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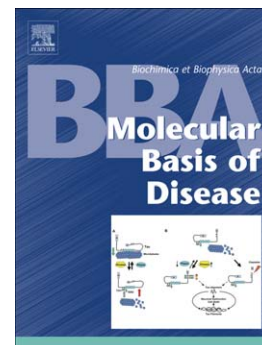
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Real-time bioluminescence imaging of polycythemia vera development in mice

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Summary

Polycythemia vera (PV) is a myeloproliferative disorder involving hematopoietic stem cells. A recurrent somatic missense mutation in JAK2 (JAK2V617F) is thought to play a causal role in PV. Therefore, targeting Jak2 will likely provide a molecular mechanism-based therapy for PV. To facilitate the development of such new and specific therapeutics, a suitable and well characterized preclinical animal model is essential. Although several mouse models of PV have been reported, the spatiotemporal kinetics of PV formation and progression has not been studied. To address this, we created a bone marrow transplant mouse model that co-expresses mutant Jak2 and Luciferase 2 (Luc2) genes. Bioluminescent imaging (BLI) was used to visualize disease cells and analyze the kinetics of PV development *in vivo*. To better understand the molecular mechanism of PV, we generated mice carrying a kinase inactive mutant Jak2 (Jak2K882E) demonstrating that the PV disease was dependent on constitutive activation of the Jak2 kinase activity. We further showed that the Jak2V617F mutation caused increased stem cell renewal activity and impaired cell differentiation which was at least in part due to deregulated transcriptional programming. The Jak2V617F-Luc2 PV mice will be a useful preclinical model to characterize novel JAK2 inhibitors for the treatment of PV.

Introduction

Myeloproliferative diseases (MPDs) including polycythemia vera (PV), essential thrombocytosis (ET) and primary myelofibrosis (PMF) are a group of diseases characterized by overproduction of terminally differentiated cells [1-3]. Recently, a somatic mutation at amino acid residue 617 in JAK2 (JAK2V617F) was discovered in approximately 99% of PV patients and about 50% of ET and PMF patients [4, 5]. Several mouse models over-expressing the mutant mouse Jak2 carrying the corresponding mutation found in human MPD patients (Jak2V617F) have been reported [4, 6, 7]. These mice develop elevated hematocrit (HCT), erythrocytosis, extramedullary hematopoiesis and splenomegaly which are characteristics of PV in humans, therefore establishing a causal role of the mutant JAK2V617F in PV. Furthermore, some of these mice have been used as preclinical animal models of PV for the development of small molecule inhibitors targeting JAK2 [8-11]. However, a longitudinal analysis of the kinetics of development of PV in these mouse models has not been reported. Moreover, although it was assumed that the constitutively activated kinase activity of Jak2 is important in exerting its function in causing the disease by activating the Jak2-Stat5 pathway, the underlying molecular mechanism has not been fully understood. To address these questions and to provide an improved preclinical model system, we developed a bone marrow transplant mouse model by combining the expression of both Jak2V617F and luciferase 2 genes (Luc2) in the mice. In this model, the transduced disease cells co-express mutant Jak2V617F and Luc2 which allowed in vivo visualization of disease burden in real time in living animals by bioluminescence imaging (BLI). Using this model system we were

able to investigate the process of disease cell proliferation and trafficking in the mice over time as well as analyze the endpoint when the disease is fully established, thereby providing insight into the kinetics of the disease development in this animal model.

Moreover, we present evidence that Jak2V617F mutation but not over-expression of the wild type Jak2 can perturb the transcriptional regulatory network resulting in impaired hematopoietic cell differentiation.

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Materials and methods

Viral production

The mouse Jak2 cDNA was cloned into the MSCV-IRES EGFP retroviral vector (a kind gift of Dr. Gary Gilliland). The V617F and kinase inactive K882E mutations in Jak2 were generated using the site-directed mutagenesis kit (Quickchange-XL; Stratagene, La Jolla, CA). The luciferase 2 cDNA was subcloned downstream of the Jak2 cDNA sequence by replacing the EGFP sequence in the same vector. All the plasmids were confirmed by restriction digestion and full-length DNA sequencing. Retroviruses were produced by transfecting 293T cells with the expression vector and packaging vector (pCL-Eco, Imgenex, San Diego, CA) at a 1:1 ratio using FuGENE (Roche, Nutley, NJ) following the manufacturer's protocol. The retroviral supernatant was harvested after 48 hours and the retroviral titer was determined by transducing NIH3T3 cells with viral supernatant plus 10 µg/mL of polybrene and analyzed either for the percentage of green fluorescent protein-positive (GFP⁺) cells by flow cytometry (for GFP-containing plasmids; FACSLSR II cytometer, BD Biosciences, San Jose, CA) or by real-time PCR (for non GFP-containing plasmids) 2 days after transduction. The high titer viruses ($5-10 \times 10^6$ U/ml) were used to transduce bone marrow cells.

Bone marrow transplantation

Female C57Bl/6 mice at age of 6-8 weeks old were purchased from Taconic (Germantown, NY). All procedures carried out on the animals were reviewed and approved by the Institutional Animal Care and Use Committee. The transplantation procedure was performed as previously described [6, 12, 13].

