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Original Research Report

Phenotypic and Functional Analysis of Human Fetal Liver Hematopoietic Stem Cells in Culture

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ABSTRACT

Steady-state hematopoiesis and hematopoietic transplantation rely on the unique potential of stem cells to undergo both self-renewal and multilineage differentiation. Fetal liver (FL) represents a promising alternative source of hematopoietic stem cells (HSCs), but limited by the total cell number obtained in a typical harvest. We reported that human FL nonobese diabetic/severe combined immunodeficient (NOD/SCID) repopulating cells (SRCs) could be expanded under simple stroma-free culture conditions. Here, we sought to further characterize FL HSC/SRCs phenotypically and functionally before and following culture. Unexpanded or cultured FL cell suspensions were separated into various subpopulations. These were tested for long-term culture potential and for in vivo repopulating function following transplantation into NOD/SCID mice. We found that upon culture of human FL cells, a tight association between classical stem cell phenotypes, such as CD34^{+}/CD38^{-} and/or side population, and NOD/SCID repopulating function was lost, as observed with other sources. Although SRC activity before and following culture consistently correlated with the presence of a CD34^{+} cell population, we provide evidence that, contrary to umbilical cord blood and adult sources, stem cells present in both CD34^{+} and CD34^{-} FL populations can sustain long-term hematopoietic cultures. Furthermore, upon additional culture, CD34-depleted cell suspensions, devoid of SRCs, regenerated a population of CD34^{+} cells possessing SRC function. Our studies suggest that compared to neonatal and adult sources, the phenotypical characteristics of putative human FL HSCs may be less strictly defined, and reinforce the accumulated evidence that human FL represents a unique, valuable alternative and highly proliferative source of HSCs for clinical applications.

INTRODUCTION

STEM CELL-BASED THERAPIES for cancer treatment or tissue repair hold great promise, but depend on our ability to identify, isolate, characterize, and manipulate stem cells. So far only adult, and to a lesser extent neonatal cord blood (CB), hematopoietic stem cells (HSCs) have been routinely used clinically. The dual ability of self-renewal and multilineage differentiation makes HSCs an essential component of hematopoietic grafts and an ideal target cell for ex-vivo manipulation (1–3).

Development of the human hematopoietic system begins in the extraembryonic yolk sac. Later, primitive hematopoiesis is replaced by the definitive multilineage blood system sustained by multipotent HSCs, first appearing in the para-aortic splanchnopleura and aorta-gonad mesonephros regions of the embryo (4). Around the 5th week of gestation, hematopoiesis starts to shift to the fetal liver (FL), which becomes the predominant site of hematopoiesis until the development of the bone marrow (BM) (5). Although traditional sources of HSCs include adult BM, mobilized peripheral blood (MPB), and CB,

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accumulated evidence suggests that FL represents a rich alternative source of "early" HSCs, possessing high proliferative and repopulating potential (6–11). Moreover, the preimmune status of FL may be important for crossing the immunological barriers, and for decreasing the incidence of serious complications such as graft-versus-host disease (12–16).

Given the positive correlation between the dose of HSC/progenitors transplanted and patient outcome (17–19), and as HSCs are rare ($\sim$1 in 10^6 CB mononuclear cells, see ref. (20), a major focus of experimental hematology has concentrated on HSC expansion (21). This is particularly important for FL and CB, where the number of hematopoietic cells is relatively small, explaining why the clinical use of FL has been limited so far mostly to in utero transplantation (16,22,23), and that of CB principally to transplantation in children (24,25). Thus, future applications of FL and CB would strongly benefit from reliable methods of HSC expansion.

One of the main barriers to the successful development of such systems has been the lack of reliable markers of HSC function. Various surface markers have been used to identify and enrich HSCs from different sources. CD34 was long thought to be a unique marker defining HSC/progenitors (26). Clinically, $CD34^+$ numbers are still routinely used to estimate the engraftment potential of hematopoietic grafts (19,27,28). $CD34^+/CD38^-$ subpopulations from human FL, CB, and adult BM were shown to contain multilineage hematopoietic cells possessing extensive proliferative capability (29–31), as well as most nonobese diabetic/severe combined immunodeficient (NOD/SCID) repopulating cells (SRCs) (32–34). However, the existence of HSCs lacking the CD34 marker has been reported (35–38). Moreover, expression of CD34 on HSCs was shown to vary depending on "age" and activation status, and therefore may be modulated by culture conditions and/or following transplantation (39–41). Thus, HSCs could be either $CD34^+$ or $CD34^-$ (42), even though the positive fraction in freshly isolated BM, CB, and FL seems to contain most SRCs (32,33,43).

A recent study also revealed functional interactions between purified CB $CD34^+$ and $CD34^-$ putative stem cells (44). Apart from CD34, other markers are thought to be present, while some, such as mature lineage markers, appear to be missing on HSCs, and they are often used to isolate subpopulations enriched in stem/progenitor cells (3,45). For example, freshly isolated murine and human hematopoietic cells possessing a side population (SP) phenotype, due to the expression of the *Bcrp1/ABCG2* gene, were shown to be highly enriched for HSCs (36,46,47). However, a combination of cell-surface markers reliably defining human HSCs, whether freshly isolated but especially following culture, has not yet been defined.

To circumvent the above problems, researchers have relied on functional xenotransplantation models to test for HSC presence in human hematopoietic suspensions. The most widely used of them has been the NOD/SCID mouse model, where sublethally irradiated animals are transplanted intravenously with bulk or purified cell populations (32,48). Engraftment is detected by analyzing the presence of human cells in the BM, blood, or other organs of transplanted animals. Although suffering from a substantial variability both between but also within samples (49), the NOD/SCID system can be used to quantitate human SRCs (20). Significant ontogeny-related differences between the repopulating potential of different freshly isolated sources of human HSCs have been demonstrated. A continuum of engraftment capacity exists, with greatest potential residing in FL cells followed by CB, adult BM, and lastly MPB (7,10). With the derivation of functional HSCs from human embryonic stem (ES) cells still in its infancy (50), FL may represent one of the best sources of HSCs for allogeneic transplantation and ex vivo expansion. Previous studies using NOD/SCID mice also revealed that upon culture of human neonatal or adult hematopoietic cells in cytokine-containing media, a dissociation between phenotype and function occurred (51–53). Thus, additional studies characterizing HSCs from FL and other sources in culture are urgently needed.

We developed simple and reproducible stroma-free culture conditions allowing long-term (over 6 months) amplification of human FL hematopoietic cells and their clonogenic progenitors (54). We then reported for the first time, using quantitative limited dilution analysis (LDA) in NOD/SCID mice, that a 10- to over 100-fold net expansion of FL SRCs could be consistently achieved after 28 days of culture (55). Here, we characterized further FL HSC/progenitors before and following expansion. Under our experimental conditions, we found that FL SRCs consistently partitioned within the $CD34^+$ population. However, hematopoietic cell suspensions that were depleted of their $CD34^+$ cells before or during culture, and were unable to positively engraft NOD/SCID mice, generated over time not only new $CD34^+$ cells possessing SRC function, but also sustained long-term cultures for at least 8 more weeks. Importantly, these properties were not found in CB or adult cells. Moreover, classical phenotypes associated with freshly isolated HSCs, such as $CD34^+/CD38^-$, SP cells, or the absence of lineage markers, were either undetectable in FL cultures or did not correlate with long-term culture potential and/or engraftment capability. Our study strengthens the accumulated evidence that FL represents a valuable alternative expandable source of HSCs for clinical applications and stem cell biology.

MATERIALS AND METHODS

Preparation of human hematopoietic cell suspensions

Human FL was harvested from aborted fetuses (gestational weeks 12–16) under approved ethical guidelines, and cellular suspensions were prepared and frozen as described (56). CB, BM, and MPB mononuclear cells were prepared as described (57,58).

CD34$^+$ and CD34$^-$ cell purification

CD34$^+$ cells present among fresh or cultured FL cells were selected by immunomagnetic positive selection (EasySep, StemCell Technologies, Vancouver, Canada), following the manufacturer's instructions. Importantly, we modified the procedure so that the usually discarded CD34$^-$ population could be used in further experiments. As the successive washing steps described by the manufacturers contained progressively more CD34$^+$ cells, only the first wash was collected. Residual CD34$^+$ cells contained in this fraction were removed by a second selection procedure, in which again only the first wash, virtually depleted of CD34$^+$ cells, was harvested. CB, BM, or MPB mononuclear cells were first enriched by centrifugation on Ficoll/Paque, before separation of the CD34$^+$ and CD34$^-$ populations as above. The purity of each fraction was estimated in all cases by fluorescence-activated cell sorting (FACS). In other studies, ABCG2-positive and -negative populations were purified from cultured FL hematopoietic cells labeled with a phycoerythrin (PE)-conjugated anti-ABCG2 monoclonal antibody by using a similar selection procedure (PE-specific EasySep beads, StemCell Technologies), following the manufacturer's instructions.

Stroma-free long-term cultures

FL hematopoietic cells, CD34$^+$, CD34$^-$, or other subpopulations were expanded as described (54). Cultures were fed twice a week and maintained at less than 2×10^6 cells/ml in 24-well plates, T12.5, T25, or T75 flasks depending on the experiment. For single-cell cultures, FL cells were deposited at 1 cell/well in 96-well plates, and proliferation was documented every 2–3 days, by counting cells under an inverted microscope. For LDA analyses, serial dilutions of cells (from ~1 to ~200) were deposited into 96-well plates (12 wells or more per dilution), split once, and fed twice a week. Growth was documented twice a week. The frequency of progenitors capable of sustaining long-term cultures was calculated using the L-Calc software (StemCell Technologies), wells being considered positive if 1000 cells or more were present after 8 weeks of culture.

