

## Critical Role of Jak2 in the Maintenance and Function of Adult Hematopoietic Stem Cells

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### ABSTRACT

**Jak2**, a member of the Janus kinase family of nonreceptor protein tyrosine kinases, is activated in response to a variety of cytokines, and functions in survival and proliferation of cells. An activating JAK2V617F mutation has been found in most patients with myeloproliferative neoplasms, and patients treated with Jak2 inhibitors show significant hematopoietic toxicities. However, the role of Jak2 in adult hematopoietic stem cells (HSCs) has not been clearly elucidated. Using a conditional Jak2 knockout allele, we have found that Jak2 deletion results in rapid loss of HSCs/progenitors leading to bone marrow failure and early lethality in adult mice. Jak2 deficiency causes marked impairment in HSC function, and the mutant HSCs are severely defective in reconstituting hematopoiesis in recipient animals. Jak2 deficiency also causes significant apoptosis and loss of quiescence in HSC-enriched LSK (Lin<sup>−</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>) cells. Jak2-deficient LSK cells exhibit elevated reactive oxygen species levels and enhanced p38 MAPK activation. Mutant LSK cells also show defective Stat5, Erk, and Akt activation in response to thrombopoietin and stem cell factor. Gene expression analysis reveals significant downregulation of genes related to HSC quiescence and self-renewal in Jak2-deficient LSK cells. These data suggest that Jak2 plays a critical role in the maintenance and function of adult HSCs. *STEM CELLS* 2014;32:1878–1889

### INTRODUCTION

Hematopoietic stem cells (HSCs) play an essential role in hematopoiesis through their unique ability to self-renew and differentiate into progenitors of all types of mature blood cells. A majority of HSCs are maintained in a state of quiescence to prevent HSC exhaustion and support long-term hematopoiesis. Understanding the mechanisms by which quiescence, survival, self-renewal, and differentiation of HSCs are regulated is critical for rational design of therapies for blood disorders.

Janus kinase 2 (Jak2) is a ubiquitously expressed nonreceptor protein tyrosine kinase which is activated in response to various growth factors and cytokines [1, 2]. Germ-line deletion of Jak2 causes impairment of fetal liver erythropoiesis leading to embryonic lethality in mice [3, 4]. Deletion of Jak2 at postnatal or adult stage results in anemia and thrombocytopenia in mice [5] suggesting a role for Jak2 in erythroid/megakaryocytic development. However, the role of Jak2 in the maintenance and function of adult HSCs has not been clearly elucidated. Also, the mechanism by which Jak2 regulates HSC function remains unknown.

An activating JAK2V617F mutation has been associated with most cases of myeloproliferative neoplasms (MPNs) [6–10]. MPNs are considered to be clonal stem cell-derived disorders, which are characterized by excessive production of myeloid/erythroid/megakaryocytic lineage cells [11, 12]. Several Jak2 inhibitors have been developed for treatment of MPNs, but most patients treated with Jak2 inhibitors exhibit significant hematopoietic toxicities [13–15]. Therefore, understanding the role of Jak2 in adult HSCs/progenitors is of considerable significance and has potential clinical implications.

In this report, we examined the role of Jak2 in adult HSCs/progenitors using conditional Jak2 knockout and MxCre mice. We found that Jak2-deficiency causes loss of quiescence, increased apoptosis, and profound defects in HSC function resulting in early deaths in adult mice. We also observe that Jak2 is cell autonomously required for HSC self-renewal. Jak2-deficiency causes impairment of Stat5, Erk, and Akt signaling mediated by thrombopoietin (TPO) and stem cell factor (SCF) in HSC-enriched Lin<sup>−</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> (LSK)

cells. Gene expression analyses also reveal significant down-regulation of HSC-related gene sets in Jak2-deficient LSK cells. Together, these results suggest an essential role for Jak2 in the maintenance and function of adult HSCs.

## MATERIALS AND METHODS

### Mice

Conditional Jak2 floxed (Jak2<sup>fl/fl</sup>) [16] mice were crossed with MxCre [17] mice to generate MxCre;Jak2<sup>fl/fl</sup> mice. Cre expression was induced by intraperitoneal injection of five doses of 300 µg of polyinosine-polycytosine (pI;pC, GE Healthcare, Pittsburgh, PA, <http://www.gelifesciences.com/>). C57BL6/J (CD45.2) and BL6.SJL-Ptprca Pep3b/BoyJ (CD45.1) mice were purchased from the Jackson Laboratory, Bar Harbor, ME, <http://www.jax.org/>. All animal studies were approved by the Institutional Animal Care and Use Committee of SUNY Upstate Medical University.

### Blood and Tissue Analysis

Peripheral blood (PB) cell counts were determined using Hema-vet 950FS (Drew Scientific, Dallas, TX, <http://www.drew-scientific.com/>). Blood smears were stained with Wright-Giemsa. For histopathologic analysis, mouse tissue specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin. Tissue sections (4 µm) were stained with hematoxylin and eosin.

### Flow Cytometry

Single-cell suspensions were prepared from bone marrow (BM) and red cells were lysed with red cell lysis solution. Cells were washed and resuspended in phosphate buffered saline (PBS) plus 2% fetal bovine serum (FBS). For HSC/progenitor analysis, BM cells were stained for 1 hour on ice with antibodies against c-Kit, Sca-1, CD34, CD16/32 (FcγR II/III), CD41, CD48, CD150, CD135, and antibodies against lineage (Lin) markers including CD3, CD4, CD8, CD19, B220, Gr-1, CD127, and Ter119. Flow cytometry antibodies were purchased from eBioscience, San Diego, CA, <http://www.ebioscience.com/>, BioLegend, San Diego, CA, <http://www.biolegend.com/>, or BD Biosciences, San Jose, CA, <http://www.bdbiosciences.com/>. BrdU incorporation in LSK was determined using the FITC BrdU Flow Kit (BD Biosciences) according to the manufacturer's protocol. For Hoechst 33342 (HO; Sigma-Aldrich, St. Louis, MO, <http://www.sigmaaldrich.com/>) and Pyronin Y (PY; Sigma-Aldrich) staining, Lin<sup>−</sup> cells were first enriched by magnetic-activated cell sorting (MACS; Miltenyi, San Diego, CA, <https://www.miltenyibiotec.com/>) and then stained with LSK surface markers along with HO/PY in the presence of verapamil (Sigma-Aldrich). To assess apoptosis, BM cells were stained with LSK surface markers and Annexin V followed by flow cytometric analysis. To assess signaling in LSK cells, MACS-enriched Lin<sup>−</sup> BM cells were starved for 1 hour before stimulation with SCF (50 ng/ml) or TPO (50 ng/ml) for 5 or 15 minutes. Cells were fixed with 4% paraformaldehyde, permeabilized with acetone, and then stained with LSK surface markers along with Alexa488-conjugated phospho-specific antibodies (Cell Signaling Technology, Danvers, MA <http://www.cellsignal.com/>). Flow cytometry was performed with an LSRII (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR, <http://www.treestar.com/>).

### Colony-Forming Assays

BM ( $2 \times 10^4$ ) cells were plated in duplicates in complete methylcellulose medium (MethoCult M3434; StemCell Technologies, Vancouver, Canada, <http://www.stemcell.com/>). Burst-forming units-erythroid (BFU-E), granulocyte-macrophage colony-forming units (CFU-GM), colony-forming units-granulocyte, erythrocyte macrophage, and megakaryocyte (CFU-GEMM) colonies were scored on day 7. To detect colony-forming units-erythroid (CFU-E) colonies, BM cells were plated in MethoCult M3234 medium (StemCell Technologies) in the presence of erythropoietin (Epo). CFU-E colonies were counted after 2 days by staining with benzidine solution (Sigma-Aldrich). To detect colony-forming units-megakaryocyte (CFU-Mk), BM cells were plated in collagen-based MegaCult medium (StemCell Technologies) in the presence of IL-6, IL-11, and Tpo. CFU-Mk colonies were scored after 7–8 days according to manufacturer's protocol.

### BM Transplantation Assays

For noncompetitive BM transplantation (BMT) assay, BM cells from pI;pC-induced MxCre;Jak2<sup>fl/fl</sup> or littermate control (Jak2<sup>fl/fl</sup>) mice (CD45.2<sup>+</sup>) were transplanted into lethally irradiated CD45.1<sup>+</sup> recipient mice by retro-orbital injection without competitor. For competitive reconstitution assay, BM cells from control (Jak2<sup>fl/fl</sup>) or MxCre;Jak2<sup>fl/fl</sup> mice (CD45.2<sup>+</sup>) were mixed with CD45.1<sup>+</sup> competitor BM cells at 1:1 or 10:1 (CD45.2/CD45.1) ratio and transplanted into lethally irradiated CD45.1<sup>+</sup> recipients by retro-orbital injection. In some cases, Jak2 was deleted after the BMT in hematopoietic compartments of recipient animals by injection with 5 doses of pI;pC at 4 weeks after transplantation. Chimerism was measured in PB and BM of transplanted animals by CD45.2 and CD45.1 expression.

