Development of ET, primary myelofibrosis, and PV in mice expressing JAK2 V617F

Running title: JAK2 V617F causes classical MPDs

Kotaro Shide[1)], Haruko K Shimoda[1)], Takashi Kumano[2)], Kennosuke Karube[3)], Takurou Kameda[1)], Katsuto Takenaka[2)],Seido Oku[2)], Hiroo Abe [1)], Keiko S Katayose[1)], Youko Kubuki[1)], Kazunori Kusumoto[1)], Satoru Hasuike[1)], Yoshihiro Tahara[1)], Kenji Nagata[1)], Tadashi Matsuda[4)], Koichi Ohshima[3)], Mine Harada[2)], and Kazuya Shimoda[1)]§

1) Department of Gastroenterology and Hematology, Faculty of Medicine, Miyazaki University, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan
2) Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka, Fukuoka 812-8582, Japan
3) Department of Pathology, Kurume University, Asahimachi 67, Kurume, Fukuoka 830-0011, Japan
4) Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-Ku Kita 12 Nishi 6, Sapporo 060-0812, Japan

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§ Correspondence should be addressed to Kazuya Shimoda, MD,
Department of Gastroenterology and Hematology, Faculty of Medicine, Miyazaki University,
5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan
Tel.: 81-985-85-9121; Fax: 81-985-85-5194; E-mail: kshimoda@med.miyazaki-u.ac.jp

Abstract

An acquired JAK2 V617F mutation is found in most patients with polycythemia vera (PV), and about half of patients with essential thrombocythemia (ET) or primary myelofibrosis (PMF). Mice transplanted with bone marrow cells in which JAK2 V617F was retrovirally expressed developed PV-like features, but not ET or PMF. To address the contribution of this mutation to the pathogenesis of these three MPDs, we generated two lines of JAK2 V617F transgenic mice. One line showed granulocytosis after 4 months of age. Among 43 mice, 8 (19%) showed polycythemia and 15 (35%) showed thrombocythemia. The second line showed extreme leukocytosis and thromobocytosis. They showed anemia that means Hb value from 9 to 10 g/dL at one month old. Myeloid cells and megakaryocytes were predominant in the bone marrow of these animals, and splenomegaly was observed. The expression of JAK2 V617F mRNA in bone marrow cells was 0.45 and 1.35 that of endogenous wild-type JAK2 in the two lines. *In vitro* analysis of bone marrow cells from both lines showed constitutive activation of ERK1/2, STAT5, and AKT, and augmentation of their phosphorylations by cytokine stimulation. We conclude that *in vivo* expression of JAK2 V617F results in ET-like, PMF-like, and PV-like disease.

Keywords

myeloproliferative diseases, JAK2 V617F, transgenic mice

Introduction

Janus Kinase 2 (JAK2) is a member of the Janus family of non-receptor tyrosine kinases, and is required for signaling from type I cytokine receptors(1, 2). In erythropoietin (EPO) intracellular signaling pathway, for example, the binding of EPO to cellular surface EPO receptor activates JAK2, followed by the activation of STAT5. Phosphorylated STAT5 translocate from the cytoplasm into the nucleus, and induce transcription of their target genes within a short period of time(3, 4). This JAK-STAT signaling pathway is essential for cytokine function, although cytokines activate several intracellular signaling pathway(3-5). Deletion of JAK2 in mice causes embryonic lethality due to severe anemia(2), a similar phenotype to that observed in EPO-, EPO receptor-(6), and STAT5-deficient mice(7). A G to T point mutation resulting in the substitution of phenylalanine for valine at position 617 (V617F) in JAK2 has been reported in myeloproliferative disease (MPD) patients(8-12). This mutation is found in most patients with polycythemia vera (PV) and in approximately half of patients suffering from essential thrombocythemia (ET) or primary myelofibrosis (PMF)(8-12). The expression of JAK2 V617F in cytokine-dependent cell lines confers cytokine-independent growth(9, 11, 13) by constitutively activating STAT5, Akt, and ERK. Therefore, JAK2 V617F is thought to be related to the development of PV, ET, and PMF.

X-inactivation-based analysis of women with MPDs and FISH analysis in MPD patients having specific chromosal abnormalities have demonstrated that MPDs are clonal stem cell disorders(14-16) with common features such as hypercellular bone marrow, cytokine-independent colony formation and cytokine hypersensitivity of bone marrow cells(17, 18), and hepatosplenomegaly with extramedullary hematopoiesis. However, MPDs have clinical differences, as erythroid hyperplasia is prevalent in PV, and megakaryocytic hyperplasia is prevalent in ET and PMF(19). It remains to be determined how a unique mutation can be involved in such phenotypic pleiotropy. To address this question, transplantation experiments using bone marrow cells transduced by a retrovirus encoding JAK2 V617F have been performed(20-23). Expression of this mutant caused mice to develop human PV-like disease, including erythrocytosis, neutrophilia, trilineage hyperplasia in bone marrow, and extramedullary hematopoiesis resulting in splenomegaly. Symptoms progressed to anemia and bone marrow fibrosis with time, resembling the “spent phase” of PV. Although megakaryocyte hyperplasia in bone marrow was observed in recipient mice, thrombocytosis rarely occurred(20, 22, 23), and was very mild and transient in its rare occurrences (21). To determine whether ET or PMF would develop due to expression of JAK2 V617F *in vivo*, we generated transgenic mice carrying this allele.

Materials and Methods

Materials

The murine Jak2 cDNA was kindly provided by Dr. J. Ihle (St. Jude Children’s Research Hospital, Memphis, TN). We used oligonucleotide-directed mutagenesis to substitute phenylalanine for valine at residue 617 of JAK2 as previously described(13).

Generation of transgenic mice The pSP65-H2K-i-LTR vector(24) was kindly provide by Dr. Weissman (Stanford University School of Medicine, Stanford, CA). We engineered the H2K-V617F JAK2 transgenic

construct by introducing the murine V617F JAK2 cDNA into the NotI subcloning site of the pSP65-H2K-i-LTR vector. The construct was isolated from vector sequences as an NaeI/PvuI fragment and purified. Transgenic mice were produced by standard oocyte injection using BDF1 mouse-derived fertilized eggs(25). All procedures were approved by the local Miyazaki University ethics committee. Transgenic mice were identified by PCR using oligonucleotide primers specific for the murine JAK2 cDNA (Primer-1: 5'-CTTCCACATAGACGAGTCAACA-3', Primer-2: 5'-GTCCTGTTCTGTCAGTGTCTCAC -3')(Figure 1A).

Determination of flanking sequences using bubble linker mediated PCR for chromosomal mapping of transgene insertion sites

