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FLT3-ITD Knock-in Impairs Hematopoietic Stem Cell Quiescence/Homeostasis, Leading to Myeloproliferative Neoplasm

S. Haihua Chu^{1,*}, Diane Heiser^{1,4,*}, Li Li¹, Ian Kaplan^{1,2}, Michael Collector¹, David Huso^{1,3}, Saul J Sharkis¹, Curt Civin⁴, and Don Small^{1,2}

¹Department of Oncology; Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore MD 21231, USA

²Pediatric Oncology; Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore MD 21231, USA

³Molecular and Comparative Pathobiology; Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore MD 21231, USA

⁴Center for Stem Cell Biology & Regenerative Medicine, University of Maryland School of Medicine, Baltimore MD 21201, USA

SUMMARY

Internal tandem duplication (ITD) mutations within the FMS-Like Tyrosine Kinase gene (FLT3) render the receptor constitutively active, driving proliferation and survival in leukemic blasts. Expression of Flt3-ITD from the endogenous promoter in a murine knock-in model results in progenitor expansion and a myeloproliferative neoplasm. In this study, we show that this expansion begins with over-proliferation within a compartment of normally quiescent long-term hematopoietic stem cells (LT-HSCs), which become rapidly depleted. This depletion is reversible upon treatment with the small molecule inhibitor Sorafenib, which also ablates the disease. Although the normal LT-HSC has been defined as Flt3-negative by flow cytometric detection, we demonstrate that Flt3 is capable of playing a role within this compartment by examining the effects of constitutively activated Flt3-ITD. This indicates an important link between stem cell quiescence/homeostasis and myeloproliferative disease while also giving novel insight into the emergence of FLT3-ITD mutations in the evolution of leukemic transformation.

INTRODUCTION

Internal tandem duplications (ITD) in FMS-like tyrosine kinase-3 (FLT3) comprise a group of the most common molecular mutations in acute myelogenous leukemia (AML) (Gilliland DG, 2002; Levis M, 2003; Stirewalt DL, 2003). Found in approximately 23% of AML cases, an ITD mutation abrogates the negative regulatory function of the juxtamembrane domain, rendering the receptor constitutively active, independent of its natural ligand

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Correspondence: Dr Don Small, Department of Oncology, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University SOM, CRB 251, 1650 Orleans Street, Baltimore, MD 21231, USA. donsmall@jhmi.edu.

*these authors contributed equally to this work

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(Fenski R, 2000; Kiyoi H, 1998; Mizuki M, 2000). This oncogenic mutation activates canonical receptor tyrosine kinase signaling, most prominently via STAT5, RAS/MAPK and PI3K, stimulating proliferation and anti-apoptotic pathways (Hayakawa F, 2000; Kiyoi H, 1998; Mizuki M, 2000; Tse KF, 2001).

The presence of a FLT3-ITD mutation is a poor prognostic feature in AML, predicting increased relapse rates and reduced overall survival (Frohling S, 2002 Kottaridis PD, 2001; Levis M, 2003; Meshinchi S, 2001; Nakao M, 1996; Thiede C, 2002). Though there are many small molecule FLT3 tyrosine kinase inhibitors (TKI) in various stages of clinical trials, responses have been largely heterogeneous and transient (Grundler R, 2003; Knapper S, 2006; Levis M, 2011; Stone RM, 2005; Weisberg E, 2002). After an initial response, patients relapse, suggesting that leukemia-initiating stem cells may be escaping TKI-induced cytotoxicity.

There has been increasing evidence linking FLT3-ITD with leukemic stem cells (LSCs), including the presence of the mutation within the CD34⁺/CD38⁻ leukemia initiating cell fraction in most cases (Lapidot T, 1994; Levis M, 2005). The FLT3-ITD mutation also remains present at relapse in most cases, suggesting its presence in the cells that escape therapy (Cloos J, 2006; Shih LY, 2002). Furthermore, AML cases that have the FLT3-ITD mutation were shown to have the highest engraftment capacity in NOD/SCID mice (Lumkul R, 2002; Rombouts WJ, 2000a,b). Thus, understanding the role of FLT3-ITD mutations in LSCs is of great therapeutic interest.

FLT3 is an important molecule in normal hematopoietic development as well as in leukemia (Gilliland DG, 2002). FLT3 is expressed on common lymphoid progenitors (CLPs) and a minor fraction of common myeloid progenitors (CMPs) and is implicated in dendritic cell development (D'Amico A, 2003). Additionally, FLT3 is expressed during the early stages of hematopoiesis, indicating a potential involvement in stem cell function or maintenance. In murine hematopoiesis, where cell surface marker expression during stages of differentiation has been extremely well-defined, hematopoietic stem cell (HSC) activity is restricted to a small subset of the KSL compartment (Lin⁻ c-Kit⁺ Sca-1⁺) (Ikuta K, 1992; Spangrude G, 1988; Okada S, 1992; Katayama N, 1993). Yet, within this compartment, Flt3 is thought to be expressed only on multipotent progenitor cells (MPPs) (Christensen JL, 2001; Adolfsson J, 2001). Of the KSLs, MPPs have the lowest capacity for self-renewal and higher rates of proliferation (Passegué E, 2005), while the compartment defined as Flt3⁻ contains HSCs capable of short-term (CD34⁺) or long-term (CD34⁻) reconstitution of all hematopoietic lineages (Morrison SM, 1994; Osawa M, 1996; Randall, 1996). Thus the most primitive stem cell in the murine hematopoietic hierarchy, the Long-Term HSC (LT-HSC), has been classically defined by its lack of Flt3 expression. Flt3 has been previously reported to be dispensable for HSC maintenance and myeloid development (Sitnicka E, 2002) and Flt3 and Flt3-ligand (FL) knockout mice have only minor defects in HSC function (Mackarehshchian K, 1995; McKenna HJ, 2000). However, knockout studies fail to take into account the effects of overlapping or compensatory pathways, and few studies to date have addressed the effects of constitutive Flt3 activity on normal hematopoiesis or stem cell function.

