The human hematopoietic stem cell compartment is heterogeneous for CXCR4 expression

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The chemokine stromal derived factor-1α (SDF-1α) has been implicated recently in the chemotaxis of primitive human hematopoietic cells, suggesting that pluripotent human stem cells express the SDF-1α receptor, CXCR4. By using flow cytometry and confocal microscopy, we have identified and isolated primitive subsets of human CXCR4$^+$ and CXCR4$^-$ cells. Distinctions in the progenitor content and response to SDF-1α *in vitro* indicate that CXCR4$^+$ and CXCR4$^-$ cells represent discrete populations of primitive blood cells. The i.v. transplantation of these subfractions into immune-deficient mice established that both possess comparable engraftment capacity *in vivo*. Human myeloid, lymphoid, and primitive CD34$^+$ CXCR4$^+$ cells were present in chimeric animals transplanted with either subset, indicating that CXCR4$^+$ and CXCR4$^-$ stem cells have equivalent proliferative and differentiative abilities. Our study indicates that the human stem cell compartment is heterogeneous for CXCR4 expression, suggesting that the relationship between CXCR4 expression and stem cell repopulating function is not obligatory.

stromal-derived factor-1α | xenotransplantation

Human tissues, including neural, muscle, and hematopoietic, are ultimately derived from stem cells with immense proliferative and differentiative capacities (1–3). Human stem cells provide promise for cellular based therapy of muscular, hematopoietic, and neurological disorders, the success of which depends on the ability of donor-derived stem cells to home and reconstitute their derived tissues after i.v. transplantation. Although little is known about the migratory ability of neural and muscle stem cells, homing and migration of hematopoietic reconstituting cells has been well characterized. During early development, hematopoietic stem cells can be found in various anatomical locations, including the *para*-aortic region, fetal liver, and bone marrow (BM), illustrating that primitive blood stem cells are capable of extensive migration (4). The migratory ability of human hematopoietic stem cells (HSCs) in the adult is demonstrated further in clinical transplantation procedures, by which primitive human cells seed and sustain donor hematopoiesis in the BM of recipient patients (5). In addition, treatment of patients with granulocyte colony-stimulating factor causes the mobilization of human HSCs from the BM to the peripheral blood and is now used routinely for the isolation of peripheral blood stem cells for autologous transplantation (6). The underlying mechanisms responsible for the migration and retention of human blood stem cells is unknown.

Chemokines are strong candidate regulators of human stem cell chemotaxis and have been shown to regulate the migration of human blood progenitor cells (7). Of the large family of chemokines, stromal-derived factor-1α (SDF-1α) was the first identified as an attractant of immature hematopoietic cells expressing the primitive blood cell marker CD34 and has been suggested to play a role in chemically induced mobilization of HSC in humans (8). Recent studies using human umbilical cord blood and adult CD34$^+$ cells have shown that populations with the ability to migrate toward SDF-1α contain cells capable of repopulating the BM of nonobese diabetic (NOD)/severe combined immunodeficient (SCID) mice (9). Genetic evidence for the role of SDF-1α and its receptor CXCR4 in the regulation of murine hematopoietic stem cells has been examined by using SDF-1$\alpha^{-/-}$ and CXCR4$^{-/-}$ knockout mice (10–12). Null mutations of either ligand or receptor are lethal during embryonic development; however, transplantation of CXCR4$^{-/-}$ fetal liver stem cells into normal recipients permits BM engraftment with eventual defects in both myelopoiesis and B-lymphopoiesis. Despite the evidence accumulated from these murine studies, the expression of CXCR4 on human stem cells and the role that SDF-1α/CXCR4 interactions may have in BM reconstitution remains unclear.

Transplantation of human hematopoietic cells into NOD/SCID mice has proven to be a reliable model for the detection of human regenerative stem cells of the hematopoietic system (13, 14). Because candidate human stem cells are transplanted by i.v. injection, this procedure provides a model to understand the engraftment process of human stem cells that home and seed recipient tissues. Human hematopoietic repopulating cells identified in this assay have been shown to be highly enriched among extremely rare CD34$^+$ CD38$^-$ subfractions of lineage depleted (Lin$^-$) cells. These primitive human blood cells have been operationally defined as SCID-repopulating cells (SRC; refs. 13 and 15). Additional evidence from gene-marking studies have demonstrated that the human SRC is biologically distinct and more primitive than human hematopoietic progenitors detected *in vitro* (16). Here, we have identified previously undetected CXCR4$^+$ and CXCR4$^-$ subfractions among neuronal, muscle, and primitive hematopoietic tissues shown to contain human stem cells. Based on the availability of human–mouse xenotransplantation assays for hematopoietic stem cells, the functional properties of hematopoietic subfractions were analyzed after the isolation of CXCR4$^+$ and CXCR4$^-$ subsets comprising the human CD34$^+$ CD38$^-$Lin$^-$ population. As demonstrated by clonogenic progenitor assays and defined liquid cultures, these subpopulations possess distinct progenitors with diverse-lineage commitment programs and unique regulatory capacity of CXCR4 in response to the chemokine SDF-1α. *In vivo* transplantation of CD34$^+$ CD38$^-$Lin$^-$CXCR4$^+$ and CD34$^+$ CD38$^-$Lin$^-$CXCR4$^-$ cells demonstrated that these populations possess equivalent engraftment ability in immune-deficient NOD/SCID mice. Our data illustrate that CXCR4 expression is not indicative of human stem cell phenotype or repopulating ability,

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Abbreviations: BM, bone marrow; HSC, hematopoietic stem cells; SDF-1α, stromal-derived factor-1α; SCID, severe combined immunodeficiency; NOD, nonobese diabetic; Lin$^-$, lineage depleted; SRC, SCID-repopulating cells; CB, cord blood; PE, phycoerythrin; FACS, fluorescence-activated cell sorter; SF, serum-free; NOD/SCID, NOD/LtSz-scid/scid; CFU, colony-forming unit.

