MINIREVIEW

Pluripotent Hemopoietic Stem Cells in Mice and Humans (44473)

SUSUMU IKEHARA[1]

First Department of Pathology, Transplantation Center, Kansai Medical University, Moriguchi, Osaka 570–8506, Japan

Abstract. **Although it has been reported previously that pluripotent hemopoietic stem cells (P-HSCs) express c-kit, the receptor for stem cell factor (steel factor), we and other groups have recently shown that P-HSCs do not express c-kit. In this review, we provide evidence that c-kit$^{<low}$ P-HSCs in mice have long-term-reconstituting activity (LTRA > 2 years) and the capacity to form colony-forming units in spleen (CFU-S) on Day 16, although c-kit^{low} HSCs or c-kit^{+} HSCs have LTRA less than 1.5 years and the capacity to form CFU-S on Day 14 or on Day 10, respectively. In addition, we have found that there is a major histocompatibility complex (MHC) restriction between P-HSCs and stromal cells; normal P-HSCs can proliferate and differentiate efficiently in collaboration with MHC class I-compatible (but not MHC class I-incompatible) stromal cells. In humans, we also show that c-kit$^{<low}$ P-HSCs can differentiate into c-kit^{low} cells, then c-kit^{+} cells *in vitro*.** [P.S.E.B.M. 2000, Vol 223]

Pluripotent hemopoietic stem cells (P-HSCs) are defined as cells with the capacity to self-renew and differentiate eternally into all hemopoietic lineage cells. Purification of P-HSCs has been carried out on the basis of the retention of rhodamine 123 (1, 2) and/or the expression of Sca-1 (3–6), Thy-1 (3–6), CD4 (7), Mac-1 (7), and CD34 (8).The expression of c-kit molecules (the receptor for stem cell factor) has also been a marker to purify P-HSCs. In earlier studies, P-HSCs in the mouse bone marrow (9–12) and in the adult liver (13) have been reported to be c-kit^{+}. However, recent mouse and human studies have provided evidence that P-HSCs are c-kit^{low}; mouse hemopoietic progenitors (in the dormant stage) and human CD34^{+} primitive progenitors are enriched in c-kit^{low} cells when assayed by colony formation and long-term culture-initiating cells (14, 15). We have very recently shown that P-HSCs are c-kit$^{<low}$ (phenotypically c-kit^{-}, but the c-kit message is only detectable by reverse-transcriptase-polymerase chain reaction [RT-PCR]) by assessing LTRA activity after serial bone marrow transplantation (BMT). This was very recently confirmed by Oritz *et al.* (16). This minireview provides evidence that P-HSCs are c-kit$^{<low}$ in mice and also humans.

Mouse P-HSCs

LTRA. The first step was to examine which cells (c-kit^{low} or c-kit$^{<low}$ cells) have LTRA. HSCs (Lin^{-}/CD71^{-}/class I^{high} cells) were fractionated on the basis of the expression of c-kit. Figure 1a shows the flow cytometric profile of Lin^{-}/CD71^{-} cells stained with anti-H-2K^{b} and anti-c-kit mAbs. H-2K$^{b\ high}$ cells were gated, and two cell populations—c-kit^{low} and c-kit$^{<low}$ (which are comparable to the negative control level)—were fractionated by sorting on the basis of c-kit expression. Sorting gates and histogram are shown in Figures 1a and 1b; the sorting gate of c-kit$^{<low}$ cells was set not to overlap that of c-kit^{low} cells. The FACS profile is very similar to that reported by Katayama *et al.*

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[1] To whom requests for reprints should be addressed at First Department of Pathology, Kansai Medical University, 10–15 Fumizono-cho, Moriguchi City, Osaka 570–8506, Japan. E-mail: ikehara@takii.kmu.ac.jp

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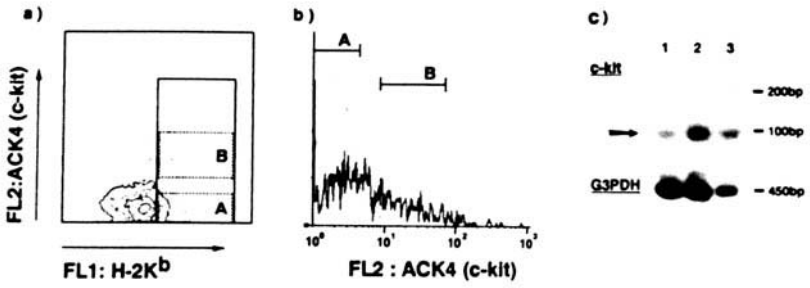

Figure 1. (a, b) Staining profiles and (c) RT-PCR analyses of Lin$^-$/CD71$^-$/class I^{high}/c-kit$^{<low}$ cells. Lin$^-$/CD71$^-$ cells were stained with anti-H-2K^b and antic-kit mAbs, and cells with a high expression of MHC class I were collected (represented in a). These cells were further divided into two populations based on the expression of c-kit [a and b (histogram)], and (A) c-kit$^{<low}$ and (B) c-kit^{low} cells were sorted by a FACStar. The dotted line in b represents a negative control stained by isotype-matched rat mAb. (c) RT-PCR-amplified products from (upper) a c-kit gene and (lower) a glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene as an internal standard were electrophoresed, transferred to a nylon membrane, and probed with internal oligonucleotides. Lanes: 1, Lin$^-$/CD71$^-$/class I^{high}/c-kit$^{<low}$ cells; 2, Lin$^-$/CD71$^-$/class I^{high}/c-kit^{low} cells; and 3, Lin$^-$/CD71$^-$ unsorted BMCs.

(15); the c-kit^{low} and c-kit$^{<low}$ populations were identified as shown in Figures 1a and 1b. Purified Lin$^-$/CD71$^-$/class I^{high}/c-kit^{low} cells or Lin$^-$/CD71$^-$/class I^{high}/c-kit$^{<low}$ cells were ≈ 0.006% or 0.008% of the 5-FU-treated whole BMCs, respectively.