### Microarray Analysis

LSK cells were sorted from the BM of control (Jak2<sup>fl/fl</sup>) or MxCre;Jak2<sup>fl/fl</sup> mice 4 weeks after pI;pC injection. Total RNA was extracted from the LSK cells using Qiagen, Valencia, CA, <http://www.qiagen.com/>, RNeasy Mini kit (Qiagen). RNA samples were processed using the Ovation Pico WTA System V2 (NuGEN Technologies, San Carlos, CA, [www.nugen.com/](http://www.nugen.com/)), and biotin labeled with the Encore Biotin Module (NuGEN Technologies). Microarray experiments were performed in the SUNY Upstate Medical University Microarray Core Facility using the GeneChip Mouse Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, <http://www.affymetrix.com/>). Arrays were scanned with the Affymetrix GeneChip Scanner 7G Plus. The threshold for considering genes differentially expressed was 1.4-fold difference with a *p* value < .05. Heat maps were generated using MultiExperiment Viewer software available from (<http://www.tm4.org/mev.html>). Gene set enrichment analysis [18] was performed with gene-set enrichment analyses (GSEA) v2.08 software available from the Broad Institute (<http://www.broad.mit.edu/gsea>). Microarray data were also analyzed with Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, <http://www.ingenuity.com/>).

### Quantitative Real-Time PCR

Total RNA was extracted from sorted LSK cells obtained from control (Jak2<sup>fl/fl</sup>) or MxCre;Jak2<sup>fl/fl</sup> mice by RNeasy Mini Kit (Qiagen) and reverse transcribed using QuantiTect Reverse

Transcription kit (Qiagen). Quantitative real-time PCR was performed using SYBR Green PCR master mix (Roche Diagnostics, Roche Applied Science, Indianapolis, IN, <http://www.roche-applied-science.com/>). The data were normalized to 18S and fold-change determined by the  $\Delta\Delta C_t$  method.

### Statistical Analysis

Results are expressed as mean  $\pm$  SEM, and data were analyzed by the two-tailed Student *t* test. A value of *p* less than .05 was considered to be statistically significant.

## RESULTS

### Deletion of Jak2 Results in Fatal BM Failure in Adult Mice

To assess the function of Jak2 in the adult hematopoietic system, we crossed Jak2 floxed (Jak2<sup>fl/fl</sup>) mice with MxCre mice which express Cre recombinase in all hematopoietic compartments including HSCs in response to interferon or polyinosine-polycytosine (pI:pC) treatment [17]. We conditionally deleted Jak2 in MxCre;Jak2<sup>fl/fl</sup> mice by administration of five doses of pI:pC at 4 weeks after birth. As shown in Figure 1A, Jak2 was almost completely deleted in the BM of MxCre;Jak2<sup>fl/fl</sup> mice upon pI:pC induction. All induced MxCre;Jak2<sup>fl/fl</sup> (hereafter Jak2-deficient) mice became moribund or died between 25 and 44 days with the mean survival of 36 days after first pI:pC injection (Fig. 1B). Therefore, all analyses were performed at 4 weeks after pI:pC induction unless otherwise specified. Mice with homozygous Jak2 deletion exhibited significantly reduced numbers of white blood cell, neutrophil, red blood cell, hematocrit, and platelet counts in their PB compared with control animals (Supporting Information Fig. S1). Mice with heterozygous Jak2 deletion, however, did not exhibit any significant decrease in their PB counts compared with control animals (Supporting Information Fig. S1). H&E staining of the BM sections from Jak2-deficient animals showed severe BM aplasia (Fig. 1C). Together, these data suggest that deletion of Jak2 results in severe defects in the hematopoietic compartment.

The fatal BM failure in Jak2-deficient mice prompted us to examine the HSC/progenitor compartments in the BM of these animals. We observed a marked decrease in the frequencies and absolute numbers of HSC-enriched Lin<sup>−</sup>Sca-1<sup>+</sup>c-kit<sup>+</sup> (LSK) and early progenitors including common lymphoid progenitors, common myeloid progenitors, granulocyte-macrophage progenitors, and megakaryocyte-erythroid progenitors in the BM of Jak2-deficient mice (Fig. 1D). Further analysis of the HSC compartment revealed a marked reduction in the number of LT-HSC (defined as Lin<sup>−</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>CD34<sup>−</sup>CD135<sup>−</sup> or Lin<sup>−</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>CD150<sup>+</sup>CD41<sup>−</sup>CD48<sup>−</sup>), ST-HSC (Lin<sup>−</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>CD34<sup>+</sup>CD135<sup>−</sup>), and multipotent progenitors (MPP) (Lin<sup>−</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>CD34<sup>+</sup>CD135<sup>+</sup>) [19, 20] in Jak2-deficient BM (Fig. 1E, 1F), indicating a defect at the earliest stage of adult hematopoietic development.

Next, we performed colony-forming unit (CFU) assays to assess HSC/progenitor function. Consistent with the flow cytometric data, Jak2-deficient BM cells produced markedly reduced numbers of CFU-E, BFU-E, CFU-GM, CFU-GEMM, and CFU-Mk colonies compared with control BM cells (Fig. 1G).

Similar results were obtained when sorted LSK cells were used in colony assays (Fig. 1H). Thus, loss of Jak2 results in decrease in the number and differentiation potential of the HSCs/progenitors.

To determine whether the loss of HSCs in Jak2-deficient animals is cell autonomous, BM cells from uninduced MxCre;Jak2<sup>fl/fl</sup> mice were transplanted into lethally irradiated congenic CD45.1<sup>+</sup> WT animals. Four weeks after transplantation, recipients were injected with pI:pC, and the percentages of donor-derived (CD45.2<sup>+</sup>) HSCs were assessed at 8 weeks after pI:pC induction using flow cytometry. As shown in Figure 1I, the number of donor-derived LT-HSCs was significantly reduced in recipient mice transplanted with MxCre;Jak2<sup>fl/fl</sup> BM compared with control BM, similar to those observed in primary Jak2-deficient animals. Furthermore, transplantation of WT CD45.1<sup>+</sup> BM into MxCre;Jak2<sup>fl/fl</sup> (CD45.2<sup>+</sup>) recipient mice showed that donor-derived HSCs were not reduced upon induction of Jak2 deletion in the mutant recipients (Fig. 1J), indicating that deletion of Jak2 from a nonhematopoietic compartment does not contribute to the HSC phenotype. These data strongly suggest that the reduction of HSCs in Jak2-deficient mice is cell autonomous.

### Jak2 Deficiency Impairs Long-Term Engraftment and Self-Renewing Capacity of Adult HSCs

To examine whether Jak2-deficient HSCs are intrinsically capable of engraftment and/or differentiation, different doses of BM cells from control or Jak2-deficient mice were transplanted into lethally irradiated recipients in the absence of competitor cells. Whereas control BM cells ( $1 \times 10^5$ ) were able to efficiently engraft, Jak2-deficient BM cells failed to engraft even at cell doses 20 times higher than controls ( $2 \times 10^6$  total BM cells) and all recipients of Jak2-deficient BM died within 18 days after transplantation (Fig. 2A).