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indicating that the role of SDF-1α/CXCR4 interactions in reconstitution remains to be elucidated.

Materials and Methods

Human Cells. Samples of full-term and early gestational human umbilical-cord blood (CB), human fetal neural tissue, and human skeletal muscle from the tibialus anterior were obtained in conjunction with local ethical and biohazard authorities of the University of Western Ontario and London Health Sciences Center. Human neural and muscle tissue was disassociated with 0.5% collengenase treatment in Hepes buffer. Whole blood was diluted (1:3) in α-MEM (GIBCO/BRL) or PBS, and mononuclear cells were collected by centrifugation on Ficoll-paque (Amersham Pharmacia) as shown (17).

Purification of Primitive Cell Populations. Lin^- cells were isolated from human hematopoietic sources by using a standard immunomagnetic separation protocol (15, 17). Lin^- cells were stained with one of two distinct anti-human CXCR4 clones; 12 g5 (PharMingen) or 44717.111 (R&D Systems) conjugated to phycoerythrin (PE) together with CD34 and CD38 conjugated to FITC and allophycocyanin (Becton Dickinson) and sorted on a Vantage SE (Becton Dickinson) to isolate $CXCR4^+$ and $CXCR4^-$ subfractions from the $CD34^+$ $CD38^-Lin^-$ cell fraction. Sorting gates were established on Lin^- cells stained with IgG1 conjugated to the appropriate fluorochromes (Becton Dickinson).

Confocal Microscopy. $CD34^+$ $CD38^-Lin^-CXCR4^+$, $CD34^+$ $CD38^-Lin^-CXCR4^-$ populations, and $CD34^+$ $CD38^-Lin^-$ cells stained with IgG1-PE were purified and mounted on glass slides. Cell surface fluorescence was visualized on a Zeiss LSM-410 laser scanning microscope. Emission from $CD34^-$ FITC (515–540 nm) and either $CXCR4^-$PE or IgG1-PE (560 nm) was detected after excitation with a 488-nm and 533-nm laser, respectively.

Colony-Forming Cell Assays. Human clonogenic progenitor assays were performed by plating equal numbers of $CD34^+$ $CD38^-Lin^-CXCR4^+$ and $CD34^+$ $CD38^-Lin^-CXCR4^-$ cells with Methocult H4434 (Stem Cell Technologies). Differential colony counts were assessed after incubation for 10–14 days at 37°C and 5% CO_2 in a humidified atmosphere.

Treatment with SDF-1α in Serum-Free (SF) Cultures. SF cultures contained 9,500 BIT media (StemCell Technologies) supplemented with 10^{-4} M β-mercaptoethanol, 2 mM L-glutamine (GIBCO/BRL), and growth factors consisting of 300 ng/ml rhu-stem cell factor, 50 ng/ml rhu-granulocyte colony-stimulating factor (Amgen Biologicals), 300 ng/ml rhu-Flt-3, 10 ng/ml rhu-IL-3, 10 ng/ml rhu-IL-6 (R&D Systems). Cultured subfractions were incubated for 24 h at 37°C and 5% CO_2 in the presence or absence of 1 μg/ml rhu-SDF-1α (R&D Systems). As a control for nonspecific binding, Lin^- cells were stained with IgG-PE, analyzed by FACS, and seeded in the same manner. Cells were harvested and either analyzed directly or stained with anti-human CXCR4, or IgG1-PE (controls), before analysis.

Transplantation of Purified Human Hematopoietic Cells into NOD/LtSz-scid/scid (NOD/SCID) Mice. NOD/SCID mice (8 weeks old) were derived from breeding pairs originally obtained from The Jackson Laboratory and maintained under defined flora in the animal facility of the Robarts Research Institute at the University of Western Ontario. Mice were sublethally irradiated at 355 cGy by using a 137Cs γ-irradiator before i.v. injection of purified human cells. Mice transplanted with 10,000 cells or less were cotransplanted with 100,000 Lin^+ irradiated (1,500 rad) accessory cells (18).

Analysis of Human Cell Engraftment. Genomic DNA was extracted from the BM, liver, lung, spleen, kidney, and skeletal muscle of NOD/SCID mice killed 6–8 weeks after transplant, and 1 μg of DNA was digested with *Eco*RI, transferred, and probed on a Southern blot by using a human chromosome-17-specific α-satellite probe (p17H8) (14). Quantitation was performed by comparison of the 2.7-kb band to standards of known mouse and human DNA mixtures, with a level of resolution equal to 0.1% human DNA representing the lower limit of detection for human chimerism.

Flow Cytometric Analysis of Murine BM. BM cells were stained with fluorochrome-conjugated antibody specific to human CD45 (a panleukocyte marker) and CD38 (Becton Dickinson Immunocytometry Systems) and analyzed by flow cytometry. Murine BM cells from engrafted animals were further stained with CD45 allophycocyanin (PharMingen) and gated to analyze human cells only in combination with either CD20 FITC and CD19 PE, CD33 FITC (Becton Dickinson Immunocytometry Systems) and CD13 PE (Immunotech), CD34 FITC and CD38 PE (Becton Dickinson Immunocytometry Systems) or CXCR4 PE (R&D Systems) monoclonal antibodies for multilineage analysis.

