Physiological *Jak2V617F* Expression Causes a Lethal Myeloproliferative Neoplasm with Differential Effects on Hematopoietic Stem and Progenitor Cells

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SUMMARY

We report a *Jak2V617F* knockin mouse myeloproliferative neoplasm (MPN) model resembling human polycythemia vera (PV). The MPN is serially transplantable and we demonstrate that the hematopoietic stem cell (HSC) compartment has the unique capacity for disease initiation but does not have a significant selective competitive advantage over wild-type HSCs. In contrast, myeloid progenitor populations are expanded and skewed toward the erythroid lineage, but cannot transplant the disease. Treatment with a JAK2 kinase inhibitor ameliorated the MPN phenotype, but did not eliminate the disease-initiating population. These findings provide insights into the consequences of JAK2 activation on HSC differentiation and function and have the potential to inform therapeutic approaches to *JAK2V617F*-positive MPN.

INTRODUCTION

The *JAK2V617F* mutation is the most common molecular abnormality in *BCR-ABL*-negative MPN and is present in approximately 95% of patients with PV and in approximately 50% of patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF) (Baxter et al., 2005). *JAK2V617F* is present at low frequency in other myeloid malignancies and is not observed at all in lymphoid neoplasms (Steensma et al., 2005; Levine et al., 2005a). This acquired point mutation in the *JAK2* gene results in a valine to phenylalanine substitution at position 617 and constitutive activation of JAK2 kinase signaling (James et al., 2005; Kralovics et al., 2005; Baxter et al., 2005; Levine et al.,

2005b). Overexpression of *JAK2V617F* confers interleukin-3 (IL-3) independence to Ba/F3 cells that coexpress a homodimeric type I cytokine receptor, such as the erythropoietin receptor (EpoR) (Lu et al., 2005). Transplantation of *JAK2V617F*-overexpressing hematopoietic cells into mice is sufficient to recapitulate a PV disease phenotype (Wernig et al., 2006; Lacout et al., 2006; Zaleskas et al., 2006). In aggregate, JAK2 therefore represents an excellent therapeutic target in MPN patients.

So that MPN could be cured in human patients, it may be necessary to eradicate all *JAK2V617F* mutated hematopoietic cells that have the capacity to self-renew and thus maintain disease. In this context, it is therefore imperative to understand the precise role and function of the *JAK2V617F* allele as it relates to hematopoietic

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Significance

The *JAK2V617F* mutation is a promising candidate for molecularly targeted therapy in MPN. Early data from JAK2 inhibitor clinical trials have called into question the capacity of these compounds to alter the natural history of *JAK2V617F*-mediated MPN. Determining the effect of JAK2 inhibitors on the disease-initiating population requires a model in which the *JAK2V617F* allele is expressed at physiological levels in hematopoietic stem and progenitor cells, as it is in humans. Our model demonstrates that *JAK2V617F* causes expansion of erythroid progenitors but that only the HSC compartment can initiate disease in a transplanted mouse. We further demonstrate that the HSC compartment, the definitive target for curative therapy of *JAK2V617F*-mediated MPN, is resistant to treatment with a JAK2 inhibitor.

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stem and multipotent progenitor cells (HSPCs). In MPN patients, *JAK2V617F* is detectable in CD34^{+} CD38^{−} hematopoietic stem cells (HSCs) (Jamieson et al., 2006) and in all mature cell lineages (Ishii et al., 2006; Delhommeau et al., 2007). However, detailed functional characterization of *JAK2V617F* HSPCs has been limited in existing retroviral and transgenic murine models (Wernig et al., 2006; Lacout et al., 2006; Zaleskas et al., 2006; Tiedt et al., 2008; Xing et al., 2008; Shide et al., 2008) and has been described only in the nonobese diabetic-severe combined immunodeficient (NOD/SCID) murine model to date (James et al., 2008). Although the retroviral *JAK2V617F* model has been informative (Wernig et al., 2006; Lacout et al., 2006; Zaleskas et al., 2006), it is subject to the problems inherent to retroviral-mediated transduction, including the identity of the transduced cells, with preferential transduction of mitotic progenitor cells relative to quiescent long-term HSCs and the nonphysiologic level of oncogene expression. These factors may ultimately affect the resultant biological and phenotypic outcome in these models (Ren, 2004). Transgenic model systems also have nonphysiologic expression of the oncogene resulting from increased copy number and the use of exogenous promoters.

Over the course of a decade of treating chronic myelogenous leukemia (CML) with imatinib, it has become evident that cure is difficult to achieve as a consequence of a reservoir of disease-initiating cells contained within the quiescent HSC compartment (Holyoake et al., 1999; Graham et al., 2002; Michor et al., 2005). Furthermore, preliminary results from small molecule JAK2 kinase inhibitor trials suggest that curative therapy of *BCR-ABL*-negative MPN may prove even more difficult (Pardanani et al., 2009; Verstovsek et al., 2009). An accurate knowledge of the early changes that occur in the HSPC compartment directly as a result of acquisition of the *JAK2V617F* mutation is therefore critical in determining the curative potential of therapies that target this molecular event.

We describe a *Jak2V617F* knockin model in which expression of *Jak2V617F* is under the control of the endogenous murine *Jak2* promoter; we provide detailed analysis of the effects of the *Jak2V617F* allele on hematopoietic stem and progenitor cells; and we evaluate the efficacy of a small molecule JAK2 inhibitor in vivo.

RESULTS

Expression of *Jak2V617F* from Its Endogenous Promoter Results in a Lethal MPN Resembling Human Polycythemia Vera

We generated a *Jak2V617F* conditional knockin allele by gene targeting in mouse embryonic stem cells (Figure 1A). The resulting chimeric animals were crossed with wild-type (WT) C57Bl/6 mice to generate floxed heterozygous *Jak2V617F* mice. Floxed heterozygous *Jak2V617F* animals were then crossed with E2ACre transgenic mice to induce germline *Jak2V617F* expression during early mouse embryogenesis (Lakso et al., 1996). Inversion of the floxed knockin allele and excision of the wild-type exon were confirmed by PCR (Figure 1B), and expression of the G1849T mutation (that results in a valine to phenylalanine substitution at amino acid position 617, *Jak2V617F*) was confirmed by cDNA sequencing (Figure 1C). These heterozygous *Jak2V617F* germline expressing animals (Jak2[+/VF]) devel-

oped a lethal MPN that was 100% penetrant with a median survival of 146 days (Figure 1D). The MPN was characterized by an elevated hematocrit (HCT) (Figure 1F), splenomegaly (Figure 1G; Figure S1A available online), and prominent splenic extramedullary erythropoiesis (Figure 1E). Flow-cytometric analysis showed increased CD71^{+} Ter119^{+} erythroid precursors in Jak2[+/VF] bone marrow (BM) (Figure S1B) and spleen (Figure 1H). Histopathology demonstrated marked erythroid and mild megakaryocytic hyperplasia within the Jak2[+/VF] splenic red pulp with overall effacement of the normal splenic architecture (Figure 1I). The Jak2[+/VF] BM showed a milder increase in erythroid elements compared to the spleen but demonstrated megakaryocytes with atypical nuclear features and prominent emperipolesis (Figure 1I). CD41^{+} cells were increased in Jak2[+/VF] BM (p = 0.05), platelet counts were not increased, and no differences were observed in megakaryocyte ploidy between Jak2[+/VF] and Jak2^{+/+} mice (Figures S1C–S1E). WBC counts were increased in Jak2[+/VF] mice (p < 0.0001) although we did not observe an increase in Mac1^{+}Gr1^{+} or Mac1^{+} cells relative to total Jak2[+/VF] BM cells (Figures S1F–S1H). Reticulin fibrosis was absent in both Jak2[+/VF] BM and spleen, even in mice that were 6 months old (Figure 1J), and the development of acute leukemia was not observed in any animals. In aggregate, these findings demonstrate that Jak2[+/VF] knockin mice develop a MPN reminiscent of human PV with a short disease latency and reduced survival.