In order to determine whether the Flt3-ITD mutation affects HSC function, our lab has generated a Flt3-ITD knock-in mouse model that has an 18bp insertion in the juxtamembrane domain of murine Flt3 (Li L, 2008). These mice express the germline ITD mutation under control of the endogenous murine Flt3 promoter and develop a myeloproliferative neoplasm (MPN) characterized by splenomegaly, leukocytosis and myeloid hypercellularity with a median survival of 10 months. Here, we describe isolation of an MPN-initiating stem cell within the signaling lymphocytic activation molecule (SLAM) compartment (Lin⁻CD48⁻CD150⁺) (Kiel M 2005) of Flt3-ITD knock-in marrow

that is capable of full engraftment and complete recapitulation of the MPN phenotype. Furthermore, we demonstrate that Flt3 transcripts and Flt3 protein are detectable in the SLAM-defined LT-HSC compartment and constitutive activation significantly perturbs HSC quiescence/homeostasis. We find that Flt3-ITD expression under the endogenous promoter results in changes in gene expression within the LT-HSC compartment including molecules involved in increased proliferation and activation of canonical FLT3 signaling pathways. Moreover, we demonstrate *in vivo* restoration of normal HSC function and simultaneous ablation of the MPN by treatment of mice with the FLT3 small molecule inhibitor Sorafenib. These data suggest a previously unrecognized role of Flt3 in LT-HSC homeostasis and demonstrates an intrinsic link between normal stem cell quiescence/homeostasis and MPN development as well as providing novel insights into the occurrence of Flt3 mutations in leukemogenesis.

RESULTS

Physiological expression of Flt3-ITD results in changes in composition of the hematopoietic stem-progenitor cell (HSPC) compartment

Bone marrow was harvested from mice expressing the Flt3-ITD mutation under the control of its endogenous promoter. When stained for cell surface marker expression, Flt3-ITD bone marrow consistently displayed a 2-10-fold expansion in the total number of lineage-negative progenitor cells as compared to wildtype (WT) littermate controls (Figure 1A,B, and F). Within this expanded compartment, Flt3-ITD marrow showed increased numbers of cells expressing both c-Kit and Sca-1, resulting in an approximate 4-8-fold increase in total number of KSLs - a population enriched for multipotent HSCs (Figure 1C,D) (Spangrude G, 1992; Okada S, 1992; Katayama N, 1993). Upon further breakdown of the KSL compartment by Flt3 and CD34 expression, Flt3-ITD mice appeared to have an increase in the frequency of multipotent progenitors (MPPs) as well as an uncharacterized population of cells expressing Lin⁻ Kit⁺ Sca⁺ Flt3⁺ CD34⁻ not observed at a significant frequency in WT littermates (Figure 1E).

An alternative method for phenotypic identification of HSCs eliminates Flt3 as a necessary surface marker by utilizing SLAM marker expression to identify these rare cells. SLAM-defined HSCs (Lin⁻ CD48⁻ CD150⁺) have previously been described as highly enriched for long-term engraftment capacity (Kiel M, 2005). Strikingly, Flt3-ITD mice consistently displayed a 2-5-fold decrease in this SLAM population despite increases observed in total bone marrow cellularity (Figure 1 F-H). This decrease was even greater for the KSL-SLAM compartment (Figure 1I,J) – believed to be even further enriched for LT-HSCs (Kiel M, 2005). This observed progenitor expansion with concomitant decrease in phenotypic LT-HSCs suggests that the expression of Flt3-ITD might perturb normal HSC homeostasis.

Another method for HSC identification is the frequency of side population (SP) cells as determined by MDR1-mediated Hoechst dye efflux, commonly associated with functional HSC activity (Goodell MA, 2004). Again Flt3-ITD bone marrow displayed a consistent 5-fold reduction in number of SP-defined HSCs (Figure 2A,B). When examined for overlap with cell surface marker phenotype, we observed that a significantly smaller proportion of Flt3-ITD KSL cells were SP cells, as compared to results in WT littermate controls (Figure 2C), suggesting that fewer Flt3-ITD KSLs were functional stem cells.