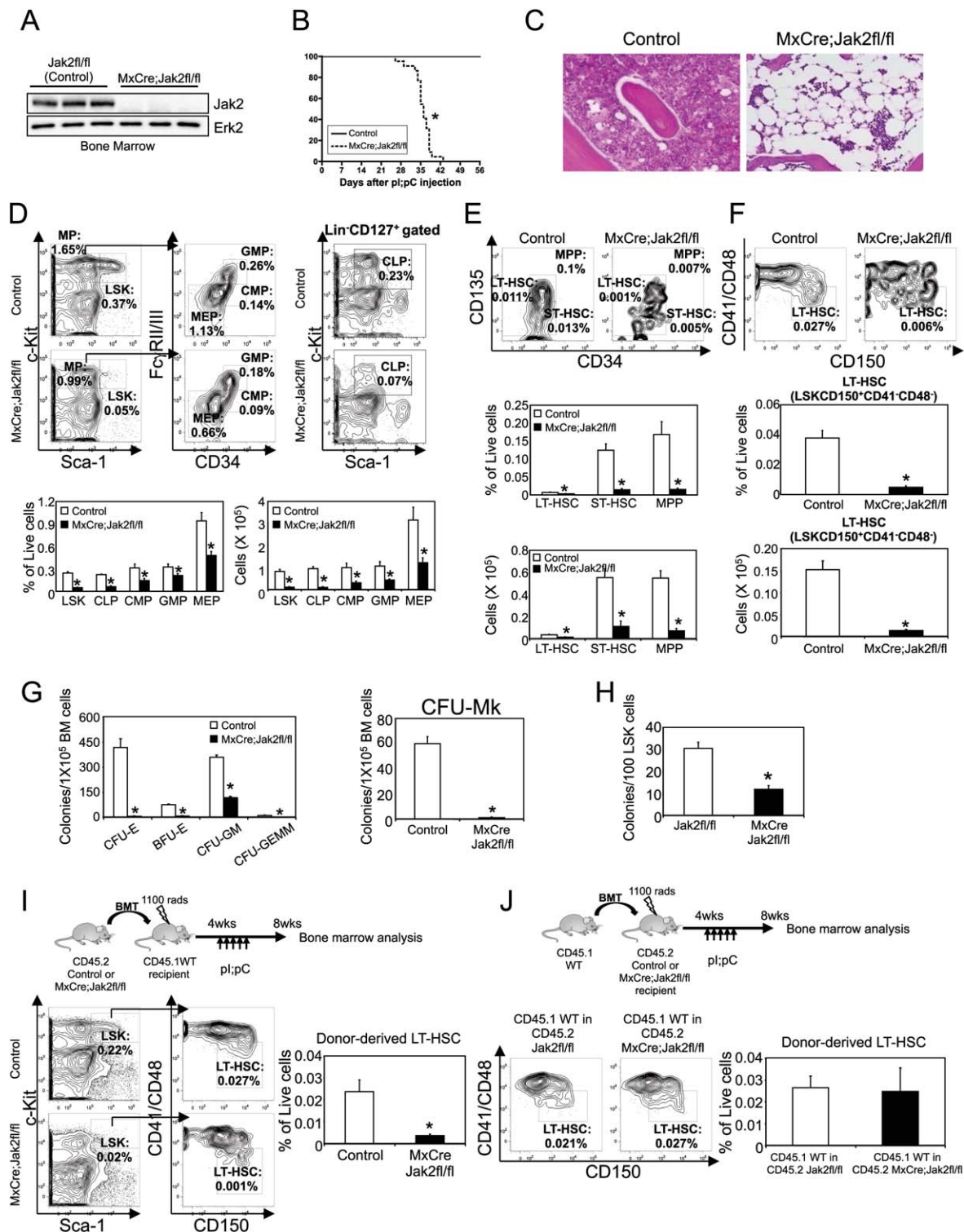
We next performed competitive repopulation assays to further evaluate the function of Jak2-deficient HSCs in hematopoietic reconstitution. BM cells from pI:pC-induced control or Jak2-deficient mice (CD45.2<sup>+</sup>) were mixed with CD45.1<sup>+</sup> competitor BM cells at a ratio of 1:1 or 10:1 and then transplanted into lethally irradiated congenic recipient animals (CD45.1<sup>+</sup>). Chimerism analysis in PB of transplanted animals revealed that BM cells from Jak2-deficient mice were completely unable to compete with WT BM cells even when transplanted at a 10-fold higher dose of mutant (Jak2-deficient) cells than competitor cells (Fig. 2B). The percentages of mutant (Jak2-deficient) donor-derived (CD45.2<sup>+</sup>) myeloid, B, and T cells were significantly lower than those derived from control (Jak2<sup>fl/fl</sup>) BM donor (Fig. 2B). At 12 weeks after transplantation, mutant donor-derived (CD45.2<sup>+</sup>) LSK cells were almost undetectable in the BM of recipient animals, whereas more than 50% LSK cells in recipients that had received control BM were donor-derived (CD45.2<sup>+</sup>) (Fig. 2C). These data suggest that Jak2-deficient HSCs are functionally defective in hematopoietic reconstitution.

To exclude the possibility that the functional defects observed in Jak2-deficient HSCs are not only due to poor engraftment of Jak2-deficient HSCs, we also performed competitive repopulation assays using uninduced BM cells from MxCre;Jak2<sup>fl/fl</sup> or control (Jak2<sup>fl/fl</sup>) mice (CD45.2<sup>+</sup>) along with CD45.1<sup>+</sup> WT competitor BM cells at a ratio of 1:1 and



transplanted into lethally irradiated congenic CD45.1 animals. Four weeks after transplantation (to allow establishment of steady-state hematopoiesis), recipients were injected with five doses of pl;pC. All mice receiving control (Jak2fl/fl) or MxCre;Jak2fl/fl BM showed comparable levels of CD45.2 engraftment in the myeloid, B, and T cells before pl;pC injection (Fig. 2D).

Whereas recipients of control (Jak2fl/fl) BM maintained more than 50% CD45.2<sup>+</sup> myeloid, B, and T cells in their PB for 16 weeks after pl;pC treatment, the percentage of CD45.2<sup>+</sup> cells in the PB was progressively and significantly reduced after pl;pC induction in mice that had received MxCre;Jak2fl/fl BM (Fig. 2D). At 16 weeks after pl;pC induction, MxCre;Jak2fl/fl



BM-derived (CD45.2<sup>+</sup>) LSK cells were markedly diminished in the recipient animals, whereas ~50% LSK cells in recipients that had received control (Jak2fl/fl) BM were CD45.2<sup>+</sup> (Fig. 2E). These data strongly suggest that Jak2 deficiency leads to severe functional impairment in stem cells.

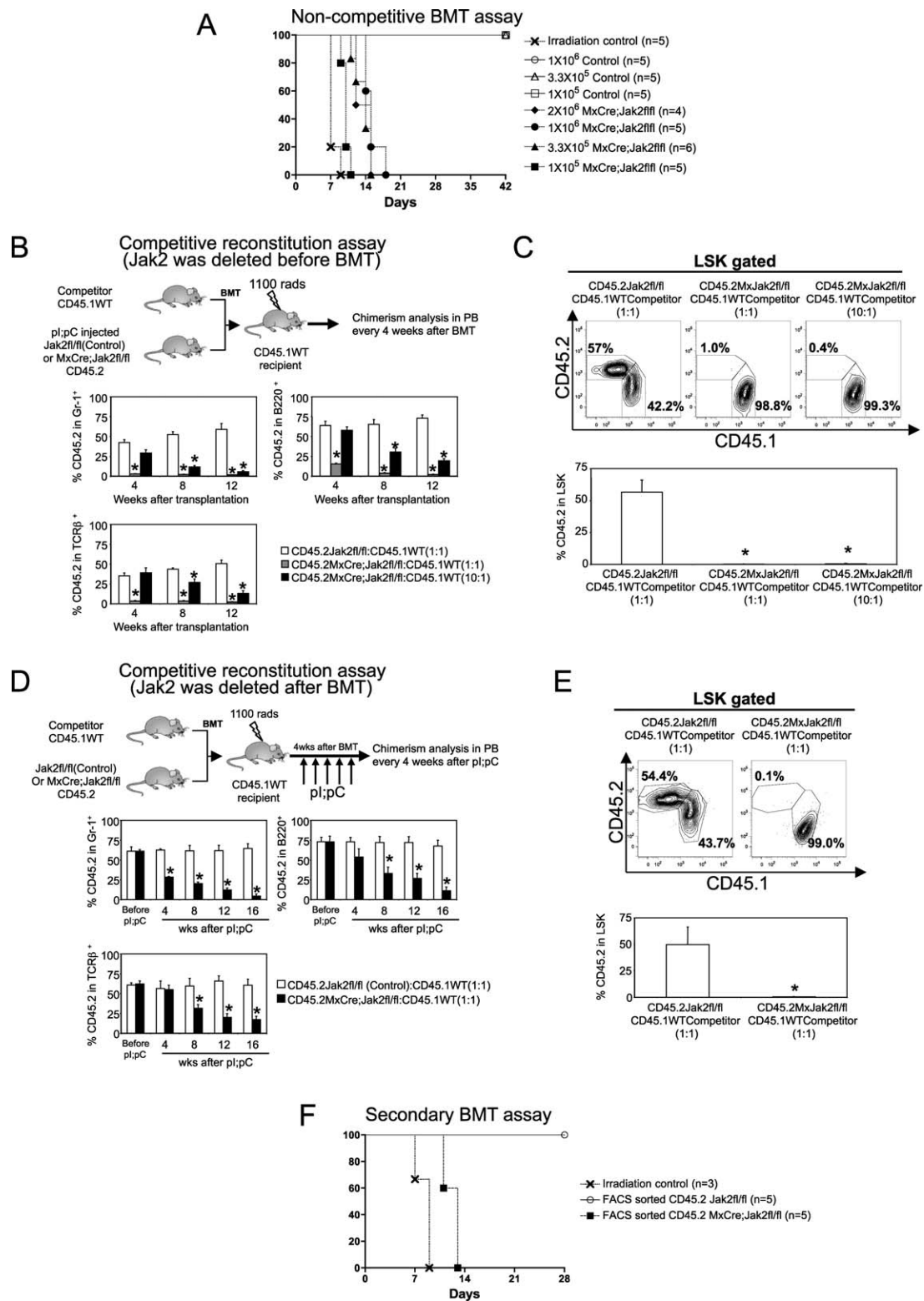
Since deletion of Jak2 in the BM leads to exhaustion of stem cells and profound defects in hematopoietic reconstitution in the primary transplant recipients (Fig. 2A, 2C, 2E), we examined whether Jak2 is required for HSC self-renewal using a secondary transplantation experiment. Jak2 deletion in primary mice (before the BMT) severely impaired hematopoietic reconstitution (Fig. 2A, 2B) and thus cannot be used for secondary transplantation experiment. So, we used the BM from the recipient animals in which Jak2 was deleted from hematopoietic compartments by pl;pC induction after the hematopoietic reconstitution (as in Fig. 2D). BM cells from uninduced control (Jak2fl/fl) or MxCre;Jak2fl/fl (CD45.2<sup>+</sup>) cells were transplanted into CD45.1 recipients along with competitor (CD45.1<sup>+</sup>) cells. Four weeks after BMT, pl;pC was injected to induce Jak2 deletion in hematopoietic compartments of the recipient animals (as in Fig. 2D). At 12 weeks after pl;pC injection, BM cells were harvested and sorted for the donor CD45.2 marker. Sorted CD45.2<sup>+</sup> (donor-derived) cells were then transplanted ( $5 \times 10^5$  cells/recipient) into lethally irradiated secondary recipients (CD45.1) without the competitor cells. Whereas CD45.2<sup>+</sup> control cells were able to efficiently engraft and reconstitute hematopoiesis in secondary recipients, Jak2-deficient cells were unable to engraft secondary recipients and all mice receiving CD45.2<sup>+</sup> Jak2-deficient cells died within 14 days after transplantation (Fig. 2F). These data suggest that Jak2 is absolutely required for self-renewal of adult HSCs.