Results

Identification of Primitive $CXCR4^+$ and $CXCR4^-$ Subsets in Human Hematopoietic Tissue. To examine the expression of the chemokine receptor CXCR4 on stem cell containing populations in the human, single-cell suspensions of human skeletal muscle and neural cells as well as primitive Lin^- cells from neonatal hematopoietic tissue were stained with PE-conjugated monoclonal antibodies recognizing human CXCR4 and analyzed by flow cytometry. Cells expressing CXCR4 comprised 22.4 $\pm$ 5.6% and 0.6 $\pm$ 0.3% of human neural and muscle tissue, respectively, whereas primitive CB cells were 73 $\pm$ 3.4% $CXCR4^+$ (Fig. 1*Ai–Aiii*). These data illustrate the global nature of CXCR4 and the potential importance of CXCR4/SDF-1 interactions in a variety of human tissues.

Determining the expression of CXCR4 on human stem cells and its significance to properties of homing and migration for tissue regeneration requires the use of functional assays that allow stem cell detection. Mainly because of the development of well defined *in vitro* and *in vivo* xenotransplantation assays available, human hematopoietic stem cells are arguably the most rigorously tested human stem cells to date. Because similar assays have yet to be created for human muscle and neural regenerative stem cells, the potential of CXCR4 expression on human stem cells was addressed by using the hematopoietic compartment. Because isolation of rare target cells requires highly purified fractionation before functional properties can be evaluated, we assessed the ability of two PE-conjugated antibodies, recognizing distinct epitopes of CXCR4, to efficiently select $CXCR4^-$ (Fig. 1*Aiii*, gated R1) and $CXCR4^+$ (Fig. 1*Aiii*, gated R2) subfractions by FACS. Lin^- cells contained two subsets of $CXCR4^-$ and $CXCR4^+$ cells with either 12 g5 or 44717.111 monoclonal antibodies (Fig. 1*Aiii*). Fluorescence of $CXCR4^-$ subpopulations was equal to unstained or isotype control cells, whereas $CXCR4^+$ cell fluorescence was approximately two orders of magnitude higher (Fig. 1*Aiii*). Primitive $CD34^+$ $CD38^-Lin^-$ cells (Fig. 1*Aiv*, gated R3) were selected based on CXCR4 expression and contained approximately 81.6 $\pm$ 5.1% of $CXCR4^+$ and 16.8 $\pm$ 1.9% $CXCR4^-$ cells ($n = 6$). Isolated $CD34^+$ $CD38^-Lin^-CXCR4^-$ and $CD34^+$ $CD38^-Lin^-CXCR4^+$ cells were reanalyzed by FACS analysis and examined by confocal microscopy, verifying purities of >98% (Fig. 1*B*). As a control for nonspecific fluorescence in the PE channel, $CD34^+$ $CD38^-Lin^-$ cells from the same parent population were stained and isolated with mouse IgG1 conjugated to PE (Fig. 1*Bi*) and compared with the $CXCR4^-$ and $CXCR4^+$ subsets (Fig. 1 *Bii* and *Biii*) selected with $CXCR4^-$PE antibodies. Confocal microscopy demonstrated similar fluorescent properties between $CD34^+$ $CD38^-Lin^-CXCR4^-$ and isotype IgG-PE-stained $CD34^+$ $CD38^-Lin^-$ cells (Fig. 1, compare *Bi* and *Bii*),

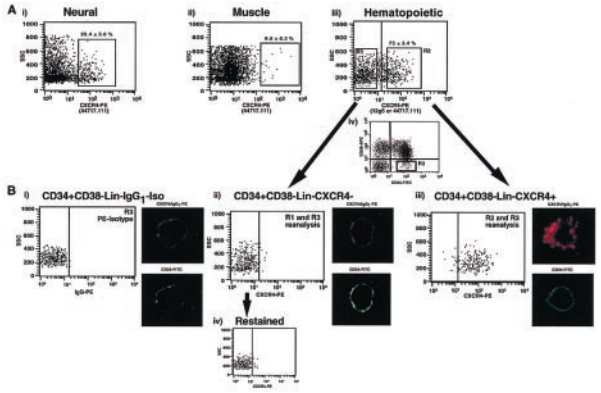

Fig. 1. Isolation of CXCR4$^+$ and CXCR4$^-$ subfractions within primitive human hematopoietic populations. (*A*) A representative FACS analysis of human neuronal (*i*), muscle (*ii*), and CB cells (Lin$^-$) (*iii*), stained with either 12 g5 or 44717.111 anti-human CXCR4 antibodies conjugated to PE. The percentage of CXCR4$^+$ cells detected is given above each positive gate as the mean ± SEM of four (neural), two (muscle), and six (hematopoietic) different samples. CB Lin$^-$ cells stained with mouse IgG1 served as isotype to establish sorting gates for the purification of CXCR4$^-$ and CXCR4$^+$ hematopoietic subpopulations indicated as R1 and R2, respectively (*iii*). Subpopulations of CD34$^+$ CD38$^-$Lin$^-$ cells were isolated by using the R3 sorting gate (*iv*). (*B*) CD34$^+$ CD38$^-$Lin$^-$ cells stained with IgG1-PE were analyzed by FACS and confocal to serve as a control (*i*). Fluorescence of control (*i*) was compared with purified CD34$^+$ CD38$^-$Lin$^-$CXCR4$^-$ (R1 and R3) and CD34$^+$ CD38$^-$Lin$^-$CXCR4$^+$ (R2 and R3) cells, a measure of sort purity (*ii* and *iii*). Selected CXCR4$^-$ cells were restained for CXCR4 by using 12 g5 or 44717.111 and reanalyzed to verify the absence of any CXCR4$^+$ cells (*iv*). For confocal microscopy, primitive CD34$^+$ CD38$^-$Lin$^-$ cells stained with isotype control, CD34$^+$ CD38$^-$Lin$^-$CXCR4$^-$, and CD34$^+$ CD38$^-$Lin$^-$CXCR4$^+$ cells were isolated by FACS and visualized with a Zeiss LSM-410 confocal microscope at random z-planes. Representative photographs depicting the PE and FITC fluorescence from CD34$^+$ CD38$^-$Lin$^-$ isotype control (*i*), CD34$^+$ CD38$^-$Lin$^-$CXCR4$^-$ (*ii*), and CD34$^+$ CD38$^-$Lin$^-$CXCR4$^+$ (*iii*) cells are presented along side each corresponding population reanalyzed by FACS ($n = 4$).

