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Identification of Flt3+ Lympho-Myeloid Stem Cells Lacking Erythro-Megakaryocytic Potential: A Revised Road Map for Adult Blood Lineage Commitment

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Summary

All blood cell lineages derive from a common hematopoietic stem cell (HSC). The current model implicates that the first lineage commitment step of adult pluripotent HSCs results in a strict separation into common lymphoid and common myeloid precursors. We present evidence for a population of cells which, although sustaining a high proliferative and combined lympho-myeloid differentiation potential, have lost the ability to adopt erythroid and megakaryocyte lineage fates. Cells in the Lin$^-$Sca-1$^+$c-kit$^+$ HSC compartment coexpressing high levels of the tyrosine kinase receptor Flt3 sustain granulocyte, monocyte, and B and T cell potentials but in contrast to Lin$^-$Sca-1$^+$c-kit$^+$Flt3$^-$ HSCs fail to produce significant erythroid and megakaryocytic progeny. This distinct lineage restriction site is accompanied by downregulation of genes for regulators of erythroid and megakaryocyte development. In agreement with representing a lymphoid primed progenitor, Lin$^-$Sca-1$^+$c-kit$^+$CD34$^+$Flt3$^+$ cells display upregulated IL-7 receptor gene expression. Based on these observations, we propose a revised road map for adult blood lineage development.

Introduction

All blood cell lineages derive from a common hematopoietic stem cell (HSC) responsible for life-long and balanced blood cell production, in man amounting to millions of cells per second in steady state (Ogawa, 1993). Although molecular pathways (cytokine receptors and transcription factors) regulating the development of the different blood cell lineages have been identified (Metcalf, 1993; Shivdasani and Orkin, 1996; Zhu and Emerson, 2002), the role of these and other pathways in governing hematopoietic lineage commitment remains elusive.

Developments in state of the art technologies enabling identification and prospective purification of HSCs and downstream progenitor cells at different stages of commitment (Akashi et al., 2000; Kondo et al., 1997; Matsuzaki et al., 2004; Osawa et al., 1996) have and will continue to play a key role in identifying the molecular mechanisms governing HSC self-renewal as well as lineage fate determination. The identification of common myeloid and lymphoid progenitors (CMPs and CLPs, respectively; Akashi et al., 2000; Kondo et al., 1997) lends support to the classical and currently prevailing model for hematopoietic commitment and blood lineage development, implicating that the first and decisive lineage commitment step of adult HSCs results in an immediate and complete separation of myelopoiesis and lymphopoiesis (Reya et al., 2001). Importantly, this model proposes that short-term HSCs (ST-HSCs) and multipotent progenitors (MPPs), although having reduced self-renewal capacity, sustain the full lympho-myeloid lineage potentials of long-term HSCs (LT-HSCs) (Reya et al., 2001).

Virtually all LT-HSC, ST-HSC, and MPP activities in adult mouse bone marrow (BM) have been shown to reside in the small Lin$^-$Sca-1$^+$c-kit^{hi} (LSK) HSC compartment (0.1% of all BM cells; Ikuta and Weissman, 1992; Li and Johnson, 1995; Spangrude et al., 1988; Weissman et al., 2001). Within the adult LSK compartment, LT-HSCs have been demonstrated to lack expression of CD34 as well as the cytokine tyrosine kinase receptor Flt3 (Adolfsson et al., 2001; Christensen and Weissman, 2001; Osawa et al., 1996), whereas ST-HSCs are LSKCD34$^+$Flt3$^-$ (Osawa et al., 1996; Yang et al., 2005). The LSKCD34$^+$Flt3$^-$ ST-HSC population gives rise to a third population of LSK cells, all coexpressing Flt3 and CD34 and representing as much as 50% of the LSK HSC compartment (Adolfsson et al., 2001). Upon transplantation LSKCD34$^+$Flt3$^+$ (hereafter called LSK Flt3$^+$) cells give primarily rise to rapid and robust lymphoid reconstitution (Adolfsson et al., 2001). A role for Flt3 in early lymphoid development has been further substantiated in studies of mice deficient in expression of Flt3 or its ligand, as these mice have reductions in early B and T cell progenitors as well as the CLPs (Mackarehtschian et al., 1995; McKenna et al., 2000; Sitnicka et al., 2002). Furthermore and in line with the documented synergistic interaction between Flt3 ligand (FL) and IL-7 on uncommitted progenitors (Veiby et al., 1996), mice double deficient in FL and interleukin-7 receptor α (IL-7Rα) expression lack mature B cells and evidence for B cell commitment in fetal as well as adult hematopoiesis (Sitnicka et al., 2003).

Although having a lymphoid-dominated short-term reconstitution potential, LSK Flt3$^+$ cells possess, at the single-cell level, a combined myeloid (granulocyte-monocyte; GM) and lymphoid (B and T cell) differentiation potential (Adolfsson et al., 2001) and should, therefore, according to the current model for HSC lineage commitment (Reya et al., 2001), also possess a megakaryocyte (Mk) and erythroid (E) differentiation potential. However, in contrast to LSKCD34$^+$Flt3$^-$ ST-HSCs and megakaryocyte/erythroid progenitors (MkEPs), LSK Flt3$^+$ cells lack in vivo day 8 clonogenic colony-forming unit spleen (CFU-S$_{d8}$) activity (Yang et al., 2005), typical for reconstituting cells capable of rapidly reconstituting myelo-erythropoiesis following lethal irradiation (Akashi

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et al., 2000; Na Nakorn et al., 2002; Osawa et al., 1996; Till and McCulloch, 1961).

Since the CD45 (common leukocyte antigen) congenic mouse model typically used to assess HSC reconstitution and lineage potentials (Bryder and Jacobsen, 2000) prevents direct evaluation of Mk and E lineage potentials (as platelets and erythrocytes do not express CD45), we here applied a number of complimentary in vitro and in vivo approaches to directly evaluate the Mk and E potentials of LSK Flt3$^+$ cells. Herein, we provide compelling evidence for a novel route for early blood lineage commitment and development, in that LSK Flt3$^+$ cells, although sustaining a high proliferative and combined lympho-myeloid differentiation potential, have lost the ability to significantly adapt Mk and E lineage fates and in agreement with this downregulate expression of a number of genes critically involved in development of these lineages.

Results

Single LSK Flt3$^+$ Lympho-Myeloid Stem/Progenitor Cells Lack Significant In Vitro Megakaryocyte and Erythroid Differentiation Potentials

We recently demonstrated that LSK Flt3$^+$ BM cells, although predominantly and efficiently reconstituting lymphopoiesis in vivo, have a combined B, T, and myeloid differentiation potential (Adolfsson et al., 2001). Specifically, using clonal assays, 95% of single LSK Flt3$^+$ cells possessed an M, 85% G, and 45% a combined B and T cell potential, demonstrating that a large fraction of LSK Flt3$^+$ cells have a combined macrophage, granulocyte, B, and T cell potential (Adolfsson et al., 2001). Here, to directly confirm their lympho-myeloid potential, highly purified (>97%) LSK Flt3$^+$ cells (25% highest Flt3 expressing LSK cells; Figure 1A; Adolfsson et al., 2001) were investigated at the single cell level for their combined B cell and GM potential. As much as 67% of all single LSK Flt3$^+$ cells demonstrated sufficient clonal growth to be evaluated in this assay, of which 70% revealed a combined B and GM potential (Figure 1B).

We also investigated the B cell potential of LSK Flt3$^+$ cells on the OP9 cell line that efficiently supports B cell development (Vieira and Cumano, 2004). When cultured on OP9 for 14–28 days, all single LSK Flt3$^+$-derived clones (40% cloning efficiency) produced B220$^+$CD19$^+$ B cells (Figure 1C).