### Jak2 Is Required for the Maintenance of Quiescence and Survival of LSK Cells

Most HSCs remain in the quiescent state (G<sub>0</sub>) for long-term maintenance of the HSC pool in adult BM. Reduced numbers of HSCs and progenitors in Jak2-deficient mice could be due to loss of quiescence and/or increased cell death. To determine whether Jak2 is required for the maintenance of HSC quiescence, we assessed the cell cycle distribution of HSC-enriched LSK cells in control and Jak2-deficient mice at 4 weeks after pl;pC induction using BrdU incorporation assay. BrdU was injected into control and Jak2-deficient mice 4 hours prior to BM harvest. We observed significantly more BrdU incorporation in Jak2-deficient LSK with a much higher proportion in the S phase and lower proportion in the G<sub>0</sub>/G<sub>1</sub> phase compared with control LSK (Fig. 3A). Further analysis of the cell cycle by Hoechst 33342/Pyronin Y staining revealed that significantly fewer number of Jak2-deficient LSK cells were in the quiescent G<sub>0</sub> phase, with a higher proportion in S/G<sub>2</sub>/M (Fig. 3B). Notably, deletion of Jak2 did not induce proliferation or any significant change in cell cycle within myeloid progenitors (Lin<sup>-</sup>c-kit<sup>+</sup>) (Supporting Information Fig. S2). These results suggest that Jak2 deficiency results in HSC-specific loss of quiescence, which may contribute to rapid exhaustion of HSCs in Jak2-deficient animals.

To assess whether Jak2 is required for survival of HSCs, we performed annexin V staining of LSK cells from control and Jak2-deficient mice. Annexin V staining showed significantly (approximately ninefold) increased apoptosis in Jak2-deficient LSK compared with control LSK (Fig. 3C), suggesting that Jak2 also plays an important role in survival of HSC pool. Collectively, our data indicate that reduced quiescence and increased apoptosis may account for loss of HSCs in Jak2-deficient mice.

**Figure 1.** Deletion of Jak2 leads to fatal bone marrow (BM) failure and loss of HSCs/progenitors. **(A):** The expression of Jak2 in the BM of Jak2fl/fl (control) and MxCre;Jak2fl/fl (Jak2-deficient) mice was determined by immunoblotting at 4 weeks after pl;pC injection. Erk2 was used as a loading control. **(B):** Kaplan-Meier survival analysis of MxCre;Jak2fl/fl ( $n = 24$ ) and littermate control (Jak2fl/fl) ( $n = 24$ ) mice after pl;pC treatment. MxCre;Jak2fl/fl mice have a median survival of 36 days after first pl;pC injection; \*,  $p < .05$ , log-rank test. **(C):** H&E staining of the BM sections ( $\times 200$ ) demonstrates pancytopenia and severe aplasia in Jak2-deficient BM. **(D):** Flow cytometric analysis of LSK compartment (Lin<sup>-</sup>Sca1<sup>+</sup>c-kit<sup>+</sup>) and myeloid progenitors including CMP (Lin<sup>-</sup>Sca1<sup>+</sup>c-kit<sup>+</sup>CD34<sup>+</sup>FcγRII/III<sup>lo</sup>), CLP (Lin<sup>-</sup>CD127<sup>+</sup>Sca1<sup>+</sup>c-kit<sup>+</sup>), GMP (Lin<sup>-</sup>Sca1<sup>+</sup>c-kit<sup>+</sup>CD34<sup>+</sup>FcγRII/III<sup>hi</sup>), and MEP (Lin<sup>-</sup>Sca1<sup>+</sup>c-kit<sup>+</sup>CD34<sup>+</sup>FcγRII/III<sup>-</sup>) in the BM from control or Jak2-deficient (MxCre;Jak2fl/fl) mice ( $n = 10$ ) at 4 weeks after pl;pC injection. The percentages and absolute numbers of LSK, CLP, CMP, GMP, and MEP are shown in bar graphs as mean  $\pm$  SEM. \*,  $p < .05$ . **(E):** Flow cytometric analysis of LT-HSC (Lin<sup>-</sup>Sca1<sup>+</sup>c-kit<sup>+</sup>CD34<sup>+</sup>CD135<sup>-</sup>), ST-HSC (Lin<sup>-</sup>Sca1<sup>+</sup>c-kit<sup>+</sup>CD34<sup>+</sup>CD135<sup>-</sup>), and MPP (Lin<sup>-</sup>Sca1<sup>+</sup>c-kit<sup>+</sup>CD34<sup>+</sup>CD135<sup>+</sup>) in the BM from control or Jak2-deficient (MxCre;Jak2fl/fl) mice at 4 weeks after pl;pC injection ( $n = 7$ ). Representative contour plots are shown in the top. The percentages and absolute numbers of LT-HSC, ST-HSC, and MPP are shown in bar graphs as mean  $\pm$  SEM. \*,  $p < .05$ . **(F):** Flow cytometric analysis of SLAM-HSC (Lin<sup>-</sup>Sca1<sup>+</sup>c-kit<sup>+</sup>CD150<sup>+</sup>CD41<sup>-</sup>CD48<sup>-</sup>) in the BM from control or Jak2-deficient (MxCre;Jak2fl/fl) mice at 4 weeks after pl;pC injection ( $n = 5$ ). Representative contour plots are shown in the top. The percentages and absolute numbers of SLAM-HSC are shown in bar graphs as mean  $\pm$  SEM; \*,  $p < .05$ . **(G, H):** Hematopoietic progenitor colony assays. Total BM cells ( $2 \times 10^6$ ) **(G)** or sorted LSK cells (100) **(H)** from control or Jak2-deficient (MxCre;Jak2fl/fl) mice were plated in methylcellulose medium MethoCult 3434 (Stem-Cell Technologies) with cytokines. BFU-E, CFU-GM, and CFU-GEMM colonies were scored 7–8 days after plating. To assess CFU-E colonies, BM cells were plated in methylcellulose medium in the presence of Epo. CFU-E colonies were scored 3 days after plating. To assess CFU-Mk colonies, BM cells were plated in collagen-based MegaCult media with IL-6, IL-11, and Tpo. Colonies were counted after 7–8 days. Note that deletion of Jak2 significantly reduces hematopoietic progenitor colonies in the total BM or sorted LSK cells from MxCre;Jak2fl/fl mice ( $n = 5$ ). \*,  $p < .05$ . **(I):** Cell autonomous BM transplantation (BMT) assay. BM cells ( $1 \times 10^6$  cells) from uninduced MxCre;Jak2fl/fl or littermate control (Jak2fl/fl) mice (CD45.2<sup>+</sup>) were transplanted into lethally irradiated CD45.1 recipients. Deletion of Jak2 was induced into the donor-derived hematopoietic cells of recipient animals by injecting pl;pC 4 weeks after BMT. Note that donor-derived (CD45.2<sup>+</sup>) HSC (LSKCD150<sup>+</sup>CD41<sup>-</sup>CD48<sup>-</sup>) was significantly reduced in recipients of Jak2-deficient BM ( $n = 5$ , \*,  $p < .05$ ). **(J):** BM cells ( $1 \times 10^6$  cells) from CD45.1 WT mice were transplanted into lethally irradiated CD45.2 control (Jak2fl/fl) or MxCre;Jak2fl/fl recipients. Four weeks after BMT to allow establishment of steady-state hematopoiesis, Jak2 was deleted from nonhematopoietic cells in the mutant recipients with five doses of pl;pC injection. Note that CD45.1<sup>+</sup> WT donor-derived HSCs (LSKCD150<sup>+</sup>CD41<sup>-</sup>CD48<sup>-</sup>) were not reduced upon induction of Jak2 deletion in mutant recipients ( $n = 3$ ). Abbreviations: CFU, colony-forming unit; HSC, hematopoietic stem cell; LSK, Lin<sup>-</sup>Sca1<sup>+</sup>c-kit<sup>+</sup>.





