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Flk2/Flt3 promotes both myeloid and lymphoid development by expanding non–self-renewing multipotent hematopoietic progenitor cells

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Abstract

Defining differentiation pathways is central to understanding the pathogenesis of hematopoietic disorders, including leukemia. The function of the receptor tyrosine kinase Flk2 (Flt3) in promoting myeloid development remains poorly defined, despite being commonly mutated in acute myeloid leukemia. We investigated the effect of Flk2 deficiency on myelopoiesis, focusing on specification of progenitors between HSC and mature cells. We provide evidence that Flk2 is critical for proliferative expansion of multipotent progenitors that are common precursors for all lymphoid and myeloid lineages, including megakaryocyte/erythroid (MegE) cells. Flk2 deficiency impaired the generation of both lymphoid and myeloid progenitors by abrogating propagation of their common upstream precursor. At steady state, downstream compensatory mechanisms masked the effect of Flk2 deficiency on mature myeloid output, whereas transplantation of purified progenitors revealed impaired generation of all mature lineages. Flk2 deficiency did not affect lineage choice, thus dissociating the role of Flk2 in promoting cell expansion and regulating cell fate. Surprisingly, despite impairing myeloid development, Flk2 deficiency afforded protection against myeloablative insult. This survival advantage was attributed to reduced cell cycling and proliferation of progenitors in Flk2-deficient mice. Our data support the existence of a common Flk2⁺ intermediate for all hematopoietic lineages and provide insight into how activating Flk2 mutations promote hematopoietic malignancy by non–Flk2-expressing myeloid cells.

The receptor tyrosine kinase Flk2 (Flt3) has been implicated in many cellular processes in normal hematopoiesis, including proliferation, self-renewal, survival, and lineage specification [1–6]. Activating mutations in Flk2 are present in greater than 30% of patients with acute myeloid leukemia (AML), and the use of Flk2 inhibitors for the treatment of AML is under intense investigation [7]. In humans, Flk2-driven myeloid leukemias could be explained by Flk2 expression by hematopoietic stem cells (HSCs) [8,9]. However, mouse

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Conflict of interest disclosure

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models of Flt3-ITD also develop myeloproliferative disease [10,11], despite a lack of Flk2 expression by HSC or myeloid-restricted progenitors. Thus, determining the role of Flk2 in myelopoiesis is critical for understanding normal differentiation pathways, as well as leukemia origin and propagation.

Controversies regarding the role of Flk2⁺ populations in myeloid development were clarified recently by Flk2-Cre lineage-tracing approaches, demonstrating that all hematopoietic lineages, including MegE lineages, develop through an Flk2⁺ intermediate population [12–14]. The role of Flk2 itself in myeloid differentiation, however, remains unclear as previous examination of hematopoiesis in the Flk2 receptor and ligand (Fl) deficient mouse models led to opposite conclusions regarding the regulation of myelopoiesis by Fl signaling [15–17]. The more obvious defects in B cell development in both Flk2^{-/-} and Fl^{-/-} mice [15–19] and the robust expression of Flk2 on lymphoid-competent progenitors have contributed to the notion that Flk2 signaling drives lymphoid specification, potentially at the expense of myelopoiesis, particularly megakaryopoiesis.

Because it is difficult to reconcile these data, we aimed to provide more definitive answers to the role of Flk2 in multilineage differentiation. We took advantage of advances in understanding hematopoietic development that have been made since the original report of hematopoiesis in Flk2^{-/-} mice [16], including the discovery that mouse HSCs do not express Flk2 [12,13,20,21], identification of additional functionally distinct progenitor populations [22–24], and more sensitive methods for lineage readout that enable assessment of erythroid progenitors and platelets. The data from these studies expand our understanding of the function of Flk2 in regulating self-renewal and lineage specification, and they support a critical role of Flk2 in driving expansion of a non-self-renewing multipotent progenitor.

Methods

Mice

Mice were housed in the University of California–Santa Cruz (UCSC) vivarium and all animal experiments were approved by the UCSC IACUC. Flk2^{-/-}, mTmG, and FlkSwitch mice (Flk2-Cre crossed to mTmG mice), were described previously [12,16,30]. Flk2^{-/-} mice were backcrossed for 10 or more generations to the C57Bl/6 background (wild type [WT] or mTmG reporter mice). All experimental mice were 8–12 weeks old at the initiation of each experiment unless noted otherwise.

