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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. PORADA¹, JOÃO L. ASCENSÃO³, ESMAIL D. ZANJANI¹, JOAQUIM M.S. CABRAL², and GRAÇA ALMEIDA-PORADA^{1,*}

¹Department of Animal Biotechnology, University of Nevada, Reno, Nevada

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

³Department of Medicine, V.A. Medical Center and the George Washington University School of Medicine, Washington, District of Columbia

Abstract

Human hematopoietic stem/progenitor cells (HSC) isolated based upon specific patterns of CD34 and CD38 expression, despite phenotypically identical, were found to be functionally heterogeneous, raising the possibility that reversible expression of these antigens may occur 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 24 h 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 ninth generation, whereas BM-cells were mostly distributed between the fifth and seventh 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 $60.8 \pm 6.3\%$ to $30.6 \pm 6.7\%$ 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.

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; Healy et al., 1995; Lanza et al., 2001; Drew et al., 2005; 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 (Zanjani et al., 1998, 2003; Tajima et al., 2001; McKenzie et al., 2007). 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 (Sato et al., 1999; Dooley et al., 2004; McKenzie et al., 2007). 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 (Zocchi et al., 1998; Deaglio et al., 2002), 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 (Tajima et al., 2001; Dooley et al., 2004; McKenzie et al., 2007). 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 (Traycoff et al., 1995; Luens et al., 1998).

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 downmodulated 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. Our present studies thus provide evidence that expression by HSC of two of the principal markers used for identification of these cells, namely CD34 and CD38, fluctuates over time in culture, is closely linked to their proliferative status, and differs depending on the source of HSC. Traditionally, the purification of stem and progenitor cells and confirmation of their continued presence in expansion cultures is often based upon expression of these two cell surface markers. Importantly, our findings suggest that investigators should use caution when relying on cell surface phenotype as the sole means of assessing the presence/maintenance of HSC in culture, since results of such an analysis will likely vary as a function of both cell source and cycling status of the HSC, potentially leading the investigators to the erroneous quantification of the stem cell content in hematopoietic grafts to be used in a transplant setting.

Materials 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 et al., 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) and washed twice in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco Laboratories, Grand Island, NY). BM MNC and CB MNC from each donor were enriched for CD34⁺ cells using magnetic cell sorting (Miltenyi Biotec Inc., Auburn, CA).

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-1⁺, Gly-A⁻, CD45⁻) were then cultured in gelatin-coated T-25 flasks with MSCGM media (Poietics™, BioWhittaker, Walkersville, MD). 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% CO₂ 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 fluorphores 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 (Brandt et al., 1999; Huang et al., 1999; Almeida-Porada et al., 2000).

Ex vivo expansion of CD34+ enriched cells

BM ($5.3\text{--}8.9 \times 10^6$ cells) and CB ($3.7\text{--}5.2 \times 10^5$ 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) 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). 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 (Brandt et al., 1999; Huang et al., 1999; Almeida-Porada et al., 2000). Flow cytometric analyses were performed in a FACScalibur cytometer (Becton Dickinson) 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 min. 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 $\pm$ standard error of the mean (S.E.M.). 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, that is 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 stroma-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). 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. The viability 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 10th generation.

Analysis of the percentage of undivided cells, that is 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 ($96.2 \pm 0.8\%$) and day 2 ($59.6 \pm 12.3\%$) ($P < 0.05$) (Fig. 2). By contrast, in the cultured BM cells there was at least a 24 h delay in the initiation of cellular division, with the percentage of undivided cells remaining largely unchanged from day 0 ($96.9 \pm 0.4\%$) to day 2 ($86.9 \pm 9.9\%$). It was only by day 3 that the percentage of undivided BM cells decreased significantly ($38.2 \pm 7.6\%$) ($P < 0.05$) (Fig. 2).

Figure 3A–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 1–10) that BM and CB have undergone at each point in culture.

