DIFFERENCES AMID BONE MARROW AND CORD BLOOD HEMATOPOIETIC STEM/PROGENITOR CELL DIVISION KINETICS

Cláudia Lobato da Silva[1,2], Raquel Gonçalves[1,2], Christopher D. Porada[1], João L. Ascensão[3], Esmail D. Zanjani[1], Joaquim M.S. Cabral[2], Graça Almeida-Porada[1*]

[1]Department of Animal Biotechnology, University of Nevada, Reno, NV, USA

[2]IBB – Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Portugal

[3] V.A. Medical Center and the George Washington University School of Medicine, Washington DC USA

[*]Graça Almeida-Porada, M.D., Ph.D.
Department of Animal Biotechnology
University of Nevada, Reno
Mail Stop 202
Reno NV 89557-0104
Phone:(775)-784-1705
Fax: (775)-784-1375
e-mail: galmeida@cabnr.edu

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ABSTRACT

Phenotypically identical human hematopoietic stem/progenitor cells (HSC) isolated and characterized based upon specific patterns of CD34 and CD38 expression were found to be functionally heterogeneous, raising the possibility that reversible expression of these antigens occurs during cellular activation and/or proliferation. In these studies, we combined PKH67 tracking with CD34/CD38 immunostaining to compare cell division kinetics between human bone marrow (BM) and cord blood (CB)-derived HSC expanded in a serum-free/stromal-based system for 14 days(d), and correlated CD34 and CD38 expression with the cell divisional history. CB cells began dividing 24h earlier than BM cells, and significantly higher numbers underwent mitosis during the time in culture. By d10, over 55% of the CB-cells reached the 9^{th} generation, whereas BM-cells were mostly distributed between the 5^{th} and 7^{th} generation. By d14, all CB cells had undergone multiple cell divisions, while 0.7-3.8% of BM $CD34^+$ cells remained quiescent. Furthermore, the percentage of BM cells expressing CD34 decreased from 60.8±6.3 to 30.6±6.7% prior to initiating division, suggesting that downmodulation of this antigen occurred before commencement of proliferation. Moreover, with BM, all primitive $CD34^+CD38^-$ cells present at the end of culture arose from proliferating $CD34^+CD38^+$ cells that downregulated CD38 expression, while in CB, a $CD34^+CD38^-$ population was maintained throughout culture. These studies show that BM and CB cells differ significantly in cell division kinetics and expression of CD34 and CD38, and that the inherent modulation of these antigens during *ex vivo* expansion may lead to erroneous quantification of the stem cell content of the expanded graft.

INTRODUCTION

Identification and characterization of human hematopoietic stem/progenitor cells (HSC) has relied upon the detection of specific patterns of antigenic expression such as CD34 (Civin et al., 1984) and CD38 (Breard et al., 1980). Since seminal studies showed that autologous BM $CD34^+$ cells were able to engraft in baboons (Berenson et al., 1988) and in humans (Berenson et al., 1991), CD34 expression became the hallmark for selection of human HSC, and $CD34^+$ cells have subsequently been widely used both for human autologous and allogeneic transplantations, resulting in a rapid reconstitution of all blood lineages (Shpall et al., 1994; Stewart et al., 2001).

While the precise biological role of CD34 is still unknown, functions such as adhesion, homing, proliferation and differentiation have been attributed to this molecule (Baumhueter et al., 1994; Drew et al., 2005; Healy et al., 1995; Lanza et al., 2001; Salati et al., 2008). Despite these proposed roles, a definite association between CD34 expression and biological function has not thus far been found, and the question remains whether or not all human HSC express CD34 (Dao et al., 2003; Nakamura et al., 2007). In addition, it was found that CD34 expression by human stem cells is a reversible process, and that CD34 negative cells also contain HSC progenitors with clonogenic potential *in vitro* and long-term multilineage hematopoietic reconstitution ability *in vivo* (McKenzie et al., 2007; Tajima et al., 2001; Zanjani et al., 1998; Zanjani et al., 2003). This apparent controversy regarding the presence or absence of CD34 on HSC has been explained by the assumption that CD34 expression reflects activation, growth commencement, and/or cycling state of the primitive cells (Dooley et al., 2004; McKenzie et al., 2007; Sato et al., 1999). Thus, these collective findings have not precluded the clinical use of $CD34^+$ cells in HSC transplantation, but rather, have initiated a series of studies aimed at correlating CD34 modulation with stem cell function.

In addition to CD34, another marker that has been associated with stem cell differentiation and growth enhancement (Deaglio et al., 2002; Zocchi et al., 1998), CD38, has also been linked to

regulation of cell cycle and/or proliferation, with quiescent HSC increasing CD38 expression prior to entry into cell cycle (Dooley et al., 2004; McKenzie et al., 2007; Tajima et al., 2001). These patterns of expression and activation of CD34 and CD38 have now been shown to occur similarly in HSC from a variety of sources, including bone marrow (Tajima et al., 2001), cord blood (McKenzie et al., 2007), and mobilized peripheral blood (Dooley et al., 2004). However, a direct comparison between proliferative responses (*i.e.* cell division) and patterns of expression of CD34 and CD38 amongst different HSC sources during *in vitro* expansion has not yet been described. Since important functional differences have been demonstrated between CB, BM, and mobilized PB HSC (Hao et al., 1995; Holyoake et al., 1999; Theunissen and Verfaillie, 2005), the understanding of the type, frequency, and maturation status of dividing HSC from each cell source could help defining strategies in stem cell expansion, optimize graft composition and determine a window during which HSC are suitable for targeting with gene therapy (Luens et al., 1998; Traycoff et al., 1995).

