the use of the serum-free medium (QBSF-60) than when IMDM+10% FBS medium was used. The results suggested that the cytokine cocktail used in the expansion stage had a substantial impact on the maturation level of Mks during the differentiation stage. In fact, CD34$^+$ cells expanded with the use of Z9 cytokine cocktail and then differentiated toward Mk lineage had a significantly higher content of CD42b$^+$ cells with higher polyploid content (DNA

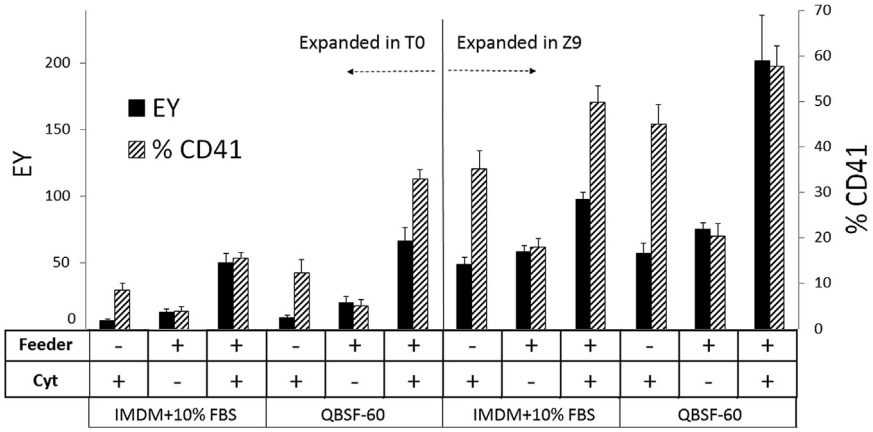

Figure 3. Impact of TPO addition in the expansion stage, co-culture with BM-MSC (Feeder), differentiation culture medium formulation (IMDM+10% FBS and QBSF-60) and cytokines addition (Cyt) during differentiation stage on the megakaryocytic differentiation of UCB CD34$^+$-enriched cells. Results were evaluated in terms of EY and %CD41. Conditions on the right and left sides refer to the cytokine cocktails used (Z9 and T0 cocktails, respectively) during the expansion stage ($n \geq 3$).

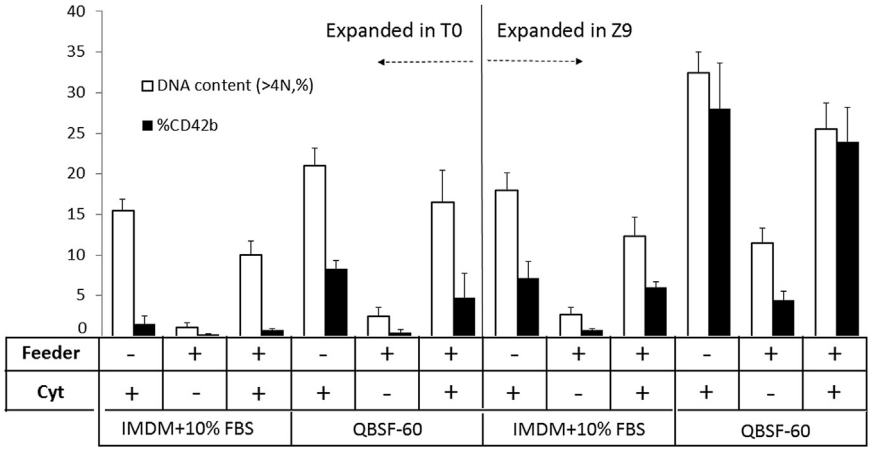

Figure 4. Impact of TPO addition in the expansion stage, co-culture with BM-MSC (Feeder), differentiation culture medium formulation (IMDM+10% FBS and QBSF-60) and cytokines addition (Cyt) during differentiation stage on the megakaryocytic differentiation of UCB CD34$^+$-enriched cells. Results were evaluated in terms of DNA content (>4N, %) and %CD42b. Conditions on the right and left sides refer to the cytokine cocktails used (Z9 and T0 cocktail, respectively) during the expansion stage ($n \geq 3$).

content >4N, %) than cells expanded in the T0 condition ($P < 0.05$), (Figure 4).

Ploidy analysis revealed that CD34$^+$-enriched cells expanded in the same conditions and then differentiated with the use of only Cyt addition resulted in polyploid content (DNA content >4N, %) at comparable levels of the ones obtained with the use of the Cyt+Feeder condition ($P > 0.05$) but with significantly higher polyploid content than with the use of only the Feeder condition ($P < 0.05$). The same tendency was observed when different conditions were compared in terms of %CD42b (ie, Cyt>Feeder and Cyt+Feeder>Feeder; $P < 0.05$). For example, CD34$^+$ cells expanded in the Z9 condition and then differentiated with the use of QBSF-60 displayed a polyploid content of 32% ± 2.5% (Cyt) and 11% ± 1.8% (Feeder) and, with the same trend, the values of %CD42b were 28% ± 5.7% (Cyt) and 4.5% ± 1.1% (Feeder) (both $P < 0.05$). The polyploid content (DNA content >4N, %) and percentage of CD42b$^+$ population were lower (Figure 4) for expanded cells differentiated in serum-containing medium (IMDM+10% FBS) compared with the ones differentiated in QBSF-60 ($P < 0.05$), although the CD34$^+$ cells expanded in the same expansion condition (Z9 or T0).

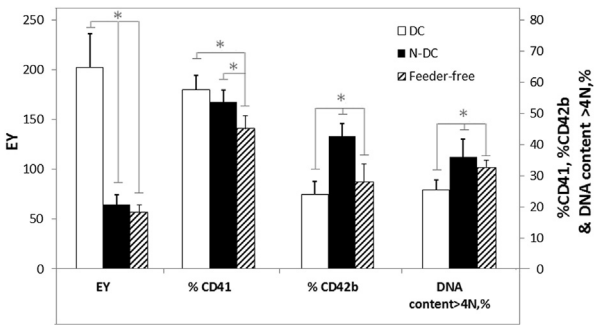

Figure 5. Impact of cell-cell contact on the megakaryocytic differentiation of UCB CD34$^+$-enriched cells. Results are presented in terms of EY, %CD41, %CD42b and DNA content (>4N, %). CD34$^+$-enriched cells were expanded in Z9 cocktail in direct contact (DC) with BM-MSC and were then differentiated with the use of QBSF-60 supplemented with Cyt in DC and non-direct contact (N-DC) with BM-MSC feeder layer or in Feeder-free configuration (Feeder-free). Results are presented as average ± SEM ($n \geq 3$). *$P < 0.05$.

