CD34 expression on long-term repopulating hematopoietic stem cells changes during developmental stages

Sahoko Matsuoka, Yasuhiro Ebihara, Ming-jiang Xu, Takefumi Ishii, Daisuke Sugiyama, Hiroshi Yoshino, Takahiro Ueda, Atsushi Manabe, Ryuhei Tanaka, Yasuo Ikeda, Tatsutoshi Nakahata, and Kohichiro Tsuji

The CD34 antigen serves as an important marker for primitive hematopoietic cells in therapeutic transplantation of hematopoietic stem cells (HSC) and gene therapy, but it has remained an open question as to whether or not most HSC express CD34. Using a competitive long-term reconstitution assay, the results of this study confirm developmental changes in CD34 expression on murine HSC. In fetuses and neonates, CD34 was expressed on Lin$^-$c-Kit$^+$ long-term repopulating HSC of bone marrow (BM), liver, and spleen. However, CD34 expression on HSC decreased with aging, and in mice older than 10 weeks, HSC were most enriched in the Lin$^-$c-Kit$^+$CD34$^-$ marrow cell fraction. A second transplantation was performed from primary recipients who were transplanted with neonatal Lin$^-$c-Kit$^+$CD34^{high} HSC marrow. Although donor-type HSC resided in CD34-expressing cell fraction in BM cells of the first recipients 4 weeks after the first transplantation, the stem cell activity had shifted to Lin$^-$c-Kit$^+$CD34$^-$ cells after 16 weeks, indicating that adult Lin$^-$c-Kit$^+$CD34$^-$ HSC are the progeny of neonatal CD34-expressssing HSC. Assays for colony-forming cells showed that hematopoietic progenitor cells, unlike HSC, continue to express CD34 throughout murine development. The present findings are important because the clinical application of HSC can be extended, in particular as related to CD34-enriched HSC and umbilical cord blood HSC. (Blood. 2001;97:419-425)

Introduction

CD34 is a glycoprotein expressed on hematopoietic cells, vascular endothelium, and embryonic fibroblasts.[1-3] Based on in vitro studies identifying cells with the capacity to differentiate into various hematopoietic lineages in clonal culture[4] and to generate hematopoietic progenitor cells (HPC) in long-term culture,[5] CD34 has served as the most important marker for primitive hematopoietic cells. However, despite the widespread clinical use of CD34 antibodies for enumeration and isolation of human primitive hematopoietic cells in therapeutic hematopoietic stem cell (HSC) transplantation and gene therapy, it has remained an open question as to whether or not all HSC express CD34. Krause and colleagues[6,7] and Morel and coworkers[8] reported that murine adult bone marrow (BM) CD34$^+$ cells were capable of long-term hematopoietic reconstitution and allow survival of lethally irradiated mice. Conversely, Osawa and associates[9] reported that CD34$^{low/-}$ rather than CD34^{high} cells in the lineage markers-negative (Lin$^-$) c-Kit$^+$Sca-1$^+$ cell fraction possess the most long-term reconstitution ability, but not short-term radioprotection ability, as determined in a competitive long-term reconstitution analysis, although it recently has been shown that a small population of c-Kit$^-$ dormant HSC are present in adult BM.[10] Goodell and colleagues[11] carried out a dual-wavelength flow cytometric analysis of BM cells stained with the fluorescent DNA-binding dye Hoechst 33342 and found that adult HSC capable of reconstituting lethally irradiated recipients express low or undetectable levels of CD34. In humans, engraftment models using immunodeficient mice[12-14] and preimmune fetal sheep,[15] and the clinical transplantation of CD34-enriched cell populations[16] indicated long-term repopulating ability of CD34$^+$ HSC. However, recent studies raised doubts as to whether the CD34$^+$ cell fraction includes all human stem cell activity.[11,17,18]

A great deal of progress has been made in characterizing murine fetal HSC. It has been shown that all the stem cell activity required to reconstitute adult hematopoiesis resides in the AA4.1$^+$c-Kit$^+$CD34$^+$ population at 14 days postcoitum (dpc) fetal liver cells and that the AA4.1$^+$c-Kit$^+$CD34$^-$ population does not repopulate.[19] Transplantation experiments in adult mice also indicated that all HSC in 11 dpc aorta-gonad-mesonephros region and fetal liver reside in a c-Kit$^+$CD34$^+$ population.[20] The presence of CD34$^+$ HSC capable of reconstituting hematopoiesis of pretreated neonate, but not adult, recipients in 9 dpc para-aortic splanchnopleura and yolk sac was demonstrated.[21] Thus, in contrast to reports on adult murine HSC, CD34 expression on fetal murine HSC at early stages is in agreement, irrespective of their location, which means that CD34 expression on murine HSC may vary during development from fetus to adult. Indeed some phenotypic variations between fetal and adult HSC have been reported for CD45RB,[22] major histocompatibility complex class I,[23] and Mac-1 antigens.[24]

We investigated CD34 expression on HSC in the murine fetus, neonate, and adult at various ages, using competitive long-term reconstitution analyses. Our data showed that HSC from the fetus express CD34 at late gestational stages and continue to express it in neonates and younger adults, but that this expression decreases with aging. Second transplantation experiments showed that the

From the Department of Clinical Oncology, Institute of Medical Science, University of Tokyo, Japan, and the Division of Hematology, Department of Internal Medicine, Keio University, Tokyo, Japan.

Submitted July 8, 1999; accepted August 26, 2000.

Reprints: Kohichiro Tsuji, Department of Clinical Oncology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan; e-mail: tsujik@ims.u-tokyo.ac.jp.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

decrease in CD34 expression on HSC occurs with a similar time course, even in older recipients engrafted with neonatal Lin$^-$c-Kit$^+$CD34$^+$ HSC. The present findings have important implications for the clinical applications of HSC, in particular CD34-enriched HSC transplantation and umbilical cord blood transplantation.

Materials and methods

Mice

C57BL/6 Ly-5.1 mice were kindly provided by Dr Koichi Ikuta (Kyoto University, Kyoto, Japan) and C57BL/6 Ly-5.2 mice were obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan). These mice were bred and maintained in a specific pathogen-free microisolator environment. For experiments on fetuses, one or 2 female mice were caged with a male for 2 hours late in the afternoon and then examined for vaginal plugs. The appearance of the vaginal plug was designated as day 0 of gestation. Mice within 24 hours from birth were used as neonatal mice. In the transplantation experiments, recipient mice were given neomycin (1.1 g/1000 mL) in the drinking water for the first month after irradiation and transplantation.

