

FLT3 receptor and ligand are dispensable for maintenance and posttransplantation expansion of mouse hematopoietic stem cells

Natalija Buza-Vidas,^{1,2} Min Cheng,¹ Sara Duarte,^{1,2} Hojjatollah Nozad Charoudeh,¹ *Sten Eirik W. Jacobsen,^{1,2} and *Ewa Sitnicka¹

¹Hematopoietic Stem Cell Laboratory, Lund Strategic Research Center for Stem Cell Biology and Cell Therapy, Lund University, Lund, Sweden; and

²Haematopoietic Stem Cell Laboratory, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Headington, United Kingdom

Originally cloned from hematopoietic stem cell (HSC) populations and its ligand being extensively used to promote ex vivo HSC expansion, the FMS-like tyrosine kinase 3 (FLT3; also called FLK2) receptor and its ligand (FL) were expected to emerge as an important physiologic regulator of HSC maintenance and expansion. However, the role of FLT3 receptor and ligand in HSC regulation remains unclear and disputed. Herein,

using *Fl*-deficient mice, we establish for the first time that HSC expansion in fetal liver and after transplantation is FL independent. Because previous findings in *Flik2*^{-/-} mice were compatible with an important role of FLT3 receptor in HSC regulation and because alternative ligands might potentially interact directly or indirectly with FLT3 receptor, we here also characterized HSCs in *Flik2*^{-/-} mice. Advanced phenotypic as well as func-

tional evaluation of *Flik2*^{-/-} HSCs showed that the FLT3 receptor is dispensable for HSC steady-state maintenance and expansion after transplantation. Taken together, these studies show that the FLT3 receptor and ligand are not critical regulators of mouse HSCs, neither in steady state nor during fetal or posttransplantation expansion. (Blood. 2009;113:3453-3460)

Introduction

Although hematopoietic stem cells (HSCs) are mostly quiescent and sustained at relatively constant numbers in steady-state adult hematopoiesis, mechanisms are in place to promote extensive HSC expansion during fetal development and after transplantation of limiting HSC numbers into myeloablated recipients.¹⁻³ Extracellular cues are thought to be involved in HSC regulation, as shown for the hematopoietin receptor c-mpl and its ligand thrombopoietin and the cytokine tyrosine kinase receptor C-KIT and its ligand.⁴⁻⁹

Another cytokine tyrosine kinase receptor, the FMS-like tyrosine kinase 3 (FLT3; also termed FLK2), having originally been cloned from highly enriched HSC populations,¹⁰ was also expected to emerge as a key HSC regulator. However, despite extensive investigations, it remains unclear as to what degree FLT3 and its ligand (FL) are involved in regulation of HSCs, whereas its role in the earliest stages of lymphoid-primed multipotent progenitors (LMPPs) and lymphoid development is well established.¹¹⁻¹⁹

Previous studies showed that FL-deficient (*Fl*^{-/-}) mice have normal HSC numbers in adult steady-state bone marrow (BM),¹³ suggesting that FL has little or no role in regulating steady-state HSC numbers. However, those studies did not preclude or investigate a role of FL in regulating HSCs during development and in regenerating BM, when they expand extensively.^{2,3} In fact, previous studies suggested that the mouse FLT3 receptor is up-regulated on cycling HSCs in fetal liver and adult BM,²⁰⁻²² and yet other studies provided strong evidence in support of FL promoting in vitro expansion of HSCs.^{15,22-26} Although those studies pointed to potential stimulatory effects of FL on cycling HSCs, they did not establish (or explore) a potential physiologic role of FL in

regulation of HSC expansion, such as during development and after transplantation.

In studies of FLK2 receptor-deficient (*Flik2*^{-/-}) mice, Mackaretschian et al¹¹ reported a multilineage reconstitution defect of *Flik2*^{-/-} BM cells after transplantation, a finding compatible with a deficiency of *Flik2*^{-/-} HSCs. However, whether the observed deficiency in generation of mature blood cells of multiple lineages reflected a role in HSC maintenance, in progenitor regeneration and differentiation, or in both could not be established. Further, because the *FLK2* deficiency is cell intrinsic, it could affect not only steady-state HSC maintenance but potentially also their posttransplantation expansion, maintenance, or differentiation.

The seeming discrepancy between the apparent HSC phenotype in FLT3 receptor (*Flik2*^{-/-}), but not FLT3 ligand-deficient mice,^{11,13} could potentially also be explained by other (yet to be identified) ligands that could directly bind to and activate the FLT3 receptor in *Fl*^{-/-} mice. Alternatively, the FLT3 receptor in *Fl*^{-/-} mice could potentially be transactivated by other signaling pathways or ligands that would not bind to the FLT3 receptor. In support of such a possibility, evidence has recently been provided for such transactivation as an explanation for the more severe lymphoid phenotype in IL-7 receptor- than IL-7 ligand-deficient mice.²⁷

Resolving the conflicting results with regard to a potential role of FLT3 receptor and ligand in regulation of mouse HSCs has considerable implications for normal and leukemic hematopoiesis in man. Several studies have suggested that FLT3 is expressed on candidate normal human HSCs,²⁸⁻³⁰ and greater than 30% of acute myeloid leukemias (AMLs) have activating mutations in the FLT3 receptor.^{31,32} Thus, it is important to establish the normal cellular

Submitted August 12, 2008; accepted December 19, 2008. Prepublished online as *Blood* First Edition paper, February 2, 2009; DOI 10.1182/blood-2008-08-174060.

*S.E.W.J. and E.S. contributed equally to this study.

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 USC section 1734.