whereas CD34$^+$ CD38$^-$Lin$^-$CXCR4$^+$ cells displayed a much higher intensity of CXCR4 expression (Fig. 1*Biii*). This analysis confirmed the absence of CXCR4 on the surface of purified CXCR4$^-$ cells. Expression of CD34 detected in the FITC (green) channel was uniformly distributed along the cell membrane at similar levels in all purified subpopulations (Fig. 1 *Bi–Biii*), illustrating equivalent cell surface expression of a protein independent of CXCR4. To confirm that contaminating CXCR4$^+$ cells were not present within purified CXCR4$^-$ subsets because of inefficiencies in antibody staining or isolation, sorted cells were restained with either 12 g5 or 44717.111 antibodies and reanalyzed. As demonstrated in the initial FACS selection (Fig. 1*Bii*), 98% purity was maintained (Fig. 1*Biv*). In addition, no CXCR4$^+$ cells were detected in purified CD34$^+$ CD38$^-$Lin$^-$CXCR4$^-$ populations restained with the alternate antibody to that used for isolation of CXCR4 subsets (data not shown), suggesting that differences in the epitope specificity of these antibodies had no effect on the ability to isolate CXCR4$^-$ or CXCR4$^+$ subfractions of CD34$^+$ CD38$^-$Lin$^-$ cells. Our analysis of primitive human CD34$^+$ CD38$^-$Lin$^-$ blood cells by flow cytometry and confocal microscopy illustrates the presence of CXCR4$^+$ and CXCR4$^-$ subpopulations within this primitive fraction, and demonstrates that these subsets can be isolated into distinct, highly purified populations for functional comparison.

Progenitor Capacity and Response to SDF-1α of CD34$^+$ CD38$^-$Lin$^-$CXCR4$^+$ and CD34$^+$ CD38$^-$Lin$^-$CXCR4$^-$ Cells *in Vitro*. Primitive CD34$^+$ CD38$^-$Lin$^-$ cells are highly enriched for clonogenic progenitors detected by colony-forming cell (CFC) assays (19–21). To determine the clonogenic capacity of CXCR4$^+$ and CXCR4$^-$ subfractions within the CD34$^+$ CD38$^-$Lin$^-$ population, cells were plated in methylcellulose media containing human cytokines. The frequency of CFC was equal in both populations, suggesting that selection of subpopulations based on CXCR4 expression does not bias primitive progenitor content (Fig. 2*A*, *Inset*). A significantly higher percentage of granulocytic precursors (CFU-G) were found among CD34$^+$ CD38$^-$Lin$^-$CXCR4$^+$ cells ($P < 0.01$), whereas a higher percentage of erythroid precursors (burst-forming units E; $P < 0.05$) was demonstrated among CD34$^+$ CD38$^-$Lin$^-$CXCR4$^-$ cells (Fig. 2). These *in vitro* data provide further evidence of the purity of CXCR4$^+$ and CXCR4$^-$ subfractions and indicate that selection of primitive CB cells based on CXCR4 expression enriches for progenitors with myeloid potential.

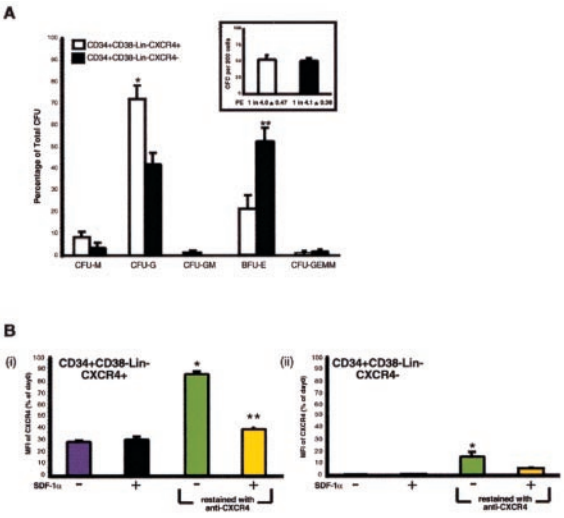

Fig. 2. Distribution of clonogenic progenitor subtypes and regulation of CXCR4 in response to SDF-1α within CXCR4$^+$ and CXCR4$^-$ fractions derived from the CD34$^+$ CD38$^-$Lin$^-$ population. Primitive CXCR4$^+$ and CXCR4$^-$ subfractions of CD34$^+$ CD38$^-$Lin$^-$ CB cells were cultured in methylcellulose media containing human cytokines, and the colony number and type were enumerated after 10–14 days. CXCR4$^+$ and CXCR4$^-$ subfractions contained the same frequency and plating efficiency (PE) of CFU as represented by the number of colonies per 200 cells (*A*, *Inset*). The proportion of CFU-M, CFU-G, CFU-granulocyte/macrophage, burst-forming units E, and CFU-GEMM contributing to the total number of CFU detected within primitive CXCR4$^+$ and CXCR4$^-$ subfractions is given as a relative percentage based on mean values ± SEM, $n = 5$. Significant differences were found between the percentage of CFU-G ($*P < 0.01$) and burst-forming units E ($**P < 0.05$) detected in CXCR4$^+$ and CXCR4$^-$ subfractions. Purified CD34$^+$ CD38$^-$Lin$^-$CXCR4$^+$ cells (*Bi*) and CD34$^+$ CD38$^-$Lin$^-$CXCR4$^-$ cells (*Bii*) were cultured for 24 h in SF conditions shown to sustain human repopulating cells in the presence or absence of SDF-1α and analyzed for the level of CXCR4 expression by FACS. Values are expressed as the percentage of mean fluorescence intensity (MFI) as compared with uncultured cells. Changes in the amount of cell surface CXCR4 detected on SDF-1α treated (black) and untreated (purple) populations were determined by direct reanalysis after *in vitro* culture of CD34$^+$ CD38$^-$Lin$^-$CXCR4$^+$ (*Bi*) and CD34$^+$ CD38$^-$Lin$^-$CXCR4$^-$ (*Bii*) cells. Cells restained with anti-human CXCR4$^-$PE in the absence (green) or presence (yellow) of SDF-1α were reanalyzed. Average MFI and SEM values are based on analysis of purified samples from at least three independent samples. Significant differences are indicated with asterisks ($*P < 0.001$, $**P < 0.005$).