Antibodies

The antibodies used in immunofluorescence staining included 49E8 (anti-CD34, kindly provided by Dr Hirohumi Hamada, Cancer Chemotherapy Center, Tokyo, Japan), ACK45 (anti–c-Kit), A20 (anti–Ly-5.1), and 104 (anti–Ly-5.2). Lineage marker antibodies included RB6-8C5 (anti–Gr-1), M1/70 (anti–Mac-1), RA3-6B2 (anti-CD45R/B220), 30-H12 (anti–Thy-1.2), L3T4 (anti-CD4), 53-6.72 (anti-CD8a), and TR-119 (anti–TER-119). All the antibodies except 49E8 were purchased from Pharmingen (San Diego, CA). Antibodies for Mac-1 and Thy-1, markers for macrophage/monocyte lineage cells and T lymphocytes, respectively, were omitted from the cocktail of lineage marker antibodies, as their expression on HSC has been reported.[22,25] All antibody incubations were carried out for 30 minutes on ice.

Cell preparation

The BM cells were flushed from femurs and tibiae of fetal, neonatal, and adult mice. Liver and spleen cells were obtained by rubbing tissue between 2 pieces of glass and repeated pipetting. Cell suspensions were then filtered through a sterile 40-μm Cell Strainer (No. 2340; Falcon, Lincoln Park, NJ), stained with biotinylated antilineage markers, and enriched for cells not expressing the lineage markers (Lin$^-$), using streptavidin-conjugated magnetic beads (PerSeptive Biosystems, Framingham, MA). Lin$^-$ cells were then stained with fluorescein isothiocyanate (FITC)–anti-CD34 and phycoerythrin (PE)–anti–c-Kit, and sorting was performed on a FACS Vantage (Becton Dickinson, Mountain View, CA).[26,27] In second transplantation experiments, Lin$^-$ cells prepared by a method using immunomagnetic beads coated with sheep antirat IgG (Dynal AS, Oslo, Norway)[28,29] were stained with biotinylated anti–Ly-5.2, followed by FITC–anti-CD34, PE–anti–c-Kit, and PE–cyanine 5–succinimidylester-streptavidin.

Transplantation and analysis of recipients

Varying numbers of sorted cells from Ly-5.2 mice were injected into sublethally irradiated Ly-5.1 mice together with 1×10^5 unfractionated BM cells from Ly-5.1 mice. In a preliminary experiment, we determined that 1×10^5 BM cells was the minimum dose of cells required for more than 95% recipient survival. Eight to 10 weeks after transplantation, peripheral blood (PB) was collected from the tail veins of the recipient mice. Red blood cells were removed, and the nucleated PB cells were stained with FITC–anti–Ly-5.2 and PE-antimyeloid cells (Mac-1 and Gr-1), anti-B lymphocytes (B220), or anti-T lymphocytes (Thy-1), and analyzed on a FACScan (Becton Dickinson). The mice in which donor-derived (Ly-5.2$^+$) cells made up more than 1% of all B220$^+$, Thy-1$^+$, and Mac-1$^+$/Ga-1$^+$ cells in PB were scored as positive for successful reconstitution. Stable chimerism was maintained for over 6 months in all engrafted mice, although the reconstitution of T lymphocytes was slightly late compared with that of B lymphocytes or myeloid cells. The secondary transplants into Ly-5.1 mice were carried out using Ly-5.2 cells sorted from BM cells of the primary recipients (Ly-5.1 mice) 4 and 16 weeks after the first transplantation of Ly-5.2 mouse HSC.

Assay for colony-forming cells

Clonal cell culture was done in triplicate, as described.[30,31] Briefly, 1 mL culture mixture containing 2.5×10^2 cells sorted from BM cells of Ly-5.2 mice at various developmental stages, α-modified Eagle medium (Flow Laboratories, Rockville, MD), 1.2% methylcellulose (Shinetsu Chemical, Tokyo, Japan), 30% fetal bovine serum (Hyclone Laboratories, Logan, UT), 1% deionized fraction V bovine serum albumin (Sigma Chemical, St. Louis, MO), 10^{-4} M mercaptoethanol (Eastman Organic Chemicals, Rochester, NY), 100 ng/mL rat stem cell factor (SCF; Amgen, Thousand Oaks, CA) and human interleukin (IL)-6 (Tosoh, Kanagawa, Japan), 20 ng/mL mouse IL-3 (Kirin Brewery, Tokyo, Japan) and human thrombopoietin (Tpo) (Kirin), 2 U/mL of human erythropoietin (Epo) (Kirin), and 10 ng/mL of human granulocyte colony-stimulating factor (G-CSF) (Kirin) was plated in each 35-mm suspension culture dish (No. 171099; Nunc, Naperville, IL), which was incubated at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. Colony types were determined on days 7 to 14 of incubation by in situ observation using an inverted microscope and according to the criteria described.[30,32] Abbreviations for the colony types are as follows: GM, granulocyte and/or macrophage colonies; E, erythroid bursts; MK, megakaryocyte colonies; and Mix, mixed hematopoietic colonies.

Results

CD34 expression on HSC in neonates

Most stem cell activity resides in the Lin$^-$c-Kit$^+$ cell fraction in the murine fetus[19,20] and adult,[33] although a small population of c-Kit$^-$ dormant HSC in adult BM has been reported.[10] Therefore, we first examined CD34 expression on Lin$^-$c-Kit$^+$ HSC in neonatal BM cells of Ly-5.2 mice. Figure 1A shows a flow cytometric analysis of c-Kit and CD34 expression on Lin$^-$ BM cells of a murine neonate. Although most of the Lin$^-$c-Kit$^-$ cells (57.2%) did not express CD34, Lin$^-$c-Kit$^+$ cells (42.8%) revealed various levels of CD34 expression. The Lin$^-$c-Kit$^+$ cells were fractionated into 3 subsets on the basis of CD34 expression; CD34$^-$ (6.6%, the average of 4 mice), CD34^{low} (58.3%), and CD34^{high} (35.1%) (R1, 2, and 3, respectively, in Figure 1A). Cells from CD34$^-$, CD34^{low}, and CD34^{high} fractions of Ly-5.2 mouse BM cells were injected into Ly-5.1 recipients together with 1×10^5 unfractionated BM cells of Ly-5.1 mice. Eight to 10 weeks later, PB of the recipients was analyzed for Ly-5.2–expressing Gr-1$^+$/Mac-1$^+$ myeloid cells, B220$^+$ B lymphocytes, and Thy-1$^+$ T lymphocytes. Figure 1B shows the results of transplantation experiments. All 8 mice transplanted with 1×10^3 Lin$^-$c-Kit$^+$CD34^{high} cells and 2 of 4 mice transplanted with 1×10^2 Lin$^-$c-Kit$^+$CD34^{high} cells had Ly-5.2$^+$ myeloid and lymphoid cells in the PB. Figure 1C shows a representative PB profile of a mouse transplanted with 1×10^3 Lin$^-$c-Kit$^+$CD34^{high} cells (R3), where 84.8% of Gr-1$^+$/Mac-1$^+$ cells, 98.4% of B220$^+$ cells, and 41.3% of Thy-1$^+$ cells were Ly-5.2$^+$. Although the proportion of Ly-5.2$^+$ cells depended on the number of cells injected into the recipient mice, all the mice had a higher proportion of Ly-5.2$^+$ cells in B220$^+$ B lymphocytes than in other lineages.