In these studies, we combined cell tracking with the membrane dye PKH67 and immunostaining for CD34 and CD38 antigens to characterize the differences in the cell division kinetics of $CD34^+$ enriched cells from BM and CB cultured in a serum-free stromal-based culture system (da Silva et al., 2005) and correlated CD34 and CD38 expression with the cell divisional history. We found that BM and CB cells differed significantly in cell division kinetics and expression of CD34 and CD38 during *in vitro* expansion. Specifically, we showed that BM $CD34^+$ cells down-modulated the CD34 antigen before initiation of cell division, upregulating expression again after commencing cycling. In addition, we demonstrated that the resulting BM-derived $CD34^+CD38^-$ population present at the end of culture arose from cells that had become $CD38^+$ during the first expansion period, while in CB cultures, a $CD34^+CD38^-$ population was maintained throughout culture. These studies may have significant implications in the quantification of the stem cell content in hematopoietic grafts to be used in a transplant setting.

MATERIAL AND METHODS

Human donor cell preparation. Heparinized human bone marrow (BM) was obtained from healthy donors after informed consent, according to guidelines from the Office of Human Research Protection at the University of Nevada, Reno. Umbilical cord blood (CB) samples were a kind gift of Crioestaminal – Saúde e Tecnologia, S.A., (Cantanhede, Portugal). After obtaining maternal donor consent, fresh blood that was going to be discarded was collected from the umbilical cord vein using the method previously described (Chrysler GR, 2004). Low density BM mononuclear cells (BM MNC) or CB mononuclear cells (CB MNC) were separated by a Ficoll density gradient (1.077 g/ml) (Sigma, St Louis, MO, USA) and washed twice in Iscove's Modified Dulbecco's Medium (IMDM), (Gibco Laboratories, Grand Island, NY, USA). BM MNC and CB MNC from each donor were enriched for $CD34^+$ cells using magnetic cell sorting (Miltenyi Biotec Inc. Auburn, CA, USA).

Human bone marrow stromal cell cultures. Isolated BM MNC obtained from healthy human donors were magnetically sorted based on Stro-1 positivity as previously described (Gonçalves et al., 2006). The mesenchymal stem cells obtained (MSC) ($Stro\text{-}1^+$, $Gly\text{-}A^-$, $CD45^-$) were then cultured in gelatin-coated T-25 flasks with MSCGM media (Poietics™, BioWhittaker, USA). Stromal layers were obtained after culture for 10 days and then γ-irradiated (14 Gy) on a ^{137}-Cs source. The irradiated stromal layers were maintained at 37 °C under 5 % CO_2 in humidified air and used within 1-5 days to support hematopoietic expansion.

PKH67 fluorescent dye labeling. Freshly isolated $CD34^+$ enriched cells were labeled with the lipophilic membrane dye, which fluoresces in the FITC channel, PKH67 (Sigma), according to the manufacturer's instructions. PKH dyes consist of flurophores attached to an aliphatic carbon backbone that bind irreversibly to the lipid layer of cell membrane (Huang et al., 1999). Since the dye is stably incorporated in the cell membrane, the molecules are equally distributed between daughter cells during cell division; each generation of cells is therefore half as fluorescent as the previous one allowing the determination of both the time point at which cultured cells commence

cell division and precise quantitation of the number of divisions the cells have undergone at any point in culture (Almeida-Porada et al., 2000; Brandt et al., 1999; Huang et al., 1999).

Ex vivo expansion of CD34$^+$ enriched cells. BM (5.3-8.9×10^6 cells) and CB (3.7-5.2×10^5 cells) CD34$^+$ enriched cells labeled with PKH67 were cultured in T-25 flasks (5 ml) for 14 days in QBSF-60 serum free medium (Quality Biological, Gaithersburg, MD, USA) over irradiated human stromal layers with the following cytokines: stem cell factor (SCF) (100 ng/ml), basic-fibroblast growth factor (bFGF) (5 ng/ml), leukemia inhibitory factor (LIF) (10 U/ml) (Leinco Technologies, Inc. St. Louis, MO) and Fms-like tyrosine kinase 3 ligand (Flt-3) (100 ng/ml) (Peprotech, Rocky Hill, NJ, USA). Cell sampling was performed daily throughout the culture period; cultures were fed every 2-3 days by adjusting to initial volume (5 ml) with fresh medium in order to compensate for the volume harvested with daily cell sampling.

Evaluation of proliferation and phenotypic analysis. Cultures were evaluated for cell numbers and viability by using Trypan Blue Stain 0.4% solution (GibcoBRL). Cells were analyzed by flow cytometry for stem/progenitor cell content using monoclonal antibodies against CD34 and CD38 (Becton Dickinson Immunocytometry Systems, San Jose, CA), as well as PKH dye fluorescence intensity as previously described (Almeida-Porada et al., 2000; Brandt et al., 1999; Huang et al., 1999). Flow cytometric analyses were performed in a FACScalibur cytometer (Becton Dickinson, San Jose, CA, USA) at each time point of culture, by incubating harvested cells with different fluorescent conjugated monoclonal antibodies, including appropriate isotype controls, at room temperature for 15 minutes. The cells were then washed in PBS containing 0.1% sodium azide (Sigma), and fixed with 2% paraformaldehyde (Sigma). A minimum of 10000 events was collected for each sample. Undivided (PKH^{High}) and divided (PKH^{Low}) cell populations were identified by differences in PKH dye fluorescence intensity, and analyzed for CD34 and CD38 expression by a multiple gating strategy using the CellQuest software (Becton Dickinson).