Depletion of lineage-committed cells

Lineage-negative (lin$^-$) cells from FL suspensions before or following culture were purified using the StemSep system according to the manufacturer's recommendations (human primitive progenitor enrichment cocktail, Catalog number 14057, Stem Cell Technologies). Lin$^-$ cells were then cultured as above ($2–5 \times 10^4$ cells/ml in 24-well plates). In parallel, control cultures were established with total cells from the same sample and with lin$^+$ cells washed off the demagnetized column.

Phenotypical analysis and clonogenic potential of FL cells

Cells were stained with a panel of fluorescein isothiocyanate (FITC) or PE-conjugated antibodies, and analyzed as described (54,57,58), using a FACScan and the Cell Quest Software (Becton-Dickinson). SP cells in FL or control BM were detected following Hoechst 33342 staining (36,46), using a FACS Vantage SE (Becton-Dickinson). The frequency of colony forming cells (CFC) in various FL hematopoietic suspensions was monitored by classical semisolid CFC assays as described (54).

NOD/SCID repopulating (SRC) assays

Six- to eight-week-old NOD/LtSz-*scid/scid* (NOD/SCID) mice were sublethally irradiated, and hematopoietic cells to be tested were injected into the lateral tail vein 4–24 h later. Mice were killed ~8 weeks post-transplantation, and their BM were harvested and analyzed for human engraftment by FACS. In most cases, mouse BM was first stained with an anti-human CD45 antibody, and multilineage lymphomyeloid engraftment was verified on positively engrafted mice as described (32,55).

RESULTS

Dissociation between phenotype and function in human FL hematopoietic cultures

Numerous studies attempted to characterize human HSCs with a set of markers correlating with repopulating function. Most transplantable SRCs in fresh FL, CB, and adult BM were shown to be contained within a CD34$^+$/CD38$^-$ subpopulation (32–34). Although CD34$^+$/CD38$^-$ cells were easily detected in freshly thawed, unexpanded FL samples, they were undetectable in FL suspensions cultured for 28 days that engrafted mice in the SRC assay (55). Later in culture, CD34$^+$/CD38$^-$ cells reappeared, their proportion being highly variable between samples, making up to 7% of the total population. However, no correlation was observed between the relative number of CD34$^+$/CD38$^-$ cells in a given sample and their repopulating capability.

Likewise, although SP cells from unexpanded FL were shown to be highly enriched in HSCs/SRCs (33), FL hematopoietic cultures from three different specimens expanded for 28 days and possessing robust SRC activity (55) were totally devoid of SP cells. These were, however, easily detected in the same unexpanded FL samples and/or in normal human or mouse BM controls (data not shown). These observations demonstrate a lack of association between function (NOD/SCID repopulation) and the presence of a defined phenotype (CD34$^+$/CD38$^-$ and/or SP) in cultured FL cells, and is consistent with reports using BM, MBP, or CB (51–53). Thus, additional studies on the phenotypic/functional characterization of human HSCs expanded in culture are needed.

Immunomagnetic separation of human FL CD34$^+$ and CD34$^-$ cell populations

Toward these goals, we first separated FL cell suspensions into CD34$^+$ and CD34$^-$ fractions, and tested the capacity of both, compared to total unseparated cells, to sustain long-term culture and to engraft NOD/SCID mice. For this, we relied on the EasySep technology. Most importantly, as described in Materials and Methods, a slight modification of this positive selection technique, where the negative fraction is usually discarded, allowed us to also harvest CD34$^-$ cells with little or no contamination with CD34$^+$ cells. An example of such separation experiments from cultured FL hematopoietic cells is shown in Fig. 1. CD34$^+$ cells were routinely purified to >99 % homogeneity (Fig. 1, third row of panels). The depleted fraction was passed again into the selection procedure to reduce further the number of contaminating CD34$^+$ cells, resulting in a suspension (called thereafter CD34$^-$) virtually depleted of CD34$^+$ cells (right panels). Thus, this technique allowed us to separate efficiently, with little loss of cells, CD34$^+$ and CD34$^-$ populations for further studies, as described below.

SRCs in unexpanded human FL are found in the CD34$^+$ population

CD34$^+$ and CD34$^-$ populations were separated from unexpanded FL cell suspensions and transplanted sepa-

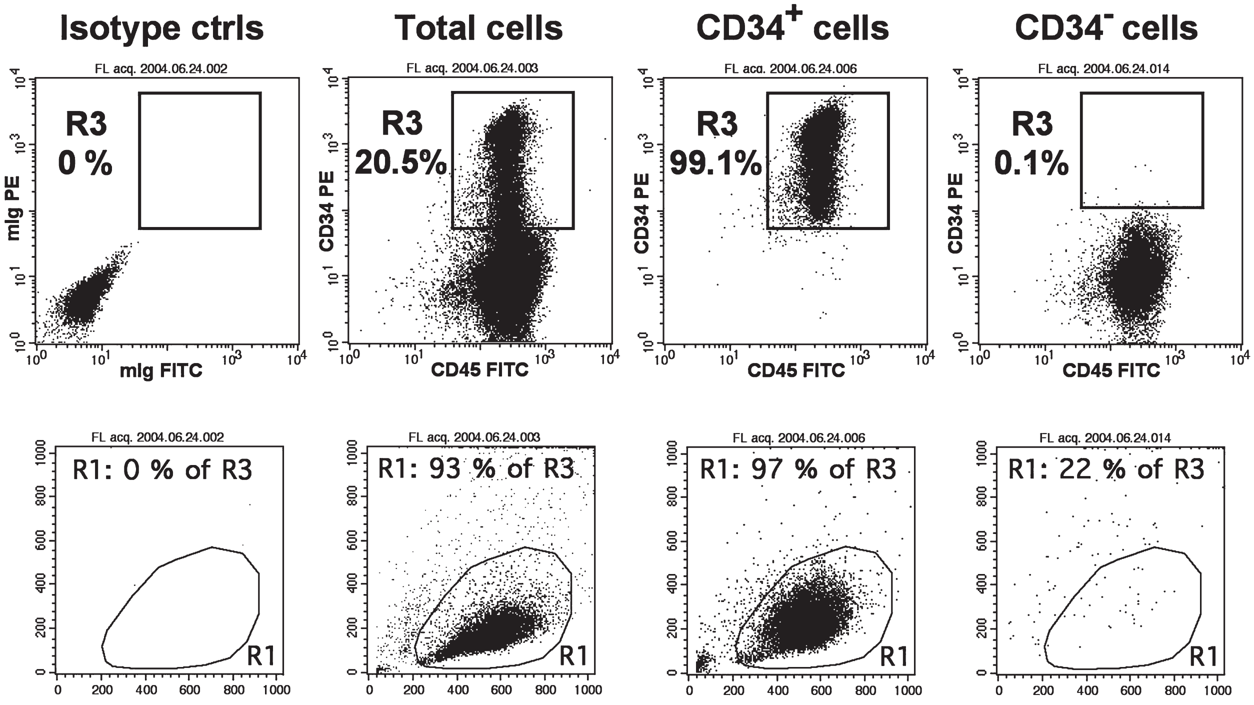

FIG. 1. Separation of CD34$^+$ and CD34$^-$ populations from human FL. A 28-day-old culture of FL#852 was stained with CD45–FITC and CD34-PE and analyzed by flow cytometry. The vast majority of the cultured cells were CD45$^+$, and in this example, about 20% of them were also positive for CD34 (*second upper panel*, gate R3). When backgated to a forward versus side-scatter plot, most of the double-positive cells in R3 fell within a viable lymphocyte-like gate (R1 in *second lower panel*). Following immunomagnetic CD34 separation using the Easy Sep technology, both the CD34$^+$ and CD34$^-$ cell populations from this FL hematopoietic culture were purified and analyzed as above. CD34$^+$ cells were reproducibly purified to >99% homogeneity (*third row of panels*), whereas a slight modification of the selection technique (see Materials and Methods) allowed us to recuperate a CD34$^-$ population virtually free of CD34$^+$ cells (*right panels*). An example of isotype control staining of the total cell population is shown in the *left panels*.

rately into NOD/SCID mice. One of the specimens used, FL#873, was previously tested for SRC activity (55), and found by quantitative LDA to contain ~1 SRC in 370,000 unexpanded total nucleated cells (TNCs). An example of positive engraftment is shown in Fig. 2A. In this unexpanded FL sample, ~13% of the cells express CD45, and about a third of those also expressed CD34 (Fig. 2B).

Cohorts of mice were transplanted with either $CD34^+$ or $CD34^-$ cells purified from a vial of unexpanded FL#873. All mice receiving $\sim 2 \times 10^5$ $CD34^+$ cells (n = 5) were engrafted (Fig. 2C), whereas none of those that received 10 times more ($\sim 2 \times 10^6$) $CD34^-$ cells (n = 5) showed detectable human hematopoietic engraftment ~8 weeks post transplantation (Fig. 2D). These results are in agreement with a previous report (33), and indicate that under our experimental conditions, most if not all SRC activity in unexpanded FL is found in the $CD34^+$ cell population.

Segregation of FL SRCs with the $CD34^+$ population in culture

We next asked whether upon culture in conditions allowing their expansion (54,55), FL SRC would also specifically partition with the $CD34^+$ population. Three different FL samples were expanded for 4–12 weeks, the $CD34^+$ and $CD34^-$ subpopulations were then purified, and both fractions tested for SRC activity. One representative series of data is shown in Fig. 3. In this example, expansion of FL#873 for 42 days resulted in a culture containing ~20% $CD34^+$ cells. As expected, mice transplanted with 20×10^6 TNCs ($n = 5$) were all engrafted (Fig. 3A), whereas none of those injected with 20×10^6 $CD34^-$ cells from the same culture ($n = 5$) contained detectable human cells (Fig. 3B). In sharp contrast, all mice ($n = 5$) that received 3×10^6 $CD34^+$ cells were engrafted (Fig. 3C). Similar results were obtained with two other FL samples expanded for different periods (~4, 6, 9, and 12 weeks). These data demonstrated that in FL hematopoietic cells cultured under our conditions most if not all SRC activity partitioned with $CD34^+$ cells.