Flt3-ITD expression results in an observed reduction in HSC activity

To investigate the functional effects of the Flt3-ITD mutation on HSCs, we transplanted several cell surface marker-defined hematopoietic populations. Unfractionated, lineage-depleted and KSL bone marrow populations from Flt3-ITD mice demonstrated decreased

engraftment capacity in comparison to WT marrow (Figure 2D). The presence of the ITD mutation did not confer any abnormal long-term engraftment capacity to any progenitor population examined, including CMP, GMP, MPP, ST-HSC, or the uncharacterized KSL $\text{Flt3}^+\text{CD34}^-$ compartment (Table S1).

To explore the possibility that Flt3-ITD expression was affecting engraftment primarily by altering the homing ability of HSCs, intrafemoral injections were performed to bypass the need for HSCs to traffic to the bone marrow. As seen with intravenous injection, intrafemorally injected Flt3-ITD Lin^- cells resulted in significantly reduced engraftment levels when compared to WT controls (Figure S1). This data suggests that homing to the bone marrow is not the primary mechanism behind FLT3-ITD engraftment defects.

The Flt3-ITD MPN-initiating cell is contained within the SLAM compartment

As SLAM-defined HSCs have been previously shown to be highly enriched for cells with high long-term engraftment capacity (Kiel M, 2005), we hypothesized that the observed engraftment defect may be due to the depletion of this SLAM population (Figure 1G,H). Once sorted by lineage, CD150 and CD48 expression, WT and Flt3-ITD SLAM cells demonstrated equivalent engraftment capacity, suggesting that in this model, SLAM cells best represent the functional HSC (Figure 3A).

We next sought to determine whether Flt3-ITD SLAM cells were capable of recapitulating the MPN observed and previously reported in mice harboring the knocked-in mutation (Li L, 2008). Transplant recipients of either Flt3-ITD or WT sorted SLAM cells were sacrificed 8 weeks post-transplant and examined for signs of disease. Flt3-ITD SLAM recipients exhibited both splenomegaly and myeloid expansion (Figure 3B,D). In addition, transplant recipients recapitulated the stem cell immunophenotypic abnormalities observed in primary Flt3-ITD knock-in animals. Flt3-ITD SLAM recipients displayed a significant expansion in lineage-negative and myeloid progenitor cells, as observed in the donor mice (Figure 3C, D). Interestingly, despite receiving an equivalent number of donor SLAM cells, Flt3-ITD recipients demonstrated a 2-4 fold depletion of the SLAM compartment by 8 weeks post-transplant (Figure 3E). These data reveal that Flt3-ITD SLAM cells have the ability not only to engraft, but also to autonomously lead to the development of a MPN.

Flt3 mRNA and Flt3 protein are present at detectable levels in long-term engrafting SLAM cells

In mice, the long-term hematopoietic stem cell has been classically defined as having a $\text{Lin}^-\text{Kit}^+\text{Sca}^+\text{CD34}^-\text{Flt3}^-$ immunophenotype (Christensen JL, 2001; Osawa M, 1996). Due to the observed defect in engraftment in the Flt3-ITD knock-in mice and the correlated depletion of the SLAM compartment, we hypothesized that although Flt3 surface protein may not be abundant enough for detection by flow cytometry in the LT-HSC, low levels of expression may still play a role in stem cell homeostasis. To determine whether Flt3 was transcribed in the long-term engrafting and MPN-initiating stem cell, SLAM cells from both WT and Flt3-ITD mice were sorted and *Flt3* transcript levels were quantified by qRT-PCR. *Flt3* transcripts were detectable in both WT and Flt3-ITD SLAM cells, with an approximate 4-fold increase in *Flt3* mRNA expression in the Flt3-ITD SLAM compartment (Figure 4A).

As Flt3 protein is thought to be absent or undetectable on the cell surface of classically defined primitive LT-HSCs we were interested in determining if the observed levels of *Flt3* mRNA resulted in detectable Flt3 protein. When we measured the levels of total Flt3 protein (including intracellular) in the SLAM-defined HSC compartments of WT and Flt3-ITD mice, we were able to detect Flt3 protein in a fraction of both SLAM compartments (Figure 4B).

The effect of Flt3-ITD on LT-HSC homeostasis is cell autonomous

We next sought to determine whether our observed Flt3-ITD phenotype in the LT-HSC was due to its direct expression within these primitive stem cells or if the effect was an indirect microenvironmental one (i.e. through cytokine/growth factor release from Flt3-ITD expressing cells in the bulk bone marrow or stromal environment). We transplanted recipient mice with a mixture of WT and Flt3-ITD Lin⁻ bone marrow cells that resulted in approximately equal numbers of WT and Flt3-ITD SLAM cells. We could then examine the effects on both stem cell populations in a single environment (in the presence of both WT and Flt3-ITD marrow). At 10 weeks post-transplant, bone marrow from these mice was examined for the presence of WT (CD45.1) and Flt3-ITD (CD45.2) SLAM cells. Strikingly, only the Flt3-ITD SLAM cells were depleted, with the frequency of WT SLAM cells remaining at a normal level (Figure 4C). Within a single recipient, WT SLAM cells were present at frequencies ranging from 3 to 20-fold higher than Flt3-ITD SLAM cells (Table S2), suggesting that the observed SLAM depletion was a direct cell autonomous effect.

