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Acceleration of Bcr-Abl$^+$ leukemia induced by deletion of JAK2

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Gene discovery efforts and functional studies have credentialed the JAK-STAT signaling pathway as an important pathway contributing to malignant transformation.[1] On the basis of these observations, JAK2 kinase inhibitors have been developed for clinical use, and ruxolitinib, a JAK2/JAK1 inhibitor is approved for primary myelofibrosis.[2,3] More importantly, JAK2 inhibitors with different pharmacokinetic properties and off-target effects have entered clinical trials for use in chronic myeloid leukemia (CML), acute lymphoblastic T-cell leukemia and myelodysplastic syndrome patients (http://clinicaltrials.gov/ct2/results?term=ruxolitinib&Search=Search). Recent studies have suggested a role for JAK2 kinase inhibition for patients with breast cancer, lung cancer and pancreatic tumors[4] and clinical trials are evaluating the role of JAK2 inhibitors in epithelial tumors.

Although there is a strong rationale for JAK2 kinase inhibition in JAK2-mutant malignancies to date, these agents have not led to molecular or pathologic remissions at clinically achievable doses. More importantly, different JAK inhibitors have had different toxicity profiles in the clinic, including anemia/thrombocytopenia, gastrointestinal side effects and more recent reports of neurologic side effects with newer-generation JAK2 inhibitors.[5] As such, there is a need to better delineate the therapeutic window of JAK2 kinase inhibition and to better understand the requirement for JAK2 signaling in normal tissue homeostasis and in different malignant contexts.

We recently reported that the initiation and progression of BCR-ABL^{p210}-driven myeloproliferative neoplasms (MPN) is independent of the presence of JAK2 using an acute, rapidly terminal retroviral disease model of CML.[6] We asked how the absence of JAK2 in a chronic phase CML model would alter disease progression. To test this, we performed bone marrow (BM) transplantation experiments in non-irradiated NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ (NSG) mice, where we lowered the total number of donor cells from 2×10^7 to 1×10^6. We transduced *Jak2*$^{+/+}$ and *Jak2*$^{f/f}$*Mx1Cre*$^+$ cells with BCR-ABL^{p210} retrovirus followed by transplantation into NSG recipients (see Supplementary Information). At the time point of transplantation, the proportion of BCR-ABL-positive cells was ~7% for both genotypes (data not shown). 24 h before injection, interferon-β was administered *in vitro* to delete *Jak2*. Under these conditions—when disease evolves slowly—the absence of *Jak2* drastically accelerated disease development with increased white blood cell counts and severe splenomegaly (Figures 1a–c). The BM of diseased *Jak2*$^{\Delta/\Delta}$ animals contained at least 70% GFP$^+$ cells (Figure 1d); GFP$^+$ cells were not restricted to the myeloid compartment but extended to CD19$^+$ B cells and Ter119$^+$ erythroid cells (Figure 1e). We observed an expanded GFP$^+$ LSK (Lin$^-$Sca1$^+$ c-Kit$^+$) compartment in the *Jak2*$^{\Delta/\Delta}$ cohort (Figure 1f). We hypothesized that this might be indicative for an advantage for BCR-ABL in stem/progenitor cells over normal hematopoietic stem cells (HSCs) in the absence of *Jak2*.