The recent development of the OP9 cell line expressing the notch ligand delta like1 (OP9-DL1) has dramatically enhanced the efficiency by which cells with T cell potential can be detected from uncommitted progenitors in vitro (Schmitt and Zuniga-Pflucker, 2002; Vieira and Cumano, 2004). When cultured on OP9-DL1, as much as 91% of LSK Flt3$^+$ single cell-derived clones (50% cloning efficiency) contained CD3ϵ-, CD4-, and CD8α-expressing cells (Figure 1C). Noteworthy, in clones investigated, we concomitantly to T cell development frequently also observed production of Mac-1$^+$ myeloid cells (data not shown). In further support of their T cell potential, we also investigated by PCR the single LSKflt3$^+$-derived clones on OP9-DL1 for expression of the early T cell-restricted genes *CD3ϵ* and *LcK*. Whereas uncultured LSK Flt3$^+$ cells and OP9-DL1 cells lacked detectable expression of these genes, as much as 15 out of 16 (94%) investigated single LSK Flt3$^+$ clones derived on OP9-DL1 expressed both of these genes (Figure 1D), demonstrating efficient commitment of LSK Flt3$^+$ cells along the T-lineage pathway. We also analyzed the recombination status at the *TCRβ* locus and found that 11 out of 12 (92%) analyzed LSK Flt3$^+$-derived clones showed DJ rearrangements. These were mainly D_2-J$\beta_{2.1}$ but other recombination events could be detected as well (Figure 1E). Thus, a majority of LSK Flt3$^+$ cells possess a combined granulocyte, monocyte, B, and T cell potentials.

The current model for hematopoietic development suggests that the first lineage commitment step of HSCs leads to a strict separation of myelopoiesis (GM and megakaryocyte/erythroid; Mk and E lineages) and lymphopoiesis (Akashi et al., 2000; Kondo et al., 1997; Reya et al., 2001). According to this model, LSK Flt3$^+$ cells having a combined B, T, and GM potential should also possess a Mk and E developmental potential. Since the Mk and E lineages upon differentiation cease to express the pan-hematopoietic marker CD45, typically used to track all other blood cell lineages in vivo (Bryder and Jacobsen, 2000), we here first compared the in vitro Mk potential of highly purified LSK Flt3$^+$ and LSK Flt3$^-$ cells containing LSKCD34$^-$Flt3$^-$ LT-HSCs but predominantly LSKCD34$^+$Flt3$^-$ ST-HSCs (Yang et al., 2005). Strikingly, whereas as much as 57% of single LSK Flt3$^-$ cells produced Mk in response to a cytokine combination that included thrombopoietin (TPO), the primary regulator of Mk production (Gurney et al., 1994), only 2% of purified LSK Flt3$^+$ cells produced Mk under the same conditions (KL + FL + TPO + IL-3; KFT3) (Figure 2A). Furthermore, whereas almost 40% of LSK Flt3$^-$ cells produced Mk also in the absence of TPO (KL + IL-3 + EPO + IL-11; K3E11), no Mk development was observed from LSK Flt3$^+$ cells (Figure 2A). Testing of other cytokine combinations and culture conditions efficiently promoting Mk development of LSK Flt3$^-$ cells also failed to promote significant Mk development from LSK Flt3$^+$ cells (J.A. and S.E.J., unpublished data). The inability of LSK Flt3$^+$ cells to generate Mk in vitro was not due to different lineage differentiation kinetics of LSK Flt3$^+$ and LSK Flt3$^-$ cells, as single LSK Flt3$^-$ cells at high frequencies efficiently produced Mk by 8 and 10 days (Figure 2B) as well as 12–15 days (Figure 2A) following initiation of culture, whereas few or no Mk were observed at any time point from LSK Flt3$^+$ cells.

The Mk and E lineages are developmentally closely linked and share a common progenitor (Akashi et al., 2000). Thus, experiments were next designed to also investigate the erythroid differentiation potential of LSK Flt3$^+$ cells in vitro. To pursue this, we developed a new efficient and highly specific assay for directly investigating the erythroid potential of uncommitted progenitor/stem cells at the single cell level (see the Experimental Procedures). Single LSK Flt3$^-$ and LSK Flt3$^+$ cells were cultured under conditions and in a cytokine combination (KFT3E) efficiently supporting myelo-erythroid development, and after 14–15 days, clonally derived cells were investigated by FACS for generation of erythroid (TER119$^+$Gr-1/Mac-1$^-$) progeny. As much as 53% of single LSK Flt3$^-$ cells generated TER119$^+$Gr-1/

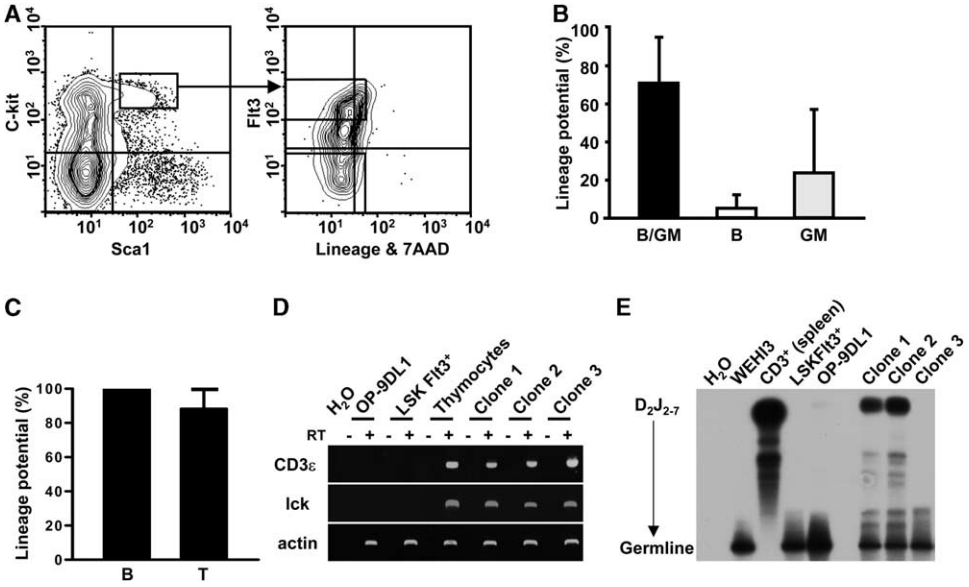

Figure 1. Combined Lympho-Myeloid Differentiation Potential of LSK Flt3+ Cells

(A) Sca-1+ BM cells expressing high levels of c-kit (left) were sorted into Lin−Flt3+ (with highest 25% Flt3 expression) and Lin−Flt3− populations as indicated (right).

(B) Frequencies of clonal single LSK Flt3+ cells demonstrating combined GM and B cell, B cell only, and GM potential only. Mean (SD) values of two experiments.

(C) B and T cell potential of single LSK Flt3+ cells evaluated after 14–28 days of culture on OP9 and OP9-DL1 stroma cells, respectively. Mean (SD) results from FACS analysis of a total of 34 clones on OP9 (from two experiments) and 104 clones grown on OP9-DL1 (from four experiments). Clones were picked at day 14–28 and analyzed by FACS. Clones, grown on OP9 stroma, which contained cells expressing both B220 and CD19 were considered to have B cell potential, and clones grown on OP9-DL1 stroma, if containing CD3ε-, CD4-, and CD8α-expressing cells were considered to have T cell potential. As much as 40% and 50% of LSK Flt3+ cells grown on OP9 and OP9-DL1, respectively, formed large enough clones to be analyzed.

(D) Ethidium bromide-stained agarose gel with PCR products of *ACTIN*, *LcK*, and *CD3ε* message from 1000 OP9-DL1 cells, 1000 uncultured LSK Flt3+ cells, thymocytes, or one twentieth of three representative (out of 16 analyzed) LSK Flt3+ single-cell clones derived on OP9-DL1.

(E) Autoradiogram of *TCRβ D-J2* rearrangements in WEHI-3 (myelomonocytic) cells, sorted CD3+ spleen cells, uncultured LSK Flt3+ cells, OP9-DL1 cells, and three representative (out of 12 analyzed) LSK Flt3+ clones derived on OP9-DL1.