Constitutive Flt3 activation leads to increased proliferation within the normally quiescent LT-HSC compartment

In an effort to determine the mechanism of Flt3-ITD-driven SLAM cell depletion, we hypothesized that the oncogenic mutation may drive these normally quiescent cells to proliferate. To further investigate, we measured HSC proliferation rates in WT and Flt3-ITD bone marrow through *in vivo* BrdU incorporation assays. Bone marrow was harvested 4 hours post BrdU injection and co-stained with cell surface markers to examine proliferation in multiple HSPC subsets. Despite observed expansion of the Lin⁻ subset, Flt3-ITD mice unexpectedly displayed decreased BrdU incorporation in this fraction (Figure 5A-C). In striking contrast however, subsets more highly enriched for LT-HSCs from Flt3-ITD mice demonstrated an unusually high level of BrdU incorporation. Flt3-ITD KSLs and KSL-SLAM cells had a greater than 2-fold increase in BrdU incorporation rate over WT controls. To further establish whether the Flt3-ITD mutation was driving normally quiescent HSCs to proliferate, we examined cell cycle status by measuring PI staining in stem cell subsets. While WT and FLT3-ITD Lin⁻ progenitors demonstrated equivalent numbers of cells in S +G2/M phase, Flt3-ITD SLAM cells showed significant increases in cycling cells as compared to WT littermates (Figure 5D).

Downstream targets of Flt3 are abnormally activated in Flt3-ITD SLAM cells

To further validate that the observed expression of Flt3-ITD in the hematopoietic stem cell resulted in a functional signal, we measured the activation of known Flt3 downstream signaling molecules in these cells. As FLT3-ITD signaling is known to activate the STAT5 transcription factor (Hayakawa F, 2000), we measured the expression of Stat5 targets by qRT-PCR in Flt3-ITD SLAM cells (Basham B, 2008). When compared to WT SLAM cells, several Stat5 targets were significantly overexpressed in the Flt3-ITD SLAM compartment - most prominently *Fos*, *JunB*, *Mkp-1*, and *Socs3* (Figure 5E). Additionally, we examined several genes that have been found to be upregulated by FLT3-ITD signaling in CD34⁺ cells transduced with Flt3-ITD (Kim KT, 2007; Li L, 2007). We found that *Pim1* was overexpressed on average 5-fold within the Flt3-ITD SLAM compartment compared to WT SLAM cells. Finally, a positive regulator of cell cycle progression *Cyclin F* was found to be overexpressed in the Flt3-ITD SLAM compartment, while negative cell cycle regulator *p57* was downregulated. This data indicates that constitutively activated Flt3 alters gene expression patterns and proliferation status within the most primitive compartment of HSCs.

Inhibition of Flt3-ITD signaling using the small molecule inhibitor Sorafenib leads to restoration of normal stem cell activity and ablation of the MPN

As the depletion of LT-HSCs in the ITD mice is driven by Flt3-ITD signaling, we hypothesized that attenuating Flt3-ITD signaling may restore normal stem cell activity in the knock-in mice. Sorafenib has been shown to have significant activity against FLT3 (Auclair D, 2007; Zhang W, 2008), and is approved for use in targeting Raf-1 in renal cell carcinoma (Escudier B, 2007). Several clinical trials involving Sorafenib have shown promising activity in AML patients with FLT3 mutations (Safaian NN, 2009; Tong FK, 2006; Ravandi F, 2010; Crump M, 2010). Sorafenib also appears to have selective inhibitory effects on mutant FLT3-ITD over WT FLT3 (Zhang W, 2008). Treating mice with Sorafenib has been shown to induce regression of MV4;11 (a human AML cell line expressing FLT3-ITD) tumors in nude mice (Auclair D, 2007).

To determine whether the loss of LT-HSCs observed in Flt3-ITD mice could be prevented, we sought to attenuate Flt3-ITD signaling early in development. WT females were bred to males heterozygous for the mutation, and pregnant mothers were treated with Sorafenib (20mg/kg) beginning at day 15 of pregnancy. Mothers were continually treated during a 2-week feeding period after giving birth, enabling pups to receive Sorafenib doses through breast milk. At 2 weeks of age, Flt3-ITD pups from untreated mothers displayed splenomegaly (Figure 6A), myeloid expansion (Figure 6B, C), a 5-fold reduction in KSL-SLAM cells and a 2-fold reduction in SLAM cells. In contrast, Flt3-ITD pups from treated mothers displayed no clinical signs of disease and their bone marrow contained equivalent numbers of SLAM and KSL-SLAM cells to those of WT littermates (Figure 6D, E).

Having demonstrated that stem cell numbers could be preserved and MPN prevented if mice were treated during development of the hematopoietic system, we next examined whether HSC numbers and function could be restored after the MPN was already established in adult mice. Adult heterozygous mice were treated with 10mg/kg Sorafenib for two weeks. Post-treatment, these mice demonstrated near complete resolution of the MPN phenotype, including a reduction in spleen size (Figure 7A) and a decrease in progenitor numbers within the Lin⁻ and KSL compartments to levels comparable to WT controls (Figure 7B, C). Strikingly, the numbers of SLAM and KSL-SLAM cells were also largely restored to the levels of WT controls (Figure 7D, E). Histopathology of the BM and spleen also demonstrated the restoration of cellularity and architecture in these tissues in the Flt3-ITD treated mice (Figure 7G). In spleens from Flt3-ITD mice treated with Sorafenib, we observed reductions in overall cellularity, nucleated cells and white pulp areas as well as a restoration of red pulp areas. The marrow of Flt3-ITD treated mice displayed decreased cellularity, collapsing venous sinuses and restoration of the number of mature red blood cells in the marrow (Figure 7G). Thus, small molecule inhibition of Flt3-ITD simultaneously led to reversal of disease phenotype and restoration of normal stem cell homeostasis in adult mice.