Cell isolation and analysis

Bone marrow (BM) and peripheral blood (PB) cells were isolated and processed as described previously [21,41] using a four-laser FACS Aria or LSRII (BD Biosciences, San Jose, CA, USA). Analysis and display of fluorescence-activated cell sorting (FACS) data was accomplished using FlowJo analysis software (Tree Star, Ashland, OR, USA). Definitions of cell populations can be found in the Supplementary Methods (online only, available at www.exphem.org).

Transplantation assays

HSCs, ST-HSC, and multipotent progenitor (MPP) transplants were performed by retroorbital injection of 100, 200, or 2000 cells, respectively, double-sorted from Flk2^{-/-} or WT mice expressing the mTmG allele into sublethally irradiated recipients (750 rads). BM and PB chimerism of recipients was analyzed by flow cytometry for Tom fluorescence after transplantation. For CFU-S analysis, MPP, ST-HSC, and HSC (500, 200, or 100 cells per mouse) were double-sorted from the same mice and injected retroorbitally into lethally irradiated mice (1036 rad). For direct spleen injections, mice were anesthetized with Avertin, and spleens were exposed through the peritoneal cavity. Ten HSCs or 50 MPPs were injected directly into the spleen in a 50-μL volume. Spleens were dissected and colonies were enumerated 11 (for MPP) or 12 (for ST-HSC and HSC) days later.

Short-term homing

One hundred thousand single-sorted KLS cells from FlkSwitch mice [12] were injected retroorbitally into mice 24 hours after administration of a lethal dose of irradiation (1037 rad, split dose). Cell localization was analyzed 3 hours after injection as described previously [41].

5-Fluorouracil treatment

Wild type and Flk2^{-/-} mice (8–10 weeks old) received weekly injections of 110 mg/kg 5-fluorouracil (5-FU; Sigma) dissolved in 5-methylbutane and distilled water. Injection volume was determined individually for each mouse based on weight, measured weekly. Mice received treatment weekly for 4 weeks, and survival was monitored daily for 8 weeks.

5-Ethynyl-2'-deoxyuridine incorporation

Flk2^{-/-} and WT mice expressing the mTmG allele were injected with 100 μL of 5-ethynyl-2'-deoxyuridine (EdU; 1 mg/mL; Click-iT EdU Alexa Fluor 488 Imaging Kit; Life Technologies) 1 hour before sacrificing and harvesting of bone marrow. HSCs, ST-HSCs, MPPs, and MyPro were double-sorted and placed on poly-lysine coated slides and fixed in 2% PFA. EdU labeling was performed per the manufacturer's instructions. Images were taken and analyzed on a Keyence microscope using BZ-II image processing software.

Cell cycle analysis

Bone marrow cells were isolated from Flk2^{-/-} and WT mice, stained with antibodies, and fixed with 1% PFA. HSCs, ST-HSCs, MPPs, and myeloid progenitors (Lin⁻, Sca1⁻, c-Kit⁺) were isolated by FACS and incubated with 40 μg/mL propidium iodide (PI) and 100 μg/mL RNase A in phosphate-buffered saline supplemented with 0.03% saponin for 30 minutes at 37°C, followed by analysis for DNA content by FACS.

Results

Separation of stem and progenitor cells into Flk2⁻ and Flk2⁺ fractions in the absence of Flk2

To investigate the effect of Flk2 deficiency on specific stem and progenitor cells within the KLS compartment, an alternative staining strategy was devised that did not rely on Flk2 as a surface marker. Both CD150 (SLAMf1) [25,26] and ESAM1 [25,27,28] have been previously used to positively identify the HSC population, because neither CD150⁻ nor ESAM1⁻ cells support long-term reconstitution. Surface staining of KLS progenitors with antibodies to CD150 and ESAM1 identified three distinct populations with cleanly separable Flk2 expression; Flk2 expression analysis in WT mice revealed that CD150⁺/ESAM1⁺ KLS did not express Flk2 at the cell surface, whereas CD150⁻/ESAM1⁺ and CD150⁻/ESAM1⁻ KLS expressed intermediate and high cell surface levels of Flk2, respectively (Fig. 1A and B). These phenotypic data, combined with functional analysis upon transplantation (discussed later), led us to designate these populations as HSCs (CD150⁺/ESAM1⁺ KLS), ST-HSCs (CD150⁻/ESAM1⁺ KLS), and MPPs (CD150⁻/ESAM1⁻ KLS).