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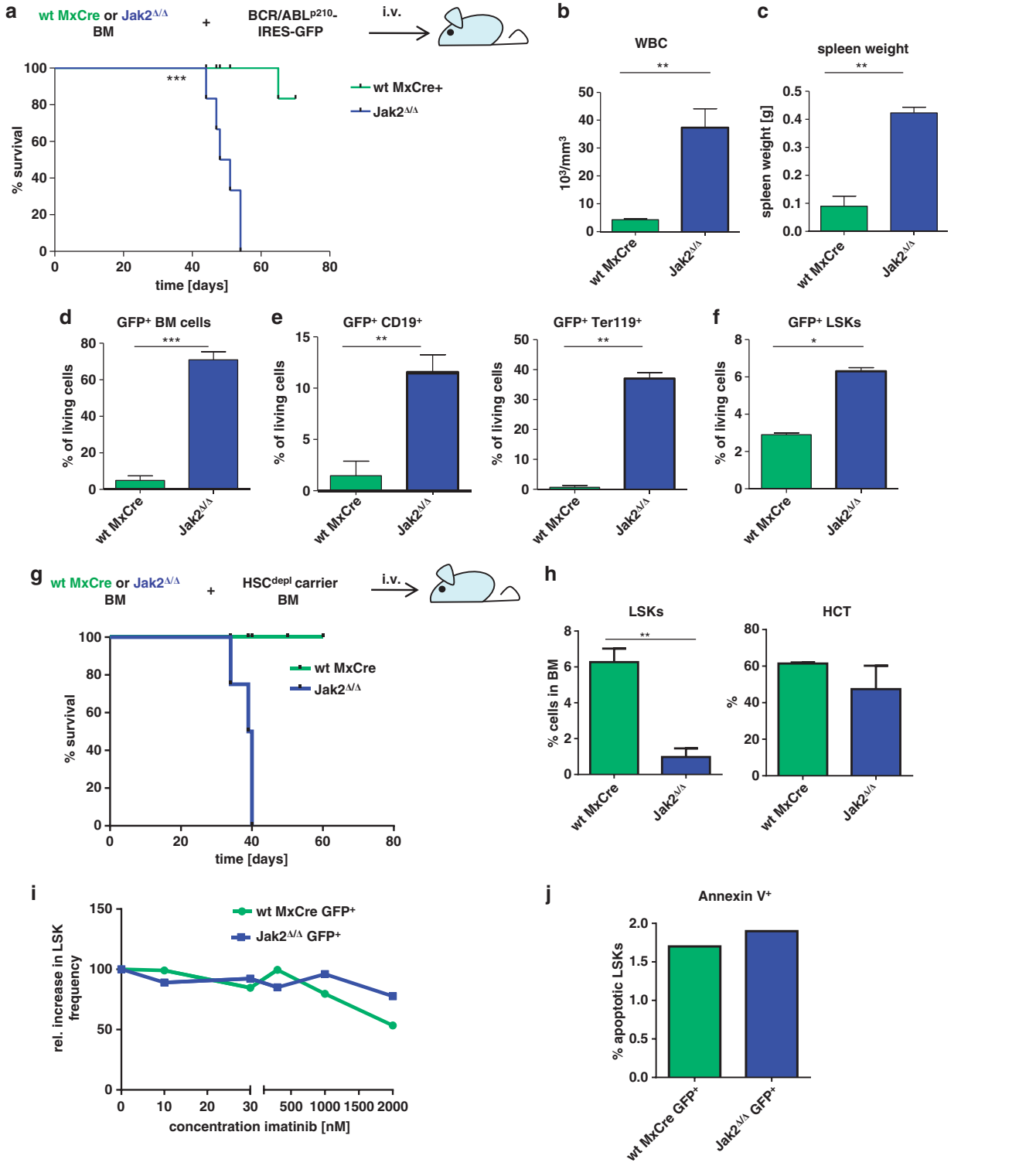
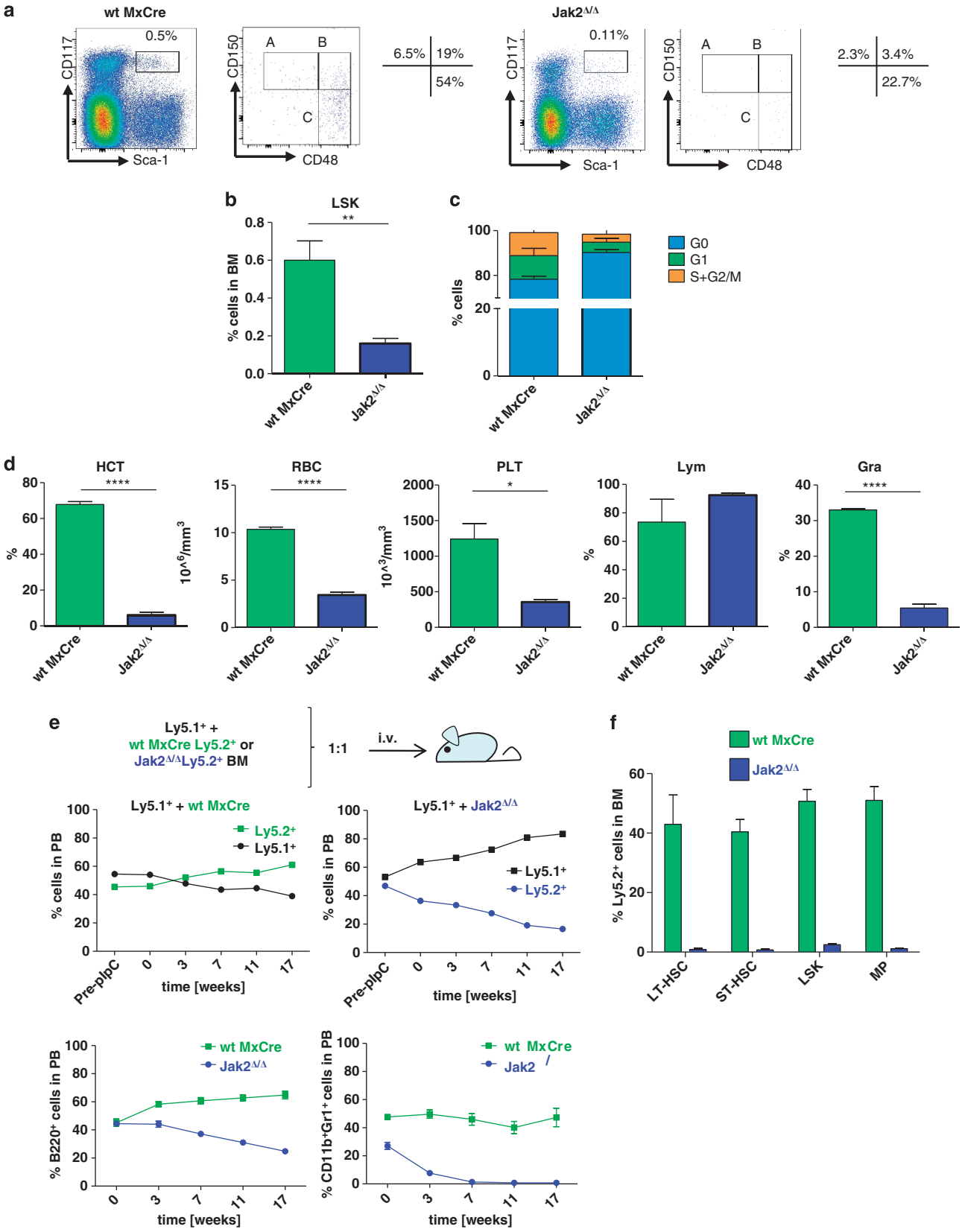