Erythroid Skewing in the Myeloid Progenitor Compartment of *Jak2V617F* Mice

Having demonstrated that Jak2[+/VF] mice developed elevated HCT and expanded erythroid precursor cells, we undertook a quantitative evaluation of the BM myeloid progenitor compartment of Jak2[+/VF] or Jak2^{+/+} mice. We found that immunophenotypically defined myeloid progenitor cells (Lineage[low] Sca1^{−}cKit[high]) were increased in Jak2[+/VF] mice (Figure S2A, p = 0.002) mainly as a result of expansion of the megakaryocytic/erythroid progenitor (MEP) population within this compartment (Figure 2C; Figures S2B and S2C). The ratio of common myeloid progenitor (CMP) and granulocyte/macrophage progenitors (GMP) cells to total BM cells was unchanged when comparing Jak2[+/VF] and Jak2^{+/+} mice (Figures S2D and S2E). We then performed a more detailed examination of megakaryocytic and erythroid progenitor populations via the additional markers CD150, CD41, and CD105 (Pronk et al., 2007). These studies showed an increase in lineage^{low}cKit^{high} CD150^{+}CD41^{−}CD105^{+} Pre-CFU-E cells (that have only erythroid lineage potential), relative to lineage^{low}cKit^{high}CD150^{+}CD41^{−} CD105^{−} Pre-MegE cells (that have mixed erythroid/megakaryocytic potential) and lineage^{low}cKit^{high}CD41^{+} MkP cells (that are lineage restricted to a mature megakaryocytic fate) (Figures 2A, 2B, and 2D), in Jak2[+/VF] mice compared with Jak2^{+/+} mice. The Jak2[+/VF] expanded CD71^{+} Ter119^{+} proerythroblast population is contained within the CD150^{−}, CD105^{+} compartment. These results demonstrate that *Jak2V617F* causes marked erythroid skewing of progenitor populations: disproportionately increasing MEP cells over other myeloid progenitors and increasing Pre-CFU-E cells relative to megakaryocyte progenitors.

One of the pathognomonic features of PV is hypersensitivity of erythroid progenitors to erythropoietin (EPO) and growth
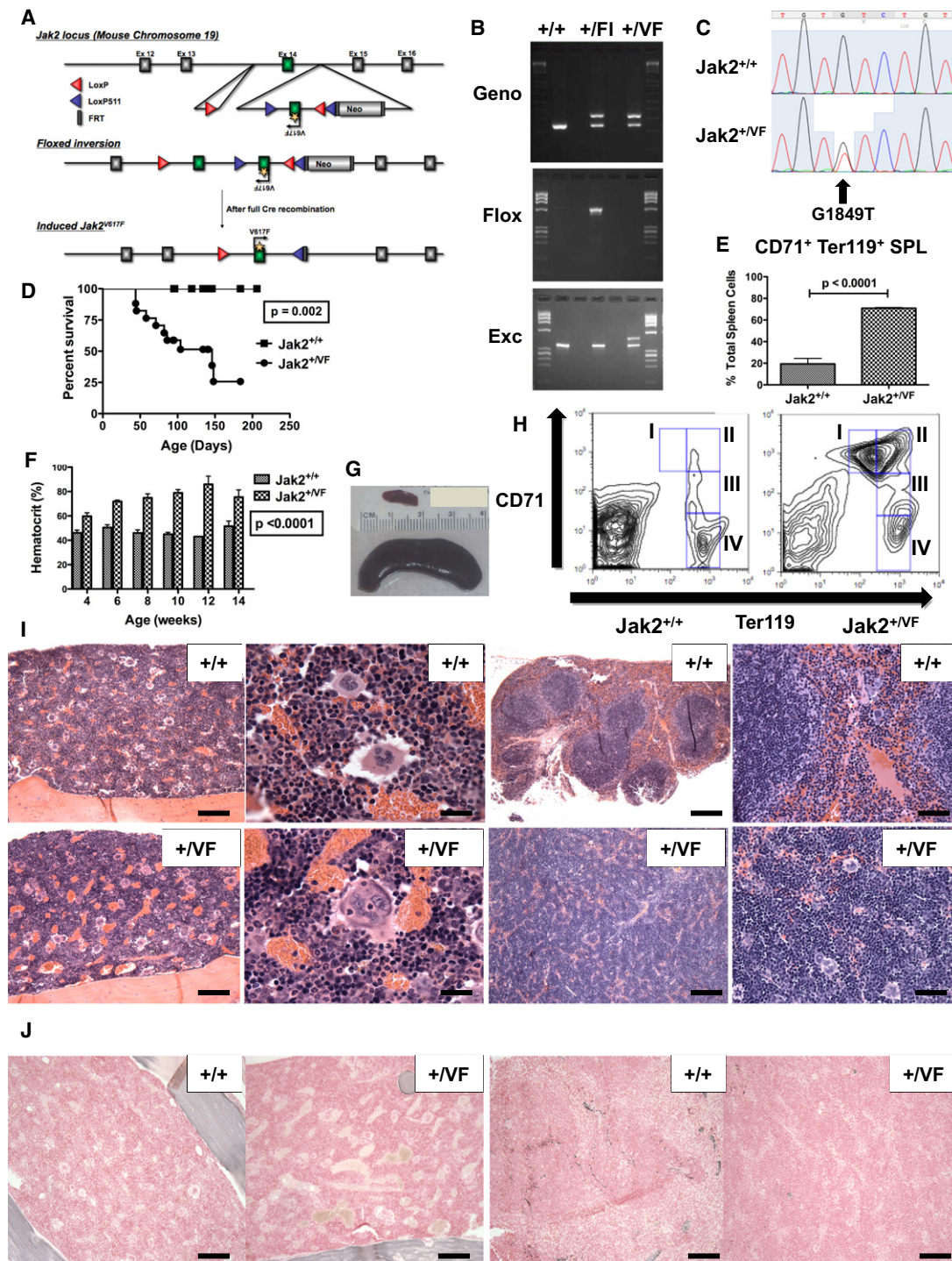
Figure 1. Physiological *Jak2V617F* Expression Causes a Lethal MPN Resembling PV

(A) Targeting vector schema for the *Jak2V617F* conditional knockin mouse.

(B) PCR analysis of tail DNA from Jak2$^{+/+}$, Jak2$^{+/Fl}$, and Jak2$^{+/VF}$ mice for genotyping (Geno), detection of floxed band (Flox), and detection of excised band (Exc); (Fl = Floxed).

(C) Jak2 sequencing analysis of unfractionated BM cells from Jak2$^{+/+}$ and Jak2$^{+/VF}$ mice.

(D) Kaplan-Meier analysis of a cohort of Jak2$^{+/VF}$ mice (n = 17) and littermate controls (n = 13).

(E) Composite data from age-matched littermates of Jak2$^{+/+}$ and Jak2$^{+/VF}$ mice (unpaired two-tailed t test; mean ± SEM; n = 4 in each group) demonstrating increased in CD71$^+$, Ter119$^+$ erythroid precursors in Jak2$^{+/VF}$ spleens.

(F) Hematocrits of age-matched littermate Jak2$^{+/+}$ and Jak2$^{+/VF}$ mice aged 4–14 weeks (mean ± SEM; n = 16 in each group).

with a low plating efficiency even in the absence of EPO. We plated unfractionated BM cells or highly purified MEP cells derived from Jak2$^{+/VF}$ or Jak2$^{+/+}$ mice in media containing growth factors supplemented with decreasing concentrations of EPO. We observed EPO hypersensitivity in both Jak2$^{+/VF}$ unfractionated BM and Jak2$^{+/VF}$ MEPs, although we did not observe endogenous erythroid colony formation (Figures 2E and 2F). Consistent with this, we also found that Jak2$^{+/VF}$ MEP cells demonstrated increased phospho-Stat5 signaling in response to stimulation with EPO and interleukin-3 (IL3) as compared with Jak2$^{+/+}$ MEP cells (Figure S2F).