Study of Cell Proliferation by analysis of FACS data using ModFit software. FACS data were analyzed using the Proliferation Wizard module of the *ModFit* software (Becton Dickinson), since it allows the determination of the number of cellular generations in culture. This program is based on histograms of fluorescence intensity and applies deconvolution algorithms to determine the proportion of proliferating cells at each cell division (Givan et al., 1999). The standard options were chosen to perform analysis on the gated population based on forward-scatter and side-scatter properties (Liu et al., 2006). The Generation Number was set at 9. For each experiment, the Parent Generation was set as the median fluorescent intensity using the respective sample for day 0.

Statistical analysis of data. Results are expressed as mean ± standard error of the mean (SEM). The two-sided Wilcoxon Rank Sum Test was used to perform statistical analyses for BM and CB comparison, as well as within BM and CB experiments, considering a p value <0.05 to be significant. In particular, the statistical test was used to determine when cell division begins, *i.e.* what constitutes a true generation of cellular proliferation, within BM and CB experiments, based on *ModFit* data.

RESULTS

BM and CB cells exhibit distinctive divisional histories in vitro.

We have previously described a human stromal-based serum free culture system that allows the effective expansion of BM and CB-$CD34^+$ cells *in vitro,* while maintaining their clonogenic and engrafting potential (da Silva et al., 2005; Gonçalves et al., 2006). We reasoned that, since the majority of transplants performed today using enriched hematopoietic progenitor cells are performed using $CD34^+$ enriched cells, the derivation of methods for expanding the number of long-term engrafting cells within this population would be of direct clinical utility. Our cocktail of cytokines includes Flt-3 and SCF, known to positively influence self-renewal, proliferation and preservation from apoptosis of the more primitive hematopoietic cells, especially in synergy with several other

cytokines, as well as LIF, which acts indirectly on HSC by mechanisms mediated by stromal cells and bFGF to support the maintenance of the feeder layer under serum-free conditions (da Silva et al., 2005). This culture method was thus suitable for the present studies, in which we aimed to investigate and characterize the differences between human BM- and CB-HSC divisional histories. To accomplish this objective, CB and BM $CD34^+$ enriched cell populations were labeled with PKH67 and cultured over stromal layers for 14 days. We then performed flow cytometric evaluation of the gradual loss of PKH dye fluorescence intensity during the time in culture and analysis with *ModFit* software to determine initiation and kinetics of cell division of CB and BM cells. Viabilities of cultured PKH-labeled cells always exceeded 94 and 92 % for BM and CB cells, respectively.

Figure 1 shows representative dot plot analyses of PKH67 *versus* CD34 expression for BM (A) and CB (B) cell populations during time in culture (n=4). Arbitrary quadrants for both cell populations were established at day 0 using freshly isolated, live-gated cells. In order to calculate the percentage of cells in each divisional cohort, we set BM and CB cells, prior to culture, as the parent generation, and *ModFit* software was then employed to automatically calculate the percentage of cells in each generation, up to the 10^{th} generation.

Analysis of the percentage of undivided cells, *i.e.*, belonging to the first generation (Parent Generation), for both BM and CB cells showed that CB cells had already undergone cell division after 2 days of culture, with a statistically significant decrease in the percentage of undivided cells between day 0 (96.2±0.8%) and day 2 (59.6±12.3%) ($p<0.05$) (Figure 2). By contrast, in the cultured BM cells there was at least a 24h delay in the initiation of cellular division, with the percentage of undivided cells remaining largely unchanged from day 0 (96.9±0.4%) to day 2 (86.9±9.9%). It was only by day 3 that the percentage of undivided BM cells decreased significantly (38.2±7.6%) ($p<0.05$) (Figure 2).

Figure 3 (A-G) shows the divisional history of BM (black bars) and CB (white bars) cells in culture. Each bar represents the percentage of cells in each doubling generation, which is the precise number of divisions (Generation 1 to 10) that BM and CB have undergone at each point in culture.

By day 2 of culture (Figure 3B), 36±8% of CB cells had already reached generation 2, and by day 3 (Figure 3C) 42.2±5.2% had reached the 3rd and 9.3±4.3% the 4th generation. By contrast, most of the BM cells by day 2 still belonged to Generation 1, and by day 3, 38.2±7.6% of BM cells continued to be in the first generation. While 23.1±7.6% of BM cells had reached Generation 2, and 36.9±19.0% were in Generation 3, almost none had reached Generation 4 (<1%) by day 3. At day 4, the percentage of cells in CB that had reached the 5th generation was 15.0±8.7%, whereas only 2.2±0.8% had reached this generation in BM cultures (Figure 3D). Furthermore, at this time point, 3.6±2.6 and 3.7±2.8% of the CB cells had already reached Generations 6 and 7, respectively. The differences in kinetics of division between BM and CB cells were even more pronounced at day 8 (Figure 3F), when almost no CB cells remained undivided (less than 0.08% of cells belonging to Parent Generation) and more than 24.3±9.2% had already reached the 9th generation. By contrast, at this same time point, most of the BM cells were only distributed between the 5th and 6th generation, 32.0±2.3 and 38.2±12.0%, respectively. At day 10 (Figure 3G), no CB cells remained undivided and more than 56.0±5.6% had reached the 9th generation, whereas for BM cells, less than 5% had attained Generation 8, and most of the cells were still in generation 6 (47±1.8%) with a smaller percentage being in Generation 7 (25±1.4%).