Potential data correlations within the set of parameters of the differentiation stage (EY, % CD41, %CD42b and content of polyploid cells) were analyzed for monotonic (Spearman's method) and linear correlation (Pearson's method) (Supplementary Table S1). Average values of those parameters were plotted against each other (Supplementary Figure S1). Both methods revealed positive correlation between each pair of the above-mentioned parameters. The strongest significant correlation was found between %CD42b and % polyploid cells (>4N, %) (Pearson's coefficient: 0.87; Spearman's coefficient: 0.96; $P < 0.001$ for both methods). A significant correlation was also found between EY and %CD41$^+$ cells (Pearson's coefficient: 0.82, $P = 0.001$; Spearman's coefficient: 0.80; $P = 0.001$). There were significant correlations between %CD42b and %CD41 and between %CD41 and content of polyploid cells (>4N, %; all $P < 0.02$). However, no significant correlation was found between EY and content of polyploid cells ($P > 0.05$) or significant monotonic correlation between EY and %CD42b$^+$ cells ($P > 0.2$).

Impact of direct contact with the BM-MSC feeder layer on the megakaryocytic differentiation of expanded UCB CD34$^+$-enriched cells (stage II)

An additional set of experiments was performed to further investigate the effect of the co-culture with the BM-MSC feeder layer on the fate of the expanded UCB CD34$^+$-enriched cells, namely, to address the role of cell-cell contact between hematopoietic and feeder cells as well as released trophic factors on the megakaryocytic differentiation. Therefore, UCB CD34$^+$-enriched cells were expanded in Z9 cytokine cocktail in direct contact with a BM-MSC feeder layer and were then differentiated toward Mk lineage with the use of QBSF-60 culture medium supplemented with Cyt either (i) in direct contact (DC) with the BM-MSC feeder layer or (ii) in non-direct contact (N-DC) (ie, with the use of a transwell insert with a porous membrane). As a control, the result was compared with the feeder-free configuration (no feeder layer, only Cyt). Cell populations, in direct, non-direct contact and feeder-free conditions, displayed high cell viabilities (>80%) during the whole differentiation culture period (7 days).

Expanded CD34$^+$-enriched cells presented significantly higher EY values when differentiated in DC with the BM-MSC feeder layer than when cultured in N-DC culture configuration (Figure 5, $P < 0.05$). However, similar %CD41 was obtained for different cell-cell contact configurations (DC and N-DC). Co-culture with the feeder layer (DC and N-DC) always resulted in a significantly higher EY and %CD41$^+$ population than feeder-free configuration (Figure 5) ($P < 0.05$). Direct contact with the feeder layer resulted in a significantly less %CD42b$^+$ and polyploid content (DNA content >4N, %) than N-DC culture configuration (Figure 5) ($P < 0.05$). This inhibition was so effective that the values of % CD42b and polyploidy (DNA content >4N, %) for the DC condition were significantly less than for the feeder-free configuration ($P < 0.05$).

Cell characterization

Considering the efficient megakaryocytic differentiation of UCB CD34$^+$-enriched cells observed in DC configuration, UCB CD34$^+$-enriched cells, expanded in the Z9 condition and then differentiated with the use of QBSF-60 supplemented by Cyt in DC with a BM-MSC feeder layer, were collected for characterization. TEM images of culture-derived Mks shows the normal features of a mature Mk with the Dm, granules and adjacent nucleus (Figure 6A). Such mature Mks were able to produce long-branched pro-platelet particles, as shown in an inverted microscopy image (Figure 6B). TEM images confirm that the *ex vivo*—generated platelets produced by the current two-stage protocol displayed the same size and morphological features as in human PB-derived platelets (Figure 6C,D).

Functional assessment of ex vivo—generated platelets

Non-activated platelets from UCB culture-derived, neonatal (fresh-UCB) and adult (PB) sources were compared in terms of light-scattering properties through the use of flow cytometry analysis (Figure 7). Fresh UCB platelet populations had less debris compared with PB-derived platelets (Figure 7A). Therefore, a reference gate was set on the basis of the forward (FSC) and side (SSC) scattering properties of fresh UCB platelets. Logarithmic scale for FSC and SSC of platelets was used, and the different platelets were compared in terms of their geometric mean fluorescence intensities (GMFI) [27]. Flow cytometry analysis demonstrated that there were no significant differences in GMFI values between fresh UCB platelets (FSC: 26 ± 3.7, SSC: 32 ± 3.5) and UCB culture-derived platelets (FSC: 23 ± 2.5, SSC: 35 ± 2.4) ($P > 0.05$, $n \geq 3$). Likewise, PB-derived platelets have FSC and SSC GMFI values of 28 ($n = 1$).

Platelets express both CD41 and CD42b surface markers when non-activated. The expression of these two markers in UCB-derived (fresh and culture-derived) and PB-derived platelets was compared. Histograms, including isotype and surface marker expression, were plotted for each condition by the use of flow cytometry (Figure 7C). All platelets presented a high expression of both CD41 (>89%) and CD42b (>86%) markers.

The activation state of the *ex vivo*—generated UCB-cultured platelets was compared with PB and fresh UCB platelets in terms of CD62P (P-selectin) and CD63 expression (Figure 8), with thrombin used as an agonist. The results demonstrated that the *ex vivo*—produced platelets can be activated, aggregating in the presence of thrombin and having similar activation profiles compared with the other sources. Moreover, no platelet aggregation in the non-activated platelet condition was observed by means of inverted microscopy (Supplementary Figure S2C).

