Critical requirement for Stat5 in a mouse model of polycythemia vera

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A somatic point mutation (V617F) in the JAK2 tyrosine kinase has been found in ~95% patients with polycythemia vera (PV) and 50%-60% of cases with essential thrombocytosis (ET) and primary myelofibrosis (PMF). The JAK2V617F mutant is a constitutively active protein-tyrosine kinase, which can transform factor-dependent hematopoietic cell lines and progenitors to cytokine independence. Studies using bone marrow transplantation, transgenic or knockin mouse models of JAK2V617F have shown that Jak2V617F is directly responsible and sufficient to cause PV, and may contribute to ET and PMF. The discovery of the JAK2V617F mutation in a majority of patients with MPNs has led to the development of inhibitors of JAK2, and several of these JAK2 inhibitors are currently undergoing phase 1/2 clinical trials. Recent results from the clinical trials suggest that JAK2 inhibitor therapy can reduce splenomegaly and constitutional symptoms, but cause significant hematologic toxicities in MPN patients. It is becoming clear that complete remissions to those seen in chronic myeloid leukemia (CML) with the BCR-ABL inhibitor imatinib cannot be achieved with the JAK2 inhibitors. Moreover, drug resistance may emerge in some patients treated with JAK2 inhibitors. These challenges underscore the need to better understand the role of downstream signaling events, and identify new pharmacologic targets in JAK2V617F-induced MPNs.

JAK2V617F activates multiple signaling molecules/pathways, including Stat5, Stat3, Erk/MAP kinase, and PI3 kinase/Akt pathways, but which of these signaling pathway(s) is critical for the induction of MPNs is unknown. It has been shown that expression of an EpoR mutant lacking the Stat5-binding site, or knockdown of Stat5, inhibited JAK2V617F-mediated transformation of Ba/F3 cells and impaired tumor formation in nude mice implanted with JAK2V617F-expressing Ba/F3 cells. Although these studies provided some evidence of the possible role of Stat5 in survival and proliferation of cell lines expressing JAK2V617F, the role of Stat5 in JAK2V617F-evoked transformation of actual hematopoietic progenitors and induction of MPNs remained unclear. We have previously reported the generation of a conditional JAK2V617F knockin mouse, which exhibits all the clinical features of human PV. These findings also provide strong support for the development of Stat5 inhibitors as targeted therapies for the treatment of PV and other JAK2V617F-positive MPNs.

Methods

Mice

Conditional JAK2V617F knockin⁶ and floxed Stat5 (Stat5fl/fl) mice have been described earlier. MxCre mice (purchased from The Jackson Laboratory) were crossed to JAK2V617F and Stat5fl/fl mice to generate JAK2V617F-deficient Jak2V617F knockin mice completely rescued the defects in transformation of hematopoietic progenitors and induction of MPNs. We have used this JAK2V617F knockin mouse to determine the in vivo role of Stat5 in JAK2V617F-induced MPNs. Our results show that Stat5 plays a critical role in polycythemia vera induced by JAK2V617F.

Plasmids

pMX-puro (empty vector), pMX-puro-Stat5a, and pBabeX-dominant-negative Stat3 (DN-Stat3) constructs were kindly provided by Dr Toshio Kitamura (University of Tokyo, Tokyo, Japan). DN-Stat3 was subcloned into pMX-puro.
vector at EcoRI and NotI sites, and confirmed by sequencing. MSCV-p210BCR-ABL and MSCV-puro-KrasG12D constructs were kindly provided by Dr Richard Van Etten (Tufts University School of Medicine, Boston, MA) and Dr Kevin Shannon (University of California, San Francisco, CA), respectively.

Retroviral transduction and transplantation

High-titer retroviral stocks of pMX-puro (vector), pMX-puro-Stat5a, and pMX-puro DN-Stat3 were prepared by transient transfection of 293T cells as described previously.\textsuperscript{22} Bone marrow cells from 5-fluorouracil (5-FU)–primed MxCre:Jak2V617F/+;Stat5f/f mice were transduced with retroviruses expressing vector alone or Stat5a by 2 rounds of spin infection.\textsuperscript{22} Transduced bone marrow cells (10\textsuperscript{6}) were injected into retro-orbital veins of lethally irradiated (2 × 550 cGy) C57/Bl6 recipient mice. Mice were maintained on acidified water. To determine the requirement of Stat5 in transformation mediated by different oncogenes associated with myeloid malignancy, MSCV vector-based retroviruses expressing Jak2V617F, p210BCR/ABL and KrasG12D were produced as described.\textsuperscript{22}

Colony-forming assays

Bone marrow (BM; 2 × 10\textsuperscript{6}) or spleen (1 × 10\textsuperscript{6}) cells were plated in duplicate in complete methylcellulose medium (Methocult M3434, StemCell Technologies). BFU-E, CFU-GM, and CFU-GEMM colonies were scored on day 7. To detect