Figure 3H depicts only BM cells at day 12 and 14 of culture, since computation of results using *ModFit* software was not possible for CB cells as the percentage of Generation 1 cells had become 0 after day 10. By day 14, 41.0±4.1% of BM cells belonged to Generation 7 and 22.4± 5.6% to Generation 8. By contrast with CB, even after 14 days of culture, 0.7-3.8% of BM cells remained in the Parent Generation, *i.e*. undivided.

BM and CB cultures display different modulation of CD34$^+$ prior to/during initiation of cell division

We next evaluated the correlation between cell division and expression of the CD34 antigen. As previously referred to, Figure 1 depicts flow cytometric analysis of a representative experiment for CD34$^+$ enriched cells from BM (A) and CB (B), evidencing gradual loss of PKH fluorescence intensity (y-axis) during time in culture among CD34$^+$ and CD34$^-$ cells (CD34 expression along x-axis). The percentage of cells expressing CD34 within the undivided, *i.e.* quiescent, and dividing fractions for BM and CB experiments is represented in Figure 4A and 4B, respectively. While BM cells did not divide significantly between day 0 and 2 (as depicted above), the percentage of BM cells expressing CD34 decreased from 60.8±6.3% at day 0 to 30.6±6.7% at day 2 ($p < 0.05$) (n=4). Furthermore, analysis of the CD34 global mean fluorescence intensity in BM cells showed a decrease from 63.0±4.5 at day 0 to 57.4±4.2 at day 2 (Table 1). By day 3, after cell division had occurred, the total percentage of cells expressing CD34 increased to almost 50%, with 27.3±7.8% and 22.3±7.0% of the cells being positive for CD34$^+$ in the non-divided and in the divided cell fraction, respectively (Figure 4A). This data suggests that there is down-regulation of expression of CD34 in BM cells prior to initiation of cell division. By contrast, in CB, the total percentage of cells expressing CD34 remained relatively constant during the first days of culture (Figure 4B). At day 0, 90.6±2.14% of CB cells expressed CD34, and at day 2, 68.1±7.0% of the cells in the undivided fraction and 17.3±6.5% in the divided fraction expressed CD34$^+$ (n=4). Furthermore, since CD34 global mean fluorescent intensity in CB cells showed a significant increase from day 0 (129±21.3) to day 2 (228±61.7), it is possible that CB cell expression of CD34 was upregulated during the first 2 days of culture (Table 1).

Global mean intensity of CD34 expression decreased progressively with time in culture as CB and BM cells divided; however, after 8 days in culture, the remaining BM and CB CD34$^+$ cells maintained their CD34 global mean intensity until the end of culture (Table 1).

Of note is that after 14 days, 2.0±1.3% of the total BM $CD34^+$ population had remained quiescent, never dividing throughout the entire culture period (Figure 4A), whereas during the same period of time all of the CB $CD34^+$ cells had undergone cell division (Figure 4B).

Differential CD38 expression during division of $CD34^+$ cells in BM and CB cultures

Having established the dynamics of CD34 expression during expansion of CB- and BM-derived cells in culture, we next evaluated differences in CD38 expression of the $CD34^+$ fraction from these two cell sources during the 14-day culture period. The results of these experiments are summarized in Figure 5. As can be seen in this figure, the percentages of $CD34^+CD38^-$ cells in BM (Figure 5A) and CB (Figure 5B) were essentially equivalent at day 0 of culture (2.24±0.07 and 1.73±0.70%, respectively). By day 2, in the CB-derived cultures, the percentage of $CD34^+CD38^-$ cells increased to 24.5±4.5% with only 4.25±1.8% of these becoming PKH low as a result of cell division (black bars Figure 5B). The majority of CB $CD34^+CD38^-$ present at day 2 of culture (24.5±4.5% of total population) were still PKH high (white bars Figure 5B), seeming to have emerged from down-regulation of the CD38 antigen as shown in Figure 6B. After day 2, the CB $CD34^+CD38^-$ cells divided steadily throughout the 14-day course of the experiment, such that essentially no undivided $CD34^+CD38^-$ cells remained in these cultures after day 6, with all of the 32.1±7.0% of $CD34^+CD38^-$ cells that were present at day 14 being comprised of divided cells. Thus, in CB, all of the $CD34^+CD38^-$ cells present at the end of culture were derived from cells that had undergone division while maintaining a $CD34^+CD38^-$ phenotype (Figure 6B). By contrast, the number of $CD34^+CD38^-$ BM-derived cells did not increase significantly between day 0 (2.24±0.07%) and day 2 (3.78 ±0.54%). Of note is the fact that, in BM cultures, $CD34^+CD38^-$ cells were present at very low levels between days 3 and 8 (Figure 5A). During this period of time, all the $CD34^+$ cells in culture also expressed the CD38 antigen and this population underwent consecutive divisions between days 3 and 8 (Figures 5A and 6A). However, at day 10 of culture, 6.59± 2.3% of dividing $CD34^+CD38^+$ cells became $CD34^+CD38^{-/dim}$ (Figure 6A), and by day 12 and 14, the percentages of $CD34^+CD38^{-/dim}$

cells had increased to 13.1±0.8% and 10.9±1.5%, respectively. These data thus suggest that the BM-derived $CD34^+$ cells passed through a requisite phase of CD38 positivity during division, only to revert to a $CD34^+CD38^-$ phenotype at later points in culture.