Histopathology

The spleen, liver and femur harvested from the mice were fixed in 10% neutral-buffered formalin and embedded in paraffin. Standard hematoxylin and eosin staining was performed on the spleen, liver and decalcified bone marrow. Peripheral blood was collected by retro-orbital bleeding and the blood smear was stained with Wright Giemsa. For immunohistochemistry (IHC) analysis, liver sections were incubated in goat serum blocking solution with an antibody specific for Ki67 (Neomarkers Inc., Fremont, CA).

Micro-CT imaging

Mice were injected with 200 μ l of Fenestra LC (ART, Canada) 30-60 minutes prior to CT scan. Mice were anaesthetized with a continuous flow of 2-4% isoflurane/oxygen mixture (2.5 l/min) and whole body was scanned using the eXplore Locus Ultra Pre-Clinical CT Scanner (GE Healthcare, Ontario, Canada). CT images were analyzed using Amira 4.1.1 software (Mercury Computer Systems, MA).

Bioluminescence imaging (BLI)

Mouse fur was removed and click beetle luciferin (Promega) working solution was prepared in PBS at 30 mg/ml. The luciferin was injected intraperitoneally (i.p.) at 150 mg/kg 10 min prior to imaging. Mice were anaesthetized with a continuous flow of 2-4% isoflurane/oxygen mixture (2.5 l/min) using an induction chamber XGI-8 gas anesthesia system (Caliper LifeSciences, Hopkinton, MA). Imaging was performed using IVIS Spectrum system (Caliper LifeSciences, Hopkinton, MA). The images were analyzed using the LivingImage software 2.50 (Caliper LifeSciences, Hopkinton, MA).

Tissue imaging

After in vivo imaging, mice were euthanized and tissues including thymus, heart, lung, liver, spleen, stomach, intestine, kidney, long bone, and reproductive organs were quickly dissected and rinsed in luciferin solution (150 µg/ml) for 2-3 minutes. Tissues were transferred onto a black paper and imaged with IVIS Spectrum.

Jak2V617F allele-specific real-time PCR

Genomic DNA from 30 µl of mouse peripheral blood was purified by Qiagen tissue and blood DNA kit (Valencia, CA). 10 µg of genomic DNA was used for 10 µl real-time PCR reaction in 384-well plate format using ABI 7900HT Real-Time PCR system (Foster City, CA). The primers used in the PCR reactions are: Jak2cDNAF2: CACATAGGAACTATTCAGAGTCTTTC; Jak2MutR3: AGAATGTTCTCCTCTCCACAGTA.

Western blot and IP Western blot analysis

Tissue or cell protein lysates were electrophoresed on SDS-PAGE gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes. The membranes were subsequently blotted with the indicated antibodies: anti-pStat5 (1:500; Cell Signaling, Beverly, MA), anti-pJak2 (1:500; Cell Signaling), anti-Jak2 (1:1000; Millipore, Temecula, CA), anti-Stat5 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), 4G10 (1:300; Millipore), anti-GATA1 (1:500; Abcam, Cambridge, MA), anti-PU.1 (1:500; Santa Cruz Biotechnology), anti-Sca1 (1:500; BD Biosciences, San Jose, CA),

anti-GAPDH (Quality Biological Inc., Gaithersburg, MD), anti-Actin (Santa Cruz Biotechnology) and the secondary antibody used was peroxidase-conjugated anti-rabbit immunoglobulin (1:5000; Amersham Biosciences, Piscataway, NJ). For IP Western blot analysis of pJak2, 50 mg of mouse spleen was homogenized in 1 ml RIPA buffer, pre-cleared and then incubated with 10 μ l of anti-pJak2 and 20 μ l of Protein-A+G sepharose beads on a rotator in cold room overnight. Samples were boiled in 50 μ l 2 x SDS sample buffer and separated on 8% SDS PAGE gel for 2 h and then blotted with pan phosphotyrosine antibody 4G10.

Statistics

Values are represented as mean \pm SD and the data were analyzed using 2-tailed Student t test. Statistical significance is reached when $p < 0.05$.

Results

Jak2V617F-Luc2 bone marrow transplant mice develop hallmarks of PV

Consistent with the previously reported Jak2V617F-GFP mice [4, 6, 7], by 4 weeks after transplantation, Jak2V617F-Luc2 mice developed elevated HCT and maintained this increased level of HCT for at least 3 months. The complete peripheral blood counts also showed increased RBC, Hb and WBC similar to the earlier reports [4, 6, 7] (Supplementary Table 1). In contrast, Jak2WT-GFP, Jak2WT-Luc2 and Jak2K882E-Luc2 mice had normal complete blood counts and HCT (Fig. 1A) throughout the course of the study. Jak2V617F-Luc2 mice also developed splenomegaly with enlarged spleen volumes evident by micro-CT imaging (Fig 1B). Splenomegaly was confirmed by directly weighing spleens harvested from the sacrificed Jak2V617F-Luc2 mice (Fig 1B,C). The bone marrow, spleen, liver and peripheral blood samples were collected and subject to histopathological analysis. As shown in Fig 2A, blood smears from the Jak2V617F mice showed marked reticulocytosis. In contrast, the blood smear from Jak2WT transplanted mice appeared normal. In the bone marrow, while the Jak2WT mice showed normal marrow architecture, the Jak2V617F mice exhibited increased erythrocytosis and maturing myeloid cells. Histopathological analysis of the enlarged spleen of the Jak2V617F mice revealed predominantly increased differentiated erythroid cells resulting in the expansion of red pulp disrupting the white pulp architecture. Hyperplasia of erythroid lineage cells was observed in the liver of Jak2V617F but not Jak2WT mice. Increased cell proliferation was evidenced by strong Ki67 positive staining in the liver from Jak2V617F mice (Fig 2B). Similar to Jak2WT-

Luc2 mice, Jak2K882E-Luc2 mice did not show histopathological abnormalities (data not shown).