### Increased Reactive Oxygen Species and p38 MAPK Activation in Jak2-Deficient LSK Cells

It has been shown that elevated reactive oxygen species (ROS) promotes loss of quiescence and limits the lifespan of HSCs through p38 MAPK activation [21]. Therefore, we assessed whether the defects in HSC maintenance in Jak2-deficient mice were associated with increased levels of ROS and p38 MAPK activation. Intracellular concentration of ROS was measured in control and Jak2-deficient LSK cells by chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate staining. We observed a significant increase in ROS levels in Jak2-deficient LSK cells compared with control LSK cells (Fig. 4A). Activation of p38 MAPK was assessed in LSK cells by flow cytometry following intracellular staining with Alexa488-conjugated phospho-p38 MAPK antibody. We observed significantly (approximately threefold) increased p38 MAPK phosphorylation in Jak2-deficient LSK cells compared with control LSK cells (Fig. 4B).

Next, we asked if reduction of ROS levels by treatment with ROS scavenger *N*-acetyl L-cysteine (NAC) could improve HSC function and prolong the lifespan of Jak2-deficient HSCs. We observed that ex vivo treatment with NAC partially, albeit significantly, rescued the hematopoietic reconstitution defects in Jak2-deficient HSCs (Fig. 4C). Whereas <1% donor-derived (CD45.2<sup>+</sup>) LSK cells were detected in the BM of recipient animals (CD45.1) that had received PBS-treated Jak2-deficient BM at 4 weeks after transplantation, ~20% LSK cells in recipients that had received NAC-treated Jak2-deficient BM were CD45.2<sup>+</sup> (Fig. 4C). Together, these data suggest that Jak2-deficiency leads to elevated ROS levels, which may contribute to impairment in HSC maintenance.

### Perturbed TPO and SCF Signaling in Jak2-Deficient LSK Cells

TPO and SCF are required for HSC maintenance in vivo [22–26], and Jak2 has been shown to interact with TPO receptor myeloproliferative leukemia virus oncogene (Mpl) and SCF receptor Kit [27, 28]. Therefore, we investigated the effects of Jak2-deficiency in signaling mediated by TPO and SCF in HSC-enriched LSK cells. Intracellular phospho flow cytometric analysis revealed that TPO-evoked Stat5, Erk, and Akt activation were markedly reduced in Jak2-deficient LSK cells compared

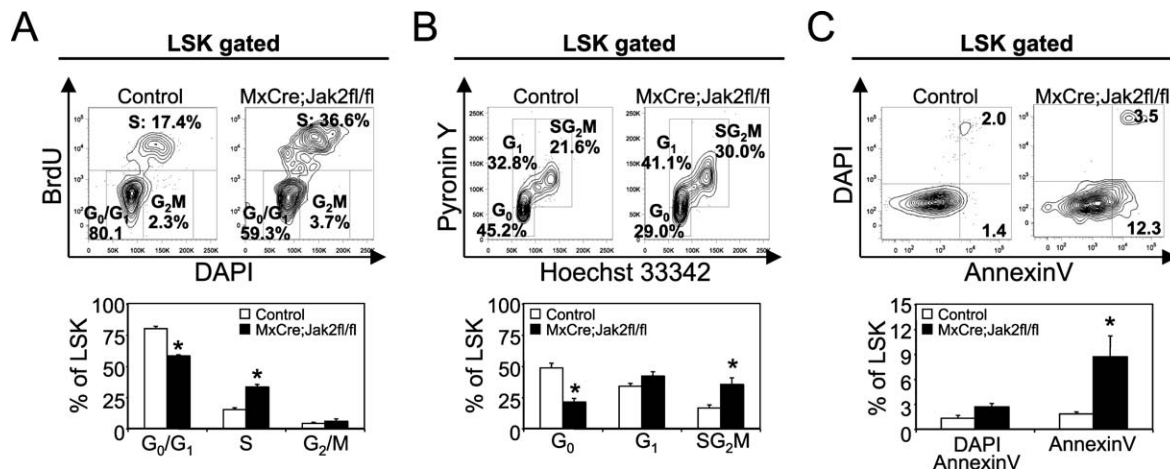
with control LSK cells (Fig. 5A). Notably, control and Jak2-deficient LSK cells express similar levels of TPO receptor Mpl (Fig. 5B). Although SCF stimulation did not cause significant activation of Stat5 in either control or Jak2-deficient LSK cells, SCF-evoked Erk and Akt activation were substantially reduced in Jak2-deficient LSK cells compared with control LSK cells (Fig. 5C). Unlike Mpl, Kit expression was significantly reduced in Jak2-deficient Lin<sup>−</sup>Sca-1<sup>+</sup>CD41<sup>−</sup>CD48<sup>−</sup>CD150<sup>+</sup> cells (Fig. 5D), indicating that Jak2 may regulate Kit expression. Thus, the HSC phenotype in Jak2-deficient mice can be attributed at least in part to perturbed TPO/Mpl and SCF/Kit signaling.

### Jak2 Deficiency Impairs Multiple Pathways Responsible for HSC Maintenance

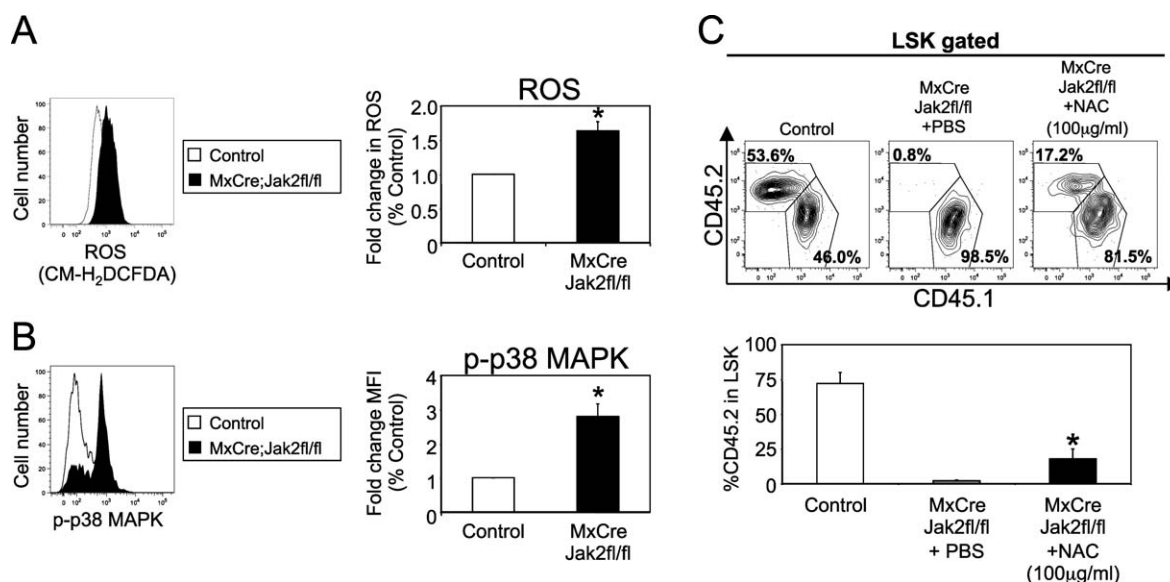
To identify the Jak2-dependent genes and pathways regulating HSC maintenance, we performed global gene expression profiling analyses. We used HSC-enriched LSK instead of LT-HSC for microarray analyses since the number of LT-HSC was extremely low in Jak2-deficient mice. Three independent sets of microarray were performed using sorted LSK cells from control and Jak2-deficient mice (15 Jak2-deficient mice were used). GSEA [18] on microarray data revealed significant downregulation of HSC-specific genes in Jak2-deficient LSK cells (Fig. 6A and Supporting Information Table S1). GSEA also revealed a strong correlation of genes downregulated in the absence of Jak2 with gene sets that identify the Jak/Stat signaling pathway, and various other signaling pathways including TPO, MAPK, PI3 kinase, and mTOR pathways (Fig. 6A), which have been implicated in HSC maintenance [22, 23, 29–32]. GSEA and ingenuity pathway analysis (IPA) further revealed enrichment for cell cycle-related and cell death and survival-related genes (Fig. 6A, 6B and Supporting Information Table S1), consistent with the loss of quiescence and increased apoptosis observed in Jak2-deficient LSK. Expression of several genes including c-Jun, Pim-1, Gfi-1, Mecom, Pbx1, Foxo3, Atm, and p21<sup>Cip1</sup>, which were shown to play important roles in the maintenance of quiescence and/or self-renewal of HSCs [20, 33–39], was significantly downregulated in Jak2-deficient LSK compared with control LSK (Fig. 6C). Microarray results were further validated by quantitative real-time PCR in sorted LSK cells (Fig. 6D).