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Figure 1. Deletion of Stat5 inhibits the development of MPN in Jak2V617F knockin mice. (A) Immunoblot analysis for total Stat5 protein show efficient deletion of Stat5 in the BM of MxCre:Jak2V617F/+;Stat5f/f mice after induction with pI:pC. Peripheral blood HCT (B), Hb (C), RBC (D), MCV (E), WBC (F), NE (G), and PLT (H) counts were assessed at 4, 8, and 12 weeks after pI:pC induction in control, MxCre:Jak2V617F/+; and MxCre:Jak2V617F/+;Stat5f/f mice (n = 15 for control; n = 17 for MxCre:Jak2V617F/+; n = 28 for MxCre:Jak2V617F/+;Stat5f/f mice at all time points). All the peripheral blood parameters in MxCre:Jak2V617F/+;Stat5f/f mice were within normal range and comparable with those in controls. Spleen weight/size (I) was significantly reduced in MxCre:Jak2V617F/+;Stat5f/f mice compared with MxCre: Jak2V617F/+; n = 15). Asterisks indicate significant differences by 1-way ANOVA (*P < .05; **P < .005). Data are shown as mean ± SEM.
cytokine-independent CFU-E and CFU-GM colonies, BM (1 x 10^5) or spleen (1 x 10^5) cells were plated in duplicate in methylcellulose medium without any cytokine (Methocult M3234; StemCell Technologies). CFU-E colonies were counted after 2 days in culture after staining with benzidine solution (Sigma-Aldrich). CFU-GM colonies were scored on day 7.

**Flow cytometry**

Single-cell suspensions were prepared from BM and spleen, and red cells were lysed with red cell lysis solution. Cells were washed and resuspended in PBS plus 2% FBS, and stained for 20 minutes on ice with directly conjugated (either PE or APC) monoclonal antibodies specific for Ter119, CD71, CD41, CD61, Mac-1, Gr-1, B220, or Thy-1. For HSC/progenitor analysis, cells were stained for 30 to 60 minutes on ice with antibodies against c-Kit, Sca-1, Flk2 (CD135), CD34, CD16/32 (FcγR II/III), and antibodies against lineage (Lin) markers including CD3, CD4, CD8α, CD19, B220, Gr-1, Ter119, and IL-7R (CD127). All antibodies were purchased from BD Biosciences, eBioscience, or BioLegend. Flow cytometry was performed with an LSRII (BD Biosciences) and analyzed using FlowJo Version 8.8.6 software (TreeStar).

**Immunoblotting**

BM cells were factor-deprived for 15 hours in IMDM medium containing 0.5% BSA at 37°C and lysed by direct boiling in 2x sample buffer. Immunoblotting was performed using phopspecific antibodies against Stat5 or p70S6 kinase (Cell Signaling Technology), or antibodies against total Stat5, p70S6 kinase, Bcl-2, Cyclin D2, Pim-1, or Erk2 (Santa Cruz Biotechnology).

**Blood and tissue analysis**

Peripheral blood counts were determined using Hemavet 950 FS (Drew Scientific). 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 H&E and reticulin stain.

**Statistical analysis**

Results are expressed as mean ± SEM, and statistical significance was determined by ANOVA or student t test using GraphPad Version 5 software. *P < .05 was considered to be statistically significant.

**Results**

**Deficiency of Stat5 inhibits the development of polycythemia vera in Jak2V617F knockin mice**

Conditional Jak2V617F knockin mice were crossed with floxed Stat5 (Stat5 fl/fl)20 and MxCre transgenic21 mice to generate the MxCre:Jak2V617F/+ and MxCre:Jak2V617F/+;Stat5fl/fl mice. The expression of Jak2V617F and the deletion of Stat5 were simultaneously induced in hematopoietic progenitors of these mice at 4 weeks after birth by intraperitoneal injection of polyinosine-polycytosine (pI:pC). Three groups of mice were analyzed: control (V617F/+ or Stat5fl/fl); Jak2V617F-expressing (MxCre; Jak2V617F/+); and Stat5-deleted Jak2V617F-expressing (MxCre; Jak2V617F/+; Stat5fl/fl).
Jak2V617F/+;Stat5fl/fl) mice. As shown in Figure 1A, Stat5 was almost completely deleted in all MxCre;Jak2V617F/+;Stat5fl/fl mice on induction with plpC. Next, we addressed whether the deficiency of Stat5 might influence the development of MPN induced by Jak2V617F. As expected, all mice expressing Jak2V617F (MxCre;Jak2V617F/+ ) developed a PV-like MPN, including markedly elevated hematocrit (HCT), hemoglobin (Hb), and red blood cells (RBC), and reduced mean corpuscular volume (MCV) in the peripheral blood of Jak2V617F-expressing mice within 4 weeks after plpC induction (Figure 1B-E). White blood cell (WBC), neutrophil (NE), and platelet (PLT) counts were also significantly increased in the MxCre;Jak2V617F/+ mice compared with controls (Figure 1F-H). Strikingly, the deletion of Stat5 in mice expressing Jak2V617F (MxCre;Jak2V617F/+;Stat5fl/fl) normalized all the blood parameters within 4 weeks after plpC injection (Figure 1B-H). Blood parameters remained normal in MxCre; Jak2V617F/+;Stat5fl/fl mice for more than 1 year (data not shown).