The genomic sequences flanking the transgenes were determined by bubble linker-mediated PCR (LM-PCR)(26, 27). Aliquots of tail DNA from transgenic lines were digested with SpeI or SacI (Takara Bio Inc., Tokyo, Japan), and the fragments were ligated to a double-stranded bubble linker. For samples digested with SpeI, 5'-CTAGGAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAACGGACGAGAGAAGGGAGAG-3' and 5'-GACTCTCCCTTCTCGAATCGTAACCGTTCGTACGAGAATCGCTGTCCTCTCCTTC-3' were used, and for samples digested with SacI, 5'-GAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAACGGACGAGAGAAGGGAGAG-3' and 5'-GACTCTCCCTTCTCGAATCGTAACCGTTCGTACGAGAATCGCTGTCCTCTCCTTCAGCT-3' were used. Bubble LM-PCR consisted of two PCR amplification steps. The primary PCR was performed on ligation products using a linker-specific vectorette primer (5'-CGAATCGTAACCGTTCGTACGAGAATCGCT-3') and a transgene-specific primer (transgene-A: 5'-TCGTGGTGTCACGCTCGTCGTTTGGTATGG-3') under the following conditions: 1 cycle of 95°C for 5 min, 25 cycles of 95°C for 30 sec, 57°C for 30 sec, and 72°C for 3 min, and 1 cycle of 72°C for 90 sec. The primary PCR mixture was then diluted and used as the template for PCR amplification with a nested transgene-specific primer (transgene-B: 5'-CGGTTCCCAACGATCAAGGCGAGTTACATG-3') and the same linker-specific vectorette primer as was used in the primary PCR, with the following conditions: 1 cycle of 95°C for 5 min, 35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 3 min, and 1 cycle of 72°C for 90 sec. All PCR amplifications were performed with AmpliTaq Gold, a hot-start DNA polymerase, and a GeneAmp 9700 (Applied Biosystems). The nested PCR products were separated by electrophoresis on 2% agarose gels in TAE buffer. The nested PCR bands were excised and purified using QIAquick Gel Extraction kits (QIAGEN). The nucleotide sequences of PCR bands were determined by direct sequencing using an ABI 310 Genetic analyzer. The transgene insertion sites in chromosomes were determined using a BLAST search of the flanking sequences in the Ensembl genome database via the Internet (http://www.ensembl.org).

Locus-specific genotyping of Tg by a flanking primer method

For locus-specific genotyping and zygosity checks, we performed PCR analysis using flanking primers with sequences based on the sequences flanking the transgenes(28) (Figure 1A). Primer-3

(5’-GCATCGTGGTGTCACGCTCGTC-3’) at the 3’-end of the transgene was used for both lines. Flanking primers were designed for each line using 5’- and 3’-flanking sequences at the transgene insertion site. Primer-3, Primer-4 (5’-GAGAAGAGGGTTTCATAGCACATGGCC-3’), and Primer-5 (5’-GGATAAGCATCCATCGTGCTATCACAGC-3’) were used for line 1. Primer-3, Primer-6 (5’-CACATGACTCACCTGAAGATGTGGAAG-3’), and Primer-7 (5’-CCACATACAGCTCAGACCTAGCAGG-3’) were used for line 2. Genotypes were determined by PCR analysis of tail DNA under the following conditions: 1 cycle of 95°C for 10 min, 30 cycles of 94°C for 1 min, 61°C for 1 min, and 72°C for 2 min, and 1 cycle of 72°C for 16 min.

Isolation of RNA, reverse transcription, quantitative PCR, and sequencing

BM RNAs were extracted using the RNeasy mini kit (QIAGEN). For quantitative PCR, reverse transcription was carried out with 250 ng of total RNA using a QuantiTect reverse transcription kit (QIAGEN). TaqMan Universal PCR Master Mix and adequate primers were used for quantitative PCR amplification of the total JAK2 cDNA including both endogenous wild-type JAK2 cDNA and transgene JAK2 V617F cDNA, and tubulin cDNA as a control. Quantitative PCR was performed on an ABI 7000 (Applied Biosystems) and analyzed with the associated software. For sequencing, reverse transcription was carried out with 250 ng of total RNA using a TaKaRa RNA PCR kit (Takara Bio Inc.). We amplified the JAK2 cDNA, 395bp segment including V617F mutation using the primers 5’-AATATTTAATGAAAGTCTTGGCCAAGGTAC-3’ and 5’-TAGCACACACATTCCCATGAATAAGGGATT-3’. The PCR products were sequenced directly using an ABI Genetic analyzer.

Protein lysates and western immunoblotting

Cells were lysed as previously described(29) and centrifuged at 12,000 x g for 15 min to remove debris. Total cell lysates were resolved by SDS-PAGE and transferred to PVDF nitrocellulose membranes (Amersham, Uppsala, Sweden). Membranes were probed using the appropriate antibodies and visualized by ECL (Amersham). Anti-phospho-STAT5, -phospho-ERK1/2, -ERK1/2, -phospho-Akt, -Akt antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-Jak2, -STAT5 antibodies were purchased from Santa Cruz Biotechnology(Santa Cruz,CA).

Pathological examination

For histological evaluation, tissue samples were fixed in formalin, paraffin embedded, and cut for hematoxylin and eosin staining or Gomori silver staining according to standard protocols.

Progenitor cell assays

To detect CFU-E colonies, bone marrow cells from wild-type and JAK2 V617F line 1 and line 2 mice were plated in semisolid methylcellulose. To test the cytokine-independent colony formation, 1 x 10^5 cells were plated in methylcellulose (M3234: StemCell Technologies) in the presence or absence of 3 U of EPO. Clusters were counted after 2 days in culture. To detect other types of colony (BFU-E, CFU-G, CFU-M,

CFU-GM, CFU-GEMM), 1 x 10^5 BM cells from wild-type, JAK2 V617F line 1, or line 2 mice were plated in methylcellulose (M3434: StemCell Technologies) containing 3U/mL EPO, 10 ng/mL recombinant murine interleukin-3 (rmIL-3), 10 ng/mL rmIL-6, and 50 ng/mL recombinant murine stem cell factor according to the manufacturer's protocol. Colonies were counted on day 7 in culture.

Flow cytometoric analysis

Cells were washed in phosphate-buffered saline (PBS) + 1% fetal bovine serum (FBS), blocked with Fc-block (BD PharMingen, San Diego, CA) for 4 min on ice, and stained with monoclonal antibodies in PBS + 1% FBS for 40 min on ice. Antibodies used were fluorescein isothiocyanate (FITC)-conjugated CD71, B220, CD41, and Mac-1, and phycoerythrin(PE)-conjugated TER119, CD3, and Gr-1 rat anti-mouse antibodies (BD PharMingen). After washing, cells were resuspended in PBS + 1%FBS and analyzed with a FACScan cytometer (BD Biosciences, San Jose, CA). At least 20,000 events were acquired, and data were analyzed using CellQuest software (BD biosciences).

Statistical analysis

Results are presented as mean ± standar error of the mean (SEM) and data were analyzed with the 2-tailed Student's *t*-test. Where data was repeatedly analyzed, p values were corrected according to the Bonferroni method. The correlation between Hb level and T/G ratio was estimated by the Pearson correlation. The value of the correlation coefficient (r) is given, in addition to the level of significance (p).

Results

Introduction of JAK2 V617F in mice, and analysis of integration sites.

We generated two lines of transgenic mice expressing a murine JAK2 cDNA that encoded phenylalanine at residue 617 rather than valine (JAK2 V617F), under the control of the H-2Kb promoter. The integration sites of the transgene were analyzed using bubble linker-mediated PCR. As shown in Figure 1A, the transgene integrated in intron 12 of *Dcc* (*Deleted in colorectal carcinoma*) on chromosome 18qE2 in the first line, and in the region between *Mef 2a* (*myocyte enhancer factor 2A*) and *Lrrc 28* (*leucine rich repeat containing 28*) on chromosome 7qD1 in the second line. We termed them as line 1 mice and line 2 mice. PCR primers as indicated in Figure 1A were used to detect the transgene. As shown in Figure 1B, mice carrying JAK2 V617F were born at the expected Mendelian ratio. All mice carrying the JAK2 V617F transgene detected by internal primers (primer sets 1 and 2 in Figure 1A) were positive by PCR using primers external to the transgene (primer sets 3, 4, and 5 for line 1, and 3, 6, and 7 for line 2 in Figure 1A), indicating there was only one integration site in each line.

Expression of the V617F mutant in the transgenic lines.