To ensure that the observed increase in phenotypic SLAM cells was also indicative of a restoration of stem cell function, Lin⁻ progenitors from treated and untreated WT and Flt3-ITD mice were transplanted into lethally irradiated recipients. Recipient mice were treated with 10 mg/kg Sorafenib for an additional 2 weeks after irradiation to continue inhibition of Flt3-ITD signaling during the engraftment period. At 4 weeks post-transplant, in contrast to untreated Flt3-ITD marrow, treated Flt3-ITD Lin⁻ bone marrow demonstrated greatly increased engraftment capacity, equivalent to that of WT controls (Figure 7F).

DISCUSSION

This model, in which the Flt3-ITD mutation is knocked-in to the murine gene under the control of its endogenous promoter, provides a powerful tool to study the function of constitutive FLT3 activity in the murine hematopoietic system. Our results demonstrate that the Flt3-ITD mutation results in major perturbations in HSPC composition as well as functional defects in stem cell homeostasis and reconstitution capacity. The observed functional alterations in the LT-HSCs point to a previously unrecognized role for Flt3 in governing the dynamics of this population.

These results are somewhat surprising in light of the previous paradigm that Flt3 protein is undetectable by flow cytometry on the cell surface of LT-HSCs. Flt3 knock-out mice display minor defects in stem cell fitness, revealed only through serial competitive transplant experiments (Mackarehshian K, 1995). Additionally, previous studies seem to rule out Flt3 and its ligand as critical regulators of HSC maintenance and post-transplantation expansion in Flt3 and FL knockout mice (Buza-Vidas N, 2009). However, Flt3 signaling is known to partially overlap with other receptors (i.e. c-Kit) and compensatory regulation by these other pathways may explain why the role of Flt3 in stem cell function has been masked in previous knock-out studies.

Multipotent progenitors with detectable surface Flt3 are thought to have less self-renewal capacity, yet give rise to all hematopoietic lineages including myeloid progenitors as well as lymphoid-primed multipotent progenitors (Christensen JL, 2001; Adolfsson J, 2001; Boyer SW, 2011). However, recent data from Flt3-cre driven fate mapping has suggested that *Flt3* transcriptional activity initiates in the phenotypic KSL-SLAM HSC compartment (Buza-Vidas N, 2011). This raises the possibility that Flt3 can be expressed and potentially functional without abundant cell surface protein present, as the limit of sensitivity for conventional FACS detection is typically 2000 molecules/cell (Zola H 2004). These and our data do not preclude the possibility that these Flt3⁺ cells within the SLAM and KSL-SLAM compartments are non-self-renewing progenitors. Rather, our results demonstrate that *Flt3* mRNA, total Flt3 protein (including intracellular), and activation of Flt3 signaling are all detectable within SLAM-defined LT-HSCs. These results are congruent with the previous paradigm defining LT-HSCs as Flt3-negative, in that Flt3 cell surface protein may not be present at a level detectable by flow cytometry in these cells. Our mouse model demonstrates a comparison of Flt3 constitutive activity in the HSC to that of genetically identical siblings and that Flt3 is capable of playing a functional role in this compartment, despite its low expression levels.

WT Flt3 must homodimerize in the presence of ligand to propagate downstream signaling. Flt3-ITD molecules in contrast, are constitutively active and can homodimerize or heterodimerize with WT molecules, resulting in activation of downstream effectors in the absence of ligand (Kiyoi H, 2002, Koch S, 2008; Choudhary C, 2009). This allows even small amounts of the activated mutant receptor to exert strong effects on signaling. This property of Flt3-ITD may explain the strong phenotype seen in our mouse model despite low levels of cell surface protein, as well as why the role of Flt3 in LT-HSCs would be difficult to observe in the absence of a constitutively-active mutant.

Our model demonstrates that Flt3-ITD is acting within the LT-HSC itself as opposed to exerting its effects on this population through the microenvironment. After a mixed bone marrow transplant we produced recipients harboring both WT and Flt3-ITD hematopoiesis within a single bone marrow environment. We observed depletion of only the ITD SLAM cells and not the WT SLAM cells from this mixed environment. This data suggests that the

depletion of the SLAM compartment in ITD mice is not the result of secondary environmental effects but rather a direct effect of the mutation in this compartment.