Since c-Jun was one of the most highly downregulated genes in Jak2-deficient LSK, we asked if ectopic expression of c-Jun could rescue the defects in Jak2-deficient HSC using

**Figure 2.** Defective stem cell function in Jak2-deficient mice. **(A):** Noncompetitive BMT assay. Different doses of bone marrow (BM) cells from control ( $1 \times 10^5$  to  $1 \times 10^6$  cells) or MxCre;Jak2fl/fl ( $1 \times 10^5$  to  $2 \times 10^6$  cells) mice were transplanted into lethally irradiated CD45.1 recipient mice in absence of competitor cells. Note that Jak2-deficient BM cells failed to reconstitute hematopoiesis in recipient animals even when transplanted 20-times more mutant BM cells than control BM cells. **(B):** Competitive reconstitution assay with Jak2 deletion before BMT. Total BM cells from pl;pC-induced control or MxCre;Jak2fl/fl (CD45.2) mice were transplanted into lethally irradiated CD45.1 recipient mice in competition with BM from CD45.1 WT mice, at different ratios (1:1 or 10:1) of donor versus competitor. The percentages of donor-derived (CD45.2<sup>+</sup>) myeloid cells (Gr-1<sup>+</sup>), B cells (B220<sup>+</sup>), and T cells (TCRβ<sup>+</sup>) in peripheral blood at different time points after transplantation are shown as mean ± SEM ( $n = 5$ , \*,  $p < .05$ ). **(C):** The percentages of donor-derived (CD45.2<sup>+</sup>) LSK in the BM of the recipient animals are shown at 12 weeks after transplantation ( $n = 5$ ). **(D):** Competitive reconstitution assay with Jak2 deletion after BMT. Total BM cells from uninduced MxCre;Jak2fl/fl or littermate control (CD45.2) mice were transplanted into lethally irradiated CD45.1 recipient mice in competition with BM from CD45.1 WT mice at 1:1 ratio. Four weeks after BMT, recipient mice were treated with five doses of pl;pC to induce Jak2 deletion after hematopoietic reconstitution. The percentages of donor-derived (CD45.2<sup>+</sup>) myeloid cells (Gr-1<sup>+</sup>), B cells (B220<sup>+</sup>), and T cells (TCRβ<sup>+</sup>) in peripheral blood were measured every 4 weeks after pl;pC injection. **(E):** The percentages of donor-derived (CD45.2<sup>+</sup>) LSK in the BM of the recipient animals at 20 weeks after transplantation (16 weeks after pl;pC injection) are shown as mean ± SEM ( $n = 5$ , \*,  $p < .05$ ). **(F):** Survival curves of secondary transplanted animals. CD45.2<sup>+</sup> (donor-derived) cells from primary transplanted mice (as in Fig. 2D) were fluorescence-activated cell sorted 12 weeks after pl;pC injection, and injected into lethally irradiated CD45.1 secondary recipient mice ( $5 \times 10^5$  CD45.2<sup>+</sup> cells/recipient) ( $n = 5$ ). Note that Jak2-deficient cells failed to engraft secondary recipients and all secondary recipients that had received Jak2-deficient cells died within 2 weeks after transplantation. Abbreviations: BMT, bone marrow transplantation; LSK, Lin<sup>−</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>.

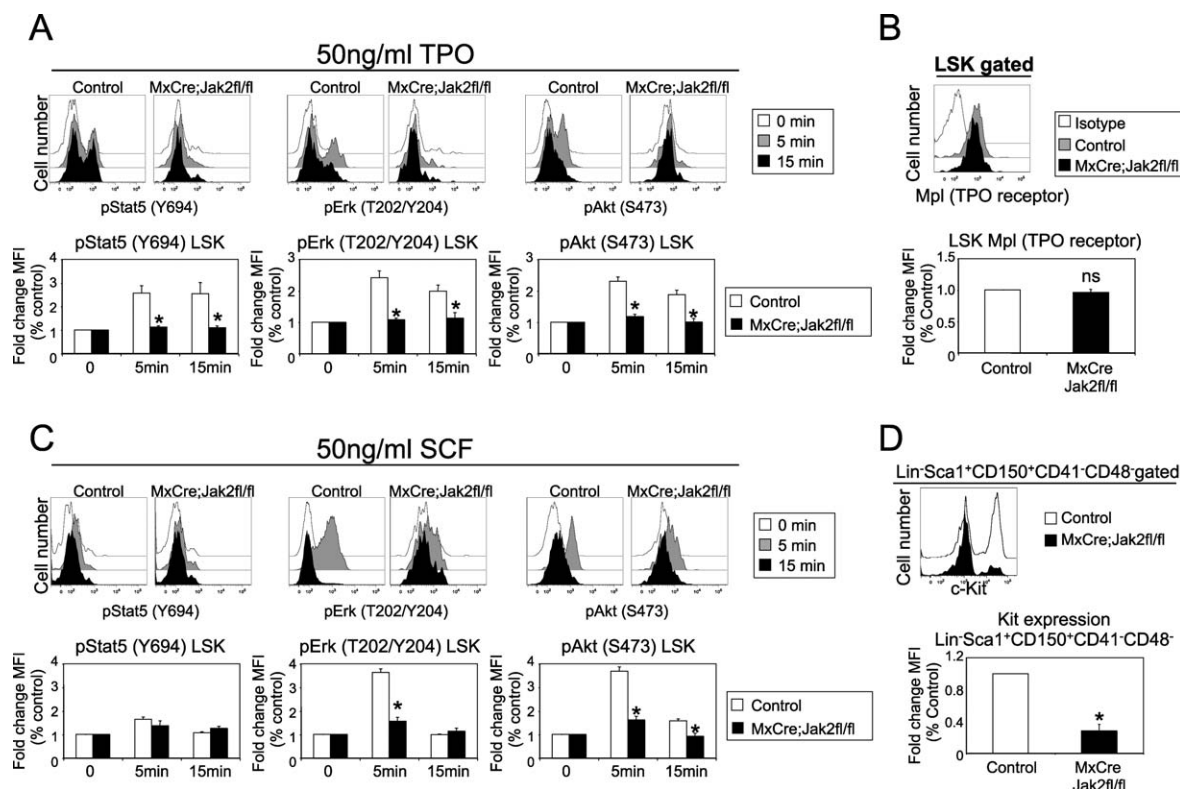


**Figure 3.** Reduced quiescence and increased apoptosis in Jak2-deficient LSK cells. **(A):** Control or Jak2-deficient mice were injected with BrdU and sacrificed 4 hours later. Representative fluorescence-activated cell sorting plots (in the top panel) illustrate BrdU/DAPI staining in LSK cells. Bar graphs in the bottom panel show percentages of BrdU<sup>+</sup> LSK cells in G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M. Data from five independent experiments are shown as mean  $\pm$  SEM. \*,  $p < .05$ . **(B):** Flow cytometric analysis of Hoechst and Pyronin Y staining on LSK cells from control and Jak2-deficient mice bone marrow. Representative contour plots are shown on the top panel. Bar graphs in the bottom panel show percentages of LSK cells in G<sub>0</sub>, G<sub>1</sub>, and S/G<sub>2</sub>/M. Data from five independent experiments are shown as mean  $\pm$  SEM. \*,  $p < .05$ . **(C):** Apoptosis in LSK cells from control or Jak2-deficient mice was determined using Annexin V and DAPI staining. Representative contour plots are shown on the top. Bar graphs (in the bottom) represent percentages of apoptotic LSK cells in control or Jak2-deficient mice. Data from five independent experiments are shown as mean  $\pm$  SEM. \*,  $p < .05$ . Abbreviation: LSK, Lin<sup>−</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>.