This specific segregation of FL SRCs with $CD34^+$ cells allowed us to test “late” cultures of FL hematopoietic cells for SRC maintenance. For example, transplantation of 2.5×10^6 $CD34^+$ cells from a 12-week-old culture of FL#841 (containing ~7 % $CD34^+$ cells) resulted in a high level of human engraftment, demonstrating that our conditions maintained FL SRCs for at least 12 weeks.

FL but not CB or adult $CD34^-$ cells can sustain long-term hematopoietic cultures

If most FL SRC activity is contained within the $CD34^+$ subpopulation, as our data suggest, then the $CD34^-$ population, devoid of SRC activity, should not be able to sustain long-term culture. Instead, cultures of $CD34^-$ cells, consisting mainly of committed hematopoietic cells at various stages of differentiation, would not be expected to last more than a few weeks. Indeed, that is exactly what happened under our conditions with CB, BM, or MPB hematopoietic cells following depletion of $CD34^+$ cells. Although $CD34^+$ cells isolated from these sources were consistently able to generate cultures lasting for at least 6 weeks, no culture initiated with $CD34^-$ cells from these sources lasted more than 2 weeks, with a minimal to absent total cellular expansion, followed by a rapid decline in total cell number.

In contrast, cultures initiated with $CD34^-$ cells separated from unexpanded FL specimens could be reproducibly maintained for extended periods, despite an initial decline. Importantly, when cultures were seeded with $CD34^-$ cells purified following 4 weeks of total cell expansion as described previously (54,55), they behaved in a similar manner, as shown in a typical example in Fig. 4. In this experiment, the curves representing the estimated total cell number of such FL $CD34^-$ cultures after ~1 week, and of clonogenic progenitors after ~3–4 weeks, could be practically superposed to those of control cultures of TNCs. Interestingly, they were also extremely similar to those of cultures of $CD34^+$ cells purified from the same original cell population (Fig. 4), demonstrating that selection of $CD34^+$ cells does not provide an advantage for long-term expansion of FL hematopoietic cells and their progenitors. Similar results were obtained with two additional FL specimens.

To exclude the possibility that the proliferation observed in bulk cultures of FL $CD34^-$ cells originated from a few remaining contaminating $CD34^+$ cells, single-cell cultures from FL TNCs, $CD34^+$ and $CD34^-$ cells were also established. Under our conditions, the frequency of wells where proliferation from a single cell continuously generated thousands of progeny was exceedingly rare, precluding large-scale experiments such as transplantation into NOD/SCID mice. Cell growth usually happened in waves, sometimes following a latency period of several days or even weeks. Proliferation generally occured sooner in wells seeded with $CD34^+$ cells as compared to $CD34^-$ cells, confirming our observations in bulk cultures. To estimate the frequency of HSC/progenitors in a given cell population capable of sustaining long-term cultures, we relied on LDA experiments, seeding 96-well plates with various numbers of FL cells and monitoring growth twice a week. Wells containing 10^3 cells or more after 8 weeks of culture were considered positive for LDA. For example, when TNC, $CD34^+$ and $CD34^-$ subpopulations from a 28-day-old culture of FL#873 were used in LDA, the frequency of progenitors sustaining long-term culture was found to be about 84 per 10^5 TNCs (95% confidence limits: 42–173

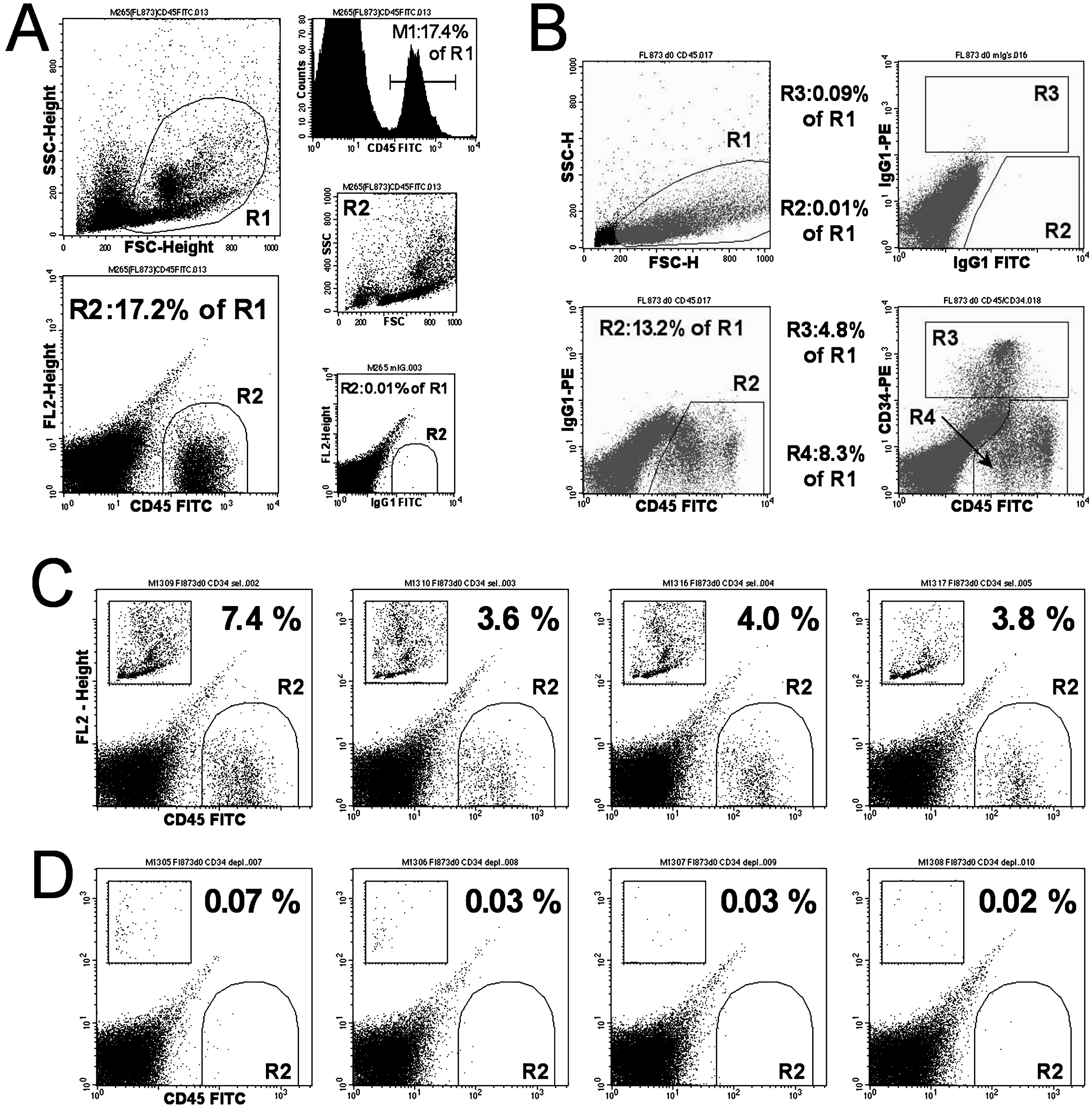

FIG. 2. Engraftment of unexpanded human FL cells into NOD/SCID mice. (**A**) Representative FACS analysis of the BM of a positively engrafted mouse transplanted eight weeks before with ~3×10^6 unexpanded FL#873 cells. Upon gating most viable cells (R1 in *upper left panel*), about 17% of the cells stained positively for the human pan-leukocyte marker CD45 (*lower left panel*, gate R2, and *upper right panel*, region M1). Once backgated to an FSC/SSC plot, these human cells mostly fell within a typical viable, heterogeneous cell population (*right middle panel*). The bottom right panel represents the same mouse BM cell suspension stained with an isotypic control (mouse IgG1) antibody, showing only a few rare events falling in gate R2. (**B**) FACS analysis of freshly thawed unexpanded FL#873 cells. Within a lymphoid-like gate (R1, *upper left panel*), about 13% of the cells are $CD45^+$ (*lower left panel*), with about a third of them also staining positively for the CD34 antigen (*lower right panel*). Control stainings of the same cell suspension with isotypic antibodies are also shown (*upper right panel*). (**C,D**) SRC in unexpanded human FL are found within the CD34 positive population. Sublethally irradiated NOD/SCID mice were transplanted with either ~2×10^5 $CD34^+$ cells (**C**) or ~2×10^6 $CD34^-$ cells (**D**) purified from unexpanded FL#873, and their BM analyzed 8 weeks later for CD45 human cell engraftment. As depicted in more detail in **A,** cells falling within the human $CD45^+$ gate (R2) were backgated to classical FSC/SSC plots, shown as insets in the upper left corner of each panel.