As further evidence of Flt3 activity within this extremely rare LT-HSC population, we noted a significant increase in known downstream signaling, including increased expression of Stat5 targets and cell cycle promoters, along with a decrease in cell cycle inhibitor *p57*. The result of Flt3-ITD signaling in the LT-HSC is a depletion of the pool of primitive stem cells. This oncogenic mutation disrupts HSC homeostasis, driving increased proliferation within this normally quiescent compartment and causing a greater proportion of HSCs to enter the cell cycle. This result is even more striking within the context of an overall decreased proliferation rate in Flt3-ITD Lin⁻ bulk progenitors. The general decreased proliferation may be due to negative feedback signals resulting from an abundance of myeloid progenitors present in the blood and marrow. Aberrant over-proliferation of the normally quiescent stem cell compartment leads to HSC exhaustion (Orford KW, 2008; Akala OO, 2008). In our model, it also results in the overproduction of various lineages (particularly of myeloid origin) that give rise to our MPN phenotype, highlighting once again the essential link between quiescence, HSC homeostasis and disease progression.

When Flt3-ITD signaling is attenuated through treatment with the small molecule inhibitor Sorafenib, the intricate link between stem cell homeostasis and disease becomes even more apparent. The restoration of stem cell numbers after only 2 weeks of treatment demonstrates the surprising resiliency and plasticity of hematopoietic stem cells. Although LT-HSCs normally divide only once every five months, there is growing evidence that this dynamic population is capable of rapidly responding to injury and then quickly returning to a largely quiescent steady state (Wilson A, 2008). The rapid response of Flt3-ITD-harboring LT-HSCs to Sorafenib treatment reveals a surprising level of sensitivity within the hematopoietic system. While Flt3 inhibition appears non-toxic to normal HSCs, we demonstrate that Sorafenib is capable of targeting signaling within a rare population of MPN-initiating SLAM cells in Flt3-ITD mice, indicating the drug may be capable of targeting FLT3-ITD-containing leukemic stem cells in AML patients as well.

While the role of FLT3 is less completely understood in human hematopoiesis than in murine hematopoiesis, there is growing evidence that FLT3 is present in both normal human HSCs and AML LSCs (Kikushige Y, 2008; Sitnicka E, 2003; Small D, 1994; Levis M, 2005). While FLT3-ITD mutations are present in only about 5% of MDS cases, the mutation is more frequently observed at the time of progression to AML, suggesting a role in transformation from MDS to AML (Cloos J, 2006; Shih LY, 2002; Pinheiro RF, 2008). In the majority of AML cases, ITD mutations are present in *de novo* disease with normal cytogenetics. These patients achieve similar remission rates to patients lacking the mutation, yet FLT3-ITD patients are more likely to relapse, with a median survival of less than 5 months post-relapse (Kottaridis PD, 2001; Frohling S, 2002; Schnittger S, 2002; Thiede C, 2002; Ravandi, 2010; Levis, 2011). While FLT3-ITD alone does not fully transform a cell to leukemia, growing evidence implicates this mutation in the process of disease progression and LSC survival and maintenance (Pinheiro RF, 2008; Yoshimoto G, 2009). We and others have shown that the combination of Flt3-ITD with other oncogenic mutations can lead to the development of a full-blown leukemia in mice (Stubbs MC, 2008; Greenblatt S, 2012) lending credence to the “two-hit” model of leukemogenesis (Gilliland G, 2002). Our knock-in model studies suggest that one reason Flt3-ITD mutations are not sufficient to induce a leukemia may be due to ITD-driven depletion of the LT-HSC compartment. Thus, ITD mutations in an otherwise normal LT-HSC are unlikely to result in leukemia as they would instead lead to rapid elimination of the clone, with strong selective pressure against stem cells bearing this mutation. In other stages of development or in the presence of additional genetic lesions however, FLT3-ITD may instead cooperate to confer proliferative

advantages (Stubbs MC, 2008). This may explain why Flt3 mutations do not seem to be early hits in primitive HSC compartments but rather acquired in later stages of leukemogenesis in human AML. This may also explain recent findings suggesting Flt3-ITD mutations are not present in human pre-leukemic clones, further implicating this mutation as a secondary hit (Jan M, 2011).

Novel insight into the role of FLT3 activating mutations in the overall etiology of transformation to leukemia may reveal new potential therapeutic strategies. In order to effectively target this molecule in AML patients it becomes necessary to consider the function of FLT3 in both disease development and stem cell maintenance, as well as the oncogenic dependence of leukemic blasts on FLT3 signaling for continued survival and proliferation. In LSCs strongly dependent on FLT3 signaling, inhibition may allow opportunities for blast clearance. In other cases FLT3 inhibition with sorafenib may simply return LSCs to a more normal proliferative state. As such, in the case of human disease, FLT3 may be considered for long-term combination therapy or even chronic inhibition, increasing the likelihood of durable remissions.

EXPERIMENTAL PROCEDURES

Mice

Generation of mice harboring a Flt3-ITD mutation knocked-in to the endogenous Flt3 locus has been previously described (Li L, 2008). The mice used in these experiments were bred with a CMV-Cre line to flox out the neo selection cassette. Except for Sorafenib treated pups that were 2 weeks of age, all mice were evaluated at 6-10 weeks of age with age-matched, littermate controls. All experiments were performed in accordance with IACUC-approved protocols.

Bone Marrow Isolation

Bone marrow was harvested by crushing tibias, femurs, hips and spine of each mouse with a mortar and pestle (Lo Celso, 2007). Red blood cell lysis was performed according to the manufacturer's protocol using RBC lysis buffer (eBioscience).

