Myeloid-lymphoid initiating cells (ML-IC) are highly enriched in the rhodamine-c-kit⁺CD33⁻CD38⁻ fraction of umbilical cord CD34⁺ cells

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Objective. The study of hematopoietic stem cells (HSC) is limited by lack of specific markers for HSC. Rhodamine 123 (Rho) is one of the substrates of P-glycoprotein (Pgp), and the presence of active Pgp can be shown by the efflux of Rho. Rho can also be used to measure the mitochondrial transmembrane potential (energy state) of a cell. We reasoned that selection of hematopoietic progenitors using a combination of Rho efflux and phenotypic markers might be superior to use of phenotypic markers alone.

Materials and Methods. We used the myeloid-lymphoid initiating cell (ML-IC) assay as functional measure of primitive progenitors. Umbilical cord blood CD34⁺CD33⁺CD38⁻, CD34⁺CD33⁺CD38⁻Rho⁻, and CD34⁺CD33⁻CD38⁻Rho⁻c-kit⁺ cells were sorted singly onto AFT024 feeders to assess their capacity to become ML-IC.

Results. The frequency of ML-IC in CD34⁺CD33⁻CD38⁻Rho⁻ cells was significantly higher (15 ± 0.4%) than that in CD34⁺CD33⁻CD38⁻ cells (6.2 ± 0.9%, p < 0.05). However, the frequency of long-term culture-initiating cells (LTC-IC) (17 ± 3% vs 12 ± 1.5%) and natural killer culture-initiating cells (NK-IC) (25 ± 3% vs 20 ± 4%) was similar in the two populations. Following the treatment of CD34⁺CD33⁺CD38⁻Rho⁻ cells with verapamil, which blocks Pgp function, no increase in ML-IC was detected compared with CD34⁺CD33⁻CD38⁻ cells (6 ± 0.7%), suggesting that differences in the energy state, which is reflected by Rho staining after verapamil treatment, cannot be used as a criterion to identify human HSC. Further selection of CD34⁺CD33⁻CD38⁻Rho⁻ cells based on expression of c-kit significantly increased the frequency of ML-IC, LTC-IC, and NK-IC by 1.75-, 1.3-, and 1.8-fold, respectively.

Conclusion. Combining the function of Pgp and phenotypic features of hematopoietic progenitors enriches the frequency of cord blood ML-IC to greater than 25%. Use of such enriched populations will allow us to characterize the biological behavior of human HSC.
[10], size differences (elutriation) [11], or the drug-resistant nature of HSC [12].

Drug resistance is mediated by multiple mechanisms. Most commonly, drug resistance is mediated by the P-glycoprotein (Pgp), product of the multidrug-resistance gene (MDR1), which belongs to the family of ATP-binding cassette supergenes [13]. The activity of Pgp can be measured by efflux of rhodamine-123 (Rho) and can be specifically blocked by the calcium-channel inhibitor, verapamil, which blocks Pgp function. Rho is also used to measure the mitochondrial transmembrane potential or energy state of a cell. Zijlmans et al showed that murine long-term repopulating HSC can be enriched by sorting cells based on low Rho labeling after the treatment of verapamil, suggesting that murine HSC are in a low-energy state [14]. Human HSC also express Pgp. However, HSC are not the only cells expressing Pgp; for instance, other human hematopoietic cells such as natural killer (NK) cells also express this protein [15].

In this study, we wanted to enrich umbilical cord blood (UCB) primitive hematopoietic progenitors by a combination of Rho efflux and phenotypic selection. The in vitro ML-IC assay, described by Punzel et al, was used to test whether the frequency of primitive progenitors with myeloid and lymphoid differentiation potential can be significantly increased by both functional and phenotypic features of UCB progenitors [8].

Materials and methods
Isolation of mononuclear cells and CD34+ cells
UCB was obtained from full-term delivered infants after informed consent using guidelines approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota. Mononuclear cells (MNC) were separated by Ficoll-Hypaque (1.077 g/ml) density-gradient centrifugation. CD34+ cells were isolated from MNC by passing over a MACS column twice following the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA, USA). The purity of CD34+ cells was more than 95% as estimated by flow cytometry.

Preparation of AFT024 stromal feeders
The murine fetal liver–derived stromal feeders, AFT024 (a kind gift from Dr. I.R. Lemischka, Princeton University), were irradiated with 2000 rad after being grown to confluence in 96-well plates with Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, Rockville, MD, USA) supplemented with 20% fetal calf serum (FCS) (HyClone, Logan, UT, USA) [16].