Mac-1− erythroid progeny (Figures 2C and 2D). In contrast, whereas LSK Flt3+ cells efficiently generated myeloid (GM; Gr-1/Mac-1+TER119−) progeny, only 3% (one out of 34 cells investigated) produced erythroid progeny (Figures 2C and 2D). Thus, purified LSK Flt3+ cells lack (beyond the frequency of contaminating LSK Flt3− cells; 1%–3%; Experimental Procedures) signficant in vitro Mk and E differentiation potential.

LSK Flt3+ Lympho-Myeloid Stem/Progenitor Cells Lack Significant In Vivo Megakaryocyte and Erythroid Developmental Potential

The inability of most LSK Flt3+ cells to produce megakaryocytic and erythroid progeny in vitro could potentially be due to the utilized conditions not providing the optimal/unique signals required for Mk and E development from this stem/progenitor cell population. Thus, we next designed in vivo experiments to further investigate the Mk and E potentials of LSK Flt3+ cells. Since Mk progenitors, but not platelets or mature Mk, express the panhematopoietic marker CD45, we used the CD45 congenic mouse model (Bryder and Jacobsen, 2000) to compare the ability of transplanted LSKCD34+Flt3− ST-HSCs and LSK Flt3+ cells to generate Mk progenitors (CFU-Mk) following lethal myeloablation (Figure 3A). Importantly, complete myeloablation results in severe/lethal thrombocytopenia (Kempf et al., 1980; Uchida et al., 1998), thereby activating pathways promoting Mk development. One week following transplantation of 10,000 LSKCD34+Flt3− or LSK Flt3+ cells (CD45.1) in competition with 200,000 unfractionated BM cells (CD45.2), the spleens (and BMs; data not shown) of recipient mice (CD45.2) were highly and comparably reconstituted by (CD45.1+) LSK Flt3+ (mean reconstitution 77%) and LSKCD34+Flt3− (mean reconstitution 75%) cells. However, whereas more than 80 Mk progenitors were generated per transplanted LSKCD34+Flt3− cell at this time, Mk progenitors were almost undetectable among the cells derived from reconstituting LSK Flt3+ cells (Figure 3B), and mice transplanted with low numbers of LSKCD34+Flt3− cells, corresponding to the contamination within sorted LSK Flt3+ cells (1%–3% as determined by reanalysis), demonstrated that the very low numbers of Mk progenitors generated from purified LSK Flt3+ cells could be derived from contaminating LSKCD34+Flt3− cells (J.A. and S.E.J., unpublished data). In contrast, LSK Flt3+ cells produced GM progenitors following transplantation (>100 per transplanted cell), although less than LSK Flt3− cells (Figure 3C).

Experiments were next designed to also investigate

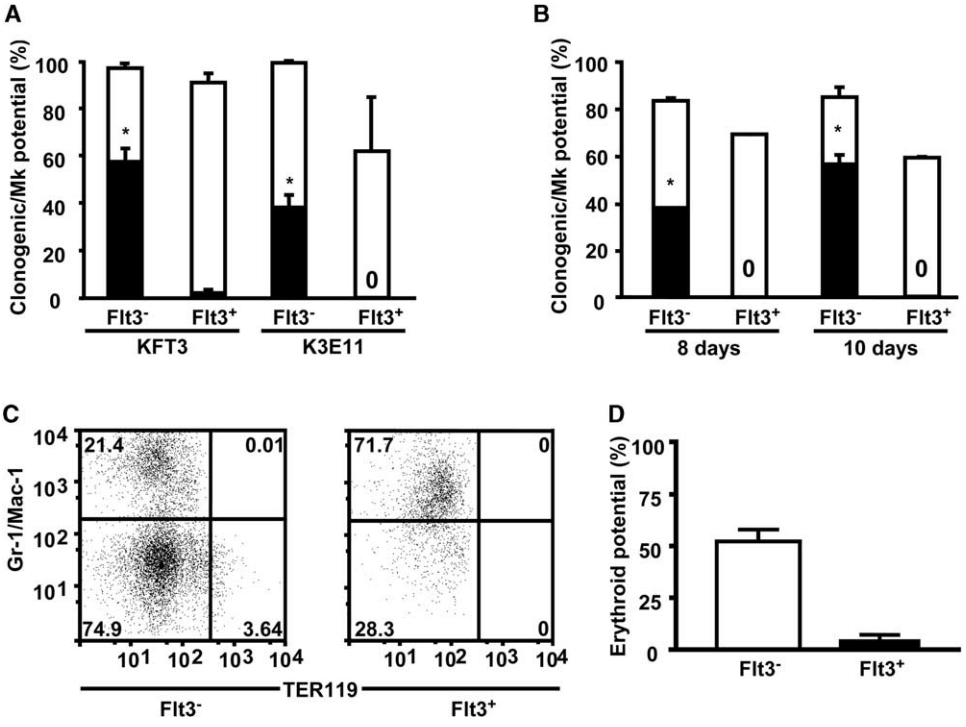

Figure 2. Lack of In Vitro Megakaryocyte and Erythroid Potential of LSK Flt3$^+$ Cells

(A) Single LSK Flt3$^+$ and LSK Flt3$^-$ cells were cultured for 12–15 days in the presence of the indicated cytokine combinations (Experimental Procedures). Bars show frequencies of LSK Flt3$^+$ and LSK Flt3$^-$ cells forming colonies (open bars) and Mks (filled bars). Results represent mean (SEM) values from 3–11 experiments.

(B) Clonal and Mk potential of single LSK Flt3$^+$ and LSK Flt3$^-$ cells cultured in KFT3 for 8 and 10 days. Mean (SD) values of two experiments. * $p < 0.05$, LSK Flt3$^+$ versus LSK Flt3$^-$ cells. 0 = no Mk formed.

(C) Representative profiles of TER119 and GR-1/Mac-1 expression on progeny of single LSK Flt3$^-$ (left) and LSK Flt3$^+$ (right) cells cultured for 14 days in the presence of KFT3E.

(D) Frequencies of single LSK Flt3$^-$ and LSK Flt3$^+$ cells producing erythroid (TER119$^+$Gr-1/Mac-1$^-$) progeny in vitro. Results represent mean (SD) values from 2 experiments, with a total of 30 and 34 clones, investigated for LSK Flt3$^-$ and LSK Flt3$^+$ cells, respectively.

the erythroid differentiation potential of LSK Flt3$^+$ cells in vivo, again taking advantage of lethally irradiated mice developing severe cytopenia and providing an environment permissive for E development. However, since mature erythrocytes lack expression of CD45, we utilized congenic mice expressing different hemoglobin isoforms as donors (Hbb^s) and recipients (Hbb^d) of LSK Flt3$^+$ and LSKCD34$^+$Flt3$^-$ cells (Figures 4A and 4B). Two

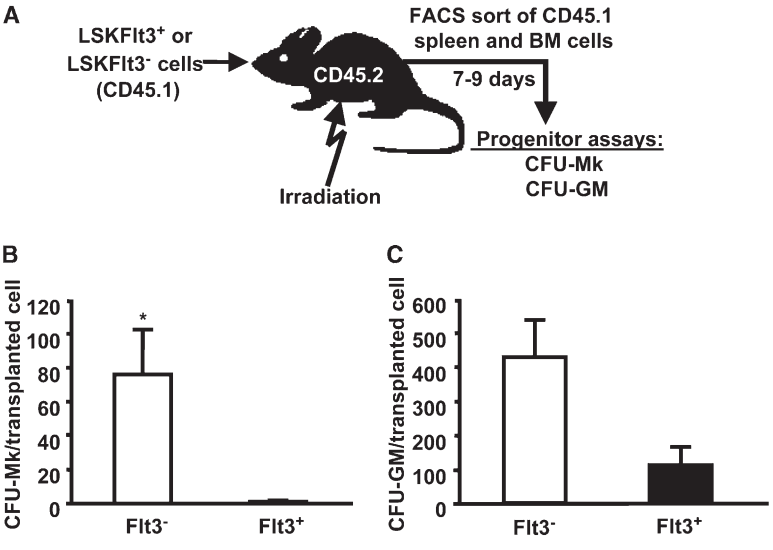

Figure 3. In Vivo Megakaryocyte and Granulocyte/Macrophage Potential of LSK Flt3$^+$ Cells

(A) Experimental design. Lethally irradiated (CD45.2) mice were transplanted with FACS-sorted LSK Flt3$^+$ or LSKCD34$^+$Flt3$^-$ (CD45.1) cells and congeneic (CD45.2) BM support cells. CD45.1$^+$ cells were sorted from spleen and BM 7–9 days posttransplantation and cultured in methylcellulose for an additional 8–12 days and scored for Mk (B) and GM (C) progenitors (CFU-Mk and CFU-GM, respectively). Mean (SEM) values of four experiments. * CFU-Mk $p < 0.05$, LSK Flt3$^+$ versus LSKCD34$^+$Flt3$^-$ cells. The difference in CFU-GM formation of LSK Flt3$^+$ and LSKCD34$^+$Flt3$^-$ cells did not reach statistical significance ($p = 0.11$).