© 2009 by The American Society of Hematology

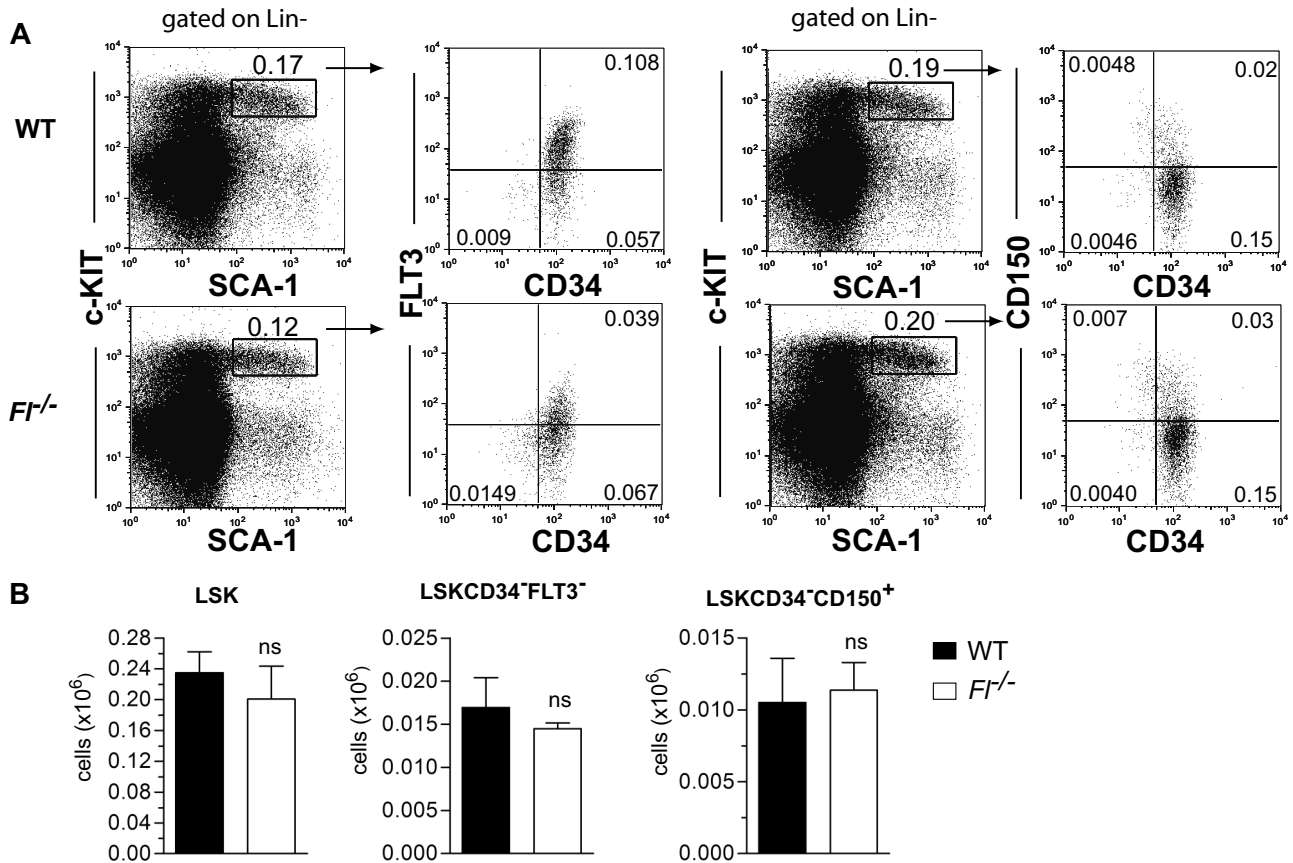


Figure 1. FLT3 ligand is dispensable for steady-state maintenance of adult HSCs. (A) Representative fluorescence-activated cell sorting (FACS) plots for analysis of LSKCD34⁻FLT3⁻ and LSKCD34⁻CD150⁺ cells in 9- to 10-week-old WT and *Fl*^{-/-} mice. Numbers indicate frequencies of populations within indicated gates or quadrants of total BM cells. Cells have first been gated on Lin⁻ cells as indicated. (B) Mean (SEM) numbers of LSK, LSKCD34⁺FLT3⁻, and LSKCD34⁺CD150⁺ cells in BM (per 2 tibiae and 2 femora) of 9- to 10-week-old WT and *Fl*^{-/-} mice (n = 5 per genotype). ns indicates not significant.

targets of FLT3 mutations, because, if these include HSCs, it might affect not only the biology but also the therapeutic targeting and resistance of FLT3-mutated leukemic clones.³³

Herein, to establish whether FL plays an important role in regulation of the HSC expansion that takes place during normal development and after transplantation,^{2,3} we used distinguishable *Fl*^{-/-} HSC donor and recipient mice. Through this approach we could establish that *Fl* deficiency does not significantly affect engraftment or fetal or posttransplantation expansion of mouse HSCs. Further, HSC numbers were also not affected in *Flk2*^{-/-} mice, neither in steady state nor after transplantation, supporting that neither the FLT3 ligand nor receptor is critically involved in regulation of HSCs in mice.

Methods

Animals

Fl^{-/-} mice on a pure C57BL/6 (CD45.2) background were previously described.¹² *Fl*^{-/-} CD45.1 mice were obtained by backcrossing *Fl*^{-/-} CD45.2 with wild-type (WT) C57BL/6 CD45.1 mice. *Flk2*^{-/-} mice were generated as previously reported¹¹ and backcrossed with C57BL/6 (CD45.2) WT mice for 9 generations. All offspring from *Fl*^{-/-} and *Flk2*^{-/-} breeders used in these studies were genotyped to establish homozygosity for *Fl*^{-/-} and *Flk2*^{-/-}, respectively, as previously described.^{11,12} All animal protocols were approved by the local ethics committee at Lund University.

Antibodies

All antibodies were from Becton Dickinson (Franklin Lakes, NJ) unless otherwise indicated. Monoclonal antibodies (conjugated with different fluorochromes) used to stain cell-surface antigens were CD16/32 (clone 2.4G2), B220 (RA3-6B2), CD11b/Mac-1 (M1/70), CD4 (H129.9), CD8 α (53-6.7), Ter-119 (LY-76), SCA-1 (E13-161.7), C-KIT (2B8), CD45.1 (A20), CD45.2, (104) FLT3 (AZF10.1), CD34 (RAM34), CD150 (TC15-12F12.2), Gr-1 (RB6-8C5), CD5 (53-7.3), CD3 (17A2), NK1.1 (PK136), and anti-IgM (R6-60.2). Biotinylated antibodies were visualized with streptavidin-PE or streptavidin-PECy7, and purified lineage (lin) antibodies were visualized with polyclonal goat anti-rat-Tricolor (both Invitrogen, Carlsbad, CA). In some experiments 7-amino-actinomycin D (7-AAD; Sigma-Aldrich, Poole, United Kingdom) was added to exclude dead cells from the analysis.