DISCUSSION

In the present studies, we combined PKH67 tracking with CD34/CD38 immunostaining to compare cell division kinetics between human bone marrow (BM) and cord blood (CB)-derived HSC and correlated CD34 and CD38 expression with the cell divisional history. Published studies using light and phase-contrast microscopy demonstrated that there is no significant difference in cell morphology between PKH-labeled and unlabeled HSC from fetal liver, BM and CB (Huang et al., 1999), and that PKH staining does not adversely affect early and late progenitor cell function (Traycoff et al., 1995; Young et al., 1996). In agreement with these prior reports, herein, BM and CB $CD34^+$ enriched cells that were labeled with PKH were expanded with equal efficiency and behaviorally indistinguishable from their unlabeled counterparts that we studied in detail previously (da Silva et al., 2005; Gonçalves et al., 2006), thus verifying that the results obtained in these studies were not influenced by PKH labeling. In addition, in order to accurately determine the appropriate threshold beyond which the decrease observed in PKH intensity corresponded to true cell division, we used a non-parametric statistical analysis based on flow cytometry data generated by the Proliferation Wizard of *ModFit* software. We found that at day 0, before CB and BM cultures were initiated, a negligible percentage of cells were located at the peak intensity for the 2^{nd}-3^{rd} generation (Figure 3A), due perhaps to either the slightly less than 100% labeling efficiency of those cells, or dilution of the PKH labeling as a result of dye leaking to the feeder cells. Nevertheless, throughout the remainder of the time in culture, *ModFit* software fitted the data into clearly visible peaks corresponding to cellular division numbers. We found that HSC derived from CB started cell division before day 2 of the culture, with a statistically significant decrease (p <0.05) in the

percentage of undivided cells within this time interval, and that BM cells only started intense cell division between days 2 and 3 of culture. CB continued to divide rapidly during the time in culture, such that by day 10, no CB cells remained undivided, and more than 56±5.6% had reached the 9^{th} generation, whereas for BM cells, less than 5% had attained Generation 8, and most of the cells (47±1.8%) were still in Generation 6. Our results are in agreement with other studies that compared BM and CB derived HSC and showed that the latter exhibit a higher proliferation and expansion potential, possibly due to their ability to exit more rapidly from G_0-G_1, (Mayani and Lansdorp, 1998; Traycoff et al., 1994) and/or autocrine production of hematopoietic cytokines (Schibler et al., 1994; Watari et al., 1994). Alternatively, the functional disparity seen between BM and CB HSC could be attributed to the longer telomere length seen in CB cells, which may correlate with higher replicative capacity (Lansdorp, 1995a; Lansdorp, 1995b; Vaziri et al., 1994)

Since we have shown that all the CB-derived cells underwent at least one cell division in the first week of culture of culture while only a small percentage of the BM-derived HSC underwent division during the same period of time, and even those cells that did cycle, the turnover was slower, these results have important implications for HSC-based gene therapy strategies. They suggest that HSC derived from CB could be more efficiently transduced with viral vectors requiring mitosis for genomic integration. Our results are thus consistent with, and provide a possible explanation for, the findings of Moritz and collaborators (Moritz et al., 1993), who reported that CB stem/progenitor cells are more efficiently transduced via retroviral-mediated gene transfer than adult BM-derived cells. Combining our findings with those of Moritz and co-workers suggests that CB may have inherent advantages in some protocols of human gene therapy.

Our studies also show that, prior to culture, $CD34^+CD38^-$ cells represent a small fraction of total CB $CD34^+$ cells and that, by day 2, the percentage of $CD34^+CD38^-$ cells increased in the CB-derived cultures to almost 25% of the total, with only 4.25±1.8% of these cells becoming PKH-low as a result of cell division. Thus, the majority of $CD34^+CD38^-$ cells present at day 2 of CB culture

(24.5±4.5% of total population) were still PKH-high, and the rising number of cells within this primitive population is likely the result of downregulation of the CD38 antigen by cells that were initially $CD38^+$ as shown in Figure 6B. The evident ability of $CD34^+CD38^+$ cells to downregulate CD38 expression during the early points in culture may explain the results by Mckenzie *et al.*, showing that both the $CD34^+CD38^-$ and $CD34^+CD38^+$ CB populations contained primitive HSC with *in vivo* repopulating ability (McKenzie et al., 2007).

In contrast to their CB counterparts, BM-derived HSC seem to have different dynamics of expression of both CD34 and CD38. For example, during culture, BM $CD34^+$ cells downmodulate the expression of this antigen prior to commencing cell division. Though BM cultures started with a lower $CD34^+$ cell enrichment compared to CB experiments (60 *versus* 90%, respectively), we believe the differences observed in terms of CD34 downmodulation are not dependent on initial $CD34^+$ purity. Corroborating our statement are our recent findings that within the same CB experiment (for different CB units), starting with different initial $CD34^+$ enrichments (within a 90 to 20% range), no downmodulation of CD34 antigen is observed early in culture, prior to cell division, irrespectively of initial progenitor purity (manuscript under preparation).