The next step was to examine the expression of c-kit transcripts in these cells. The c-kit transcripts in Lin$^-$/CD71$^-$ cells, Lin$^-$/CD71$^-$/class I^{high}/c-kit^{low} cells, and Lin$^-$/CD71$^-$/class I^{high}/c-kit$^{<low}$ cells were therefore compared by RT-PCR analyses. As shown in Figure 1c, substantial expression of c-kit mRNA was detected even in the class I^{high}/c-kit$^{<low}$ cells, although the level in these cells was much lower than that in class I^{high}/c-kit^{low} cells. Based on these findings, we defined the cells as "c-kit$^{<low}$" instead of "c-kit$^-$," although the cells appeared to be "c-kit$^-$" in FACS analyses, as shown in Figures 1a and 1b.

Five hundred cells of each fraction from 5-FU-treated B6 Ly5.1 mice were intravenously (iv) injected into irradiated B6 Ly5.2 recipients along with B6 Ly5.2 compromized cells. Six months after the transplantation, cells from several organs were double-stained with a panel of mAbs against mature myeloid/lymphoid cells and anti-Ly5.1 mAb to check for donor-derived cells. Ly5.1$^+$ donor-derived mature cells (B cells, T cells, granulocytes, and macrophages) were detected in both recipients that received either Lin$^-$/CD71$^-$/class I^{high}/c-kit^{low} cells or Lin$^-$/CD71$^-$/class I^{high}/c-kit$^{<low}$ cells, in all organs tested such as the bone marrow, thymus, spleen, lymph nodes, peripheral blood, and peritoneal cavity (data not shown).

To examine the LTRA of these two cell populations, 1 × 10^6 BMCs either from recipients of Lin$^-$/CD71$^-$/class I^{high}/c-kit^{low} cells or Lin$^-$/CD71$^-$/class I^{high}/c-kit$^{<low}$ cells were re-transplanted into irradiated B6 Ly5.2 mice. Six months later, several organs were again examined to check for original donor-derived Ly5.1$^+$ cells. In contrast to the results observed in the primary recipients, original donor-derived Ly5.1$^+$ cells could be detected only in secondary recipients originally reconstituted with Lin$^-$/CD71$^-$/class I^{high}/c-kit$^{<low}$ cells; Ly5.1$^+$ cells were observed in the myeloid and lymphoid cells in all organs tested (Fig. 2a). However, in the secondary recipients of BMCs from the mice that originally received Lin$^-$/CD71$^-$/class I^{high}/c-kit^{low} cells, no Ly5.1$^+$ donor-derived cells were detected at all (Fig. 2b). These findings clearly show that P-HSCs with LTRA are Lin$^-$/CD71$^-$/class I^{high}/c-kit$^{<low}$ cells, but not Lin$^-$/CD71$^-$/class I^{high}/c-kit^{low} cells.

Tertiary transplantation was carried out to confirm the LTRA of the original Lin$^-$/CD71$^-$/class I^{high}/c-kit$^{<low}$ cells. Six months after the secondary transplantation, BMCs (1 × 10^6) from the secondary recipients were again transplanted into irradiated B6 Ly5.2 tertiary recipients. All the tertiary recipients of BMCs from secondary recipients originally reconstituted with Lin$^-$/CD71$^-$/class I^{high}/c-kit^{low} cells died within 10 days. However, all the tertiary recipients of BMCs from secondary recipients originally reconstituted with Lin-/CD71$^-$/class I^{high}/c-kit$^{<low}$ cells showed donor (Ly5.1$^+$)-derived cells in their peripheral blood; cells expressing Ly5.1 phenotype were detected in the bone mar-

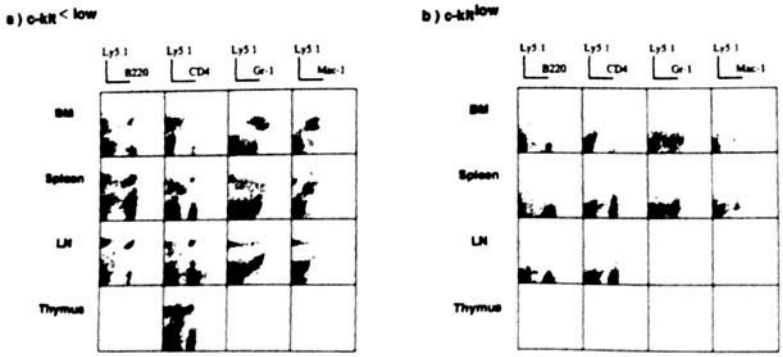

Figure 2. Detection of donor-derived cells in the secondary recipients. Six months after the primary transplantation, BMCs from the primary recipients were transferred to the irradiated secondary B6 Ly5.2 recipients. Six months later, cells from various organs were stained with a panel of mAbs and donor-specific anti-Ly5.1 mAb to determine whether the original donor-derived cells could be detected among the multilineage cells.