**Figure 4.** Elevated ROS levels and increased p38 MAPK activation in Jak2-deficient LSK cells. **(A):** LSK cells from control (Jak2fl/fl) and Jak2-deficient mice were stained with CM-H<sub>2</sub>DCFDA to measure intracellular ROS. Jak2-deficient LSK cells showed increased levels of ROS compared with control LSK. Representative fluorescence-activated cell sorting (FACS) plots are shown on the left. Mean values  $\pm$  SEM are shown on the right ( $n = 3$ , \*,  $p < .05$ ). **(B):** Flow cytometric analysis for phospho-p38 MAPK in LSK cells. MACS-enriched Lin<sup>−</sup> cells from control or Jak2-deficient mice bone marrow (BM) were starved for 1 hour in serum-free media at 37°C. Cells were fixed, permeabilized, and stained with appropriately conjugated antibodies against LSK surface markers and Alexa488 conjugated phospho-p38 MAPK. Representative FACS plots on intracellular phospho-p38 MAPK staining in LSK cells are shown on the left. The fold change in MFI of phospho-p38 MAPK in Jak2-deficient LSK is shown in bar graphs (on the right) as mean  $\pm$  SEM ( $n = 4$ , \*,  $p < .05$ ). **(C):** NAC treatment of Jak2-deficient mice BM partially rescued the defects in hematopoietic reconstitution. Lin<sup>−</sup> BM cells from control or Jak2-deficient mice (CD45.2<sup>+</sup>) were treated with PBS or NAC ex vivo for 48 hours in StemSpan medium (Stem Cell Technologies) containing SCF (50 ng/ml) and Tpo (50 ng/ml) and then  $1 \times 10^6$  cells were transplanted in the presence of  $2 \times 10^5$  helper BM cells (CD45.1<sup>+</sup>) into lethally irradiated CD45.1 recipient mice. The percentages of donor-derived (CD45.2<sup>+</sup>) LSK in the BM of recipient animals were determined at 4 weeks after transplantation by flow cytometry. Representative contour plots are shown on the top panel. Bar graphs (in the bottom) show percentages of donor-derived (CD45.2<sup>+</sup>) LSK cells in recipient mice that had received PBS-treated or NAC-treated Jak2-deficient BM or control BM ( $n = 4$  in each group, \*,  $p < .05$ ). Abbreviations: LSK, Lin<sup>−</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>; MFI, mean fluorescent intensity; NAC, N-acetyl L-cysteine; ROS, reactive oxygen species.





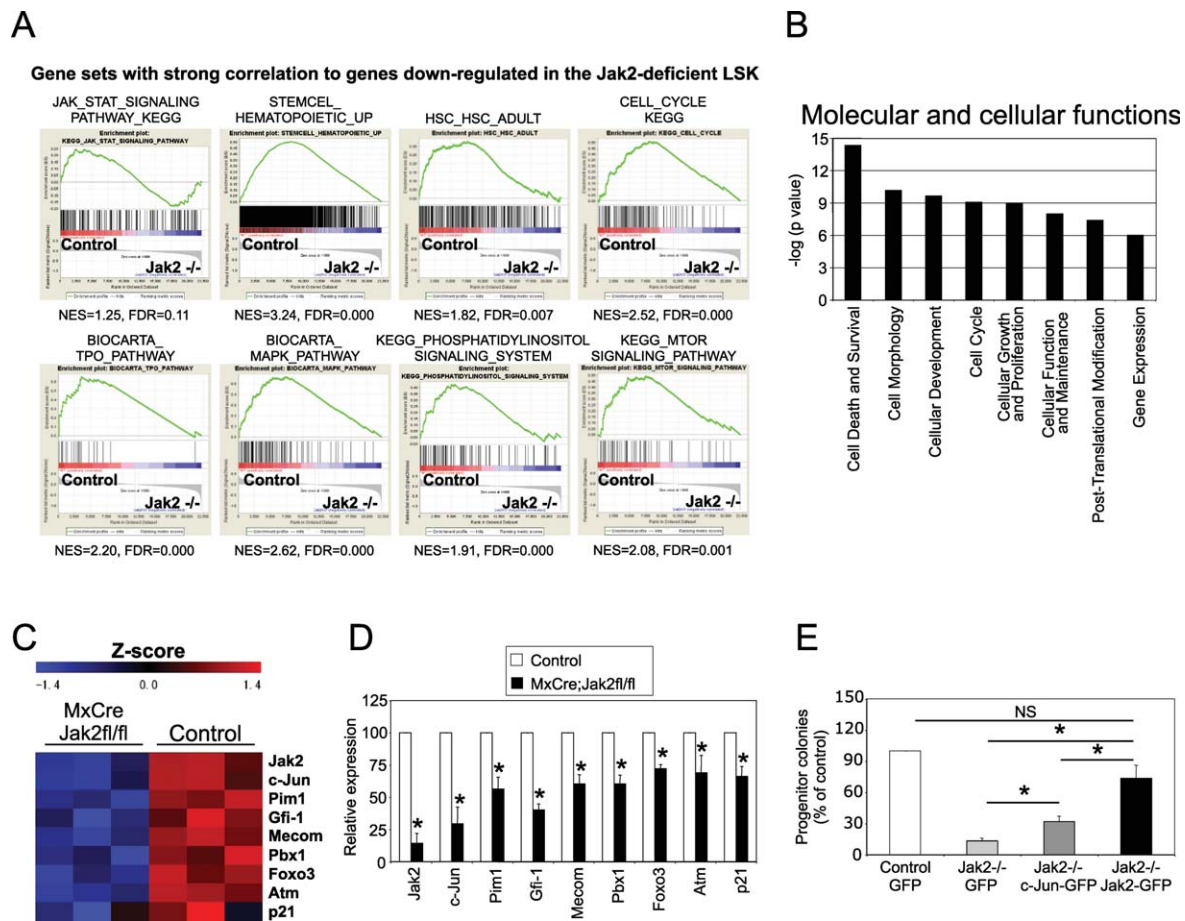
**Figure 5.** Signaling defects in Jak2-deficient LSK cells. **(A):** Lin<sup>+</sup> bone marrow (BM) cells from control or Jak2-deficient mice were starved for 1 hour in serum-free media at 37°C before they were either left untreated or stimulated with 50 ng/ml Tpo for the indicated time. Cells were fixed, permeabilized, and stained with appropriately conjugated antibodies against LSK surface markers and Alexa488 conjugated phospho-stat5, phospho-Erk1/2, or phospho-Akt antibodies. Representative histograms are shown on the top panels, and the fold change in MFI in phospho-stat5, phospho-Erk1/2, or phospho-Akt in Jak2-deficient LSK cells is shown in bar graphs (in the bottom panels) as means  $\pm$  SEM ( $n = 3$ , \*,  $p < .05$ ). **(B):** Representative histograms on Mpl (TPO receptor) expression in control or Jak2-deficient LSK cells are shown (top panel), and the fold change in MFI in Mpl expression from four independent experiments are shown (in the bottom panel) as means  $\pm$  SEM. No significant (ns) difference was observed in Mpl expression between control and Jak2-deficient LSK cells. **(C):** Lin<sup>+</sup> BM cells from control or Jak2-deficient mice were starved for 1 hour in serum-free media at 37°C before they were either left untreated or stimulated with 50 ng/ml SCF for the indicated time. Cells were fixed, permeabilized, and stained for LSK surface markers and phospho-stat5, phospho-Erk1/2, or phospho-Akt as in panel (A). Representative histograms are shown on the top panels, and the fold change in MFI in phospho-stat5, phospho-Erk1/2, or phospho-Akt in Jak2-deficient LSK cells is shown in bar graphs (in the bottom panels) as means  $\pm$  SEM ( $n = 3$ , \*,  $p < .05$ ). **(D):** Relative expression of Kit in SLAM-HSC (Lin<sup>+</sup>Sca1<sup>+</sup>CD150<sup>+</sup>CD41<sup>+</sup>CD48<sup>+</sup>). Representative histograms are shown on the top panels, and the fold change in MFI in Kit expression is shown (in the bottom panel) as means  $\pm$  SEM ( $n = 4$  in each group, \*,  $p < .05$ ). Note that Kit expression was significantly reduced in Jak2-deficient HSCs. Abbreviations: LSK, Lin<sup>+</sup>Sca1<sup>+</sup>c-Kit<sup>+</sup>; MFI, mean fluorescent intensity; SCF, stem cell factor; TPO, thrombopoietin.

hematopoietic progenitor colony formation assay. Whereas wild-type Jak2 expression almost completely rescued the defects in progenitor colony formation, overexpression of c-Jun only partially (although significantly) rescued the defects in progenitor colony formation in Jak2-deficient LSK cells (Fig. 6E). This suggests that multiple genes and pathways related to HSC maintenance and function are perturbed in the absence of Jak2.

## DISCUSSION

Although Jak2 has been studied in various cell lines and tissues, genetic data that directly address the role of Jak2 in HSC maintenance and function are not available. In this report, we demonstrate that Jak2 plays a crucial role in the maintenance and function of adult HSCs. Previous studies showed that germ-line deletion of Jak2 perturbed fetal liver erythropoiesis leading to embryonic lethality in mice [3, 4].

Park et al. showed that tamoxifen-induced deletion of Jak2 in the whole body of young adult animals impaired erythropoiesis and thrombopoiesis [5]. In this study, we developed MxCre;Jak2fl/fl mice to conditionally delete Jak2 in hematopoietic compartments of adult animals. We observed that deletion of Jak2 resulted in BM aplasia with severe anemia and thrombocytopenia in adult animals (Fig. 1 and Supporting Information Fig. S1). In contrast to the previous report by Park et al., in which tamoxifen-induced deletion of Jak2 in young adult animals resulted in only 20% mortality [5], we observed that hematopoietic deletion of Jak2 in adult animals results in 100% mortality (median survival 36 days after induction of Jak2 deletion) (Fig. 1B). The lower rate of mortality observed in tamoxifen-induced adult Jak2 mutant mice was due to incomplete deletion and subsequent repopulation with Jak2-expressing hematopoietic cells [5]. Our data reveal a more profound requirement for Jak2, as complete deletion of Jak2 leads to dramatic loss of HSCs/progenitors in adult animals (Fig. 1D–1F).