Discussion

The current study evaluated the combinatorial effects of different biological factors, such as stromal/accessory cells and biochemical cues that mimic the

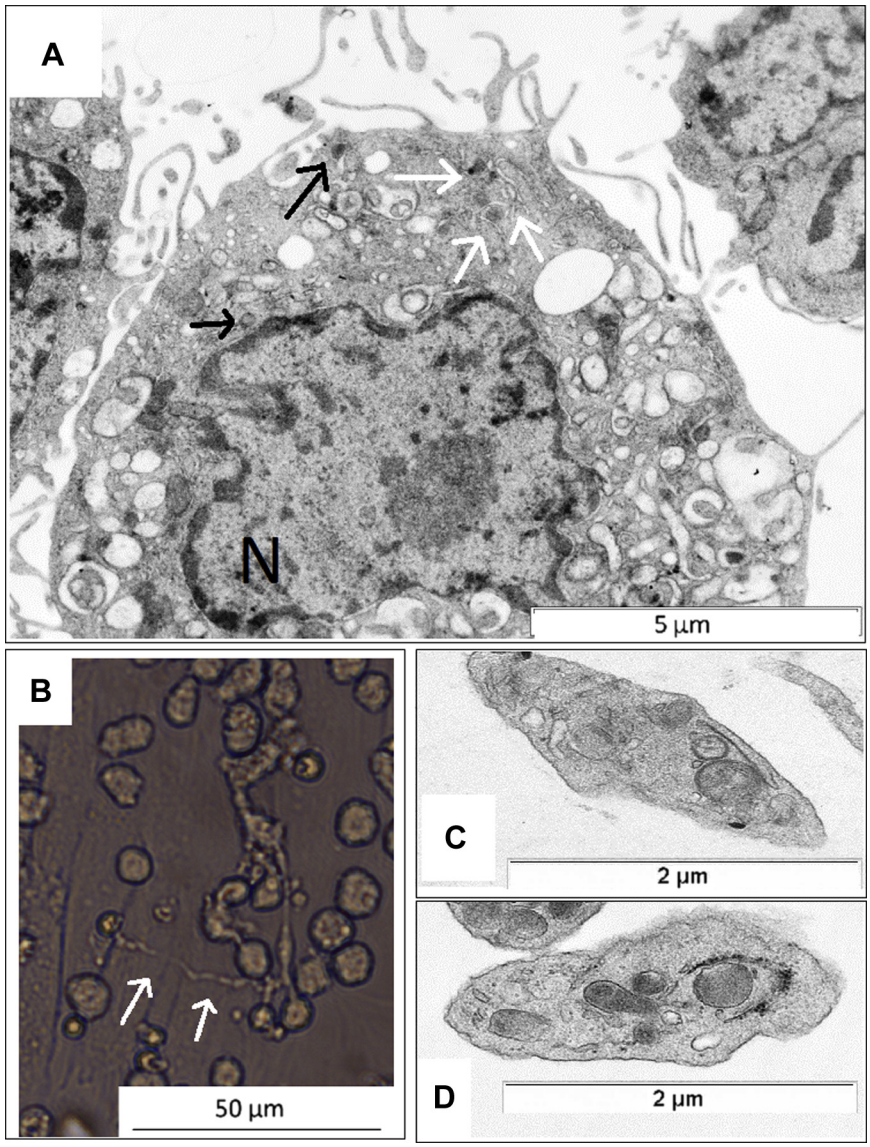

Figure 6. (A) Representative TEM image of a UCB culture-derived Mk. Nucleus (N), demarcation membrane system (Dm; white arrows) and granules (black arrows) are presented. (B) Inverted microscopy image of culture-derived cells. Pro-platelet–bearing particles with extended cytoplasmic branches are identified by white arrows. BM-MSC feeder layer (with spindle-shaped morphology) is presented in the background of the image. TEM images of (C) an *ex vivo*–generated UCB-derived platelet and (D) human PB-derived platelets. All culture-derived images refer to the Mks generated from UCB $CD34^+$-enriched cells expanded with the Z9 cocktail and differentiated with the use of QBSF-60 supplemented by TPO (100 ng/mL) and IL-3 (10 ng/mL) in the direct contact with BM-MSC.

BM niche, on the megakaryocytic commitment of UCB $CD34^+$-enriched cells to establish an efficient megakaryo/thrombopoiesis process *ex vivo*. Different parameters were systematically studied, namely, (i) the impact of TPO addition in the expansion cocktail, (ii) the use of a serum-free medium rather than an FBS-containing medium in the differentiation stage and (iii) a co-culture system with a BM-MSC feeder layer in the differentiation stage, either with or without direct cell-cell contact, in combination with exogenously addition of TPO and IL-3 on the megakaryo/thrombopoiesis process of UCB $CD34^+$-enriched cells.

We hypothesized that TPO inclusion in the expansion cocktail could affect the extent of megakaryocytic commitment on the differentiation stage. Therefore T0, a TPO-free cytokine cocktail, was tested against Z9 during the expansion stage. The FI-$CD34^+$ values obtained experimentally are in agreement with the predicted values of the mathematical model. The higher FI-$CD34^+$ obtained with the use of the Z9 cocktail, compared with the T0 (Figure 2A), corroborates the crucial role of TPO on the proliferation and survival of $CD34^+$ cells during the expansion stage. TPO was shown not only to affect the differentiation and maturation of megakaryocytic cells but also to