By day 2 of culture (Fig. 3B), $36 \pm 8\%$ of CB cells had already reached generation 2, and by day 3 (Fig. 3C) $42.2 \pm 5.2\%$ had reached the third and $9.3 \pm 4.3\%$ the fourth generation. By

contrast, most of the BM cells by day 2 still belonged to Generation 1, and by day 3, $38.2 \pm 7.6\%$ of BM cells continued to be in the first generation. While $23.1 \pm 7.6\%$ of BM cells had reached Generation 2, and $36.9 \pm 19.0\%$ 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 fifth generation was $15.0 \pm 8.7\%$, whereas only $2.2 \pm 0.8\%$ had reached this generation in BM cultures (Fig. 3D). Furthermore, at this time point, 3.6 ± 2.6 and $3.7 \pm 2.8\%$ 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 (Fig. 3F), when almost no CB cells remained undivided (less than 0.08% of cells belonging to Parent Generation) and more than $24.3 \pm 9.2\%$ had already reached the ninth generation. By contrast, at this same time point, most of the BM cells were only distributed between the fifth and sixth generation, $32.0 \pm 2.3\%$ and $38.2 \pm 12.0\%$, respectively. At day 10 (Fig. 3G), no CB cells remained undivided and more than $56.0 \pm 5.6\%$ had reached the ninth generation, whereas for BM cells, less than 5% had attained Generation 8, and most of the cells were still in generation 6 ($47 \pm 1.8\%$) with a smaller percentage being in Generation 7 ($25 \pm 1.4\%$).

Figure 3H depicts only BM cells at days 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, $41.0 \pm 4.1\%$ of BM cells belonged to Generation 7 and $22.4 \pm 5.6\%$ to Generation 8. By contrast with CB, even after 14 days of culture, $0.7\text{--}3.8\%$ of BM cells remained in the Parent Generation, that is 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, that is quiescent, and dividing fractions for BM and CB experiments is represented in Figure 4A and B, respectively. While BM cells did not divide significantly between days 0 and 2 (as depicted above), the percentage of BM cells expressing CD34 decreased from $60.8 \pm 6.3\%$ at day 0 to $30.6 \pm 6.7\%$ 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 $27.3 \pm 7.8\%$ and $22.3 \pm 7.0\%$ of the cells being positive for CD34 in the non-divided and in the divided cell fraction, respectively (Fig. 4A). This data suggests that there is downregulation 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 (Fig. 4B). At day 0, $90.6 \pm 2.14\%$ of CB cells expressed CD34, and at day 2, $68.1 \pm 7.0\%$ of the cells in the undivided fraction and $17.3 \pm 6.5\%$ 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, $2.0 \pm 1.3\%$ of the total BM CD34⁺ population had remained quiescent, never dividing throughout the entire culture period (Fig. 4A), whereas during the same period of time all of the CB CD34⁺ cells had undergone cell division (Fig. 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 (Fig. 5A) and CB (Fig. 5B) were essentially equivalent at day 0 of culture ($2.24 \pm 0.07\%$ and $1.73 \pm 0.70\%$, respectively). By day 2, in the CB-derived cultures, the percentage of CD34⁺CD38⁻ cells increased to $24.5 \pm 4.5\%$ with only $4.25 \pm 1.8\%$ 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 ($24.5 \pm 4.5\%$ of total population) were still PKH high (white bars Fig. 5B), seeming to have emerged from downregulation 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 $32.1 \pm 7.0\%$ 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 (Fig. 6B). By contrast, the number of CD34⁺CD38⁻ BM-derived cells did not increase significantly between day 0 ($2.24 \pm 0.07\%$) and day 2 ($3.78 \pm 0.54\%$). Of note is the fact that, in BM cultures, CD34⁺CD38⁻ cells were present at very low levels between days 3 and 8 (Fig. 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 (Figs. 5A and 6A). However, at day 10 of culture, $6.59 \pm 2.3\%$ of dividing CD34⁺CD38⁺ cells became CD34⁺CD38^{-dim} (Fig. 6A), and by day 12 and 14, the percentages of CD34⁺CD38^{-dim} cells had increased to $13.1 \pm 0.8\%$ and $10.9 \pm 1.5\%$, 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 second to third generation (Fig. 3A), due perhaps to either the slightly less than 100% labeling efficiency of those 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 $56 \pm 5.6\%$ had reached the ninth generation, whereas for BM cells, less than 5% had attained Generation 8, and most of the cells ($47 \pm 1.8\%$) 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 G₀–G₁ (Traycoff et al., 1994; Mayani and Lansdorp, 1998) 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 (Vaziri et al., 1994; Lansdorp, 1995a,b).