Previous studies have suggested that interactions between SDF-1α and CXCR4 are necessary for the homing and retention of primitive human CD34$^+$ cells within the BM (8). Because engraftment of repopulating cells is established within 24 h after transplantation (22),**†† we used SF culture conditions that maintain

Traycoff, C. M., Leonard, E., Plett, A., Tong, X. & Srour, E. (1999) *Blood* **94, Suppl., 41a (abstr.).

††Cashman, J., Nicolini, F. & Eaves, C. (1999) *Blood* **94,** Suppl. 651a (abstr.).

primitive quiescent cells without inducing mitotic division for 24 h (17, 19, 23, 24) to determine whether novel $CXCR4^+$ and $CXCR4^-$ subfractions of $CD34^+$ $CD38^-Lin^-$ were able to regulate CXCR4 in response to SDF-1α. Purified $CD34^+$ $CD38^-Lin^-CXCR4^+$ and $CD34^+$ $CD38^-Lin^-CXCR4^-$ populations were analyzed by flow cytometry to obtain initial "day-0" levels of CXCR4 expression as measured by mean fluorescence intensity (MFI). The MFI was determined by using saturating concentrations of CXCR4 antibody optimized in the subset isolation experiments shown in Fig. 1. Purified cells were cultured in the presence or absence of rhu-SDF-1α for 24 h and then harvested for direct analysis by flow cytometry or restained with anti-human CXCR4 before analysis. Direct analysis of $CD34^+$ $CD38^-Lin^-CXCR4^+$ cells showed a 70% decrease in surface CXCR4 expression when cultured in the absence or presence of SDF-1α (Fig. 2*Bi*, purple and black). However, the restaining of cells cultured in the absence of SDF-1α indicated that CXCR4 expression was maintained as compared with *de novo*-isolated cells (Fig. 2*Bi*, green), whereas the presence of SDF-1α inhibited maintenance of CXCR4 cell surface expression (Fig. 2*Bi*, yellow). These data indicate that only $CXCR4^+$ cells cultured in the absence of SDF-1α were able to demonstrate CXCR4 expression equivalent to that of *de novo*-isolated $CXCR4^+$ cells and originally expressed (antibody-bound) CXCR4 was down-regulated to the same extent in cultures with or without SDF-1α. We suggest that levels of surface CXCR4 remain at a steady state on primitive $CXCR4^+$ cells in the absence of SDF-1α, but these levels are significantly decreased in its presence. These observations may be the consequence of multiple regulatory mechanisms common to G-coupled protein receptors, such as CXCR4, that include recycling, internalization, and down-regulation (25).

In the absence or presence of SDF-1α, CXCR4 remained undetectable on cultured $CD34^+$ $CD38^-Lin^-CXCR4^-$ cells relative to *de novo*-isolated populations (Fig. 2*Bii*, purple and black). In the absence of SDF-1α, restained $CXCR4^-$ cells demonstrated low but detectable levels of CXCR4 (Fig. 2*Bii*, green), whereas addition of SDF-1α suppressed this modest increase (Fig.2*Bii*, yellow). Therefore, although $CD34^+$ $CD38^-Lin^-CXCR4^-$ cells regulate CXCR4 expression in response to SDF-1α in a similar manner to $CD34^+$ $CD38^-Lin^-CXCR4^+$ cells, the extent of this response, as measured by changes in level of CXCR4 expression, illustrates that these two subsets represent physiologically distinct populations. Because no increase in the level of CXCR4 expression was detected immediately after isolation (Fig. 1 *Bii* and *Biv*), or 1 h subsequent to culture in the absence of SDF-1 (data not shown), we suggest that primitive $CXCR4^-$ cells are unable to up-regulate CXCR4 within a time frame determined to be critical for stem cell engraftment *in vivo* (9).**††