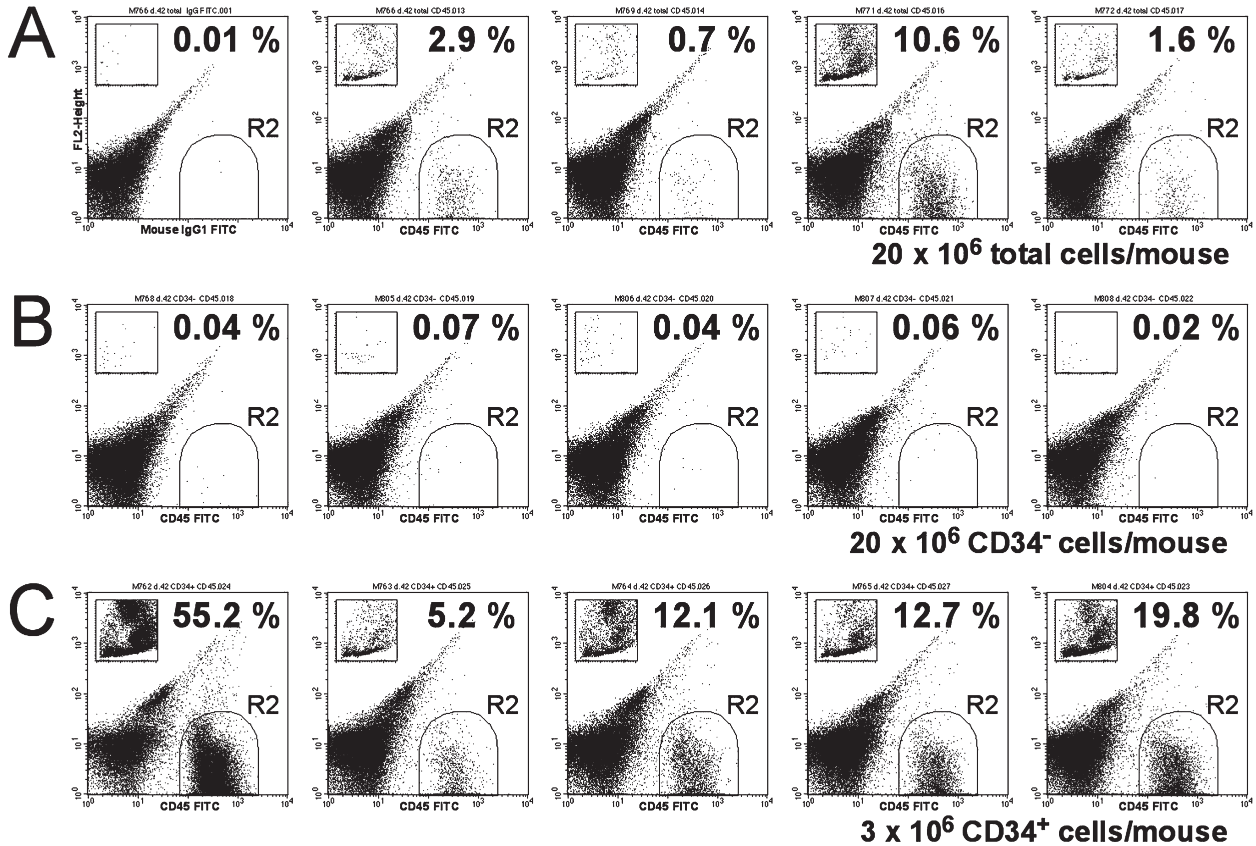

FIG. 3. Segregation of human FL SRC with the $CD34^+$ population in culture. FL#873 hematopoietic cells were expanded as described in Materials and Methods for 6 weeks, and the repopulation potential of the total culture ($\sim 20 \times 10^6$ cells/mouse, **A,** with a representative isotypic control staining shown in the *first panel at left*), and of the purified $CD34^-$ ($\sim 20 \times 10^6$ $CD34^-$ cells/mouse, **B**) and $CD34^+$ ($\sim 3 \times 10^6$ $CD34^+$ cells/mouse, **C**) subpopulations tested in the SRC assay as in Fig. 2.

per 10^5 TNCs), 43 per 10^5 $CD34^+$ cells (95% confidence limits: 15–114 per 10^5 $CD34^+$ cells), and 42 per 10^5 $CD34^-$ cells (95% confidence limits: 16–116 per 10^5 $CD34^-$ cells). These data suggested that the frequency of such HSC/progenitors was similar in both $CD34^+$ and $CD34^-$ subpopulations, and demonstrated that a fraction of FL $CD34^-$ cells (as opposed to contaminating $CD34^+$ cells) were indeed able to proliferate under our experimental conditions.

FL $CD34^-$ cells in culture regenerate $CD34^+$ cells possessing SRC activity

We next asked whether $CD34^-$ cells, purified from unexpanded FL samples or from 4-week-old cultures of FL total cells, both shown above to be devoid of SRC activity, could upon further culture generate $CD34^+$ cells and/or SRCs. First, $CD34^-$ cells from an unexpanded vial of FL#889 were cultured for 28 days. Parallel expansions were established with purified $CD34^+$ cells and TNCs from the same sample, and all three hematopoietic suspensions were tested for CD34 expression and SRC activity. As shown in a representative example in Fig. 5, although very few $CD34^+$ cells were detected after a week of culture of FL#889 $CD34^-$ cells (left panels), 3 more weeks of expansion generated a hematopoietic suspension containing a distinct $CD34^+$ population (about 4.4 % in this example, middle panels), that positively engrafted NOD/SCID mice (Fig. 6A). Control cultures of TNC and purified $CD34^+$ done in parallel from the same FL cellular suspension both retained a substantial percentage of $CD34^+$ cells and also possessed SRC activity, as expected (Fig. 5, right panels and data not shown). Similar results were obtained with two other FL samples, and a few examples obtained with FL#873 are shown in Fig. 6B.

In a second step, 28-day-old FL cultures initiated with TNCs and shown to be rich in SRCs (55) were separated into $CD34^+$ and $CD34^-$ cells as before. Both populations were then expanded for an additional 4 weeks and tested for CD34 expression and SRC activity, in parallel to control cultures consisting of the same 4-week-old cell population also expanded for 4 more weeks, but without CD34 separation. As before, these cultures of $CD34^-$

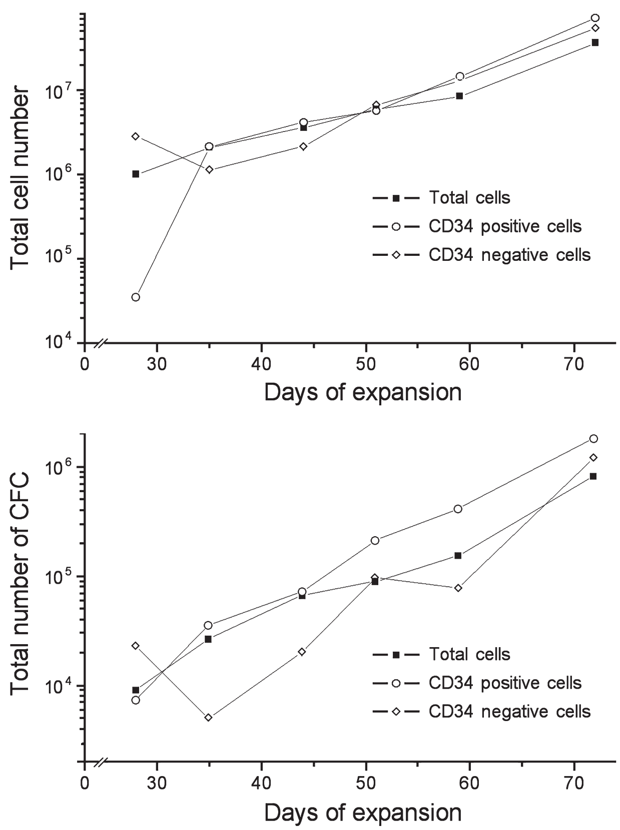

FIG. 4. Long-term expansion of hematopoietic cells and clonogenic progenitors from FL CD34$^+$ and CD34$^-$ populations. Total nucleated cells from FL#852 were cultured for 28 days, after which the CD34$^+$ and CD34$^-$ populations were purified and replaced in long-term culture. For comparison, an aliquot of 10^6 total cells from the same 28-day-old culture was also further expanded. At various intervals, the extrapolated total number of hematopoietic cells (*upper panel*) and progenitors detected by semisolid assays (CFC, *lower panel*) were calculated taking into account the weekly demi-depopulation (54).

cells were able to generate new CD34$^+$ cells, and SRC were also found to be present. Thus, whereas CD34 is a valid marker for FL SRCs in culture, it is also clear that in the FL CD34$^-$ population, whether purified from unexpanded cell suspensions or following culture, some HSCs/progenitors capable of sustaining long-term hematopoietic cultures and generating SRC are present.

Depletion of FL lineage-negative cells does not prevent long-term culture potential

Most freshly isolated HSCs/progenitors are thought to reside within the lin$^-$ fraction of BM, MPB, CB, and FL (3,32,45). If that was also true in culture, FL hematopoietic cells depleted of their lin$^-$ cells and expanded in our culture conditions would be expected to display only reduced, short-term proliferative potential. This was not the case, as described below.

In a first step, we used a 28 day-old culture of FL#841, rich in SRC [~1 in 350,000 TNC by LDA, see ref. 55], and purified lin$^-$ cells by immunomagnetic negative selection. From about 1.2×10^8 total cells, containing ~14 % CD34$^+$ cells, ~1.3×10^5 lin$^-$ cells were isolated. Interestingly, ~90 % of the purified lin$^-$ cells were CD34$^-$/CD38$^-$, although a minor population of CD34$^+$/CD38$^-$ was also clearly detectable by FACS. lin$^-$ cells contained virtually no CD38$^+$ cells, demonstrating an efficient depletion of lineage-positive cells, because an anti-CD38 was part of the antibody cocktail used in the selection. lin$^-$ cells were then expanded in parallel with total cells and also lin$^+$ cells detached from the column. Surprisingly, all cultures proliferated immediately and equally well for at least 6 months, after which the experiment was terminated. As shown in Fig. 7A, the growth curves representing total cellular expansion from all cultures were after a few weeks practically superposable. Similarly, classical CFC assays were performed on all cultures, and the expansion curves of clonogenic progenitors could also be superposed after a few weeks of culture.