We first measured total (endogenous wild-type and transgenic mutant) JAK2 mRNA to estimate the expression of JAK2V617F mRNA *in vivo* by real-time PCR. Total JAK2 mRNA in bone marrow cells from JAK2 V617F line 1 and line 2 mice measured 1.45- and 2.35- fold, respectively, compared with that

from wild-type mice. This means that the expression of JAK2 V617F mRNA in bone marrow cells was 0.45 and 1.35 that of endogenous wild-type JAK2 in the two lines. Sequencing of PCR products of JAK2 cDNA from bone marrow cells from line 1 mice showed a peak of mutant JAK2 (T peak identifies the mutation) that was about half of wild-type endogenous JAK2 (G peak identifies wild-type), and a peak of mutant JAK2 in bone marrow cells from line 2 mice was slightly higher than that of wild-type endogenous JAK2 (Figure 2A). The expression of JAK2 V617F protein in them was estimated by measuring the amount of total JAK2 protein (endogenous wild-type JAK2 plus exogenous JAK2 V617F). As shown in Figure 1C, total amount of JAK2 from JAK2 V617F Tg mice was slightly higher than that from wild-type mice.

To determine the kinase activity of JAK2 V617F *in vivo*, we performed western blot analysis of extracts from bone marrow cells from wild-type, JAk2 V617F line 1, and line 2 mice. Constitutive STAT5 and AKT phosphorylation in the absence of cytokine stimulation were observed in JAK2 V617F bone marrow cells from either line (Figure 1D). ERK was weakly but constitutively activated in cells from JAK2 V617F line 2 mice without cytokine stimulation, while very small amount of phosphorylation was observed in cells from line 1 mice. Upon cytokine (Epo + WEHI conditioned medium) stimulation *in vitro*, the phosphorylation of ERK1/2, STAT5, and AKT was much stronger in cells from line 1 and line 2 mice than in cells from wild-type mice (Figure 1D).

Changes in the peripheral blood in JAK2 V617F expressing mice.

In the JAK2 V617F line 1 mice, a total of 57 mice were examined for peripheral blood count, and results at 3 months and older were obtained for 43 of the 57. White cell number, Hb value, and platelet number varied by individual. Fifteen of the 43 mice showed leukocytosis (difined as white blood cell number > 2 x 10^{10}/L) (Figure 2A), with a predominance of granulocytes (Figure 2B). The mean Hct value at one month after birth was 49.9 ± 2.9 %, and that at 6 months old was 46.8 ± 6.7 %. Eight of the 43 mice showed polycythemia (defined as Hb > 18 g/dL) three months after birth, and the highest observed Hb level was greater than 20 g/dL. Fifteen mice displayed thrombocytosis (defined as platelets > 140 x 10^{10}/L), and the highest number of platelets observed was greater than 300 x 10^{10}/L. As for the white cell number, two of the 8 mice with polycythemia and nine of the 15 mice with thrombocytosis concomitantly showed leukocytosis. Two mice with polycythemia showed thrombocytosis during subsequent examinations. A total of 21 of the 43 mice (49%) showed polycythemia and/or thromobocytosis. Hb value and platelet count in the remaining 22 mice were comparable to those in wild-type mice, and 4 of them showed leukocytosis.

Peripheral blood features in the JAK2 V617F line 2 mice were more simple and dramatic. These animals showed severe leukocytosis at only one month after birth, and leukocyte numbers elevated as the animals aged. Leukocytosis peaked 5 months after birth at a mean leukocyte number of 30 x 10^{10}/L, with some mice exhibiting values close to 50 x 10^{10}/L. Leukocyte number subsequently decreased slightly, remaining high at 20 x 10^{10}/L at 8 months after birth. The majority of the increased number of leukocytes comprised mature neutrophils with some immature cells (Figure 2B). Marked thrombocytosis was also observed in line 2 mice at one month, with a mean platelet number of 250 x 10^{10}/L and some mice

exhibiting values close to 500 x 10^{10}/L. Platelet count slowly declined as time passed, and returned to near-normal values 8 months after birth. Platelets from these mice aggregated easily, and giant platelets were observed (Figure 2B and C). These mice also exhibited anemia, with Hb levels of 10 g/dL at one month. This declined as time passed, reaching 9 g/dL at 7 months old. The drop in Hb levels corresponded to decreased volume and number of RBCs. The mean Hct at one month after birth was 42 ± 4.5 %, and it decreased to 32 ± 6.6 % at 6 months old. The number of RBCs was decreased at 1 month after birth, and peaked at 605 x 10^{10}/L 9 months after birth. The Hct value and RBC number in wild-type mice at 1 month were 50 ± 2.4 % and 945 ± 45.5, respectively. Polychromatophilia and RBCs with Howell-Jolly bodies were observed in line 2 mice. In addition, erythroblasts and myeloblasts were observed in the peripheral blood (Figure 2C).

Pathological characterization of bone marrow (Figure 3A).

Histopathological examination of femurs from JAK2 V617F line 1 mice showed hypercellular bone marrow with maturing myeloid cells, erythrocytes in many stage of differentiation, and mature megakaryocytes. The number of megakaryocytes/10 high power fields was 217 ± 76 in line 1 mice, and it was statistically larger than that in wild-type mice (82 ± 12) ($p<0.01$). Emperipolesis of neutrorphils in megakaryocyte cytoplasm was observed (63 ± 36 /10 high power fields), although that was scarcely observed in wild-type mice. Reticulin fibrosis was not observed by Gomori silver staining, nor increment of bone trabeculae (Figure 3A (M-O)).

Femurs from JAK2 V617F line 2 mice showed prominent populations of maturing myeloid cells with moderately increased numbers of megakaryocytes. The number of megakaryocytes/10 high power fields was 231 ± 20 in line 2 mice, and it was larger than that in wild-type mice (82 ± 12) ($p<0.01$). Megakaryocytes of many sizes were usually clustered and displayed unusual morphology, including aberrant nuclear to cytoplasmic ratio, emperipolesis of neutrophils in megakaryocyte cytoplasm (80 ± 27/10 high power fields), and bulbous or irregularly folded nuclei. Gomori silver staining showed a mild accumulation of fibers in the intercellular space of the marrow from 6-month-old mice (Figure 3A (Q)). Bone cortical thickness was also mildly increased, with newly formed bony trabeculae. This phenomenon became more obvious as the mice aged. Fibrosis of the bone marrow and increment of bone trabeculae and intercellular matrix were observed in 9-month-old mice (Figure 3A (R)). Maturation along the granulocyte and erythroid lineages was normal.

The number of nucleated cells collected from femurs of both lines of JAK2 V617F mice was lower than the number collected from wild-type mice (Figure 4A), although the bone marrow specimens from both lines of JAK2 V617F mice looked hypercellular (Figure 3A). The percentage of neutrophil precursor cells was greater, and that of erythroid precursor cells was les ser in both lines of JAK2 V617F mice than in wild-type mice. Consistent with these findings, flow cytometric analysis of both JAK2 V617F line 1 and 2 mice showed increased numbers of CD41 positive cells and Gr-1/Mac1 double positive cells (Figure 5). The population of CD71/TER119 double positive cells was unchanged in line 1 mice, but was decreased in line 2 mice. The percentage of B220 positive B cells decreased dramatically in both lines.

JAK2 V617F induces splenomegaly.