The *Jak2V617F* MPN-Initiating Population Is Contained within the HSC-Enriched LSK Population

Existing murine MPN models have generally demonstrated poor transplantability of the primary disease (Chan et al., 2004, 2009; Braun et al., 2004; Lee et al., 2007), limiting the evaluation of the disease-initiating population in these models. To assess the transplantability of *Jak2V617F*-evoked MPN, we first transplanted unfractionated BM cells from diseased Jak2$^{+/VF}$ mice into lethally or sublethally irradiated littermate recipients and unfractionated spleen cells from diseased Jak2$^{+/VF}$ mice into lethally irradiated littermate recipients. In all cases, a MPN developed in secondary recipients, characterized by elevated HCT as early as 4 weeks posttransplantation (Figure 3A) and a median survival of 342 days in this mixed group of transplants.

We next sought to identify the hematopoietic developmental stage that contains the disease-initiating cell. Highly purified BM LSK, MEP, or GMP cell populations from Jak2$^{+/VF}$ and Jak2$^{+/+}$ animals, respectively, were transplanted into lethally irradiated congenic secondary recipients and full donor engraftment was confirmed in all recipients. Recipient mice that received Jak2$^{+/VF}$ LSK cells developed a MPN that was similar to that which developed in primary mice, characterized by elevated HCT (Figure 3B), increased WBC and platelet counts (Figures S3A and S3B), prominent extramedullary erythropoiesis (Figure 3C), increased MEP cells (Figure S3C), and erythroid and megakaryocytic hyperplasia in the BM (Figures 3D and 3E). In contrast, animals that received either the Jak2$^{+/VF}$ MEP or Jak2$^{+/VF}$ GMP-committed progenitor populations did not develop MPN with >6 months of followup (Figures 3B, 3F, and 3G). Furthermore, lethally irradiated tertiary recipients of unfractionated BM from mice that were transplanted with Jak2$^{+/VF}$ LSK cells 6 months earlier also developed elevated HCT (Figure 3B), and upon sacrifice, histopathologic evidence of MPN was evident, demonstrating that the disease is serially transplantable (Figures S3D and S3E). These experiments demonstrate that *Jak2V617F*-expressing LSK cells, but not committed myeloid progenitors, are able to initiate and maintain the MPN in vivo.

Jak2V617F Directs Differentiation within the LSK Compartment but Has Otherwise Modest Effects on LSK Cells

We observe that the MPN-initiating population is contained within the LSK compartment, and the *JAK2V617F* mutation has been identified in HSCs from human MPN patients (Jamieson et al., 2006). Based on these insights, we further assessed the LSK compartment that contains long-term (LT) and short-term (ST) HSCs (Spangrude et al., 1988), as well as multipotent progenitor cells (MPPs). We found no significant quantitative differences between Jak2$^{+/VF}$ and Jak2$^{+/+}$ LSK cells (Figure S4A). Similarly, we did not observe quantitative differences in immunophenotyically defined LT-HSCs (CD150$^+$CD48$^-$ LSKs) (Kiel et al., 2005) when comparing Jak2$^{+/VF}$ and Jak2$^{+/+}$ BM (Figure S4B). In an assessment of the functional effects of the *Jak2V617F* mutation in LSK cells, we did not observe any significant differences between Jak2$^{+/+}$ and Jak2$^{+/VF}$ LSK cells with regard to cell cycle status (Figure 4A).

These observations suggested that JAK-STAT signal transduction might not be differentially activated between Jak2$^{+/VF}$ and Jak2$^{+/+}$ LSK cells. To assess this possibility, we used flow cytometry to assess phospho-Stat5 levels within Jak2$^{+/VF}$ and Jak2$^{+/+}$ LSK cells via a phospho-specific Stat5 antibody (Figure 4B). Consistent with our colony-forming cell (CFC) data (described above), we observed no difference in basal Stat5 phosphorylation signaling after serum starvation between Jak2$^{+/VF}$ and Jak2$^{+/+}$ LSK cells. Similarly, there was no statistically significant difference in Stat5 activation between these populations in response to stimulation with EPO plus IL3 (Figure 4C). We also assessed Stat5 activation in Jak2$^{+/VF}$ or Jak2$^{+/+}$ LSK cells in response to low-dose EPO and IL3 stimulation and obtained similar results to those obtained at the higher doses (Figure S4C). As expected, Stat5 activation after cytokine stimulation was inhibited by in vitro treatment with the JAK2 inhibitor TG101348 (300 nM), although phospho-Stat5 did not return to basal levels under these experimental conditions (Figure 4B).

To further explore the functional consequences of *Jak2V617F* expression in the LSK compartment, and in view of the recently described role of JAK2 as an epigenetic regulator through phosphorylation of Tyr 41 (Y41) on histone H3 (Dawson et al., 2009), we analyzed Jak2$^{+/VF}$ or Jak2$^{+/+}$ LSK cells by gene expression profiling. In a supervised analysis of Jak2$^{+/VF}$ versus Jak2$^{+/+}$ LSK cells, no individual genes were significantly differentially expressed between the two states (FDR < 0.05). Given that we observed expansion and erythroid skewing of the myeloid

(G) Photograph of spleens from Jak2$^{+/+}$ (upper; weight = 0.08 G) and Jak2$^{+/VF}$ (lower; weight = 3.1 G) mice demonstrating marked splenomegaly in Jak2$^{+/VF}$ mouse.

(H) Representative flow-cytometric analysis of single cell suspensions of spleen from age-matched littermate Jak2$^{+/+}$ and Jak2$^{+/VF}$ mice, indicating expansion of CD71$^+$ Ter119$^+$ cells (subpopulations I and II) in Jak2$^{+/VF}$ mice.

(I) Histopathologic H&E sections of bone marrow (BM) and spleen (SPL) from representative Jak2$^{+/+}$ and Jak2$^{+/VF}$ mice demonstrating megakaryocyte hyperplasia with clustering and emperipolesis in Jak2$^{+/VF}$ BM and marked erythroid hyperplasia with complete effacement of white pulp in Jak2$^{+/VF}$ SPL when compared with unremarkable BM and SPL from Jak2$^{+/+}$ controls (BM, first column [scale bars represent 250 μM], second column [scale bars represent 50 μM]; SPL, third column [scale bars represent 500 μM], fourth column [scale bars represent 125 μM]).