In the transplantation of Lin$^-$c-Kit$^+$CD34^{low} cells, 1 of 5, and 1 of 2 mice injected with 1×10^3, and 5×10^3 cells, respectively, had Ly-5.2$^+$ myeloid and lymphoid cells. By contrast, all 6 mice

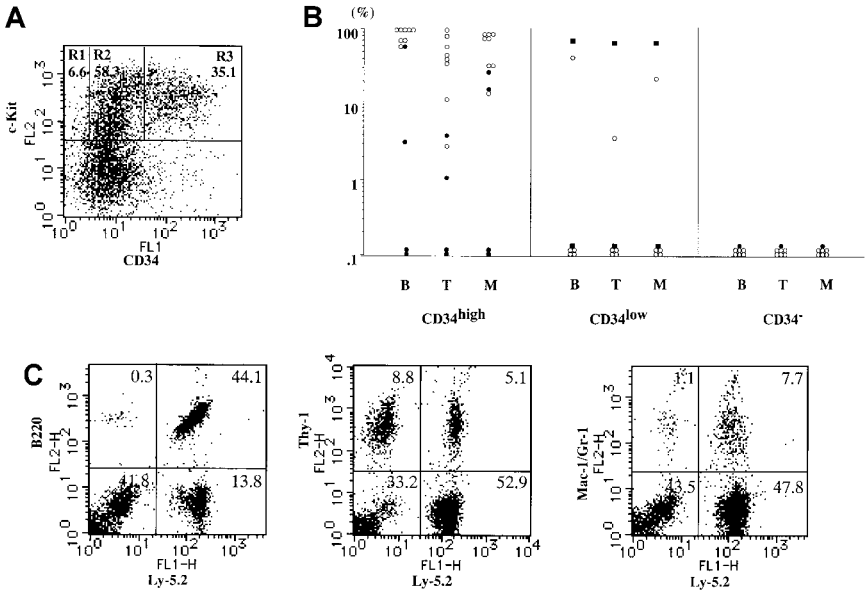

Figure 1. Flow cytometric analysis and transplantation results. (A) Expression of c-Kit and CD34 on Lin$^-$ BM cells of a neonatal mouse (Ly-5.2). Lin$^-$c-Kit$^+$ cells were fractionated into CD34$^-$ (R1), CD34^{low} (R2), and CD34^{high} (R3) cells on the basis of CD34 expression. The averages of the proportions of the 3 fractions in Lin$^-$c-Kit$^+$ cells (n = 4) are shown in each window. (B) The percentages of Ly-5.2$^+$ cells in B220$^+$ cells (B), Thy-1$^+$ cells (T), and Gr-1/Mac-1$^+$ cells (M) in Ly-5.1 recipient PB 8 to 10 weeks after transplantation. The recipients were transplanted with CD34$^-$ (R1), CD34^{low} (R2), and CD34^{high} (R3) cells (1×10^2 [●] 1×10^3 [○] and 5×10^3 cells [■]) in BM of neonatal mice (Ly-5.2). (C) A representative PB profile of a recipient mouse (Ly-5.1) engrafted with 1×10^3 Lin$^-$c-Kit$^+$CD34^{high} cells (R3) sorted from Ly-5.2 mouse BM. Ly-5.2$^+$ cells were present in all of B220$^+$ cells, Thy-1$^+$ cells, and Gr-1/Mac-1$^+$ cells in the PB.

transplanted with 1×10^3 Lin$^-$c-Kit$^+$CD34$^-$ cells had no detectable Ly-5.2$^+$ PB leukocytes. Thus, long-term repopulating HSC were most enriched in the Lin$^-$c-Kit$^+$CD34^{high} cell fraction in neonatal BM cells. Because CD34$^-$ cells accounted for only one fifteenth of the neonatal Lin$^-$c-Kit$^+$ BM cells, these results indicate that most neonatal BM HSC express CD34.

We then examined CD34 expression on HSC existing in neonatal liver and spleen. As shown in Figure 2, although the proportion of CD34^{high} cells in Lin$^-$c-Kit$^+$ cells was smaller in neonatal liver or spleen than in BM, most long-term repopulating HSC were also included in the CD34-expressing cell fraction of neonatal liver and spleen cells, indicating that neonatal HSC express CD34, irrespective of the hematopoietic tissue in which they reside.