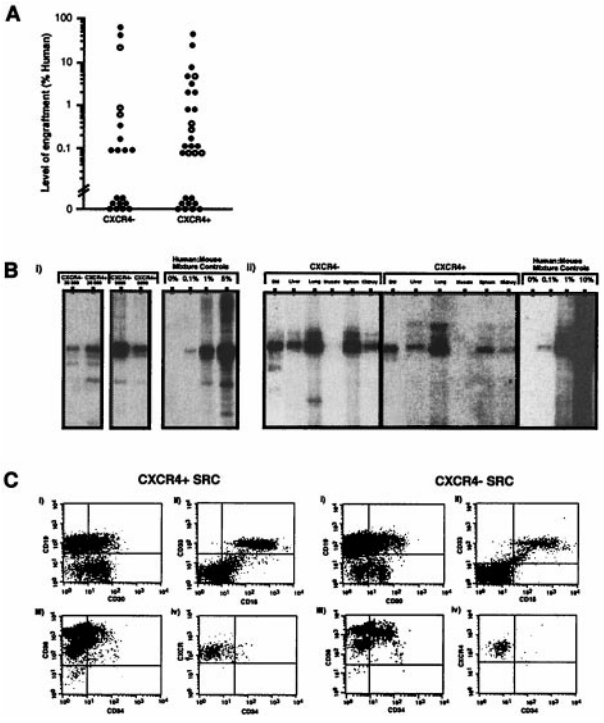

Fig. 3. NOD/SCID repopulating cells with equivalent engraftment and multilineage proliferation capacity exist in highly purified $CXCR4^+$ and $CXCR4^-$ populations. (*A*) Summary of the level of human cell engraftment in the BM of NOD/SCID mice transplanted with CB-derived $CD34^+CD38^-CXCR4^-Lin^-$ and $CD34^+CD38^-CXCR4^+$ Lin^- cell populations (●) or $CD34^+$ Lin^-CXCR4^+ and $CD34^+$ Lin^-CXCR4^- (○). The percentage of human cells present in the murine BM 8 weeks after transplantation was determined by flow cytometric analysis of human CD45 and by Southern blot analysis for human α-satellite DNA sequences. (*Bi*) Representative Southern blot analysis of mice transplanted with 20,000 or 5,000 purified $CD34^+$ $CD38^-Lin^-CXCR4^-$ and $CD34^+$ $CD38^-Lin^-CXCR4^+$ cells. (*Bii*) Detection of human chimerism in BM, liver, lung, muscle, spleen, and kidney from two individual NOD/SCID mice transplanted with primitive $CXCR4^+$ and $CXCR4^-$ cells. (*C*) Representative FACS analysis of human cells in the BM of NOD/SCID mice transplanted 8 weeks earlier with $CXCR4^+$ or $CXCR4^-$ subfractions of $Lin^-CD34^+CD38^-$ cells derived from CB. Cells were removed from the femur, tibia, and iliac crests of transplanted NOD/SCID mice and stained with the human-specific monoclonal antibody against panleukocyte-marker CD45 in combination with other human differentiated cell markers indicated. Surface-marker profiles of human cells were obtained by gating on the human cell component ($CD45^+$) of BM-derived cells. Dot plots show CD19 and CD20 B-cell markers (*Ci*), CD33 and CD13 myeloid cell markers (*Cii*), CD38 and CD34 primitive cell markers (*Ciii*), and CD34 primitive cell marker and the CXCR4 chemokine receptor (*Civ*).

Human $CXCR4^+$ and $CXCR4^-$ Cells Are Capable of Reconstituting Function with Equivalent Engraftment and Developmental Capacity *in Vivo*. The NOD/SCID mouse has been used as a model system to understand seeding efficiencies and navigation of human stem cells after *in vivo* transplantation (22, 26).**†† To identify whether CXCR4 expression of *de novo*-isolated cells correlated with engraftment and repopulating function, $CXCR4^+$ and $CXCR4^-$ subpopulations were isolated from human full-term and earlier gestational umbilical CB and transplanted by i.v. injection into NOD/SCID mice. $CXCR4^-$ and $CXCR4^+$ cells were restained with anti-human CXCR4 and resorted to ensure a high degree of purity as shown in Fig. 1. Individual NOD/SCID mice were transplanted with highly purified $CXCR4^+$ or $CXCR4^-$ subsets of either $CD34^+$ $CD38^-Lin^-$ or $CD34^+$ Lin^- populations, and the percentage of engrafted mice (human chimerism $\geq$0.1% by FACS and Southern blot analysis) was compared between each subset. When transplanted at doses ranging from 330 to 300,000 cells, both primitive human $CXCR4^+$ ($n = 29$) and $CXCR4^-$ ($n = 20$) subsets were capable of engrafting NOD/SCID mice at frequencies of 69% and 55%, respectively. These results are summarized in Fig. 3*A*. A representative example of human chimerism detected by Southern blot analysis of DNA extracted from the BM of mice transplanted with 20,000 and 5,000 purified cells illustrates that comparable levels of engraftment result from either $CXCR4^-$ or $CXCR4^+$ stem cells (Fig. 3*Bi*). Furthermore, transplantation of equal numbers of $CXCR4^+$ and $CXCR4^-$ cells resulted in similar levels of human chimerism in NOD/SCID mice demonstrating equivalent proliferative capacity of these two stem cell subsets (Table 1). Because the degree of human chimerism in individual NOD/SCID mice partly depends on the number of stem cells transplanted, the actual number of $CXCR4^-$ and $CXCR4^+$ stem cells that result in a single engrafted NOD/SCID mouse cannot be determined. Accordingly, limiting-dilution analysis was performed to quantitate the number and frequency of repopulating cells within $CXCR4^+$ and $CXCR4^-$ subpopulations. NOD/SCID mice were transplanted over a dose range which resulted in nonengraftment in a fraction of mice (Table

Table 1. Frequency of SRCs in $CD34^+CD38^-Lin^-$ cells within the $CXCR4^+$ and $CXCR4^-$ fractions of primary human hematopoietic tissue by limiting-dilution analysis

Cell number transplanted into NOD/SCID mice	$CD34^+CD38^-Lin^-CXCR4^-$		$CD34^+CD38^-Lin^-CXCR4^+$	
	Frequency of engraftment	Level of human chimerism	Frequency of engraftment	Level of human chimerism
50,000 to >10,000	(100%) 3/3	15.3% ± 14.8	(100%) 6/6	9.0% ± 6.6
10,000 to >5,000	(75%) 3/4	0.23% ± 0.08	(66%) 2/3	0.1% ± 0.00
5,000 to ≥1,000	(40%) 2/5	0.2% ± 0.00	(43%) 3/7	0.6% ± 0.5
<1,000	(20%) 1/5	0.1% ± 0.00	(14%) 1/7	0.1% ± 0.00