Flk2 deficiency results in decreased cellularity of multipotent, lymphoid, and myeloid progenitors

To determine the effect of Flk2 deficiency on hematopoiesis at steady state, we examined the cellularity of stem, progenitor, and mature cells in Flk2^{-/-} mice and WT controls, focusing on early progenitors. Flk2 deficiency did not significantly affect whole BM cellularity (data not shown). Examination of the KLS compartment revealed that the relative cellularity of both HSCs and ST-HSCs were unaffected by Flk2 deficiency, whereas cellularity of MPPs, defined by high surface levels of Flk2, were significantly decreased by approximately 60% ($p < 0.0001$; Fig. 1C, Supplementary Figure E1A [online only, available at www.exphem.org]). Flk2 deficiency also significantly decreased the cellularity of the lymphoid compartment (Fig. 1D; Supplementary Figures E1B, E1C [online only, available at www.exphem.org]). Common lymphoid progenitors (CLPs) [23] exhibited an approximately 70% reduction in Flk2^{-/-} mice ($p < 0.0001$; Fig. 1D, Supplementary Figure E1B). Cellularity of pro-B progenitors and mature B cells was also significantly reduced in response to Flk2 deficiency ($p < 0.001$; Fig. 1D, Supplementary Figure E1C [online only, available at www.exphem.org]), although the reduction was not as severe as that observed for the CLP (Pro-B vs. CLP, $p < 0.001$; B cell vs. CLP, $p < 0.01$). Despite the significantly reduced numbers of MPPs and CLPs, the likely precursors to mature T cells [29], T cell cellularity in the BM was unaffected in Flk2^{-/-} mice.

Interestingly, Flk2 deficiency also affected the generation of myeloid-committed progenitors (Fig. 1E, Supplementary Figure E1D [online only, available at www.exphem.org]). Using two alternative staining strategies for previously defined myeloid progenitor subpopulations [22,24], we found that the total cell numbers for CMP, PreGM, and PreMegE progenitors were significantly reduced by 20%, 60%, and 20%, respectively, compared with WT controls ($p < 0.01$, $p < 0.0001$, and $p < 0.05$, respectively; Fig. 1E, Supplementary Figure E1D [online only, available at www.exphem.org]). Cellularity of unipotent and mature myeloid cells was unaffected in Flk2^{-/-} mice.

Transplanted Flk2-deficient HSC exhibit impaired reconstitution of all lineages

Previous whole BM transplant experiments yielded contradictory conclusions regarding myeloid output from Flk2^{-/-} cells [15,16]; therefore, we performed kinetic analysis by transplanting purified stem and progenitor cells to provide a more definitive answer on the role of Flk2 in myeloid differentiation. To assess the effect of Flk2 deficiency on MegE production, we crossed Flk2^{-/-} mice with the mTmG reporter mice to label plts, as well as all nucleated cells [30]. We transplanted highly purified Tomato-expressing WT and Flk2^{-/-} HSC, ST-HSC, and MPP into sublethally irradiated recipients.

Examination of multilineage reconstitution in the PB at 4, 8, 12, and 16 weeks after transplantation of highly purified Flk2^{-/-} or WT HSC revealed that donor contribution to platelets and GM cells was significantly different between Flk2^{-/-} and WT cells throughout the 4–16-week analysis period (Fig. 2A and B). Reduced contribution to B and T cell lineages in the PB was also evident at 8 weeks and beyond. Representative FACS plots for PB donor contribution at 16 weeks are shown in Supplementary Figure E2 (online only, available at www.exphem.org). Lineage distribution analysis of donor-derived cells at 16 weeks showed that Flk2^{-/-} HSCs were drastically more defective in their ability to generate B lineages ($p < 0.001$) compared with the GM lineage ($p < 0.05$; Fig. 2B and C; Supplementary Figure E2 [online only, available at www.exphem.org]).

Examination of BM reconstitution 17–18 weeks after transplantation revealed that, similar to steady-state hematopoiesis, chimerism within the HSC and ST-HSC compartments was not significantly different for transplanted Flk2^{-/-} HSCs compared with WT HSCs. In contrast, stark differences were observed in chimerism of MPP and all downstream myeloid and lymphoid lineages (Fig. 2D). Collectively, these data imply that cell expansion downstream of Flk2^{-/-} HSCs was impaired, thereby resulting in a reduction in the number of progeny of all mature lineages.