Figure 1. *Jak2* deletion accelerates BCR-ABL^{p210+} leukemia in mice but leads to a reduction of LSKs in normal hematopoiesis. *Jak2*$^{\Delta/\Delta}$ and *Jak2*$^{f/f}$*MxCre*$^{+}$ BM cells (1×10^6) were injected in non-irradiated NSG mice ($n = 12$) (**a**) Mice that received *Jak2*$^{\Delta/\Delta}$ BCR-ABL^{p210}-transformed BM succumb prematurely to leukemia. Short lines indicate individual mice that were killed as control. Mice of the *Jak2*$^{\Delta/\Delta}$ cohort display **(b)** increased peripheral WBCs and (**c**) spleen weights. (**d**) Percentages of BCR-ABL^{p210+}/GFP^{+} cells are increased in BMs of mice that received *Jak2*$^{\Delta/\Delta}$ BCR-ABL^{p210}-transformed BM compared with control animals. (**e**) *Jak2*$^{\Delta/\Delta}$ BCR-ABL^{p210+}/GFP^{+} cells contribute to B-cell and erythroid lineages. (**f**) Increased percentages of BCR-ABL^{p210+}/GFP^{+} LSKs in mice that received a *Jak2*$^{\Delta/\Delta}$ transplant. (**g**) Supplementation of *Jak2*$^{\Delta/\Delta}$ BM with HSC-depleted carrier BM leads to premature death. Non-transformed *Jak2*$^{\Delta/\Delta}$ BM was mixed with high-purity sorted HSC-depleted C57BL/6 J BM cells and injected into lethally irradiated recipients ($n = 8$). Scheme depicts experimental setup. (**h**) Numbers of LSKs are severely reduced in mice that received a mixture of *Jak2*$^{\Delta/\Delta}$ BM and HSC-depleted carrier cells. Hematocrit (HCT) levels remained unaltered upon JAK2 loss. (**i**) Dose-response curves of BCR-ABL^{p210+} LSKs incubated for 24 h in the presence of ruxolitinib (300 nM) and increasing doses of imatinib (ranging from 10 nM to 2 μM). (**j**) Frequencies of apoptotic (Annexin V^{+}) LSKs upon imatinib treatment (48 h incubation; 2 μM). Asterisks denote level of statistical significance as determined by an unpaired *t*-test: *$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.001$.