The PV phenotype observed in Jak2V617F knockout mice (MxCre;Jak2V617F/+ ) was also associated with marked splenomegaly. The average spleen weight in MxCre;Jak2V617F/+ mice was 7-fold higher than that of controls (Figure 1I). In contrast, the spleen size and weight were dramatically reduced in Stat5-deleted Jak2V617F-expressing mice (MxCre;Jak2V617F/+;Stat5fl/fl), and were almost identical to those of controls (Figure 1I). Thus, deficiency of Stat5 reduces the extramedullary hematopoiesis in mice expressing Jak2V617F.

Histopathologic analyses also revealed that Stat5 deficiency blocked the development of PV in mice expressing Jak2V617F. Peripheral blood smears showed increased RBC, reticulocytes, and platelets in mice expressing Jak2V617F (Figure 2A). BM sections showed hypercellularity with trilineage hyperplasia, whereas spleen sections exhibited attenuation of white pulp and marked expansion of red pulp with clusters of megakaryocytes and immature erythroid precursors in Jak2V617F-expressing mice (Figure 2B-C). Noticeably, blood smears, BM and spleen sections from Stat5-deleted Jak2V617F-expressing mice were normal and indistinguishable from those of control mice (Figure 2A-C). Reticulin staining indicated the presence of fibrosis in the BM, and spleen of older MxCre;Jak2V617F/+ mice (24 weeks after induction; Figure 2D-E). Deletion of Stat5, however, blocked the development of fibrosis in the BM and spleen of mice expressing Jak2V617F (MxCre;Jak2V617F/+;Stat5fl/fl; Figure 2D-E). Together, these results suggest that deficiency of Stat5 inhibits the development of PV-like MPN induced by Jak2V617F.

**Effects of Stat5-deficiency on hematopoietic progenitors expressing Jak2V617F**

Flow cytometric analysis of the BM and spleens from Jak2V617F mice showed marked increase in CD71 + Ter119 + erythroid precursors compared with control mice (Figure 3A). Expansion of the CD71 + Ter119 + population was greater in the spleen (~20-fold) than in the BM (~2-fold) of Jak2V617F-expressing mice compared with control animals (Figure 3A). In addition, spleens from Jak2V617F-expressing mice showed an increase in myeloid cells (Gr-1 + Mac-1 +; supplemental Figure 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article), as we previously observed. However, deletion of Stat5 with concomitant expression of Jak2V617F inhibited the expansion of CD71 + Ter119 + as well as Gr-1 + Mac-1 + populations in the BM and spleens of MxCre;Jak2V617F/+;Stat5fl/fl mice, and they were comparable with those observed in control animals (Figure 3A, supplemental Figure 1).

Next, we examined the effects of Stat5 deficiency on hematopoietic stem cell (HSC)/progenitor compartments in Jak2V617F-expressing mice. Expression of Jak2V617F resulted in significant increase in the Lin - Sca1 + c-kit + (LSK; containing HSC) and megakaryocyte/erythroid progenitor (MEP) compartments (Figure 3B), as we previously observed. Concomitant deletion of Stat5 reduced the Jak2V617F-induced expansion of LSK and MEP populations in the BM and spleens of MxCre;Jak2V617F/+;Stat5fl/fl mice (Figure 3B, supplemental Figure 2A-B). Notably, the LSK and myeloid progenitor compartments in the BM of MxCre; Jak2V617F/+;Stat5fl/fl mice were comparable with those of control animals (Figure 3B).

Further analysis of the long-term HSC (LT-HSC; defined as LSKCD34 + Flk2 + ) and short-term HSC (ST-HSC; defined as LSKCD34 + Flk2 + ) within the LSK compartment revealed a significant increase in LT-HSC and ST-HSC in the BM of Jak2V617F-expressing mice (Figure 3C-D). Concomitant deletion of Stat5 reduced the Jak2V617F-induced expansion of LT-HSC and ST-HSC populations in the BM of MxCre;Jak2V617F/+;Stat5fl/fl mice and those numbers were comparable with those observed in control animals (Figure 3C-D).

Hematopoietic progenitor colony assays showed significant increase in BFU-E, CFU-GM, and CFU-GEMM colonies in the BM and spleens of Jak2V617F mice compared with control animals (Figure 3E). Deletion of Stat5, however, inhibited the increase in BFU-E, CFU-GM, and CFU-GEMM colonies in mice expressing Jak2V617F (Figure 3E). No significant difference was observed in hematopoietic progenitor colonies between MxCre; Jak2V617F/+;Stat5fl/fl and control mice (Figure 3E). We also observed a large number of Epo-independent CFU-E colonies in the BM and spleens of Jak2V617F mice (Figure 3F), which indicates the presence of endogenous erythroid colonies (EEC), a hallmark feature of PV. Deficiency of Stat5 completely inhibited Jak2V617F-induced erythroid transformation and Epo-independent EEC formation in the BM and spleens of MxCre;Jak2V617F/+;Stat5fl/fl mice (Figure 3F). Together, these data establish that Stat5 is required in HSC/progenitors for Jak2V617F to induce PV.