Flk2^{-/-} ST-HSCs exhibit significantly impaired reconstitution

As the number of HSCs was unaffected by Flk2 deficiency both at steady state and after transplantation, we aimed to pinpoint the stage at which Flk2 deficiency impaired multilineage output. We therefore investigated PB reconstitution from Flk2^{-/-} and WT ST-HSCs. Flk2^{-/-} ST-HSCs displayed an immediate impairment in readout of all mature lineages, including platelets (Fig. 3A). This robust difference was sustained at 4 weeks, but disappeared at 8 weeks for GM and Plt lineages, at which point reconstitution from both WT and Flk2^{-/-} ST-HSC was negligible. Differences in B cell repopulation were sustained through 8 weeks, after which B cell readout was exhausted from both WT and Flk2^{-/-} ST-HSCs. Similarly, T cell reconstitution from Flk2^{-/-} ST-HSCs was significantly impaired through 12 weeks.

B cell readout, but not total reconstitution, is impaired from transplanted Flk2^{-/-} MPP

Our results suggested that the effect of Flk2 deficiency on multilineage repopulation from HSCs and ST-HSCs was due primarily to an inability to generate adequate numbers of Flk2⁺ MPPs, a critical developmental intermediate for all mature lineages. To test this hypothesis, we transplanted equivalent numbers of WT and Flk2^{-/-} MPPs into sublethally irradiated

hosts and monitored PB reconstitution. Remarkably, readout from Flk2^{-/-} MPPs closely resembled that of WT MPPs (Fig. 3B), with the exception of a transient difference in B cell readout at 2 weeks ($p < 0.01$). Neither GM nor platelet readout was significantly different between WT and Flk2^{-/-} MPP at any time examined. Very little T cell readout was observed at these early times because of the extended time required for generation of mature T cells; however, examination over 4 months revealed that Flk2 deficiency did not significantly impair T or B cell readout from MPP at later times (data not shown). These data suggest that although Flk2 is important for generating MPPs from HSCs, once cells reach the MPP stage only B cell production is significantly affected by Flk2 deficiency.

Flk2 deficiency does not affect CFU-S efficiency of HSCs or MPPs

To examine the effect of Flk2 deficiency on generation of MegE progenitors and mature cells, we examined colony-forming unit of the spleen (CFU-S) efficiency of transplanted Flk2-deficient stem and progenitor cells. We confirmed that the CFU-S assay is a clonal measure of erythroid potential, as the vast majority (>99%) of HSC-and MPP-derived CFU-S cells were erythroid (Fig. 4A). Flk2 deficiency had no effect on the frequency of CFU-S derived from HSCs, ST-HSCs, or MPPs (Fig. 4B). However, consistent with previous results [21], we observed a lower frequency of CFU-S arising from MPPs compared with HSCs and ST-HSCs, regardless of Flk2 genotype.

Homing to the spleen partially accounts for the differential CFU-S efficiency between HSCs and MPPs

The difference in CFU-S formation between HSCs and MPPs could be due to differences in the ability to home to the spleen. We tested spleen homing efficiency by performing short-term localization analysis after injection of HSCs and MPPs into lethally irradiated hosts. For donor cells, we used KLS cells isolated from the recently characterized FlkSwitch model [12,13]; in mice with high floxing efficiency, almost all (>96%) MPPs express GFP, whereas all HSCs express Tomato. Although HSCs (Tomato⁺ cells) and MPPs (GFP⁺ cells) were recovered at similar frequencies from the BM and blood 3 hours later, significantly fewer MPPs were recovered from the spleen ($p = 0.02$; Fig. 4C).

We also compared CFU-S frequency of HSCs and MPPs injected either intravenously (IV) or directly into the spleen (intrasplenic [IS]; Fig. 4 E–G), with the assumption that direct spleen injection would circumvent inherent differences in the ability to home to the spleen. Direct spleen injection resulted in significantly higher CFU-S frequency for both HSCs and MPPs (Fig. 4D and E). Importantly, CFU-S frequency from MPPs injected IS (1/37; Fig. 4E) was comparable to the frequency observed for HSCs injected IV (1/28; Fig. 4D), indicating that MPPs possess significant MegE potential in the CFU-S assay. Despite the increase observed for MPP upon IS injection, a substantial difference in CFU-S frequency was still observed between HSCs and MPPs (Fig. 4G).