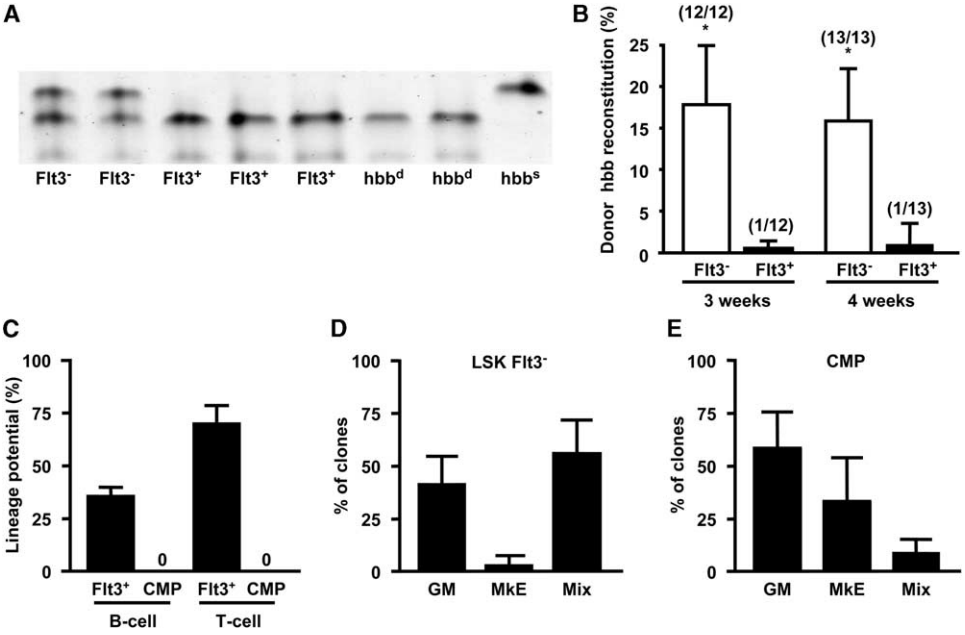

Figure 4. Myeloid Potentials of LSK Flt3+ Cells and CMPs

(A) Lethally irradiated (CD45.2, Hbb^d) mice were transplanted with 200 purified LSK Flt3+ or LSKCD34+Flt3− (CD45.1, Hbb^s) cells and 200,000 congeneic (CD45.2, Hbb^d) support BM cells. PB samples were analyzed at 3 and 4 weeks posttransplantation for recipient (Hbb^d) and donor (Hbb^s) hemoglobin reconstitution by electrophoresis. One representative example of electrophoresis gel with PB from two mice transplanted with 200 LSKCD34+Flt3− cells, three mice transplanted with 200 LSK Flt3+ cells, and three PB samples from nontransplanted mice of donor (Hbb^s) and recipient (Hbb^d) types.

(B) Mean (SD) values from two experiments with 12–13 mice in each group and time point. Numbers above bars show frequency of transplanted mice positive for donor-derived (Hbb^s) erythroid reconstitution. * $p < 0.05$, LSK Flt3+ versus LSKCD34+Flt3− cells.

(C) In vitro B cell (B220+CD19+) and T cell (CD4+CD8+) potential of single LSK CD34+Flt3+ cells and 10 Flt3− CMPs evaluated at 21–28 days of culture on OP9 and OP9-DL1 cell lines, respectively. Results are presented as mean (SD) percentages of single cells with B and T cell potential from three to five experiments. A total of 180 (OP9) and 300 (OP9-DL1) single LSK Flt3+ cells and 1440 (OP9) and 1440 (OP9-DL1) CMPs were investigated.

(D and E) Combined in vitro MkE and GM potential of single LSK Flt3− cells (D) and Flt3− CMPs (E). In each experiment, 360 to 600 single CMPs and LSK Flt3− cells were plated into conditions promoting both MkE and GM development. Results show mean percentages (SD) of analyzed clones containing GM or MkE lineages alone or in combination (Mix), from eight LSK Flt3− and five Flt3− CMPs experiments.

hundred LSK Flt3+ or LSKCD34+Flt3− (Hbb^s) cells were transplanted in competition with 200,000 BM cells (Hbb^d) into lethally irradiated recipients (Hbb^d). Three and four weeks posttransplantation, LSKCD34+Flt3− cells contributed substantially to E reconstitution in all (13/13) mice by as much as 18% and 16%, respectively, whereas only one of thirteen mice showed detectable E reconstitution from transplanted LSK Flt3+ cells (Figures 4A and 4B), although the contribution to other blood lineages (B, T, and myeloid cells) was as much as 14% ± 5% and comparable to that of LSKCD34+Flt3− cells (22% ± 14%). Thus, in contrast to LSKCD34+Flt3− cells, most LSK Flt3+ cells lack in vitro as well as in vivo Mk and E development potential.

Our finding that LSK Flt3+ cells sustain B, T, M, and G potential but lack significant Mk and E potential could be compatible with CMPs' (Akashi et al., 2000; Miyamoto et al., 2002) predominantly being derived from LSK Flt3− (or potentially LSK Flt3^{low}) cells that also have Mk and E potential. Thus, we next compared the combined GM and MkE potentials of single LSK Flt3− cells and CMPs. As recent studies had demonstrated that only the Flt3− fraction of CMPs is myeloid restricted (D'Amico and Wu, 2003), we purified Flt3− CMPs (Lin−IL-7Rα−Sca-1−c-kit+CD34+FcR−Flt3−; see Figure S1 in the Supplemental Data available with this article online). Importantly, no analyzed Flt3− CMPs generated B or T cells on OP9 (1440 cells total; n = 3) and OP9-DL1 (1440 cells total; n = 3), respectively (Figure 4C). Using culture conditions promoting both MkE and GM differentiation (Experimental Procedures and Figure 4), as much as 83% (n = 8) and 60% (n = 5) of single LSK Flt3− cells and Flt3− CMPs formed clones, respectively. Noteworthy, whereas 56% of analyzed LSK Flt3− clones had a mixed MkE and GM composition (Figure 4D), pure GM (59%) and MkE (33%) colonies dominated among Flt3− CMP clones analyzed, although 8% of Flt3− CMPs revealed a combined MkE and GM potential (Figure 4E). Another striking observation was that as much as 40% (n = 5) of all Flt3− CMP-derived clones represented small colonies (too small for cytospin analysis) composed exclusively of large megakaryocytes.