Transplantation assays

Lethally irradiated (900-975 cGy) CD45.2 WT or *Fl*^{-/-} primary recipient mice were transplanted with 2×10^5 unfractionated BM (9- to 11-week-old) or day 14.5 unfractionated fetal liver cells from CD45.1 WT or *Fl*^{-/-} mice, respectively. At 16 weeks after transplantation, 5×10^6 unfractionated BM cells from primary recipients were transplanted into secondary lethally irradiated WT (CD45.2) recipients along with 5×10^5 WT competitor BM cells (CD45.2). In other experiments, lethally irradiated (900-975 cGy) CD45.1 WT recipient mice were transplanted with 10^6 unfractionated BM cells from (10-14 weeks old) CD45.2 WT or *Flk2*^{-/-} mice together with 10^6 unfractionated WT CD45.1 BM cells, respectively. At 16 weeks after transplantation, 20×10^6 unfractionated BM cells from primary recipients were transplanted into secondary lethally irradiated WT

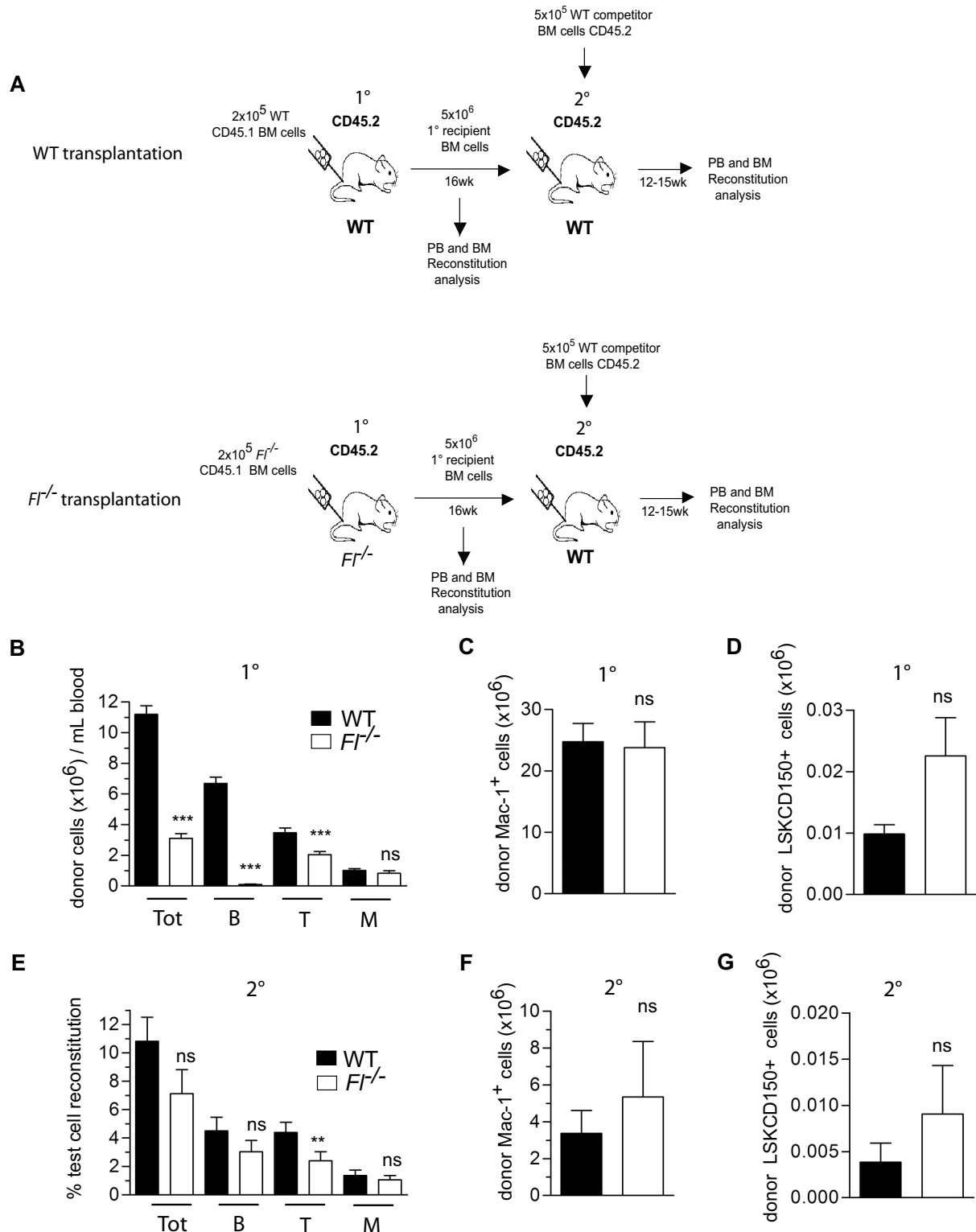


Figure 2. FLT3 ligand is dispensable for adult HSC reconstitution and expansion after BM transplantation. (A) Experimental design for HSC reconstitution and expansion experiments: 2 × 10⁵ BM cells from 9- to 11-week-old WT and Flt^{-/-} mice (CD45.1) were transplanted into lethally irradiated (900-975 cGy) adult WT and Flt^{-/-} primary (1°) recipients (CD45.2), respectively. At 16 weeks after transplantation, 5 × 10⁶ BM cells from 1° recipients were transplanted into secondary (2°) lethally irradiated WT (CD45.2) recipients along with 5 × 10⁵ WT competitor BM cells (CD45.2). (B) Test cell–derived total (Tot), B, T, and myeloid (M) reconstitution in PB at 16 weeks after transplantation in 1° recipients, presented as total number of each cell type per milliliter blood. Data represent mean (SEM) values from 30 to 35 recipient mice of each genotype, from 5 independent experiments. (C) Mean (SEM) total number of test cell–derived myeloid (Mac-1⁺) cells in the BM (2 tibiae and 2 femora) of WT and Flt^{-/-} 1° recipients at 16 weeks after transplantation (13 recipient mice per genotype from 2 independent experiments). (D) Mean (SEM) total number of test cell–derived LSKCD150⁺ cells in the BM (2 tibiae and 2 femora) of WT and Flt^{-/-} 1° recipients at 16 weeks after transplantation (7-8 recipient mice per genotype). (E) Test cell–derived PB total, B-, T-, and M-cell reconstitution 12 to 15 weeks after transplantation of 2° recipients. Data are expressed as mean (SEM) percentage of test cell reconstitution (20-22 recipient mice per genotype, from 3 independent experiments). (F) Mean (SEM) total number of test cell–derived myeloid (Mac-1⁺) cells in the BM (2 tibiae and 2 femora) of WT and Flt^{-/-} 2° recipients at 14 weeks after transplantation (7-8 recipient mice per genotype). (G) Mean (SEM) total number of test cell–derived LSKCD150⁺ cells in the BM (2 tibiae and 2 femora) of WT and Flt^{-/-} 2° recipients at 14 weeks after transplantation (7-8 recipient mice per genotype). ns indicates not significant; **P < .01, ***P < .001.