In agreement with our findings of CD34 downmodulation by BM $CD34^+$ cells are studies by Dooley *et al*., in which the authors reported that PB-derived $CD34^+$ cells lost CD34 expression while in a quiescent state for 2-3 days, only to begin re-expressing CD34 upon initiation of growth (Dooley et al., 2004). This up- and downmodulation of CD34 expression may explain the results found by numerous groups showing that BM $CD34^-$ cells contain stem cell potential (Bhatia et al., 1998; Dao et al., 2003; Osawa et al., 1996; Zanjani et al., 1998). It is possible that at a specific point in culture, a certain percentage of BM HSC will downregulate CD34, thus reducing CD34-mediated aggregation and allowing the cells to enter a phase of cycling (Bullock et al., 2007). Thus, cells harvested at that specific stage will contribute to the CD34- fraction with stem cell properties. Furthermore, since *Salati et al* showed that CD34 silencing enhanced HSC differentiation into the

granulocytic and megakaryocytic pathways while reducing erythroid maturation (Salati et al., 2008), downregulation of CD34 prior to cycling may be necessary to prime the cells for differentiation along a specific lineage. Our studies also demonstrate another important difference between BM and CB HSC with respect to expression of CD38. All $CD34^+CD38^-$ CB cells present at the end of culture were derived from cells that had undergone division while maintaining a $CD34^+CD38^-$ phenotype, while BM-derived $CD34^+CD38^-$ cells present at the end of culture passed through a requisite phase of CD38 positivity during division. While it is possible that these $CD34^+CD38^-$ cells no longer correspond to long-term engrafting HSC, we have shown that BM-derived HSC cultured for 14 days under the same culture conditions not only exhibited multilineage clonogenic potential, but they were also able to engraft fetal sheep (Gonçalves et al., 2006). Thus, we feel it is highly likely that at least some of the $CD34^+CD38^-$ generated from $CD34^+CD38^+$ cells in the present study possessed stem cell-like activity.

Our studies have highlighted the functional differences that exist between CB and BM HSC and added insight into the heterogeneity that exists within HSC populations harboring the same CD34 and/or CD38 phenotype. Since these markers are frequently used to quantify the absolute numbers of stem cells present in hematopoietic grafts prior to transplant and/or assess the number of stem cells obtained after *ex-vivo* expansion, it is important to realize the limitations of the use of these markers as the sole means of stem cell enumeration and how they change depending on the ontogenic source of the hematopoietic cells.

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REFERENCES

Almeida-Porada G, Brown RL, MacKintosh FR, Zanjani ED. 2000. Evaluation of serum-free culture conditions able to support the ex vivo expansion and engraftment of human hematopoietic stem cells in the human-to-sheep xenograft model. J Hematother Stem Cell Res 9(5):683-693.

Baumhueter S, Dybdal N, Kyle C, Lasky LA. 1994. Global vascular expression of murine CD34, a sialomucin-like endothelial ligand for L-selectin. Blood 84(8):2554-2565.

Berenson RJ, Andrews RG, Bensinger WI, Kalamasz D, Knitter G, Buckner CD, Bernstein ID. 1988. Antigen CD34+ marrow cells engraft lethally irradiated baboons. J Clin Invest 81(3):951-955.

Berenson RJ, Bensinger WI, Hill RS, Andrews RG, Garcia-Lopez J, Kalamasz DF, Still BJ, Spitzer G, Buckner CD, Bernstein ID, et al. 1991. Engraftment after infusion of CD34+ marrow cells in patients with breast cancer or neuroblastoma. Blood 77(8):1717-1722.

Bhatia M, Bonnet D, Murdoch B, Gan OI, Dick JE. 1998. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. Nature medicine 4(9):1038-1045.

Brandt JE, Bartholomew AM, Fortman JD, Nelson MC, Bruno E, Chen LM, Turian JV, Davis TA, Chute JP, Hoffman R. 1999. Ex vivo expansion of autologous bone marrow CD34(+) cells with porcine microvascular endothelial cells results in a graft capable of rescuing lethally irradiated baboons. Blood 94(1):106-113.

Breard J, Reinherz EL, Kung PC, Goldstein G, Schlossman SF. 1980. A monoclonal antibody reactive with human peripheral blood monocytes. J Immunol 124(4):1943-1948.

Bullock TE, Wen B, Marley SB, Gordon MY. 2007. Potential of CD34 in the regulation of symmetrical and asymmetrical divisions by hematopoietic progenitor cells. Stem cells (Dayton, Ohio) 25(4):844-851.

Chrysler GR MD, Shierman T, Kadidlo D, Askari S, Miller J, Clay M and McCullough J. 2004. Umbilical cord blood banking. In: H.E. B, editor. Cord Blood - Biology, Immunology, Banking and Clinical Transplantation: AABB Press.

Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, Shaper JH. 1984. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. J Immunol 133(1):157-165.

da Silva CL, Goncalves R, Crapnell KB, Cabral JM, Zanjani ED, Almeida-Porada G. 2005. A human stromal-based serum-free culture system supports the ex vivo expansion/maintenance of bone marrow and cord blood hematopoietic stem/progenitor cells. Experimental hematology 33(7):828-835.

Dao MA, Arevalo J, Nolta JA. 2003. Reversibility of CD34 expression on human hematopoietic stem cells that retain the capacity for secondary reconstitution. Blood 101(1):112-118.

Deaglio S, Zubiaur M, Gregorini A, Bottarel F, Ausiello CM, Dianzani U, Sancho J, Malavasi F. 2002. Human CD38 and CD16 are functionally dependent and physically associated in natural killer cells. Blood 99(7):2490-2498.

Dooley DC, Oppenlander BK, Xiao M. 2004. Analysis of primitive CD34- and CD34+ hematopoietic cells from adults: gain and loss of CD34 antigen by undifferentiated cells are closely linked to proliferative status in culture. Stem cells (Dayton, Ohio) 22(4):556-569.

