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

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Current procedures for the genetic manipulation of hematopoietic stem cells are relatively inefficient due, in part, to a poor understanding of the conditions for ex vivo maintenance or expansion of stem cells. We report improvements in the retroviral transduction of human stem cells based on the SCID-repopulating cell (SRC) assay and analysis of Lin^- CD34^+ CD38^- cells as a surrogate measure of stem cell function. Based on our earlier study of the conditions required for ex vivo expansion of Lin^- CD34^+ CD38^- cells and SRC, CD34^-enriched lineage-depleted umbilical cord blood cells were cultured for 2 to 6 days on fibronectin fragment in MGIN (MSCV-EGFP-Neo) retroviral supernatant (containing 1.5% fetal bovine serum) and IL-6, SCF, Flt-3 ligand, and G-CSF. Both CD34^+ CD38^- cells (20.8%) and CFC (26.3%) were efficiently marked. When the bone marrow of engrafted NOD/SCID mice was examined, 75% (12/16) contained multilineage (myeloid and B lymphoid) EGFP^+ human cells composing as much as 59% of the graft. Half of these mice received a limiting dose of SRC, suggesting that the marked cells were derived from a single transduced SRC. Surprisingly, these culture conditions produced a large expansion (166-fold) of cells with the CD34^+ CD38^- phenotype (n = 20). However, there was no increase in SRC numbers, indicating dissociation between the CD34^+ CD38^- phenotype and SRC function. The underlying mechanism involved apparent downregulation of CD38 expression within a population of cultured CD34^+ CD38^- cells that no longer contained any SRC function. These results suggest that the relationship between stem cell function and cell surface phenotype may not be reliable for cultured cells.

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Introduction

The expansion of human hematopoietic repopulating cells in ex vivo culture will likely have important applications in transplantation, stem cell marking, and gene therapy. Expansion may allow the use of stem cell sources, which are available in limited quantities, such as umbilical cord blood (CB), and should permit the retroviral transduction of stem cells for the long-term correction of genetic defects. Extensive amplification of primitive progenitor cells assayed as colony-forming cells (CFC) or cobblestone area-forming cells (CAFC) / long-term culture-initiating cells (LTC-IC) in vitro has been achieved, but the repopulating activity of cells produced in these cultures was not tested. While the relationship between CAFC/LTC-IC and the stem cell remains unclear, experiments using the SCID repopulating cell (SRC) stem cell model have demonstrated that several properties distinguish SRC from LTC-IC and suggest that the former is a useful predictor of stem cell activity. Recently, this repopulation model has been applied to cells grown under ex vivo expansion conditions, revealing that SRC numbers can be increased 2- to 4-fold in 4-day serum-free suspension culture.

Culture conditions that induce stem cell cycling without causing differentiation are necessary for gene transfer using Moloney murine leukemia virus–based retroviral vectors, because mitotic activity is required for proviral integration. Early attempts to retrovirally transduce SRC were inefficient; under conditions that permitted the relatively high marking of CFC and LTC-IC, SRC were poorly infected. This result parallels that of human gene therapy trials using similar retroviral vectors, which did not achieve significant levels of gene transfer in transplanted recipients, and suggests that the SRC model is a useful predictor of clinical stem cell behavior. Recent reports have used the SRC assay to improve the efficiency of transduction using new vectors and ex vivo culture protocols. Although the conditions differ among the reports, most mice now contain marked human cells with transduction frequencies of approximately 20%. However, the growth and differentiation behavior of SRC in these culture conditions is unclear and, in general, large numbers of cells needed to be transplanted, suggesting that SRC may be lost during the transduction. Rebel et al were the first to examine this question in detail, and they found a significant decline of approximately 4-fold both in the frequency and total number of SRC under their culture conditions.