The proportion of human-engrafted mice and average level of human chimerism, at individual cell doses transplanted is expressed as a percentage for both $CD34^+CD38^-Lin^-CXCR4^+$ and $CD34^+CD38^-Lin^-CXCR4^-$ purified populations. The total number of positive mice over the total number transplanted is expressed as a fraction for each cell-dose range. Poisson statistics were applied to the single-hit model, and the frequency of SRCs calculated with the maximum likelihood estimator was determined to be 1 SRC in 1,521 $CD34^+CD38^-Lin^-CXCR4^+$ cells, $n = 23$ (95% confidence limits; 1 in 1,220 to 1 in 1945) and 1 SRC in 1844 $CD34^+CD38^-Lin^-CXCR4^-$ cells, $n = 17$ (95% confidence limits; 1 in 1,315 to 1 in 2,210).

1). Data from these limiting-dilution assays of $CXCR4^-$ and $CXCR4^+$ cells were pooled for statistical analysis, according to the method described by Porter and Berry (27, 28) applied to this system (15, 29, 30). A similar frequency of repopulating stem cells was found within $CXCR4^-$ and $CXCR4^+$ subfractions by using the single hit Poisson model (Table 1). Our data provide quantitative evidence that engraftment capacity and frequency of primitive human stem cells capable of repopulating the BM of transplanted recipients is distributed equally between $CXCR4^+$ and $CXCR4^-$ cells within the human stem cell compartment.

Recent studies have shown that in addition to the BM, functionally primitive human cells can be identified in a variety of tissue sites in the NOD/SCID mouse (22, 26). These tissues have been shown to contain human SRC by secondary transplantation 24–48 h after primary transplant, and therefore indicate that homing of human SRC is not exclusive to the murine BM microenvironment. Although high levels of human engraftment can be detected in murine tissues after 6–8 weeks after transplantation of NOD/SCID mice, this chimerism unlikely is caused by human cells in the circulation of chimeric animals, because little to no human cells can be detected in the peripheral blood of mice engrafted at high levels in the BM and tissues (31, 32). Taken together, it seems that human engraftment in the BM or tissue sites of NOD/SCID mice is caused by the immediate migration and seeding of SRC into several compartments. To further evaluate whether the *in vivo*-engraftment potential of $CXCR4^+$ and $CXCR4^-$ SRC is equivalent, tissues were removed from the lung, liver, spleen, kidney, and muscle of animals 6–8 weeks after transplantation and examined for human chimerism. Southern blot analysis representing the human DNA content in tissues removed from 4 and 6 mice, transplanted with primitive $CXCR4^-$ and $CXCR4^+$ cells, respectively, is shown in Fig. 3*Bii*. Human cells were detected in each of the organs analyzed regardless of whether they were transplanted with human $CXCR4^+$ or $CXCR4^-$ populations. Our results demonstrate that the *in vivo*-engraftment capacity of $CXCR4^+$ and $CXCR4^-$ SRC is similar, irrespective of whether engrafted tissue is associated with SDF-1 production. Based on these findings, we suggest that the human repopulating function may involve properties other than CXCR4 and SDF-1α interactions.

Human blood stem cells are defined by their ability to give rise to multiple lineages *in vivo*. Accordingly, we examined and compared the pluripotent capacity of $CXCR4^+$ and $CXCR4^-$ repopulating cells in the BM of engrafted mice by flow cytometry. The human cell component within the BM of mice transplanted with $CD34^+$ $CD38^-Lin^-CXCR4^-$ or $CD34^+$ $CD38^-Lin^-CXCR4^+$ cells at doses ranging from 40,000 to 5,000 cells was analyzed by gating the fraction of cells expressing the human specific panleukocyte marker CD45 ($n = 8$). Mice transplanted with either $CXCR4^+$ or $CXCR4^-$ primitive cell fractions contained human B-lymphoid cells, demonstrated by the presence of $CD19^+$ and $CD20^+$ cells (Fig. 3*Ci*) and myeloid cells at various stages of differentiation as shown by the expression of CD33 and CD13 (Fig.3*Cii*). Therefore, although differences exist in the proportion of myeloid progenitors within the $CXCR4^+$ and $CXCR4^-$ subfractions of $CD34^+$ $CD38^-Lin^-$ cells detected *in vitro*, repopulating cells existing within these fractions sustain similar myelopoietic potential *in vivo*. Although the granulocytic potential seems to be augmented in mice transplanted with $CXCR4^-$ SRC as compared with $CXCR4^+$ SRC, this difference was not significant. In both $CXCR4^+$ and $CXCR4^-$ transplanted mice, human cells expressing CD34 and CD38 surface receptors were also detected, including a small population of immature $CD34^+$ $CD38^-$ cells (Fig. 3*Ciii*). Surprisingly, analysis of mouse BM using anti-human CXCR4 indicated that >99% of the human cell component expressed CXCR4, and very few, if any, human cell types, including primitive $CD34^+$ cells, were devoid of CXCR4 cell surface expression (Fig. 3*Civ*). Taken together, our data demonstrate that $CXCR4^+$ and $CXCR4^-$ repopulating cells possess an equivalent proliferative and differentiative capacity *in vivo*, suggesting that the selection of human stem cells based on CXCR4 expression does not provide any advantage for human blood cell transplantation and may, in fact, result in the loss of a biologically important population of human reconstituting stem cells.