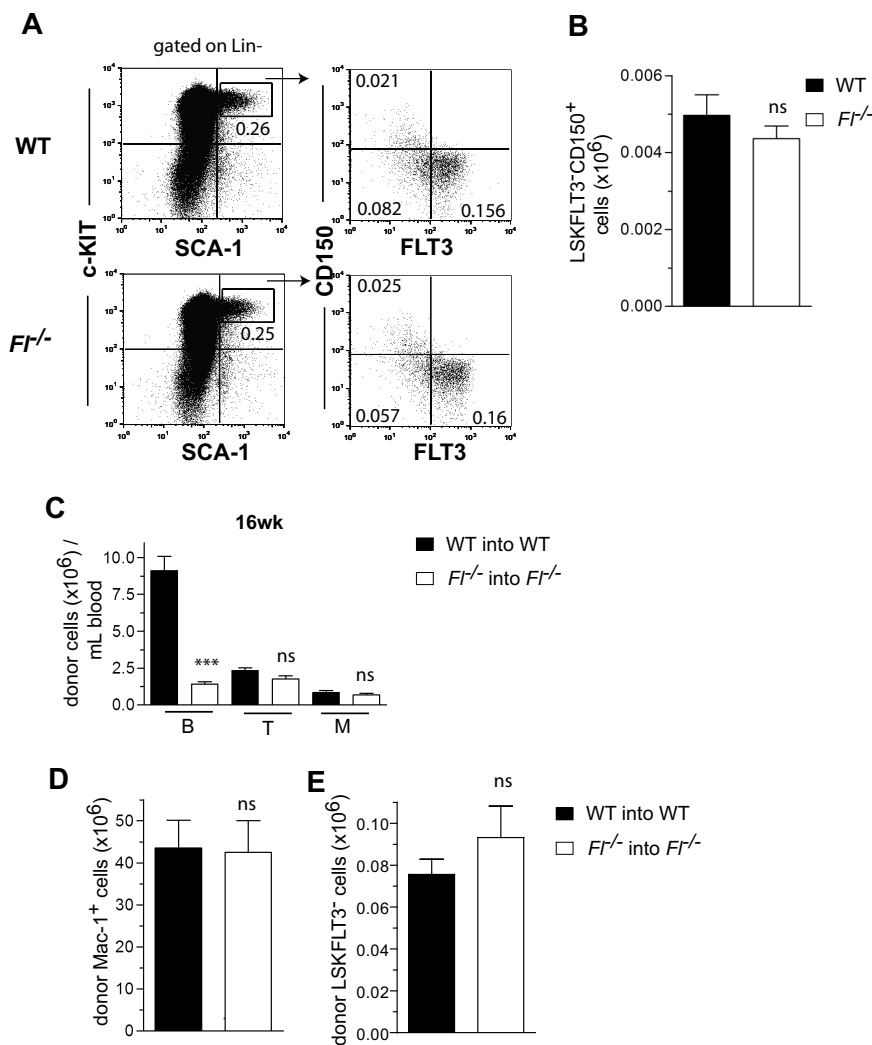


Figure 3. FLT3 ligand is dispensable for generation, reconstitution, and posttransplantation expansion of fetal liver HSCs. (A) Representative FACS plots of LSKFLT3⁻CD150⁺ cells in day 14.5 fetal liver from WT and *Fl*^{-/-} mice. Cells have first been gated as Lin⁻ as indicated. Numbers indicate frequencies of populations within indicated gates or quadrants of total fetal liver cells. (B) Mean (SEM) numbers of LSKFLT3⁻CD150⁺ cells in day 14.5 fetal liver of WT and *Fl*^{-/-} mice (n = 5 per genotype). (C-E) Liver cells (2 × 10⁵) from WT and *Fl*^{-/-} day 14.5 fetuses (CD45.1) were transplanted into lethally irradiated adult WT and *Fl*^{-/-} recipients (CD45.2), respectively. Fetuses from at least 2 different litters were used. (C) Mean (SEM) donor-derived total B, T, and myeloid (M) reconstitution in PB at 16 weeks after transplantation from 9 recipient mice of each genotype. (D,E) Mean (SEM) total number (per 2 tibiae and 2 femora) of donor-derived Mac-1⁺ myeloid cells (D) and LSKFLT3⁻ cells (E) in the BM of recipient mice (9 per genotype in 2 experiments) 16 weeks after transplantation. ns indicates not significant; ***P < .001.

(CD45.1) recipients. To determine multilineage reconstitution³⁴ nucleated peripheral blood (PB) cells or BM cells were stained with antibodies against CD45.1, CD45.2, Mac-1, Gr-1 (myeloid cells, also defined as negative for NK1.1), B220 and anti-IgM (B cells), and CD4 and CD8 α (T cells). Positively reconstituted mice were as previously described,³⁵ defined as having a minimum of 0.1% total and 0.02% of each of the B, T, and myeloid test cell contribution toward total PB cells. Donor-derived blood and BM lineage reconstitution in secondary recipients were analyzed at 12 to 28 weeks after the secondary transplantation. In addition, evaluation of the donor-derived HSC compartment was performed in recipients that received a transplant as described in "Transplantation assays."

Analysis of HSC compartment in *Fl*^{-/-} and *Fik2*^{-/-} mice by flow cytometry

BM cells were enumerated and incubated with lineage (Lin) cocktail (purified rat antibodies against B220, CD5, CD8 α , Gr-1, Ter-119, CD4, and Mac-1), stained with goat anti-rat-Tricolor, anti-mouse-FLT3-PE, or anti-mouse CD150-PE, and anti-CD34-FITC, anti-KIT-APC, anti-SCA1-biotinylated, or isotype-matched control antibodies and subsequently SA-PECy7 before analysis on FACSCalibur, FACSDiva, or FACS LSR II (Becton Dickinson) using FlowJo software (TreeStar, Ashland, OR). The same antibodies were used to evaluate HSCs in day 14.5 fetal liver except for exclusion of anti-Mac-1 and anti-CD4 from the Lin cocktail.^{1,36} In experiments evaluating donor-derived HSC reconstitution, anti-CD45.2-FITC antibody was included to detect donor cells.

Statistics

Statistical significances were determined with the use of the 2-tailed Mann Whitney test.