With the advent of retroviral vectors expressing selectable markers such as enhanced green fluorescent protein (EGFP), it will be possible to rapidly optimize expansion/transduction protocols to...
favor the efficient transduction of primitive hematopoietic cell subsets, assuming that there is a clear understanding of the cell surface phenotype that correlates with stem cell function. It is generally accepted that CD34 expression correlates inversely with hematopoietic differentiation. The membrane-bound ectoenzyme CD38 has also been used to classify the differentiation state of hematopoietic cells. Among lineage-depleted (Lin−) cells, a CD34/CD38− phenotype identifies a very rare and highly primitive hematopoietic subpopulation in both fetal tissues and adult bone marrow. The CB Lin− CD34+ CD38− subpopulation contains SRC at a frequency of 1 in 617, while Lin− CD34+ CD38− cells lack SRC function. Furthermore, Bhatia et al showed that virtually all new cells produced within 4 days in SRC expansion cultures initiated with CD34+ CD38− cells retained this phenotype, indicating that newly generated SRC are CD34+ CD38−. In contrast, the CD34+ CD38− cells generated following longer times of culture (8 days) no longer had SRC activity, providing further evidence of a link between stem cell function and cell surface phenotype. The CD34+ CD38− surface phenotype has also been used as a direct measure of primitive cell expansion in culture. Together, these publications suggest that the differentiation state of infected cells can be conveniently monitored by simultaneous measurement of CD34 and CD38 expression and gene transfer using EGFP.

In this study, we developed an ex vivo infection protocol that combines retroviral transduction of Lin− cells with ex vivo expansion culture conditions adapted from those previously reported by Bhatia et al and Conneally et al. SRC transduction was significantly improved compared with our earlier study, consistent with recent reports. Unlike other quantitative studies that have documented a loss of SRC, the ex vivo transduction conditions we used resulted in the maintenance of SRC number over 4 days of culture. In addition, these studies also showed a clear dissociation between the CD34+ CD38− cell surface phenotype and SRC function, indicating that this combination of cell surface markers may not be reliable with cultured cells.

Materials and methods

Sample collection and purification

CB samples were obtained from placental and umbilical tissues scheduled for discard according to procedures approved by the institutional review board of Mount Sinai Hospital (Toronto, Canada). Samples were collected in heparin and centrifuged on Ficoll-Paque (Pharmacia, Uppsala, Sweden) to obtain mononuclear cells. Lineage depletion and CD34+ enrichment were achieved by negative selection with the StemSep® system according to the manufacturer’s protocol (Stem Cell Technologies Inc, Vancouver, British Columbia, Canada). The antibody cocktail that was used removes cells expressing glycophorin A, CD2, CD3, CD14, CD16, CD19, CD24, CD41, CD56, or CD66b. The efficiency of primitive cell enrichment was determined by flow cytometric assessment of CD34 expression (see below).

Flow cytometry

Flow cytometric analysis was performed using a FACScan® or FACScan® Plus (Becton Dickinson, San Jose, CA). Isotype controls were mouse immunoglobulin G conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP; all Becton Dickinson), or tricolour (TC; Caltag, Burlingame, CA). The CD34 and CD38 expression characteristics of preculture and postculture CB cells were assessed using anti-CD34-PerCP with anti-CD38-PE (both Becton Dickinson), anti-CD34-PE (Becton Dickinson) with anti-CD38-FITC (Coulter, Fullerton, CA). Further characterization was performed using anti-HLADR-PE (Becton Dickinson), anti-Thy-1-PE (Pharmingen), or anti-CD13-PE (both Coulter). Marrow cells from transplanted nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were assessed using anti-CD45-PerCP, anti-CD19-PE, and anti-CD33-PE (all Becton Dickinson). EGFP fluorescence was detected using detector channel FL1 calibrated to the FITC emission profile. During quadrant analysis, coordinates were set to locate ≥99% of isotype events in the lower left quadrant.

Retrovirus production

MGIN is a murine stem cell virus (MSCV)-based retroviral vector in which EGFP and neomycin phosphotransferase (Neo) are encoded by a bicistronic transcript expressed from the MSCV long terminal repeat. PG13/MGIN producer cells, generated according to previously published procedures, exported gibbon ape leukemia virus (GALV)-psuedotyped MGIN virus at a titer of 1 × 10^6 G418-resistant colony-forming units/mL when assayed on HT1080 human fibrosarcoma cells (CCL-121; American Type Culture Collection, Manassas, VA). Retroviral supernatants were collected from subconfluent cultures 12 hours after the medium was changed to Iscove’s modified Dulbecco’s medium (IMDM; Gibco BRL, Burlington, Ontario, Canada) supplemented with 1.5% fetal bovine serum (FBS; Caisera, Redxola, Ontario, Canada).