Imaging of bone marrow reconstitution process and development of PV after bone marrow transplantation

To visualize the processes of homing, proliferation and trafficking of transplanted cells, we imaged mice transplanted with bone marrow cells transduced with Jak2V617F-Luc2, Jak2K882E-Luc2 or Jak2WT-Luc2 retroviruses at various time points post transplantation. The transplanted cells rapidly distributed in the circulation and harbored in several major organs within 1 hour post transplantation. Strong luciferase signals were observed in the lung and spleen while liver and abdominal signals were evident but at a lower level compared to the lung and spleen (Fig. 3A). After a rapid transient decline in BLI signals at day 1, the transduced cells exponentially proliferated in the first two weeks, especially in the spleen and bones such as tibia, femur, vertebra and sternum, and reached a steady level between 8 and 14 days post transplantation after initial homing into the tissues (Fig. 3A & B, Supplementary Figure 1). In a separate experiment, we started imaging 2 weeks after transplantation and also found luciferase signals had reached a steady level and were sustained over at least 8 weeks (Fig. 3A & B and data not shown). Both Jak2V617F-Luc2 and Jak2WT-Luc2 cells initially had similar kinetics during the bone marrow reconstitution process in terms of total lateral BLI signals though the signal in the Jak2V617F-Luc2 mice was slightly higher than the Jak2WT-Luc2 mice (Fig. 3C) around 2 weeks post transplantation. However, the difference was not statistically significant ($p>0.05$) at this early time point. Interestingly, this difference

continued to increase over time. By 19 weeks post transplant, a significantly stronger total lateral BLI signal was observed in Jak2V617F-Luc2 than Jak2WT-Luc2 control mice (Supplementary Figure 2). The ratios of spleen signals over total lateral signals rapidly increased during the first 1-2 weeks from 15% to 80% for both Jak2V617F-Luc2 and Jak2WT-Luc2 cells (Fig. 3B). Interestingly, mutant Jak2V617F-Luc2 cells sustained high levels (70-80%) in spleen from week 1 to at least week 5. In contrast, Jak2WT-Luc2 cells in spleen quickly declined as early as day 10 post transplantation from 80% to 40% within 5 weeks. It's intriguing that BLI revealed accumulation of labeled cells in the abdominal region over time, which were subsequently confirmed to reside in the intestine by *ex vivo* post laparotomy imaging (Fig. 3D). The precise underlying mechanism for this observation is currently unknown. However, the distribution of BLI signal in the gut in bone marrow transplant mice was not unprecedented and was previously observed in other studies [14].

Quantification of Jak2V617F mutant allele burden by real-time PCR

A Jak2 allele-specific real-time PCR assay was developed to quantify the Jak2V617F allele burden in the peripheral blood from bone marrow transplant mice. The Jak2V617F mutant allele level increased within approximately the first week and then exhibited a trend toward decrease for about a week though the decrease wasn't statistically significant. Interestingly, the allele increased significantly thereafter and the high level was maintained for at least 2 months (Fig. 4).

Downstream pathway activation caused by Jak2V617F, but not Jak2K882E in vivo

Spleens were harvested from Jak2V617F-Luc2, Jak2K882E-Luc2 and Jak2WT-Luc2 mice and protein was isolated from the splenocytes. To dissect the Jak2-Stat5 pathway, we performed Western blot and IP Western analysis using antibodies specific for the phosphorylated forms of proteins. As shown in Fig 5A, an elevated level of pJak2 was detected in Jak2V617F compared to Jak2WT mice. The Jak2K882E was expected to potentially have some dominant negative effect. However, unexpectedly the pJak2 levels were similar between the Jak2WT and Jak2K882E animals (data not shown). This could be due to limitation of Western blot sensitivity that could not detect a subtle reduction of pJak2 level. As we observed at 19 weeks post transplant, the BLI signal was lower in the Jak2K882E relative to Jak2WT mice (Supplementary Figure 2) suggesting a possible effect of Jak2K882E. The downstream effector of the Jak2 pathway, Stat5 was also activated as evidenced by higher levels of pStat5 in the Jak2V617F mice relative to the Jak2WT and Jak2K882E controls.

Jak2V617F-driven erythroid proliferation may be in part due to enhanced stem cell renewal and deregulated cell differentiation

To better understand the molecular mechanism for the Jak2V617F-driven PV, we determined the expression of several important transcription factors known to be involved in hematopoietic stem cell differentiation in the spleen where the increased erythropoiesis appeared predominant. As shown in Fig. 5B, the erythroid cell differentiation transcription factor GATA-1 [15-17], was only slightly upregulated in splenocytes from Jak2V617F compared to Jak2WT mice. In contrast, the myeloid cell differentiation transcription factor PU.1 [18-20] was substantially downregulated in the