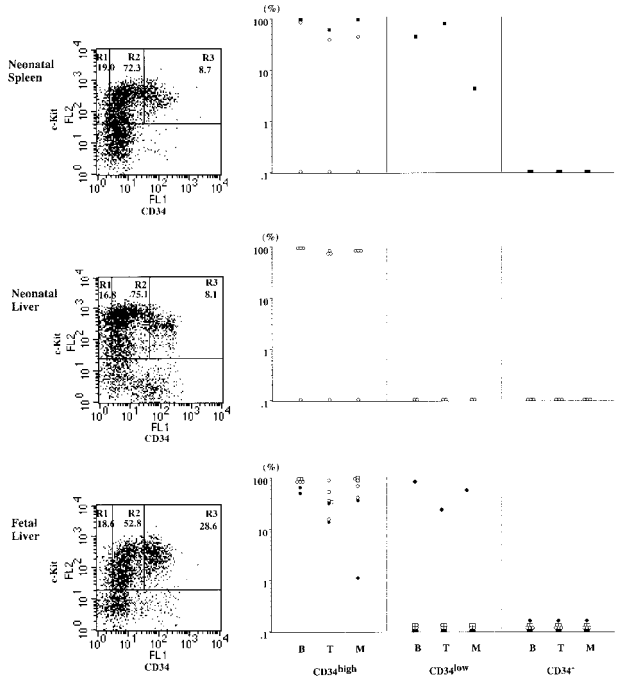

Figure 2. CD34 expression on HSC in fetal and neonatal liver and spleen. Expression of c-Kit and CD34 on Lin$^-$ BM cells of neonatal liver, neonatal spleen, and fetal liver cells (16 dpc) of Ly-5.2 mice, and the percentages of Ly-5.2$^+$ cells in B220$^+$ cells (B), Thy-1$^+$ cells (T), and Gr-1/Mac-1$^+$ cells (M) in Ly-5.1 recipient PB 8 to 10 weeks after transplantation. The recipients were transplanted with CD34$^-$ (R1), CD34^{low} (R2), and CD34^{high} (R3) cells (1×10^2 [●], 1×10^3 [○], 2×10^3 [■], 1×10^4 [□] and 2×10^4 cells [◆]) in Ly-5.2 neonatal liver, neonatal spleen, and fetal liver cells (14-18 dpc). The averages of the proportions of the 3 fractions in Lin$^-$c-Kit$^+$ cells of neonatal spleen, neonatal liver, and fetal liver cells (n = 2, 2, and 4) are shown in each window in the flow cytometry graphs.

CD34 expression on HSC in fetus

Next, CD34 expression on HSC in fetal liver at 14, 16 and 18 dpc was analyzed (Figure 2). Although the proportion of CD34^{high} cells in Lin$^-$c-Kit$^+$ cells was larger in 14 and 16 dpc fetal than in neonatal livers, Lin$^-$c-Kit$^+$ cells of 18 dpc fetal liver had a distribution of CD34-expressing cells similar to that in neonatal liver (CD34^{high} cells, 31.9%, 28.6%, 17.1%; CD34^{low} cells, 53.9%, 52.8%, 72.1%; and CD34$^-$ cells, 14.2%, 18.6%, 10.8%, in 14, 16 and 18 dpc fetal livers, respectively). In transplantation of the 3 fractions from 14, 16, and 18 dpc fetal liver cells, all 7 mice transplanted with over 1×10^2 Lin$^-$c-Kit$^+$CD34^{high} cells had Ly-5.2$^+$ myeloid and lymphoid cells at levels that depended on the number of cells injected. In mice injected with Lin$^-$c-Kit$^+$CD34^{low} cells, only one recipient receiving 2×10^4 cells from 18 dpc fetal liver showed engraftment of Ly-5.2$^+$ cells, and 6 with less than 1×10^4 cells did not. No mouse had Ly-5.2$^+$ PB leukocytes among the 8 mice transplanted with 1×10^2 to 2×10^4 Lin$^-$c-Kit$^+$CD34$^-$cells. We also carried out the transplantation using 18 dpc BM cells, in which CD34 expression in Lin$^-$c-Kit$^+$ cells revealed a similar distribution to that of neonatal BM cells (CD34^{high} cells, 49.4%; CD34^{low} cells, 42.6%; and CD34$^-$ cells, 8.0%). In the mouse injected with 3×10^3 Lin$^-$c-Kit$^+$CD34^{high} BM cells, 60% of myeloid cells, 84% of B lymphocytes, and 37% of T lymphocytes expressed Ly-5.2. The mouse injected with 3×10^3 Lin$^-$c-Kit$^+$CD34^{low} or Lin$^-$c-Kit$^+$CD34$^-$ cells had no Ly-5.2$^+$ PB leukocytes. These results indicate that most stem cell activity in the fetus at late gestational stages, as well as in neonates, resides in the CD34-expressing cell fraction.

Change of CD34 expression on HSC with aging

Competitive long-term reconstitution assays were done using BM cells sorted from mice of various ages, the objective being to examine developmental changes in CD34 expression on HSC.

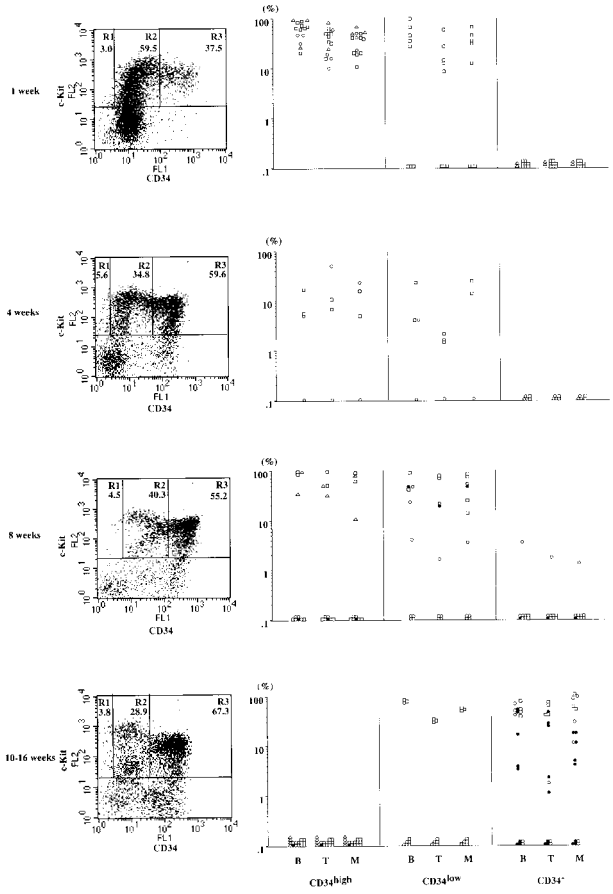

Figure 3. CD34 and c-Kit expression in BM of mice at various ages. Expression of c-Kit and CD34 on Lin$^-$ BM cells of 1-, 4-, 8-, and 16-week-old mice (Ly-5.2), and the percentages of Ly-5.2$^+$ cells in B220$^+$ cells (B), Thy-1$^+$ cells (T)- and Gr-1/Mac-1$^+$ cells (M) in Ly-5.1 recipient PB 8 to 10 weeks after transplantation. The recipients were transplanted with CD34$^-$ (R1), CD34^{low} (R2), and CD34^{high} (R3) cells (1-5 $\times$ 10^2 [●], 1-5 $\times$ 10^3 [○], 1-2 $\times$ 10^4 [□] and 1 $\times$ 10^5 cells [△]) sorted from the BM of 1-, 4-, 8-, and 16-week-old mice (Ly-5.2). The averages of the proportions of the 3 fractions in Lin$^-$c-Kit$^+$ cells of 1-, 4-, 8-, and 16-week-old mice (n = 5, 3, 7, and 10) are shown in each window in the flow cytometry graphs.