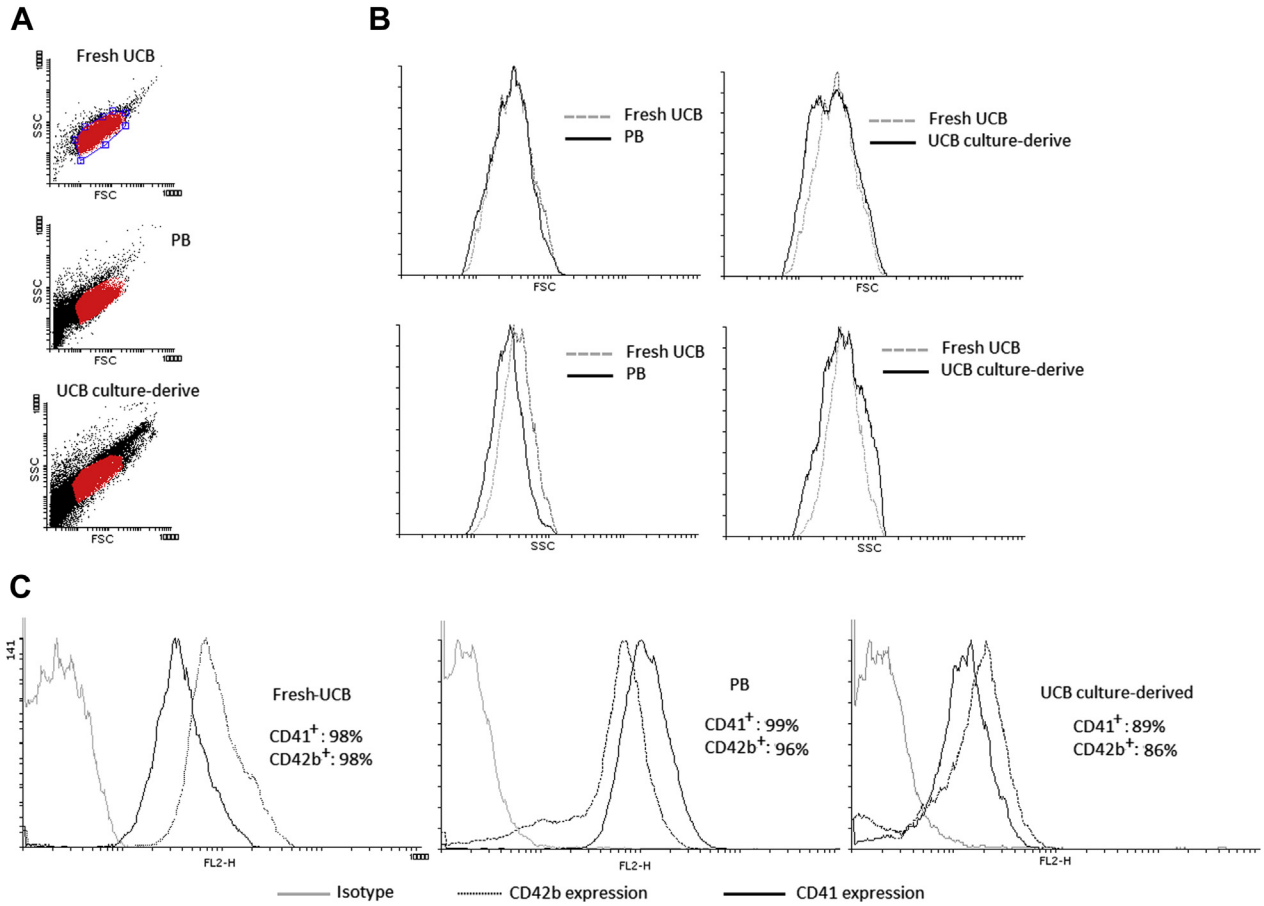

Figure 7. UCB culture-derived platelets have similar light-scattering properties compared with the human PB and fresh UCB platelets in non-activated condition. (A) Representative light-scattering properties of platelets from fresh UCB, PB and UCB culture-derived platelets. (B) Representative forward (FSC; B, top panel) and side scatter (SSC; B, bottom panel) properties of fresh UCB (background gray line), PB (solid line) and UCB culture-derived platelets (solid line) are presented. (C) CD41 (solid line) and CD42b (dotted line) expression of non-activated (Nact) platelets from fresh UCB (left), PB (middle) and UCB culture-derived platelets (right). Isotypes are presented as background gray lines.

increase the rate of Mk proliferation and expansion [28]. In the current study, UCB $CD34^+$-enriched cells expanded by means of the Z9 condition displayed a significantly higher expression of CD41 and CD42b compared with the T0 condition, highlighting a determinant role of TPO as a major driving force for megakaryocytic commitment in the expansion stage. This result is consistent with the previous observations that TPO is the primary regulator of megakaryo/thrombopoiesis *in vivo* and a main non-redundant contributor to the HSC/HPC proliferation, survival and maintenance, especially when combined with other cytokines such as IL-3 or SCF [29,30].

The concentration of Flt-3 is higher in T0 cocktail compared to the Z9 (75 versus 55 ng/mL), which could offer another possible reason for the lower megakaryocytic commitment observed in T0 compared with Z9. In fact, the addition of a high concentration of Flt-3 (100 ng/mL) to cultures of $CD34^+$-enriched mPB cells has been reported to inhibit expansion and development of Mk progenitors and favored monocytic and granulocytic cell expansion [31–33]. On the other hand, it has been reported that the addition of a limited amount of Flt-3 ($\leq$10 ng/mL) to UCB $CD34^+$-enriched cells cultured in a serum-free medium supplemented with TPO and SCF resulted in a 15-fold increase in the number of Mk cells [33]. Therefore, the precise effects of Flt-3 on the megakaryocytic commitment in our culture conditions must be further studied. The values obtained in this study are consistent with the ones previously reported [19] concerning myeloid and early lymphocytic populations for the UCB $CD34^+$-enriched cells co-cultured with BM-MSC and expanded in Z9 cocktail. Overall, in our culture conditions, the differentiation potential of UCB $CD34^+$ cells, expanded by use of the Z9 cocktail, was shifted toward myeloid lineage, as previously reported [19], with significantly higher megakaryocytic content, considerably affecting the differentiation stage. Indeed, expanded cells in the Z9 condition