(J) Histopathologic reticulin sections of bone marrow (BM) and spleen (SPL) from representative Jak2$^{+/+}$ and Jak2$^{+/VF}$ mice demonstrating absence of reticulin fibrosis (BM, first and second panels [scale bars represent 250 μM]; SPL, third and fourth panels [scale bars represent 250 μM].
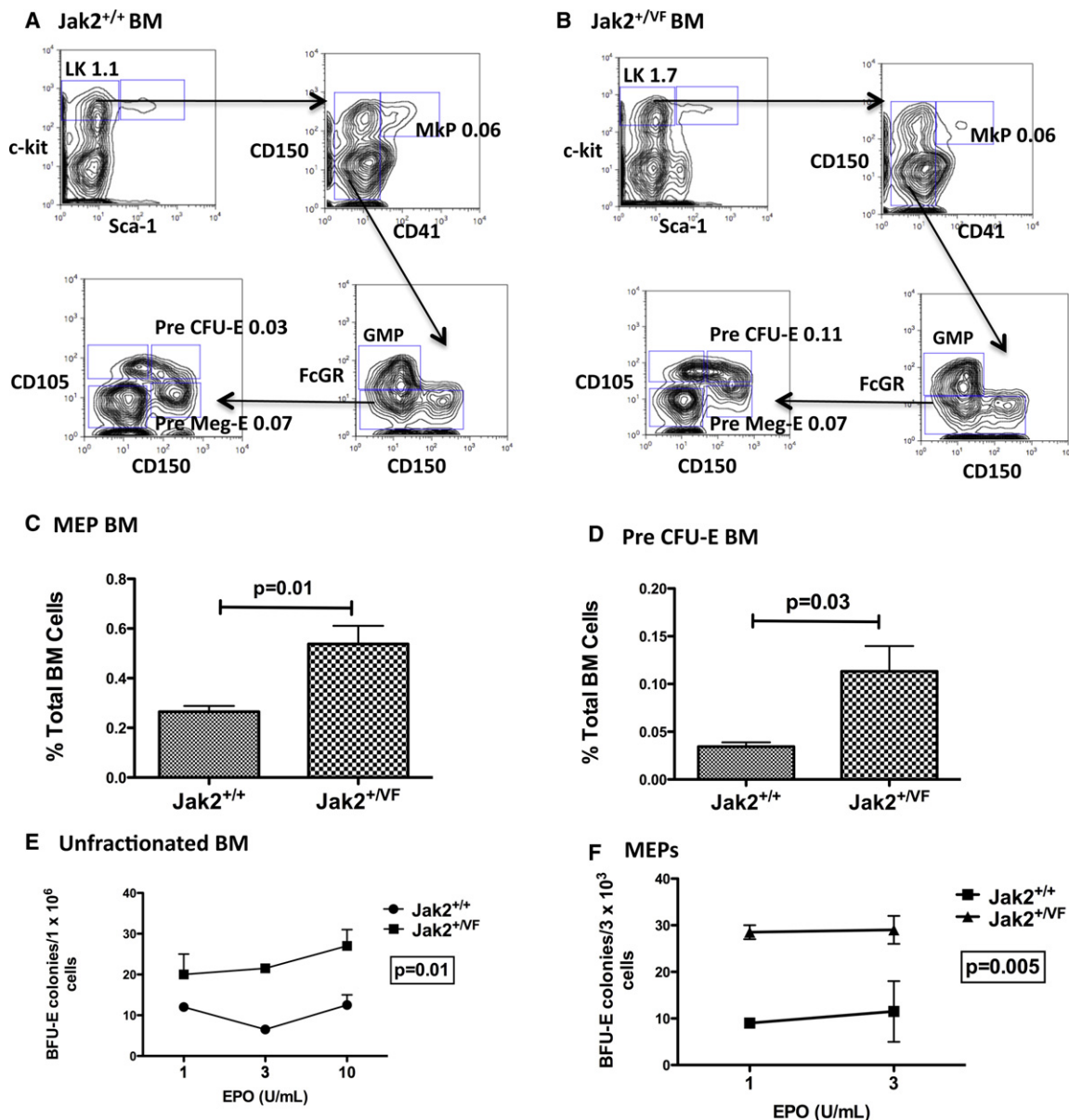
See also Figure S1.

Figure 2. Erythroid Skewing in the Myeloid Progenitor Compartment of *Jak2V617F* Mice
(A and B) Multiparameter flow cytometry of Jak2^{+/+} and Jak2[+/VF] BM. Representative contour plots indicating LK subsets (MkPs, GMPs, Pre Meg-Es, and Pre CFU-Es) are shown. All values are mean frequency of total BM cells. Representative data from one of two independent experiments (n = 4 in each group).
(C) Composite data from age-matched littermates of Jak2^{+/+} and Jak2[+/VF] mice demonstrating significantly increased numbers of MEP cells in Jak2[+/VF] BM over Jak2^{+/+} BM (unpaired two-tailed t test; mean ± SEM; n = 4 in each group).
(D) Composite data from age-matched littermates of Jak2^{+/+} and Jak2[+/VF] mice demonstrating significantly increased numbers of Pre CFU-E cells in Jak2[+/VF] BM over Jak2^{+/+} BM (unpaired two-tailed t test; mean ± SEM; n = 4 in each group).
(E and F) Unfractionated BM and MEP cells from Jak2^{+/+} and Jak2[+/VF] mice were plated and scored for BFU-E colony formation 7–10 days later. Results are the average of two independent experiments performed in duplicate (unpaired two-tailed t test; mean ± SEM).
See also Figure S2.

progenitor compartment of Jak2[+/VF] mice and that recipients of Jak2[+/VF] BM developed elevated HCT as soon as 3 weeks posttransplantation (Figures 3A and 3B), we used gene set enrichment analysis (GSEA) (Subramanian et al., 2005) to compare murine myeloid progenitor signatures in Jak2[+/VF] or Jak2^{+/+} LSK cells. We observed that MEP, CMP, and GMP differentiation signatures (Krivtsov et al., 2006) were significantly

enriched in Jak2[+/VF] LSK cells (Table S1, Figure S4D). We also found significant enrichment of erythroid and megakaryocytic progenitor differentiation signatures (Pronk et al., 2007) in Jak2[+/VF] LSK cells (Table S1). These findings indicate that although the *Jak2V617F* mutation does not expand LSK cell numbers, it directs hematopoietic differentiation within the LSK compartment.
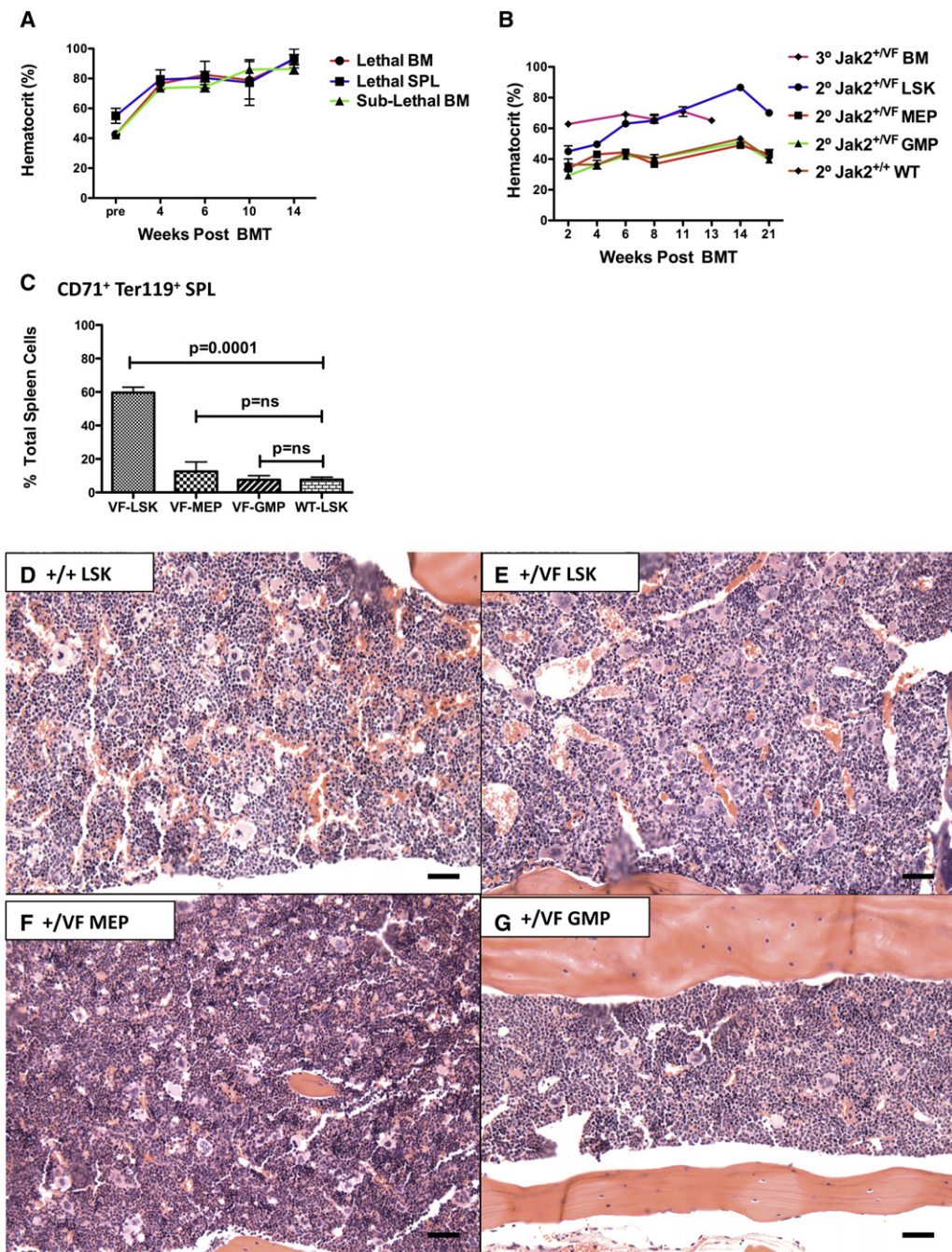
Figure 3. The *Jak2V617F* MPN-Initiating Population Is Contained within the LSK Population

(A) HCT of lethally (Lethal) or sublethally (Sublethal) irradiated littermate secondary recipients of unfractionated BM or SPL cells from diseased Jak2$^{+/VF}$ mice measured 4–14 weeks posttransplantation (mean ± SEM; n = 3 in each group).