CD34 and c-Kit expression in BM cells of 1-, 4-, 8-, and 16-week-old mice are shown in Figure 3. Although the proportion of CD34$^-$ cells in BM Lin$^-$c-Kit$^+$ cells showed no remarkable changes during aging, CD34^{low} cells decreased and CD34^{high} cells increased in BM of mice over 4 weeks of age. When transplanted with 1 $\times$ 10^3 Lin$^-$c-Kit$^+$CD34^{high}, CD34^{low}, and CD34$^-$ cells obtained from 1-week-old mouse BM cells, 8 of 8, 5 of 8, and 0 of 8 mice, respectively, showed successful engraftment, indicating that HSC were most enriched in Lin$^-$c-Kit$^+$CD34^{high} fraction. The recipients transplanted with Lin$^-$c-Kit$^+$CD34^{high} and Lin$^-$c-Kit$^+$CD34^{low} cells sorted from 4-week-old mouse BM cells revealed a similar engraftment rate. Transplantation using 8-week-old mouse BM cells showed that, although most HSC still expressed CD34, they were more enriched in Lin$^-$c-Kit$^+$CD34^{low} cells than in Lin$^-$c-Kit$^+$CD34^{high} cells. In contrast to results with BM cells obtained from mice younger than 8 weeks, HSC were found in Lin$^-$c-Kit$^+$CD34$^-$ in addition to Lin$^-$c-Kit$^+$CD34^{low} cell fractions in BM from 10- to 16-week-old mice. When 1 $\times$ 10^5 Ly-5.2$^+$Lin$^-$c-Kit$^+$CD34^{high} cells in 10- to 16-week-old mouse BM, which was the largest population in Lin$^-$c-Kit$^+$ cells, were transplanted into 4 recipients, none was successfully engrafted with Ly-5.2$^+$ cells. By contrast, only 1 to 5 $\times$ 10^2 Ly-5.2$^+$Lin$^-$c-Kit$^+$CD34$^-$cells could repopulate in 5 of 8 recipients. Because the number of Lin$^-$c-Kit$^+$CD34$^-$ cells was only one twentieth that of Lin$^-$c-Kit$^+$CD34^{high} cells in 10- to 16-week-old mouse BM, the result indicates that most hematopoietic repopulating ability is present in Lin$^-$c-Kit$^+$CD34$^-$ cell fraction in 10- to 16-week-old mice. Thus, CD34 expression on murine HSC decreases with aging.

Second transplantation of donor-derived HSC from the primary recipients

To confirm that adult Lin$^-$c-Kit$^+$CD34$^-$ HSC are the progeny of neonatal CD34-expressing HSC, we carried out a second transplantation of Ly-5.2$^+$ cells sorted from BM of Ly-5.1 primary recipients who were transplanted with 1 $\times$ 10^3 BM Lin$^-$c-Kit$^+$CD34^{high} cells from Ly-5.2 neonates. CD34 expression on Ly-5.2$^+$Lin$^-$c-Kit$^+$ BM cells of the primary recipients 4 and 16 weeks after the first transplantation of Lin$^-$c-Kit$^+$CD34^{high} cells from Ly-5.2 neonatal BM was similar to that for 4- and 16-week-old mice, respectively (Figures 4A and 5A, and compare Figure 3). We then sorted

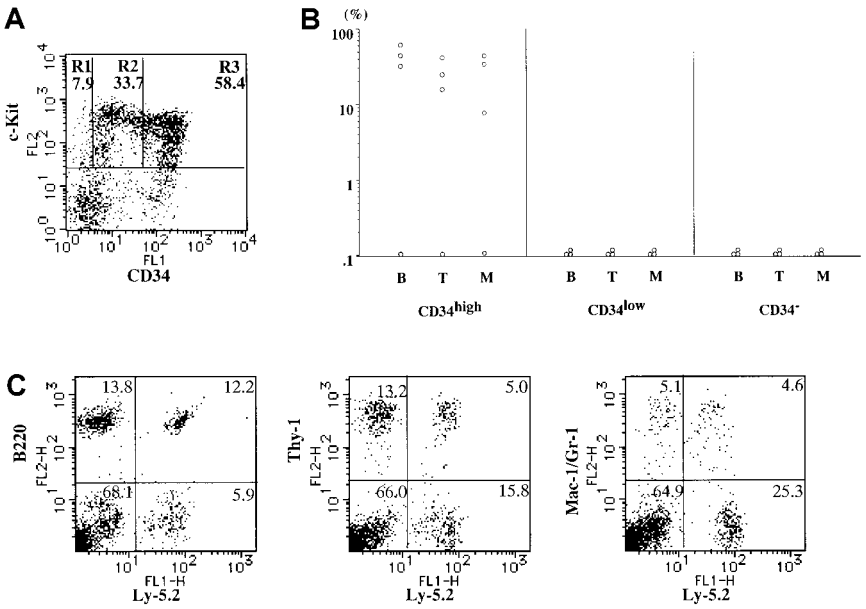

Figure 4. Second transplantation of donor-derived HSC in first recipients (4 weeks). (A) Expression of c-Kit and CD34 on Ly-5.2$^+$Lin$^-$ BM cells of the first recipient mice (Ly-5.1) engrafted with 1 $\times$ 10^3 neonatal Lin$^-$c-Kit$^+$CD34^{high} BM cells (Ly-5.2) 4 weeks after transplantation. Lin$^-$c-Kit$^+$ cells were fractionated into CD34$^-$ (R1), CD34^{low} (R2), and CD34^{high} (R3) cells on the basis of CD34 expression. The averages of the proportions of the 3 fractions in Lin$^-$c-Kit$^+$ cells (n = 3) are shown in each window. (B) The percentages of Ly-5.2$^+$ cells in B220$^+$ cells (B), Thy-1$^+$ cells (T), and Gr-1/Mac-1$^+$ cells (M) in Ly-5.1 second recipient PB 8 to 10 weeks after the second transplantation. The second recipients were transplanted with CD34$^-$ (R1), CD34^{low} (R2), and CD34^{high} (R3) cells (2 $\times$ 10^3 cells for each) in BM of the first recipients (Ly-5.1). (C) A representative PB profile of a second recipient (Ly-5.1) engrafted with 1 $\times$ 10^3 Lin$^-$c-Kit$^+$CD34^{high} cells (R3) sorted from the first recipient. Ly-5.2$^+$ cells were present in all B220$^+$ cells, Thy-1$^+$ cells, and Gr-1/Mac-1$^+$ cells in the PB.