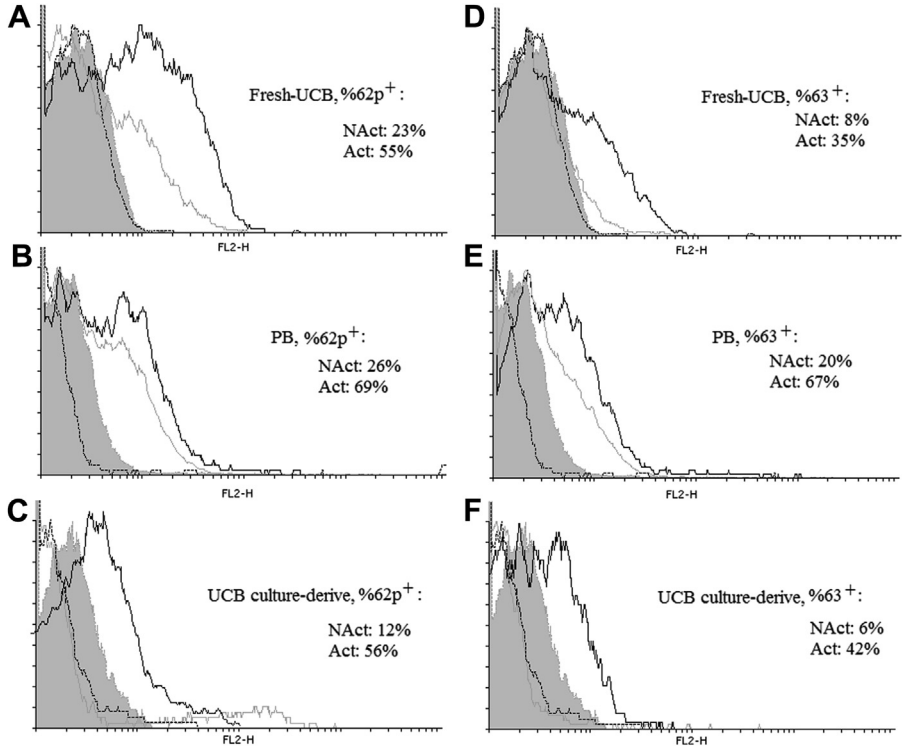

Figure 8. Activation potential of UCB culture-derived platelets. Platelets were activated by addition of thrombin (2 U/L) or kept in a non-activated condition without addition of thrombin (NAct) as a control. CD62p and CD63 expression in activated (Act; solid black line) and non-activated (dotted black line) platelets was measured by means of flow cytometry. Marker expression from fresh UCB (A, D), PB (B, E) and UCB culture-derived platelets (C, F) are presented. Isotype controls are shown for un-activated (filled gray) and activated (solid gray line) platelets. Platelets are identified as $CD41^+$ events that fell in the same scatter properties of fresh UCB platelets.

generated cell populations with significantly higher %CD41, %CD42b, EY and polyploid cells (DNA content >4N, %) than in T0 conditions.

Although FBS has been widely used as a culture medium supplement to support the growth and proliferation of several animal cells, the development of serum-free/animal origin–free culture systems is crucial to meet the requirements of the regulatory agencies [34], improving the batch-to-batch consistency and safety of cellular products [35]. In this study, QBSF-60 was assessed as a medium for differentiation stage, supplemented by Cyt and/or Feeder conditions, resulting in a significantly higher EY, %CD41 and %CD42b and higher polyploid content (>4N, %) when compared with a non-defined medium containing FBS (IMDM+10% FBS). FBS-supplemented (10%) medium contains 1.0 to 2.0 ng/mL of latent transforming growth factor (TGF)-β [36,37]. An inhibitory effect of TGF-β on the maturation and proliferation of Mk cells [38–40], as well as an inhibition of the number and polyploidy of rat Mks, were previously reported [41]. Animal serum is a source of platelet factor 4, which also inhibited megakaryopoiesis and Mk maturation *in vitro* and downregulated platelet count *in vivo* [42,43]. Taken together, the presence of TGF-β and platelet factor 4 in FBS are two possible factors that contribute to an inferior megakaryopoiesis capacity of FBS-containing medium observed in our studies.

Co-culture with feeder layers, including MSC and endothelial cells, has been shown to enhance HSC/HPC survival and expansion *ex vivo* [13,17,44]. In particular, we have demonstrated the benefit of BM-MSC co-culture for the expansion of UCB $CD34^+$-enriched cells [18,19]. It was previously shown that accessory cells such as human MSC from BM, UCB and human BM endothelial cells regulate Mk development and differentiation *in vitro* [15,45,46]. BM stromal cells provide physical support for HSC/HPC and secret several cytokines/growth factors such as granulocyte-macrophage colony-stimulating factor, IL-6 and SCF, which act together with TPO to regulate megakaryocytic commitment [15,23,24]. The results of the current study demonstrated that the highest EY (202 ± 34 Mks per seeded CD 34^+ cells) and $\%CD41^+$ content (58% ± 4.9%) in the differentiation stage were achieved by combination of Cyt addition with Feeder co-culture, with the use of a serum-free medium. The observed increase in %CD41 can result from the expansion of megakaryocytic progenitors and/or differentiation from $CD34^+$ stem/progenitor cells. A significantly higher content of $CD41^+$ and $CD42b^+$ cells with higher polyploid population (>4N, %) but

lower EY were obtained for cell populations co-cultured with Cyt (ie, only cytokines) compared with the Feeder condition (ie, co-culture with BM-MSC without exogenous addition of cytokines) in the differentiation stage. The lower EY but higher % CD42b and polyploid content observed in Cyt differentiation cultures versus Feeder might be the result of different roles of these conditions, with Cyt configuration promoting the growth and maturation of Mks and Mk progenitors, whereas the BM-MSC feeder layer supports the proliferation of HSC/HPC and Mk progenitors. Considering that the BM stromal cells have been reported to ubiquitously synthesize low levels of TPO messenger RNA at a median value of 37×10^{-3} ng/mL [47], the higher TPO concentration (100 ng/mL) in the Cyt condition must have provided a stronger signaling for megakaryocytic commitment.

Statistical tools have provided a deeper insight into the megakaryo/thrombopoiesis process by evaluating the relationship among a set of differentiation parameters. For example, a highly significant correlation between average values of polyploid content (DNA content >4N, %) and %CD42b presented in our culture conditions suggests the existence of a linear relationship between the percentage of polyploid content and maturation level of Mks, meaning that the higher content of polyploid cells presented in the culture, the more likely that the population expresses specific mature Mk markers (ie, CD42b).