Flk2 deficiency improves survival in response to 5-FU treatment

Our finding that Flk2 deficiency affects myeloid cell generation both at steady state and after HSC transplantation prompted us to examine whether Flk2 deficiency influences the ability to rapidly generate myeloid cells under conditions of hematopoietic stress. WT and

Flk2^{-/-} mice were treated with 110 mg/kg of the myeloablative agent 5-FU weekly for 4 weeks; this dose allows for survival of approximately 50% of WT mice. Survival was examined over the course of treatment and for four weeks thereafter. Surprisingly, Flk2 deficiency did not impair survival of mice in response to 5-FU (Fig. 5A). In contrast, binary analysis at the last time point using the Fisher exact test revealed enhanced survival of Flk2^{-/-} mice compared with WT mice ($p = 0.05$). Thus, not only did Flk2 deficiency fail to sensitize mice to 5-FU, but a lack of Flk2 expression afforded protection following the termination of treatment.

Flk2 deficiency impairs proliferation and cell cycle status of stem and progenitor cells

As proliferative activity influences cellular sensitivity to chemotherapeutic agents such as 5-FU [31,32], the finding that Flk2^{-/-} mice were less sensitive to 5-FU prompted us to examine proliferative activity and cell cycle status of Flk2^{-/-} progenitors. Analysis of incorporation of the nucleoside analog 5-ethynyl-2'-deoxyuridine (EdU) revealed significantly reduced EdU incorporation for Flk2-deficient MPPs as compared with WT MPPs (Fig. 5B and C; Supplementary Figure E3 [online only, available at www.exphem.org]). Additionally, analysis of cell cycle status via quantification of DNA content in sorted HSCs, ST-HSCs, MPPs, and myeloid progenitors revealed a reduced proportion of HSCs, ST-HSCs, and MPPs in S-G2-M phase in Flk2^{-/-} as compared with WT cell populations, whereas the cell cycle status of myeloid progenitors was unaffected by Flk2 deficiency (Fig. 5D and E). Cycling of MPPs was most affected by Flk2 deficiency (40% reduced; $p < 0.001$) as compared with HSCs and ST-HSCs (21% and 14% reduced, respectively; $p < 0.05$, Fig. 5E), consistent with the highest level of Flk2 expression in WT MPP and the observed reduction in EdU incorporation. These data suggest that Flk2^{-/-} mice exhibit higher survival because more KLS cells, protected by quiescence, remain to support hematopoiesis after 5-FU treatment.

Discussion

Flk2 deficiency affects both myeloid and lymphoid lineages

Although the role of Flk2 signaling in hematopoietic specification has received considerable attention, conflicting findings in both the Flk2^{-/-} and Fl^{-/-} mouse models have left several key aspects unresolved. In the present study, thorough investigation of hematopoiesis in Flk2-deficient mice with a focus on myelopoiesis demonstrated a role of Flk2 in multilineage differentiation downstream of HSC. Separation of the stem and progenitor cells using a novel surface staining strategy (Fig. 1A and B) enabled us to assess the effects of Flk2 deficiency on Flk2⁻ HSC and Flk2⁺ MPP without the confounding use of Flk2 as a surface marker, as well as myeloid and lymphoid progenitors and mature cells. Both steady-state and transplantation experiments revealed impairment across all myeloid lineages (Figs. 1–3). Because Flk2 is not expressed in myeloid progenitors or mature cells, the observed impairment in myeloid development can be attributed to attenuated MPP expansion in Flk2^{-/-} mice. Impaired expansion of the MPP stage is consistent with the observed reduction in proliferation of stem and progenitor cells in Flk2^{-/-} mice (Fig. 5), as well as the well-documented role for Flk2 in cell proliferation during normal hematopoiesis and in leukemias [33–35]. These results indicate that Flk2 promotes the generation of all

hematopoietic lineages, and that its role is particularly important during acute needs for cell replacement, such as transplantation into irradiated hosts.