At 3-5 months, JAK2 V617F mice showed marked splenomegaly and had higher average spleen weights (1.01 ± 0.34 g in line 1 and 1.57 ± 0.31 g in line 2 mice) than wild-type mice (0.11± 0.05g) (Figure 4B). In line 1, not all mice had splenomegaly, and mice having splenomegaly showed at least one of erythrocytosis, thrombocytosis, or leukocytosis. The architecture of spleens from JAK2 V617F line 1 mice was barely preserved, and white pulp was obviously decreased (Figure 3B (A-C)). Red pulp was expanded due to invasion by nonlymphoid cells, megakaryocytes, erythroblasts, and maturing myeloid cells (Figure 3B (G-I)). Megakaryocytes were large in size and had multilobulated nuclei. Spleen sections from JAK2 V617F line 2 mice exhibited complete effacement of normal splenic architecture and the invasion of nonlymphoid cells. The white pulp was blended throughout and was partially preserved (Figure 3B (D-F)). The red pulp was expanded by an atypical population of maturing myeloid cells and megakaryocytes (Figure 3B (J-L)). Few erythroblasts were observed. In JAK2 V617F line 2 mice, Gomori silver staining showed a mild accumulation of fibers of the spleen from 6-month old mice (Figure 3B (Q)), and fibrosis was observed in spleen from 9-month old mice (Figure 3B (R)). The spleen weight was 1.57 ± 0.31, 1.61 ± 0.45, and 1.42 ± 0.29 g in 3-5 months old, 6-9 months old, and 10- month old JAK2 V617F line 2 mice, respectively. There is no statistical difference of spleen weight in three age groups from JAK2 V617F line 2 mice, although fibrosis became obvious as the mice aged (Figure 3B (P-R)) .

Differential cell counts showed increased numbers of mature myeloid cells accompanied by megakaryocytes in both lines (Figure 4B). The percentage of erythroblasts increased in line 1 mice and decreased in line 2 mice than in wild-type mice. FACS analysis showed an increase of Gr-1/Mac double positive cells (16.6% in line 1 and 41.6% in line 2, versus 2.4% in wild-type mice) and CD41 positive cells (18.3% in line 1 and 6.0% in line 2, versus 0.6% in wild-type mice) in JAK2 V617F mice compared with wild type mice (Figure 5). B220 positive B cells represented 64.5% in wild-type mice, and decreased to 47.5% in line 1 and to 22.2% in line 2. CD71/TER119 double positive erythroid cells increased to 8.1% in line 1 mice, and decreased to 1.0% in line 2 mice compared to 2.7% in wild-type mice.

JAK2 V617F induces expansion of hematopoietic progenitor cells.

The frequencies of progenitor cells in bone marrow among wild-type, JAK2 V617F line 1, and line 2 mice were approximately equivalent, although they had some margin of error (Figure 6A). Breakdown of colony type was comparable among the three groups; CFU-GM represented more than half of colonies. Endogenous erythroid colony formation *in vitro* is a major hallmark of chronic myeloproliferative diseases. Bone marrow cells were plated in methylcellulose in the absence of cytokines or with 3 U/mL of EPO (Figure 6B). Spontaneous formation of bone marrow-derived colonies (CFU-Es) was detected in both JAK2 V617F lines in the absence of cytokines. The addition of EPO had little or a little influence on the number of CFU-E in bone marrow cells from both lines, although CFU-Es appeared in bone marrow cells from wild type mice following EPO treatment.

Expression levels of the V617F mutant in line1 mice

As some displayed thrombocytosis and/or erythrocytosis, and the others did not show the increment of Hb value and platelet count (MPD-free mice) in line 1 mice, we compared the T/G ratio by sequencing of PCR products of JAK2 cDNA from line 1 mice. As T peak identifies the mutation and G peak identifies wild-type, T/G ratio represents the JAK2 mutation / wild-type ratio. The T/G ratio in mice with erythrocytosis was higher than that in mice with thrombocytosis ($p < .05$) or MPD-free mice ($p < .01$), although there was no statistically difference in T/G ratio between MPD-free mice and mice with thrombocytosis (Figure 7). In addition, the Hb value moderately correlated with the T/G ratio in line 1 mice ($r = 0.459$, $p < .01$).

Discussion

The pathogeneses of MPDs, with the exception of CML, have not been clarified. JAK2 V617F has recently been identified by several groups as an acquired somatic mutation present in the majority of patients with PV and large proportions of patients with ET and PMF(8-12). JAK2 V617F has constitutive activity in cell lines, and can transform IL-3-dependent cells, such as Ba/F3, to cytokine independence(9, 11, 13). This cytokine-independent growth may depend on the proper expression of cytokine receptors, such as IL-3 receptor, Epo receptor, G-CSF receptor, or thrombopoietin receptor(30). Although JAK2 mutations have often been observed in MPD patients, it remains unknown whether the JAK2 V617F mutation is sufficient to cause MPD. If this is the case, it is also unclear how a single active mutant allele might cause 3 clinically distinct, though related, myeloproliferative diseases. In murine transplantation experiments, retroviral expression of JAK2 V617F in BM cells caused key features of PV, including erythrocytosis, low serum erythropoietin levels, splenomegaly, and cytokine-independent colony formation *in vitro*(20-23). Transplanted mice also showed striking leukocytosis and neutrophilia. In contrast to polycythemia and leukocytosis, JAK2 V617F had either no effect(20, 23) or a small and transient effect(21, 22) on platelet counts, despite the detection of proviral gene expression in megakaryocytes(23). At later stages following transplantation, mice developed fibrosis in marrow and spleen, associated with anemia, mimicking the "spent" phase of human PV. These data indicate that JAK2 V617F is able to confer a disease phenotype in mice with clinicopathologic features reminiscent of PV. However, features of ET or primary myelofibrosis did not develop following retroviral expression of JAK2 V617F in murine bone marrow transplantation models, despite the observation that half of ET and primary myelofibrosis patients have this JAK2 mutation. We thus generated transgenic mice in which JAK2 V617F was stably expressed under the H-2kb promoter, which has previously been used to drive expression of a bcl2 transgene in stem cells(24).

Two lines of JAK2 V617F transgenic mice were established. The protein level of JAK2 V617F was not so much (Figure 1C). This small amount of JAK2 V617F protein, however, was enough to constitutively phosphorylate STAT5, which is the direct substrate of JAK2, in the absence of cytokine stimulation in cells from JAK2 V617F line 1 and line 2 mice (Figure 1D), and induced the proliferation of hematopoietic cells, especially megakaryocytes. About half of JAK2 V617F line 1 mice developed

features of human PV or ET. The remaining half did not show erythrocytosis or thrombocytosis, although some showed leukocytosis (Figure 2A). The bone marrow from JAK2 V617F line 1 mice was hypercelluar with increased numbers of large megakaryocytes (Figure 4A). JAK2 V617F line 2 mice showed the complete spectrum of clinicopathological features of human primary myelofibrosis, including clustered megakaryocytes of many sizes and unusual morphology, an accumulation of fibers in the bone marrow, and bone cortical thickness with newly formed bony trabeculae in the intercellular space of the marrow (Figure 3A). They also showed extreme anemia (Figure 2A), leukoerythroblastosis in the peripheral blood (Figure 2C), and splenomegaly (Figure 4B). Both lines presented cytokine-independent colony formation *in vitro*, a characteristic of MPDs (Figure 6B). The phenotypes of JAK2 V617F line 1 and line 2 were not the same, and were not consistent with the features in mice transplanted with BM cells transduced with JAK2 V617F(20-23). Importantly, the features of PV, ET, and primary myelofibrosis have now been experimentally reproduced by the enforced expression of JAK2 V617F in mice. Leukocytotis in the peripheral blood, myeloid and megakaryocyte hyperplasia in the bone marrow, and splenomegaly were commonly observed to varying degrees in JAK2 V617F line 1 mice, line 2 mice, and recipient mice in transplantation models. Cytokine-independent colony formation, a characteristic of MPDs, was also observed in bone marrow cells. Although these mice share a number of common phenotypes, there are also a number of differences among JAK2 V617F line 1 mice, line 2 mice, and recipient mice in transplantation models.