In a second step, lin$^-$ cells were purified from unexpanded FL samples. In a typical experiment, from ~40×10^6 total cells, ~6×10^5 lin$^-$ cells were purified. As before, when stained with CD34 FITC and CD38 PE, lin$^-$ cells contained virtually no CD38$^+$ cells, and on average ~70 % of them were CD34$^-$, and ~30 % CD34$^+$. Cultures of lin$^-$, lin$^+$ and TNC from the same FL cell suspension were established and found to expand equally, with the growth curves representing total cellular expansion from these populations practically superposable after ~2 weeks of culture, and remaining so up to 8 weeks when the experiment was terminated (Fig. 7B). At this time, all these cultures contained a similar percentage of CD34$^+$ cells (8–9%), suggesting that they would all engraft equally well, and had achieved an average cellular expansion of about a thousand-fold. Thus, lineage-positive cells from human FL also contained HSC/progenitors capable of long-term proliferation.

DISCUSSION

Expansion of human HSCs has been the subject of intense studies (18,21). Achieving HSC amplification in culture while retaining their multilineage differentiation properties is important for FL and CB, two hematopoetic sources for transplantation (12,16,24,25) limited in HSC numbers. We reported simple and reproducible long-term culture conditions (54), allowing a true expansion of SRCs found within frozen unpurified human FL suspensions (55). Although thought to represent one of the most

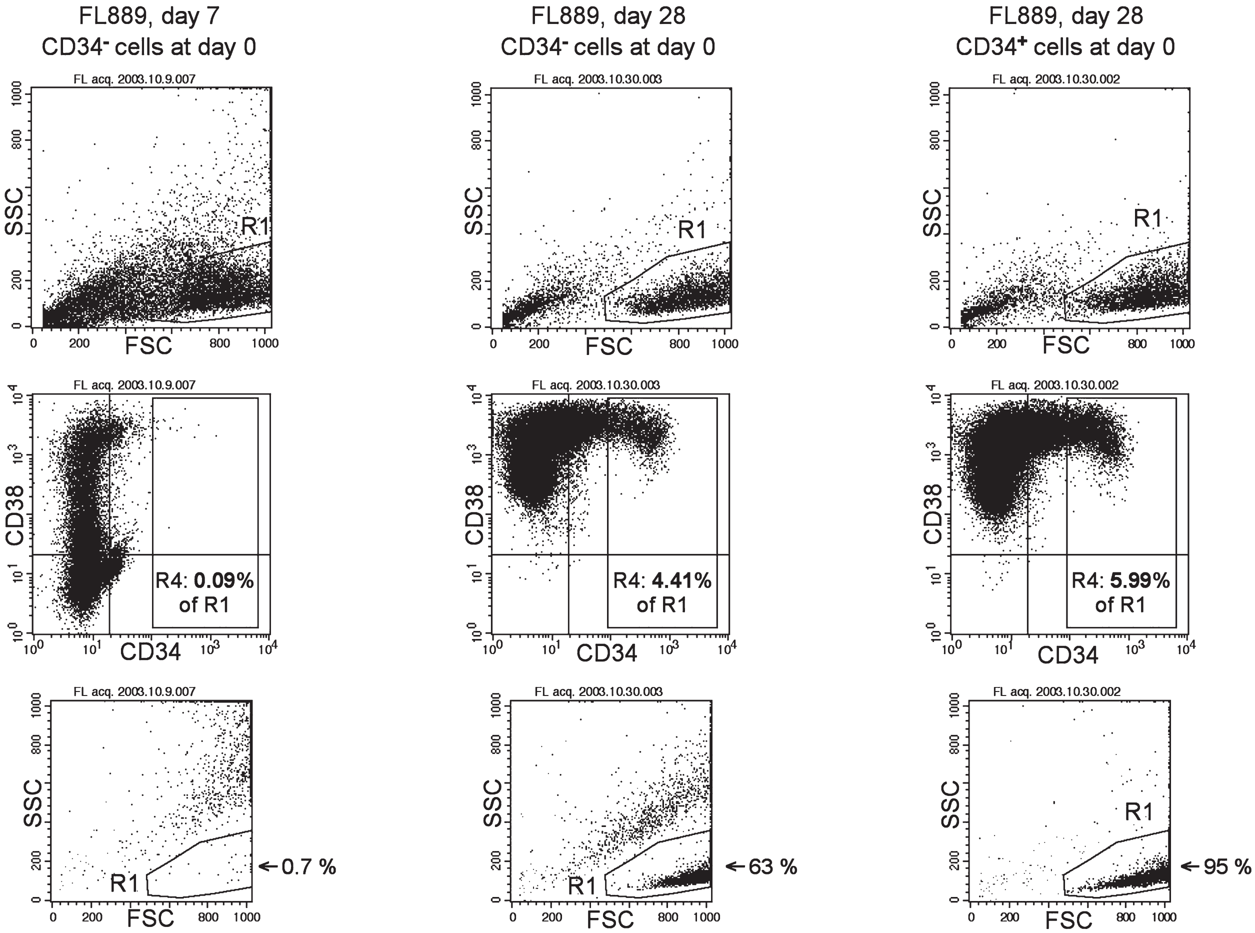

FIG. 5. Regeneration of CD34$^+$ cells from CD34$^-$ in culture. CD34$^+$ and CD34$^-$ cell populations were purified from a freshly thawed vial of unexpanded FL#889 and placed in culture. Every week, the cell suspension was stained with CD34-FITC and CD38-PE and analyzed by FACS. Shown here are the timepoints at 1 and 4 weeks for the culture seeded with CD34$^-$ cells (*left and middle panels*, respectively), and the timepoint at 4 weeks for the culture initiated with purified CD34$^+$ cells (*right panels*). In the CD34/CD38 panels, cells are gated on a large lymphoid-like gate (R1 in *upper panels*); CD34$^+$ cells are shown in gate R4 (*middle panels*), with their frequency expressed as a percentage of R1. In the *lower panels*, the above CD34$^+$ cells are backgated to forward versus side-scatter dot plots.

primitive human HSC/progenitors (48), SRCs may not be equivalent to conceptually defined HSCs, capable of long-term self-renewal and multilineage differentiation. Indeed, contrary to the mouse where long-term repopulation of the hematopoietic system of a lethally irradiated recipient can be achieved by a single, prospectively isolated HSC (35,59,60), human adult and neonatal SRC compartments were shown to be heterogeneous, being composed of various classes of HSCs possessing different proliferative and repopulating potentials (61–63). Additionally, a direct comparison of HSCs contained within baboon BM CD34$^+$ cells suggested that a distinct, more mature population was responsible for NOD/SCID repopulation as compared to autologous engraftment in this primate (64). Although commonly used and accepted for human HSC enumeration, the NOD/SCID system may therefore be biased toward the detection of already committed HSC/progenitors. Thus, a significant obstacle in basic and clinical research remains the detailed characterization and the prospective isolation of true HSCs capable of both self-renewal and multilineage differentiation. Accordingly, purification of specific subpopulations enriched in human HSCs/progenitors has been the subject of intensive studies (3,45).

Various markers and/or functional attributes defining HSCs have been proposed. Among those, the SP phenotype, measuring Hoechst 33342 DNA dye efflux (36,46), and due to *Bcrp1/ABCG2* expression (47), may represent a novel marker common to stem cells from various tissues, including fresh human FL (33). Although easily detected in unexpanded FL suspensions, SP cells were absent in 4-week-old FL hematopoietic cultures possessing robust SRC activity (55). Analyzed for the first time in culture, these data suggested that SP cells may not rep-