Downregulation of the Expression of Genes for Nonredundant Regulators of Megakaryocyte and Erythroid Development in LSK Flt3+ Cells

A number of cytokine receptors and transcription factors have been demonstrated to be critically involved in Mk and E development (Cantor and Orkin,

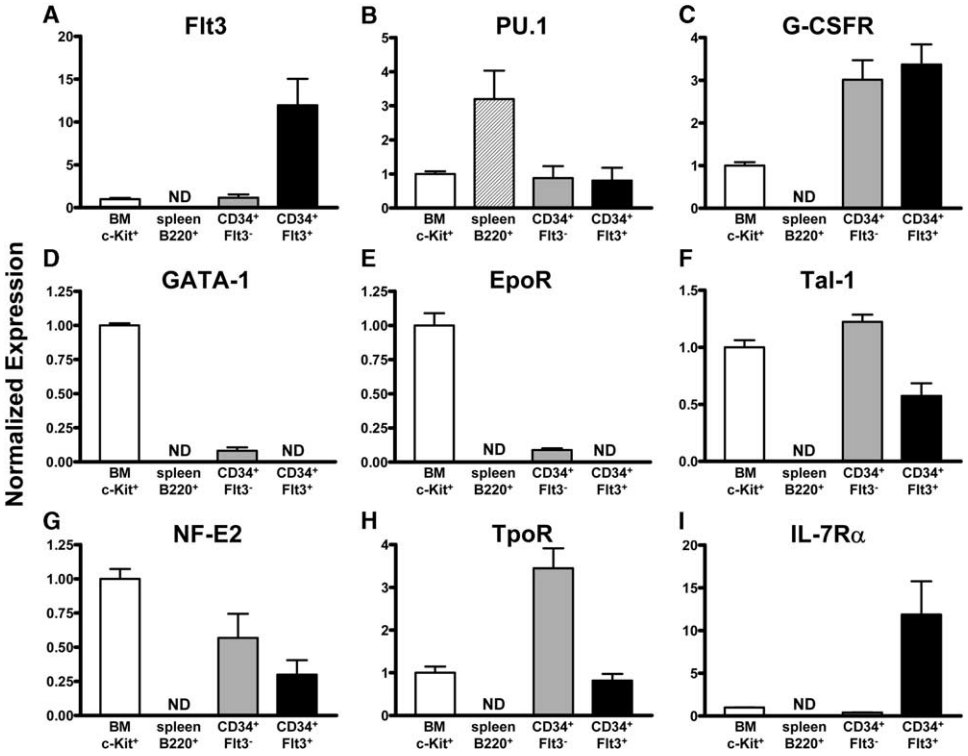

Figure 5. Downregulation of Essential Erythroid and Megakaryocyte Transcription Factors and Cytokine Receptor Genes and Upregulation of *IL-7Rα* mRNA Expression in LSK Flt3$^+$ Cells

Quantitative PCR data of indicated genes in FACS-sorted LSKCD34$^+$Flt3$^-$ and LSK Flt3$^+$ cells. Lin$^-$c-kit$^+$ BM cells (containing committed progenitor cells of Mk, E, GM, and B lineages), and B220$^+$ spleen cells (mature B cells) were used as controls for the PCR analysis, with the expression levels in Lin$^-$c-kit$^+$ cells set to 1. All data were normalized to the expression of *HPRT*. Results represent mean (SEM) values from at least two independent experiments, with PCR analysis in each experiment performed in triplicates. ND = no detectable expression after 45 cycles of PCR.

2002; Shivdasani and Orkin, 1996; Zhu and Emerson, 2002). In particular, Mk as well as E development are strictly dependent on the transcription factors *GATA-1* and *SCL/TAL-1* (Mikkola et al., 2003; Orkin et al., 1998; Shivdasani and Orkin, 1996). Importantly, the expression of these genes has not only been reported in E- and Mk-committed progenitors, but also in multipotent lympho-myeloid HSC populations with E and Mk potential, as has expression of the genes for the nonredundant hematopoietin receptors for Epo and Tpo (EpoR and TpoR, respectively) (Akashi et al., 2000; Miyamoto et al., 2000; Terskikh et al., 2003). Thus, any multipotent stem/progenitor cell population downstream of HSCs with E and Mk potential would be expected to express these genes. Using quantitative RT-PCR, we found in agreement with their sustained GM potential that LSK Flt3$^+$ as LSKCD34$^+$Flt3$^-$ ST-HSCs expressed high levels of mRNA for the transcription factor *PU.1* as well as the *G-CSFR* (Figures 5B and 5C). However, in support of their loss of E and Mk developmental potential, LSK Flt3$^+$ cells, in contrast to LSKCD34$^+$Flt3$^-$ cells, revealed no detectable gene expression of *GATA-1* and *EpoR* (Figures 5D and 5E) and had reduced expression of *SCL/TAL-1*, *NF-E2*, and *TpoR* (Figures 5F–5H). Most noteworthy, whereas LSKCD34$^+$Flt3$^-$ ST-HSCs as expected expressed little or no mRNA for *IL-7Rα,* the expression was clearly upregulated in LSK Flt3$^+$ cells (Figure 5I).

We also performed a multiplex single-cell RT-PCR analysis (Hu et al., 1997) to investigate whether LSK Flt3$^+$ cells that had turned on *IL-7Rα* expression might still represent multipotent progenitors with sustained myeloid potential and gene expression or rather more lymphoid-restricted progenitors. Whereas a majority of single LSKCD34$^+$Flt3$^-$ cells were found to express *G-CSFR* mRNA, none were *IL-7Rα*$^+$ (Figure 6A). In contrast, 9% of single LSK Flt3$^+$ cells were found to express detectable *IL-7Rα* mRNA (Figure 6B), and, noteworthy, the vast majority of these continued to coexpress the gene for the nonredundant myeloid *G-CSFR* (Figures 6B and 6C).

Thus, in contrast to upstream pluripotent LSKCD34$^+$ Flt3$^-$ ST-HSCs and downstream committed E and Mk progenitors, LSK Flt3$^+$ cells downregulate expression of transcription factors and cytokine receptors critically involved in development of the E and Mk lineages, sustain expression of GM-associated genes, and upregulate expression of the *IL-7Rα*, required for B and T cell development (Peschon et al., 1994).

Discussion

In the present studies, we provide compelling evidence for the existence in adult hematopoiesis of a prominent and potent lympho-myeloid stem/progenitor cell population within the LSK HSC compartment, which lacks

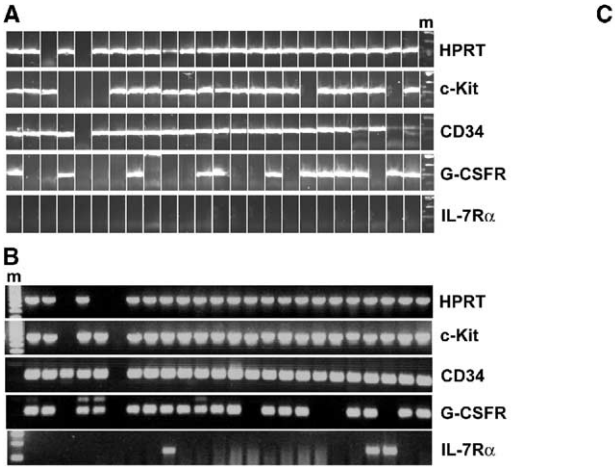

Figure 6. Multiplex Single-Cell RT-PCR Demonstrates Coexpression of *G-CSFR* and *IL-7Rα* in LSK Flt3+ Cells

Representative gel analysis data for expression of *HPRT*, *CD34*, *c-kit*, *G-CSFR*, and *IL-7Rα* in single LSKCD34+Flt3− cells (A) and LSK Flt3+ cells (B).