Jak2 mutant compared to the wild-type cells. Moreover, Sca1, a marker present in hematopoietic stem cells [21, 22] was dramatically upregulated in the Jak2V617F mutant cells suggesting a possible defective differentiation process in the Jak2V617F cells. STAT5 has been shown to play an important role in stem cell renewal [23, 24]. In the Jak2V617F-Luc2 mice, we observed elevated pStat5 in the bone marrow and spleens compared to the Jak2WT-Luc2 mice (Fig. 5A) suggesting possible increased stem cell renewal activity in the mutant mice. Interestingly, in a Western assay using an antibody that recognizes the N-terminus of the Stat5 protein, we found a shorter form of the Stat5 protein present only in the Jak2V617F cells isolated from the mutant mice, but not in the Jak2WT mice (Fig. 5C). A truncated version of Stat5 has been reported as a marker in undifferentiated cells and this truncated protein was not detected in differentiated cells [25-27]. Consistent with previous reports [6, 7], we observed significantly increased erythroid progenitor cells in Jak2V617F than Jak2WT control mice (Supplementary Figure 3). Taken together, these results were in agreement with the hypothesis that the cellular differentiation process was impaired in the Jak2V617F cells.

Discussion

PV is a myeloproliferative disorder involving hematopoietic stem cells, primarily affecting the erythroid lineage. A recurrent somatic missense mutation in the pseudokinase domain of JAK2 (JAK2V617F) is believed to play a causal role in PV [4, 5]. Therefore, targeting Jak2 represents a novel molecular mechanism-based therapeutic approach for PV. To facilitate development of a specific treatment for the disease, we developed a bone marrow transplant mouse model in which the transplanted cells co-express both the mutant Jak2 and Luciferase 2 (Luc2) genes. The luciferase reporter provides an internal light source for *in vivo* bioluminescence imaging (BLI) to indicate the number of cells and relative anatomical locations. This model system allows us to investigate the spatiotemporal kinetics of bone marrow reconstitution and PV development in the context of either wild type or mutant Jak2 in the transplanted mice. One of the advantages of this approach is that the non-invasive BLI modality allowed us to repetitively image the same animals over time. Furthermore, such a model system enabled us to better understand the mechanism, development and maintenance of PV. These mice will also be useful to study the pharmacological properties of novel JAK2 inhibitors for the treatment of PV.

Previous studies have shown that the hematopoietic stem cells reside in several organs after bone marrow transplantation [28, 29]. However, it is not known if the V617F mutation in Jak2 has effect on the initial homing and trafficking of the donor cells after they are intravenously injected into the recipient mice. To address this question, we

analyzed the BLI signal longitudinally at multiple time points in the Jak2-Luc2 model system. Our results showed that although the initial homing and distribution of the cells appeared to be similar between Jak2V617F and Jak2WT mice, the high level BLI signal was maintained in the Jak2V617F mice while the signal rapidly declined after a short period of time in the Jak2WT mice. These observations suggest that the Jak2V617F mutation may confer a survival advantage to the cells, allowing their sustained renewal and conferring the ability to repopulate after the initially injected mutant cells terminally differentiated or died off in the mice. This is further supported by the allele-specific PCR result in which similar kinetics of the Jak2V617F mutant allele burden was observed. The kinetics of the allele level changes over time is consistent with the BLI result suggesting that the more primitive Jak2V617F cells repopulated the hematopoietic compartment while the more differentiated Jak2V617F cells completed their life cycle. Since majority of the cells initially transplanted were more differentiated or mature cells which completed their life span and disappeared from the circulation shortly after the transplantation, this caused the transient decrease of the Jak2V617F allele and BLI level. However, as newly differentiated cells were generated and entered into the circulation from the stem cell compartment in the transplanted mice, the Jak2V617F allele and BLI levels soon returned to high level. The maintenance of steady level of BLI and allele in the Jak2V617F-Luc2 mice over long period of time suggests that the more primitive Jak2V617F cell population persisted in the mutant mice, which continued to be the source of newly differentiated cells resulting in sustained high level of BLI and Jak2V617F allele.

To better understand the mechanism for the mutant JAK2-driven disease *in vivo*, we introduced a mutant Jak2 carrying a point mutation in the ATP binding loop resulting in substitution of lysine for glutamic acid at amino acid residue 882 [30, 31] that abolishes the kinase activity of Jak2 into the mouse. We show that in contrast to the Jak2V617F mice that robustly developed PV-like disease, the Jak2K882E mice did not show PV phenotype. This finding supports the previous reports indicating the importance of the kinase activity of Jak2 in the pathogenesis of PV.

Although it was postulated that one of the cellular events during the development of PV is skewed cell differentiation, the underlying molecular mechanism remains obscure. We attempted to examine several genes important in processes by which the hematopoietic stem cells differentiate into different lineages in the mouse spleen where the increased erythropoiesis is predominant. We show that Sca-1, a marker in hematopoietic stem cells [21, 22], is highly up-regulated in the spleen of Jak2V617F mice compared to Jak2WT control animals. Furthermore, the transcription factor PU.1 [18-20] that is critical in directing cells into myeloid lineage was down-regulated in the Jak2V617F mice. On the contrary, the erythroid transcription factor GATA-1 [15-17] was slightly up-regulated in the spleens from the Jak2V617F mutant animals. Furthermore, the erythroid progenitor cells were dramatically increased in the Jak2V617F mutant mice. These findings are consistent with a recent report showing differential transcriptional regulation of the GATA-1 and PU.1 transcription factors in a human cell system [11]. These data suggest that the overwhelming overproduction of erythroid lineage cells in the spleen may be at least in part due to perturbed balance between