Purification of primitive progenitors and preparation of cells for fluorescence-activated cell sorting and analysis
Enriched CD34+ cells were resuspended in phosphate-buffered saline (PBS) containing 0.15 mg/mL Rho ( Molecular Probes, Eugene, OR, USA). After incubation at 37°C for 30 minutes, the cells were washed, resuspended, and reincubated in Iscove’s modified Dulbecco’s medium (IMDM) (Gibco BRL) with 20% FCS at 37°C for 30 minutes in the presence or absence of 50 mg/mL verapamil (Sigma) [14]. The cells were washed and kept on ice in IMDM with 20% FCS. The Rho-labeled cells were subsequently stained with APC-conjugated anti-CD34 (Becton-Dickinson [BD], San Jose, CA, USA), and PE-conjugated anti-CD33 and anti-CD38 (BD) for 30 minutes on ice. The cells were washed once with PBS and resuspended in IMDM with 20% FCS and kept on ice before fluorescence-activated cell sorting (FACS). For isolating CD34+ CD33- CD38+ Rho- c-kit+ cells, PE-conjugated anti-c-kit (BD), PerCP-conjugated anti-CD34, APC-conjugated anti-CD33, and anti-CD38 were used. Cells stained with APC-, PerCP-, or PE-conjugated IgG isotypes were used as controls.

Phenotypic analysis of Rho-labeled CD34+ cells
Enriched CD34+ cells were labeled with Rho as described above. Cells were then stained with PerCP-conjugated anti-CD34, APC-conjugated anti-CD33, APC-conjugated anti-CD38, and a panel of PE-conjugated antibodies against CD2, CD3, CD7, CD10, CD15, CD19, CD27, c-kit (CD117), HLA-DR (all purchased from BD), P-glycoprotein (clone UIC2, Immunotech, Miami, FL, USA), or Flt-3 receptor (Pharmingen, San Diego, CA, USA), respectively, on ice for 30 minutes. For analysis of expression of Thy-1 or CXCR4, Rho-labeled cells were incubated for 30 minutes with biotin-conjugated anti–Thy-1 (Pharmingen) or anti-CXCR4 (clone 12G5, R&D Systems) using concentrations recommended by the manufacturers and subsequently for 15 minutes with PE-conjugated streptavidin (Gibco BRL). Data were acquired by FACScalibur (BD) and analyzed by CellQuest (BD) or WinMDI (Scripp Institute, San Diego, CA, USA).

Pyronin-Y staining
MNC and CD34+ cells were stained with Rho, washed, and restained with 1 mg/mL pyronin-Y (Sigma) for 30 minutes. The Rho and pyronin-Y–labeled cells were washed once and stained with PerCP-conjugated anti-CD34, APC-conjugated anti-CD33, and APC-conjugated anti-CD38 for 30 minutes. Cells were analyzed as previously described [17].

Annexin-V apoptosis assay
Rho-labeled verapamil-treated cells were incubated at 37°C in IMDM with 20% FCS. After 24 hours, cells were harvested and stained with FITC-conjugated anti-annexin-V (Pharmingen), 2 mg/mL propidium iodide (PI) (Sigma), and PerCP-conjugated anti-CD34. Data were acquired and analyzed according to manufacturer’s instructions.

Cell cycle analysis of FACS-sorted CD34+ CD33- CD38-, CD34+ CD33+ CD38+ Rho+, and CD34+ CD33+ CD38- Rho- cells
FACS-sorted cells were permeabilized with ice-cold 70% ethanol for 30 minutes, washed, and stained with 50 mg/mL PI (Sigma) and 100 mg/mL RNase I (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 30 minutes before cell cycle analysis.