By 9 months of age, about 20% of line 1 mice developed PV-like features and about 30% developed ET-like features. The occurrence of ET plateaued by 5 months, whereas the occurrence of PV increased gradually over time and had not plateaued by 9 months. It is possible that the remaining half of MPD-free line 1 mice would have developed PV over a longer follow-up. Recipient mice transplanted with bone marrow cells infected with a JAK2 V617F retroviral vector all developed PV shortly after transplantation(20-23). The precise cause of the phenotypic and penetrance differences between JAK2 V617F line 1 mice and recipient mice in this transplantation model is unknown. One hypothesis is that another acquired genetic event is necessary for the development of PV or ET in addition to JAK2 V617F mutation. The observation that some line 1 mice developed PV, some developed ET, and others did not develop MPD may support this hypothesis. The requirement of another genetic event in addition to JAK2 V617F is consistent with the clinical observation that ET patients had few cells with the JAK2 V617F mutation even when they showed monoclonal hematopoiesis (31). However, this contrasts with observations that most PV patients with monoclonal hematopoiesis had a majority of cells with the JAK2 V617F mutation(31), as well as observations that almost all recipient mice developed PV shortly after transplantation with JAK2 V617F expressing bone marrow cells(20-23).

A second possible explanation is that the expression level of JAK2 V617F is important for the proliferation and differentiation of cell lineages. The expression level of JAK2 V617F mRNA in bone marrow cells from JAK2 V617F line 1 mice was 0.45-fold that of wild-type endogenous JAK2 mRNA. Meanwhile, expression of JAK2 V617F was reported to be 10 to 40-times that of endogenous wild-type JAK2 in transplant models (21). This high expression of JAK2 V617F in reported transplant models may influence hematopoiesis and lead to the development of PV. We then assessed the expression level of

JAK2 V617F among line 1 mice. As T wave derives from JAK2 V617F mRNA, T/G ratio would roughly represent the expression level of JAK2 V617F in each mouse. The T/G ratio in PV-like mice was higher than that in ET-like mice ($p < .05$) or MPD-free mice ($p < .01$) (Figure 7), and the Hb value moderately correlated with the T/G ratio in line 1 mice ($r = 0.459$, $p < .01$). Higher expression of JAK2 V617F would favor erythrocytosis, and lower one would favor thrombocytosis. This is consistent with one report that transient and very mild thrombosis was observed only in a few JAK2 V617F low-expresser mice in a transplantation model(21), although other reports indicated no effect of JAK2 V617F on platelet number(20, 22, 23). This is also supported by the clinical observations that most patients with ET are heterozygous for the JAK2 V617F mutation, while approximately 30% of patients with PV are homozygous(8-12). Homozygosity increases the expression of JAK2 V617F, and also increases the effect of JAK2 V617F because competition with wild-type JAK2 is lost (9). Homozygous line 1 mice carrying two JAK2 V617F alleles would be expected to have higher JAK2 mRNA expression. We are now crossing heterozygous JAK2 V617F line 1 mice to obtain homozygous JAK2 V617F mice. If these mice develop erythrocytosis more frequently than line 1 heterozygous mice, it would provide support to the dose effect hypothesis.

The features of JAK2 V617F line 2 mice differ significantly from those of both recipient mice transplanted with bone marrow cells infected with JAK2 V617F retroviral vector, and JAK2 V617F line 1 mice. Line 2 mice showed severe leukocytosis, thrombocytosis, and anemia from an early age (Figure 2A). Marked megakaryocyte hyperplasia in the bone marrow with dysplasia was observed (Figure 3A). Interestingly, the expression levels of JAK2 V617F mRNA in line 2 mice were about three times more than in line 1 mice. ERK1/2, STAT5, and AKT were constitutively phophorylated in cells from line 2 mice, and the extent of phosphorylation after cytokine stimulation was greater than that in wild-type mice (Figure 1D). However, it seems unlikely that a small increment of JAK2 V617F expression would promote anemia in line 2 mice, as recipient mice transplanted with bone marrow cells retrovirally transduced with JAK2 V617F showed erythrocytosis, not anemia(20-23). Whether homozygous line 1 mice, which should express higher levels of JAK2 V617F, will be anemic may shed some light on this question. A second genetic mutation in addition to JAK2 V617F might explain the phenotype of line 2 mice. Increased expression of high-mobility group protein A2 (HMGA2)(32) and FK506 binding protein 51 (FKBP51)(33), and decreased expression of retinoic acid receptor gamma 2 (RAR gamma 2)(34) mRNAs have been reported in PMF patients. It is possible that the integration sites of the transgene affected the expression of these genes, and the phenotype of JAK2 V617F line 2 mice. This issue should be resolved by generating and analyzing several additional lines of JAK2 V617F transgenic mice.

Fibrosis of the bone marrow and thickening of trabecular bone (osteosclerosis), which are characteristic of primary myelofibrosis, are thought to be due to elevated cytokines, such as TGF-beta 1(35, 36) and osteoprotegerin (OPG)(37), released from increased numbers of megakaryocytes or stromal cells. Such cytokines might directly stimulate the fibrogenic and osteogenic responses(38). We have previously shown that transgenic mice expressing TPO developed myelofibrois, and their serum TGF-beta 1 and OPG levels were high(25). It is therefore tempting to speculate that the increased numbers of megakaryocytes in line 2 mice are responsible for the production of cytokines that induce myelofibrosis and osteosclerosis. In addition, dysfunction of megakaryocytes can be responsible for TGF-beta 1 release; as

dysmegakaryopoiesis was observed in JAK2 V617F transgenic mice, both mechanisms might be involved.

In conclusion, we have shown that exogenous expression of JAK2 V617F *in vivo* induces disease with features similar to PV, ET, and PMF. In a transplantation model using a retroviral vector, JAK2 V617F was expressed much more highly than endogenous JAK2(21), and recipient mice developed only PV-like features(20-23). This may suggest that levels of JAK2 V617F expression directly determine which cell lineages increase, and this may lead to the diversity of MPD. This murine MPD model also provides a robust and reproducible *in vivo* assay system to assess the therapeutic efficacy of JAK2-selective inhibitors for JAK2 V617F-positive diseases, especially myelofibrosis, for which no specific treatment exists.

References

1. Witthuhn BA, Quelle FW, Silvennoinen O, Yi T, Tang B, Miura O, Ihle JN. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell. 1993 Jul 30;74(2):227-236.

2. Parganas E, Wang D, Stravopodis D, Topham DJ, Marine JC, Teglund S, Vanin EF, Bodner S, Colamonici OR, van Deursen JM, Grosveld G, Ihle JN. Jak2 is essential for signaling through a variety of cytokine receptors. Cell. 1998 May 1;93(3):385-395.

3. Ihle JN. Cytokine receptor signalling. Nature. 1995 Oct 19;377(6550):591-594.

4. Ihle JN. STATs: signal transducers and activators of transcription. Cell. 1996 Feb 9;84(3):331-334.

5. Ihle JN, Nosaka T, Thierfelder W, Quelle FW, Shimoda K. Jaks and Stats in cytokine signaling. Stem Cells. 1997;15 Suppl 1:105-111; discussion 112.

6. Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell. 1995 Oct 6;83(1):59-67.

7. Cui Y, Riedlinger G, Miyoshi K, Tang W, Li C, Deng CX, Robinson GW, Hennighausen L. Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation. Mol Cell Biol. 2004 Sep;24(18):8037-8047.

8. Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S, Vassiliou GS, Bench AJ, Boyd EM, Curtin N, Scott MA, Erber WN, Green AR. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet. 2005 Mar 16;365(9464):1054-1061.

9. James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, Garcon L, Raslova H, Berger R, Bennaceur-Griscelli A, Villeval JL, Constantinescu SN, Casadevall N, Vainchenker W. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature. 2005 Apr 28;434(7037):1144-1148.

10. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, Tichelli A, Cazzola M, Skoda RC. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med. 2005 Apr 28;352(17):1779-1790.

11. Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, Boggon TJ, Wlodarska I, Clark JJ, Moore S, Adelsperger J, Koo S, Lee JC, Gabriel S, Mercher T, D'Andrea A, Frohling S, Dohner K, Marynen P, Vandenberghe P, Mesa RA, Tefferi A, Griffin JD, Eck MJ, Sellers WR, Meyerson M, Golub TR, Lee SJ, Gilliland DG. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell. 2005 Apr;7(4):387-397.

12. Jones AV, Kreil S, Zoi K, Waghorn K, Curtis C, Zhang L, Score J, Seear R, Chase AJ, Grand FH, White H, Zoi C, Loukopoulos D, Terpos E, Vervessou EC, Schultheis B, Emig M, Ernst T, Lengfelder E, Hehlmann R, Hochhaus A, Oscier D, Silver RT, Reiter A, Cross NC. Widespread occurrence of the JAK2 V617F mutation in chronic myeloproliferative disorders. Blood. 2005 Sep 15;106(6):2162-2168.

13. Shide K, Shimoda K, Kamezaki K, Kakumitsu H, Kumano T, Numata A, Ishikawa F, Takenaka K, Yamamoto K, Matsuda T, Harada M. Tyk2 mutation homologous to V617F Jak2 is not found in essential thrombocythaemia, although it induces constitutive signaling and growth factor independence. Leuk Res. 2007 Aug;31(8):1085-1092.

14. Dameshek W. Some speculations on the myeloproliferative syndromes. Blood. 1951 Apr;6(4):372-375.

15. Reeder TL, Bailey RJ, Dewald GW, Tefferi A. Both B and T lymphocytes may be clonally involved in myelofibrosis with myeloid metaplasia. Blood. 2003 Mar 1;101(5):1981-1983.

16. Prchal JT, Guan YL, Prchal JF, Barany F. Transcriptional analysis of the active X-chromosome in normal and clonal hematopoiesis. Blood. 1993 Jan 1;81(1):269-271.

17. Prchal JF, Axelrad AA. Letter: Bone-marrow responses in polycythemia vera. N Engl J Med. 1974 Jun 13;290(24):1382.

18. Reid CD. The significance of endogenous erythroid colonies (EEC) in haematological disorders. Blood reviews. 1987 Jun;1(2):133-140.

19. Tefferi A, Thiele J, Orazi A, Kvasnicka HM, Barbui T, Hanson CA, Barosi G, Verstovsek S, Birgegard G, Mesa R, Reilly JT, Gisslinger H, Vannucchi AM, Cervantes F, Finazzi G, Hoffman R, Gilliland DG, Bloomfield CD, Vardiman JW. Proposals and rationale for revision of the World Health Organization diagnostic criteria for polycythemia vera, essential thrombocythemia, and primary myelofibrosis: recommendations from an ad hoc international expert panel. Blood. 2007 Aug 15;110(4):1092-1097.

20. Wernig G, Mercher T, Okabe R, Levine RL, Lee BH, Gilliland DG. Expression of Jak2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. Blood. 2006 Jun 1;107(11):4274-4281.

21. Lacout C, Pisani DF, Tulliez M, Gachelin FM, Vainchenker W, Villeval JL. JAK2V617F expression in murine hematopoietic cells leads to MPD mimicking human PV with secondary myelofibrosis. Blood. 2006 Sep 1;108(5):1652-1660.

22. Bumm TG, Elsea C, Corbin AS, Loriaux M, Sherbenou D, Wood L, Deininger J, Silver RT, Druker BJ, Deininger MW. Characterization of murine JAK2V617F-positive myeloproliferative disease. Cancer research. 2006 Dec 1;66(23):11156-11165.

23. Zaleskas VM, Krause DS, Lazarides K, Patel N, Hu Y, Li S, Van Etten RA. Molecular Pathogenesis and Therapy of Polycythemia Induced in Mice by JAK2 V617F. PLoS ONE. 2006;1:e18.

24. Domen J, Gandy KL, Weissman IL. Systemic overexpression of BCL-2 in the hematopoietic system protects transgenic mice from the consequences of lethal irradiation. Blood. 1998 Apr 1;91(7):2272-2282.

25. Kakumitsu H, Kamezaki K, Shimoda K, Karube K, Haro T, Numata A, Shide K, Matsuda T, Oshima K, Harada M. Transgenic mice overexpressing murine thrombopoietin develop myelofibrosis and osteosclerosis. Leuk Res. 2005 Jul;29(7):761-769.

26. Riley J, Butler R, Ogilvie D, Finniear R, Jenner D, Powell S, Anand R, Smith JC, Markham AF. A novel, rapid method for the isolation of terminal sequences from yeast artificial chromosome (YAC) clones. Nucleic acids research. 1990 May 25;18(10):2887-2890.

27. Schessl C, Rawat VP, Cusan M, Deshpande A, Kohl TM, Rosten PM, Spiekermann K, Humphries RK, Schnittger S, Kern W, Hiddemann W, Quintanilla-Martinez L, Bohlander SK, Feuring-Buske M, Buske C. The AML1-ETO fusion gene and the FLT3 length mutation collaborate in inducing acute leukemia in mice. J Clin Invest. 2005 Aug;115(8):2159-2168.

28. Noguchi A, Takekawa N, Einarsdottir T, Koura M, Noguchi Y, Takano K, Yamamoto Y, Matsuda J, Suzuki O. Chromosomal mapping and zygosity check of transgenes based on flanking genome sequences determined by genomic walking. Experimental animals / Japanese Association for Laboratory Animal Science. 2004 Apr;53(2):103-111.

29. Shimoda K, Feng J, Murakami H, Nagata S, Watling D, Rogers NC, Stark GR, Kerr IM, Ihle JN. Jak1 plays an essential role for receptor phosphorylation and Stat activation in response to granulocyte colony-stimulating factor. Blood. 1997 Jul 15;90(2):597-604.

30. Lu X, Levine R, Tong W, Wernig G, Pikman Y, Zarnegar S, Gilliland DG, Lodish H. Expression of a homodimeric type I cytokine receptor is required for JAK2V617F-mediated transformation. Proc Natl Acad Sci U S A. 2005 Dec 27;102(52):18962-18967.

31. Kralovics R, Teo SS, Li S, Theocharides A, Buser AS, Tichelli A, Skoda RC. Acquisition of the V617F mutation of JAK2 is a late genetic event in a subset of patients with myeloproliferative disorders. Blood. 2006 Aug 15;108(4):1377-1380.

32. Andrieux J, Demory JL, Dupriez B, Quief S, Plantier I, Roumier C, Bauters F, Lai JL, Kerckaert JP. Dysregulation and overexpression of HMGA2 in myelofibrosis with myeloid metaplasia. Genes Chromosomes Cancer. 2004 Jan;39(1):82-87.

33. Komura E, Chagraoui H, Mansat de Mas V, Blanchet B, de Sepulveda P, Larbret F, Larghero J, Tulliez M, Debili N, Vainchenker W, Giraudier S. Spontaneous STAT5 activation induces growth factor independence in idiopathic myelofibrosis: possible relationship with FKBP51 overexpression. Exp Hematol. 2003 Jul;31(7):622-630.

34. Jones LC, Tefferi A, Idos GE, Kumagai T, Hofmann WK, Koeffler HP. RARbeta2 is a candidate tumor suppressor gene in myelofibrosis with myeloid metaplasia. Oncogene. 2004 Oct 14;23(47):7846-7853.

35. Chagraoui H, Komura E, Tulliez M, Giraudier S, Vainchenker W, Wendling F. Prominent role of TGF-beta 1 in thrombopoietin-induced myelofibrosis in mice. Blood. 2002 Nov 15;100(10):3495-3503.