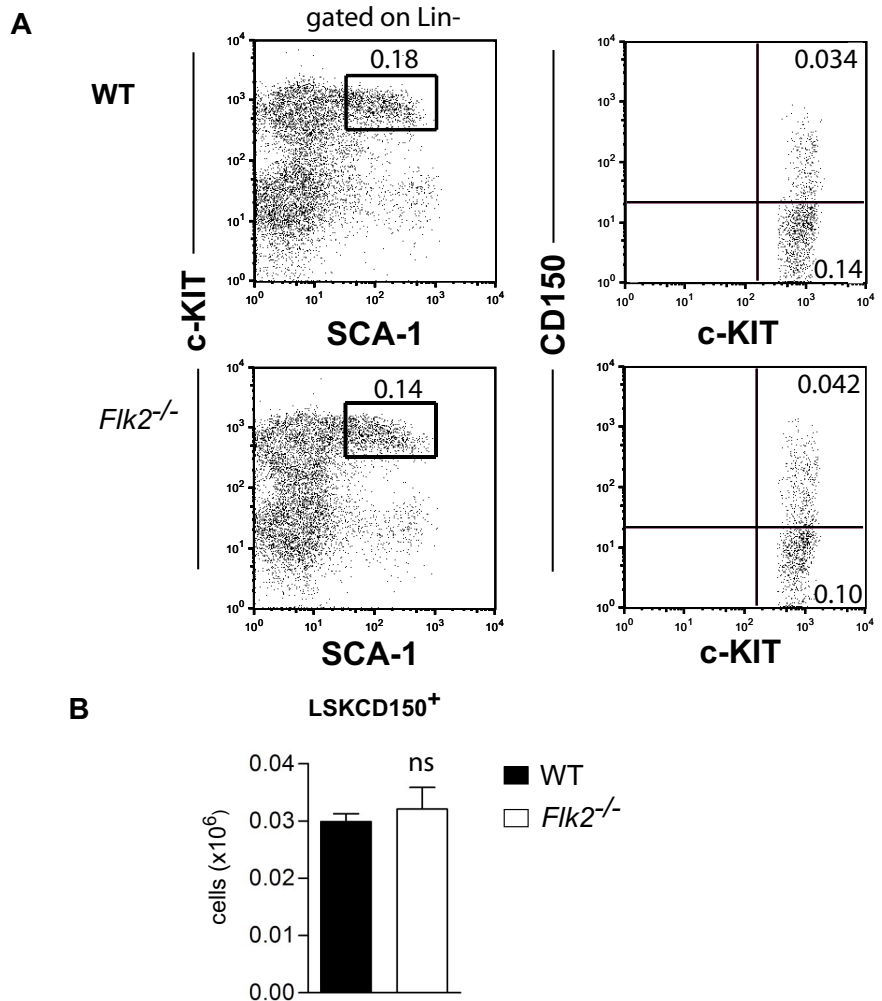
Results

Role of FLT3 ligand in HSC maintenance and expansion after transplantation

Although *in vivo* multilineage reconstitution assays remain the "gold standard," recent improvements in phenotypic identification of HSCs have made direct evaluation of HSC numbers more feasible.^{34,37} We previously showed that the size of the LIN⁻SCA1⁺KIT⁺ (LSK) compartment is unaffected in adult *Fl*^{-/-} mice,¹³ and we here analyzed more stringently defined HSCs and found LSKCD34⁻FLT3⁻ as well as LSKCD34⁻CD150⁺ cells to be present at normal levels in adult *Fl*^{-/-} mice (Figure 1A,B). Thus, maintenance of phenotypically defined HSCs in steady-state adult hematopoiesis is FL independent.

Next, to investigate the potential role of FL in promoting HSC engraftment and expansion after transplantation, lethally irradiated WT and *Fl*^{-/-} (CD45.2) recipients were transplanted with a limited number (2 × 10⁵) of WT and *Fl*^{-/-} BM cells (CD45.1), respectively (Figure 2A). It was essential to also use *Fl*^{-/-} recipients,

Figure 4. FLT3 receptor is dispensable for steady-state maintenance of adult HSCs. (A) Representative FACS plots of LSK and LSKCD150⁺ cells in BM of 9- to 18-week-old WT and *Flik2*^{-/-} mice. Numbers indicate frequencies of populations within indicated gates or quadrants of total BM cells. Cells have first been gated as Lin⁻ as indicated. In the right panels, only gated c-KIT⁺ cells are displayed. (B) Mean (SEM) numbers of LSKCD150⁺ cells in BM (per 2 tibiae and 2 femora) of 9- to 18-week-old WT and *Flik2*^{-/-} mice (n = 8-10 per genotype), from at least 2 different litters. ns indicates not significant.



because hematopoietic cells have been shown to be a major source of FL production.^{13,38,39} Notably, compared with WT mice a reduction was observed in total PB reconstitution in *Flt1*^{-/-} mice at 16 weeks after transplantation (3.6-fold; Figure 2B). This was almost entirely due to a failure to regenerate primarily B cells and partially T cells in the absence of FL, in agreement with recent studies,^{19,40} whereas myeloid reconstitution, representing the best measure of HSC activity because of the short half-life of the myeloid lineage, was not significantly reduced in peripheral blood (Figure 2B) or in BM (Figure 2C) of *Flt1*^{-/-} recipients. Furthermore, the regeneration of donor-derived LSKCD150⁺ HSC compartment was not different in *Flt1*^{-/-} and WT recipients (Figure 2D).

To more stringently assess the maintenance of HSCs in *Flt1*^{-/-} recipients, secondary competitive transplantation experiments were performed in which WT CD45.2 unfractionated BM cells were transplanted together with BM from primary recipients as a source of competitor cells (Figure 2A) into secondary WT CD45.2 recipients (Figure 2A). Importantly, also in this setting, *Flt1*^{-/-} BM cells from primary recipients were as efficient at reconstituting the myeloid, B- and T-cell lineages as those from WT primary recipients (Figure 2E). Further, there was no difference in the number of donor-derived myeloid cells (Figure 2F) or the LSKCD150⁺ HSC compartment (Figure 2G) in the BM of mice transplanted with WT and *Flt1*^{-/-} BM cells, further supporting that expansion of adult mouse HSCs after transplantation does not depend on FL.

Role of FLT3 ligand in HSC expansion during fetal development

Because HSCs in fetal liver have been suggested to express FLT3,²⁰ we next investigated whether FL might play a role in regulation of the extensive HSC expansion that takes place in the fetal liver² and/or reconstitution of fetal HSCs after transplantation. Analysis of the HSC compartment in day 14.5 fetal liver⁴¹ showed no deficiency in *Flt1*^{-/-} mice (Figure 3A,B), supporting a redundant role of FL also in regulation of fetal HSC expansion.

With exception of reduced B cells, PB reconstitution (T cell and myeloid) was unaffected in *Flt1*^{-/-} recipients of fetal liver cells at 16 weeks (Figure 3C) after transplantation, and long-term reconstitution in the BM of myeloid (Figure 3D) and LSKFLT3⁻ (Figure 3E) cells were also unaffected. These results further supported that FL is dispensable for HSC expansion during fetal development and after transplantation.