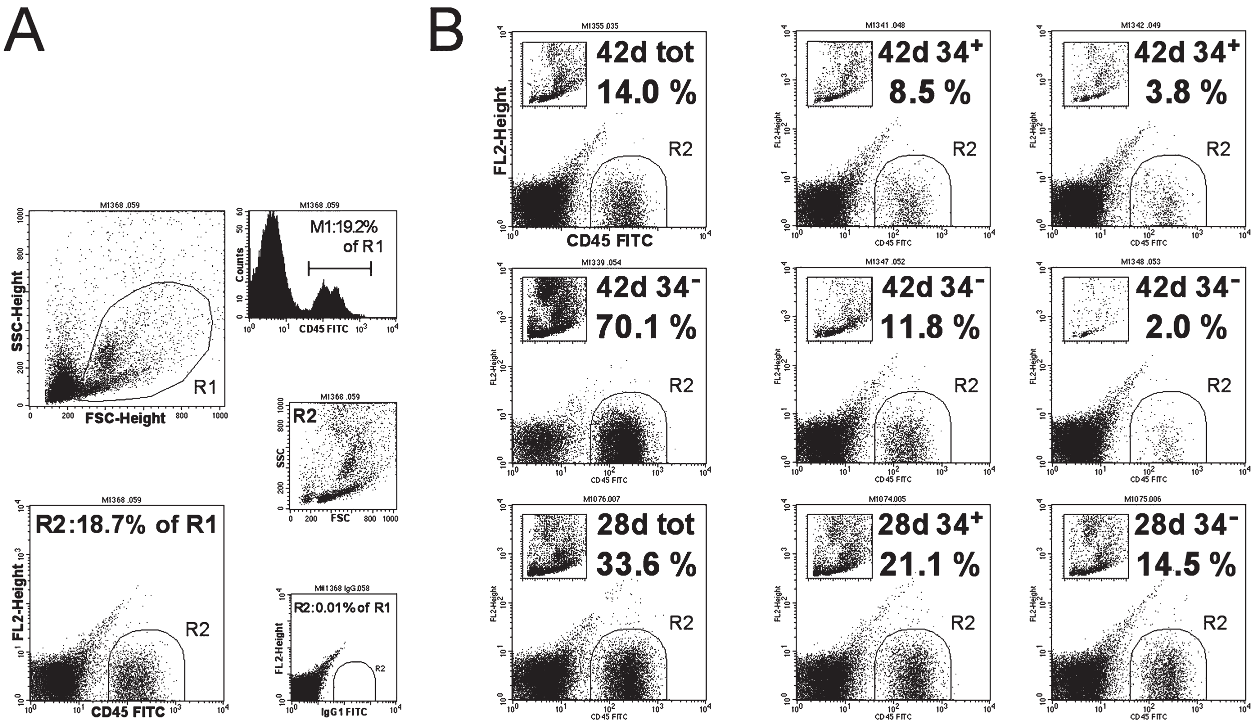

FIG. 6. Generation of SRC from cultured FL CD34$^-$ cells. (**A**) CD34$^-$ cells purified from an unexpanded vial of FL#889 were cultured for 28 days, and the hematopoietic suspension obtained (analyzed by FACS in the *middle row of panels* in Fig. 5) was tested for the presence of SRC 8 weeks after transplantation of $\sim 9 \times 10^6$ cells and found to positively engraft NOD/SCID mice. Legends are as in Fig. 2A. (**B**). Representative examples of human hematopoietic engraftment in the BM of NOD/SCID mice transplanted 8 weeks before with $\sim 20 \times 10^6$ viable cells (*upper and middle panels*) or $\sim 8 \times 10^6$ viable cells (*lower panels*) from various cultures (28 or 42 days as shown) of FL#873 seeded at day 0 with total (tot), purified CD34$^+$ (34$^+$) or CD34$^-$ (34$^-$) cells. Backgating to FSC/SSC plots of human-derived hematopoietic cells (CD45$^+$ in R2, expressed as the percentage of viable cells in R1, see **A**) is also shown in the upper left corner of each panel.

resent a valuable HSC enrichment tool in cultures devised for HSC expansion. This correlates well with a study showing that SP cells in adult murine BM represent a resting HSC population, its quiescence regulated by the Tie2/Angiopoietin-1 signaling pathway (65). When ABCG2$^+$ and ABCG2$^-$ subpopulations were separated from FL suspensions expanded for 28 days, SRCs were only found in the ABCG2-negative population and in control TNC from the same cultures, indirectly confirming the SP data discussed above. We also observed that many FL cultures of 28 days, although rich in SRCs (55), were virtually devoid of a CD34$^+$/CD38$^-$ population, shown to contain most SRCs in fresh human FL, CB, and BM cells (32–34). Thus, as described for neonatal/adult sources (51–53), a dissociation between HSC phenotype and function also occurs in FL cultures, and novel markers and/or ways of analyzing HSC function need to be developed. Interestingly, mouse HSCs were also shown to change their phenotype in culture (66).

Toward this goal, we separated FL subpopulations based on the presence or absence of specific cell-surface markers, and analyzed them for long-term culture potential (54) and SRC activity (55). We first demonstrated that most SRCs present in uncultured human FL partitioned with CD34$^+$ cells, confirming previous observations (33). This was also found to be the case following expansion, allowing us to test FL SRC maintenance in older cultures. In preliminary experiments, many NOD/SCID mice injected with $\sim 20 \times 10^6$ TNC following 60–100 days of culture were apparently not engrafted, probably because putative remaining SRCs were diluted by faster proliferating more committed cells. When purified CD34$^+$ cells from 6- to 12-week-old cultures were transplanted, multilineage engraftment was reproducibly observed, demonstrating maintenance of FL SRCs for at least 12 weeks. Whether a better expansion of FL SRCs later than 4 weeks of culture could be achieved would necessitate careful LDA analyses. However, compiling NOD/SCID data, we observed that at equivalent numbers of cells transplanted, there was no strict correlation between the percentage of CD34$^+$ cells in a given sample and the levels of human engraftment in the recipient mouse BM. This was likely due to the inherent properties of the NOD/SCID system, showing an important vari-

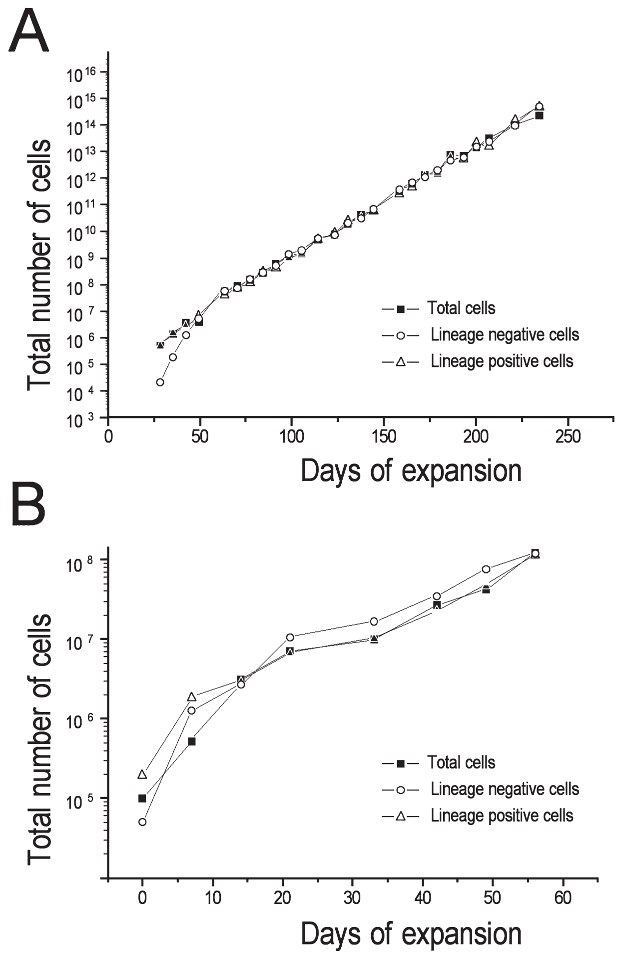

FIG. 7. Depletion of lineage-negative cells does not prevent long-term culture potential of human FL. (**A**) A 28-day-old culture of total cells from FL#841, known to be rich in SRCs (55), was passed through negative immunomagnetic selection (see Materials and Methods). lin^- and lin^+ subpopulations, as well as total cells as control, were further expanded for over 6 months. The estimated total cell number in each culture was plotted at intervals of 1–2 weeks. (**B**) Lineage-negative, positive and total cells from an unexpanded suspension of FL#873 were cultured for 8 weeks, and the estimated total cell number plotted as above.

ability both between but also within hematopoietic samples tested (49).

Next, we observed that $CD34^-$ cells purified from unexpanded but also from expanded FL suspensions and cultured again were able, following an initial decline, to support long-term cultures lasting for at least another 8 weeks. When the FACS profiles, and the estimated total cell and CFC numbers of control, $CD34^+$ and $CD34^-$ cultures were plotted, they were found to become similar after a few weeks of culture. Additionally, single-cell cultures and LDA studies suggested that the frequency of cells capable of sustaining long-term FL culture was similar in the $CD34^+$ and $CD34^-$ subpopulations, ruling out that the observed proliferation of $CD34^-$ cells was simply the result of $CD34^+$ contamination.

Importantly, in our hands, when $CD34^+$ and $CD34^-$ cell populations were separated from fresh CB, MPB, or adult BM, only the positive fractions were able to proliferate, suggesting that for these sources $CD34^+$ cells are best for supporting long-term culture, as described (67,68). Single-cell culture experiments confirmed these data, as little or no proliferation was achieved with $CD34^-$ cells from CB or adult sources, even when seeded at 10–20 cells/well. We then demonstrated for the first time that FL $CD34^-$ cells placed again in culture were able to generate both $CD34^+$ cells and SRCs. Thus, FL behaves differently from other sources, with $CD34^+$ cells not being the only cell type able to sustain long-term culture, and contrary to other sources, there is no advantage to purify the FL $CD34^+$ cell population for expansion. Whether putative FL $CD34^-$ HSCs/progenitors failed to engraft NOD/SCID mice due to impaired homing capabilities would deserve further experimentation, such as direct intra-BM transplantation, as shown for CB $CD34^-$ SRCs (63,69,70). We also attempted to use additional markers to enrich FL cultures in HSCs/progenitors. For example, as murine FL HSCs were shown to express Tie-2 (71), we searched for Tie-2^+ cells in our cultures. Although rare cells ($\leq$0.1% of the total population) expressed Tie-2 in 4-week-old FL cultures rich in SRCs, these were not $CD34^+$ and thus were not expected to engraft NOD/SCID mice. Interestingly, murine HSCs were also shown to lose Tie-2 expression in culture (66).

HSCs/primitive progenitors are thought to reside within the lin^- population (45). We used negative selection to purify lin^- and lin^+ cell populations from unexpanded FL or from 4-week-old FL cultures. FACS analyses after selection suggested that cross-contamination between both fractions was minimal. Surprisingly, both lin^- and lin^+ populations were able to sustain long-term cultures. Unfortunately, freshly separated lin^+ cells could not directly engraft NOD/SCID mice, probably because they are loaded with magnetic particles. Interestingly, we found that following another week of culture, lin^+ cells separated from a 28-day-old culture of FL#889 positively engrafted NOD/SCID mice, suggesting that SRCs were also present in lineage-committed FL hematopoietic cells.