Drew E, Merzaban JS, Seo W, Ziltener HJ, McNagny KM. 2005. CD34 and CD43 inhibit mast cell adhesion and are required for optimal mast cell reconstitution. Immunity 22(1):43-57.

Givan AL, Fisher JL, Waugh M, Ernstoff MS, Wallace PK. 1999. A flow cytometric method to estimate the precursor frequencies of cells proliferating in response to specific antigens. J Immunol Methods 230(1-2):99-112.

Gonçalves R, da Silva CL, Cabral JM, Zanjani ED, Almeida-Porada G. 2006. A Stro 1+ Universal Stromal Feeder Layer to Expand/Maintain Human Bone Marrow Hematopoietic Stem/Progenitor Cells
In a Serum-Free Culture System. Experimental hematology 34:1353-1359.
Hao QL, Shah AJ, Thiemann FT, Smogorzewska EM, Crooks GM. 1995. A functional comparison of CD34 + CD38- cells in cord blood and bone marrow. Blood 86(10):3745-3753.
Healy L, May G, Gale K, Grosveld F, Greaves M, Enver T. 1995. The stem cell antigen CD34 functions as a regulator of hemopoietic cell adhesion. Proc Natl Acad Sci U S A 92(26):12240-12244.
Holyoake TL, Nicolini FE, Eaves CJ. 1999. Functional differences between transplantable human hematopoietic stem cells from fetal liver, cord blood, and adult marrow. Experimental hematology 27(9):1418-1427.
Huang S, Law P, Francis K, Palsson BO, Ho AD. 1999. Symmetry of initial cell divisions among primitive hematopoietic progenitors is independent of ontogenic age and regulatory molecules. Blood 94(8):2595-2604.
Lansdorp PM. 1995a. Developmental changes in the function of hematopoietic stem cells. Experimental hematology 23(3):187-191.
Lansdorp PM. 1995b. Telomere length and proliferation potential of hematopoietic stem cells. J Cell Sci 108 (Pt 1):1-6.
Lanza F, Healy L, Sutherland DR. 2001. Structural and functional features of the CD34 antigen: an update. Journal of biological regulators and homeostatic agents 15(1):1-13.
Liu D, Yu J, Chen H, Reichman R, Wu H, Jin X. 2006. Statistical determination of threshold for cellular division in the CFSE-labeling assay. J Immunol Methods 312(1-2):126-136.
Luens KM, Travis MA, Chen BP, Hill BL, Scollay R, Murray LJ. 1998. Thrombopoietin, kit ligand, and flk2/flt3 ligand together induce increased numbers of primitive hematopoietic progenitors from human CD34+Thy-1+Lin- cells with preserved ability to engraft SCID-hu bone. Blood 91(4):1206-1215.
Mayani H, Lansdorp PM. 1998. Biology of human umbilical cord blood-derived hematopoietic stem/progenitor cells. Stem cells (Dayton, Ohio) 16(3):153-165.
McKenzie JL, Gan OI, Doedens M, Dick JE. 2007. Reversible cell surface expression of CD38 on CD34-positive human hematopoietic repopulating cells. Experimental hematology 35(9):1429-1436.
Moritz T, Keller DC, Williams DA. 1993. Human cord blood cells as targets for gene transfer: potential use in genetic therapies of severe combined immunodeficiency disease. J Exp Med 178(2):529-536.
Nakamura Y, Yahata T, Muguruma Y, Uno T, Sato T, Matsuzawa H, Kato S, Shirasugi Y, Hotta T, Ando K. 2007. Angiopoietin-1 supports induction of hematopoietic activity in human CD34-bone marrow cells. Experimental hematology 35(12):1872-1883.
Osawa M, Hanada K, Hamada H, Nakauchi H. 1996. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science (New York, NY 273(5272):242-245.
Salati S, Zini R, Bianchi E, Testa A, Mavilio F, Manfredini R, Ferrari S. 2008. Role of CD34 antigen in myeloid differentiation of human hematopoietic progenitor cells. Stem cells (Dayton, Ohio) 26(4):950-959.
Sato T, Laver JH, Ogawa M. 1999. Reversible expression of CD34 by murine hematopoietic stem cells. Blood 94(8):2548-2554.
Schibler KR, Li Y, Ohls RK, Nye NC, Durham MC, White W, Liechty KW, Le T, Christensen RD. 1994. Possible mechanisms accounting for the growth factor independence of hematopoietic progenitors from umbilical cord blood. Blood 84(11):3679-3684.

Shpall EJ, Jones RB, Bearman SI, Franklin WA, Archer PG, Curiel T, Bitter M, Claman HN, Stemmer SM, Purdy M, et al. 1994. Transplantation of enriched CD34-positive autologous marrow into breast cancer patients following high-dose chemotherapy: influence of CD34-positive peripheral-blood progenitors and growth factors on engraftment. J Clin Oncol 12(1):28-36.

Stewart AK, Vescio R, Schiller G, Ballester O, Noga S, Rugo H, Freytes C, Stadtmauer E, Tarantolo S, Sahebi F, Stiff P, Meharchard J, Schlossman R, Brown R, Tully H, Benyunes M, Jacobs C, Berenson R, White M, DiPersio J, Anderson KC, Berenson J. 2001. Purging of autologous peripheral-blood stem cells using CD34 selection does not improve overall or progression-free survival after high-dose chemotherapy for multiple myeloma: results of a multicenter randomized controlled trial. J Clin Oncol 19(17):3771-3779.