The interaction between stromal cells and HSC/HPC, particularly direct cell-cell contact, is important for the maintenance of the multipotential characteristics of HSCs. For instances, we have previously demonstrated that the DC between BM-MSC and UCB $CD34^+$-enriched cells enhances the expansion of $CD34^+$ and $CD34^+$ $CD38^+$ cells [13]. Zweegman *et al.* [48] studied the effect of DC with an irradiated murine fibroblastic cell line on the megakaryocytic differentiation of mPB-derived hematopoietic progenitors (from patients with hematological malignancies). In agreement with our results, these authors found that CD42b expression was lower in DC cultures compared with non-direct contact which indicates that direct cell-cell interactions with the stromal layer inhibited Mk maturation [48]. Production of trophic factors and anchorage to the marrow niche have been suggested to induce Mk proliferation and development. Indeed, EY was significantly higher in direct contact differentiation cultures, whereas there was no difference in terms of CD41 expression for both DC and N-DC conditions. Consistent with other studies [48,49] and in agreement with *in vivo* observations that Mks take a journey from endosteal (direct cell-cell contact) to vascular niches (non-direct cell-cell contact) to complete the thrombopoiesis process [50], direct cell-cell interaction in our *ex vivo* model has resulted in a less mature Mk content.

In the current study, Mks were identified by the analysis of the CD41 surface marker and ploidy level in combination with morphological and ultrastructure observations. After 7 days of differentiation in direct cell-cell contact with BM-MSC, under serum-free conditions supplemented with cytokines, 25% of cells were polyploid (DNA content >4N) when cells were previously expanded for 7 days with the use of the Z9 cocktail. Consistent with this result, Liu *et al.* [24] reported that approximately 25% of Mks (from human UCB), co-cultured with BM-MSC, had higher polyploid content (>4N) after 12 days in culture. UCB-derived Mks generated *ex vivo* in the present study have shown extended Dm and abundant presence of granules in the cytoplasm. These were able to support pro-platelet formation as well as production of functional platelets, as assessed by means of TEM imaging and functional assessment of platelets. On facing abnormality/injury in the vessels wall, platelets are activated by translocation and redistribution of α-granules to the platelet surface; therefore CD62P (P-selectin) and CD63 are expressed on the plasma membrane of activated platelets [51]. Thrombin can induce platelet aggregation by binding fibrinogen and von Willebrand factor on platelet membrane receptors and cross-linking with receptors from other platelets. Our results confirmed that fresh UCB, PB and UCB culture-derived platelets have the ability to be activated and therefore aggregated in the presence of thrombin. Fresh UCB and PB-derived platelets showed a semi-activated state in the absence of any agonists. This pre-activation state was also observed in the small fraction of UCB culture-derived platelets assessed by expression of CD62p (12% $CD62p^+$) and $CD63^+$ (6% $CD63^+$) markers, which might be due to serial centrifugation and/or interaction with ancillary materials during the experimental procedures [27,52,53]. Pre-activation of platelets is observed in many other studies in platelets produced *in vitro* from human sources and also detected in platelet units stored for adult transfusion, which may suggest that the low levels of platelet pre-activation may not significantly decrease the post-transfusion benefits [27,54,55].

In summary, this study reveals that the early Mk commitment mainly driven by TPO during the expansion stage had a significant impact on the whole process (including the differentiation stage) of Mk development and platelet generation. Our results suggest the existence of inhibitory effects of FBS addition to the culture medium on the

megakaryopoiesis *ex vivo* and provide insights on how a BM-MSC feeder layer, used as accessory cells, contribute either through direct cell-cell contact and/or soluble factor signaling to modulate proliferation, maturation and development of Mks as well as platelet production from the expanded UCB progenitors. The protocol developed benefits from the use of UCB progenitors because these are readily available from simple collection procedures, having less risk of provoking graft-versus-host disease and a high proliferative rate *in vitro* compared with adult counterparts [35]. This study provides evidence that UCB $CD34^+$-enriched cells differentiated in the presence of a BM-MSC feeder layer are able to generate polyploid Mks (DNA content >4N) and finally to produce functional platelets through the use of a serum-free system supplemented by cytokines (TPO and IL-3). This work establishes an *in vitro* model that is able to recapitulate the HSC niche by providing a suitable microenvironment to support the efficient megakaryo/thrombopoiesis *ex vivo*. The results of the current study may provide insights for a deeper understanding of different biological factors modulating megakaryo/thrombopoiesis and thus potentially contributing to the establishment of different therapeutic strategies such as transfusion of *ex vivo*—produced platelets to treat thrombocytopenia, co-transplantation of HSC/HPC with culture-derived Mk progenitors or/and mature megakaryocytic cells to avoid delayed platelet recovery in the patients undergoing UCB transplantation.

Acknowledgments

This work was financially supported by the Fundação para a Ciência e Tecnologia (FCT), Portugal, through Project PTDC/EQU-EQU/114231/2009 and MIT-Portugal Program, Bioengineering Systems focus area. This work was co-financed by BIOREG (SOE3/P1/E750)-INTERREG IV-B SUDOE Program with ERDF funds. JH, PA and FCF acknowledge FCT for granting PhD scholarship SFRH/BD/61450/2009, post-doctoral scholarship SFRH/BPD/81861/2011 and research contract IF/00442/2012, respectively. The authors thank Isabel Nogueira (MicroLab, IST, Lisboa, Portugal) for electron microscopy imaging. The authors also extend their acknowledgments to Dr Ana Paula Sousa (Instituto Português do Sangue, Lisboa, Portugal) for donation of PB-derived platelet units.

