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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+/H11545 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+/H11545 and CD34+/H11546 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,
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 (~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 quantify 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 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 × 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 FACSScan 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. How-
ever, no correlation was observed between the relative number of CD34<sup>+</sup>/H11001<sup>+</sup>/CD38<sup>+</sup>/H11002<sup>+</sup> 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<sup>+</sup>/H11001<sup>+</sup>/CD38<sup>+</sup>/H11002<sup>+</sup> 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<sup>+</sup> and CD34<sup>−</sup> cell populations**

Toward these goals, we first separated FL cell suspensions into CD34<sup>+</sup> and CD34<sup>−</sup> 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<sup>−</sup> cells with little or no contamination with CD34<sup>+</sup> cells. An example of such separation experiments from cultured FL hematopoietic cells is shown in Fig. 1. CD34<sup>+</sup> 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<sup>+</sup> cells, resulting in a suspension (called thereafter CD34<sup>−</sup>) virtually depleted of CD34<sup>+</sup> cells (right panels). Thus, this technique allowed us to separate efficiently, with little loss of cells, CD34<sup>+</sup> and CD34<sup>−</sup> populations for further studies, as described below.

**SRCs in unexpanded human FL are found in the CD34<sup>+</sup> population**

CD34<sup>+</sup> and CD34<sup>−</sup> populations were separated from unexpanded FL cell suspensions and transplanted sepa-

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**FIG. 1.** Separation of CD34<sup>+</sup> and CD34<sup>−</sup> 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<sup>+</sup>, 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<sup>+</sup> and CD34<sup>−</sup> cell populations from this FL hematopoietic culture were purified and analyzed as above. CD34<sup>+</sup> 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<sup>−</sup> population virtually free of CD34<sup>+</sup> 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 ~2 × 10^5 CD34− cells (n = 5) were engrafted (Fig. 2C), whereas none of those that received 10 times more (~2 × 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 occurred 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

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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 \( \sim 3 \times 10^6 \) 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 \( 2 \times 10^5 \) CD34\(^+\) cells (C) or \( 2 \times 10^6 \) 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.
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 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^−
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 hematopoietic 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
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<sup>+</sup> cells suggested that a distinct, more mature population was responsible for NOD/SCID reconstitution 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-
resent a valuable HSC enrichment tool in cultures de-
vised for HSC expansion. This correlates well with a
study showing that SP cells in adult murine BM repre-
sent a resting HSC population, its quiescence regulated
by the Tie2/Angiopoietin-1 signaling pathway (65).

When ABCG2\(^+\) and ABCG2\(^-\) subpopulations were sep-
arated 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 con-
firming 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\(^+\) popula-
tion, 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 poten-
tial (54) and SRC activity (55). We first demonstrated
that most SRCs present in uncultured human FL partitioned with CD34\(^+\) cells, confirming previous observa-
tions (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 \(\sim 9 \times 10^6\) viable cells
following 60–100 days of culture were apparently not engrafted,
probably because putative remaining SRCs were diluted
by faster proliferating more committed cells. When pu-
rified 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 be-
tween 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 proper-
ses of the NOD/SCID system, showing an important vari-

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

Next, we observed that CD34⁺/H11002 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⁺/H11001 and CD34⁺/H11002 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⁺/H11001 cells in our cultures. Although rare cells (≤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⁺ 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.

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 dif-

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.

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ficult to reconcile with published studies. However, func-
tional 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 expan-
sion studies of human adult and/or neonatal HSCs/pro-
genitors 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 subpopu-
lations 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 ex-
pansion, perfectly correlating with NOD/SCID repopu-
lation 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 pheno-
typic 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 uni-
fied stem cell theory (77).

Ultimately, hematopoietic grafts in the clinical setting
need to provide short- and long-term reconstitution, al-
lowing an efficient recovery of adequate levels of circu-
lating neutrophils and platelets and also the stable de-
velopment 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 rele-
vant numbers suitable for allogeneic transplantation into
adults.

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REFERENCES

2. Verfaillie CM. (2002). Hematopoietic stem cells for trans-
topoietic stem and progenitor cells: clinical and preclinical regeneration of the hematolymphoid system. Annu Rev
adult hematopoietic stem cells. Haematologica 90:100–108.
The repopulation potential of fetal liver hematopoietic stem
cells in mice exceeds that of their adult bone marrow coun-
hematopoietic stem cells from fetal tissues, umbilical cord
blood vs. adult bone marrow and mobilized peripheral
9. Goffier 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 Trans-
tional differences between transplantable human hematopoietic stem cells from fetal liver, cord blood, and adult
11. Nicolini FE, TL Holyoake, JD Cashman, PP Chu, K Lamb-
ie and CJ Eaves. (1999). Unique differentiation programs
of human fetal liver stem cells shown both in vitro and in
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.
13. Touraine JL. (1983). Bone-marrow and fetal-liver transplanta-
"


FETAL LIVER HSCs IN CULTURE

72. Piacibello W, F Sanavio, A Severino, A Dane, L Gam-
maitoni, 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

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.

74. Gupta P, TR Oegema, Jr., JJ Brazil, AZ Dudek, A Slun-
guard 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 he-
paran sulfates: requirement for optimal binding interactions
of heparan sulfate with early-acting cytokines and matrix

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 hematopoietic stem cells us-
ing 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 mul-
tiple self-renewal divisions of human in vivo repopulating
2168.

77. Quesenberry PJ, GA Colvin and JF Lambert. (2002). The
chiaroscuri stem cell: a unified stem cell theory. Blood
100:4266–4271.

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