Role of FLT3 receptor in HSC maintenance and posttransplantation expansion

Previous studies of adult *Flik2*^{-/-} mice showed a multilineage reconstitution defect,¹¹ compatible with an important role of FLT3 receptor in regulation of HSCs. Herein, we first analyzed the HSC compartment of *Flik2*-deficient mice backcrossed to CD45.2 C57BL/6 mice for 9 generations. In agreement with previous studies,¹¹ BM cellularity was not significantly affected in *Flik2*^{-/-} mice (data not shown), and phenotypic analysis showed that also

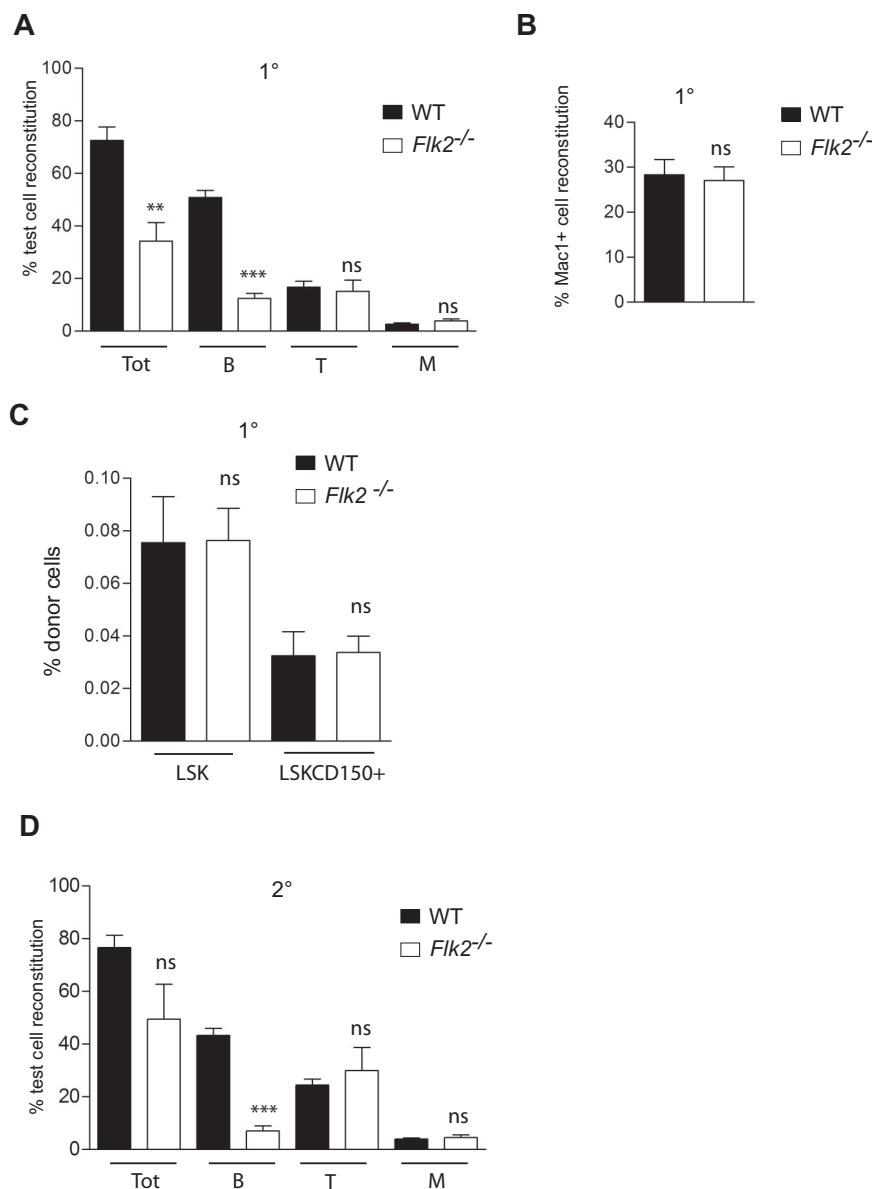


Figure 5. FLT3 receptor is dispensable for reconstitution and expansion of adult HSCs after BM transplantation. Lethally irradiated adult WT recipient (CD45.1) mice were transplanted with 10^6 unfractionated *Flk2*^{-/-} (CD45.2) or WT (CD45.2) BM cells together with 10^6 unfractionated WT CD45.1 competitor BM cells. (A) Donor-derived PB total (Tot), B-cell, T-cell, and myeloid (M) reconstitution at 16 weeks after transplantation. Mean (SEM) values from 12 to 14 primary recipient mice of each genotype, from 2 independent experiments. At 16 weeks after transplantation recipient mice were killed, and BM cells were counted and analyzed for donor-derived myeloid (Mac1⁺) reconstitution (B) as well as donor-derived regeneration of LSK and LSKCD150⁺ compartments (C). Mean (SEM) values for proportion of donor cells of 12 to 14 mice per genotype, from 2 independent experiments. (D) Test cell-derived PB total, B-, T-, and M-cell reconstitution 24 to 28 weeks after transplantation in secondary WT recipients. Data are expressed as mean (SEM) percentage of test cell reconstitution (9-10 recipient mice per genotype, from 2 independent experiments). ns indicates not significant; ** $P < .01$, *** $P < .001$.

the LSKCD150⁺ HSC compartment was unaltered in *Flk2*^{-/-} mice (Figure 4), compatible with also the FLT3 receptor being dispensable for HSC maintenance in steady-state adult hematopoiesis.

Next, a competitive transplantation experiment was performed to investigate whether the FLT3 receptor plays an important role in posttransplantation HSC expansion and maintenance. At 16 weeks after transplantation, total donor peripheral blood reconstitution by *Flk2*^{-/-} BM cells was 2.1-fold lower compared with that of WT cells (Figure 5A). However, in agreement with the studies of *Flt*^{-/-} mice, this reduction was entirely due to impaired B-cell regeneration, which was reduced as much as 4.1-fold in mice transplanted with *Flk2*^{-/-} BM cells (Figure 5A). In contrast, peripheral blood reconstitution of both T cells and myeloid cells were not significantly affected in recipients of *Flk2*^{-/-} BM cells (Figure 5A), and also myeloid reconstitution in the BM was normal (Figure 5B). *Flk2*^{-/-} BM cells were as efficient as WT cells in regenerating the LSK and LSKCD150⁺ HSC compartments (Figure 5C). Finally, after transplantation into secondary recipients, *Flk2*^{-/-} BM cells remained as efficient in sustaining long-term myeloid (and T cell) reconstitution at 24 to 28 weeks after transplantation as did WT

cells (Figure 5D). Taken together, these findings show that steady state maintenance and posttransplantation expansion and self-renewal of HSCs occur independently of FLT3 ligand and receptor.