Infection protocol

Infections of Lin− CB cells were carried out in flat-bottom 24-well plates or 35-mm dishes (Nunc, Burlington, Ontario, Canada) that were coated with CH-296 fibronectin fragment (Retronectin®; Takara Shuzo Ltd, Otsu, Japan) at 4 µg/cm^2. Cells were seeded at a density of 5 × 10^4 per well in 1 mL, when using 24-well plates and between 0.3 × 10^4 to 1.0 × 10^4 in 2 mL per 35-mm dish. Culture medium consisted of IMDM supplemented with 1.5% FBS, 20% Bovine serum albumin, insulin, and human transferrin; Stem Cell Technologies Inc), 100 µM β-mercaptoethanol, and cytokines at 37°C and 5% CO2. The cytokine mixture included 10 ng/mL interleukin (IL)-6, 10 ng/mL granulocyte colony-stimulating factor (G-CSF), 300 ng/mL stem cell factor (each provided by Amgen, Thousand Oaks, CA) and 300 ng/mL Flt-3 ligand (Immunex, Seattle, WA). After a 12-hour prestimulation, the medium was replaced with supernatant from the PG13/MGIN producer cells and supplemented with BIT, β-mercaptoethanol, and cytokines as described above. The duration of culture was 48 or 96 hours, and media were replaced with fresh retroviral supernatant every 12 hours.

Culture of sorted populations to assess postculture CD34/CD38 phenotype

Lin− CB cells were labeled with anti-CD34-Cy5PE and anti-CD38-PE and sorted to isolate CD34− CD38−, CD34+ CD38+, and CD34+ CD38+ fractions using a FACSort® Plus. CD34+ CD38− cells were seeded at 1 × 10^4 cells per well of a 96-well plate (Nunc) in 0.2 mL of culture medium (using the nutrients and growth factors described above). CD34+ CD38− or CD34+ CD38+ cells were seeded in a 24-well plate (1 × 10^5 cells per well in 1.0 mL of medium). After 4 days at 37°C and 5% CO2, cells were harvested and characterized for CD34 and CD38 expression by flow cytometry.

Fluorescence microscopy

A Leica inverted fluorescent microscope (Leica, Heerbrugg, Switzerland) was employed to examine colonies in methylcellulose (CFC assay) and cobblestone or hematopoietic areas on stroma (CAFC assay) for EGFP fluorescence.

Progenitor assays

CFC assays were performed as previously described except that 10% 5637-conditioned medium was included with uncultured and postculture CB cells. CFC assays were also performed according to the previously published protocol. Briefly, nonirradiated murine MS-5 cells (generously provided by Kirin Brewery, Tokyo, Japan) were seeded into 96-well tissue culture
plates (Nunc) 1 day prior to plating hematopoietic cells. Uncultured or postculture Lin− cells were added at 15 to 100 cells per well in 150 µL of human long-term bone marrow culture media (Stem Cell Technologies Inc). No hydrocortisone was added to the culture media. Wells containing at least 1 hematopoietic or cobblestone area were defined as positive. Positive wells were tested for EGFP fluorescence by flow cytometric analysis and examination with a fluorescent microscope. The absolute number of CAFC present in each fraction was calculated using Poisson statistics.

Analysis of SRC by NOD/SCID mouse repopulation
Primary and cultured cells were transplanted into NOD/SCID mice using a slightly modified version of our standard protocol. Following transplantation, mice received intraperitoneal injections of human IL-3 and GM-CSF (6 µg each; Amgen) on alternating days for the first week posttransplantation. The combination of brief cytokine treatment and large cell doses (>10^6 cells) has been shown to provide optimal engraftment. After 6 to 7 weeks, mice were killed and bone marrow was collected from femurs and tibiae. Human cell content was quantified by Southern analysis using a human chromosome 17-specific α-satellite probe. Flow cytometric analysis of the human-specific pan-leukocyte marker CD45 and human-specific CFC progenitor assays were also applied to each experimental animal. Only mice with ≥0.1% total human content and whose marrow contained human CFC were considered to be engrafted. Gene transfer into human myeloid (CD45+CD33+) and B lymphoid (CD45+CD19+) cells was determined by flow cytometric measurement of EGFP fluorescence; gene transfer into myeloid progenitors was measured in CFC assays with 1500 µg/mL G418 selection and verified by observation of EGFP fluorescence.

Statistical analysis
For limiting dilution assays of CAFC and SRC, Poisson statistics for the single-hit model were applied. The frequency of CAFC and SRC in cell suspensions was calculated using maximum likelihood estimator.