The data described here indicate that under our experimental conditions, the stem/progenitor cell(s) responsible for sustaining long-term cultures of human FL may not display the classical phenotypes described for freshly isolated HSCs. Our studies suggest that human FL HSCs could be either $CD34^+$ or $CD34^-$, as well as lineage-positive or -negative, and that for long-term hematopoietic expansion, cultures initiated with total unseparated human FL cells work best. Our data may first seem difficult to reconcile with published studies. However, functional differences exist between SRCs from different sources (10), and it is important to remember that with few exceptions (67,68,72–75), most stroma-free expansion studies of human adult and/or neonatal HSCs/progenitors lasted 2–3 weeks or less. Only one study besides ours reported human FL SRC expansion (76), and in this case cultures of only 5 days were used. In our hands, if we considered only the first 2–3 weeks of culture, the best proliferation was indeed achieved with subpopulations commonly associated with HSCs, such as $lin^-CD34^+CD38^-$. With CB and adult sources, our own unpublished studies showed that human cells with the above phenotype represent the best target cell for expansion, perfectly correlating with NOD/SCID repopulation studies (32,34). However, as described in detail here, we suggest that for human FL, the capacity of a given subpopulation to sustain long-term hematopoietic cultures (over 6–8 weeks) may represent a more reliable "marker" indicating the presence of HSCs than positive engraftment in SRC assays. We suggest that the phenotypic properties of FL hematopoietic stem/progenitors may not be as strictly defined, and/or may fluctuate more than those from neonatal or adult sources. Alternatively, the identity of putative FL HSCs may be masked in our unsynchronized cultures, in agreement with a recent unified stem cell theory (77).

Ultimately, hematopoietic grafts in the clinical setting need to provide short- and long-term reconstitution, allowing an efficient recovery of adequate levels of circulating neutrophils and platelets and also the stable development of a functional pool of HSCs providing mature blood cells for the patient's life time. Our studies strengthen the accumulated evidence that FL represents a valuable cell source for clinical applications. Indeed, under simple culture conditions, FL hematopoietic cells and their progenitors can be expanded to clinically relevant numbers suitable for allogeneic transplantation into adults.

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REFERENCES

1. Domen J and IL Weissman. (1999). Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. Mol Med Today 5:201–208.
2. Verfaillie CM. (2002). Hematopoietic stem cells for transplantation. Nature Immunol 3:314–317.
3. Shizuru JA, RS Negrin and IL Weissman. (2005). Hematopoietic stem and progenitor cells: clinical and preclinical regeneration of the hematolymphoid system. Annu Rev Med 56:509–538.
4. Durand C and E Dzierzak. (2005). Embryonic beginnings of adult hematopoietic stem cells. Haematologica 90:100–108.
5. Tavassoli M. (1991). Embryonic and fetal hemopoiesis: an overview. Blood Cells 17:269–281.
6. Lansdorp PM, W Dragowska and H Mayani. (1993). Ontogeny-related changes in proliferative potential of human hematopoietic cells. J Exp Med 178:787–791.
7. Rebel VI, CL Miller, CJ Eaves and PM Lansdorp. (1996). The repopulation potential of fetal liver hematopoietic stem cells in mice exceeds that of their adult bone marrow counterparts. Blood 87:3500–3507.
8. Huang S, P Law, D Young and AD Ho. (1998). Candidate hematopoietic stem cells from fetal tissues, umbilical cord blood vs. adult bone marrow and mobilized peripheral blood. Exp Hematol 26:1162–1171.
9. Golfier F, A Barcena, J Cruz, M Harrison and M Muench. (1999). Mid-trimester fetal livers are a rich source of $CD34^{+}/^{++}$ cells for transplantation. Bone Marrow Transplant 24:451–461.
10. Holyoake TL, FE Nicolini and CJ Eaves. (1999). Functional differences between transplantable human hematopoietic stem cells from fetal liver, cord blood, and adult marrow. Exp Hematol 27:1418–1427.
11. Nicolini FE, TL Holyoake, JD Cashman, PP Chu, K Lambie and CJ Eaves. (1999). Unique differentiation programs of human fetal liver stem cells shown both in vitro and in vivo in NOD/SCID mice. Blood 94:2686–2695.
12. Lucarelli G, T Izzi, A Porcellini, C Delfini, M Galimberti, L Moretti, P Polchi, F Agostinelli, M Andreani, M Manna and B Dallapiccola. (1982). Fetal liver transplantation in 2 patients with acute leukaemia after total body irradiation. Scand J Haematol 28:65–71.
13. Touraine JL. (1983). Bone-marrow and fetal-liver transplantation in immunodeficiencies and inborn errors of metabolism: lack of significant restriction of T-cell function in long-term chimeras despite HLA-mismatch. Immunol Rev 71:103–121.
14. Gale RP. (1987). Fetal liver transplantation in aplastic anemia and leukemia. Thymus 10:89–94.
15. Mehra NK, V Taneja, B Jhinghon, T Chaudhuri, S Sharma and V Kochupillai. (1987). HLA status following fetal liver transplantation in aplastic anaemia and acute myeloid leukaemia. Thymus 10:131–136.

16. Touraine JL, MG Roncarolo, R Bacchetta, D Raudrant, A Rebaud, S Laplace, P Cesbron, L Gebuhrer, MT Zabot, F Touraine et al. (1993). Fetal liver transplantation: biology and clinical results. Bone Marrow Transplant 11:119–122.
17. Mehta J, R Powles, J Treleaven, S Kulkarni, C Horton and S Singhal. (1997). Number of nucleated cells infused during allogeneic and autologous bone marrow transplantation: an important modifiable factor influencing outcome. Blood 90:3808–3810.
18. Pecora AL. (2001). Progress in clinical application of use of progenitor cells expanded with hematopoietic growth factors. Curr Opin Hematol 8:142–148.
19. Bittencourt H, V Rocha, S Chevret, G Socie, H Esperou, A Devergie, L Dal Cortivo, JP Marolleau, F Garnier, P Ribaud and E Gluckman. (2002). Association of CD34 cell dose with hematopoietic recovery, infections, and other outcomes after HLA-identical sibling bone marrow transplantation. Blood 99:2726–2733.
20. Wang JC, M Doedens and JE Dick. (1997). Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. Blood 89:3919–3924.
21. Devine SM, HM Lazarus and SG Emerson. (2003). Clinical application of hematopoietic progenitor cell expansion: current status and future prospects. Bone Marrow Transplant 31:241–252.
22. Flake AW and ED Zanjani. (1999). In utero hematopoietic stem cell transplantation: ontogenic opportunities and biologic barriers. Blood 94:2179–2191.
23. Westgren M, O Ringden, P Bartmann, TH Bui, B Lindton, J Mattsson, M Uzunel, H Zetterquist and M Hansmann. (2002). Prenatal T-cell reconstitution after in utero transplantation with fetal liver cells in a patient with X-linked severe combined immunodeficiency. Am J Obstet Gynecol 187:475–482.
24. Grewal SS, JN Barker, SM Davies and JE Wagner. (2003). Unrelated donor hematopoietic cell transplantation: marrow or umbilical cord blood? Blood 101:4233–4244.
25. Rocha V, G Sanz and E Gluckman. (2004). Umbilical cord blood transplantation. Curr Opin Hematol 11:375–385.
26. Krause DS, MJ Fackler, CI Civin and WS May. (1996). CD34: structure, biology, and clinical utility. Blood 87: 1–13.
27. Mavroudis D, E Read, M Cottler-Fox, D Couriel, J Molldrem, C Carter, M Yu, C Dunbar and J Barrett. (1996). CD34$^+$ cell dose predicts survival, posttransplant morbidity, and rate of hematologic recovery after allogeneic marrow transplants for hematologic malignancies. Blood 88: 3223–3229.
28. Jillella AP and C Ustun. (2004). What is the optimum number of CD34$^+$ peripheral blood stem cells for an autologous transplant? Stem Cells Dev 13:598–606.
29. Terstappen LW, S Huang, M Safford, PM Lansdorp and MR Loken. (1991). Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34$^+$CD38$^-$ progenitor cells. Blood 77:1218–1227.
30. Reems JA and B Torok-Storb. (1995). Cell cycle and functional differences between CD34$^+$/CD38hi and CD34$^+$/38lo human marrow cells after in vitro cytokine exposure. Blood 85:1480–1487.
31. Weekx SF, DR Van Bockstaele, J Plum, A Moulijn, I Rodrigus, F Lardon, M De Smedt, G Nijs, M Lenjou, P Loquet, ZN Berneman and HW Snoeck. (1998). CD34^{++} CD38$^-$ and CD34$^+$ CD38$^+$ human hematopoietic progenitors from fetal liver, cord blood, and adult bone marrow respond differently to hematopoietic cytokines depending on the ontogenic source. Exp Hematol 26:1034–1042.
32. Bhatia M, JC Wang, U Kapp, D Bonnet and JE Dick. (1997). Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc Natl Acad Sci USA 94:5320–5325.
33. Uchida N, T Fujisaki, AC Eaves and CJ Eaves. (2001). Transplantable hematopoietic stem cells in human fetal liver have a CD34$^+$ side population (SP) phenotype. J Clin Invest 108:1071–1077.
34. Ishikawa F, AG Livingston, H Minamiguchi, JR Wingard and M Ogawa. (2003). Human cord blood long-term engrafting cells are CD34$^+$ CD38$^-$. Leukemia 17:960–964.
35. Osawa M, K Hanada, H Hamada and H Nakauchi. (1996). Long-term lymphohematopoietic reconstitution by a single CD34$^-$ low/negative hematopoietic stem cell. Science 273:242–245.
36. Goodell MA, M Rosenzweig, H Kim, DF Marks, M DeMaria, G Paradis, SA Grupp, CA Sieff, RC Mulligan and RP Johnson. (1997). Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nature Med 3:1337–1345.
37. Bhatia M, D Bonnet, B Murdoch, OI Gan and JE Dick. (1998). A newly discovered class of human hematopoietic cells with SCID-repopulating activity. Nature Med 4: 1038–1045.
38. Zanjani ED, G Almeida-Porada, AG Livingston, AW Flake and M Ogawa. (1998). Human bone marrow CD34$^-$ cells engraft in vivo and undergo multilineage expression that includes giving rise to CD34$^+$ cells. Exp Hematol 26: 353–360.
39. Sato T, JH Laver and M Ogawa. (1999). Reversible expression of CD34 by murine hematopoietic stem cells [see comments]. Blood 94:2548–2554.
40. Matsuoka S, Y Ebihara, M Xu, T Ishii, D Sugiyama, H Yoshino, T Ueda, A Manabe, R Tanaka, Y Ikeda, T Nakahata and K Tsuji. (2001). CD34 expression on long-term repopulating hematopoietic stem cells changes during developmental stages. Blood 97:419–425.
41. Ogawa M, F Tajima, T Ito, T Sato, JH Laver and T Deguchi. (2001). CD34 expression by murine hematopoietic stem cells. Developmental changes and kinetic alterations. Ann NY Acad Sci 938:139–145.
42. Donnelly, DS and DS Krause. (2001). Hematopoietic stem cells can be CD34$^+$ or CD34. Leuk Lymphoma 40:221–234.
43. Gao Z, MJ Fackler, W Leung, R Lumkul, M Ramirez, N Theobald, HL Malech and CI Civin. (2001). Human CD34$^+$ cell preparations contain over 100–fold greater NOD/SCID mouse engrafting capacity than do CD34$^-$ cell preparations. Exp Hematol 29:910–921.
44. Hess DA, FN Karanu, K Levac, L Gallacher and M Bhatia. (2003). Coculture and transplant of purified