(B) HCT of lethally irradiated secondary (2°) recipients of purified Jak2$^{+/VF}$ BM subpopulations (LSKs, MEPs, and GMPs) and corresponding Jak2$^{+/+}$ BM subpopulations and of lethally irradiated tertiary (3°) recipients of unfractionated BM, measured 2–21 weeks posttransplantation (mean ± SEM; n = 5 in each group).

(C) Composite data from secondary recipients of purified Jak2$^{+/VF}$ BM subpopulations (LSKs, MEPs, and GMPs), demonstrating increased CD71$^+$, Ter119$^+$ erythroid precursors in spleens of secondary recipients of purified Jak2$^{+/VF}$ LSK cells (unpaired two-tailed t test; mean ± SEM; n = 3 in each group).

(D–G) Histopathologic H&E sections of BM from representative secondary recipients of purified Jak2$^{+/+}$ and Jak2$^{+/VF}$ LSK cells, Jak2$^{+/VF}$ MEP cells, and Jak2$^{+/VF}$ GMP cells, demonstrating erythroid hyperplasia, increased megakaryocytes in clusters with large forms, and emperipoliesis in Jak2$^{+/VF}$ LSK; slight increase in megakaryocytes in Jak2$^{+/VF}$ MEP and mild myeloid hyperplasia in Jak2$^{+/VF}$ GMP as compared with unremarkable BM from Jak2$^{+/+}$ LSK (BM [scale bars represent 250 μM]).
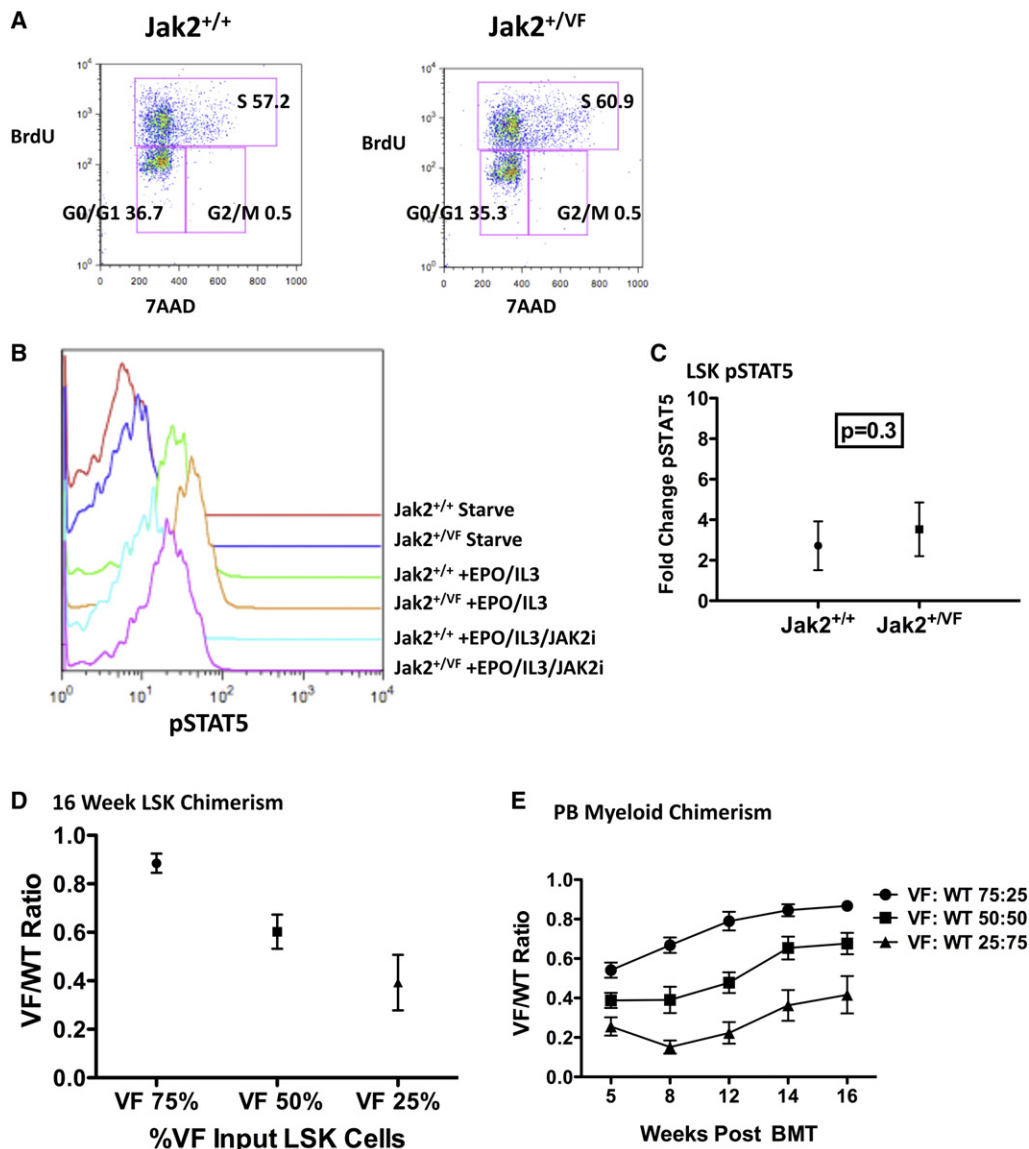
See also Figure S3.

Figure 4. *Jak2V617F* Has Modest Effects in the LSK Compartment and Does Not Confer a Significant Competitive Advantage to LSK Cells
(A) Representative multiparameter flow cytometry of LSK populations in Jak2$^{+/+}$ and Jak2$^{+/VF}$ mice demonstrating no significant differences in cell numbers in different phases of cell cycle. All values are mean frequency of total LSK cells. Representative data from one of two independent experiments (n = 4 in each group).
(B) Representative flow cytometric analysis of levels of phospho-STAT5 (p-STAT5) in LSK cells from Jak2$^{+/+}$ and Jak2$^{+/VF}$ littermate controls, after serum starvation (starve) or stimulation with EPO (3 U/mL) and IL3 (10 ng/mL) for 10 min, in the absence (EPO/IL3) or presence of the JAK2 inhibitor TG101348 (EPO/IL3/JAK2i). Representative data from one of three independent experiments.
(C) Composite data from Jak2$^{+/+}$ and Jak2$^{+/VF}$ LSK cells purified from primary mice (unpaired two-tailed t test; mean ± SEM), demonstrating no significant difference in phospho-STAT5 activation after stimulation with EPO/IL3 (n = 4 in each group).
(D) Jak2$^{+/VF}$(VF) to Jak2$^{+/+}$ (WT) chimerism ratios assessed in BM LSK compartment from lethally irradiated secondary recipients of Jak2$^{+/VF}$ and Jak2$^{+/+}$ LSK cells in 75:25, 50:50, and 25:75 ratios measured 16 weeks posttransplantation (mean ± SEM; n = 4 in each group).
(E) Jak2$^{+/VF}$ (VF) to Jak2$^{+/+}$ (WT) myeloid chimerism ratios assessed in peripheral blood (PB) from lethally irradiated secondary recipients of Jak2$^{+/VF}$ and Jak2$^{+/+}$ LSK cells in 75:25, 50:50, and 25:75 ratios measured 5–16 weeks posttransplantation (mean ± SEM; n = 6 in each group).
See also Figure S4 and Table S1.