Discussion

The FLT3 ligand and receptor have received considerable attention in hematology, in part because FLT3-activating mutations are very common in hematologic malignancies, in particular in AML³² but also because FLK2 was identified and cloned as a cytokine tyrosine kinase receptor highly expressed on enriched mouse HSCs.¹⁰ When FL was cloned, it also became evident that it through activation of FLT3 was a potent synergistic factor for in vitro growth of enriched HSCs,^{15,42-44} and FL was also reported to promote ex vivo expansion of mouse HSCs.^{15,22-26} Because through these findings FL emerged as a candidate key HSC regulator,¹⁵ it was unexpected when HSCs in steady state adult BM were found to be unaltered in *Flt*^{-/-} mice.¹³ However, several findings, including reports of FLT3 being expressed on cycling HSCs in fetal liver and regenerating

BM,²⁰⁻²² were compatible with FLT3 ligand and receptor perhaps having a distinct role in regulating actively cycling HSCs. Such a scenario could also explain why *Flik2*^{-/-} BM cells would show reduced reconstituting ability after transplantation into ablated recipients.¹¹

Herein, we demonstrate through studies of *Fl*^{-/-} and *Flik2*^{-/-} mice that both FL and FLT3 receptor are dispensable for regulation of HSCs in steady-state adult hematopoiesis in which HSCs are predominantly quiescent or slowly cycling,^{45,46} as well as during fetal development and after transplantation when HSCs expand extensively.^{2,3} These findings were corroborated by direct phenotypic analysis of the HSC compartment as well as through functional evaluation of HSC activity. Although our findings of normal HSC homeostasis in *Flik2*^{-/-} mice might appear to be in conflict with the impaired multilineage reconstitution ability of *Flik2*^{-/-} BM cells reported by Mackarehshchian et al,¹¹ there were aspects of those studies which in fact would be most compatible with FLT3 being redundant for HSC self-renewal and maintenance. Specifically, in the studies of Mackarehshchian et al¹¹ the hematopoietic activity of *Flik2*^{-/-} HSCs being transplanted appeared stable over time, and most notably serial transplantation, the hallmark assay for HSC self-renewal, failed to show any further reduction in the ability of *Flik2*^{-/-} HSCs to multilineage reconstitute secondary recipients.¹¹ Thus, the reduced multilineage reconstitution ability observed on transplantation of *Flik2*^{-/-} BM cells might rather have reflected a critical role of FLT3 in generation and maintenance of early progenitors, in particular of the lymphoid lineages,^{11,12,14,19,40} as confirmed here. Notably, the lack of a role of FLT3 receptor and ligand in HSC regulation is distinctly different from that of c-KIT, which has been shown to be critically required for maintenance of HSCs, in particular in HSC expansion after transplantation.⁶⁻⁸

Whereas our current studies show that FLT3 ligand and receptor have little or no role in regulation of mouse HSCs, previous studies have shown that FLT3 is critically involved in regulation of the earliest B- and T-cell progenitors,¹¹⁻¹³ and in fact more recent studies also show a role of FLT3 in regulation of LMPPs with combined granulocyte-monocyte, B-cell and T-cell potential but no megakaryocyte-erythroid potential.^{17,19} Thus, in mice, FLT3 recep-

tor and ligand might act at the very earliest stages in the lineage restriction pathway(s) from HSCs to lymphoid-restricted progenitors. However, it remains possible that FLT3 might be expressed and important already in the HSC compartment in man. There is in fact strong evidence in support of human candidate HSCs expressing FLT3,²⁸⁻³⁰ in contrast to mouse HSCs that are predominantly FLT3⁻.^{16,20}

Acknowledgments

We thank Dr Jacques Peschon for kindly providing the *Fl*^{-/-} mice and Drs Ihor Lemischka and Christoph Schaniel for *Flik2*^{-/-} mice. We also thank Lilian Wittmann, Christina T. Jensen, Kees-Jan Pronk, Stuart Walsh, Hong Qian, and Yanjuan Tang for their expert technical assistance and advice.

This work was supported by grants from The Swedish Paediatric Cancer Society, The Göran Gustafsson Foundation, The Swedish Research Council, and the Juvenile Diabetes Research Foundation (JDRF). E.S. has an assistant professor position from the Swedish Cancer Society. The Lund Stem Cell Center is supported by a Center of Excellence grant from the Swedish Foundation for Strategic Research. S.E.W.J. is supported by an international recruitment award from the Medical Research Council, United Kingdom.

Authorship

Contribution: N.B.-V. and E.S. designed and performed the research, analyzed data, and wrote the paper; M.C., S.D., and H.N.C. performed research and analyzed data; and S.E.W.J. designed the research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Sten Eirik W. Jacobsen, Haematopoietic Stem Cell Lab, Weatherall Institute of Molecular Medicine, University of Oxford, Headington, Oxford OX3 9DS, United Kingdom; e-mail: sten.jacobsen@imm.ox.ac.uk.

References

- 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;92:10302-10306.
- Pawliuk R, Eaves C, Humphries RK. Evidence of both ontogeny and transplant dose-regulated expansion of hematopoietic stem cells in vivo. *Blood*. 1996;88:2852-2858.
- Iscove NN, Nawa K. Hematopoietic stem cells expand during serial transplantation in vivo without apparent exhaustion. *Curr Biol*. 1997;7:805-808.
- Geissler EN, Russell ES. Analysis of the hematopoietic effects of new dominant spotting (W) mutations of the mouse, I: influence upon hematopoietic stem cells. *Exp Hematol*. 1983;11:452-460.
- Kimura S, Roberts AW, Metcalf D, Alexander WS. Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. *Proc Natl Acad Sci U S A*. 1998;95:1195-1200.
- Thoren LA, Liuba K, Bryder D, et al. Kit regulates maintenance of quiescent hematopoietic stem cells. *J Immunol*. 2008;180:2045-2053.
- Miller CL, Rebel VI, Helgason CD, Lansdorp PM, Eaves CJ. Impaired steel factor responsiveness differentially affects the detection and long-term maintenance of fetal liver hematopoietic stem cells in vivo. *Blood*. 1997;89:1214-1223.
- Sharma Y, Astle CM, Harrison DE. Heterozygous kit mutants with little or no apparent anemia exhibit large defects in overall hematopoietic stem cell function. *Exp Hematol*. 2007;35:214-220.
- Qian H, Buza-Vidas N, Hyland CD, et al. Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. *Cell Stem Cell*. 2007;1:671-684.
- Matthews W, Jordan CT, Wiegand GW, Pardoll D, Lemischka IR. A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. *Cell*. 1991;65:1143-1152.
- Mackarehshchian K, Hardin JD, Moore KA, Boast S, Goff SP, Lemischka IR. Targeted disruption of the *flik2/flt3* gene leads to deficiencies in primitive hematopoietic progenitors. *Immunity*. 1995;3:147-161.
- McKenna HJ, Stocking KL, Miller RE, et al. Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood*. 2000;95:3489-3497.
- Sitnicka E, Bryder D, Theilgaard-Monch K, Buza-Vidas N, Adolfsson J, Jacobsen SE. Key role of flt3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool. *Immunity*. 2002;17:463-472.
- Sitnicka E, Brakebusch C, Martensson I-L, et al. Complementary signaling through flt3 and interleukin-7 receptor (alpha) is indispensable for fetal and adult B cell genesis. *J Exp Med*. 2003;198:1495-1506.
- Lyman SD, Jacobsen SE. c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. *Blood*. 1998;91:1101-1134.
- Adolfsson J, Borge OJ, Bryder D, et al. Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. *Immunity*. 2001;15:659-669.
- Adolfsson J, Mansson R, Buza-Vidas N, et al. Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential: a revised road map for adult blood lineage commitment. *Cell*. 2005;121:295-306.
- Mansson R, Hultquist A, Luc S, et al. Molecular evidence for hierarchical transcriptional lineage priming in fetal and adult hematopoietic stem cells and lymphoid-primed multipotent progenitors. *Immunity*. 2007;26:407-419.