Detection of multidrug resistance–associated protein
MRP activity by the efflux of carboxy-2’,7’-dichlorofluorescein
Enriched CD34+ cells were resuspended in PBS containing 2 mM carboxy-2’,7’-dichlorofluorescein ( Molecular Probes, Eugene, OR, USA) as previously described [18]. After incubation at 37°C for 30 minutes, the cells were washed, resuspended, and reincubated in IMDM with 20% FCS at 37°C for 120 minutes in the presence or absence of 2.5 mM probenecid (Sigma). Cells were then stained on ice with PE-conjugated anti-CD34, APC-conjugated anti-CD33, APC-conjugated anti-CD38 for 30 minutes before FACS analysis.
Clonogenic assay
Rho-labeled CD34+ cells either with or without verapamil treatment were plated in clonogenic assay as previously described [19]. Briefly, 1000 cells were plated with clonogenic methylcellulose medium containing 30% FCS, 3 IU/mL erythropoietin (Amgen, Thousand Oaks, CA, USA), and 7.5% conditioned medium of 5637 bladder cancer cell line (ATCC, Manassas, VA, USA). Colony-forming cells (CFC) were scored 10–12 days after culture at 37°C with 5% CO2. CFC were visually defined by cells which generated cell clusters with more than 50 cells.

Single-cell deposition
The method of single-cell deposition has been previously described [8]. Briefly, individual cells were deposited into 96-well plates (Corning Costar, Acton, MA, USA) containing irradiated AFT024 feeders using the automatic cell deposition unit (ACDU) on a FACS-Star Plus (BD). To ensure that only a single cell was deposited, the ACDU was set up in a low-event throughput (200 events/second). The precision of the single-cell deposition was assessed by sorting fluorescent beads into 96-well plates and was estimated by fluorescence microscopy. The percentage of wells that contained 2, 1, and 0 cells were 80, 20, and 20%.

Culture media and culture of myeloid-lymphoid initiating cells
Culture medium for ML-IC (ML-IC medium) consisted of RPMI-1640 (Gibco BRL) with 20% FCS, 10 ng/mL IL-3, 20 ng/mL IL-7 and IL-15, 1% low-endotoxin serum (Hyclone), 5 μg/mL antithymocyte globulin (ATG, Connaught, Toronto, ON, Canada), and 20 ng/mL IL-3, IL-5, IL-7, IL-15, and IL-1b. The frequency of LTC-IC was determined by the total number of LTC-IC divided by total number of sorted single cells.

Culture medium for LT-IC culture (LT-IC medium) was identical to that for ML-IC but also contained 375 µg/mL hydrocortisone (Upjohn, Kalamazoo, MI, USA) to inhibit the growth of lymphoid cells.

Culture medium for natural killer culture-initiating cell (NK-IC) culture (NK-IC medium) consisted of DMEM and Ham’s F12 medium (Gibco BRL) in 2:1 (v/v) mixed supplemented with 20% heat-inactivated human AB serum (Nabi, Boca Raton, FL, USA), 5 μg/L sodium selenite, 20 mg/L ascorbic acid (Gibco BRL), 25 μmol/L 2-ME, and 50 μmol/L ethanolamine (Gibco BRL). Cytokines added on day 0 of lymphoid culture: 1000 U/mL interleukin-2 (IL-2) (a kind gift from Amgen), 5 ng/mL interleukin-3 (IL-3) (R&D Systems), 20 ng/mL IL-7, 20 ng/mL SCF, and 10 ng/mL Flt-3 ligand. Half-medium changes were done weekly with the same medium but supplemented with 10% human AB serum and identical cytokines except IL-3 [20].

Individually sorted cells were maintained on irradiated AFT024 stromal feeders with ML-IC medium for 2 weeks with half-medium change after 1 week. After 2 weeks, each individual well was harvested by trypsinization. The progeny of each well was divided equally in four secondary 96-well plates containing irradiated AFT024 feeders in such a manner that one-fourth of each single-cell progeny was deposited in the identical location of the four secondary plates (Fig. 1).

Two of the four secondary plates were maintained under myeloid conditions with LTC-IC medium for 5 weeks and then laid with clonogenic methylcellulose medium as described [8]. Plates were scored for secondary CFC after an additional 2-week culture. LTC-IC were defined as cells that were capable of forming secondary CFC after 5-week LTC-IC culture. The frequency of LTC-IC was determined by the total number of LTC-IC divided by the total number of sorted single cells.

The other two plates were maintained under lymphoid conditions with NK-IC medium for 6–7 weeks. The presence of NK cells was examined by FACS for CD56+ NK cells or CD19+ B cells. The presence of NK-IC was determined by the total number of NK-IC divided by the total number of singly sorted cells.

An ML-IC was identified when at least one of the daughter cells was an LTC-IC and at least one of the daughter cells was an NK-IC.