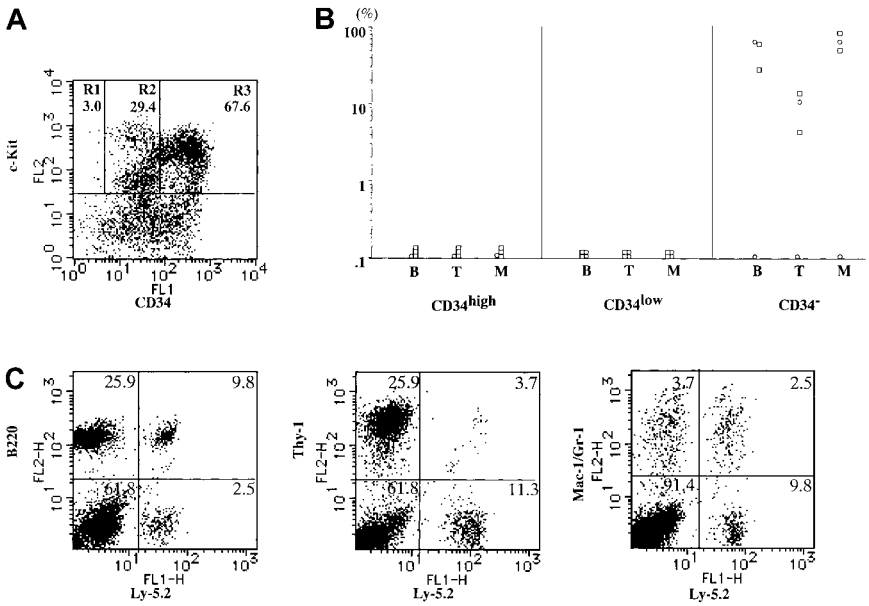

Figure 5. Second transplantation of donor-derived HSC in first recipients (16 weeks). (A) Expression of c-Kit and CD34 on Ly-5.2$^+$Lin$^-$ BM cells of the primary recipient mice (Ly-5.1) engrafted with 1×10^3 neonatal Lin$^-$c-Kit$^+$CD34^{high} BM cells (Ly-5.2) 16 weeks after transplantation. Lin$^-$c-Kit$^+$ cells were fractionated into CD34$^-$ (R1), CD34^{low} (R2), and CD34^{high} (R3) cells on the basis of CD34 expression. The averages of the proportions of the 3 fractions in Lin$^-$c-Kit$^+$ cells (n = 3) are shown in each window. (B) The percentages of Ly-5.2$^+$ cells in B220$^+$ cells (B), Thy-1$^+$ cells (T), and Gr-1/Mac-1$^+$ cells (M) in Ly-5.1 secondary recipient PB 8 to 10 weeks after the second transplantation. The secondary recipients were transplanted with CD34$^-$ (R1), CD34^{low} (R2), and CD34^{high} (R3) cells (2×10^3 [○] and 1×10^4 cells [□]) from BM of the primary recipients (Ly-5.1). (C) A representative PB profile of a secondary recipient (Ly-5.1) engrafted with 1×10^3 Lin$^-$c-Kit$^+$CD34$^-$ cells (R3) sorted from the first recipient. Ly-5.2$^+$ cells were present in B220$^+$ cells, Thy-1$^+$ cells, and Gr-1/Mac-1$^+$ cells in the PB.

Ly-5.2$^+$Lin$^-$c-Kit$^+$CD34^{high}, CD34^{low}, and CD34$^-$ cells from the primary recipients and transplanted them into Ly-5.1 secondary recipients. As shown in Figure 4B, most of the stem cell activity of Ly-5.2$^+$ cells resided in the CD34-expressing cell fraction in BM cells of the primary recipients 4 weeks after the first transplantation. Figure 4C shows a representative PB profile of a second recipient transplanted with 2×10^3 Ly-5.2$^+$Lin$^-$c-Kit$^+$CD34^{high} cells (R3) from a first recipient. As shown in Figure 5B, however, the stem cell activity was found in the Lin$^-$c-Kit$^+$CD34$^-$ cell fraction in BM cells of the primary recipient 16 weeks after transplantation. A representative PB profile of a mouse transplanted with 2×10^3 Ly-5.2$^+$Lin$^-$c-Kit$^+$CD34$^-$ cells (R1) in the primary recipient BM 16 weeks after transplantation is shown in Figure 5C. These results indicate that neonatal Lin$^-$c-Kit$^+$CD34^{high} HSC generate Lin$^-$c-Kit$^+$CD34$^-$ HSC, and that the decrease in CD34 expression on HSC of young donors occurs even in aged congeneic recipients with a time course similar to that seen in the donor.

CD34 expression on colony-forming cells

Finally, we examined CD34 expression on murine HPC, using a methylcellulose clonal culture assay. Lin$^-$c-Kit$^+$CD34^{high}, CD34^{low}, and CD34$^-$ cells (2.5×10^2 cells) sorted from BM cells of mice at various developmental stages (18 dpc to 15 weeks old) were cultured in the presence of SCF, IL-3, IL-6, G-CSF, Epo, and Tpo. As shown in Table 1, Lin$^-$c-Kit$^+$CD34^{high} cells produced the largest number of hematopoietic colonies, whereas no colonies were generated from Lin$^-$c-Kit$^+$CD34$^-$ cells at any developmental stage, indicating that HPC, unlike HSC, continue to express CD34 throughout development.

Discussion

Despite the clinical importance of CD34 antigen as a marker for primitive hematopoietic cells for HSC transplantation or gene therapy, it has been controversial whether or not all HSC express CD34 in mice or humans. We here demonstrated developmental changes in CD34 expression on murine HSC. In fetal, neonatal, and younger adult hematopoietic tissues, most of Lin$^-$c-Kit$^+$ HSC expressed CD34. However, the CD34 expression on HSC decreased with aging, and HSC were relatively enriched in the Lin$^-$c-Kit$^+$CD34$^-$ cell fraction in mice over 10 weeks of age. The evidence for a decrease of CD34 expression on HSC during development was strengthened by the second transplantation experiments showing that donor-type HSC were present in the CD34$^-$ cell fraction in recipients engrafted with neonatal Lin$^-$c-Kit$^+$CD34^{high} HSC 16 weeks after the first transplantation. This developmental change in CD34 expression on murine HSC may explain the contradictory data in previous reports regarding CD34 expression. Although Morel and coworkers[8] noted the presence of CD34 antigen on HSC in 4- to 6-week-old mice, Osawa and colleagues[9] showed, using older mice, that Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^{low/-}$ cells rather than Lin$^-$c-Kit$^+$Sca-1$^+$CD34^{high} cells possessed stem cell activity. There remains a possibility that CD34 varies among different strains of mice, since we, as well as Morel and associates and Osawa and colleagues, used C57BL/6 mice.