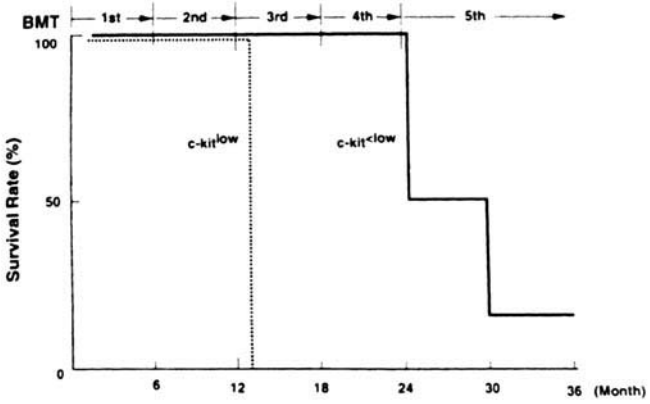

Figure 3. Survival rates in serial BMT using c-kit$^{<low}$ or c-kit^{low} cells.

row, spleen, lymph nodes, thymus, and peritoneal cavity 6 months after the tertiary BMT (17). We are now carrying out fifth BMT to examine how long c-kit$^{<low}$ cells have LTRA. Although all c-kit$^{<low}$ cells have the capacity to self-renew for 2 years, the self-renewal capacity diminishes after 2 years (Fig. 3). However, it should be noted that some (but not all) c-kit$^{<low}$ cells have the capacity to self-renew even after 3 years (Inaba M *et al.*, unpublished data).

CFU-S. We next carried out CFU-S assays using c-kit^{low} or c-kit$^{<low}$ cells. c-kit^{low} or c-kit$^{<low}$ cells (1×10^3) from male B6 Ly5.1 mice were transplanted into allotype-disparate female B6 Ly5.2 mice, and CFU-S counts were measured 8–20 days later. As shown in Figure 4, the recipients of c-kit^{low} cells formed visible CFU-S on Day 12. CFU-S counts (and also spleen weight) gradually increased and became uncountable as a result of fusion on Days 16 and 20. In contrast, no CFU-S was detected in the recipients of c-kit$^{<low}$ cells on Days 12 and 14, but first appeared on Day 16. The numbers had increased on Day 20, though each colony was small. Thus, c-kit$^{<low}$ cells can form CFU-S, although colony formation is delayed. However, c-kit^{+} cells (Lin^{-}/Thy-1^{low}/Sca-1^{+} cells) formed CFU-S on Day 10 (Fig. 4). In contrast, all the irradiated mice died within 14 days without the transplantation of HSCs (c-kit^{+}, c-kit^{low}, or c-kit$^{<low}$ cells), and no CFU-S was detected during these days after the fractionated irradiation (5.5 Gy × 2 = 11 Gy). This indicates that CFU-S in the recipients are actually donor-derived, and are not derived from the radioresistant progenitors of the recipients (18).

Using counter-current centrifugal elutriation (CCE), Oritz *et al.* (16) have very recently shown that P-HSCs are c-kit^{-} (no c-kit RNA expression even by RT-PCR), and that they do not form CFU-S on Day 12. However, as shown in the above, our P-HSCs (Lin^{-}/CD71^{-}/class I ^{high}/c-kit$^{<low}$ cells) form CFU-S on Day 16. The difference between the data from Oritz *et al.* and ours may be due to different populations, since their cell population contains a moderate number of CD4^{+} or Thy-1^{+} (not Thy-1^{low}) cells, whereas our cell population does not contain CD4^{+} or Thy-1^{+} cells.

Peripheral Blood Stem Cells in Mice. The next step was to examine whether peripheral blood stem cells (PBSCs) are true P-HSCs or not. PBSCs were mobilized in mice by treatment with cytosine-arabinoside on Day 0, followed by the administration of granulocyte colony-stimulating factor for 4 days. There were remarkable increases in the numbers of cells with Lin^{-}/c-kit^{+} markers, cells with colony-forming unit-cell (CFU-C) and CFU-S activities, and cells with marrow-repopulating ability (MRA) in the extramedullary sites (the spleen, peripheral blood, and liver) on Day 5, whereas the number of these immature hemopoietic cells decreased in the bone marrow (BM) on Day 5. This finding suggests the mobilization of immature hematopoietic cells from the BM to the extramedullary sites. Three-color flow cytometric analyses showed that CD4 antigen was not expressed on the Lin^{-}/Sca-1^{+} cells

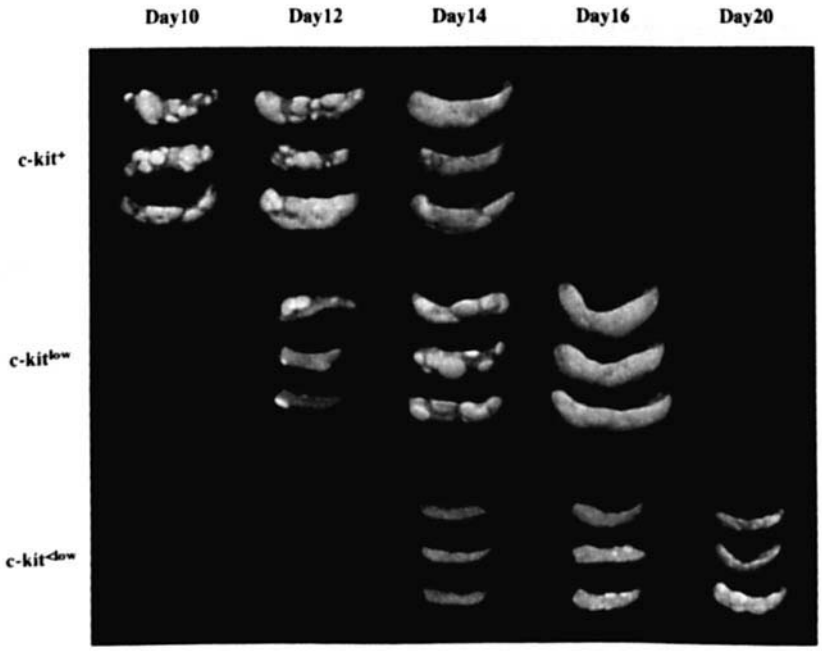

Figure 4. CFU-S assays using c-kit$^{<low}$, c-kit^{low}, or c-kit^{+} cells. The female C57BL/6 Ly 5.2 mice that received 10^3 c-kit$^{<low}$, c-kit^{low}, or c-kit^{+} cells were killed on Days 8–20; c-kit$^{<low}$ and c-kit^{low} cells were obtained from the BMCs of 5-FU-treated male Ly 5.1 mice, and c-kit^{+} cells were obtained from the BMCs of male Ly 5.1 mice without 5-FU treatment. The spleens were removed and fixed with Bouin's fixing fluid before the spleen colonies were counted.