Results
Ex vivo culture increases the number of mononuclear, CD34+CD38−, and progenitor cells
Modifications to the ex vivo culture protocols of Bhatia et al and Connelly et al were implemented to incorporate retroviral gene transfer. Lin− cells were substituted for sorted CD34+CD38− cells because the former are conveniently obtained and cell sorting reduces the final yield of primitive cells. We omitted IL-3 because evidence exists that it can contribute to the induction of differentiation in early hematopoietic progenitors. Infection was facilitated with the application of Retronectin to colocalize cells and virus and with a low (1.5%) concentration of FBS to support optimal retrovirus production by producer cells. Finally, supernatant was replaced at 12-hour intervals to introduce new retrovirus, which in turn mandated the addition of fresh growth factors and nutrients.

The differentiation characteristics of Lin− CB cells in these ex vivo infection conditions were first assessed on the basis of CD34 and CD38 expression. Figure 1 shows the flow cytometric analysis of a representative 4-day time-course experiment. The day 0 CD34/CD38 panel depicts the profile of a typical Lin− CB sample immediately following lineage depletion. CD34+ cells and CD34−CD38− cells constituted 28% to 78% (mean, 53%; n = 15) and 0.4% to 5.5% (mean, 1.7%; n = 15) of the total cell number, respectively, defined by strict gating to exclude nonspecific labeling. It should be noted that while identical flow cytometer instrument settings were used, an increase in cellular autofluorescence over the course of the culture was observed in the isotype analyses. As a result, it was necessary to adjust quadrant gating to maintain exclusion of >99% of nonspecifically labeled cells. During days 1 to 4, as illustrated in Figure 1 and listed in Table 1, the percentage of CD34+ cells increased slightly. However, following day 1 a dramatic increase of CD34+CD38− cell frequency occurred. After 4 days, the CD34+CD38− fraction represented 21% to 48% (mean, 30.6%; n = 14) of the total population. While the expression of CD34 and CD38 was routinely assayed with anti-CD34-PerCP and anti-CD38-PE on a FACScalibur, the validity of the observed phenotype was confirmed with alternate antibody combinations (including anti-CD34-PE with anti-CD38-TC and anti-CD34-PerCP with anti-CD38-FITC) and by analysis on a FACStar Plus flow sorter (data not shown).

Mean expansion values for total cell count and numbers of CD34+ and CD34−CD38− cells from all experiments are given in Table 1. Most striking is the behavior of the CD34+CD38− fraction, which exhibited a mean expansion of 166-fold after 4 days. Also shown are the results obtained from CFC and CAFC.
assays performed using preculture and postculture cells. Day 0 CFC frequencies ranged from 11% to 35% (mean, 26.5%; n = 13), and the day 4 frequencies were moderately higher, 22% to 36% (mean, 34.4%; n = 14), indicating that CFC numbers increased proportionally to the total cell number. In 2 experiments, CAFC frequencies were also assessed. At day 0, these frequencies were proportionally to the total cell number. In 2 experiments, CAFC (mean, 34.4%; n = 5) and the day 4 frequencies were moderately higher, 22% to 36% (mean, 34.4%; n = 5) and the day 4 frequencies were moderately higher, 22% to 36% (mean, 34.4%; n = 5). It was possible to assess the efficiency of gene transfer into the total cell population (Figure 2C). Total cell gene transfer efficiency was assessed by flow cytometry. The percentage of EGFP+ cells within the CD34+CD38− fraction (Figure 2B) was equal or slightly greater than that within the total cell population (Figure 2C). Total cell gene transfer efficiency averaged 20.1% (n = 13) and CD34+CD38− gene transfer efficiency, 20.8% (n = 11), as shown in Table 2. While 4 days in culture was adopted as the standard infection protocol, we also examined the efficiency of cell marking after 2 or 6 days. After 2 days (overnight prestimulation and three 12-hour infections), the expression of EGFP in the total cell population was very poor (mean, 2.4%; n = 2). This is consistent with the observation of little or no expansion of cell number by day 2 (Table 1), which suggests that few cells had entered mitosis by this time. At day 6, the efficiency of gene transfer into “expanded” cells was somewhat greater than day 4 (26.5%; n = 1). CFC and CAFC assays were performed to test the efficiency of gene transfer into clonogenic progenitor cells. As listed in Table 2, CFC were transduced by day 4 at a higher frequency (mean, 26.3%; n = 12) than that of total cells but also exhibited reduced transduction when the culture period was shortened to 2 days. CAFC transduction after 4 days (3% and 8%; n = 2) was substantially less efficient than total cell transduction. Intriguingly, in CAFC assays we were able to reproducibly generate B-cell production in addition to the commonly recognized myeloid cobblestone areas and hematopoietic areas (data not shown). In rare cases, we observed EGFP marking of these B-lymphoid cells, which are derived from a primitive lymphomyeloid precursor.39