**Figure 6.** Gene expression profile of Jak2-deficient Lin<sup>+</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> (LSK) cells. **(A):** Gene-set enrichment analyses (GSEAs) were performed on microarray data obtained from control and Jak2-deficient LSK cells. Enrichment plots of selected gene sets from GSEA analysis with NES and FDR are shown. **(B):** Ingenuity pathway analysis of microarray data revealed that most significantly affected molecular and cellular functions associated with Jak2 deficiency were cell death/survival, cell morphology, cellular development, cell cycle, cell growth, cellular function and maintenance, reflecting abnormal cell cycle, and survival in Jak2-deficient hematopoietic stem cells (HSCs). **(C):** Heat map showing differential expression of a selected list of genes related to HSC maintenance and function in control and Jak2-deficient LSK cells. **(D):** Relative expression of Jak2, c-Jun, Pim1, Gfi-1, Mecom, Pbx1, Foxo3, Atm, and p21 mRNA were determined by quantitative real-time PCR and normalized with 18S expression. Asterisks indicate significant differences by Student *t* test with *p* < .05. **(E):** Ectopic expression of c-Jun only partially rescues the defects in hematopoietic progenitor colony formation in Jak2-deficient LSK cells. Lin<sup>+</sup> BM cells from control or Jak2-deficient mice were transduced with lentiviruses expressing GFP alone or c-Jun-IRES-GFP or Jak2-IRES-GFP in StemSpan H3000 plus BIT9500 medium (StemCell Technologies) containing 50 ng/ml SCF and 50 ng/ml TPO. GFP-positive LSK cells were sorted using FACS and plated ( $1 \times 10^4$  cells/dish) in methylcellulose medium containing complete cytokines (M3434; StemCell Technologies). Progenitor colonies were counted 7–8 days after plating. Results are expressed as percentage of controls (*n* = 3; \*, *p* < .05). Note that ectopic expression of wild-type Jak2 almost completely rescued whereas c-Jun expression only partially rescued the defects in hematopoietic progenitor colony formation in Jak2-deficient LSK cells. Abbreviations: FDR, false discovery rate; NES, normalized enrichment score; TPO, thrombopoietin.

Assessment of HSC functions using noncompetitive and competitive BM transplantation assays reveals that Jak2-deficient HSCs are severely defective in reconstituting hematopoiesis in recipient animals (Fig. 2A–2E). Notably, Jak2-deficient BM cells failed to reconstitute hematopoiesis in recipient animals even when transplanted 20-times more mutant BM cells than control BM cells (Fig. 2A). Also, Jak2-deficient HSCs were unable to compete with WT HSCs and there was rapid loss of hematopoietic progenitors/precursors derived from the Jak2-deficient HSCs even when 10-times more Jak2-deficient BM cells were used in competitive transplantation assays (Fig. 2B, 2C). These data suggest an intrinsic functional defect in Jak2-deficient HSCs.

Most HSCs usually remain in the quiescent state ( $G_0$ ) to prevent HSC exhaustion. Flow cytometric data indicate that

Jak2-deficient LSK cells are driven out of quiescence into the cell cycle (Fig. 3A, 3B). Mutant HSCs probably enter into the cell cycle in an attempt to respond to the rapid depletion of hematopoietic cells in Jak2-deficient mice. Increased rate of apoptosis is also evident in Jak2-deficient LSK cells (Fig. 3C). Thus, rapid loss of HSCs in Jak2-deficient mice is possibly due to disruption of HSC quiescence and increased apoptosis.

Aberrant increase in ROS has been associated with disruption of HSC quiescence [37, 38, 40]. In our experiments, ROS levels were significantly elevated in Jak2-deficient LSK cells (Fig. 4A). It has been shown that elevation of ROS levels induces HSC-specific p38 MAPK activation in Atm-deficient mice [21]. We also observed significant (approximately threefold) activation of p38 MAPK in Jak2-deficient LSK cells compared

with control LSK cells (Fig. 4B). Furthermore, NAC treatment significantly improved the function of Jak2-deficient HSCs, as revealed by significantly increased reconstitution of hematopoiesis in recipient animals transplanted with NAC-treated Jak2-deficient BM cells (Fig. 4C). However, NAC treatment (either ex vivo or in vivo) did not completely rescue the Jak2-deficient HSC phenotype, suggesting that all phenotypic manifestations of Jak2 deficiency are not the consequence of increased ROS in the HSC compartment.

Previous studies using cultured cell lines show that Jak2 binds to Mpl and Kit, mediating signaling downstream of TPO and SCF [27, 28]. TPO and SCF are required for HSC maintenance [22–26]. We observed defective Erk and Akt activation in Jak2-deficient LSK cells in response to TPO and SCF (Fig. 5A, 5C). Disruption of Erk and Akt signaling may contribute to the HSC phenotype in Jak2-deficient mice, as Erk1/2 and Akt1/2 have been shown to play roles in the maintenance of adult HSCs [29–31]. Interestingly, whereas Mpl expression was not affected by Jak2 deficiency, Kit expression was markedly reduced in Jak2-deficient HSCs (Fig. 5B, 5D). Loss of Kit expression may also contribute to decreased quiescence and increased apoptosis in Jak2-deficient HSCs since Kit is required for HSC quiescence and survival [24, 41]. Thus, perturbation of TPO/Mpl and SCF/Kit signaling may contribute to the Jak2-deficient HSC phenotype. However, the HSC phenotype in TPO/Mpl or SCF/Kit-deficient mice [22, 23, 25, 41] is less severe than that observed in Jak2-deficient mice, suggesting that combined defects in multiple pathways contribute to the severe HSC phenotype in Jak2-deficient mice.

Gene expression analyses reveal that multiple stem cell maintenance programs are perturbed in the absence of Jak2, including genes associated with Jak/Stat, TPO, MAPK, PI3 kinase, and mTOR signaling pathways. GSEA and IPA also reveal that HSC cell cycle and cell death/survival-related genes are significantly altered in Jak2-deficient LSK (Fig. 6A, 6B), reflecting abnormal cell cycle status and loss of quiescence in Jak2-deficient HSCs. Expression of c-Jun, Pim-1, Gfi-1, Mecom, Pbx1, Foxo3, Atm, and p21<sup>Cip1</sup>, which have been implicated in HSC quiescence, survival, and/or self-renewal [20, 33–39], is significantly downregulated in Jak2-deficient LSK cells (Fig. 6C, 6D). Ectopic expression of c-Jun only partially rescued the defects in hematopoietic progenitor colony formation in Jak2-deficient LSK (Fig. 6E), suggesting that additional genes and pathways downstream of Jak2 are involved in HSC maintenance and function. It has been shown previously that Atm or Foxo3 deficiency increases ROS levels and decreases the life span of HSCs by disrupting HSC quiescence [37, 38, 40]. It is thus possible that reduced expression of Atm and Foxo3 may contribute to the elevated ROS levels and loss of quiescence in Jak2-deficient LSK cells. p21 regulates HSC quiescence [39],

whereas pim1 is important for HSC survival [34]. Pbx1 and Gfi-1 have been shown to regulate HSC quiescence [20, 35], whereas Mecom is required for HSC self-renewal [36]. Reduced expression of several of these genes may contribute to exhaustion of HSCs in Jak2-deficient mice. Thus, abnormal signaling and downregulation of multiple genes related to HSC maintenance and function likely contribute to severe HSC phenotype in Jak2-deficient mice. Further work is needed to define how Jak2 regulates the expression of these genes.

## CONCLUSIONS

In conclusion, we demonstrate that Jak2 plays an essential role in the maintenance and normal function of adult HSCs. The discovery of JAK2V617F mutation in MPNs has led to the development of Jak2 inhibitors. However, patients treated with Jak2 inhibitors show significant hematopoietic toxicities [13–15]. Current Jak2 inhibitors cannot distinguish between wild type and mutant Jak2; so both wild-type and mutant Jak2 are inhibited by Jak2 inhibitor treatment. Since Jak2 is critical for the maintenance of normal hematopoiesis, effective treatment of MPNs would require specific targeting of the mutant Jak2 or pathways downstream of Jak2V617F that are required for induction of MPNs. Such therapeutic approaches will minimize hematopoietic toxicities in treating patients with JAK2V617F mutation.

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## AUTHOR CONTRIBUTIONS

H.A.: performed research, analyzed data, and wrote the manuscript; S.A.: performed research; R.E.H.: performed histopathologic analysis; K.-U.W. and K.S.: generated and maintained the Jak2 floxed mice; G.M.: designed the research, analyzed data, and wrote the manuscript.

## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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