Discussion

Our study demonstrates the presence of CXCR4-expressing cells within human neural, muscle, and primitive hematopoietic tissue shown to contain pluripotent stem cells. Extensive purification of primitive human hematopoietic tissue determined that *de novo*-isolated $CXCR4^+$ and $CXCR4^-$ subfractions of $CD34^+$ $CD38^-Lin^-$ populations each contain pluripotent hematopoietic repopulating cells. After *in vitro* stimulation in response to human cytokines, the level of CXCR4 expressed on $CD34^+$ $CD38^-Lin^-CXCR4^-$ cells was significantly lower than $CD34^+$ $CD38^-Lin^-CXCR4^+$ cells, illustrating that these subsets are physiologically distinct. The proportion of myeloid progenitors comprising $CXCR4^-$ and $CXCR4^+$ subsets was also unique. The $CD34^+$ $CD38^-Lin^-CXCR4^+$ population was enriched for granulocytic precursors, and contained a significantly lower percentage of erythroid precursors compared with the $CD34^+$ $CD38^-Lin^-CXCR4^-$ population. However, primitive $CXCR4^+$ and $CXCR4^-$ populations were equivalent in their homing and engraftment ability to a variety of tissue sites, in addition to their proliferative and differentiative capacity *in vivo*. Our data demonstrate that subpopulations distinguished by CXCR4 expression exist within human $CD34^+$ $CD38^-Lin^-$ populations and contain a similar frequency of human repopulating cells. We suggest that the role of CXCR4 in human stem cell selection and function during *in vivo* reconstitution remains to be characterized and is more complex than previously surmised.

Studies have shown that the ability of hematopoietic cells, including primitive $CD34^+$ cells, to migrate toward SDF-1α by using

transwell assay systems does not correlate with the expression of CXCR4 (33, 34). It is therefore possible for cells to migrate to SDF-1 and be devoid of CXCR4 expression. In addition, recent evidence shows that SDF-1 may induce the repulsion of CXCR4-expressing cells, indicating that interactions between SDF-1 and CXCR4 are not concomitant with chemoattraction (35). Taken together, these studies suggest that the relationship between SDF-1-induced chemotaxis and CXCR4 remains to be defined. By using an *in vivo* approach in our study, human $CXCR4^-$ cells were capable of engrafting the BM microenviroment and several other tissue sites not previously thought to express SDF-1α, supporting the observations that $CXCR4^-$SDF-1 interactions are not critical for stem cell function. Our identification of human $CXCR4^-$ cells with multilineage repopulating capacity is in contrast to studies in which primitive subpopulations capable of migrating to the chemokine SDF-1 were shown to have repopulating ability in NOD/SCID mice (9). It is likely that transwell assays may be inefficient at separating primitive $CXCR4^+$ and $CXCR4^-$ populations, as compared with well established flow cytometric techniques classically used for stem cell isolation, and these differences in cell selection may explain the discrepancy between our current study and that of Peled *et al.* (9). The limitation in transwell assays to detect and separate rare cells at high purity, such as human stem cells, is supported by data generated by these same investigators in which human repopulating cells were detected, albeit at a lower frequency than migrating cells, among cell fractions that were unable to migrate to SDF-1. From these studies, it is evident that a greater proportion of repopulating $CD34^+$ cells exist in the initial population that have migrating ability, however, in the absence of complete frequency analysis by limiting-dilution experiments, it is difficult to conclude whether nonmigrating cells contained equal frequency of human SRC as compared with migrating cells. This conclusion is further supported by phenotypic analysis of migrating and nonmigrating fractions, because both fractions contained primitive $CD34^+$ $CD38^-$ cells and cells expressing cell surface CXCR4.[‡‡] Our demonstration that $CXCR4^-$ repopulating cells are present in the human is in agreement with two independent studies in which hematopoietic reconstituting cells from the fetal liver of CXCR4 knockout mice were able to home and reconstitute the BM of lethally irradiated adult recipients (36, 37). The progeny of $CXCR4^{-/-}$ murine repopulating cells were unable to disseminate appropriately in the recipient animals, indicating that CXCR4 may be critical to the organization and migration of mature committed progeny. This observation is similar to our analysis of transplanted NOD/SCID mice where virtually all mature human cell progeny are $CXCR4^+$. Taken together, our identification of candidate human $CXCR4^-$ HSC and the ability of $CXCR4^{-/-}$ murine stem cells to migrate to the BM of lethally irradiated recipients indicates that CXCR4 is not necessary for repopulating function.

The identification of human SRC within the $CXCR4^-$ subfraction of primitive $CD34^+$ $CD38^-Lin^-$ cells establishes that CXCR4 is not a suitable marker for purification or diagnostic analysis of human HSCs before transplantation. Furthermore, the presence of unique $CXCR4^+$ and $CXCR4^-$ subsets within the $CD34^+$ $CD38^-Lin^-$ population indicates that heterogeneity exists among repopulating cells within this relatively homogeneous population. Similar features of the human stem cell compartment have been demonstrated recently by using CD34 as a putative stem cell marker, because cells with repopulating ability have been shown also to reside in a $CD34^-$ subpopulation (30, 38–40). The relationship between these $CD34^+$ and $CD34^-$ stem cells has been more clearly illustrated recently in the mouse, where CD34 has been shown to oscillate, and appearance of CD34 on the cell surface seems to be indicative of stem cell activation (41, 42). Parallel mechanisms to CD34 expression may prove to be consistent to stem cell regulation of CXCR4. Nevertheless, removal of hematopoietic cells that lack detectable CXCR4 expression before transplantation may result in the loss of a primitive fraction of repopulating cells that contribute to patient hematopoietic reconstitution. Our study suggests that, whereas CXCR4 may play an important role in the regulation of human HSC progeny, its functional relationship to the homing and engraftment, proliferation, and differentiation of human stem cells has yet to be fully understood. These findings have critical implications in the future development of *in vivo* assays for human neural and muscle stem cells (2, 3), and suggest that chemokine receptors, in addition to CXCR4, should be evaluated in neural and muscle stem cell homing.

MEDICAL SCIENCES

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