Previous studies had indicated a role for JAK2 in survival of normal HSCs: employing a tamoxifen-inducible conditional *Jak2* knockout model (*Jak2*$^{f/f}$*Cre*^{ERT2}), Park *et al.*[7] observed a reduction of *Jak2*-deficient stem cells that is reverted with time owing to incomplete deletion. Given these basic indications, we set out to analyze the self-renewal capacity of *Jak2*-deficient stem cells



in a transplantation model. *Jak2*$^{\Delta/\Delta}$ HSCs were mixed with HSC-depleted wild-type (wt) carrier BM, transplanted into lethally irradiated recipient mice that died within 40 days (Figure 1g). By contrast, all animals engrafted with wt*MxCre* HSCs remained healthy and displayed normal long-term engraftment. Mice reconstituted with *Jak2*$^{\Delta/\Delta}$ HSCs suffered from a nearly complete loss of the LSK compartment consistent with a severe impairment of HSPCs (hematopoietic stem and progenitor cells) function (Figure 1h). In this setting, percentages of granulocytes were slightly reduced, those of lymphocytes significantly increased (data not shown). Numbers of platelets (PLTs) and red blood cells (RBCs) remained unchanged (data not shown). Even when supported by wt BM, *Jak2*$^{\Delta/\Delta}$ HSCs fail to survive and repopulate efficiently after transplantation demonstrating a cell-autonomous self-renewal defect of *Jak2*-deficient HSCs.

To see whether the functions of JAK2 and BCR-ABL^{p210} in mediating cell survival are redundant, we tested the responsiveness of BCR-ABL^{p210+} LSKs to imatinib and ruxolitinib. We found that, under a constant ruxolitinib concentration of 300 nM, the frequency of JAK2-expressing LSKs remained unaffected upon gradual increase of imatinib after 24 (Figure 1i), 48 and 72 h (data not shown). In line with this, after 48 h imatinib treatment only, JAK2 deficiency failed to increase the frequency of apoptotic LSKs (Figure 1j). These data suggest that JAK2 and BCR-ABL do not compensate for each other in mediating survival of LSKs.

To analyze the effects of JAK2 loss on HSC number and cell cycle status, we further analyzed the LSK compartment in donor mice that had received repeated doses of poly(I:C) to delete Jak2 (see Supplementary Information). *Jak2* loss led to a diffuse reduction in the overall LSK compartment consistent with a role for JAK2 in long-term/short-term stem cells and in multipotent progenitors (Figures 2a and b). Following *Jak2* deletion, LSKs remained primarily in G0, suggesting the loss of stem/progenitor cells was not due to loss of quiescence (Figure 2c). We observed significantly lowered levels of hematocrit, RBCs, PLTs and granulocytes in *Jak2*$^{\Delta/\Delta}$ animals (Figure 2d).