myeloid and erythroid cell differentiation processes. Loss of function analysis of Stat5 has demonstrated a critical role of Stat5 in hematopoiesis. Effective activation of Stat5 was shown to be important in hematopoietic stem cell renewal [23, 24]. Consistent with several previous reports [4, 6, 7], we show that the Jak2V617F mutation resulted in constitutive activation of Stat5. Interestingly, in addition to the elevated phosphorylation of the full-length Stat5 in the Jak2V617F mutant mice, we observed a smaller phosphorylated Stat5 protein which was only detected in the splenocytes isolated from Jak2V617F mutant but not Jak2WT mice. It was previously reported that there are two isoforms of Stat5 due to serine-dependent proteolysis [27]. The shorter form, termed Stat5 β , is a truncated form of the full-length Stat5 (termed Stat5 α) that lacks the COOH-terminal sequence of Stat5 α . Earlier studies demonstrated that the truncated version of Stat5 serves as a marker in undifferentiated cells and this truncated protein was not detected in differentiated cells [25-27]. It has been suggested that the truncated Stat5 may play a role in blocking differentiation and promoting survival. In addition, the majority of acute myeloid leukemia patients carry the Stat5 β isoform in their hematopoietic tissues and the presence of this isoform is associated with disease progression [27, 32]. While in myeloid progenitor cell lineages, several cytokines can activate the shorter form of Stat5, in differentiated mature myeloid cells only the full-length Stat5 is activated [25, 33]. Therefore, our observation of the activation of the shorter form of Stat5 is intriguing and the fact that this activation is only detected in the Jak2V617F mutant but not in the Jak2WT cells under identical sample preparation conditions suggests that the Jak2V617F mutation predisposes cells to a more undifferentiated state. Taken together, these observations suggest that Jak2V617F mutation caused imbalanced transcriptional

program and increased stem cell renewal activity which resulted in impaired cell differentiation leading to overproduction of erythroid lineage cells in the spleen.

In summary, such a model proved to be a very useful system to study the mechanism and kinetics of PV and may be used as a preclinical animal model to characterize novel JAK2 inhibitors. Evaluation of the JAK2 inhibitors will be greatly enhanced by the non-invasive real-time quantification of the disease burden using a BLI-based assay in the living animals.

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Figure legends

Fig. 1. Jak2V617F-Luc2 mice showed PV-like erythrocytosis and splenomegaly. A. HCTs were measured 4 wks after transplantation of cells expressing Jak2WT, Jak2K882E and Jak2V617F. HCTs were elevated in bone marrow transplant mice expressing Jak2V617F. HCTs remained normal in Jak2WT and Jak2K882E control mice throughout the study. ** indicates highly significant increase of HCTs in Jak2V617F mice compared to Jak2WT or Jak2K882E control mice ($p < 0.01$). B. μ CT imaging was used to visualize and quantify the spleen volumes in vivo. Purple; spleens. A representative image for each of the genotypes is shown on the top. Volumes measured by μ CT and post euthanasia spleen weights are indicated below the images. The values are represented as mean \pm SD. Jak2V617F vs Jak2WT: $p = 0.0138$, spleen volumes; $p = 0.00001$, spleen weight. C. Gross images of spleens isolated from Jak2WT and Jak2V617F transplanted mice showed consistent differences.

Fig. 2. Myeloid hyperplasia was seen in Jak2V617F transplanted mice. A. Histopathology of myeloid hyperplasia in the bone marrow transplant mice. Tissues from Jak2V617F and Jak2WT mice were collected at 1 month after transplantation and analyzed by H & E staining. Blood smears were stained with Giemsa. PB, peripheral blood; BM, bone marrow; W, white pulp; R, red pulp; arrow, multiple small aggregates of immature erythroid & myelocytic cells. B. Immunohistochemistry of Jak2V617F mutant and Jak2WT bone marrow transplant mice. Livers were collected at 1 m after transplantation and subject to IHC staining with Ki67 antibody. The positive Ki67

staining in the perivascular and sinusoidal regions in Jak2V617F mutant but not the wild type mouse liver is consistent with extramedullary hematopoiesis in the mutant mice.

Fig. 3. Visualization of bone marrow reconstitution process in live animals. A. Representative BLI images at various time points in Jak2V617F-Luc2 mice. B. Kinetics of bone marrow reconstitution indicated by BLI signals from the spleen as a function of the total lateral signals. *, $p < 0.05$; **, $p < 0.01$, relative to Jak2WT mice. C. BLI measurement at 14 days post BMT showing a trend toward stronger signals in Jak2V617F than Jak2WT mice. However, the difference was not statistically significant ($p = 0.366$). D. Tissue distribution of Jak2V617F-Luc2 cells. Mice were imaged 19 weeks after transplantation. Mice were euthanized using CO₂ after luciferin injection and laparotomy imaging was then performed 5-10 minutes following luciferin injection. Tissues shown were dissected and rinsed in 15 µg/ml luciferin in PBS for 2-3 minutes and imaged. Stronger BLI signal was observed in Jak2V617F than Jak2WT and Jak2K882E mice.

Fig. 4. Quantification of Jak2V617F allele burden. A mutant Jak2 allele specific real time PCR assay was developed and used to quantify the Jak2V617F allele level in peripheral blood of the bone marrow transplant mice. The mutant Jak2 allele level showed a trend of decrease around 1-2 weeks post BMT though it was not statistically significant. However, this mutant Jak2 allele level was rapidly followed by a substantial increase and the elevated mutant allele level was maintained for at least 2 months.