Jak2V617F Does Not Confer a Significant Competitive Advantage to LSK Cells

Our observations on LSK cell number and gene expression, cell cycle, and Stat5 signaling indicate that in aggregate, physiologic expression of the *Jak2V617F* allele has quite modest effects on the LSK compartment.

To further assess LSK function and to determine whether the *Jak2V617F* allele conferred a selective advantage to Jak2$^{+/VF}$ LSK cells when compared with Jak2$^{+/+}$ LSK cells, we performed competitive transplantation experiments. We transplanted LSK cells into lethally irradiated congenic recipients by using the following Jak2$^{+/VF}$ to Jak2$^{+/+}$ ratios: 75:25, 50:50, or 25:75 in

combination with 250,000 WT supportive BM cells. All recipient groups developed HCT > 55% that was sustained over 16 weeks, all developed the same degree of expansion of CD71$^+$ Ter119$^+$ cells in the spleen, and all demonstrated the same degree of splenomegaly (Figures S4E–S4G). LSK cell chimerism in the BM at 16 weeks showed that the ratios of Jak2$^{+/VF}$ to Jak2$^{+/+}$ LSK cells were mildly increased from the input Jak2$^{+/VF}$ to Jak2$^{+/+}$ ratios in all groups (Figure 4D). Peripheral blood myeloid chimerism demonstrated similar results to LSK chimerism (Figure 4E) and there was no significant difference in the percentage of peripheral blood Mac1$^+$ Gr1$^+$ myeloid cells between the groups (Figure S4H). These data demonstrate that *Jak2V617F* confers, at most, a minor selective competitive advantage on the HSC-enriched LSK population and that the presence of even a minority of Jak2$^{+/VF}$ LSK cells in the BM is sufficient to cause a marked expansion of erythroid precursors in the spleen and the development of a PV phenotype.

Inhibiting JAK2 Kinase Does Not Eradicate the MPN-Initiating Population

Together, these data indicate that *Jak2V617F* has nominal effects on the size or function of the LSK compartment. A clinically relevant prediction of these observations is that inhibition of *Jak2V617F* might be expected to have minimal effects on this compartment. If this hypothesis were correct, it could have implications for this population as a resistant reservoir of MPN-initiating cells—and for the efficacy of JAK2 inhibitors as curative rather than remitting therapy.

To first evaluate whether Jak2$^{+/VF}$ mice respond to treatment with a JAK2 inhibitor, we treated primary mice with the JAK2 kinase inhibitor TG101348 (Wernig et al., 2008) or vehicle for 6 weeks by oral gavage (60 mg/kg twice daily). Primary Jak2$^{+/VF}$ mice responded to treatment with a statistically significant reduction in spleen size (Figure 5A) and histopathological improvement of the erythroid hyperplasia in the BM and spleen (Figures 5B and 5C), compared with mice treated with vehicle. HCT remained elevated in treated mice, possibly a reflection of the long life span of red blood cells. We also treated lethally irradiated congenic tertiary recipients of unfractionated Jak2$^{+/VF}$ BM with TG101348 for 6 weeks by oral gavage (60 mg/kg twice daily). Tertiary recipients also responded to treatment and in addition to a statistically significant reduction in spleen size (Figure S4I), decreased erythroid precursors were observed in the spleens of TG101348-treated mice, compared with tertiary recipient mice treated with vehicle (Figure 5D).

To assess the effect of Jak2 inhibition on the MPN-initiating cell population, we performed serial transplantation experiments (see Figure 5F for schema). We first purified LSK cells from primary Jak2$^{+/VF}$ mice that had been treated with TG101348 or vehicle for 6 weeks by oral gavage (60 mg/kg twice daily) and transplanted equal numbers of cells into lethally irradiated congenic secondary recipients. Three weeks later, blood counts demonstrated full hematopoietic reconstitution, with elevated HCT in all secondary recipient mice (Figure 5E), demonstrating that the MPN-initiating population was not eliminated in primary treated mice. To investigate whether treatment for a longer duration could eradicate the disease-initiating population, TG101348 treatment was initiated in secondary recipients of LSK cells from a TG101348-treated primary mouse and vehicle

treatment was initiated in secondary recipients of LSK cells from a vehicle-treated primary mouse. Both the treatment and vehicle groups showed sustained elevation in HCT (Figure 5E). After 10 weeks of treatment with TG101348, unfractionated BM was then transplanted from treated secondary recipients into lethally irradiated congenic tertiary recipients, and at 3 weeks posttransplantation, these tertiary recipients also demonstrated elevated HCT (Figure 5E), indicating that the MPN-initiating population was not eliminated in secondary treated mice.

In aggregate, these data indicate that although the JAK2 kinase inhibitor TG101348 demonstrated therapeutic efficacy against serially transplanted *Jak2V617F*-evoked MPN with regard to reduction in spleen size and in erythroid precursor cell number, treatment with the drug did not eliminate the MPN-initiating population in vivo.

DISCUSSION

Eradicating the disease-initiating cells within a tumor is the definitive goal of curative cancer therapy. In murine models of leukemia, the cell populations that possess the capacity to initiate disease vary depending on the oncogene driving the leukemia (Cleary, 2009). In general, murine MPN models have been limited in their ability to address questions pertaining to the MPN-initiating cell population because of poor transplantability of the primary disease (Chan et al., 2004, 2009; Braun et al., 2004; Lee et al., 2007). We report a murine knockin model in which *Jak2V617F* is expressed from its endogenous promoter and in which the MPN that arises is serially transplantable. We demonstrate the presence of at least two distinct cellular subfractions that are essential for MPN pathogenesis. The *Jak2V617F* LSK population is enriched for disease-initiating activity but has few phenotypic attributes that distinguish it from a wild-type LSK population. In contrast, *Jak2V617F* MEP cells are expanded in number and drive the disease phenotype in vivo but do not transplant the MPN. Concordantly, treatment with a JAK2 inhibitor attenuates the disease phenotype but the MPN-initiating population retains its ability to reconstitute disease.