Expression of Stat5 can rescue the defect in induction of PV by Jak2V617F in Stat5-deficient BM

To further confirm that the failure to induce PV by Jak2V617F in MxCre;Jak2V617F/+;Stat5fl/fl mice was mainly because of the defects in signaling mediated by Stat5, we performed rescue experiments by adding Stat5 back into the BM of the Stat5-deficient Jak2V617F-expressing mice. We took 2 approaches to “rescue” the PV phenotype in Stat5-deficient BM. First, we crossed plpC induced MxCre;Jak2V617F/+;Stat5fl/fl mice (which exhibited no sign of PV disease) with Stat5 fl/wt mice. Four different genotypes of mice were analyzed: Stat5fl/fl (control), MxCre; Jak2V617F/+ (Jak2V617F-expressing diseased animals), MxCre; Jak2V617F/+;Stat5fl/fl (Stat5-deficient Jak2V617F-expressing animals), and MxCre;Jak2V617F/+;Stat5fl/flRescue (rescued animals) as outlined in supplemental Figure 3A. These 4 groups of mice were injected with plpC at 4 weeks and monitored for MPN development by periodic peripheral blood counts. As expected, the level of Stat5 expression in the BM of MxCre;Jak2V617F/+;Stat5fl/flRescue mice was almost one-half of the amount of Stat5 expressed in the BM of control or MxCre;Jak2V617F/+ mice whereas Stat5 was almost undetectable in the BM of MxCre; Jak2V617F/+;Stat5fl/fl mice after induction with plpC (supplemental Figure 3B). Whereas all blood parameters were normal in MxCre;Jak2V617F/+;Stat5fl/fl mice, expression of one allele of
Stat5 resulted in marked increase in peripheral blood HCT, Hb, and RBC counts in MxCre;Jak2V617F/+;Stat5fl/fl mice within 4 weeks after pI:pC induction (supplemental Figure 3C-E). Profound splenomegaly and large number of Epo-independent CFU-E colonies were also observed in the spleens of MxCre;Jak2V617F/+;Stat5fl/fl mice (supplemental Figure 3F-G). In addition, there was a significant increase in CD71+Ter119+ erythroid precursor populations in the spleens of MxCre;Jak2V617F/+;Stat5fl/fl mice similar to what we observed in Jak2V617F-expressing (MxCre;Jak2V617F/+;Stat5fl/wtRescue) mice (supplemental Figure 3H). Thus,
expression of one allele of Stat5 is sufficient to rescue the defect in Jak2V617F-induced PV in Stat5-deficient BM.

We also used a BM transplantation approach to rescue the defect in Jak2V617F-induced PV in Stat5-deficient mice. BM from Stat5-deficient Jak2V617F-expressing mice (which were defective in induction of PV) were transduced with retrovirus expressing Stat5a or vector control, and then transplanted into lethally irradiated C57BL/6 recipient mice as outlined in Figure 4A. Immunoblotting for Stat5 protein levels in the peripheral blood leukocytes of transplanted animals confirmed efficient expression of Stat5a and reconstitution of Stat5a-transduced BM (Figure 4B). Within 4 weeks after transplantation, we observed marked increase in recipients receiving Stat5a-transduced BM (n = 4). Blood counts were measured at 4, 8, and 12 weeks after transplantation. Data are represented as mean ± SEM. Spleen weight (F) and Epo-independent CFU-E colonies (G) were also markedly elevated in mice receiving Stat5a-transduced BM compared with those receiving vector-transduced BM. Data are presented as mean ± SEM. Asterisks show significant differences by t test (**P < .005). (H) Flow cytometric analysis demonstrates a marked increase in CD71+Ter119+ erythroid precursors in the spleens of recipient mice transplanted with Stat5a-transduced BM compared with those with vector-transduced BM. Shown are representative dot plots from 3 independent experiments.

Figure 4. Retroviral expression of Stat5a complements the deficiency of Stat5 in inducing PV by Jak2V617F. (A) Experimental design for rescue of Jak2V617F-induced PV by retroviral transduction/transplantation of Stat5a in the BM of Stat5-deficient Jak2V617F-expressing (MxCre;Jak2V617F/+;Stat5fl/fl) mice. (B) Immunoblot analysis of Stat5 protein levels in leukocytes 4 weeks after transplantation. Erk2 was used as a loading control. Peripheral blood HCT (C), Hb (D), and RBCs (E) were significantly increased in recipients receiving Stat5a-transduced BM (n = 4). Blood counts were measured at 4, 8, and 12 weeks after transplantation. Data are represented as mean ± SEM. Spleen weight (F) and Epo-independent CFU-E colonies (G) were also markedly elevated in mice receiving Stat5a-transduced BM compared with those receiving vector-transduced BM. Data are presented as mean ± SEM. Asterisks show significant differences by t test (**P < .005). (H) Flow cytometric analysis demonstrates a marked increase in CD71+Ter119+ erythroid precursors in the spleens of recipient mice transplanted with Stat5a-transduced BM compared with those with vector-transduced BM. Shown are representative dot plots from 3 independent experiments.

loss of Stat5 does not cause any major defect in normal hematopoiesis in adult mice