The function of CD34 in hematopoiesis has been elusive,

Table 1. Progenitor content of Lin$^-$ c-Kit$^+$ CD34^{high}, CD34^{low}, and CD34$^-$ BM cells of mice at various stages of development

Developmental stage	CD34 expression of Lin$^-$ c-Kit$^+$ cells	No. of colonies/250 cells*				
		GM	E	MK	Mix	Total
18 dpc	CD34^{high}	18 ± 2	1 ± 1	0	4 ± 2	23 ± 5
	CD34^{low}	9 ± 6	1 ± 1	0	0	10 ± 7
	CD34$^-$	0	0	0	0	0
Neonate	CD34^{high}	34 ± 9	25 ± 13	7 ± 2	5 ± 1	71 ± 18
	CD34^{low}	1 ± 1	2 ± 2	0	1 ± 1	3 ± 3
	CD34$^-$	0	0	0	0	0
1 wk	CD34^{high}	38 ± 9	5 ± 1	2 ± 1	5 ± 1	49 ± 9
	CD34^{low}	0	1 ± 1	0	0	1 ± 1
	CD34$^-$	0	0	0	0	0
4 wk	CD34^{high}	55 ± 15	1 ± 1	5 ± 6	9 ± 3	69 ± 15
	CD34^{low}	4 ± 5	0	0	3 ± 3	7 ± 3
	CD34$^-$	0	0	0	0	0
8 wk	CD34^{high}	67 ± 6	1 ± 1	6 ± 2	13 ± 1	87 ± 7
	CD34^{low}	3 ± 1	4 ± 2	3 ± 1	1 ± 1	11 ± 3
	CD34$^-$	0	0	0	0	0
16 wk	CD34^{high}	47 ± 20	1 ± 1	2 ± 2	16 ± 3	66 ± 26
	CD34^{low}	5 ± 3	3 ± 2	1 ± 1	2 ± 3	10 ± 3
	CD34$^-$	0	0	0	0	0

*Abbreviations for colony types are given in "Materials and methods."

although potential adhesive functions of CD34 have been reported.[34,35] Recently, 2 groups of investigators reported on hematopoiesis in CD34-deficient mice.[36,37] One group noted a decreased number of HPC in 10.5 dpc yolk sac, 14.5 dpc fetal liver, adult BM, spleen, and PB, and a poor response of adult HPC to cytokines, which suggested the involvement of CD34 in fetal and adult hematopoiesis.[36] The present observation that HPC, unlike HSC, expressed CD34 throughout murine development from fetus to adult is consistent with their results. However, neither group discussed the biologic activity of long-term repopulating HSC of CD34-deficient mice. Therefore, the function of CD34 on HSC still remains unclear. An analysis of differences in biologic activities between neonatal $Lin^-c\text{-}Kit^+CD34^{high}$ HSC and adult $Lin^-c\text{-}Kit^+CD34^-$ HSC should be instructive to define the function of CD34 on HSC.

We found a difference in differentiation potential between neonatal $Lin^-c\text{-}Kit^+CD34^{high}$ cells and adult $Lin^-c\text{-}Kit^+CD34^-$ cells. Mice transplanted with neonatal $Lin^-c\text{-}Kit^+CD34^{high}$ cells showed a predominant reconstitution of B lymphocytes, compared to adult $Lin^-c\text{-}Kit^+CD34^-$ cells. The predominant reconstitution of B lymphocytes was also found in engraftment of $Lin^-c\text{-}Kit^+CD34^{high}$ cells from fetal liver. A similar observation was made in the mice reconstituted by fetal liver $Thy\text{-}1^{low}Sca\text{-}1^+Lin^-Mac\text{-}1^+CD4^-c\text{-}Kit^+$ HSC.[38] Therefore, the predominant reconstitution of B lymphocytes may result from a strong B-lymphoid potential of HSC from fetal, neonatal, and younger adult cells.

We found no difference in proliferation potentials between neonatal $Lin^-c\text{-}Kit^+CD34^{high}$ HSC and adult $Lin^-c\text{-}Kit^+CD34^-$ HSC. Although our experiments were not designed to evaluate the proliferative potential of single HSC, stable chimerism was maintained for over 6 months in all recipients engrafted with the respective HSC phenotypes. Morrison and coworkers[38] noted indistinguishable proliferation activities of single HSC between young and old mice, yet it was also reported that the proliferation potential of HSC isolated from fetal liver is higher than that from adult BM.[24,39] Further studies are needed to clarify the difference in proliferation potentials between neonatal $Lin^-c\text{-}Kit^+CD34^{high}$ HSC and adult $Lin^-c\text{-}Kit^+CD34^-$ HSC.

Aside from the evidence for developmental changes in CD34 expression on HSC, the present study has important implications for the clinical application of HSC. Because CD34-enrichment procedures are used to prevent graft-versus-host disease or for the purging of tumor/leukemic cells in therapeutic HSC transplantation, and are also used as a target cell population for gene therapy, it is an extremely crucial issue whether human HSC express CD34. Based on the present findings, we consider that more attention should be directed to age of the donor in discussing this issue. In addition, the present findings also suggest that fetal and neonatal HSC have characteristics different from those of adult HSC. Accordingly, more detailed characterization of umbilical cord blood HSC may contribute to further development of cord blood transplantation, which is now increasingly used as an alternative to BM transplantation.