Fig. 5. Pathway activation in Jak2V617F-Luc2 mice. A. Mutant Jak2V617F induces phosphorylation of Jak2 and Stat5 *in vivo*. B. Differential regulation of GATA-1, PU.1 and Sca1 in Jak2V617F and Jak2WT cells. C. A truncated pStat5 is present in spleens of Jak2V617F but not Jak2WT mice. Both the full-length and truncated forms could be induced by cytokines (6 ng/mL IL-3, 10 ng/mL IL-6 and 10 ng/mL stem cell factor, StemCell Technologies) in Jak2V617F cells whereas only the full-length form was induced by cytokines in Jak2WT cells. Lane 1, Jak2WT; Lane 2, Jak2WT + cytokines; Lane 3, Jak2V617F; Lane 4, Jak2V617F + cytokines. Mice were used 1 month post transplant. Three independent experiments were performed and 2 mice/genotype were analyzed in each.

References

- [1] P.J. Campbell, A.R. Green, The myeloproliferative disorders, *N Engl J Med* 355 (2006) 2452-2466.
- [2] R.L. Levine, A. Pardanani, A. Tefferi, D.G. Gilliland, Role of JAK2 in the pathogenesis and therapy of myeloproliferative disorders, *Nat Rev Cancer* 7 (2007) 673-683.
- [3] A. Tefferi, Essential thrombocythemia, polycythemia vera, and myelofibrosis: current management and the prospect of targeted therapy, *Am J Hematol* 83 (2008) 491-497.
- [4] C. James, V. Ugo, J.P. Le Couedic, J. Staerk, F. Delhommeau, C. Lacout, L. Garcon, H. Raslova, R. Berger, A. Bennaceur-Griscelli, J.L. Villeval, S.N. Constantinescu, N. Casadevall, W. Vainchenker, A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera, *Nature* 434 (2005) 1144-1148.
- [5] R. Kralovics, F. Passamonti, A.S. Buser, S.S. Teo, R. Tiedt, J.R. Passweg, A. Tichelli, M. Cazzola, R.C. Skoda, A gain-of-function mutation of JAK2 in myeloproliferative disorders, *N Engl J Med* 352 (2005) 1779-1790.
- [6] G. Wernig, T. Mercher, R. Okabe, R.L. Levine, B.H. Lee, D.G. Gilliland, Expression of Jak2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model, *Blood* 107 (2006) 4274-4281.

- [7] C. Lacout, D.F. Pisani, M. Tulliez, F.M. Gachelin, W. Vainchenker, J.L. Villeval, JAK2V617F expression in murine hematopoietic cells leads to MPD mimicking human PV with secondary myelofibrosis, *Blood* 108 (2006) 1652-1660.
- [8] V.M. Zaleskas, D.S. Krause, K. Lazarides, N. Patel, Y. Hu, S. Li, R.A. Van Etten, Molecular pathogenesis and therapy of polycythemia induced in mice by JAK2 V617F, *PLoS ONE* 1 (2006) e18.
- [9] A. Pardanani, J. Hood, T. Lasho, R.L. Levine, M.B. Martin, G. Noronha, C. Finke, C.C. Mak, R. Mesa, H. Zhu, R. Soll, D.G. Gilliland, A. Tefferi, TG101209, a small molecule JAK2-selective kinase inhibitor potently inhibits myeloproliferative disorder-associated JAK2V617F and MPLW515L/K mutations, *Leukemia* 21 (2007) 1658-1668.
- [10] G. Wernig, M.G. Kharas, R. Okabe, S.A. Moore, D.S. Leeman, D.E. Cullen, M. Gozo, E.P. McDowell, R.L. Levine, J. Doukas, C.C. Mak, G. Noronha, M. Martin, Y.D. Ko, B.H. Lee, R.M. Soll, A. Tefferi, J.D. Hood, D.G. Gilliland, Efficacy of TG101348, a selective JAK2 inhibitor, in treatment of a murine model of JAK2V617F-induced polycythemia vera, *Cancer Cell* 13 (2008) 311-320.
- [11] I. Geron, A.E. Abrahamsson, C.F. Barroga, E. Kavalarchik, J. Gotlib, J.D. Hood, J. Durocher, C.C. Mak, G. Noronha, R.M. Soll, A. Tefferi, K. Kaushansky, C.H. Jamieson, Selective inhibition of JAK2-driven erythroid differentiation of polycythemia vera progenitors, *Cancer Cell* 13 (2008) 321-330.
- [12] J. Schwaller, J. Frantsve, J. Aster, I.R. Williams, M.H. Tomasson, T.S. Ross, P. Peeters, L. Van Rompaey, R.A. Van Etten, R. Ilaria, Jr., P. Marynen, D.G. Gilliland, Transformation of hematopoietic cell lines to growth-factor