Transplantation

Congenic C57BL/6 CD45.1⁺ recipient mice were irradiated with a single dose at a lethal (10 cGy) level, delivered >4 hours before injections. For *in vivo* intrafemoral injections, cells were injected into the femoral cavity of anesthetized mice with a Hamilton needle (Sigma Aldrich). Sca1 depleted helper cells were obtained from double positive CD45.1+CD45.2+ bone marrow flushed from femurs stained with Sca1-APC and depleted using anti-APC microbeads (Miltenyi Biotech) bound to LD columns (Miltenyi Biotech). Similarly, lineage depletions were conducted using biotinylated monoclonal antibodies (Ter119, Gr-1, B220, and CD3) (BD Biosciences) and depleted with anti-biotin microbeads (Miltenyi Biotech).

To create chimeric mice harboring both Flt3-ITD and WT hematopoiesis, recipients were injected with CD45.2 Flt3-ITD and CD45.1 WT Lin⁻ cells in a 4:1 (ITD:WT) ratio. Donor contributions were assessed by FACS post-transplant (Table S4).

Sorafenib treatment

Sorafenib was formulated and administered as previously described (Li L, 2011). Adult mice were orally gavaged at a dose of 10mg/kg daily for 2 weeks with a schedule of 5 days on and 2 days off and sacrificed immediately for analysis after the 2 week period. For functional studies after treatment, Lin⁻ BM was isolated by magnetic cell separation as described above and 100,000 Lin⁻ cells were injected with 1×10⁶ red blood cell lysed WT

CD45.1 WBM helper cells. For the *in utero* experiment, pregnant mothers were gavaged with 20mg/kg of Sorafenib starting at E.15 and continuing for 2 weeks after birth of pups.

Flow cytometry and cell sorting

Cell preparation, staining and analysis were performed as previously described (Li L, 2008). . Sorting of different hematopoietic subpopulations was also conducted on a FACS Aria (BD Biosciences) and analyzed using FlowJo software (Flow Jo). Side population staining was conducted as previously described with Hoechst 33342 (Invitrogen) (Goodell MA, 2004) and analyzed on a MoFlow Cytometer (Dako Cytomation).

BrdU and Flt3 intracellular staining

BrdU incorporation experiments were carried out using a BrdU Assay kit (BD Biosciences) with cell surface staining prior to fixation, permeabilization and DNase treatment. Each of the three independent experiments was conducted as per manufacturer's protocol.

Flt3 intracellular stains were conducted with the FoxP3/Transcription Factor Staining Buffer set (eBiosciences). Flt3-PE antibody (BD Biosciences) and PE Rat IgG2a κ (BD Biosciences) isotype control was used for these experiments.

RNA preparation and Quantitative Polymerase Chain Reaction

RNA was isolated by using a miRNeasy Mini RNA kit (Qiagen). cDNA synthesis and qPCR was performed as described previously using a CFX-96 RealTime PCR system (BioRad) (Li L, 2008). Primers for genes are listed in the supplemental data (Table S4). RPS16 was used as an internal loading control. Each PCR was run in triplicate and gene expression values are expressed as a fold change calculated by $2^{-\Delta C_t}$. A total of three independent PCR reactions were run for each gene target.

Statistical Analysis

All p values were calculated using a student's t-test, two tailed, unpaired analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Flt3-ITD perturbs normal mouse hematopoietic stem cell homeostasis
- Flt3 is expressed and capable of exerting functional effects in murine LT-HSCs
- Flt3-ITD leads to depletion of murine LT-HSCs through loss of quiescence
- MPN and HSC defects are simultaneously reversed by Sorafenib treatment

Figure 1. Expression of FLT3-ITD from the endogenous promoter disrupts HSPC composition in knock-in mice

Representative flow cytometric analyses and quantification of HSPC populations in bone marrow from WT and FLT3-ITD littermates. (A, B) Lineage negative compartment (C, D) KSLs (Lin⁻ c-kit⁺ sca-1⁺) (E) LT-HSC, ST-HSC, and MPP populations by CD34 and Flt3 expression within the KSL population (F) Total bone marrow cellularity (G, H) SLAM populations (Lin⁻ CD150⁺CD48⁻) (I, J) KSL-SLAM populations (Lin⁻ c-kit⁺ sca-1⁺ CD150⁺ CD48⁻) Data is representative of 3 independent experiments. p<0.05 (*)

Figure 2. FLT3-ITD bone marrow demonstrates reduced Side Population and HSC function (A) Representative flow analysis of Side Population cells by Hoechst dye efflux in WT and FLT3-ITD mice (n=6) (B) verapamil control (C) overlap of Hoechst efflux activity within KSL populations (n=6) (D) Lethally irradiated recipient mice (CD45.1) were injected with CD45.2 bone marrow cells from WT or FLT3-ITD donors. For total bone marrow (tBM) experiments 2×10^6 tBM donor cells were injected with 5×10^5 CD45.1 helper cells. For Lin⁻ and KSL transplants, 1×10^5 Lin⁻ cells or 5,000 KSLs were injected with 1×10^6 WT CD45.1 tBM helper cells (n=5 mice for each group) Mice were bled at 4 weeks post-transplant and analyzed for ratio of CD45.2 to CD45.1 in peripheral blood. Data is representative of 3 independent experiments. $p < 0.05$ (*), $p < 0.01$ (**), and not significant (ns).