**Ex vivo culture does not induce SRC expansion**

While in vitro results indicated the dramatic expansion of cells with a primitive (CD34+CD38−) phenotype and modest expansion of primitive cells with in vitro progenitor activity, we were most interested in the fate of repopulating cells. We therefore performed SRC assays in which NOD/SCID mice were injected with uncultured or ex vivo transduced cells. The dose range was selected to include 1 to 20 SRC, given that an average of 1.7% of uncultured Lin− CD38− cells are CD34+CD38− and that 1 in 617 of these cells is an SRC.39 Murine bone marrow was assessed for human cell content after 6-8 weeks. Figure 3 shows the engraftment percentage of all injected mice, using values obtained by anti-CD45 flow cytometry in the case of well-engrafted (≥5% human cells) mice and by Southern blot quantification of all others. By Poisson statistics, the SRC frequency of the uncultured cells was calculated to be approximately 1 in 2.5 × 105 (χ2 = 4.84). The SRC frequency of cells following 4 days of transduction was 1 in 8.8 × 105 (χ2 = 7.58). When this approximate 3.5-fold decline in SRC frequency is combined with an average 4.2-fold increase in total cell number, the result is maintenance—but no significant expansion—of SRC after 4 days in culture. Thus, there was a dissociation between the CD34+CD38− phenotype, which showed a dramatic increase, and SRC, whose number remained approximately constant within the same cultures.

**Gene transfer into repopulating cells**

The ex vivo transduction conditions described here were intended to induce a significant percentage of SRC to enter mitosis, and therefore become permissive to retroviral infection while retaining repopulating activity. The human cells present in engrafted NOD/SCID mice were therefore examined for evidence of gene marking by assessing both EGFP fluorescence and the presence of G418-resistant CFC. Table 3 lists mice that were engrafted with NOD/SCID mice, using values obtained by anti-CD45 flow cytometry in the case of well-engraftment (≥5% human cells) mice and by Southern blot quantification of all others. By Poisson statistics, the SRC frequency of the uncultured cells was calculated to be approximately 1 in 2.5 × 105 (χ2 = 4.84). The SRC frequency of cells following 4 days of transduction was 1 in 8.8 × 105 (χ2 = 7.58). When this approximate 3.5-fold decline in SRC frequency is combined with an average 4.2-fold increase in total cell number, the result is maintenance—but no significant expansion—of SRC after 4 days in culture. Thus, there was a dissociation between the CD34+CD38− phenotype, which showed a dramatic increase, and SRC, whose number remained approximately constant within the same cultures.

![Figure 2. Gene transfer into the “expanded” CD34+CD38− subpopulation is comparable to that into the total cell population.](image-url)

(A) After 4 days of ex vivo transduction culture, cells were labeled with anti-CD34-PerCP and anti-CD34-PE. R1 is a region defining the CD34+CD38− fraction. (B) A histogram shows EGFP fluorescence in the total cell population. (C) A histogram shows EGFP fluorescence within the CD34+CD38− fraction as defined by the R1 region shown in (A).
Table 2. Gene transfer efficiency into cell subsets and clonogenic progenitors

<table>
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<th>Component</th>
<th>Gene Transfer*</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
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<tr>
<td>Total cells</td>
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<td>CD34<em>CD38</em> cells</td>
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<td>CFU</td>
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<td>26.3% (n = 12)</td>
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<tr>
<td>CAFC</td>
<td>7.5% (n = 2)</td>
<td>5.5% (n = 2)</td>
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*Gene transfer into CFU is expressed as the ratio of G418-resistant colonies to unselected colonies; for other components, gene transfer efficiency is the percentage of cells, colonies (CFU), or hematopoietic areas (CAFC/LTC-IC) exhibiting EGFP fluorescence. ND indicates not done.

Table 3. Transduction frequencies of human cell subsets and CFC from engrafted NOD/SCID mice

<table>
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<tr>
<th>Mouse</th>
<th>Cells Injected (x10^9)</th>
<th>% Human Cells</th>
<th>% Human Myeloid Cells</th>
<th>% Human Lymphoid Cells</th>
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NA indicates not applicable; ND, not done.

*These mice received a limiting dose of postculture cells (≤8.8 x 10^9).