The *Jak2V617F* knockin murine model we report was engineered to faithfully reproduce the human situation, and the murine phenotype closely recapitulates many of the features of human PV. Jak2$^{+/VF}$ MEPs exhibit hypersensitivity to EPO stimulation as manifested by enhanced growth of erythroid colonies in methylcellulose media supplemented with low-dose EPO but do not demonstrate endogenous erythroid colony formation. These results are consistent with in vitro studies of primary human PV patient samples (Jamieson et al., 2006; Dupont et al., 2007). The absence of reticulin fibrosis in this model is compatible with the existence of strain-specific and microenvironmental modifiers of fibrosis. This phenotype is more prominent in Balb/c mice in the retroviral *JAK2V617F* model (Wernig et al., 2006) and rapidly progressive BM fibrosis is seen in the *TEL-JAK2* NOD/SCID model, in which JAK2 is activated as a result of a chromosomal translocation (Kennedy et al., 2006). Our transplantation experiments demonstrate that the development of *Jak2V617F*-evoked MPN is cell autonomous, consistent with the finding that the *JAK2V617F* mutation is not detectable in
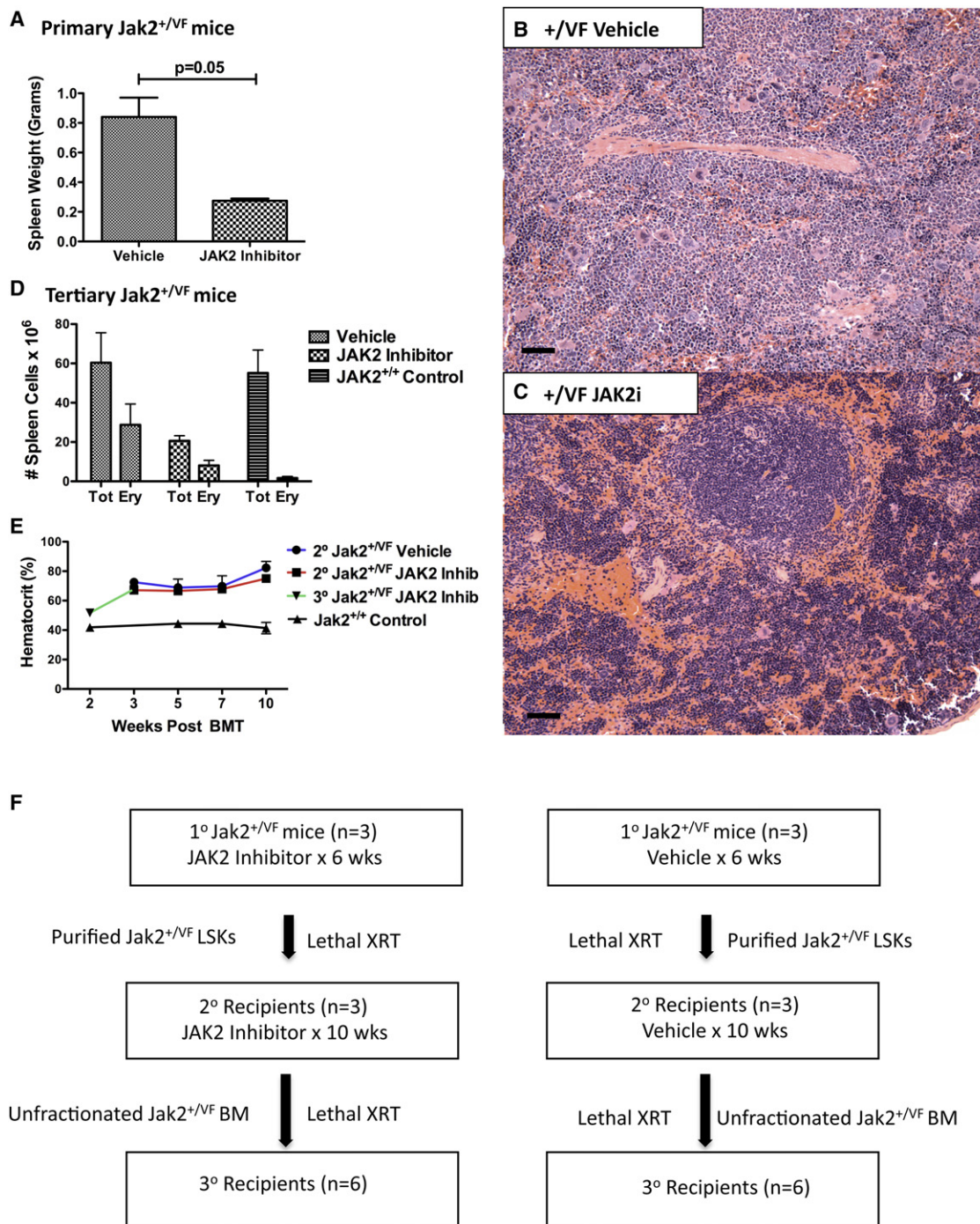
Figure 5. JAK2 Kinase Inhibition Does Not Eradicate the MPN-Initiating Population

(A) Composite data from primary Jak2$^{+/VF}$ age-matched littermates treated with vehicle or the JAK2 inhibitor TG101348 for 6 weeks, demonstrating reduced spleen weight in mice treated with TG101348 as compared with those treated with vehicle (unpaired two-tailed t test; mean ± SEM; n = 3 in each group).

(B and C) Histopathologic H&E sections of spleen from representative primary Jak2$^{+/VF}$ mice treated with vehicle or JAK2 inhibitor TG101348 for 6 weeks, demonstrating complete effacement of normal splenic architecture in vehicle-treated mice and improvement toward normal in TG101348-treated mice (SPL [scale bars represent 250 μM]).

(D) Composite data from tertiary Jak2$^{+/VF}$ recipients treated with vehicle or TG101348 for 6 weeks and from untreated Jak2$^{+/+}$ controls, demonstrating reduced splenic cellularity and reduced number of CD71$^+$ Ter119$^+$ erythroid precursor cells in mice treated with TG101348 (mean ± SEM; n = 4 in each group; Tot, total; Ery, erythroid).

(E) HCTs of lethally irradiated secondary recipients of purified Jak2$^{+/VF}$ LSKs from mice treated with vehicle or JAK2 inhibitor TG101348 for 6 weeks pretransplant and resumed 3 weeks posttransplantation. HCTs of lethally irradiated secondary recipients of purified Jak2$^{+/+}$ control LSKs, measured 2–10 weeks posttransplantation, also shown.

the stroma of patients with *JAK2V617F*-positive MPN (Pieri et al., 2008; Mercier et al., 2009).

Our preliminary observations suggest that lethality of the MPN in this model may have been related to thrombotic events. Animals died suddenly, precluding detailed histopathologic analysis, but among a small cohort of animals who had limited necropsy, gangrenous bowel was observed that would be consistent with thrombosis. We also note that thrombotic events were not observed in retroviral transduction or transgenic *JAK2V617F* models (Wernig et al., 2006; Lacout et al., 2006; Zaleskas et al., 2006; Tiedt et al., 2008; Xing et al., 2008; Shide et al., 2008) and that cardiac thrombosis was indicated as a cause of death in homozygous *Jak2V617F*-expressing animals in the recently published conditional knockin model of Akada et al. (2010). Collectively, these data suggest the possibility that physiologic expression of the *Jak2V617F* allele from the endogenous *Jak2* promoter may enable preclinical studies of thrombosis in MPN.

JAK2 was recently identified to have a previously unrecognized nuclear role, directly phosphorylating Tyr 41 (Y41) on histone H3 and preventing binding of the transcriptional repressor heterochromatin protein 1∝ (HP1∝) to H3 (Dawson et al., 2009). We did not find any significantly differentially expressed genes within the LSK compartment of Jak2$^{+/VF}$ mice, indicating that endogenous heterozygous *Jak2V617F* expression has minimal effects on global HSPC gene expression. Microarray profiling of Jak2$^{+/VF}$ LSKs demonstrated a robust enrichment of myeloid progenitor differentiation signatures including those derived from MEP (Krivtsov et al., 2006) and Pre-CFU-E cells (Pronk et al., 2007), respectively, indicating that *Jak2V617F* instructs HSC differentiation, consistent with what has been observed in vitro in HSCs from PV patients (Jamieson et al., 2006). Our demonstration that Jak2$^{+/VF}$ LSK cells have little selective advantage over Jak2$^{+/+}$ LSK cells in competitive transplant experiments are in keeping with NOD/SCID models of *JAK2V617F* PV, in which the majority of SCID repopulating cells (SRCs) are wild-type cells and transformed SRCs have no proliferative advantage over normal SRCs (James et al., 2008). Moreover, human patients with ET, who are heterozygous for the *JAK2V617F* mutation, can maintain a stable *JAK2V617F* clone over years (Gale et al., 2007).

Because we could not generate homozygote *Jak2V617F*-expressing mice in this model, we were unable to address the impact of allelic dosage on disease phenotype. However, given that the phenotype of heterozygote *Jak2V617F*-expressing mice is more consistent with PV than ET, it may be that additional somatic mutations and/or host modifiers (Pardanani et al., 2008) contribute to MPN phenotypic pleiotropy. In support of this hypothesis, another conditional *Jak2V617F* knockin model was published during the course of the preparation of this manuscript, in which both heterozygote and homozygote mice demonstrated a similar PV phenotype, with more severe disease observed in the homozygote animals (Akada et al., 2010). We observe minor phenotypic differences between the models, including the development of thrombocytosis, mild splenic

reticulin fibrosis, and expansion of the LSK population that we did not see in heterozygote *Jak2V617F*-expressing mice in our study. These differences may be explained in part by the use of Mx1Cre recombinase by Akada et al. (2010), requiring the administration of polyinosinic-polycytidylic acid (pIpC) for induction, because it has recently been demonstrated that interferon-∝ can cause proliferation of HSCs in vivo (Essers et al., 2009; Sato et al., 2009).