We also determined the effects of Stat5 deficiency on normal adult hematopoiesis. Deletion of Stat5 was induced in hematopoietic progenitors of adult (4 weeks old) MxCre;Stat5fl/fl mice by intraperitoneal injection of pI:pC. As shown in supplemental Figure 4A, Stat5 was almost completely deleted in the peripheral blood leukocytes of all MxCre;Stat5fl/fl mice by induction with pI:pC. Analysis of peripheral blood at 4 and 12 weeks after pI:pC injection showed that Stat5-deficient mice had normal numbers of WBCs, NEs, RBCs, and PLTs, with only a small decrease in HCT and Hb levels (supplemental Table 1). Unlike germ line deleted Stat5 knockout mice,25 which died perinatally, deletion of Stat5 in adult hematopoietic compartments did not result in any major abnormality and the mice survived for a long period (monitored for 1 year).
Flow cytometric analysis showed no significant difference in myeloid (Gr-1+/Mac-1+) populations in the BM and spleens of Stat5-deleted mice compared with controls (supplemental Figure 4B). The percentages of erythroid (Ter119+/CD71+) populations were increased to some extent in the BM and spleens of Stat5-deficient mice (supplemental Figure 4B), possibly because of a compensatory response to a slight decrease in HCT and Hb levels in these animals. The percentage of HSC-enriched LSK population in the BM of Stat5-deficient mice was comparable with that of control animals (supplemental Figure 4C).

Hematopoietic progenitor colony assays also did not show any significant difference in erythroid (BFU-E) and myeloid (CFU-GM) colonies in the BM and spleens of Stat5-deficient mice compared with control animals (supplemental Figure 5A-B). Spleen size of Stat5-deficient mice was normal and comparable with that of control mice (supplemental Figure 5C). Thus, deficiency of Stat5 alone did not result in any major defect in hematopoiesis in adult animals.

Stat5 is not required for hematopoietic transformation mediated by KrasG12D

To examine if Stat5 is a general requirement for hematopoietic transformation by oncogenes, we compared the ability to transform wild-type and Stat5-deficient hematopoietic progenitors by 3 different oncogenes, Jak2V617F, BCR-ABL, and KrasG12D, which have been associated with myeloid malignancies.26-28 and have shown to cause MPN-like disease in mice.5,15,29-31 We expressed Jak2V617F, p210 BCR-ABL, or KrasG12D into the BM of wild-type and Stat5-deficient mice by retroviral transduction and assessed their ability to transform myeloid and erythroid progenitors ex vivo using colony-forming assay in the absence of cytokine. Normal myeloid and erythroid progenitors require cytokines to form colonies in methylcellulose. Consistent with previous reports,30,32 expression of BCR-ABL or KrasG12D in wild-type (control) BM resulted in a large number of cytokine-independent myeloid (CFU-GM) colonies (Figure 5A). Retroviral expression of BCR-ABL also resulted in a large number of cytokine-independent erythroid colonies in the BM of control mice (Figure 5B). Expression of Jak2V617F in control BM gave rise to a small number of CFU-GM (Figure 5A), but a large number of erythroid (CFU-E) colonies in the absence of cytokine (Figure 5B). However, Stat5-deficient BM was completely refractory to myeloid and erythroid transformation mediated by Jak2V617F (Figure 5A-B). Stat5 deficiency also resulted in a marked decrease in myeloid and erythroid transformation by BCR-ABL (Figure 5A-B). In contrast, transformation of hematopoietic progenitors by KrasG12D was unaffected by Stat5 deletion (Figure 5A-B). These results suggest that the requirements for transformation by different oncogenes might be different, and Stat5 is not essential for myeloid transformation evoked by KrasG12D.

Erk, Akt, and Stat3 are dispensable for hematopoietic transformation mediated by Jak2V617F

Erk, Akt and Stat3 are also constitutively activated in hematopoietic cells expressing Jak2V617F.1,2,6,33 To determine the contribution of these signaling molecules in hematopoietic transformation mediated by Jak2V617F, we have used pharmacologic inhibitors of Erk and Akt and retroviral expression of dominant-negative Stat34,35 in hematopoietic progenitors from Jak2V617F knockin mice. Because Jak2V617F expression caused efficient transformation of erythroid progenitors (Akada et al6 and Figures 3-5), we used this erythroid transformation assay (cytokine-independent CFU-E colony formation) to determine the contribution of Erk, Akt, and Stat3 in Jak2V617F-evoked transformation. As expected, the MEK inhibitor U0126 (1-10μM) profoundly inhibited constitutive activation of Erk in the BM of Jak2V617F knockin mice (Figure 6A). However, U0126, even at a high concentration (10μM), failed to cause any significant inhibition of erythroid transformation by Jak2V617F (Figure 6A). Similarly, the doses of wortmannin (0.5-1μM), which profoundly inhibited the Akt activation in the BM of Jak2V617F knockin mice, only resulted in partial (~40%) inhibition of erythroid transformation by Jak2V617F (Figure 6B).