Compensatory mechanisms mitigate the effects of *Flk2* deficiency

In contrast to the impaired B cell generation consistently observed in *Fl^{-/-}* and *Flk2^{-/-}* mice, controversy regarding the role of Fl signaling in myeloid specification stems in part from contradictory reports on myeloid generation from steady-state analysis and whole BM transplantation studies in the same models [15,16,19,36]. Our comprehensive and quantitative steady-state analyses of stem, progenitor, and mature cells revealed a modest, but significant, decrease in myeloid progenitor numbers in *Flk2^{-/-}* mice, consistent with previous reports from *Fl^{-/-}* mice, including reduced CFU-GM activity [19] and significantly reduced numbers of CMP [17], preGM, and GMP [36]. Interestingly, the significant decrease in early myeloid-restricted progenitors, including MegE-restricted progenitors, did not translate into a reduction in the cellularity of unipotent and mature myeloid cells at steady state (Fig. 1E). These data indicate that mechanisms exist downstream of early progenitor specification to normalize production of mature cells. Similarly, downstream compensation during lymphoid specification was also observed, as cellularity of Pro-B, B, and T cells was increased relative to their common precursor, the CLP (Fig. 1D). In contrast to the downstream compensation observed at steady state, transplantation of purified *Flk2^{-/-}* HSC led to decreased reconstitution of all investigated cell types from MPPs and beyond, including erythroid progenitors (EPs) and platelets (Fig. 2D). More severe deficiency upon transplantation compared with steady state is common, and it was observed in the original report of hematopoiesis in *Flk2^{-/-}* mice [16]. The defects in reconstitution of mature cells from *Flk2*-deficient HSC upon transplantation, including EP and plt, validate the reduction observed across all lineage-restricted progenitors at steady state. Furthermore, our results demonstrate that compensatory mechanisms can be revealed by comprehensive analysis of intermediate progenitors and by assessing cell output under increasingly challenging conditions.

Flk2 is critical for progenitor expansion, but does not instruct lineage choice

In addition to revealing downstream compensation, analysis and transplantation of purified stem and progenitor cells allowed us to pinpoint the cellular stage at which *Flk2* deficiency is manifested. Not surprisingly, significant decreases in cell numbers were first observed at the MPP stage (Figs. 1C, 2), coinciding with establishment of robust *Flk2* cell surface levels (Fig. 1B). Whereas comparison of reconstitution from equivalent numbers of *Flk2^{-/-}* and WT HSC and ST-HSC revealed significant impairment in PB reconstitution of all mature lineages, including platelets (Figs. 2, 3A), transplantation of equivalent number of *Flk2^{-/-}* and WT MPPs revealed only subtle reconstitution defects that were restricted to the B lineage (Fig. 3B). The abrogation of impairment in B cell readout and the absence of an effect of *Flk2* deficiency on myeloid and T cell reconstitution when equal numbers of MPP are transplanted indicate that impaired reconstitution from *Flk2*-deficient HSCs and ST-HSCs is due primarily to an inability to expand the MPP pool.

The continued dependence on *Flk2* signaling during lymphoid development, in particular in the absence of readily detectable effects on mature myeloid cells, could easily lead to the

conclusion that Flk2 signaling regulates the decision between lymphoid and myeloid fates. If Flk2 promotes lymphoid development at the expense of myeloid cells, Flk2 deficiency would be expected to decrease lymphoid generation with a corresponding increase in the generation of myeloid cells. Instead, the observed reduction in MPPs and both myeloid and lymphoid restricted progenitors in Flk2^{-/-} mice demonstrates that Flk2 promotes expansion of Flk2-expressing cells, including MPPs, that then contribute to all downstream cell types, including MegE cells. Together, these data indicate that Flk2 is critical for expanding the pool of available MPP, which in turn must give rise to sufficient numbers of both myeloid and lymphoid lineages, but does not regulate lineage choice.

Trafficking to the spleen and CFU-S frequency are Flk2-independent

Despite robust MegE generation from MPPs upon transplantation in vivo, MPPs have reduced CFU-S readout compared with Flk2⁻ cells, including HSCs, ST-HSCs, and myeloid progenitors [21,37]. We found that Flk2 expression itself does not influence CFU-S frequency from Flk2⁺ cells, consistent with our conclusion that Flk2 does not direct lineage specification downstream of HSCs (Supplementary Figure E1, online only, available at www.exphem.org). Both the short-term homing assay (Fig. 4C) and the IV versus IS CFU-S assays (Fig. 4D–G) indicated that homing efficiency plays an essential role in CFU-S readout. However, despite significant differences in the ability of HSCs and MPPs to home to the spleen, homing alone did not account for differences in CFU-S frequency, because direct spleen injections still resulted in decreased CFU-S frequency from MPPs relative to HSCs. It remains unclear whether factors such as survival, burst size, and ability to stay within the spleen differentially affect CFU-S generation from MPPs as compared with HSCs. Interestingly, although all HSCs possess erythroid potential, IS injection of HSCs resulted in CFU-S frequency of approximately 1 of 5 cells. That the CFU-S assay underestimates erythroid potential by at least fivefold when performed IS and more than 30-fold upon IV injection provides further direct evidence for robust erythroid potential of MPP in vivo.