Figure 3. The engraftment levels of NOD/SCID recipients injected with uncultured or ex vivo–transduced cells. The percentages of human cells within the marrow of NOD/SCID mice transplanted with uncultured Lin−CB or ex vivo–transduced Lin−CB are shown. Values are derived from flow cytometric analysis where engraftment exceeded 5% and Southern blot quantification of all others. The dose of injected cells is shown on the X axis. Closed circles represent mice containing EGFP+ human cells, crossed circles indicate mice in which the human cells did not contain EGFP, and open circles show mice that could not be assessed for human cell transduction.

Characterization of CD34*CD38* cells produced in culture

The combination of a large expansion of CD34*CD38* cells without SRC expansion is difficult to reconcile given the large body of evidence showing that cells with this phenotype are highly enriched for primitive cells, including SRC. To characterize these cells in more detail, multiparameter flow cytometry was performed during 4 days of ex vivo transduction culture assessing whether markers of maturation were also present on the CD34*CD38* cells. Although a proportion of the CD34*CD38* cells that started culture expressed CD13 and CD33, the level of expression on each cell was very low as monitored by the fluorescence channel. A progressive increase in the frequency of CD34*CD38* cells that also express high cellular levels of CD13 (early myelo/monocytic marker) or CD33 (early myeloid marker) was observed; by day 4, 95% and 93% of the CD34*CD38* cells derived from cultured CD34*CD38* cells expressed CD13 and CD33, respectively (Figure 5). Thus, the major CD34*CD38* cells derived from cultured cells appear to be fundamentally different compared with uncultured cells because they show evidence of significant maturation.

Because a progressive decrease in the frequency of CD34*CD38* cells was observed while CD34*CD38* cells were increasing in frequency, we hypothesized that the expression of CD38 in relatively mature CD34*CD38* cells was subject to downregulation during culture. Prior studies have shown that both
fresly isolated CD34+CD38+ cells and CD34+CD38+ cells that are generated in ex vivo culture from Lin−CD34+CD38− cells lack SRC activity.5,11 Therefore, the loss of CD38 expression on these cells would result in their classification as CD34+CD38− diluting SRC activity in this fraction. To assess this possibility, sorted Lin−CD34+CD38− cells were cultured for 4 days under the same conditions as shown for Figure 1. In addition, we wanted to examine the fate of isolated Lin−CD34+CD38− and Lin+CD34−CD38+ cells under these conditions because all 3 fractions of cells are present within the lineage-depleted samples used in our transduction experiments, and both of these fractions have been shown to contain SRC.9,33 Figure 6 illustrates the phenotypic assessment of each population in a representative experiment. Interestingly, the phenotype of Lin−CD34+CD38+ cells (Figure 6A) changed dramatically after 4 days in culture (Figure 6B). By day 4, 23% of the cells adopted a CD34+CD38− phenotype while cell number increased by an average of 16-fold (n = 3). Therefore, approximately 4 × 10^5 “CD34+CD38−” cells were produced after an initial seeding of 1 × 10^5 CD34+CD38+ cells, representing a yield of 400% in the CD34+CD38− fraction. When 1 × 10^5 sorted Lin−CD34+CD38− cells (Figure 6D) were cultured, approximately 5% acquired CD34 expression (Figure 6E) consistent with our recent studies33 (Bonnet et al, in preparation); because the total cell number declined by an average of 2-fold in these cultures (n = 3), approximately 2.5 × 10^5 CD34+CD38− cells (a 2.5% yield) were present on day 4. Nearly all Lin−CD34+CD38− cells (Figure 6G) retained the same phenotype while expanding by approximately 4.1-fold (410% yield; n = 3) after 4 days in culture (Figure 6H) consistent with our earlier study11 under somewhat different culture conditions. The values obtained in these experiments with isolated subpopulations can be extrapolated to an unsorted Lin−CB as follows. A typical uncultered Lin− CB sample of 1 × 10^6 cells contains 5.13 × 10^5 (51.3%) CD34+CD38+ cells, 1.6 × 10^5 (16%) CD34−CD38+ cells, and 1.7 × 10^5 (17%) CD34+CD38− cells. With the yields calculated above, after 4 days in culture the fraction that was originally CD34+CD38− should have generated 2 × 10^6 “CD34+CD38−” cells, the original CD34−CD38+ fraction should have produced 4 × 10^3 “CD34−CD38+” cells, and the initial CD34−CD38− fraction should have given rise to 7 × 10^4 “CD34−CD38−” cells. Therefore, 96.4% of the “CD34+CD38−” cells present at day 4 are predicted to have been derived from CD38 downregulation in mature Lin−CD34+CD38+ cells that contain no SRC activity.