Overexpression of dominant-negative Stat3 (DN-Stat3) has been shown to cause significant inhibition of Stat3 activation in hematopoietic cells.34,35 We have expressed DN-Stat3 in the BM of Jak2V617F knockin mice by retroviral transduction. The transduced BM cells were cultured in a StemPro medium containing SCF, FLT3 ligand, and TPO to maintain them in a stem cell-like condition.36 Transduced cells were selected by puromycin for 2 days before plating in methylcellulose medium without any cytokine. CFU-E colonies were counted 2 days after plating. As shown in Figure 6C, expression of DN-Stat3 in the BM of Jak2V617F knockin mice almost completely inhibited the activation of Stat3 as determined by immunoblotting with phospho-Stat3 antibody (Figure 6C). However, expression of DN-Stat3 in BM progenitors resulted in only partial (~40%) inhibition of erythroid transformation mediated by Jak2V617F (Figure 6C). Together, these results suggest that Erk, Akt, and Stat3 are dispensable for Jak2V617F-evoked transformation of hematopoietic progenitors.

The effects of Stat5 deficiency on signaling mediated by Jak2V617F in hematopoietic cells

To investigate the effects of Stat5 deficiency on Jak2V617F-mediated hematopoietic signaling, we performed Western blot analysis on BM extracts from control, Jak2V617F-expressing (MxCre;Jak2V617F+/+) and Stat5-deficient Jak2V617F-expressing (MxCre;Jak2V617F+/+;Stat5fl/fl) mice. As expected, constitutive activation of Stat5 was observed in Jak2V617F-expressing BM, whereas no activation of Stat5 was observed in control or...
Stat5-deficient Jak2V617F-expressing mice BM (Figure 7). Interestingly, we observed marked increase in phosphorylation of p70S6 kinase in Jak2V617F-expressing BM compared with control BM (Figure 7). Constitutive phosphorylation of p70S6 kinase was significantly inhibited in Stat5-deficient Jak2V617F-expressing BM (Figure 7), indicating that p70S6 kinase is a downstream target of Jak2V617F-Stat5 signaling. No significant difference was observed in the activation of Akt and Erk 1/2 between Jak2V617F-expressing and Stat5-deficient Jak2V617F-expressing BM (data not shown), suggesting that Akt and Erk 1/2 are independent of the Stat5 signaling pathway. Furthermore, expression of Bcl-xL, Cyclin-D2, and Pim-1 was significantly induced in the BM of Jak2V617F-expressing mice compared with controls (Figure 7). In contrast, deficiency of Stat5 markedly reduced expression of Bcl-xL, Cyclin-D2, and Pim-1 mediated by Jak2V617F (Figure 7). Thus, p70S6 kinase, Bcl-xL, Cyclin-D2, and Pim-1 are downstream targets of Jak2V617F-Stat5 signaling. These proteins are known to regulate cell growth/survival. Therefore, it is plausible that inhibition of p70S6 kinase phosphorylation and reduced expression of Bcl-xL, Cyclin-D2, and Pim-1 caused by Stat5 deletion might contribute to the defects in development of PV in Stat5-deficient Jak2V617F-expressing mice.

Discussion

The identification of the JAK2V617F mutation in the majority of patients with PV, ET, and PMF has advanced our understanding of the pathogenesis of MPNs and led to the development of small molecule inhibitors of JAK2. However, recent results from clinical trials indicate that JAK2 inhibitor therapy alone cannot provide complete remissions to MPN patients. Moreover, significant hematopoietic toxicities including anemia and thrombocytopenia were observed in a majority of patients treated with JAK2 inhibitors. Therefore, identifying additional new therapeutic targets would be valuable in developing effective treatment of patients with MPNs. Here, we have used the conditional Jak2V617F knockin and the floxed Stat5 mice to determine the requirement of Stat5 in PV-like MPN induced by Jak2V617F. We showed earlier that knockin mice expressing Jak2V617F resulted in all the features of PV. In this study, we observed that deficiency of Stat5 completely blocked the development of PV phenotype evoked by Jak2V617F. All blood parameters including WBCs, RBCs, Hb, HCT, and PLTs were normal in Stat5-deficient Jak2V617F-expressing mice and were comparable with those observed in control animals (Figure 1B-H). Splenomegaly caused by Jak2V617F expression was also significantly reduced on deletion of Stat5 (Figure 1I). These results clearly indicate a critical role for Stat5 in induction of PV by Jak2V617F. A more definitive proof came from the demonstration that adding back 1 allele of Stat5 by genetic cross or expression of Stat5a by retroviral transduction/transplantation in Stat5-deficient BM completely rescued the defect in induction of PV by Jak2V617F (Figure 4 and supplemental Figure 3). Thus, Stat5 signaling is absolutely required for the pathogenesis of PV induced by Jak2V617F.

We showed earlier that knockin mice expressing Jak2V617F resulted in all the features of PV. In this study, we observed that deficiency of Stat5 completely blocked the development of PV phenotype evoked by Jak2V617F. All blood parameters including WBCs, RBCs, Hb, HCT, and PLTs were normal in Stat5-deficient Jak2V617F-expressing mice and were comparable with those observed in control animals (Figure 1B-H). Splenomegaly caused by Jak2V617F expression was also significantly reduced on deletion of Stat5 (Figure 1I). These results clearly indicate a critical role for Stat5 in induction of PV by Jak2V617F. A more definitive proof came from the demonstration that adding back 1 allele of Stat5 by genetic cross or expression of Stat5a by retroviral transduction/transplantation in Stat5-deficient BM completely rescued the defect in induction of PV by Jak2V617F (Figure 4 and supplemental Figure 3). Thus, Stat5 signaling is absolutely required for the pathogenesis of PV induced by Jak2V617F.