Statistical analysis
The results of experimental points from different experiments were reported as mean ± standard error of the mean (SEM). Significance levels were determined by two-sided Student’s t-test. Correlation between ML-IC, LTC-IC, and NK-IC was analyzed by correlation analysis using StatView 5.0 (SAS Institute, Chicago, IL, USA).

Results
Rhodamine efflux is a good selection marker for primitive hematopoietic progenitors
Because CD33 and CD38 have been used to enrich human primitive hematopoietic cells, we first examined the expression of CD33 and CD38 on UCB CD34+ cells and found that 94.06 ± 15.6% of UCB CD34+ cells express CD33 (Fig. 2A) (n = 3). Analysis of enriched CD34+ cells based on Rho efflux showed higher percentage of Rho− cells in the
most primitive CD34+33+38− population (11.2% in R3 of Fig. 2B) than in the less primitive CD34+33+38− (6.7% in R2 of Fig. 2B) and the mature CD34+33+38− populations (3.7% in R1 of Fig. 2B) (n = 3). We then evaluated the expression of lineage markers on CD34+CD33−CD38− Rho− cells. CD34+CD33+CD38− Rho− cells were negative for CD2, CD3, CD7, CD19, and CD27. However, of the CD34+CD33−CD38− Rho− cells, 9.2 ± 2.7%, 78.2 ± 5.1%, 55.3 ± 3.2%, and 75.8 ± 6.7% were positive for CD10, HLA-DR, c-kit, and Flt-3 receptor, respectively (n = 3) (Fig. 3). All CD34+CD33−CD38− Rho− cells were Thy-1medium−low. The expression of CXCR4 on CD34+CD33−CD38− Rho− cells was variable (Fig. 3). Only 5.2 ± 1.3% of the CD34+CD33−CD38− cells were positive for Pgp. CD34+CD33−CD38− Rho− cells did not stain with the RNA stain, pyronin-Y, suggesting that the cells are metabolically inactive (Fig. 4A). These metabolically inactive cells were also quiescent as cell cycle analysis using propidium iodide showed that 98.4% of CD34+ cells, 99.1% of CD34+CD33−CD38− Rho+ cells, and 100% of CD34+CD33−CD38− Rho− cells were in the G0/G1 phase, respectively (Fig. 4B).

**Use of rhodamine efflux can significantly enrich ML-IC, LTC-IC, and NK-IC**

Single CD34−CD33−CD38− cells with or without selection based on Rho efflux were deposited onto irradiated AFT024 feeders to assess the frequency of LTC-IC, NK-IC, and ML-IC. The inclusion of Rho efflux significantly increased the frequency of ML-IC (2.43-fold, p < 0.05), but to a much lesser extent, LTC-IC (1.38-fold, p = 0.26) and NK-IC (1.28-fold, p = 0.38) (Fig. 5). The majority of LTC-IC, NK-IC, and ML-IC were present in the CD34−CD33−CD38− Rho− cells but not in the CD34+CD33−CD38− Rho+ cells (Fig. 5). Subselection of CD34−CD33−CD38− Rho− cells based on their c-kit

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**Figure 2.** Rhodamine staining reflects the primitive state of hematopoietic cells. (A) The expression of CD33 and Rho staining profile of the CD34+CD38− population. Enriched CD34+ cells were labeled with CD38-FITC, CD33-PE, and CD34-APC and analyzed for the expression of CD33 in the gated CD34+33−38− cells. Ninety-four percent of CD34+33−38− cells were CD33+. (B) The Rho efflux in 3 different CD34+−enriched populations. Enriched CD34− cells were stained with Rho, CD33-PE, CD38-PE, and CD34-APC and gated to assess the Rho efflux in different populations (upper panel). The percentage of Rho− cells was higher in the most primitive CD34+33+38− hematopoietic progenitors (R3 in the lower panel, 11.2%) than in the less primitive CD34−33−38− cells (R2 in the lower panel, 6.7%) and the mature CD34+33−38− cells (R1 in the lower panel, 3.7%).