To directly compare self-renewal of *Jak2*$^{\Delta/\Delta}$ HSCs and control HSCs *in vivo*, we next performed competitive transplantation studies (see Supplementary Information). We mixed *Jak2*$^{+/+}$ Ly5.1 and *Jak2*$^{f/f}$*Mx1Cre*$^{+}$ Ly5.2^{+} BM cells in a 1:1 ratio and reconstituted lethally irradiated Ly5.1^{+} recipient animals. Controls included transplantation of Ly5.1^{+} and Ly5.2^{+} wt cells. *Jak2* was deleted via poly(I:C) injections after documenting successful engraftment and equal chimerism. Serial analysis for 17 weeks following somatic *Jak2* deletion revealed a continuous decrease in the proportion of *Jak2*$^{\Delta/\Delta}$ Ly5.2^{+} cells in the peripheral blood (Figure 2e). The marked reduction of *Jak2*$^{\Delta/\Delta}$ Ly5.2^{+} cells affected all lineages, including B220^{+} B cells, and was most prominent for CD11b^{+}Gr1^{+} cells, which were no longer detectable after 7 weeks (Figure 2e). After 17 weeks the mice were killed; fluorescence-activated cell sorting analysis confirmed the near-complete absence of Ly5.2^{+} HSCs and progenitors in recipients (Figure 2f).

Taken together, our data demonstrate a critical role for JAK2 for HSC maintenance, survival and function. Our data contrast findings from clinical trials with JAK2 inhibitors, in which context JAK2 kinase inhibition leads to dose-dependent anemia and thrombocytopenia. However, it is important to note that dose escalation of JAK inhibitors in MPN patients was titrated based on limiting anemia and thrombocytopenia, and as such the degree of target inhibition has been less than complete in preclinical and clinical studies.[2,3,8,9] However, current trials in acute leukemias and solid tumors are allowing dose escalation in an effort to inhibit the target more completely. Our approach suggests that more complete inhibition of JAK2 will result in dense inhibition of hematopoietic stem cell function and may even support leukemic stem cells outgrowth that is not dependent on JAK2. It may be possible that intermittent, complete JAK2 inhibition can spare normal HSPC function (as indicated by Park *et al.*[7] that show a long-term recovery of HSC numbers); alternative-dosing approaches should be considered to increase the therapeutic window of JAK kinase inhibitors.

All available inhibitors of JAK2 interfere with the ATP-binding pocket and thus block JAK2 kinase activity and the resultant activation of the STAT1, STAT3 and STAT5 pathways. None of the phenotypes observed in respective knockout mice matches our findings or explains the pronounced effects of *Jak2* deletion.[10–12] Our data suggest that *Jak2* loss leads to profound defects in HSC function due to coordinate inhibition of STAT1, STAT3 and STAT5, or due to STAT-independent effects of JAK2 on other signaling pathways.

Alternatively it is possible that JAK2 exerts its effects on stem/progenitor effects through kinase-independent and non-canonical functions in HSCs. Exposure of MPN cells with type I JAK2 kinase inhibitors results in increased JAK2 protein expression and transactivation of JAK2 by other JAK kinases.[13] It is currently unclear whether scaffolding functions of JAK2 contribute to the different effects of *Jak2* deletion and inhibition of JAK2 kinase activity on adult hematopoiesis as recently suggested.[14] In addition, the emerging appreciation of JAK2's role in directly regulating the chromatin state suggests that direct epigenetic effects of JAK2 may contribute to the role of JAK2 in regulating stem cell self-renewal.[15] Our data and previous studies suggest that JAK2 has a complex and multilayered role in HSCs. The role of JAK2 in thrombopoietin signaling likely impacts HSC function *in vivo*. Most importantly, our data demonstrate that BCR-ABL^{p210} overcomes the requirement for JAK2, and that the absence of JAK2 enables BCR-ABL^{+} leukemia-initiating cells to outcompete regular HSCs. These data enhance the doubt whether JAK2 inhibition will indeed be an effective treatment strategy to eradicate LSCs in CML, and that malignant cells with