Consistent with our previous report, the conditional Jak2V617F knockin mice result in significant expansion of the LSK, LT-HSC, ST-HSC, and MEP compartments in the BM of Jak2V617F-expressing mice. This expansion is partially rescued by adding back 1 allele of Stat5 by genetic cross or expression of Stat5a by retroviral transduction/transplantation in Stat5-deficient BM. These results suggest that Stat5 signaling is critical for the pathogenesis of PV induced by Jak2V617F.
observed that VavCre-mediated expression of Jak2V617F in mice rescued the defects in transformation and gave rise to a Stat5a into the BM cells of Stat5-deficient Jak2V617F-expressing mice (Figure 4A-H). However, retroviral expression of Stat5-deficient Jak2V617F-expressing mice was reasonably well tolerated; these mice survived for a long time and hematopoiesis was mostly preserved.41,42 Consistent with the later reports, we also did not observe any major defect in normal adult hematopoiesis in Stat5-deficient mice. Peripheral blood RBC, WBC, NE, and PLT counts in Stat5-deficient mice were within normal range although slightly lower than those in control mice (supplemental Table 1). In addition, there was no significant difference in the HSC-enriched LSK population, hematopoietic progenitor colony formation and the spleen size between control and Stat5-deficient mice (supplemental Figures 4-5). Only HCT and Hb levels were slightly below the normal range in Stat5-deficient mice (supplemental Table 1), and they survived for a long period without any significant problem (observed for 1 year).

We have previously shown that the PV phenotype observed in the Jak2V617F knockin mice (Figure 3B-D). However, Mullaney et al did not observe any significant increase in LSK and LT-HSC on E2ACre-induced germ line Jak2V617F expression.15 Similar to MxCre, we observed that VavCre-mediated expression of Jak2V617F in the hematopoietic compartments also resulted in significant increase in LSK, LT-HSC, ST-HSC, and MEP in the BM of our Jak2V617F knockin mice (data not shown). The reasons for discrepancy between our mouse model and the mouse model reported by Mullaney et al are not clear, but could be related to different targeting strategies, different Cre recombinase used or different Cre recombination efficiencies.15 Deficiency of Stat5 significantly reduced the Jak2V617F-evoked expansion of LSK, LT-HSC, ST-HSC, and MEP compartments and normalized these populations to that observed in control animals (Figure 3B-D). Furthermore, deletion of Stat5 completely blocked the Jak2V617F-evoked expansion and transformation of erythroid progenitors in the BM and spleens (Figure 3E-F), suggesting that Stat5 is required by Jak2V617F in hematopoietic stem cells and progenitors to induce PV.

We have previously shown that the PV phenotype observed in the Jak2V617F knockin mice is cell autonomous.5 In this study, we have shown that the transplanted animals receiving BM cells from Stat5-deficient Jak2V617F-expressing mice failed to produce PV-like disease (Figure 4A-H). However, retroviral expression of Stat5a into the BM cells of Stat5-deficient Jak2V617F-expressing mice rescued the defects in transformation and gave rise to a PV-like disease in recipient animals (Figure 4C-H). Thus, Stat5 mediation of the Jak2V617F-induced PV is cell autonomous.

The effect of Stat5 deficiency on normal hematopoiesis has been previously studied in several mouse models,25,39-41 and some discrepancies were observed in these studies. The effect of germ-line deletion of Stat5 could be different from the hematopoietic deletion of Stat5 because Stat5 may also have some important function in nonhematopoietic cells.39 In addition, the use of different Cre lines might have contributed to different phenotypes observed. Teglund et al observed normal erythropoiesis and no significant defect in hematopoietic colony formation in response to cytokines that activate Stat5 proteins in germ-line Stat5-deleted adult mice.39 In contrast, Socolovsky et al observed fetal anemia and reduced number of erythroid progenitors in Stat5-deficient embryos.40 Thus, the requirement for Stat5 in fetal and adult hematopoietic development could be different. More recently, using a conditional Stat5 knockout mouse, Zhu et al observed reduced levels of HCT and anemia on Tie2Cre-mediated deletion of Stat5.25 Other investigators, however, observed that conditional deletion of Stat5 in the adult hematopoietic system using MxCre mice was reasonably well tolerated; these mice survived for a long time and hematopoiesis was mostly preserved.41,42 Consistent with the later reports, we also did not observe any major defect in normal adult hematopoiesis in Stat5-deficient mice. Peripheral blood RBC, WBC, NE, and PLT counts in Stat5-deleted mice were within normal range although slightly lower than those in control mice (supplemental Table 1). In addition, there was no significant difference in the HSC-enriched LSK population, hematopoietic progenitor colony formation and the spleen size between control and Stat5-deficient mice (supplemental Figures 4-5). Only HCT and Hb levels were slightly below the normal range in Stat5-deficient mice (supplemental Table 1), and they survived for a long period without any significant problem (observed for 1 year).