Flk2 affects cell cycle status and animal survival in response to chemotherapy

Despite reduced numbers of MPPs and myeloid progenitors, Flk2^{-/-} mice were less sensitive than WT mice to the myeloablative agent 5-FU (Fig. 5A). Specifically, more Flk2^{-/-} than WT mice survived after termination of treatment. Consistent with this finding, a previous study examining cell recovery after a single treatment of 5-FU in FL-deficient mice revealed impaired B cell recovery but normal myeloid cell recovery [38]. How can mice with demonstrated impairments in the propagation of myeloid progenitors be less sensitive to myeloablation? Examination of proliferation and cell cycle status revealed reduced proliferative activity and altered cell cycle distribution of Flk2-deficient stem and progenitor cells, with the greatest differences observed in the MPP compartment (Fig. 5B–E). As 5-FU preferentially affects proliferating cells [31,32], this relative quiescence likely spared a greater fraction of KLS cells in Flk2^{-/-} mice, with the higher number of remaining cells conferring enhanced survival. Because cells with activating Flk2 mutations may be more sensitive to 5-FU, and activating Flk2 mutations are present in 30%–35% of AML cases [39], these findings bear significant implications for therapeutic approaches to treatment of Flk2-driven leukemias.

Flk2⁺ progenitors contribute to all mature blood lineages

Our new data converge with previous reports on a model in which Flk2 is dispensable for HSC maintenance, including self-renewal, which is consistent with our recent functional validation that HSCs do not express detectable surface levels of Flk2 [13]. Gradually increased Flk2 expression—as HSCs differentiate into ST-HSCs and then MPPs—coincides with increased dependence on Flk2 to promote cell proliferation. Thus, Flk2 acts to promote expansion of progenitor cells that gives rise to both myeloid and lymphoid lineages, rather than mediating fate decisions between lineages. Once a sufficient number of MPPs have been established, Flk2 expression is no longer needed to generate myeloid or T cells. However, optimal B cell generation is dependent on sustained Flk2-mediated proliferation, and B cell numbers are therefore particularly affected by Flk2 loss. These conclusions are consistent with recent reports using Flk2-Cre lineage tracing, demonstrating that all lymphoid and myeloid lineages, including MegE cells, derive from Flk2⁺ progenitors in vivo [12–14]. Recently, using a gain-of-function model on a Runx-1 hypomorphic background, Mead and colleagues suggested that the Flt3-ITD aberrantly induces myeloid bias by altering downstream signaling [40]. However, our examination of normal hematopoiesis suggests that activating mutations of Flk2/Flt3 might contribute to AML by further promoting expansion of a precursor that normally gives rise to myeloid lineages. These data underscore the importance of understanding differentiation pathways and examining effects on intermediate precursors, because they reveal novel insights into mechanisms regulating HSC function, hematopoietic specification, and how constitutively active mutations in Flk2 contribute to the development of leukemia in non-Flk2-expressing myeloid cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Flk2 deficiency decreases cellularity of multipotent, lymphoid, and myeloid progenitors. **(A)** Flow cytometry gating strategy used to identify Flk2⁺ HSCs, Flk2^{lo} ST-HSCs, and Flk2^{hi} MPPs in Flk2^{-/-} mice. **(B)** Relative distribution of Flk2 expression levels among the three populations gated in **(A)**. **(C)** Relative cellularity of multipotent stem and progenitor cells in WT and Flk2^{-/-} mice. **(D)** Relative cellularity of the lymphoid compartment in WT and Flk2^{-/-} mice. **(E)** Relative cellularity of the myeloid compartment in WT and Flk2^{-/-} mice. Data represent mean $\pm$ SEM of total cell number normalized to WT average for each cell

type. Differences were analyzed with two-tailed Student *t* test. *n* = 11 per genotype representing three independent experiments. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

Figure 2.