Figure 3. Transplantation of SLAM cells recapitulates MPN phenotypes
 (A) Transplantation of either 500 WT or ITD CD45.2 SLAM sorted cells ($\text{Lin}^- \text{CD150}^+ \text{CD48}^-$) into lethally irradiated recipients (CD45.1) with 100,000 Sca-1 depleted CD45.1 helper cells (n=4 for WT mice, n=5 ITD mice). Peripheral blood was measured for CD45.2 contribution at each time point shown. These data are representative of 2 independent experiments. (B) Representative spleen weights from primary transplants (C) Percent of WBM staining negative for Lineage markers (D) Percent of WBM positive for myeloid markers Mac-1 and Gr-1 (E) SLAM cell percentage of WBM. Mice were sacrificed and analyzed for disease progression 8 weeks post-transplant. These data are representative of 2 independent experiments with n= 4 mice. $p < 0.05$ (*) and $p < 0.01$ (**)

Figure 4. Flt3 is detected in SLAM cells and capable of exerting functional effects in LT-HSCs (A) *Flt3* expression measured by quantitative PCR in sorted SLAM cells from WT or Flt3-ITD mice. Results shown are the average of three independent experiments + SEM, normalized to internal control RPS16. Data is normalized to baseline *Flt3* expression in WT SLAM cells. $p < 0.01$. (B) Representative flow analysis of Lin⁻ and SLAM cell compartment in for expression of total Flt3 protein (surface and intracellular). Results shown are compared to isotype controls. Data is representative of 3 independent experiments. $p < 0.01$ (Lin⁻), $p < 0.05$ (SLAM) (C) Representative flow analysis of SLAM cell frequency in bone marrow of chimeric mice containing both WT (CD45.1) and Flt3-ITD (CD45.2) bone marrow (n=4 mice). Data is representative of 3 independent experiments, see also Table S2. $p < 0.05$

Figure 5. Flt3-ITD HSCs demonstrate increased proliferation and cell cycle entry
 Bone marrow was harvested from (A) WT and (B) Flt3-ITD mice 24 hours after BrdU injection. FACS plots represent 2-3 pooled mice and are representative of data from two independent experiments. Lineage-depleted marrow was stained for KSL and SLAM markers as well as for BrdU incorporation. (C) Quantification of BrdU incorporation in Lin⁻, KSL, and KSL-SLAM stem cell subsets. (D) PI staining of Lin⁻ progenitors or SLAM cells from one representative experiment of WT vs. Flt3-ITD mice. Gates shown represent percentage of cells in S+G2/M phase. Data is representative of three independent experiments and summarized with SEM. (E) Quantitative PCR of predicted FLT3 targets, Stat5 targets and cell cycle regulators. SLAM cells were sorted from pools of 3-4 WT or Flt3-ITD mice, and results shown are the average of three independent experiments + SEM. All readings were normalized to internal control RPS16 and set to a baseline of 1 for expression in WT SLAM cells. p<0.05(*), p<0.01(**) and not significant (ns).

Figure 6. Early treatment with Sorafenib prevents MPN and preserves stem cell numbers
 WT females were bred to a heterozygous Flt3-ITD male and treated with Sorafenib starting at E.15 through weaning of pups at 2 weeks of age.. Data is representative of three independent experiments. (A) Spleens harvested from untreated pups vs. those treated with Sorafenib. Flt3-ITD mice are noted with (#). Spleen weight is quantified by genotype in right panel. (B, C, D, E) Quantification of percentage of bone marrow comprising (B) Lin⁺ progenitors, (C) KSLs, (D) SLAM cells, or (E) KSL-SLAM cells in treated vs. untreated WT and Flt3-ITD mice. p<0.05 (*) and p<0.01 (**).

Figure 7. Treatment of adult mice with Sorafenib restores stem cell activity and reverses signs of disease

Adult mice 8 weeks of age were treated daily with Sorafenib (sb) for 2 weeks. Data represents averages of 4 mice per group and is representative of two independent experiments. (A) Quantification of spleen weight for treated vs. untreated adult mice. (B, C, D, E) Quantification of percentage of bone marrow comprising (B) Lin⁻ progenitors, (C) KSLs, (D) SLAM cells, or (E) KSL-SLAM cells in treated vs. untreated adult mice. (F) 100,000 CD45.2 lineage-depleted donor cells from treated or untreated WT or Flt3-ITD mice were transplanted along with 1×10^6 CD45.1 whole bone marrow helper cells into lethally irradiated CD45.1 recipients. Peripheral blood was analyzed for percent donor engraftment 4 weeks post-transplant. p value of WT and WT+sb was not significant ($p > 0.05$). (G) H&E histopathology showing bone marrow (top row) and spleen (bottom row) architecture in treated and untreated adult WT and Flt3-ITD mice (WP = White Pulp, RP = Red Pulp). $p < 0.05$ (*), $p < 0.01$ (**) and not significant (ns)