19. Sitnicka E, Buza-Vidas N, Ahlenius H, et al. Critical role of FLT3 ligand in IL-7 receptor independent T lymphopoiesis and regulation of lymphoid primed multipotent progenitors. *Blood*. 2007;110:2955-2964.
20. Christensen JL, Weissman IL. Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. *Proc Natl Acad Sci U S A*. 2001;98:14541-14546.
21. Hasumura M, Imada C, Nawa K. Expression change of Flk-2/Flt-3 on murine hematopoietic stem cells in an activating state. *Exp Hematol*. 2003;31:1331-1337.
22. 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;84:2422-2430.
23. Ku H, Hirayama F, Kato T, et al. Soluble thrombopoietin receptor (Mpl) and granulocyte colony-stimulating factor receptor directly stimulate proliferation of primitive hematopoietic progenitors of mice in synergy with steel factor or the ligand for Flt3/Flk2. *Blood*. 1996;88:4124-4131.
24. Matsunaga T, Kato T, Miyazaki H, Ogawa M. Thrombopoietin promotes the survival of murine hematopoietic long-term reconstituting cells: comparison with the effects of FLT3/FLK-2 ligand and interleukin-6. *Blood*. 1998;92:452-461.
25. Yonemura Y, Ku H, Lyman SD, Ogawa M. In vitro expansion of hematopoietic progenitors and maintenance of stem cells: comparison between FLT3/FLK-2 ligand and KIT ligand. *Blood*. 1997;89:1915-1921.
26. Miller CL, Eaves CJ. Expansion in vitro of adult murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability. *Proc Natl Acad Sci U S A*. 1997;94:13648-13653.
27. Jahn T, Sindhu S, Gooch S, et al. Direct interaction between Kit and the interleukin-7 receptor. *Blood*. 2007;110:1840-1847.
28. Sitnicka E, Buza-Vidas N, Larsson S, Nygren JM, Liuba K, Jacobsen SE. Human CD34+ hematopoietic stem cells capable of multilineage engrafting NOD/SCID mice express flt3: distinct flt3 and c-kit expression and response patterns on mouse and candidate human hematopoietic stem cells. *Blood*. 2003;102:881-886.
29. Kikushige Y, Yoshimoto G, Miyamoto T, et al. Human flt3 is expressed at the hematopoietic stem cell and the granulocyte/macrophage progenitor stages to maintain cell survival. *J Immunol*. 2008;180:7358-7367.
30. Ebihara Y, Wada M, Ueda T, et al. Reconstitution of human haematopoiesis in non-obese diabetic/severe combined immunodeficient mice by clonal cells expanded from single CD34+CD38- cells expressing Flk2/Flt3. *Br J Haematol*. 2002;119:525-534.
31. Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. *Blood*. 2002;100:1532-1542.
32. Levis M, Small D. FLT3: ITDoes matter in leukemia. *Leukemia*. 2003;17:1738-1752.
33. Huntly BJ, Gilliland DG. Leukaemia stem cells and the evolution of cancer-stem-cell research. *Nat Rev Cancer*. 2005;5:311-321.
34. Yang L, Bryder D, Adolfsson J, et al. Identification of Lin(-)Sca1(+)/kit(+)/CD34(+)/Flt3- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. *Blood*. 2005;105:2717-2723.
35. Buza-Vidas N, Antonchuk J, Qian H, et al. Cytokines regulate postnatal hematopoietic stem cell expansion: opposing roles of thrombopoietin and LNK. *Genes Dev*. 2006;20:2018-2023.
36. Ogawa M, Tajima F, Ito T, Sato T, Laver JH, Deguchi T. CD34 expression by murine hematopoietic stem cells. Developmental changes and kinetic alterations. *Ann NY Acad Sci*. 2001;938:139-145.
37. Kiel MJ, Yilmaz OH, Iwashita T, Yilmaz OH, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell*. 2005;121:1109-1121.
38. Chklovskaya E, Nissen C, Landmann L, Rahner C, Pfister O, Wodnar-Filipowicz A. Cell-surface trafficking and release of flt3 ligand from T lymphocytes is induced by common cytokine receptor gamma-chain signaling and inhibited by cyclosporin A. *Blood*. 2001;97:1027-1034.
39. Lisovsky M, Braun SE, Ge Y, et al. Flt3-ligand production by human bone marrow stromal cells. *Leukemia*. 1996;10:1012-1018.
40. Buza-Vidas N, Cheng M, Duarte S, Nozad H, Jacobsen SE, Sitnicka E. Crucial role of FLT3 ligand in immune reconstitution after bone marrow transplantation and high-dose chemotherapy. *Blood*. 2007;110:424-432.
41. Kim I, He S, Yilmaz OH, Kiel MJ, Morrison SJ. Enhanced purification of fetal liver hematopoietic stem cells using SLAM family receptors. *Blood*. 2006;108:737-744.
42. Jacobsen SE, Okkenhaug C, Myklebust J, Veiby OP, Lyman SD. The FLT3 ligand potently and directly stimulates the growth and expansion of primitive murine bone marrow progenitor cells in vitro: synergistic interactions with interleukin (IL) 11, IL-12, and other hematopoietic growth factors. *J Exp Med*. 1995;181:1357-1363.
43. Hirayama F, Lyman SD, Clark SC, Ogawa M. The flt3 ligand supports proliferation of lymphohematopoietic progenitors and early B-lymphoid progenitors. *Blood*. 1995;85:1762-1768.
44. Hudak S, Hunte B, Culpepper J, et al. FLT3/FLK2 ligand promotes the growth of murine stem cells and the expansion of colony-forming cells and spleen colony-forming units. *Blood*. 1995;85:2747-2755.
45. Bradford GB, Williams B, Rossi R, Bertoncello I. Quiescence, cycling, and turnover in the primitive hematopoietic stem cell compartment. *Exp Hematol*. 1997;25:445-453.
46. Cheshier SH, Morrison SJ, Liao X, Weissman IL. In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proc Natl Acad Sci U S A*. 1999;96:3120-3125.