Tajima F, Deguchi T, Laver JH, Zeng H, Ogawa M. 2001. Reciprocal expression of CD38 and CD34 by adult murine hematopoietic stem cells. Blood 97(9):2618-2624.

Theunissen K, Verfaillie CM. 2005. A multifactorial analysis of umbilical cord blood, adult bone marrow and mobilized peripheral blood progenitors using the improved ML-IC assay. Experimental hematology 33(2):165-172.

Traycoff CM, Abboud MR, Laver J, Clapp DW, Srour EF. 1994. Rapid exit from G0/G1 phases of cell cycle in response to stem cell factor confers on umbilical cord blood CD34+ cells an enhanced ex vivo expansion potential. Experimental hematology 22(13):1264-1272.

Traycoff CM, Kosak ST, Grigsby S, Srour EF. 1995. Evaluation of ex vivo expansion potential of cord blood and bone marrow hematopoietic progenitor cells using cell tracking and limiting dilution analysis. Blood 85(8):2059-2068.

Vaziri H, Dragowska W, Allsopp RC, Thomas TE, Harley CB, Lansdorp PM. 1994. Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric DNA with age. Proc Natl Acad Sci U S A 91(21):9857-9860.

Watari K, Lansdorp PM, Dragowska W, Mayani H, Schrader JW. 1994. Expression of interleukin-1 beta gene in candidate human hematopoietic stem cells. Blood 84(1):36-43.

Young JC, Varma A, DiGiusto D, Backer MP. 1996. Retention of quiescent hematopoietic cells with high proliferative potential during ex vivo stem cell culture. Blood 87(2):545-556.

Zanjani ED, Almeida-Porada G, Livingston AG, Flake AW, Ogawa M. 1998. Human bone marrow CD34- cells engraft in vivo and undergo multilineage expression that includes giving rise to CD34+ cells. Experimental hematology 26(4):353-360.

Zanjani ED, Almeida-Porada G, Livingston AG, Zeng H, Ogawa M. 2003. Reversible expression of CD34 by adult human bone marrow long-term engrafting hematopoietic stem cells. Experimental hematology 31(5):406-412.

Zocchi E, Daga A, Usai C, Franco L, Guida L, Bruzzone S, Costa A, Marchetti C, De Flora A. 1998. Expression of CD38 increases intracellular calcium concentration and reduces doubling time in HeLa and 3T3 cells. J Biol Chem 273(14):8017-8024.

FIGURE LEGENDS

Figure 1: *FACS analysis of CD34$^+$ enriched cells cultured in serum-free conditions over human stromal layers during 14 days.*

Dot plots display representative experiments for (A) BM (n=4) and (B) CB cells (n=4), with PKH67 intensity measured along the Y axis (FL1) and CD34 expression along the X axis (FL3). Quadrants were set at day 0 using live-gated undivided cells and maintained in the dot plot analysis of cell populations during time in culture. Loss of PKH fluorescence indicates cell division.

Figure 2: *Percentage of undivided cells – Parent Generation Cells - during time in culture*.

BM (black bars) and CB (white bars) cells were labeled with PKH67 and cultured in serum-free conditions as described in the Material and Methods section. Data was acquired using flow cytometry and analyzed using the Proliferation Wizard of *ModFit* software. The mean percentages ± SEM of cells in Generation 1 are presented (BM: n=4; CB: n=4). (*) CB: statistically significant for $p<0.05$; (**) BM: statistically significant for $p<0.05$.

Figure 3: *Divisional history of BM and CB cells in culture.*

BM (black bars) and CB (white bars) cells were labeled with PKH67 and cultured for 14 days. Data was acquired by flow cytometry and analyzed using the Proliferation Wizard of *ModFit* software. Each bar represents the percentage of cells in each doubling generation, which is the precise number of divisions (Generation 1 to 10) that BM and CB have undergone at each point in culture. The mean percentages ± SEM of cells in each generation (from 1 to 10) are presented (BM: n=4; CB: n=4). Days 0, 2, 3, 4, 6, 8 and 10 (A, B, C, D, E, F and G, respectively) are represented for both BM and CB experiments. Days 12 and 14 are presented for BM experiments only (H), since computation of results using *ModFi*t software is not possible when the percentage of Generation 1 cells becomes 0.

Figure 4: *Percentage of cells expressing CD34 within the undivided, i.e. quiescent, and dividing fractions of BM and CB HSC.*

$CD34^+$ expression in the undivided PKH^{High} (black bars) and dividing PKH^{Low} (white bars) cells are presented as mean value ± SEM for BM (A) and CB (B) experiments (BM: n=4; CB: n=4).

Figure 5*: Percentage of BM and CB $CD34^+CD38^-$ cells in the undivided and dividing fraction during time in culture.*

Percentage of $CD34^+CD38^-$ cells in the undivided PKH^{High} (white bars) and the dividing PKH^{Low} (black bars) fractions are presented as mean value ±SEM for BM (A) and CB (B) experiments.(BM:n=3;CB:n=3)

Figure 6: *Differential CD38 expression during division of $CD34^+$ cells in BM and CB cultures.*

Dot plots display representative experiments for (A) BM and (B) CB cells, with PKH67 intensity measured along the Y axis (FL1) and CD38 expression along the X axis (FL2), gated for $CD34^+$ cells. Quadrants were set at day 0 using live-gated undivided cells and maintained in the dot plot analysis of cell populations during time in culture. Loss of PKH fluorescence indicates cell division. (BM: n=3; CB: n=3).