Since we have shown that all the CB-derived cells underwent at least one cell division in the first week 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 $4.25 \pm 1.8\%$ 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 ($24.5 \pm 4.5\%$ 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. (2007), showing that both the CD34⁺CD38⁻ and CD34⁺CD38⁺ CB populations contained primitive HSC with in vivo repopulating ability.

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. Although the enrichment in CD34⁺ cells was lower in BM cultures than in CB (60% vs. 90%, respectively), we believe that the differences observed with CD34 downmodulation are not due to the different initial CD34⁺ purities. Indeed, corroborating our statement are our findings that, in CB cultures with initial lower CD34⁺ enrichment purities, even in the 20% range, no downmodulation of CD34 antigen was observed prior to cell division.

Also, in agreement with our findings of CD34 downmodulation by BM CD34⁺ cells, are studies by Dooley et al. (2004), 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. This up- and downmodulation of CD34 expression may explain the results found by numerous groups showing that BM CD34⁺ cells contain stem cell potential (Osawa et al., 1996; Bhatia et al., 1998; Zanjani et al., 1998; Dao et al., 2003). 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. (2008) showed that CD34 silencing enhanced HSC differentiation into the granulocytic and megakaryocytic pathways while reducing erythroid maturation, 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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Fig. 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 they-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.

Fig. 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 $\pm$ S.E.M. of cells in Generation 1 are presented (BM: n = 4; CB: n = 4). (*) CB: statistically significant for $P < 0.05$; (**) BM: statistically significant for $P < 0.05$.

Fig. 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 (Generations 1–10) that BM and CB have undergone at each point in culture. The mean percentages $\pm$ S.E.M. 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 *ModFit* software is not possible when the percentage of Generation 1 cells becomes 0.

Fig. 4.

Percentage of cells expressing CD34 within the undivided, that is quiescent, and dividing fractions of BM and CBHSC. CD34⁺ expression in the undivided PKH^{High}(black bars)and dividing PKH^{Low}(white bars) cells are presented as mean value $\pm$ S.E.M. for BM(A)and CB(B)experiments (BM: n = 4; CB: n = 4).

Fig. 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 $\pm$ S.E.M. for BM (A) and CB (B) experiments (BM: n = 3; CB: n = 3).

Fig. 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).

TABLE 1Global CD34 mean intensity BM and CB CD34⁺ enriched cells during time in culture

Day of culture	BM cells	CB cells
0	63.0 ± 4.5	129 ± 21.3
2	57.4 ± 4.2	228 ± 61.7
3	56.2 ± 2.4	169 ± 33.9
4	54.0 ± 3.8	87.5 ± 12.1
6	44.7 ± 7.2	75.4 ± 16.1
8	40.5 ± 6.9	64.5 ± 9.3
10	35.9 ± 5.5	59.7 ± 13.1
12	35.1 ± 4.4	59.1 ± 6.2
14	36.2 ± 3.6	69.5 ± 7.5

Freshly isolated BM and CB CD34⁺ enriched cells were labeled with PKH67 membrane dye and cultured in serum-free medium supplemented with SCF, bFGF, LIF and Flt-3L, over a human stromal layer for 14 days. Results are expressed as the mean of CD34 mean intensity values given by histogram analysis ± S.E.M. (BM: n =4; CB: n =4).