Figure 2. *Jak2*-deficient BM harbors reduced numbers of HSCs and shows a selective disadvantage in competitive transplantations. *Jak2*$^{f/f}$*MxCre*$^{+}$ and wt*Mx1Cre*$^{+}$ mice were treated with poly(I:C) every 3 days for 2 weeks to induce gene deletion (**a**–**c**; $n = 8$ for each genotype). (**a**) Loss of HSCs upon *Jak2* deletion. Representative fluorescence-activated cell sorting (FACS) plots indicate reduction of HSC numbers upon *Jak2* deletion. After 2 weeks, BM of *Jak2*$^{\Delta/\Delta}$ cells displayed significantly reduced numbers of LSK and fraction A, B and C cells. Upper panels: Representative FACS plot depicting LSK cells that were further subdivided by CD150 and CD48 expression. Percentages of cells belonging to fraction A, B or C are provided next to the plot. (**b**) Summary of LSK numbers in *Jak2*$^{\Delta/\Delta}$ and wt*Mx1Cre*$^{+}$ mice. BM cells were analyzed as described in (**a**). (**c**) Higher numbers of HSCs in G0 phase in *Jak2*$^{\Delta/\Delta}$ mice. BM cells of *Jak2*$^{\Delta/\Delta}$ and wt*Mx1Cre*$^{+}$ mice were analyzed for Ki67 and 4′,6-diamidino-2-phenylindole incorporation. The majority of *Jak2*$^{\Delta/\Delta}$ cells underwent the G0 phase of the cell cycle. (**d**) Analyses of peripheral blood (PB) of poly(I:C)-treated wt*Mx1Cre*$^{+}$ and *Jak2*$^{\Delta/\Delta}$ mice. Hematocrit (HCT), RBCs and PLT counts as well as percentages of lymphocytes and granulocytes are summarized in bar graphs. (**e**) BM of Ly5.1^{+} mice was mixed with either *Jak2*$^{f/f}$*MxCre*$^{+}$ or wt*Mx1Cre* (both Ly5.2^{+}) BM cells and injected intravenously into lethally irradiated Ly5.1^{+} mice. Post transplantation, mice received poly (I:C) to delete *Jak2*. Upper panels: percentages of peripheral blood cells expressing Ly5.1 or Ly5.2 of mice that received a *Ly5.1/*wt*Mx1Cre (*left panel) or a *Ly5.1/Jak2*$^{f/f}$*MxCre*$^{+}$ (right panel) mixture of cells ($n = 9$ each). Lower panels: percentages of CD11b^{+}Gr-1^{+}Ly.5.2^{+} (left panel) and B220^{+}Ly5.2^{+} (right panel) cells in the peripheral blood of mice that received a *Ly5.1/*wt or a *Ly5.1/Jak2*$^{f/f}$*MxCre*$^{+}$ mixture of cells are shown. (**f**) Numbers of *Jak2*$^{\Delta/\Delta}$ HSCs and MPs are markedly reduced in a competitive setting with wt cells. Asterisks denote level of statistical significance as determined by an unpaired *t*-test: *$P \leqslant 0.05$; **$P \leqslant 0.01$; ****$P \leqslant 0.0001$.

JAK-independent constitutive signaling will derive a competitive advantage in the setting of potent JAK2 inhibition. A combined application of imatinib and ruxolitinib is unlikely to solve that issue as JAK2 deficiency fails to enhance sensitivity to imatinib in BCR-ABL$^+$ LSKs.

However, we hypothesize that in some cases, JAK2 inhibitor-mediated effects on stem cells might be advantageous; for example, as an adjunct to stem cell transplantation. However, in summary our data support the notion that JAK2 has a critical role in normal hematopoiesis, and current and future therapeutic approaches aimed at targeting JAK2 need to consider effects on stem cell function and the relative inhibitory effects on normal and malignant cells, which might limit, or even abrogate, therapeutic efficacy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Circulating miRNA markers show promise as new prognosticators for multiple myeloma

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One approach to improve risk prognostication in patients with multiple myeloma (MM) is to use new technologies to stratify patients based on distinct outcomes.[1] Outcome prediction for most patients is based on the International Staging System (ISS) and the presence or absence of specific fluorescent *in-situ* hybridization abnormalities. Additional biomarkers are necessary to improve precision, and there is evidence that small non-coding RNAs, microRNAs (miRNAs), are involved in MM pathogenesis,[2–4] but the predictive role of circulating miRNAs remains to be fully evaluated.

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