Flk2 deficiency impairs HSC reconstitution capacity and lineage output after transplantation.

(A) Quantitative and kinetic analysis of donor-derived Tomato⁺ white blood cells (WBCs) and Plt production in PB of sublethally irradiated mice transplanted with 100 WT or Flk2^{-/-} HSCs. (B) Fold difference in GM, B cell, T cell, and Plt PB reconstitution measured 16 weeks after transplantation for the same mice as in (A). (C) Relative contribution of each mature WBC lineage (GM, B, and T) to total PB reconstitution measured 16 weeks after transplantation for same mice as in (A). (D) Total donor chimerism of each stem, progenitor,

and mature cell population in the BM 17–18 weeks after transplantation in the same mice as in (A). Data represent mean $\pm$ SEM of the frequency of donor-derived cells for each population. n = 16 per genotype, representing three independent experiments. Differences were analyzed with two-tailed Student *t* test. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

Figure 3.

Flk2 deficiency impairs multilineage reconstitution from ST-HSCs but not MPPs.

Multilineage reconstitution kinetics of transplanted WT and Flk2^{-/-} progenitors. **(A)**

Quantitative and kinetic analysis of Tomato⁺ GM, Plt, B, and T cells derived from 200 WT or Flk2^{-/-} ST-HSCs transplanted into sublethally irradiated recipients (n = 14, representing two independent experiments). **(B)** Quantitative and kinetic analysis of Tomato⁺ total GM, Plt, B, and T cells derived from 2000 WT or Flk2^{-/-} MPPs transplanted into sublethally irradiated recipients. n = 9 per genotype, representing two independent experiments. Note the difference in x axis scale in panel A (weeks) and B (days). Differences were analyzed with two-tailed Student *t* test. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

Figure 4.

Flk2 expression does not account for differences in CFU-S efficiency between HSCs and MPPs. **(A)** Frequency of GM, megakaryocytes (Meg), and erythroid progenitors (EP) on day 11 CFU-S formed from transplanted HSCs or MPPs. **(B)** CFU-S frequency was not significantly different between transplanted Flk2^{-/-} and WT HSCs (100 cells; n = 9–10, representing two independent experiments), ST-HSCs (200 cells; n = 22, representing three separate experiments), or MPPs (500 cells; n = 18–19, representing three independent experiments). **(C)** Percent cell recovery in blood, spleen, and BM 3 hours after transplantation of HSCs (Tom⁺) and MPPs (GFP⁺) into lethally irradiated recipients (n = 13, representing three independent experiments). **(D)** Frequency of CFU-S observed on day 12 following injection of WT HSCs IV (100 cells) or IS (10 cells) into lethally irradiated recipients. **(E)** Frequency of CFU-S observed on day 11 following injection of WT MPPs IV (500 cells) or IS (50 cells) into lethally irradiated recipients. **(F)** Comparison of colony frequency of WT HSCs and MPPs following IV injection. **(G)** Comparison of CFU-S frequency of WT HSCs and MPPs following IS injection. n = 12–14 per condition,

representing three independent experiments. Differences were analyzed with two-tailed Student *t* test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 5.

Flk2 deficiency enhances survival in response to 5-FU, but impairs proliferation. (A) Percent survival of Flk2^{-/-} and WT mice over 8 weeks in response to treatment with 110 mg/kg 5-FU. Arrows indicate time of dosing. More Flk2^{-/-} mice survived after 8 weeks as compared with WT mice (Fisher exact test, $p < 0.05$). $n = 32$ – 33 per genotype, representing four independent experiments. (B) Representative microscope images of FACS-sorted HSCs, MPPs (WT and Flk2^{-/-}), and MyPro labeled with EdU 1 hour after injection. (C) Quantification of the percentage of cells labeled with EdU for each progenitor subtype.

Significantly less proliferation was observed in Flk2-deficient MPPs ($p < 0.01$, paired Student t test). $n = 6$ per genotype, representing three independent experiments. **(D)** Representative FACS plots of DNA content for WT and Flk2^{-/-} HSCs, ST-HSCs, MPPs, and myeloid progenitors (MyPro) as determined by propidium iodide staining. Brackets demarcate the percentage of cells in S/G2/M phase. **(E)** Quantification of the percentage of cells in S/G2/M phase for each progenitor subtype at steady state. $n = 5-7$ mice per genotype, representing three independent experiments. Differences were analyzed with two-tailed Student t test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.