36. Roberts AB, Sporn MB. Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta). Growth Factors. 1993;8(1):1-9.

37. Chagraoui H, Tulliez M, Smayra T, Komura E, Giraudier S, Yun T, Lassau N, Vainchenker W, Wendling F. Stimulation of osteoprotegerin production is responsible for osteosclerosis in mice overexpressing TPO. Blood. 2003 Apr 15;101(8):2983-2989.

38. Yan XQ, Lacey D, Fletcher F, Hartley C, McElroy P, Sun Y, Xia M, Mu S, Saris C, Hill D, et al. Chronic exposure to retroviral vector encoded MGDF (mpl-ligand) induces lineage-specific growth and differentiation of megakaryocytes in mice. Blood. 1995 Dec 1;86(11):4025-4033.

Titles and legends to figures

Figure 1. **The generation of JAK2 V617F transgenic mice.**

(A) The transgene integration sites were analyzed using bubble linker-mediated PCR. In line 1, the transgene integrated in intron 12 of *Dcc* (*Deleted in colorectal carcinoma*) on chromosome 18qE2. In line 2, the transgene integrated between *Mef 2a* (*myocyte enhancer factor 2A*) and *Lrrc 28* (*leucine rich repeat containing 28*) on chromosome 7qD1.

(B) PCR analysis to detect mice carrying the transgene. P1 mouse has transgene, and it mates with wild-type P2 mouse. Tail DNA was prepared from parent mice and their offspring. PCR was performed using both internal primers (primer sets 1 and 2) and external primers (primer sets 3, 4, and 5 for line 1, and 3, 6, and 7 for line 2) as indicated in (A). W; water. M; marker.

(C) JAK2 protein level. Bone marrow cells from wild-type, line 1, and line 2 mice were immunoprecipitated with anti-JAK2 antibody, and analyzed by Western blot with JAK2 antibody.

(D) Phosphorylation of ERK1/2, STAT5, and AKT in bone marrow cells. Bone marrow cells from wild-type, line 1, and line 2 mice were stimulated with or without cytokine (EPO + WEHI conditioning medium) for 30 min. Total cell lysates were analyzed by Western blot analysis with the indicated antibodies.

Figure 2. **Peripheral blood in JAK2 V617F mice.**

(A) Peripheral blood counts from line 1 and line 2 mice. Horizontal axis indicates the age (month). Black dots represent the value from wild-type mice, and red dots from JAK2 V617F mice. ** $p<.01$ and * $p<.05$ compared with wild-type mice.

(B) Smears of peripheral blood from wild-type, line 1, and line 2 mice. Granulocytosis and platelet aggregation were obvious in line 1 and 2 mice.

(C) Smear of peripheral blood of line 2 mice. Erythroblasts (black arrow), myeloblasts (arrow head), and giant platelets (white arrow) appeared in the peripheral blood. Polychromatophilia and RBCs with Howell-Jolly bodies (white arrow head) were also observed.

Figure 3. **Histopathological characterization of JAK2 V617F mice.**

(A) Bone marrow changes over time in line 1 and 2 mice. Hematoxilin and eosin stains (A-L) and Gomori silver staining (M-R). In femurs from line 1 mice, megakaryocytes were greater in number, and emperipolesis of neutrophils in megakaryocyte cytoplasm (white arrow) was observed (H). In femurs from line 2, megakaryocytes of many sizes, with bizarre nucleus (arrowhead) were usually clustered (L). An accumulation of fibers in the intercellular space of the marrow and bone cortical thickness was present in JAK2 V617F line 2 mice (Q, R), whereas they were absent in JAK2 V617F line 1 mice (O).

(B) Spleen changes over time in line 1 and 2 mice. Hematoxilin and eosin stains (A-L) and Gomori silver staining (M-R). The architecture of spleen from line 1 mice was profoundly affected, with decreaed

white pulp (arrow) and red pulp infiltrated by nonlymphoid cells (C, H, I). In line 2 mice, red pulp was expanded by an atypical population of maturing myeloid cells and megakaryocytes (J, K, L), and the white pulp was scarcely observed (E, F). Silver staining showed mild to moderate fibrosis in spleens from JAK2 V617F mice (O, R).

Figure 4. **Bone marrow and spleen analysis.**

(A) Bone marrow cellularity and differential cell counts. (upper) Mice were arbitrarily divided into the same age groups 3- to 5- month-old mice (□) and 6- to 9- month-old ones (■). Bone marrow nucleated cells were collected from femurs from both lines of JAK2 V617F mice. The numbers of bone marrow mononuclear cells from line 1 and 2 mice were lower than in the number from wild-type mice. ** $p<.01$ and * $p<.05$ compared with wild-type mice. (lower) The percentage of neutrophil precursor cells increased, and the percentage of erythroid precursor cells decreased in JAK2 V617F line 1 and line 2 mice.

(B) Spleen weights and differential cell counts. (upper) Mice grouping was as same as (A). Average spleen weights in both lines of JAK2 V617F mice were higher than in wild-type animals. ** $p<.01$ compared with wild-type mice. (lower) Differential cell counts showed increased numbers of mature myeloid cells in JAK2 V617F line 1 and line 2 mice. The percentage of erythroblasts was higher in line 1 mice but lower in line 2 mice compared to wild-type mice.

Figure 5. **Immunophenotype of hematopoietic cells obtained from BM and spleen in JAK2 V617F transgenic mice.**

The plots show representative flow cytometric analysis of BM and spleen cells from line 1 and line 2 mice in comparison with cells from wild-type mice. JAK2 V617F both lines show an increase of CD41 positive cells and Gr-1/Mac1 double positive myeloid cells, and the decrease of B220 positive B cells in bone marrow and spleen. The population of CD71/TER119 double positive erythroid cells remained unchanged in line 1 mice, but it decreased in line 2 mice in bone marrow. In spleen, the population of CD71/TER119 double positive erythroid cells increased in line 1 mice, but decreased in line 2 mice.

Figure 6. **Frequency of progenitor cells and endogenous erythroid colony formation.**

(A) CFU assays using bone marrow cells from wild-type, line 1, and line 2 mice. 1×10^5 cells were plated in methylcellulose containing EPO, IL-3, IL-6, and stem cell factor. Three independent experiments were done in triplicate, and the mean value was presented.

(B) Endogenous erythroid colony formation *in vitro* and BFU-E. Bone marrow cells were plated in methylcellulose in the absence (■) or presence (□) of 3 U/mL EPO, and the colony number was counted on day 3. Two experiments were done in triplicate, and each bar represents one animal.