in the mobilized PB cells (PBCs), although $CD71^{-}$ $CD4^{low}$ cells were found in those of normal BM cells. Lin^{-}/c-kit^{+} cells in the mobilized PBCs contained more cells with immature phenotypes (Sca-1^{+}/Thy1.2^{low}/$CD71^{-}$/$Rh123^{dull}$) than in normal BMCs, indicating an alteration of the hierarchical composition of the Lin^{-}/c-kit^{+} cells. We could find neither Lin^{-}/Sca-1^{+}/c-kit^{low} cells nor Lin^{-}/Sca^{+}/c-$kit^{<low}$ cells in the PBCs. Electron microscopic studies of these cells in the mobilized PBCs showed that only 10% to 20% of these cells had a thin rim of cytoplasm with poorly developed organelles (Fig. 5a). In contrast, P-HSCs present in the bone marrow had a large nucleus with narrow cytoplasm. Their chromatin pattern was dispersed, but small aggregates appeared at nuclear margins. There were few cytoplasmic organelles but abundant free ribosomes (Fig. 5b). It should be noted that P-HSCs possess microvilli; they show active movement like neutrophils when observed on video tape. Thus, PBSCs seem to be more mature than P-HSCs in the bone marrow (19).

The next step was to examine by allogeneic PBSC transplantation (PBSCT) whether PBSCs have LTRA. Allogeneic PBSCT [B6→C3H] showed long-term reconstituting activity across the MHC barrier within 1 year of transplantation. However, all the [B6→C3H] mice with PBSCT died 60 weeks after PBSCT, although the [B6→C3H] mice with allogeneic BMT showed a 70% survival rate (Fig. 6). These findings suggest that PBSCs are not true P-HSCs (Yamamoto Y *et al.*, unpublished data).

Human P-HSCs

In humans, HSCs have been characterized as $CD34^{+}$cells; they possess colony-forming activity when bone marrow (BM), cord blood (CB), and mobilized peripheral blood are cultured (20–23). Other immunophenotypes defining primitive HSCs have been reported to be

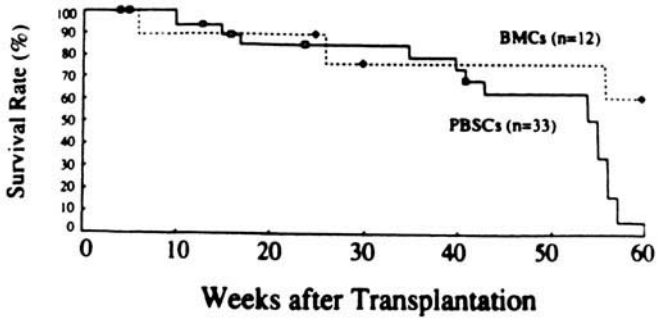

Figure 6. Survival rate in [B6→C3H] chimeric mice reconstituted with normal BMCs (1.5×10^{7} cells) or PBSCs (7×10^{6} cells).

$CD34^{+}$/$CD38^{-}$, $CD34^{+}$/Thy-1^{low+}, $CD34^{+}$/Lin^{-}/CD41a^{-}, and $CD34^{+}$/HLA-DR^{+}, based on the assays for high proliferative potential colony-forming cells (HPP-CFCs) or long-term culture-initiating cells (LTC-ICs). $CD34^{+}$/$CD38^{-}$ cells can generate colony-forming unit-culture (CFU-C) in LTC-IC assay (24). Most LTC-IC, cobblestone area–forming cells, and cells capable of reconstituting human hemopoiesis in severe combined immunodeficiency mice are contained in the $CD34^{+}$/Thy-1^{low} population (25–27).

Another functionally and phenotypically important molecule is c-kit, which plays an important role in the early stage of hemopoiesis (28–31). In previous studies, it was found that the $CD34^{+}$/c-kit^{low} population contained the majority of cycle-dormant progenitors, multilineage colony-forming cells, colony-forming unit granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM) and LTC-ICs (14, 32, 33). In addition, LTRA was found to be enriched in the $CD34^{+}$/c-kit^{low} population using an *in utero* transplantation system (34). In contrast, $CD34^{+}$/c-kit^{-} cells were rejected because of their low proliferative responses or the absence of such responses to several stimuli (30, 32–35), in spite of the existence of these cells in the human BM and