- independence and induction of a fatal myelo- and lymphoproliferative disease in mice by retrovirally transduced TEL/JAK2 fusion genes, *Embo J* 17 (1998) 5321-5333.
- [13] L.M. Kelly, Q. Liu, J.L. Kutok, I.R. Williams, C.L. Boulton, D.G. Gilliland, FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model, *Blood* 99 (2002) 310-318.
- [14] V.H. Nguyen, R. Zeiser, D.L. Dasilva, D.S. Chang, A. Beilhack, C.H. Contag, R.S. Negrin, In vivo dynamics of regulatory T-cell trafficking and survival predict effective strategies to control graft-versus-host disease following allogeneic transplantation, *Blood* 109 (2007) 2649-2656.
- [15] F. Morceau, C. Dupont, V. Palissot, P. Borde-Chiche, C. Trentesaux, M. Dicato, M. Diederich, GTP-mediated differentiation of the human K562 cell line: transient overexpression of GATA-1 and stabilization of the gamma-globin mRNA, *Leukemia* 14 (2000) 1589-1597.
- [16] T. Stopka, D.F. Amanatullah, M. Papetti, A.I. Skoultchi, PU.1 inhibits the erythroid program by binding to GATA-1 on DNA and creating a repressive chromatin structure, *Embo J* 24 (2005) 3712-3723.
- [17] G.R. Aispuru, M.V. Aguirre, J.A. Aquino-Esperanza, C.N. Lettieri, J.A. Juaristi, N.C. Brandan, Erythroid expansion and survival in response to acute anemia stress: the role of EPO receptor, GATA-1, Bcl-xL and caspase-3, *Cell Biol Int* 32 (2008) 966-978.

- [18] M. Papetti, A.I. Skoultschi, Reprogramming leukemia cells to terminal differentiation and growth arrest by RNA interference of PU.1, *Mol Cancer Res* 5 (2007) 1053-1062.
- [19] M. Dijon, F. Bardin, A. Murati, M. Batoz, C. Chabannon, C. Tonnelle, The role of Ikaros in human erythroid differentiation, *Blood* 111 (2008) 1138-1146.
- [20] Y. Fukuchi, M. Ito, F. Shibata, T. Kitamura, H. Nakajima, Activation of CCAAT/enhancer-binding protein alpha or PU.1 in hematopoietic stem cells leads to their reduced self-renewal and proliferation, *Stem Cells* 26 (2008) 3172-3181.
- [21] N. Suzuki, O. Ohneda, N. Minegishi, M. Nishikawa, T. Ohta, S. Takahashi, J.D. Engel, M. Yamamoto, Combinatorial Gata2 and Sca1 expression defines hematopoietic stem cells in the bone marrow niche, *Proc Natl Acad Sci U S A* 103 (2006) 2202-2207.
- [22] R. Kumar, V. Fossati, M. Israel, H.W. Snoeck, Lin-Sca1+kit- bone marrow cells contain early lymphoid-committed precursors that are distinct from common lymphoid progenitors, *J Immunol* 181 (2008) 7507-7513.
- [23] Y. Kato, A. Iwama, Y. Tadokoro, K. Shimoda, M. Minoguchi, S. Akira, M. Tanaka, A. Miyajima, T. Kitamura, H. Nakauchi, Selective activation of STAT5 unveils its role in stem cell self-renewal in normal and leukemic hematopoiesis, *J Exp Med* 202 (2005) 169-179.
- [24] A.T. Wierenga, E. Vellenga, J.J. Schuringa, Maximal STAT5-induced proliferation and self-renewal at intermediate STAT5 activity levels, *Mol Cell Biol* 28 (2008) 6668-6680.

- [25] M. Azam, C. Lee, I. Strehlow, C. Schindler, Functionally distinct isoforms of STAT5 are generated by protein processing, *Immunity* 6 (1997) 691-701.
- [26] J. Meyer, M. Jucker, W. Ostertag, C. Stocking, Carboxyl-truncated STAT5beta is generated by a nucleus-associated serine protease in early hematopoietic progenitors, *Blood* 91 (1998) 1901-1908.
- [27] Z. Xia, R.R. Salzler, D.P. Kunz, M.R. Baer, L. Kazim, H. Baumann, M. Wetzler, A novel serine-dependent proteolytic activity is responsible for truncated signal transducer and activator of transcription proteins in acute myeloid leukemia blasts, *Cancer Res* 61 (2001) 1747-1753.
- [28] S. Aizawa, M. Tavassoli, Molecular basis of the recognition of intravenously transplanted hemopoietic cells by bone marrow, *Proc Natl Acad Sci U S A* 85 (1988) 3180-3183.
- [29] Y.A. Cao, A.J. Wagers, A. Beilhack, J. Dusich, M.H. Bachmann, R.S. Negrin, I.L. Weissman, C.H. Contag, Shifting foci of hematopoiesis during reconstitution from single stem cells, *Proc Natl Acad Sci U S A* 101 (2004) 221-226.
- [30] D. Watling, D. Guschin, M. Muller, O. Silvennoinen, B.A. Witthuhn, F.W. Quelle, N.C. Rogers, C. Schindler, G.R. Stark, J.N. Ihle, et al., Complementation by the protein tyrosine kinase JAK2 of a mutant cell line defective in the interferon-gamma signal transduction pathway, *Nature* 366 (1993) 166-170.
- [31] J. Feng, B.A. Witthuhn, T. Matsuda, F. Kohlhuber, I.M. Kerr, J.N. Ihle, Activation of Jak2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop, *Mol Cell Biol* 17 (1997) 2497-2501.