(C) Frequency (SD) of *G-CSFR* coexpression on *IL-7Rα*-expressing LSK Flt3+ cells. Only cells in which expression of *CD34* and *c-kit* as well as the housekeeping gene *HPRT* were detected were included for further analysis of *IL-7Rα* and *G-CSFR* expression. Mean (SD) values from two independent experiments, representing a total of three and two 96-well plates analyzed for LSKCD34+Flt3+ and LSKCD34+Flt3− cells, respectively. M = marker.

significant Mk and E developmental potential, contrary to what would be predicted from the classical and prevailing model for hematopoietic lineage commitment in adult hematopoiesis (Akashi et al., 2000; Kondo et al., 1997; Reya et al., 2001). That LSK(CD34+)Flt3+ cells (25% highest Flt3-expressing LSK cells), although sustaining a combined myeloid and lymphoid potential at the single cell level, have already undergone a first step of HSC lineage restriction through loss of Mk and E potential was substantiated by several lines of experimental evidence. First, in vitro and in vivo biological assays efficiently promoting Mk and E development from LSKCD34+Flt3− HSCs, including myeloablative conditioning activating endogenous pathways for Mk and E production, failed to promote significant development of these lineages from LSK Flt3+ cells beyond what could be expected from sorting impurities (1%–3%). Furthermore, in contrast to the LSKCD34+Flt3− ST-HSCs, LSK Flt3+ cells lacked detectable mRNA expression for *GATA-1* and *EpoR* and downregulated gene expression for *SCL/TAL1*, *NF-E2*, and *TpoR*. This combined with the fact that not only committed MkEPs (Akashi et al., 2000; Miyamoto et al., 2002) but also upstream HSCs (in agreement with previous studies; Miyamoto et al., 2002) express the genes for critical regulators of Mk and E development strongly support that LSK Flt3+ cells have undergone an irreversible lineage commitment step through downregulation of Mk and E development programs. That the first HSC lineage restriction step might involve simultaneous loss of Mk and E potentials is not so surprising in light of Mk and E lineages sharing a common progenitor, MkEPs (Akashi et al., 2000), as well as critical regulators of lineage development such as GATA-1 and SCL/TAL-1 (Cantor and Orkin, 2002; Mikkola et al., 2003; Shivdasani and Orkin, 1996). In contrast and in agreement with their sustained G and M potentials, LSK Flt3+ cells maintained similar expression levels of *PU.1* and *G-CSFR* as LSK Flt3− HSCs.

Contrary to the current model for hematopoietic development (Figure 7A), our findings do not support that the earliest lineage restriction or commitment event of adult HSCs results exclusively in a strict separation into common lymphoid and common myeloid differentiation pathways. The generation of LSK Flt3+ cells with G, M, B, and T cell, but little or no Mk and E potentials, would be more compatible with a model in which MkEPs and LSK Flt3+ cells are primarily generated through asymmetric cell divisions of LSKCD34+Flt3−/low HSCs (Figure 7B; Yang et al., 2005). A similar model has in fact previously been proposed based on a hierarchy of transcription factors, with PU.1-deficient HSCs giving rise to Mk and E progenitors, but not progenitors of the G, M, B, and T cell lineages (Singh, 1996). Recent studies of paired daughter cells of purified HSCs also support that MkEPs might develop from HSCs without a CMP intermediate (Takano et al., 2004). Furthermore, such a model is also compatible with the observation of myeloid (erythroid- and macrophage-like cells) emerging earlier in evolution (Hansen and Zapata, 1998) and ontogeny (Cumano and Godin, 2001) than lymphoid cells.

Alternative and more complex models of HSC lineage commitment are needed to reconcile the evidence for CMPs (Akashi et al., 2000; Miyamoto et al., 2002) and our findings of LSK Flt3+ lymphoid-primed multipotent progenitors (LMPPs). In one such model, LSKCD34+Flt3−/low ST-HSCs could upon asymmetrical cell divisions give rise to CMPs and LMPPs (Figure 7C). Importantly, our studies support the existence of myeloid-restricted CMPs with a combined MkE and GM potential (Akashi et al., 2000). However, when compared to LSK Flt3−, cells very few Flt3− CMPs were found to give rise to mixed MkE and GM colonies, and in agreement with recent studies (Nutt et al., 2005), the Lin−IL-7Rα−Sca-1−c-kit+CD34+FcR−Flt3− candidate CMP population gave rise to primarily pure GM or MkE colonies. Thus, although Flt3− CMPs represent one pathway for generation of Mk, E, and GM progenitors, it might not represent an obligate or even dominating intermediate for myeloid development from HSCs. Rather than a strict hierarchical model, our findings might be equally compatible with a commitment process occurring on a probabilistic or stochastic basis (Nakahata et al., 1982; Siminovitch et al., 1963) in which the probability of different commitment fates can be altered by intrinsic and extrinsic cues, differentially expressed within the LSK HSC hierarchy. If so, LSKFlt3+/high cells might potentially possess some residual MkE potential, although our findings of little or no MkE development in the utilized assays, would suggest that such a potential must be very restricted.

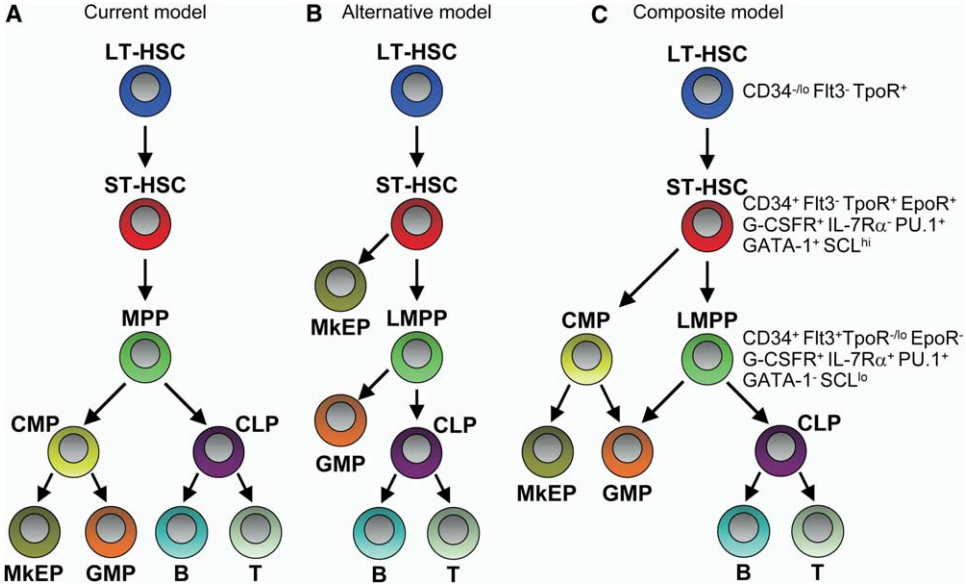

Figure 7. Current and Alternative Models for Hematopoietic Stem Cell and Blood Lineage Commitment

(A) Current model (Reya et al., 2001) for hematopoietic lineage commitment and development, postulating that the first lineage commitment step of HSCs results in a strict separation of myleopoiesis and lymphopoiesis as supported through the identification of CMPs and CLPs, respectively (Akashi et al., 2000; Kondo et al., 1997).

(B) Alternative model, based on the present studies, in which a pluripotent HSC upon loss of Mk and E potential develops into a lymphoid primed multipotent progenitor (LMPP) that upon loss of GM potential generates the CLP (Adolfsson et al., 2001).

(C) Composite model, incorporating the experimental evidence for models (A) and (B). Also shown are expression of key genes in ST-HSCs and LMPPs based on Q-PCR data.

LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; MPP, multipotent progenitor; LMPP, lymphoid-primed multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/macrophage pronenitor; MkEP, megakaryocyte/erythroid progenitor; B, B cell; T, T cell.

Our findings are particularly intriguing in light of recent gene profiling studies of HSCs (with full myeloid and lymphoid lineage differentiation potentials), revealing wide expression of myeloid (MkE and GM) but not lymphoid gene programs (Delassus et al., 1999; Miyamoto et al., 2002). Combined with our finding, this observation strongly supports that HSCs might initially be primed to undergo myeloid (Mk, E, and subsequently GM) commitment (Figure 7B) and that lymphoid commitment through an LSK Flt3$^+$ LMPP stage depends on subsequent activation of lymphoid genes. In that regard, it is noteworthy that LSK Flt3$^+$ cells upregulate gene expression for *FLT3* and *IL-7R*α, the two cytokine receptors critically involved in lymphoid and B cell commitment (Peschon et al., 1994; Sitnicka et al., 2002). Furthermore, in agreement with their sustained G and M potential, multiplex single cell PCR analysis demonstrates that *IL-7R*α^+ LSK Flt3$^+$ cells also sustain expression of the *G-CSFR*.