**Discussion**

In this report, we have developed conditions that result in both efficient retroviral transduction of human SRC and their preservation during short-term ex vivo culture. In addition, the use of the EGFP marker gene together with flow cytometry enabled assessment of changes in the cell surface phenotype of specific hematopoietic subfractions during ex vivo culture and of the efficiency of gene transfer. Unexpectedly, the ex vivo transduction cultures generated large numbers of CD34+CD38− cells without a concomitant increase in the number of SRC. This dissociation of the CD34+CD38− cell surface phenotype, previously associated exclusively with the stem cell compartment, and stem cell function within the cultured cells indicates that caution should be used when using flow cytometric methods to optimize stem cell transduction and inferring stem cell function based on cell surface phenotypes that are valid for uncultered cells.
In our earlier study, we concluded that SRC were relatively difficult to transduce after finding no gene-marked cells in half of the recipient mice and low levels (0.1%-3%) in the rest, even though all mice contained high numbers of human cells. Only in rare (3 of 50) mice could high levels (>20%) of marking be detected. In an attempt to improve these results, many changes were made to the transduction protocol. The conditions for cell culture were adapted from serum-free conditions that we and others had earlier shown resulted in 2- to 4-fold expansion of SRC. The use of fibronectin as a cell support and cell/virus colocalizer eliminated the need for a stromal layer and its high serum requirements, although optimal virus production required the presence of 1.5% FBS. A different retroviral vector (MSCV) was employed that was designed for optimal expression within hematopoietic cells. In addition, the retrovirus was pseudotyped with GALV rather than the amphotropic envelope gene, because hematopoietic cells appear to express higher levels of this receptor. With the new protocol, EGFP+ human cells were found in 75% of engrafted mice and up to 57% of the human cells were marked (mean 18%), indicating a significant improvement in SRC transduction. Interestingly, half of the mice containing marked human cells had been injected with a limiting dose of SRC. The myeloid and lymphoid cell marking in the human graft of these mice indirectly suggests that the marked cells were derived from a transduced SRC rather than 2 marked, lineage-committed precursor cells. Recently, van Hennek et al used an EGFP-expressing retroviral vector to transduce SRC, and other groups have reported similar efficiency of SRC gene marking using a variety of different vectors and culture protocols. Together, these studies provide strong support for new human clinical trials based on a new generation of SRC transduction methodology.

Because mitosis is required for retroviral infection and the marked human cells observed in engrafted mice are derived from transduced SRC, it is reasonable to conclude that some of the SRC placed in ex vivo transduction culture underwent self-renewal divisions. Nevertheless, no significant increase in the number of SRC occurred in culture. The most significant difference between the transduction protocol reported here and that of the expansion protocols of Bhatia et al and Conneally et al, who each observed a net expansion of SRC, is likely to be the presence of 1.5% FBS throughout the protocol. Serum is known to contain factors that promote cell differentiation, and its exclusion may be a key element of the successful SRC expansion protocols developed to date. Unfortunately, the withdrawal of serum compromises the viability and virus production of retroviral producer lines and thus may limit the efficiency of gene transfer. The exclusion of serum by virus concentration or use of a serum substitute has been shown to permit the serum-free transduction of human hematopoietic cells. The results of Schilz et al show that even in the absence of serum a significant loss of SRC can occur. It is possible, however, that the replacement of FBS with a serum substitute may enhance SRC survival; efforts to explore this are in progress.