CD34(+)Lin(-) and CD34(−)Lin(−) cells reveals functional interaction between repopulating hematopoietic stem cells. Leukemia 17:1613–1625.
45. Wognum AW, AC Eaves and TE Thomas. (2003). Identification and isolation of hematopoietic stem cells. Arch Med Res 34:461–475.
46. Goodell MA, K Brose, G Paradis, AS Conner and RC Mulligan. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183:1797–1806.
47. Zhou S, JD Schuetz, KD Bunting, AM Colapietro, J Sampath, JJ Morris, I Lagutina, GC Grosveld, M Osawa, H Nakauchi and BP Sorrentino. (2001). The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nature Med 7:1028–1034.
48. Larochelle A, J Vormoor, H Hanenberg, JC Wang, M Bhatia, T Lapidot, T Moritz, B Murdoch, XL Xiao, I Kato, DA Williams and JE Dick. (1996). Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. Nature Med 2:1329–1337.
49. Ballen KK, H Valinski, D Greiner, LD Shultz, PS Becker, CC Hsieh, FM Stewart and PJ Quesenberry. (2001). Variables to predict engraftment of umbilical cord blood into immunodeficient mice: usefulness of the non-obese diabetic—severe combined immunodeficient assay. Br J Haematol 114:211–218.
50. Kyba M and GQ Daley. (2003). Hematopoiesis from embryonic stem cells: lessons from and for ontogeny. Exp Hematol 31:994–1006.
51. Dorrell C, OI Gan, DS Pereira, RG Hawley and JE Dick. (2000). Expansion of human cord blood CD34(+)CD38(−) cells in ex vivo culture during retroviral transduction without a corresponding increase in SCID repopulating cell (SRC) frequency: dissociation of SRC phenotype and function. Blood 95:102–110.
52. Danet GH, HW Lee, JL Luongo, MC Simon and DA Bonnet. (2001). Dissociation between stem cell phenotype and NOD/SCID repopulating activity in human peripheral blood CD34(+) cells after ex vivo expansion. Exp Hematol 29:1465–1473.
53. Donaldson C, P Denning-Kendall, B Bradley and J Hows. (2001). The CD34(+)CD38(neg) population is significantly increased in haemopoietic cell expansion cultures in serum-free compared to serum-replete conditions: dissociation of phenotype and function. Bone Marrow Transplant 27:365–371.
54. Peters R, S Leyvraz, E Faes-Van't Hull, P Jaunin, S Gerber and P Rollini. (2002). Long-term *ex vivo* expansion of human fetal liver primitive haematopoietic progenitor cells in stroma-free cultures. Br J Haematol 119:792–802.
55. Rollini P, S Kaiser, E Faes-van't Hull, U Kapp and S Leyvraz. (2004). Long-term expansion of transplantable human fetal liver hematopoietic stem cells. Blood 103: 1166–1170.
56. Zwicky C, S Gerber, D Gasparini, F Forestier, P Hohlfeld, JD Tissot and P Schneider. (2000). Preparation and analysis of fetal liver extracts. Bone Marrow Transplant 26:667–671.
57. Peters R, S Leyvraz and L Perey. (1998). Apoptotic regulation in primitive hematopoietic precursors. Blood 92: 2041–2052.
58. Perey L, R Peters, S Pampallona, P Schneider, N Gross and S Leyvraz. (1998). Extensive phenotypic analysis of CD34 subsets in successive collections of mobilized peripheral blood progenitors. Br J Haematol 103:618–629.
59. Wagers AJ, RI Sherwood, JL Christensen and IL Weissman. (2002). Little evidence for developmental plasticity of adult hematopoietic stem cells. Science 297:2256–2259.
60. Camargo FD, R Green, Y Capetenaki, KA Jackson and MA Goodell. (2003). Single hematopoietic stem cells generate skeletal muscle through myeloid intermediates. Nat Med 9:1520–1527.
61. Guenechea G, OI Gan, C Dorrell and JE Dick. (2001). Distinct classes of human stem cells that differ in proliferative and self-renewal potential. Nature Immunol 2:75–82.
62. Hogan CJ, EJ Shpall and G Keller. (2002). Differential long-term and multilineage engraftment potential from subfractions of human $CD34^+$ cord blood cells transplanted into NOD/SCID mice. Proc Natl Acad Sci USA 99:413–418.
63. Mazurier F, M Doedens, OI Gan and JE Dick. (2003). Rapid myeloerythroid repopulation after intrafemoral transplantation of NOD-SCID mice reveals a new class of human stem cells. Nature Med 9:959–963.
64. Horn PA, BM Thomasson, BL Wood, RG Andrews, JC Morris and HP Kiem. (2003). Distinct hematopoietic stem/progenitor cell populations are responsible for repopulating NOD/SCID mice compared with nonhuman primates. Blood 102:4329–4335.
65. Arai F, A Hirao, M Ohmura, H Sato, S Matsuoka, K Takubo, K Ito, GY Koh and T Suda. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 118:149–161.
66. Zhang CC and HF Lodish. (2005). Murine hematopoietic stem cells change their surface phenotype during ex vivo expansion. Blood 105:4314–4320.
67. Piacibello W, F Sanavio, L Garetto, A Severino, D Bergandi, J Ferrario, F Fagioli, M Berger and M Aglietta. (1997). Extensive amplification and self-renewal of human primitive hematopoietic stem cells from cord blood. Blood 89:2644–2653.
68. Gammaitoni L, S Bruno, F Sanavio, M Gunetti, O Kollet, G Cavalloni, M Falda, F Fagioli, T Lapidot, M Aglietta and W Piacibello. (2003). Ex vivo expansion of human adult stem cells capable of primary and secondary hemopoietic reconstitution. Exp Hematol 31:261–270.
69. Wang J, T Kimura, R Asada, S Harada, S Yokota, Y Kawamoto, Y Fujimura, T Tsuji, S Ikehara and Y Sonoda. (2003). SCID-repopulating cell activity of human cord blood-derived $CD34^-$ cells assured by intra-bone marrow injection. Blood 101:2924–2931.
70. Yahata T, K Ando, T Sato, H Miyatake, Y Nakamura, Y Muguruma, S Kato and T Hotta. (2003). A highly sensitive strategy for SCID-repopulating cell assay by direct injection of primitive human hematopoietic cells into NOD/SCID mice bone marrow. Blood 101:2905–2913.
71. Hsu HC, H Ema, M Osawa, Y Nakamura, T Suda and H Nakauchi. (2000). Hematopoietic stem cells express Tie-2 receptor in the murine fetal liver. Blood 96:3757–3762.

72. Piacibello W, F Sanavio, A Severino, A Dane, L Gammaitoni, F Fagioli, E Perissinotto, G Cavalloni, O Kollet, T Lapidot and M Aglietta. (1999). Engraftment in nonobese diabetic severe combined immunodeficient mice of human CD34($^{+}$) cord blood cells after ex vivo expansion: evidence for the amplification and self-renewal of repopulating stem cells. Blood 93:3736–3749.
73. Gilmore GL, DK DePasquale, J Lister and RK Shadduck. (2000). Ex vivo expansion of human umbilical cord blood and peripheral blood CD34($^{+}$) hematopoietic stem cells. Exp Hematol 28:1297–1305.
74. Gupta P, TR Oegema, Jr., JJ Brazil, AZ Dudek, A Slungaard and CM Verfaillie. (2000). Human LTC-IC can be maintained for at least 5 weeks in vitro when interleukin-3 and a single chemokine are combined with O-sulfated heparan sulfates: requirement for optimal binding interactions of heparan sulfate with early-acting cytokines and matrix proteins. Blood 95:147–155.
75. Lazzari L, S Lucchi, P Rebulla, L Porretti, G Puglisi, L Lecchi and G Sirchia. (2001). Long-term expansion and maintenance of cord blood haematopoietic stem cells using thrombopoietin, Flt3–ligand, interleukin (IL)-6 and IL-11 in a serum-free and stroma-free culture system. Br J Haematol 112:397–404.
76. Glimm H and CJ Eaves. (1999). Direct evidence for multiple self-renewal divisions of human in vivo repopulating hematopoietic cells in short-term culture. Blood 94:2161–2168.
77. Quesenberry PJ, GA Colvin and JF Lambert. (2002). The chiaroscuro stem cell: a unified stem cell theory. Blood 100:4266–4271.

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