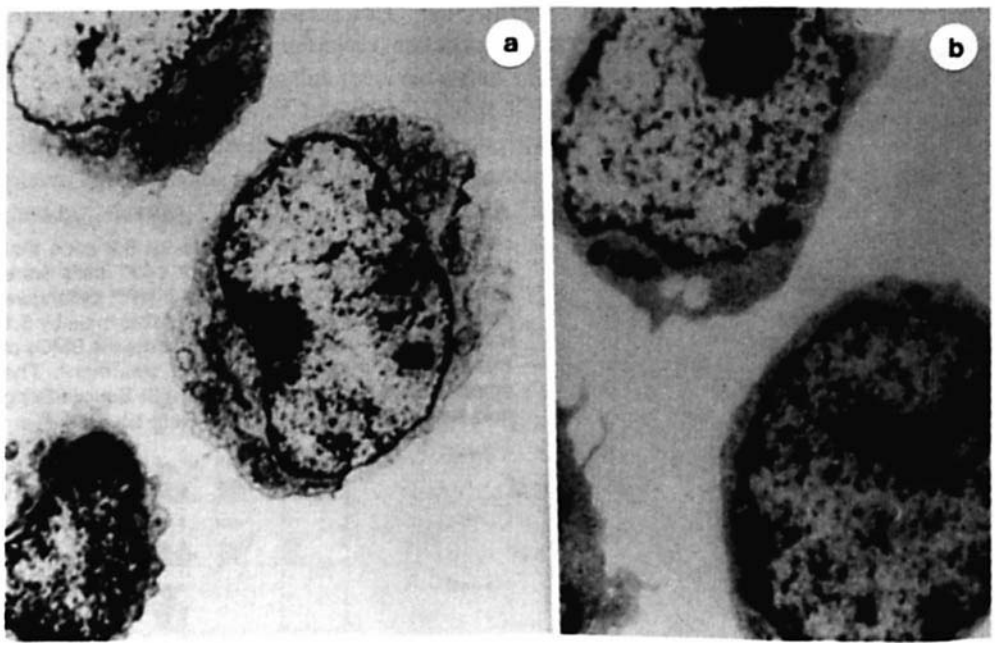

Figure 5. Electron microscopic (EM) findings in (a) PBSCs (Lin^{-}/Sca-1^{+}/c-kit^{+} cells) and (b) P-HSCs in the bone marrow. P-HSCs show large nuclei with narrow cytoplasm and microvilli, whereas PBSCs show a more spacious cytoplasm with more abundant mitochondrias than P-HSCs.

CB. However, it has also been shown that the expression of c-kit molecules on HSCs is dependent on the differentiation steps (30, 35, 36). Therefore, there is a possibility that the induction of c-kit molecules is a first step in the differentiation of P-HSCs, being accompanied by the acquirement of reactivity to SCF. Furthermore, taking the dormancy of HSCs into consideration, it would be logical for HSCs not to express c-kit on their surface and so protect them from stimuli that would induce them to enter the cell cycle. In this sense, $CD34^+/c\text{-}kit^{low}$ cells could be more primitive HSCs.

These findings prompted us to examine whether human P-HSCs are $c\text{-}kit^{<low}$, $c\text{-}kit^{low}$, or $c\text{-}kit^+$. We purified $CD34^+/c\text{-}kit^{<low}$ cells (phenotypically c-kit-negative but only detectable at the message level) from the human cord blood and examined their maturational steps in relation to the expression of c-kit molecules. When the $CD34^+/c\text{-}kit^{<low}$ cells were cultured with cytokines (flt3-ligand, IL-6, and IL-7) plus immobilized anti-CD34 monoclonal antibody (mAb) (to cross-link CD34 molecules), c-kit molecules were clearly induced within 24 hr (Fig. 7). The c-kit expression gradually increased until Day 8. Although the colony-forming ability of sorted $CD34^+/c\text{-}kit^{<low}$ cells is less than that of the other two populations ($CD34^+/c\text{-}kit^+$ and $CD34^+/c\text{-}kit^{low}$), when $CD34^+/c\text{-}kit^{low}$ or $CD34^+/c\text{-}kit^+$ cells that had been induced from $CD34^+/c\text{-}kit^{<low}$ cells were re-sorted and re-cultured using a methylcellulose culture system, they showed the same colony-forming ability as the freshly isolated $CD34^+/c\text{-}kit^{low}$ or $CD34^+/c\text{-}kit^+$ cells, respectively. These data indicate that the changes in the expression of c-kit molecules reflect the changes not only in their immunophenotypes but also in the functional maturation of the $CD34^+/c\text{-}kit^{<low}$ cells. This is in accordance with the report by Gunji *et al.* (14) where: (i) $CD34^+/c\text{-}kit^{high}$ cells were induced from $CD34^+/c\text{-}kit^{low}$ cells after four-week culture with stromal cells and (ii) LTC-ICs were enriched in the $CD34^+/c\text{-}kit^{low}$ population, but not in differentiated $CD34^+/c\text{-}kit^{high}$ cells with the ability to form CFU-GM. However, they only reported the induction of differentiation from $c\text{-}kit^{low}$ to $c\text{-}kit^{high}$. Our purified $CD34^+/c\text{-}kit^{<low}$ or ^{low} and $CD34^+/c\text{-}kit^+$ cells may correspond to $CD34^+/c\text{-}kit^-$ and $CD34^+/c\text{-}kit^{low}$ cells, respectively, in the previous reports, based on FCM pattern and colony-forming ability (32, 34). Therefore, our results clearly show that the changes in the intensity of c-kit molecules are closely related to the earliest maturational phase of hematopoiesis. Furthermore, $CD34^+/c\text{-}kit^{<low}$ cells have a similar hemopoietic potential to $CD34^+/c\text{-}kit^{low}$ cells in assays for LTC-IC and CFU-C generated from long-term cultures. These findings suggest that $CD34^+/c\text{-}kit^{<low}$ cells differentiate into $CD34^+/c\text{-}kit^{low}$ and $CD34^+/c\text{-}kit^+$ cells and acquire the reactivity to various humoral hemopoietic stimuli. Moreover, $CD34^+/c\text{-}kit^{<low}$ cells showed a low level of rhodamine 123 (Rh123) retention, suggesting that these cells have multidrug resistance. Electron microscopic studies revealed the presence of P-HSCs, similar to those seen in the mouse bone marrow (Fig. 5b) (37) in the cord blood (Fig. 8). Therefore, we conclude that human cord blood contains $CD34^+/c\text{-}kit^{<low}$ cells that correspond to mouse P-HSCs (38).