Disclosure of interests: The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

References

[1] Stroncek DF, Rebulla P. Platelet transfusions. Lancet 2007;370:427–38.
[2] Italiano JE. Unraveling Mechanisms that Control Platelet Production. Semin Thromb Hemost 2013;39:15–24.
[3] Lee EJ, Godara P, Haylock D. Biomanufacture of human platelets for transfusion: Rationale and approaches. Exp Hematol 2014;42:332–46.
[4] Reems JA, Pineault N, Sun S. In Vitro Megakaryocyte Production and Platelet Biogenesis: State of the Art. Transfus Med Rev 2010;24:33–43.
[5] Avanzi MP, Mitchell WB. Ex vivo production of platelets from stem cells. Br J Haematol 2014;165:237–47.
[6] Lu SJ, Li F, Yin H, Feng Q, Kimbrel EA, Hahm E, et al. Platelets generated from human embryonic stem cells are functional in vitro and in the microcirculation of living mice. Cell Res 2011;21:530–45.
[7] Feng Q, Shabrani N, Thon JN, Huo H, Thiel A, Machlus KR, et al. Scalable generation of universal platelets from human induced pluripotent stem cells. Stem Cell Reports 2014;3:817–31.
[8] Thon JN, Mazutis L, Wu S, Sylman JL, Ehrlicher A, Machlus KR, et al. Platelet bioreactor-on-a-chip. Blood 2014;124:1857–67.
[9] British Committee for Standards in Haematology and Blood Transfusion Task Force (Chairman P. Kelsey). Guidelines for the use of platelet transfusions. Brit J Haematol 2003;122:10–23.
[10] Patel SR, Hartwig JH, Italiano JE Jr. The biogenesis of platelets from megakaryocyte proplatelets. J Clin Invest 2005;115:3348–54.
[11] Yoshida M, Tsuji K, Ebihara Y, Muraoka K, Tanaka R, Miyazaki H, et al. Thrombopoietin alone stimulates the early proliferation and survival of human erythroid, myeloid and multipotential progenitors in serum-free culture. Br J Haematol 1997;98:254–64.
[12] Stasi R, Bosworth J, Rhodes E, Shannon MS, Willis F, Gordon-Smith EC. Thrombopoietic agents. Blood Rev 2010;24:179–90.
[13] da Silva CL, Gonçalves R, dos Santos F, Andrade PZ, Almeida-Porada G, Cabral JM. 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. J Tissue Eng Regen Med 2010;4:149–58.
[14] Bilko NM, Votyakova IA, Vasylovska SV, Bilko DI. Characterization of the interactions between stromal and haematopoietic progenitor cells in expansion cell culture models. Cell Biol Int 2005;29:83–6.
[15] Cheng LZ, Qasba P, Vanguri P, Thiede MA. Human mesenchymal stem cells support megakaryocyte and proplatelet formation from $CD34^+$ hematopoietic progenitor cells. J Cell Physiol 2000;184:58–69.
[16] Lewis ID, Almeida-Porada G, Du J, Lemischka IR, Moore KA, Zanjani ED, et al. Umbilical cord blood cells capable of engrafting in primary, secondary, and tertiary xenogeneic hosts are preserved after ex vivo culture in a noncontact system. Blood 2001;97:3441–9.
[17] da Silva CL, Gonçalves R, Crapnell KB, Cabral JM, Zanjani ED, Almeida-Porada G. A human stromal-based serum-free culture system supports the ex vivo expansion/maintenance of bone marrow and cord blood hematopoietic stem/progenitor cells. Exp Hematol 2005;33:828–35.
[18] Goncalves R, Lobato da Silva C, Cabral JM, Zanjani ED, Almeida-Porada G. A Stro-1^+ human universal stromal feeder layer to expand/maintain human bone marrow