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**Figure 3.** Phenotypic analysis of CD34+33+38− Rho− cells. Enriched CD34+ cells were first stained with rhodamine-123 and labeled with antibodies against CD2, CD3, CD7, CD10, CD19, HLA-DR, Thy-1, Flt-3 receptor, c-kit, P-glycoprotein (Pgp), and CXCR-4. CD34+33+38− cells were gated and analyzed for their expression of different antigens and intensity of rhodamine. X-axis represents the different lineage markers; y-axis, rhodamine intensity.
expression further increased the frequency of LTC-IC (1.8-fold, p < 0.05), NK-IC (1.3-fold, p < 0.05), and ML-IC (1.75-fold, p < 0.05). Addition of 50 mg/mL verapamil to block Pgp decreased LTC-IC, NK-IC, or ML-IC in CD34+ CD33+ CD38− Rho+ cells to the level in CD34+ CD33+ CD38− cells (statistically not significant compared with those from CD34−CD33− CD38− cells), suggesting that the active state of Pgp defines the function of multipotent hematopoietic progenitor cells. The reduction of ML-IC in the verapamil-treated CD34+ CD33+ CD38− Rho+ cell population was not due to toxic effects of verapamil as there was no difference in the percentage of annexin-V+ cells in verapamil-treated cells compared with untreated cells after 24 and 48 hours of culture. There was also no difference in the frequency of CFC between the verapamil-treated and untreated CD34+ cells. As it was possible that the treatment with verapamil only selected the most primitive progenitors which were not detected by either conventional LTC-IC or our ML-IC assay, we tested whether the frequency of extended LTC-IC (secondary colony-forming cells generated in LTC-IC in culture for more than 10 weeks) was increased in the verapamil-treated CD34+CD33+ CD38− Rho− cells. However, extended LTC-IC frequency was lower in verapamil-treated CD34+CD33+ CD38− Rho− cells than untreated CD34+ CD33+ CD38− Rho− cells (data not shown).

**The rhodamine efflux is mediated by Pgp, not by the multidrug resistance–associated protein**
P-glycoprotein is not the only drug-resistance gene in mammalian cells. Other proteins in the P-glycoprotein family are also responsible for drug resistance. Therefore, efflux of Rho could be mediated by other energy-dependent pumps, e.g., the multidrug resistance–associated protein, MRP. MRP activity can be measured by an MRP-specific dye, carboxy-2′,7′-dichlorofluorescein (CDF). Efflux of CDF in MRP-expressing cells can be blocked by the specific inhibitor, probenecid. We examined whether MRP contributed to the Rho efflux of CD34+ cells. Rho efflux could be blocked by verapamil but not by probenecid (Fig. 6). The Rho efflux in the presence of probenecid showed two separate peaks. The reason for the change in Rho efflux mediated by probenecid is currently unknown. Conversely, the CDF efflux in CD34+CD33+ CD38− cells was not affected by either verapamil or probenecid. Therefore, efflux of Rho from CD34+CD33+ CD38− cells is mediated by Pgp, not MRP.

**Discussion**
In this study, we show that primitive UCB hematopoietic progenitors, ML-IC, can be significantly enriched by combining rhodamine efflux and phenotypic selection. We found

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that CD34⁺CD33⁺CD38⁺Rho⁻c-kit⁺ cells have the highest frequency of ML-IC, LTC-IC, and NK-IC, followed by CD34⁺CD33⁺CD38⁺Rho⁺, CD34⁺CD33⁺CD38⁻, and CD34⁺CD33⁺CD38⁺Rho⁺ cells. The frequency of ML-IC in verapamil-treated CD34⁺CD33⁺CD38⁺Rho⁻ cells is similar to that in CD34⁺CD33⁺CD38⁻ cells.

Consistent with other reports, our results show that Rho staining is a useful tool to select primitive progenitors and that Rho intensity correlates with the stage of differentiation in different subsets of hematopoietic cells. Ratajczak has shown that CD34⁺c-kit⁺Rho⁻ cells are highly enriched for scid-repopulating cells in human bone marrow [21]. However, the mechanisms underlying such low Rho staining in human HSC is not clear. The efflux of Rho could be caused by dye extrusion mediated by P-glycoprotein, an important protein for drug resistance. However, Rho is also used to measure mitochondrial transmembrane potential and several reports have shown that low Rho staining reflects the low-energy state of cells. Kim et al showed that low Rho staining of HSC in young mice is due to low mitochondrial activation but not dye efflux [22]. Zijlmans et al showed that murine HSC exist only in WGA⁻Lin⁻Rho⁻ cells [14]. The early phase of engraftment is mediated by Rho⁻ cells while the late phase of engraftment is mediated by verapamil-treated Rho⁺ cells, suggesting that the low Rho staining in murine long-term engrafting HSC reflects the low-energy state but not dye efflux. However, our results show that the low Rho staining in primitive UCB hematopoietic progenitors is caused by efflux via the multidrug-resistance ATP-binding protein, Pgp, and not due to mitochondrial activity. Cell-cycle analysis of CD34⁺CD33⁺CD38⁻Rho⁻ cells was analyzed by FACS.