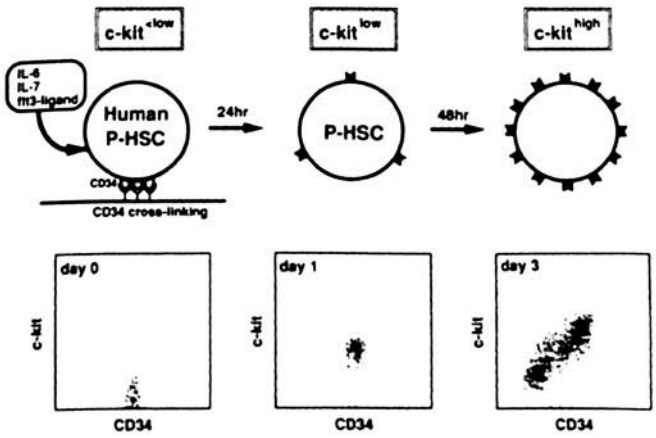

Figure 7. Induction of human $c\text{-}kit^+$ cells from $c\text{-}kit^{<low}$ cells *in vitro*.

MHC Restriction Exists Between Normal P-HSCS and Stromal Cells

In vitro studies revealed that P-HSCs cannot proliferate in the presence of putative cytokines such as GM-CSF, stem cell factor (SCF), and IL-3, whereas they can do so by direct interaction with stromal cells without adding any cytokines (Fig. 9). This finding indicates that the direct interaction of P-HSCs with stromal cells is essential for P-HSCs to proliferate.

We have found previously that donor-derived stromal cells play a crucial role in successful BMT across MHC barriers (39, 40). This finding prompted us to examine

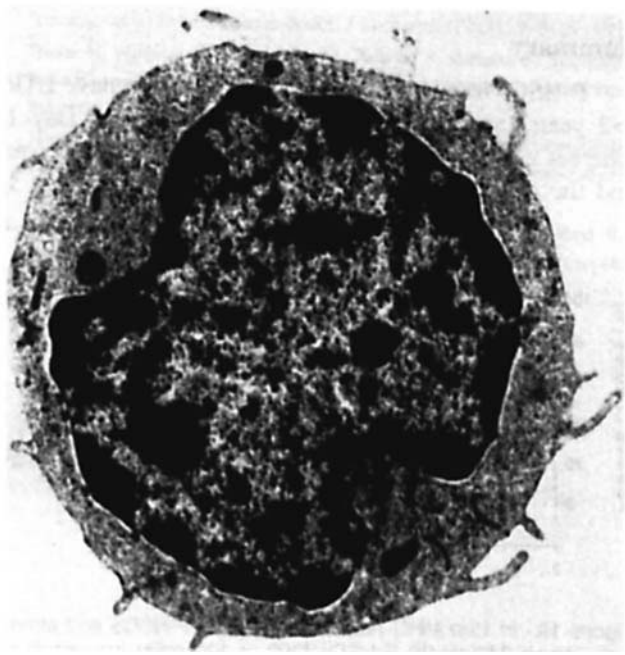

Figure 8. Electron microscopic findings in human P-HSCs ($CD34^+/c\text{-}kit^{<low}$). A human P-HSC shows a large nucleus with narrow cytoplasm and microvilli, as seen in mouse P-HSCs (see Fig. 5).

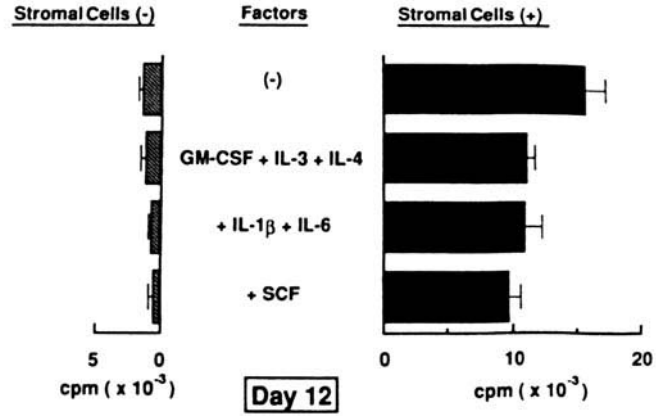

Figure 9. Necessity of stromal cells for P-HSCs to proliferate. When P-HSCs were cocultured with stromal cells, the P-HSCs proliferated without the addition of cytokines, whereas they did not without stromal cells.

whether there is MHC restriction between P-HSCs and stromal cells. Hemopoiesis was observed only in the bone marrow engrafted with the BALB/c bone when BALB/c BMCs (T cell-depleted and adherent cell-depleted) were i.v. injected into irradiated C3H/HeN mice that had been engrafted with bones of C3H/HeN, B6, and BALB/c mice or with a Teflon tube as a control. This was confirmed in *in vitro* experiments; when B10 (H-2^b) P-HSCs were cocultured with B10 stromal cells, the P-HSCs proliferated, whereas when B10 P-HSCs were cocultured with B10D2 (H-2^d) stromal cells, the P-HSCs showed poor proliferative responses (Fig. 10) (41). These findings indicate that an MHC restriction exists between P-HSCs and stromal cells not only *in vivo* but also *in vitro*. Recently, we have found that there is an MHC class I (D and S loci) restriction between them (42).

Summary

P-HSCs in mice are c-kit$^{<low}$ cells, which have LTRA (>2 years) and the capacity to form CFU-S on Day 16, whereas c-kit^{low} and c-kit$^+$ cells have LTRA (<1.5 years) and the capacity to form CFU-S on Day 12 or Day 10, respectively. MHC class I-compatible stromal cells are essential for P-HSCs to proliferate and differentiate. In humans, CD34$^+$/c-kit$^{<low}$ are also found to differentiate into c-kit^{low} cells, then c-kit$^+$ cells *in vitro*. Based on these findings, we believe that c-kit$^{<low}$ cells are P-HSCs in both mice and humans. From mouse experiments, we recommend the recruitment of donor-derived stromal cells for allogeneic BMT across MHC barriers to succeed.