hematopoietic stem/progenitor cells in a serum-free culture system. Exp Hematol 2006;34:1353–9.
[19] Andrade PZ, dos Santos F, Almeida-Porada G, da Silva CL, S Cabral JM. Systematic delineation of optimal cytokine concentrations to expand hematopoietic stem/progenitor cells in co-culture with mesenchymal stem cells. Mol Biosyst 2010;6:1207–15.
[20] Lambert MP, Sullivan SK, Fuentes R, French DL, Poncz M. Challenges and promises for the development of donor-independent platelet transfusions. Blood 2013;121:3319–24.
[21] Deutsch VR, Tomer A. Advances in megakaryocytopoiesis and thrombopoiesis: from bench to bedside. Br J Haematol 2013;161:778–93.
[22] Majumdar MK, Thiede MA, Mosca JD, Moorman M, Gerson SL. Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. J Cell Physiol 1998;176:57–66.
[23] Bruno E, Miller ME, Hoffman R. Interacting cytokines regulate in vitro human megakaryocytopoiesis. Blood 1989;73:671–7.
[24] Liu M, Yang SG, Shi L, Du WT, Liu PX, Xu J, et al. Mesenchymal stem cells from bone marrow show a stronger stimulating effect on megakaryocyte progenitor expansion than those from non-hematopoietic tissues. Platelets 2010;21:199–210.
[25] Hatami J, Andrade PZ, Bacalhau D, Cirurgião F, Ferreira FC, Cabral JMS, et al. Proliferation extent of $CD34^+$ cells as a key parameter to maximize megakaryocytic differentiation of umbilical cord blood-derived hematopoietic stem/progenitor cells in a two-stage culture protocol. Biotechnology Reports 2014;4:50–5.
[26] Mattia G, Vulcano F, Milazzo L, Barca A, Macioce G, Giampaolo A, et al. Different ploidy levels of megakaryocytes generated from peripheral or cord blood $CD34^+$ cells are correlated with different levels of platelet release. Blood 2002;99:888–97.
[27] Panuganti S, Schlinker AC, Lindholm PF, Papoutsakis ET, Miller WM. Three-stage ex vivo expansion of high-ploidy megakaryocytic cells: toward large-scale platelet production. Tissue Eng Part A 2013;19:998–1014.
[28] Kaushansky K. Thrombopoietin: understanding and manipulating platelet production. Annu Rev Med 1997;48:1–11.
[29] Kaushansky K, Broudy VC, Lin N, Jorgensen MJ, McCarty J, Fox N, et al. Thrombopoietin, the Mp1 ligand, is essential for full megakaryocyte development. Proc Natl Acad Sci U S A 1995;92:3234–8.
[30] Sitnicka E, Lin N, Priestley GV, Fox N, Broudy VC, Wolf NS, et al. The effect of thrombopoietin on the proliferation and differentiation of murine hematopoietic stem cells. Blood 1996;87:4998–5005.
[31] Case J, Hicks C, Trickett A, Kwan YL, Manoharan A. The expansion of megakaryocyte progenitors from $CD34^+$-enriched mobilized peripheral blood stem cells is inhibited by Flt3-L. J Interferon Cytokine Res 2006;26:76–82.
[32] Sigurjonsson OE, Gudmundsson KO, Haraldsdóttir V, Rafnar T, Gudmundsson S. Flt3/Flk-2-ligand in synergy with thrombopoietin delays megakaryocyte development and increases the numbers of megakaryocyte progenitor cells in serum-free cultures initiated with $CD34^+$ cells. J Hematother Stem Cell Res 2002;11:389–400.
[33] Proulx C, Boyer L, Hurnanen DR, Lemieux R. Preferential ex vivo expansion of megakaryocytes from human cord blood $CD34^+$-enriched cells in the presence of thrombopoietin and limiting amounts of stem cell factor and Flt-3 ligand. J Hematother Stem Cell Res 2003;12:179–88.
[34] Reflection paper on stem cell-based medicinal products. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/02/WC500101692.pdf; 2011.
[35] Andrade PZ, Santos FD, Cabral JM, da Silva CL. Stem cell bioengineering strategies to widen the therapeutic applications of haematopoietic stem/progenitor cells from umbilical cord blood. J Tissue Eng Regen Med 2013. http://dx.doi.org/10.1002/term.1741.
[36] Oida T, Weiner HL. Depletion of TGF-beta from fetal bovine serum. J Immunol Methods 2010;362:195–8.
[37] Danielpour D, Kim KY, Dart LL, Watanabe S, Roberts AB, Sporn MB. Sandwich enzyme-linked immunosorbent assays (SELISAs) quantitate and distinguish two forms of transforming growth factor-beta (TGF-beta 1 and TGF-beta 2) in complex biological fluids. Growth Factors 1989;2:61–71.
[38] Fortunel NO, Hatzfeld A, Hatzfeld JA. Transforming growth factor-beta: pleiotropic role in the regulation of hematopoiesis. Blood 2000;96:2022–36.
[39] Jackson H, Williams N, Westcott KR, Green R. Differential effects of transforming growth factor-beta 1 on distinct developmental stages of murine megakaryocytopoiesis. J Cell Physiol 1994;161:312–8.
[40] Berthier R, Valiron O, Schweitzer A, Marguerie G. Serum-free medium allows the optimal growth of human megakaryocyte progenitors compared with human plasma supplemented cultures: role of TGF beta. Stem Cells 1993;11:120–9.
[41] Kuter DJ, Gminski DM, Rosenberg RD. Transforming growth factor beta inhibits megakaryocyte growth and endomitosis. Blood 1992;79:619–26.
[42] Han ZC, Sensébe L, Abgrall JF, Brière J. Platelet factor 4 inhibits human megakaryocytopoiesis in vitro. Blood 1990;75:1234–9.
[43] Lambert MP, Rauova L, Bailey M, Sola-Visner MC, Kowalska MA, Poncz M. Platelet factor 4 is a negative autocrine in vivo regulator of megakaryopoiesis: clinical and therapeutic implications. Blood 2007;110:1153–60.
[44] Kelly SS, Sola CB, de Lima M, Shpall E. Ex vivo expansion of cord blood. Bone Marrow Transplant 2009;44:673–81.
[45] Gao L, Chen XH, Feng YM, Zhang X, Yu SC, Gao L, et al. Human umbilical cord blood-derived stromal cells: multifaceted regulators of megakaryocytopoiesis. Cell Cycle 2010;9:1342–53.
[46] Rafii S, Shapiro F, Pettengell R, Ferris B, Nachman RL, Moore MA, et al. Human bone marrow microvascular endothelial cells support long-term proliferation and differentiation of myeloid and megakaryocytic progenitors. Blood 1995;86:3353–63.
[47] Guerriero A, Worford L, Holland HK, Guo GR, Sheehan K, Waller EK. Thrombopoietin is synthesized by bone marrow stromal cells. Blood 1997;90:3444–55.
[48] Zweegman S, Veenhof MA, Debili N, Schuurhuis GJ, Huijgens PC, Dräger AM. Megakaryocytic differentiation of human progenitor cells is negatively influenced by direct contact with stroma. Leukemia 1999;13:935–43.
[49] Zweegman S, Veenhof MA, Huijgens PC, Schuurhuis GJ, Dräger AM. Regulation of megakaryocytopoiesis in an in vitro stroma model: preferential adhesion of megakaryocytic progenitors and subsequent inhibition of maturation. Exp Hematol 2000;28:401–10.
[50] Nagahisa H, Nagata Y, Ohnuki T, Osada M, Nagasawa T, Abe T, et al. Bone marrow stromal cells produce thrombopoietin and stimulate megakaryocyte growth and maturation but suppress proplatelet formation. Blood 1996;87:1309–16.
[51] Kamath S, Blann AD, Lip GY. Platelet activation: assessment and quantification. Eur Heart J 2001;22:1561–71.
[52] Bagamery K, Kvell K, Landau R, Graham J. Flow cytometric analysis of CD41-labeled platelets isolated by the rapid, one-

step OptiPrep method from human blood. Cytometry A 2005;65:84–7.
[53] Michelson AD, Barnard MR, Krueger LA, Frelinger AL 3rd, Furman MI. Evaluation of platelet function by flow cytometry. Methods 2000;21:259–70.
[54] Sullenbarger B, Bahng JH, Gruner R, Kotov N, Lasky LC. Prolonged continuous in vitro human platelet production using three-dimensional scaffolds. Exp Hematol 2009;37:101–10.
[55] Chen TW, Yao CL, Chu IM, Chuang TL, Hsieh TB, Hwang SM. Large generation of megakaryocytes from serum-free expanded human CD34$^+$ cells. Biochem Biophys Res Commun 2009;378:112–7.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcyt.2014.12.010.