**Figure 5.** ML-IC and to a lesser extent LTC-IC and NK-IC are enriched by rhodamine dye efflux and selection using CD33, CD38, and c-kit antibody. Different subsets of FACS-sorted cells were deposited singly onto AFT024 feeders and cultured as shown in Figure 1 to assess the frequency of (A) LTC-IC, (B) NK-IC, and (C) ML-IC. Each bar represents data from 3 to 4 independent experiments. Error bar represents SEM. #: p < 0.05. Y-axis represents percent frequency.

**Figure 6.** CD34⁺ cells have active MDR but not MRP. CD34⁺ cells were treated with (A) rhodamine or (B) carboxy-2',7'-dichlorofluorescein (CDF), an MRP-specific dye, with or without verapamil or probenecid. Cells were then labeled with PE-conjugated anti-CD34, APC-conjugated anti-CD33, and APC-conjugated anti-CD38. The efflux of rhodamine and CDF by CD34⁺CD33⁺CD38⁻ cells was analyzed by FACS.
and transduction of mouse hematopoietic cells with MDR-1 increases the percentage of SP cells, suggesting that P-glycoprotein also plays an important role in Hoechst 33342 extrusion [24]. Zhou et al recently reported that expression of another ATP-binding cassette protein Bcrp1/ABCG2 is responsible for the SP phenotypes in both mouse and rhesus monkey [25]. Different from murine SP cells that do not express CD34, human lineage-depleted SP cells in UCB express CD34, suggesting that CD34 is still a useful marker for UCB hematopoietic progenitors [26].

Our results also show the importance of c-kit expression as CD34+CD33−CD38−Rho−c-kit+ cells have twofold higher content of ML-IC than CD34+CD38−Rho− cells. Mice deficient in c-kit show severe defects in hematopoiesis. Domen et al recently showed that HSC need both bcl-2 and c-kit to overcome apoptosis [27]. The c-kit ligand provides a strong proliferative stimulus to HSC. However, c-kit− pluripotent stem cells have also been described in mice [11]. Such c-kit− cells are slow in response to cytokine stimulation. Recent studies further show that CD34+Lin− cells can engrat into cardiac muscle, indicating that c-kit may be present not only on hematopoietic progenitors but also on other types of stem cells [28]. In addition, c-kit is also found on more mature hematopoietic cells. For instance, combining Rho staining and c-kit selection can be used to enrich T-cell progenitors. In contrast to what we have shown here, cells enriched for T-cell progenitors are c-kit−Rhobright [29]. Therefore, our data support the approach that combining c-kit and Rho staining is essential to enrich primitive hematopoietic progenitors.

Our results on CXCR4 are less clear. In murine studies, CD34+CD38low/CXCR4+ cells are capable of engrafting primary and secondary recipient mice. In vivo stimulation of CD34+CD38+ cells by SCF upregulates the expression of CXCR4 and potentiates their homing capacity. We could not identify a distinct population of cells expressing CXCR4. The chemokine stroma-derived factor-1α (SDF-1α) has been implicated in the migration of primitive hematopoietic cells, suggesting that HSC express the SDF-1α receptor, CXCR4. However, consistent with our finding, Rosu-Myles et al recently showed that human HSC are heterogeneous in CXCR4 expression [30]. It is possible that the expression of CXCR4 correlates with the homing capacity instead of the multilineage potential, a characteristic not tested in the ML-IC assay [31].

We found that combining antibodies against CD33 and CD38 eliminates the majority of lineage-positive cells, with the exception of CD10. CD10 is thought to be a marker for a common BM lymphoid progenitor. Human CD34+Lin−CD10+ cells can generate T, B, NK, and dendritic cells [32]. However, such progenitors also express CD38. Therefore, it remains to be tested whether removal of CD10+ cells will further enrich ML-IC.

In summary, we show that a combination of phenotypic selection, especially c-kit and Rho efflux, provides an efficient way to enrich primitive cells. These highly enriched subpopulations should allow us to further characterize the behavior of HSC both in vitro and in vivo. In addition, use of these purified subsets of cells in combination with modern genetic analysis should lead to a better understanding of the genetic control of hematopoiesis.

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