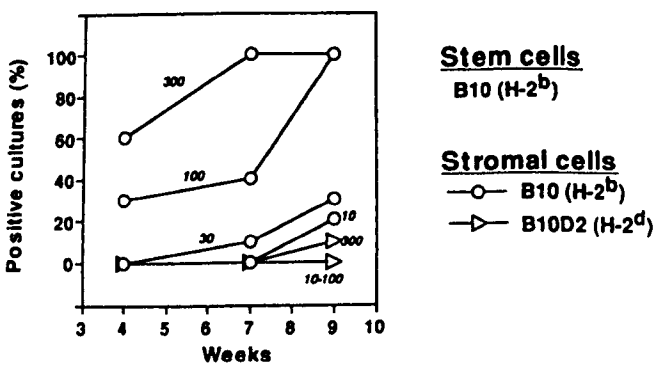

Figure 10. *In vitro* MHC restriction between P-HSCs and stromal cells. When B10 (H-2^b) P-HSCs (300 or 100 cells) are cocultured with B10 stromal cells, the P-HSCs proliferate. In contrast, when B10 P-HSCs are cocultured with B10D2 (H-2^d) stromal cells, the P-HSCs (10–300 cells) show poor proliferative responses.

These experiments were carried out in collaboration with researchers who appear in the references of this paper. I would like to express my deep appreciation to them. I would also like thank Mr. Hilary Eastwick-Field and Ms. Keiko Ando for preparing this manuscript.