References

1. Krause DS, Fackler MJ, Civin CI, May WS. CD34: structure, biology, and clinical utility. Blood. 1996; 87:1.
2. Fina L, Molgaard HV, Robertson D, et al. Expression of the CD34 gene in vascular endothelial cells. Blood. 1998;75:2417.
3. Baumheter S, Singer MS, Henzel W, et al. Binding of L-selectin to the vascular sialomucin CD34. Science. 1993;262:436.
4. Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, Shaper JH. Antigenic analysis of hematopoiesis, III: a hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. J Immunol. 1984;133:157.
5. Sutherland HJ, Eaves CJ, Eaves AC, Dragowska W, Lansdorp PM. Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. Blood. 1989;74:1563.
6. Krause DS, Ito T, Fackler MJ, et al. Characterization of murine CD34, a marker for hematopoietic progenitor and stem cells. Blood. 1994;84:691.
7. Donnelly DS, Zelterman D, Sharkis S, Krause DS. Functional activity of murine $CD34^+$ and $CD34^-$ hematopoietic stem cell populations. Exp Hematol. 1999;27:788.
8. Morel F, Szilvassy SJ, Travis M, Chen B, Galy A. Primitive hematopoietic cells in murine bone marrow express the CD34 antigen. Blood. 1996;88: 3774.
9. Osawa M, Hanada K, Hamada H, Nakauchi H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science. 1996;273:242.
10. Ortiz M, Wine JW, Lohrey N, Ruscetti FW, Spence SE, Keller JR. Functional characterization of a novel hematopoietic stem cell and its place in the c-Kit maturation pathway in bone marrow cell development. Immunity. 1999;10:173.
11. Goodell MA, Rosenzweig M, Kim H, et al. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med. 1997;3:1337.
12. Baum CM, Weissman IL, Tsukamoto AS, Buckle AM, Peault B. Isolation of a candidate human hematopoietic stem-cell population. Proc Natl Acad Sci U S A. 1992;89:2804.
13. Larochelle A, Vormoor J, Hanenberg H, et al. Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. Nat Med. 1996;2:1329.
14. Xu MJ, Tsuji K, Ueda T, et al. Stimulation of mouse and human primitive hematopoiesis by murine embryonic aorta-gonad-mesonephros-derived stromal cell lines. Blood. 1998;92:2032.
15. Kawashima I, Zanjani ED, Almaida-Porada G, Flake AW, Zeng H, Ogawa M. $CD34^+$ human marrow cells that express low levels of Kit protein are enriched for long-term marrow-engrafting cells. Blood. 1996;87:4136.
16. Berenson RJ, Bensinger WI, Hill RS, et al. Engraftment after infusion of $CD34^+$ marrow cells in patients with breast cancer or neuroblastoma. Blood. 1991;77:1717.
17. Zanjani ED, Almeida-Porada G, Livingston AG, Flake AW, Ogawa M. Human bone marrow $CD34^-$ cells engraft in vivo and undergo multilineage expression that includes giving rise to $CD34^+$ cells. Exp Hematol. 1998;26:353.
18. Bhatia M, Bonnet D, Murdoch B, Gan OI, Dick JE. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. Nat Med. 1998;4:1038.
19. Zeigler FC, Bennett BD, Jordan CT, et al. Cellular and molecular characterization of the role of the flk-2/flt-3 receptor tyrosine kinase in hematopoietic stem cells. Blood. 1994;84:2422.
20. Sanchez MJ, Holmes A, Miles C, Dzierzak E. Characterization of the first definitive hematopoietic stem cells in the AGM and liver of the mouse embryo. Immunity. 1996;5:513.
21. Yoder MC, Hiatt K, Dutt P, Mukherjee P, Bodine DM, Orlic D. Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. Immunity. 1997;7:335.
22. Rebel VI, Miller CL, Thornbury GR, Dragowska WH, Eaves CJ, Lansdorp PM. A comparison of long-term repopulating hematopoietic stem cells in fetal liver and adult bone marrow from the mouse. Exp Hematol. 1996;24:638.
23. Huang H, Auerbach R. Identification and characterization of hematopoietic stem cells from the yolk sac of the early mouse embryo. Proc Natl Acad Sci U S A. 1993;90:10110.
24. Morrison SJ, Hemmati HD, Wandycz AM, Weissman IL. The purification and characterization of fetal liver hematopoietic stem cells. Proc Natl Acad Sci U S A. 1995;92:10302.
25. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science. 1988;241:58.
26. Tajima S, Tsuji K, Ebihara Y, et al. Analysis of interleukin 6 receptor and gp130 expressions and proliferative capability of human $CD34^+$ cells. J Exp Med. 1996;184:1357.
27. Yang FC, Watanabe S, Tsuji K, et al. Human granulocyte colony-stimulating factor (G-CSF) stimulates the in vitro and in vivo development but not commitment of primitive multipotential progenitors from transgenic mice expressing the human G-CSF receptor. Blood. 1998;92:4632.
28. Sui X, Tsuji K, Tajima S, et al. Erythropoietin-independent erythrocyte production: signals through gp130 and c-kit dramatically promote erythropoiesis from human $CD34^+$ cells. J Exp Med. 1996;183:837.
29. Okumura N, Tsuji K, Ebihara Y, et al. Chemotactic and chemokinetic activities of stem cell factor on murine hematopoietic progenitor cells. Blood. 1996;87:4100.

30. Nakahata T, Ogawa M. Identification in culture of a class of hemopoietic colony-forming units with extensive capability to self-renew and generate multipotential hemopoietic colonies. Proc Natl Acad Sci U S A. 1982;79:3843.
31. Akasaka T, Tsuji K, Kawahira H, et al. The role of mel-18, a mammalian Polycomb group gene, during IL-7-dependent proliferation of lymphocyte precursors. Immunity. 1997;7:135.
32. Koike K, Nakahata T, Takagi M, et al. Synergism of BSF-2/interleukin 6 and interleukin 3 on development of multipotential hemopoietic progenitors in serum-free culture. J Exp Med. 1988;168:879.
33. Ikuta K, Weissman IL. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. Proc Natl Acad Sci U S A. 1992;89:1502.
34. Baumhueter S, Dybdal N, Kyle C, Lasky LA. Global vascular expression of murine CD34, a sialomucin-like endothelial ligand for L-selectin. Blood. 1994;84:2554.
35. Puri KD, Finger EB, Gaudernack G, Springer TA. Sialomucin CD34 is the major L-selectin ligand in human tonsil high endothelial venules. J Cell Biol. 1995;131:261.
36. Cheng J, Baumhueter S, Cacalano G, et al. Hematopoietic defects in mice lacking the sialomucin CD34. Blood. 1996;87:479.
37. Suzuki A, Andrew DP, Gonzalo JA, et al. CD34-deficient mice have reduced eosinophil accumulation after allergen exposure and show a novel crossreactive 90-kD protein. Blood. 1996;87:3550.
38. Morrison SJ, Wandycz AM, Akashi K, Globerson A, Weissman IL. The aging of hematopoietic stem cells. Nat Med. 1996;2:1011.
39. Rebel VI, Miller CL, Eaves CJ, Lansdorp PM. The repopulation potential of fetal liver hematopoietic stem cells in mice exceeds that of their liver adult bone marrow counterparts. Blood. 1996;87:3500.