JAK2 small molecule inhibitors are currently in early phase clinical trials in patients with advanced myelofibrosis (Pardanani et al., 2009; Verstovsek et al., 2009). Although treated patients have generally been noted to experience rapid, marked decreases in spleen size and hematological responses, these findings have not been consistently associated with a reduction in *JAK2V617F* allelic burden (Verstovsek et al., 2008). Furthermore, patients without *JAK2* mutations who were enrolled in these trials have also demonstrated clinical responses, indicating that clonal *JAK2V617F*-positive disease is not being selectively targeted by these compounds (Verstovsek, 2009). The clinical trial results are consistent with our study, where we observed a statistically significant reduction in spleen weight and reduced erythroid precursor cell numbers in Jak2$^{+/VF}$ mice treated with TG101348, but this was not associated with elimination of the MPN-initiating population. Although we cannot rule out that treatment with a higher dose of TG101348 or for a longer duration could potentially diminish or possibly even eliminate the MPN-initiating population, we observed clear evidence of differential sensitivity to JAK2 kinase inhibition in vivo between different Jak2$^{+/VF}$ cellular compartments, with JAK2 selective effects on Jak2$^{+/VF}$ progenitors as compared with Jak2$^{+/VF}$ LSKs. Furthermore, our demonstration that *Jak2V617F* has modest effects on LSK phenotype suggests that JAK2 inhibitors, regardless of efficacy, dose, or treatment duration, are unlikely to have a curative therapeutic index in *JAK2V617F*-mediated MPN.

The therapeutic eradication of MPN-initiating cells will require insights into the differential molecular circuitry of normal and *JAK2V617F* mutant stem cells. Maintenance of *JAK2V617F* disease-initiating cells may require normal self-renewal pathways in a manner analogous to the dependence of CML stem cells on the Wnt-β-catenin (Zhao et al., 2007) and Hedgehog (Dierks et al., 2008; Zhao et al., 2009) signaling pathways. However, unlike chronic-phase CML, which appears to be a monoallelic neoplasm driven by *BCR-ABL*, there is accumulating evidence that additional genetic lesions occur within the HSC compartment of *BCR-ABL*-negative MPN. At least 50% patients with a *JAK2V617F*-positive MPN transform to a *JAK2V617F*-negative acute leukemia, suggesting that *JAK2V617F* may not be the disease-initiating mutation in many cases of MPN (Campbell et al., 2006; Theocharides et al., 2007). Recently, inactivating acquired mutations in *TET2* (Delhommeau et al., 2009), *ASXL1* (Gelsi-Boyer et al., 2009), and *IDH1/2* (Abdel-Wahab et al., 2010; Green and Beer, 2010) have been identified in multipotent MPN progenitors. CD34$^+$ HSCs with mutations in *TET2* in addition to the *JAK2V617F* mutation show an increased capacity over *JAK2V617F* single mutant CD34$^+$ HSCs to repopulate

HCTs of tertiary recipients of unfractionated Jak2$^{+/VF}$ BM from treated secondary recipients measured 2–3 weeks posttransplantation are also shown (mean ± SEM; n = 3 in each group for secondary recipients; n = 6 for tertiary recipients).
(F) Schema outlining schedule for primary, secondary, and tertiary Jak2$^{+/VF}$ mice treated with TG101348 or vehicle.

NOD-SCID mice (Delhommeau et al., 2009) and both *TET2* and *IDH1/2* mutations have been associated with leukemic transformation in MPN (Abdel-Wahab et al., 2010; Green and Beer, 2010), suggesting that these mutations may enhance HSC self-renewal. Little is known about the function of TET2, but it was recently shown that TET1 has an enzymatic function in epigenetic regulation (Tahiliani et al., 2009). Identifying strategies to target TET2 therapeutically in the HSC compartment will require the development of preclinical genetic murine models that integrate *TET2* loss into the *Jak2V617F* context. Such models will enable the interrogation of the therapeutic susceptibilities of MPN alleles within different hematopoietic compartments.

In conclusion, we report a *Jak2V617F* heterozygote knockin mouse model that develops a MPN resembling human PV. We found that the LSK compartment contained the MPN-initiating cell population but that *Jak2V617F* had modest effects on the size of the LSK compartment or its subpopulations and on cell cycle, signal transduction, or gene expression of LSK cells. Furthermore, in functional studies employing competitive repopulation assays, *Jak2V617F* did not confer a significant competitive advantage to the LSK compartment. Concordant with this, the disease-initiating population was not eradicated with therapeutic dosing of a JAK2 kinase inhibitor. This model will be valuable in evaluating the curative potential of MPN therapies. Effective elimination of MPN disease-initiating cells will require insights into the differential molecular dependencies of normal and *JAK2V617F* mutant HSCs.

EXPERIMENTAL PROCEDURES

Generation of *Jak2V617F* Knockin Mice
The *Jak2V617F* knockin construct was generated via the recombineering method (Liu et al., 2003; Copeland et al., 2001) (see Supplemental Information for full details). Exposure to Cre recombinase results in inversion of the mutant exon followed by an excision reaction that removes the WT exon, one LoxP site, and one LoxP511 site, fixing the inversion in place (Figure 1A). All results described pertain to *Jak2V617F* germline expressing heterozygous mice. We did not obtain any viable germline homozygous *Jak2V617F*-expressing animals and as a result we believe germline homozygosity for *Jak2V617F* to be embryonically lethal. We were unable to generate homozygous floxed animals (Jak2$^{Fl/Fl}$) because the targeting construct appeared to interfere with WT Jak2 expression such that Jak2$^{Fl/Fl}$ functioned as a Jak2 null which is known to be embryonically lethal (Parganas et al., 1998; Neubauer et al., 1998). Heterozygous floxed mice (Jak2$^{+/Fl}$) had no phenotype. All experiments were conducted with an IUCAC-approved animal protocol at our institution.

Survival Analysis
Jak2$^{+/VF}$ or age-matched Jak2$^{+/+}$ control mice were monitored biweekly and were sacrificed when visibly ill (hunched posture, weight loss). Statistical analysis was performed with Prism 5 software (GraphPad, San Diego, CA) via the Kaplan-Meier method.

Histopathology
Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin or with reticulin to assess for fibrosis. Images of histological slides were obtained on a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) equipped with a SPOT RT color digital camera model 2.1.1 (Diagnostic Instruments, Sterling Heights, MI).

Flow Cytometry
BM cells were isolated from age-matched littermates between 1 and 6 months of age backcrossed to C57BL/6 and were flushed from hind leg bones with

PBS (GIBCO) + 2% FBS + penicillin/streptomycin (Cambrex, Biowhittaker), lysed on ice with red blood cell (RBC) lysis solution (Puregene), and washed in PBS (GIBCO) + 2% FBS. Single-cell suspensions of spleen were prepared by pressing tissue through a cell strainer and lysed and washed in the same manner as BM. Cells were stained with monoclonal antibodies in 2% FBS/PBS for 30 min on ice.

ACCESSION NUMBERS

Gene expression data are available in GEO database with the accession number GSE21842.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures (flow cytometry, megakaryocyte ploidy analysis, cytokine stimulation and intracellular phosphoprotein analysis, cell cycle, colony-forming assays, gene expression profiling, bone marrow transplantation, and JAK2 inhibitor studies), four figures, and one table and can be found with this article online at doi:10.1016/j.ccr.2010.05.015.

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Note Added in Proof

While this paper was in press, two additional *JAK2V617F* knockin mouse models were reported elsewhere.

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