The retrovirus used in this study contained the EGFP gene, making it convenient to monitor both the consequence of ex vivo culture on various primitive cell fractions as well as gene marking of these cells. Because our earlier studies had provided strong evidence for a tight association between the Lin-CD34+CD38- cell surface phenotype and SRC function, it was anticipated that flow cytometric analysis would provide a useful surrogate assay with which to optimize transduction of the stem cell compartment in a manner less cumbersome than long-term repopulation. Indeed, in these cultures CD34+CD38- cells were readily marked with EGFP. However, both the proportion and total number of cells with a CD34+CD38- phenotype were dramatically increased (166-fold, by number) with a concomitant reduction in CD34+CD38- cells. The combination of a large increase in the number of CD34+CD38- cells in culture and no increase in SRC number was surprising given that the Lin-CD34+CD38- fraction is known to be highly enriched for SRC activity. This suggested that the CD34+CD38- cells that appeared after 4 days were fundamentally different from uncultured cells with the same phenotype, specifically with respect to SRC function. This idea was supported by the demonstration that day 4 CD34+CD38- cells expressed high levels of myeloid differentiation markers (Figure 5). Examination of the kinetics of CD4 and CD38 expression in culture revealed a gradual decrease in the proportion of CD34+CD38- cells, resulting in a “migration” of events from the CD34+CD38- quadrant to the CD34+CD38- quadrant when assessed by flow cytometry (Figure 1). This mechanism was confirmed by the emergence of a substantial CD34+CD38- population when sorted CD34+CD38- cells are cultured under identical conditions (Figures 6A and 6B). Quantitative analysis suggests that most (96.4%) phenotypically CD34+CD38- cells present at day 4 are actually derived from comparatively mature “CD34+CD38-” cells in which CD38 expression has been downregulated. Since neither uncultured CD34+CD38- cells nor those generated in expansion cultures contain SRC, these cells almost certainly contribute to the apparent “expansion” of CD34+CD38- cells without providing additional SRC activity. Similar to our earlier studies, the culture of Lin-CD34+CD38- cells in the transduction culture showed maintenance of the original phenotype, suggesting that the maintenance of SRC in the original Lin-CD34- cells used for the transduction studies came from this fraction. It is also possible that at least some of the SRC also were derived from Lin-CD34+CD38- cells and/or the Lin-CD34+CD38- cells that develop during 4 days of culture. In our earlier studies, SRC activity was found in both cultured and uncultured Lin-CD34- cells. As shown in Figures 6D and 6E, a few CD34-CD38- cells are able to acquire CD34 expression in culture and appear as CD34+CD38- cells on day 4. Because CD34+CD38- cells typically comprise approximately 16% of a Lin-CD38+ sample and only 5% of CD34+CD38- cells are phenotypically CD34+CD38- after 4 days (Figure 6E), the frequency of SRC within these cells cannot be distinguished.

While, to our knowledge, the downregulation of CD38 on Lin-CD34+CD38- cells has not been previously described, we note that in a flow cytometry analysis published by Reems and Torok-Storb, a significant percentage of sorted CD34+CD38- cells appear to lose CD38 expression while retaining CD34 expression after 6 days in culture. More recently, McCowage et al examined the posttransduction phenotype of Lin-CD38- cells and showed that under serum-free conditions a large expansion of CD34+CD38- cells occurred. While repopulating activity was not assayed in these experiments, the published posttransduction CD34+CD38- flow cytometry data are similar to what we have observed. Interestingly, the work of Rebel et al using primitive murine hematopoietic cells provides a striking parallel with our own results. These researchers were able to expand primitive murine Lin-Sca-1+ WGA+ cells up to 1000-fold in culture without any expansion of repopulating activity. This phenomenon may be the result of a phenotype change, as with CD38, or may reflect the selective
expansion of a subset of Lin- Sca-1+ WGA+ cells that lacks repopulating activity.

It is unclear why CD38 should be downregulated on hematopoietic cells during ex vivo culture. CD38 is a multifunctional membrane ectoenzyme that is known to be downregulated as T-cell precursors mature from the CD4+CD8+ stage into CD4+CD8- or CD4+CD8+ T cells and when CD45RA+ T cells differentiate into CD45RO+ memory cells (reviewed by Shubinsky and Schlesinger45). CD38 downregulation also occurs during the maturation of B lymphocytes and has been shown to be mediated by the activity of serine/threonine kinases during IL-4 signaling.46

As a functional test whose endpoint is the detection of engrafted human cells, the SRC assay does not readily distinguish between the quality and quantity of repopulating units. It is conceivable that the number of SRC present in these culture conditions has in fact been expanded but that a proportion of them has been impaired with respect to graft durability, multipotentiality, or the capacity for homing to the recipient bone marrow. With the identification of Lin-CD34+SRC, which can be functionally distinguished from CD34+SRC, it has become clear that multiple types of human SRC exist.33 The recent discovery of the chemokine receptor CXCR4 as a candidate homing molecule47 has revealed another level of regulation that should be amenable to experimental manipulation. The cytokines (IL-6 and SCF) that were shown to up-regulate CD38 downregulation also occurs during the maturation of T cells into CD4+CD8+. CD38 downregulation also occurs during the maturation of B lymphocytes and has been shown to be mediated by the activity of serine/threonine kinases during IL-4 signaling.46

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References


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

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