1. Wolf NS, Kone A, Priestley GV, Bartelmez SH. *In vivo* and *in vitro* characterization of long-term repopulating primitive hematopoietic stem cells isolated by sequential hoechst 33342-rhodamine 123 FACS selection. Exp Hematol **21**:614–622, 1993.
2. Spangrude GJ, Brooks DM, Tumas DB. Long-term repopulation of irradiated mice with limited numbers of purified hematopoietic stem cells: *In vivo* expansion of stem cell phenotype but not function. Blood **85**:1006–1016, 1995.
3. Spangrude GJ, Brooks DM. Mouse strain variability in the expression of the hematopoietic stem cell antigen Ly-6A/E by bone marrow cells. Blood **82**:3327–3332, 1993.
4. Uchida N, Weissman IL. Searching for hematopoietic stem cells: Evidence that Thy-1.1^{low}Lin$^-$Sca-1$^+$ cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. J Exp Med **175**:175–184, 1992.
5. Spangrude GJ, Brooks DM. Phenotype analysis of mouse hematopoietic stem cells shows a Thy-1-negative subset. Blood **80**:1957–1964, 1992.
6. Chung LL, Johnson GR. Long-term hemopoietic repopulation by Thy-1^{low}, Lin$^-$, Ly6A/E$^+$ cells. Exp Hematol **20**:1309–1315, 1992.
7. Morrison SJ, Weissman IL. The long-term hemopoietic repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity **1**:661–673, 1994.
8. Krause DS, Ito T, Fackler MJ, Smith OM, Collector MI, Sharkis SJ, May WS. Characterization of murine CD34, a marker for hematopoietic progenitors and stem cells. Blood **84**:691–701, 1994.
9. Orlic D, Fischer R, Nishikawa S-I, Nienhuis AW, Bodine DM. Purification and characterization of heterogeneous pluripoent hematopoietic stem cell populations expressing high levels of c-kit receptor. Blood **2**:762–770, 1993.
10. Ikuta K, Weissman IL. Evidence that hematopoietic stem cells express c-kit, but do not depend on steel factor for their generation. Proc Natl Acad Sci U S A **89**:1502–1506, 1992.
11. Ogawa M, Matsuzaki Y, Nishikawa S, Hayashi S-I, Kunisada T, Sudo T, Kina T, Nakauchi H, Nishikawa S-I. Expression and function of c-kit in hemopoietic progenitor cells. J Exp Med **174**:63–71, 1991.
12. Li CL, Johnson GR. Murine hematopoietic stem and progenitor cells: I. Enrichment and biologic characterization. Blood **85**:1472–1479, 1995.
13. Taniguchi H, Toyoshima T, Fukao K, Nakauchi H. Presence of hematopoietic stem cells in the adult liver. Nat Med **2**:198–203, 1996.
14. Gunji Y, Nakamura M, Osawa H, Nagayoshi K, Nakauchi H, Miura Y, Yanagisawa M, Suda T. Human primitive hematopoietic progenitor cells are more enriched in KIT^{low} cells than in KIT^{high} cells. Blood **82**:3283–3289, 1993.
15. Katayama N, Shih J-P, Nishikawa S-I, Kina T, Clark SC, Ogawa M. Stage-specific expression of c-kit protein by murine hematopoietic progenitors. Blood **82**:2353–2360, 1993.
16. Oriz 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 **10**:173–182, 1999.
17. Doi H, Inaba M, Yamamoto Y, Taketani S, Mori S-I, Sugihara A, Ogata H, Toki J, Hisha H, Inaba K, Sogo S, Adachi M, Matsuda T, Good RA, Ikehara S. Pluripotent hemopoietic stem cells are c-kit^{low}. Proc Natl Acad Sci U S A **94**:2513–2517, 1997.
18. Lian Z, Feng B, Sugiura K, Inaba M, Yu C, Jin T, Fan T, Cui Y, Yasumizu R, Toki J, Adachi Y, Hisha H, Ikehara S. c-kit^{low} pluripotent hemopoietic stem cells form CFU-S on Day 16. Stem Cells **17**:39–44, 1999.
19. Yamamoto Y, Yasumizu R, Amou Y, Watanabe N, Nishio N, Toki J, Fukuhara S, Ikehara S. Characterization of peripheral blood cells in mice. Blood **88**:445–454, 1996.
20. Bernstein ID, Andrewa RG, Zsebo KM. Recombinant human stem cell factor enhances the formation of colonies by CD34$^+$ and CD34$^+$lin$^-$ cells, and the generation of colony-forming cell progeny from CD34$^+$lin$^-$ cells cultured with interleukin-3, granulocyte colony-stimulating factor, or granulocyte-macrophage colony-stimulating factor. Blood **77**:2316–2321, 1991.
21. Xiao M, Leemhuis T, Broxmeyer HE, Lu L. Influence of combinations of cytokines on proliferation of isolated single cell–sorted human bone marrow hematopoietic progenitor cells in the absence and presence of serum. Exp Hematol **20**:276–279, 1992.
22. Lu L, Xiao M, Shen R-N, Grigsby S, Broxmeyer HE. Enrichment, characterization, and responsiveness of single primitive CD34 human umbilical cord blood hematopoietic progenitors with high proliferative and replating potential. Blood **81**:41–48, 1993.
23. Siena S, Bregni M, Brando B, Ravagnani F, Bonadonna G, Gianni AM. Circulation of CD34$^+$ hematopoietic stem cells in the peripheral blood of high-dose cyclophosphamide-treated patients: Enhancement by intravenous recombinant human granulocyte-macrophage colony-stimulating factor. Blood **74**:1905–1914, 1989.
24. Hao Q-L, Shah AJ, Thiemann FT, Smogorzewaska EM, Crooks GM. A functional comparison of CD34$^+$CD38$^-$ cells in cord blood and bone marrow. Blood **86**:3745–3753, 1995.
25. Murray L, Chen B, Galy A, Chen S, Tushinski R, Uchida N, Negrin R, Tricot G, Jagannath S, Vesole D. Enrichment of human hematopoietic stem cell activity in the CD34$^+$Thy-1$^+$Lin$^-$ subpopulation from human mobilized peripheral blood. Blood **85**:368–378, 1995.
26. Craig W, Kay R, Cutler RL, Lansdorp PM. Expression of Thy-1 on human hematopoietic progenitor cells. J Exp Med **177**:1331–1342, 1993.
27. Mayani H, Lansdorp PM. Thy-1 expression is linked to functional properties of primitive hematopoietic progenitor cells from human umbilical cord blood. Blood **83**:2410–2417, 1994.
28. Katayama N, Clark SC, Ogawa M. Growth factor requirement for survival in cell-cycle dormancy of primitive murine lymphohematopoietic progenitors. Blood **8**:610–616, 1993.
29. Tsuji K, Lyman SD, Sudo T, Clark SC, Ogawa M. Enhancement of murine hematopoiesis by synergistic interactions between steel factor (ligand for c-kit), interleukin-11, and other early acting factors in culture. Blood **79**:2855–2860, 1992.
30. Simmons PJ, Aylett GW, Niutta S, To LB, Juttner CA, Asman LK. c-Kit is expressed by primitive human hematopoietic cells that give rise to colony-forming cells in stroma-dependent or cytokine-supplemented culture. Exp Hematol **22**:157–165, 1994.
31. Gore SD, Amin S, Weng L-J, Civin CI. Steel factor supports the cycling of isolated human CD34+ cells in the absence of other growth factors. Exp Hematol **23**:413–421, 1995.
32. Laver JH, Abboud MR, Kawashima I, Leary AG, Ashman LK, Ogawa M. Characterization of c-kit expression by primitive hematopoietic progenitors in umbilical cord blood. Exp Hematol **23**:1515–1519, 1995.
33. Briddell RA, Broudy VC, Bruno E, Brabdt JE, Srour EF, Hoffman R. Further phenotypic characterization and isolation of human hematopoietic progenitor cells using a monoclonal antibody to the c-kit receptor. Blood **79**:3159–3167, 1992.
34. 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 **87**:4136–4142, 1996.
35. De Jong MO, Wagemaker G, Wognum AW. Separation of myeloid and erythroid progenitors based on expression of CD34 and c-kit. Blood **86**:4076–4085, 1995.
36. Uoshima N, Ozawa M, Kimura S, Tanaka K, Wada K, Kobayashi Y, Kondo M. Changes in c-kit expression and effects of SCF during differentiation of human erythroid progenitor cells. Br J Haematol **91**:30–36, 1995.
37. Miyama-Inaba M, Ogata H, Toki J, Kuma S, Sugiura K, Yasumizu R, Ikehara S. Isolation of murine pluripotent hemopoietic stem cells in the Go phase. Biochem Biophys Res Commun **147**:687–694, 1987.
38. Sogo S, Inaba M, Ogata H, Hisha H, Adachi Y, Mori S-I, Toki J, Yamanishi K, Kanzaki H, Adachi M, Ikehara S. Induction of c-kit molecules on human CD34$^+$/c-kit^{low} cells: Evidence for CD34$^+$/c-kit^{low} cells as primitive hematopoietic stem cells. Stem Cells **15**:420–429, 1997.
39. Ishida T, Inaba M, Hisha H, Sugiura K, Adachi Y, Nagata N, Ogawa R, Good RA, Ikehara S. Requirement of donor-derived stromal cells in the bone marrow for successful allogeneic bone marrow transplantation: Complete prevention of recurrence of autoimmune diseases in MRL/MP-*lpr*-*lpr* mice by transplantation of bone marrow plus bone (stromal cells) from the same donor. J Immunol **152**:3119–3127, 1994.
40. Hisha H, Nishino T, Kawamura M, Adachi S, Ikehara S. Successful bone marrow transplantation by bone grafts in chimeric-resistant combination. Exp Hematol **23**:347–352, 1995.
41. Hashimoto F, Sugiura K, Inoue K, Ikehara S. Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells *in vivo*. Blood **89**:49–54, 1997.
42. Sugiura K, Inaba M, Hisha H, Borisov K, Sardi-a EE, Good RA, Ikehara S. Requirement of major histocompatibility complex-compatible microenvironment for spleen colony formation (CFU-S on Day 12 but not on Day 8). Stem Cells **15**:461–468, 1997.