In fetal (but not adult) hematopoiesis, a number of previous observations have implicated the potential existence of lympho-myeloid lineage-restricted progenitors, primarily with a combined B cell, monocyte (Cumano et al., 1992; Mebius et al., 2001), and in some cases also T cell (Lacaud et al., 1998; Lu et al., 2002) potential. However, as emphasized by others (Ema and Nakauchi, 2003; Katsura, 2002), definitive conclusions have been difficult to reach based on these studies, since they have been limited by the fact that the candidate progenitors in question have not been prospectively purified and characterized and therefore present at low frequencies and assayed along with other progenitors showing additional lineage potentials in the utilized assays. Thus, under such conditions it has not been possible to conclude whether or not progenitors showing restricted lineage development are in fact lineage restricted or whether it rather reflects the inability of the utilized assays to efficiently support the full lineage potentials of all progenitors investigated. For instance, the granulocyte potential of a multipotent progenitor might go unnoticed if investigated at the wrong time or under suboptimal conditions, as granulocytes are very short lived in vitro, whereas monocytes accumulate with time in such cultures. Thus, to prove the existence of other lineage-restricted progenitors, prospective purification must be combined with characterization of their lineage potentials in efficient in vitro and in vivo assays for all lineage potentials, as demonstrated for LSK Flt3$^+$ cells in the present studies. Prospective purification of such populations will also be required to obtain meaningful genetic information regarding the genetic determinants of lineage commitment.

In conclusion, we have in the adult mouse bone marrow LSK HSC compartment identified a prominent LSK Flt3$^+$ LMPP, which in contrast to true ST- and LT-HSCs lacks significant Mk and E potential but sustains other blood lineage developmental potentials. This repre-

sents the earliest known lineage commitment/restriction step of HSCs, leading us to propose that an alternative or complimentary road map to that of the classical hematopoietic hierarchy must guide adult blood cell lineage commitment and development. Its exact identification should greatly facilitate delineation of the molecular pathways regulating these and other HSC fate decisions.

Experimental Procedures

Mice

Wild-type C57BL/6(Hbb^s) mice were from M&B (Ry, Denmark) and C57BL/6-Hbb^d mice from Jackson Laboratories (Maine). All mice procedures were performed with consent from the local ethics committee at Lund University.

Hematopoietic Growth Factors

All cytokines were used at predetermined optimal concentrations, and all human cytokines utilized have been shown to be fully cross-reactive with mouse cells. For detailed information on cytokines see the Supplemental Experimental Procedures.

FACS Purification of Subpopulations of Lin$^-$Sca-1$^+$c-kit^{hi} Bone Marrow Cells and CMPs

All sorts were performed by immunomagnetic-based preenrichment followed by multicolor flow cytometric sorting as previously described (Adolfsson et al., 2001; Bryder and Jacobsen, 2000). Briefly, bone marrow cells were incubated in a cocktail of predetermined optimal concentrations of lineage (Lin) antibodies: purified RA3-6B2 (B220), RB6-8C5 (Gr-1), M1/70 (Mac-1), 53-6.7 (CD8), 53-7 (CD5), H129.19 (CD4), and Ter119, all from PharMingen (San Diego, California). Lin$^+$ cells were then depleted using sheep anti-rat IgG (Fc)-conjugated immunomagnetic beads (Dynal, Oslo, Norway). Lineage-negative/low (Lin$^{-/lo}$) cells were incubated with CyChrome-conjugated goat anti-rat antibody (Caltag Laboratories, Burlingame, California) and subsequently stained with FITC-conjugated rat anti-mouse E13-161.7 antibody (Sca-1), APC-conjugated rat anti-mouse 2B8 (c-kit), and PE- or biotin-conjugated rat anti-mouse Flt3 (A2F10.1) plus streptavidin-PE or isotype-matched control antibodies (all PharMingen). Cells were also stained with 7-amino actinomycin (7-AAD; Sigma-Aldrich Co., St. Louis, Missouri) to exclude dead cells. To exclude long-term stem cells (Osawa et al., 1996), Lin$^{-/lo}$ cells were in some experiments stained with FITC-conjugated rat anti-mouse RAM-34 antibody (CD34), APC-conjugated rat anti-mouse 2B8 (c-kit), PE-conjugated rat anti-mouse Flt3 (A2F10.1), and biotin-conjugated E13-161.7 (Sca-1) plus streptavidin-Tricolor (Caltag). For isolation of Flt3$^-$ CMPs (Akashi et al., 2000; D'Amico and Wu, 2003), Lin$^-$ BM cells were isolated and remaining Lin$^+$ cells visualized as described above. Cells were subsequently stained with 7-AAD, FITC-conjugated rat anti-mouse RAM34 (CD34), PE-conjugated rat anti-mouse 2.4G2 (CD16/32; FcγR), Tricolor-conjugated rat anti-mouse D7 (eBiosciences, San Diego, California; Sca-1) and Tricolor rat anti-mouse A7R34 (IL7Rα; eBiosciences), biotin-conjugated rat anti-mouse A2F10.1 (Flt3) plus streptavidin-PE-Texas-Red (Caltag), or isotype-matched control antibodies (all PharMingen unless otherwise indicated). To obtain high purity (reproducibly 97%–99% upon reanalysis) Lin$^{-/lo}$Sca-1$^+$c-kit^{hi}(LSK)Flt3$^{+/-}$ and Lin$^-$Sca-1$^-$c-kit$^+$IL7Rα$^-$FcγR^{lo}CD34$^+$Flt3$^-$ CMPs were sorted twice on a FACSVantage or FACSDiva Cell Sorter (Becton Dickinson).

Single-Cell Assay for Combined B Cell and Granulocyte/Macrophage Potential

LSK Flt3$^+$ cells were seeded in Terasaki plates at a concentration of one cell per well in 20 μl serum-free medium (X-vivo15; BioWhittaker, Walkersville, Maryland), supplemented with 1% detoxified Bovine Serum Albumin (StemCell Technologies Inc., Vancouver, Canada). Cells were cultured in kit ligand (KL) + Flt3 ligand (FL) + IL-7 for 6 days, at which time proliferating clones were divided, with one part of the cells continuing in KL + FL + IL-7 for an additional 9 days to establish their B cell potential through evaluation of B220$^+$CD19$^+$ cells produced in cultures (Veiby et al., 1996). The other part of the cells were switched to culture conditions promoting GM development (IMDM, BioWhittaker; supplemented with 20% FCS, BioWhittaker), and a cytokine cocktail composed of KL + FL + IL-3 + thrombopoietin (TPO) + granulocyte colony-stimulating factor (G-CSF) + granulocyte-macrophage colony-stimulating factor (GM-CSF) for an additional 9 days. GM potential was established by transferring individual colonies to slides in a cytospin centrifuge and examining cells morphologically after May-Grünwald/Giemsa-staining.

Clonogenic B and T Cell Assays on OP9 and OP9-Delta Like1 (OP9-DL1) Cell Lines

OP9 and OP9-DL1 cell lines (kindly provided by Drs. A. Cumano, Paris, and J.C. Zuniga Pflucker, Toronto) were maintained as described (Schmitt and Zuniga-Pflucker, 2002; Vieira and Cumano, 2004) in OptiMEM with L-Glutamine (Invitrogen Corporation, Carlsbad, California) supplemented with 10% FCS (Gibco Paisley, Scotland). Cell lines were trypsinized and prepared at a density of 2 × 10^4 cells/ml. Single LSK Flt3$^+$ cells and CMPs were deposited into wells containing OP9 or OP9-DL1 cell lines (supplemented with FL 50 ng/ml and IL-7 100 ng/ml in OP9 and FL 50 ng/ml in OP-DL1 cocultures and in some experiments with 25 ng/ml KL) by a single cell depositor unit coupled to a FACSDiva (Becton Dickinson), providing single cells in >98% of the wells and no wells with more than 1 cell. Clones were identified and picked at days 14, 21, and 28 and analyzed by FACS for presence of B cell (defined as B220$^+$CD19$^+$) and T cell (defined as CD3ε$^+$, CD4$^+$, and CD8α$^+$) committed progeny.

Reverse transcriptase and Polymerase chain reaction analysis of the clones generated on OP9-DL1 was preformed as described in the Supplemental Experimental Procedures.