- [32] Z. Xia, S.N. Sait, M.R. Baer, M. Barcos, K.A. Donohue, D. Lawrence, L.A. Ford, A.M. Block, H. Baumann, M. Wetzler, Truncated STAT proteins are prevalent at relapse of acute myeloid leukemia, *Leuk Res* 25 (2001) 473-482.
- [33] M. Azam, H. Erdjument-Bromage, B.L. Kreider, M. Xia, F. Quelle, R. Basu, C. Saris, P. Tempst, J.N. Ihle, C. Schindler, Interleukin-3 signals through multiple isoforms of Stat5, *Embo J* 14 (1995) 1402-1411.

Supplementary Figure Legends

Supplementary Figure 1. Close-up photographs demonstrate that transplanted cells were present in long bones such as femur and tibia in fully reconstituted Jak2V617F-Luc2 PV mice.

Supplementary Figure 2. BLI measurement at 19 weeks post transplant shows increased signal in Jak2V617F-Luc2 than Jak2WT-Luc2 mice. Jak2K882E-Luc2 mice displayed reduced BLI signal compared to Jak2WT-Luc2 mice.

Supplementary Figure 3. Increased erythroid progenitor cells in Jak2V617F-Luc2 than Jak2WT-Luc2 mice. **, $p < 0.01$.

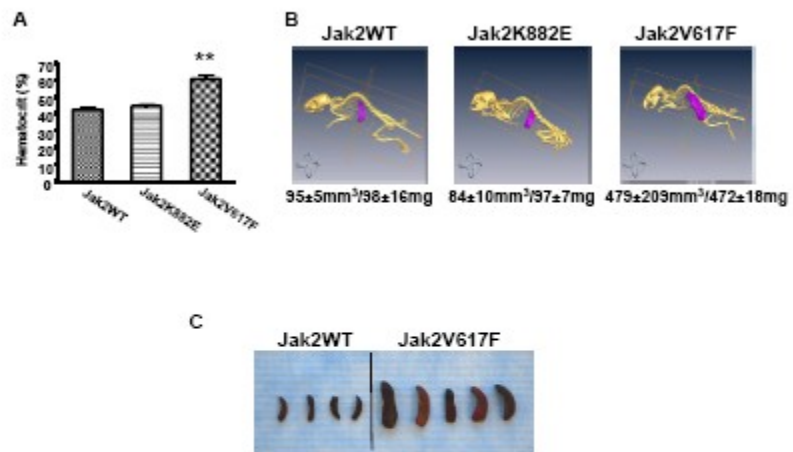


Figure 1

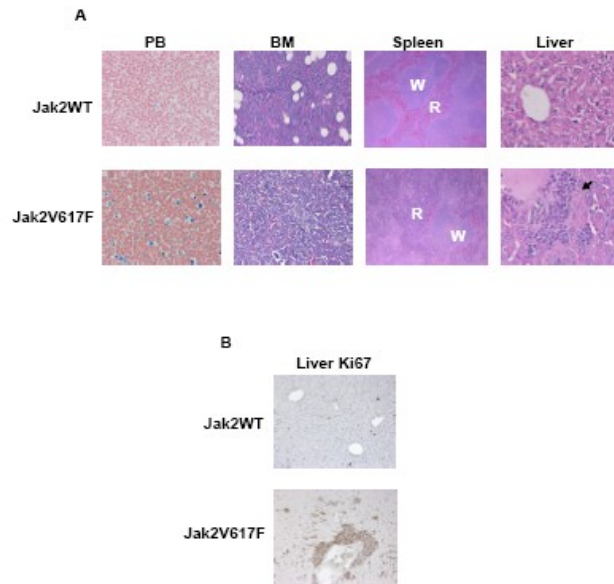


Figure 2

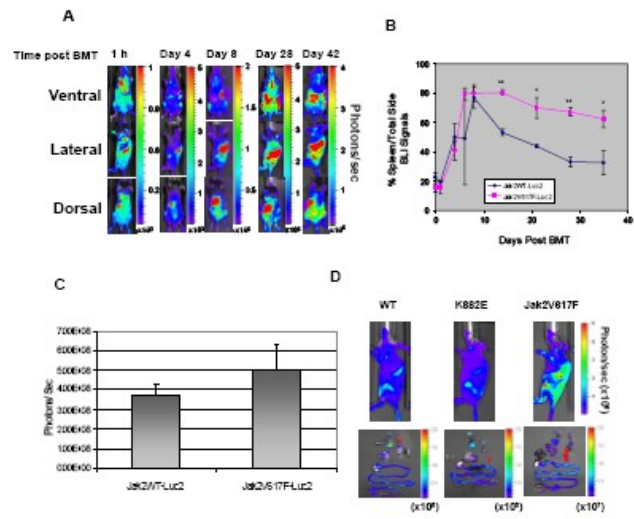


Figure 3

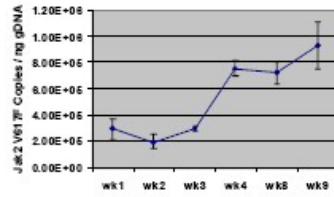


Figure 4

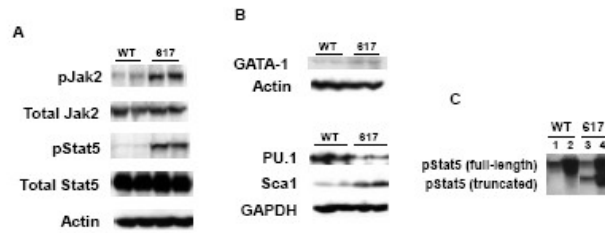


Figure 5