Deficiency of Stat5, however, inhibited Jak2V617F-evoked increase in RBCs, WBCs, NEs, and PLTs in MxCre;Jak2V617F/;Stat5f/f mice (Figure 1). All blood parameters in Stat5-deficient mice expressing Jak2V617F were within normal range and were comparable with control animals (Figure 1). This suggests that Stat5 plays a more important role in Jak2V617F-evoked transformation/MPN than in normal hematopoiesis.

Our work also provides evidence that Stat5 is not a general requirement for transformation by oncoproteins associated with myeloid malignancy. Whereas Stat5 deficiency completely blocked myeloid and erythroid transformation by Jak2V617F, BM progenitors from both control and Stat5-deficient mice were efficiently transformed by KrasG12D (Figure 5A-B). There was no significant difference in myeloid transformation between control and Stat5-deficient BM progenitors by KrasG12D (Figure 5A-B). Stat5 deficiency, however, resulted in significant inhibition of hematopoietic transformation by BCR-ABL (Figures 5A-B), consistent with the previous reports that Stat5 is required for transformation by BCR-ABL.41,43,44 Notably, the effect of Stat5 deficiency in transformation mediated by Jak2V617F is much greater than BCR-ABL (Figure 5A-B). The observation that Stat5 deficiency does not have any effect on myeloid transformation mediated by KrasG12D suggests that different oncoproteins may have different requirements for transformation, with obvious therapeutic implications.

Other signaling molecules, such as Erk, Akt, and Stat3 are also constitutively activated in hematopoietic cells expressing Jak2V617F.12,6,33 So, we have tested the contribution of these signaling molecules in transformation of hematopoietic progenitors by Jak2V617F. The doses of MEK inhibitor U0126 (10 mM) or Akt inhibitor Wortmannin (1 mM) could not inhibit the activation of Erk or Akt in the BM expressing Jak2V617F, evoked almost no or partial inhibition of Jak2V617F-induced transformation of erythroid progenitors (Figure 6A-B). Retroviral expression of
DN-Stat3 markedly inhibited the Stat3 activation in Jak2V617F-expressing BM, but only caused partial inhibition of transformation mediated by Jak2V617F (Figure 6C). Thus, Erk, Akt, and Stat3 are dispensable for transformation induced by Jak2V617F. Although Erk and Akt are interesting targets in many types of cancer and inhibitors of Erk and Akt are in clinical trials, they may not be suitable for treatment of Jak2V617F-Stat3 positive MPNs.

We have also provided some insights into the effects of Stat5 deficiency on Jak2V617F-mediated hematopoietic signaling. Direct analysis of signaling in BM cells by Western blotting revealed that Jak2V617F-induced phosphorylation of p70S6 kinase was markedly reduced on deletion of Stat5 (Figure 7). The identification of the p70S6 kinase as a target of Jak2V617F-Stat5 signaling is interesting because inhibitors of mammalian target of rapamycin (mTOR) can potentially inhibit the p70S6 kinase and several mTOR inhibitors are in clinical trials. In this regard, we have recently found that mTOR inhibitor rapamycin can efficiently inhibit the growth and transformation of Jak2V617F-expressing hematopoietic progenitors (D.Y. and G.M., unpublished observations). Thus, inhibition of p70S6 kinase or mTOR may be a useful strategy for treating PV. Expression of Jak2V617F in hematopoietic cells also results in activation of Erk and Akt.5 However, we did not observe any significant increase in activation of Erk and Akt by Jak2V617F in Stat5-deficient BM cells (data not shown), suggesting that Erk and Akt activation is independent of the Stat5 signaling pathway. Whereas Jak2V617F induced expression of Bcl-xL, Cyclin-D2, and Pim-1, concurrent deletion of Stat5 significantly reduced expression of these proteins in the BM cells (Figure 7). Bcl-xL, Cyclin-D2, and Pim-1 are known downstream targets of Jak2V617F. It has been shown that overexpression of Bcl-xL or a constitutively active mutant of Stat5 in human hematopoietic progenitors can induce endogenous erythroid colony formation.55 Our data suggest that p70S6 kinase, Bcl-xL, Cyclin-D2, and Pim-1 are downstream targets of Jak2V617F-Stat5 signaling, and they may play a role in hematopoietic transformation and PV-like disease induced by Jak2V617F.

In summary, we have demonstrated a critical role of Stat5 in the pathogenesis of PV induced by Jak2V617F. Our results validate Stat5 as a therapeutic target for treatment of PV and other MPNs induced by Jak2V617F. Future studies using this mouse model should lead to a better understanding of the molecular pathogenesis of Jak2V617F-associated MPNs and to test novel therapeutic approaches involving inhibitors of Stat5 or other downstream targets for treatment of MPNs.

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Authorship

Contribution: D.Y. performed research, analyzed data, and wrote the paper; R.E.H. performed histopathologic analysis and revised the paper; and G.M. designed and performed research, analyzed data, and wrote the paper.

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Critical requirement for Stat5 in a mouse model of polycythemia vera

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