Evaluation of In Vitro Megakaryocyte Potential of Single LSK Flt3$^{+/-}$ Cells

LSK Flt3$^+$ and LSK Flt3$^-$ cells were seeded in Terasaki plates at a concentration of one cell per well in 20 μl serum-free medium (as above) supplemented with KL + FL + TPO + IL-3 (KFT3) or KL + IL-3 + erythropoietin (EPO) + IL-11 (K3E11). Wells were scored for cell growth at different time points. Mk-containing colonies were identified by light microscopy and confirmed morphologically after transferring individual colonies to slides in a cytospin centrifuge and subsequent May-Grünwald/Giemsa-staining.

In Vitro Erythroid Potential Assay

Single LSK Flt3$^-$ and LSK Flt3$^+$ cells were seeded in Terasaki plates in 20 μl IMDM supplemented with 1% BIT 9500 (StemCell Technologies) and KL (50 ng/ml), FL (50 ng/ml), TPO (50 ng/ml), IL-3 (10 ng/ml), and EPO (5 U/ml). After 14–15 days of culture, clones were picked and stained with PE-conjugated rat anti-mouse TER119 and APC-conjugated rat anti-mouse Gr-1 (RB6-8C5) and Mac-1 (M1/70) (all from Becton Dickinson) as well as 7-AAD. Erythroid potential was defined by the presence of TER119$^+$Gr-1/Mac-1$^-$ cells. Culture-derived TER119$^+$Gr-1/Mac-1$^-$ cells were also sorted with a FACSDiva and subsequently stained with May-Grünwald/Giemsa to confirm erythroid identity by morphology (data not shown).

In Vitro Combined Myeloid Potentials of CMPs

LSK Flt3$^-$ cells and CMPs were deposited as single cells (using a single cell depositor; see above) into Terasaki plates in 20 μl medium (X-vivo15) supplemented with 10% FCS (Gibco) and 0.5% BSA, 0.1mM β-mercaptoethanol and cytokines, KL + FL + TPO (50 ng/ml each) + IL-3 (10 ng/ml) + EPO (5 U/ml), KL + FL + TPO + G-CSF (all 50 ng/ml) + IL-3 (10 ng/ml) + EPO (3 U/ml), or KL + FL + TPO + G-CSF (all 50 ng/ml) + IL-3 (10 ng/ml) + GM-CSF (20 ng/ml) + EPO (5 U/ml), all giving similar results. After initial optimization of growth conditions and kinetics, cell growth and multilineage (Mk, E, G, and M) differentiation (after May-Grünwald/Giemsa staining of clones containing over 100 cells) were established at day 13–14 for LSK Flt3$^-$ cells and at day 6–7 for CMPs.

In Vivo Megakaryocte and Granulocyte/Macrophage Potential Assay

Ten thousand LSK Flt3$^+$ or LSK Flt3$^-$ cells freshly isolated from C57BL/6 mice (CD45.1) were injected intravenously to lethally irradiated (975 cGy) C57BL/6 mice (CD45.2), along with 200,000 unfractionated congeneic (CD45.2) BM cells providing a competitor and survival population. Seven to nine days posttransplantation, spleen and BM cells were stained with CD45.1, CD45.2, and 7-AAD. LSK Flt3$^{+/-}$-derived (CD45.1$^+$CD45.2$^-$7-AAD$^-$) cells were sorted on a FACSDiva and evaluated for Mk progenitor potential in serum-free methylcellulose (M3226; StemCell Technologies Inc.) supplemented with KL + TPO and for colony forming unit-granylocyte/macrophage (CFU-GM) in FCS containing methylcellulose (M3134; StemCell Technologies Inc.) supplemented with KL + TPO + FL + IL-3 + G-CSF + GM-CSF. GM and Mk colony formation was evaluated following 8–12 days of in vitro incubation.

In Vivo Erythroid Potential Assay

Two hundred FACS-sorted LSK Flt3$^+$ or LSKCD34$^+$Flt3$^-$ cells from C57BL/6-Hbb^s (CD45.1) mice were transplanted into lethally irradiated C57BL/6-Hbb^d (CD45.2) mice, along with 200,000 unfractionated congeneic (C57BL/6-Hbb^d, CD45.2) BM cells. At 3 and 4 weeks after transplantation, peripheral blood (PB) was analyzed for Hbb^s and Hbb^d reconstitution (Harrison et al., 1988; Whitney, 1978). Briefly, PB was washed twice with 3 ml PBS (PAA Laboratories GmbH, Linz, Austria). Twenty microliters packed red blood cells were lysed and cystamine modified with 150 μl cystamine lyse solution for 30 min. The different Hbb isoforms were separated and visualized using a hemoglobin electrophoresis kit (P/N 4411780; Beckman Coulter Inc., Fullerton, California). Gel-pro analyser version 2.0 (Media Cybernetics, Silver Springs, Massachusetts) software was used for quantification of percentage hemoglobin reconstitution. In each experiment, PB from nontransplanted C57BL/6-Hbb^d (CD45.2) mice were used as a negative control and PB from nontransplanted C57BL/6-Hbb^s (CD45.1) mice as a positive control. The mean background of the electrophoresis assay was 4% (SD = 1%; n = 7).

Quantitative RT-PCR

LSKCD34$^+$Flt3$^{-/+}$ cells were FACSDiva sorted directly into 75 μl of buffer-RLT and frozen at −80°C. RNA extraction and DNase treatment was performed with the RNeasy Micro kit (Qiagen Inc., California) according to manufacturers' instructions for samples containing $\leq 10^5$ cells. Eluted RNA samples were reverse transcribed using SuperScript II and random hexamers (Invitrogen) according to protocol supplied by the manufacturer. Newly synthesized cDNA was diluted to approximately contain cDNA from 30 cells/μl and frozen at −20°C. Q-PCR reactions were performed by mixing 2× TaqMan universal PCR master mix, 20× Assays-on-Demand (primer/MGB-probe mix), RNase-free H20, and 5μl of cDNA for a final reaction volume of 25 μl. TaqMan Assays-on-Demand probes used are described in the Supplemental Experimental Procedures. All experiments were performed in triplicates, and differences in cDNA input were compensated by normalizing against HPRT expression levels.

Analysis of Single Cells by Reverse Transcriptase-Polymerase Chain Reaction

Multiplex single-cell RT-PCR analysis of LSK Flt3$^+$ and LSKCD34$^+$Flt3$^-$ cells was performed according to the methods described by Hu et al. (Hu et al., 1997). Single cells were deposited by a single-cell depositor coupled to a FACSDiva as described above (>98% of the wells contained single cells), into 96-well PCR plates containing 4 μl lysis buffer (0.4% Nonidet P-40, 65 μmol/l dNTPs, 25 μmol/l dithiothreitol, and 0.5 U/μl RNaseOUT (Invitrogene Corporation, California). Cell lysates were reverse transcribed using multiple (up to 6) pairs of gene-specific reverse primers and 50 U MMLV-RT per reaction in the buffer provided by the supplier (Invitrogene Corporation, California). The first-round PCR with 35 cycles was performed by addition of 40 μl PCR buffer and 1.25 U Taq polymerase (TaKaRa Bio Inc., Shiga, Japan). One microliter aliquots of first-round PCRs were further amplified using fully nested gene-specific primers. Aliquots of second-round PCR products were subjected to gel electrophoresis and visualized by ethidium bromide staining on ordinary agarose gels or E-gels (Invitrogene). The sequences for the external and internal oligonucleotide primers for *HPRT*, *CD34*, *c-kit*, and *G-CSFR* were kindly provided by Professor Tariq Enver, Weatherall Institute, Oxford, England. The external primer sequences used are described in the Supplemental Experimental Procedures. Empty wells were used as negative controls and never showed any signals for any of the investigated genes.

Statistics

The statistical significance of difference between groups was determined using the two-tailed paired Student's t test. For the progenitor data, the Student's t test was performed after log transformation of the data.

Supplemental Data

Supplemental Data include one figure and Supplemental Experimental Procedures and can be found with this article online http://www.cell.com/cgi/content/full/121/2/295/DC1/.

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