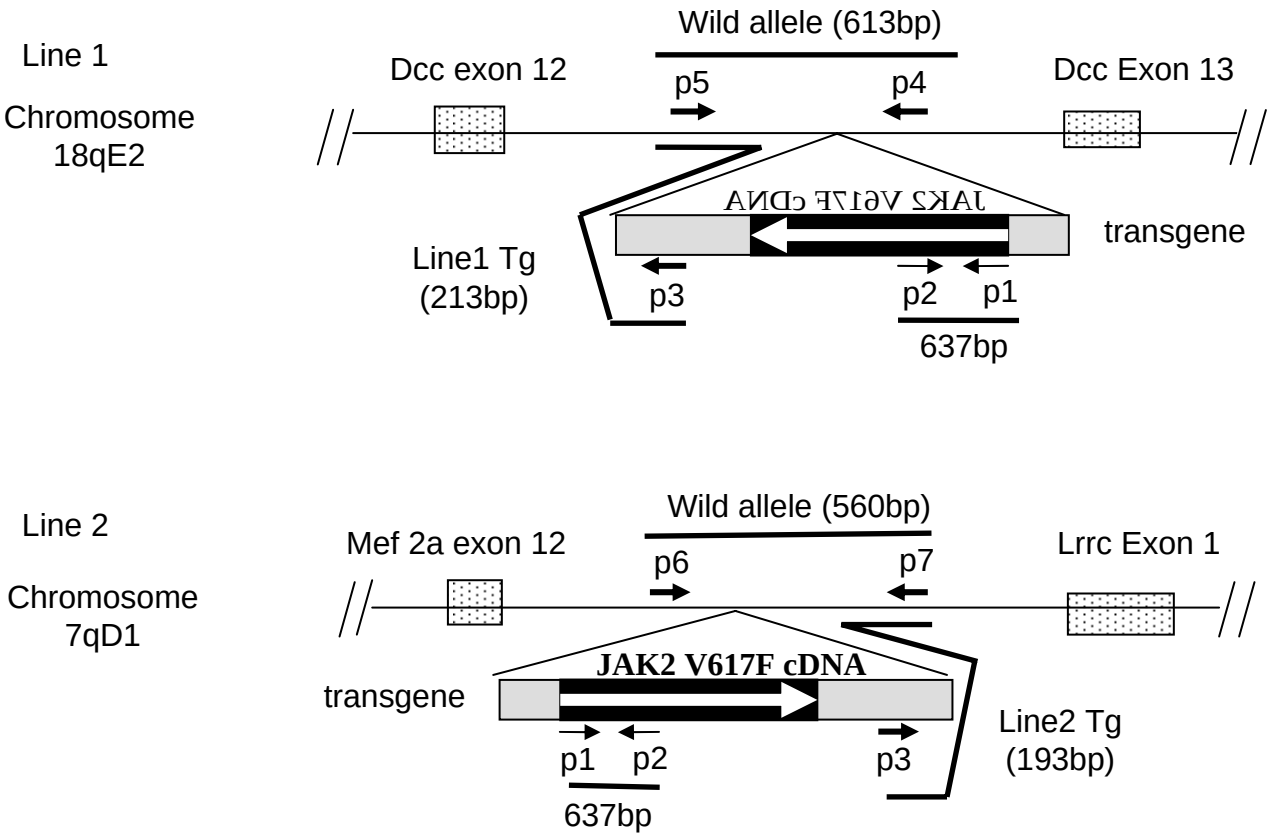
Figure 7. **The expression level of JAK2 V617F in line 1 mice**.

RT-PCR was performed on bone marrow cells from line 1 mice, and the resulting cDNAs were subjected to direct sequencing. Mutant JAK2 (JAK2 V617F) is identified by the presence of T wave, and wild-type by

G wave. T/G ratio in MPD-free, ET-like, and PV-like mice was calculated and plotted. Axis is T/G ratio.

Figure 1

(A)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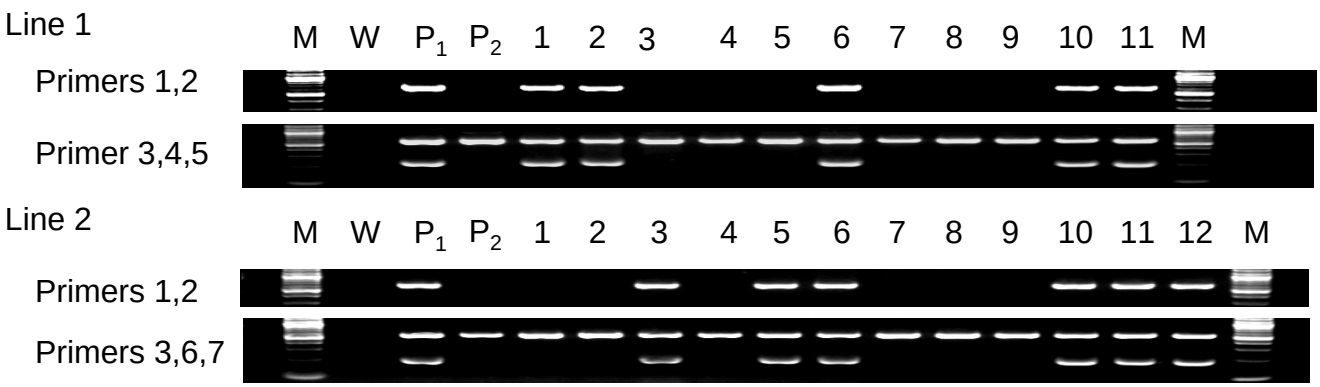
Chromosome 18qE2
Wild allele (613bp)
Dcc exon 12
p5
p4
Dcc Exon 13
JAK2 V617F cDNA
transgene
Line1 Tg (213bp)
p3
p2
p1
637bp
Line 2
Chromosome 7qD1
Wild allele (560bp)
Mef 2a exon 12
p6
p7
Lrrc Exon 1
JAK2 V617F cDNA
transgene
Line2 Tg (193bp)
p1
p2
p3
637bp

(B)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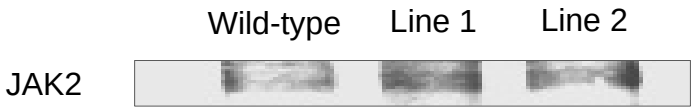
M W P1 P2 1 2 3 4 5 6 7 8 9 10 11 M
Primers 1,2
Primer 3,4,5
Line 2
M W P1 P2 1 2 3 4 5 6 7 8 9 10 11 12 M
Primers 1,2
Primers 3,6,7

Figure 1

(C)


Wild-type
Line 1
Line 2
JAK2

(D)

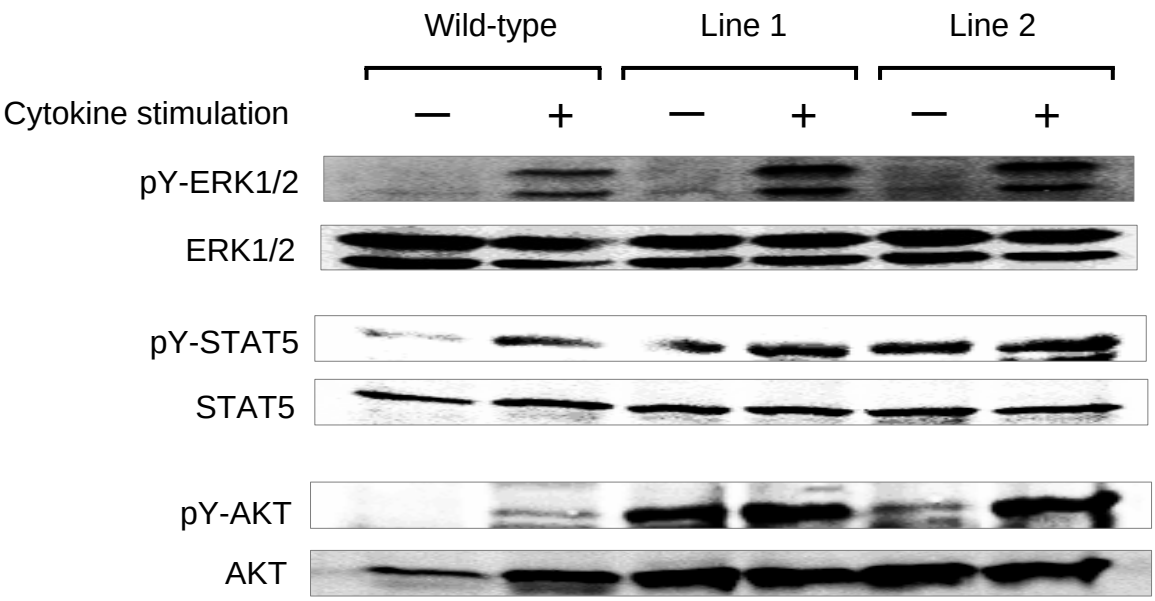
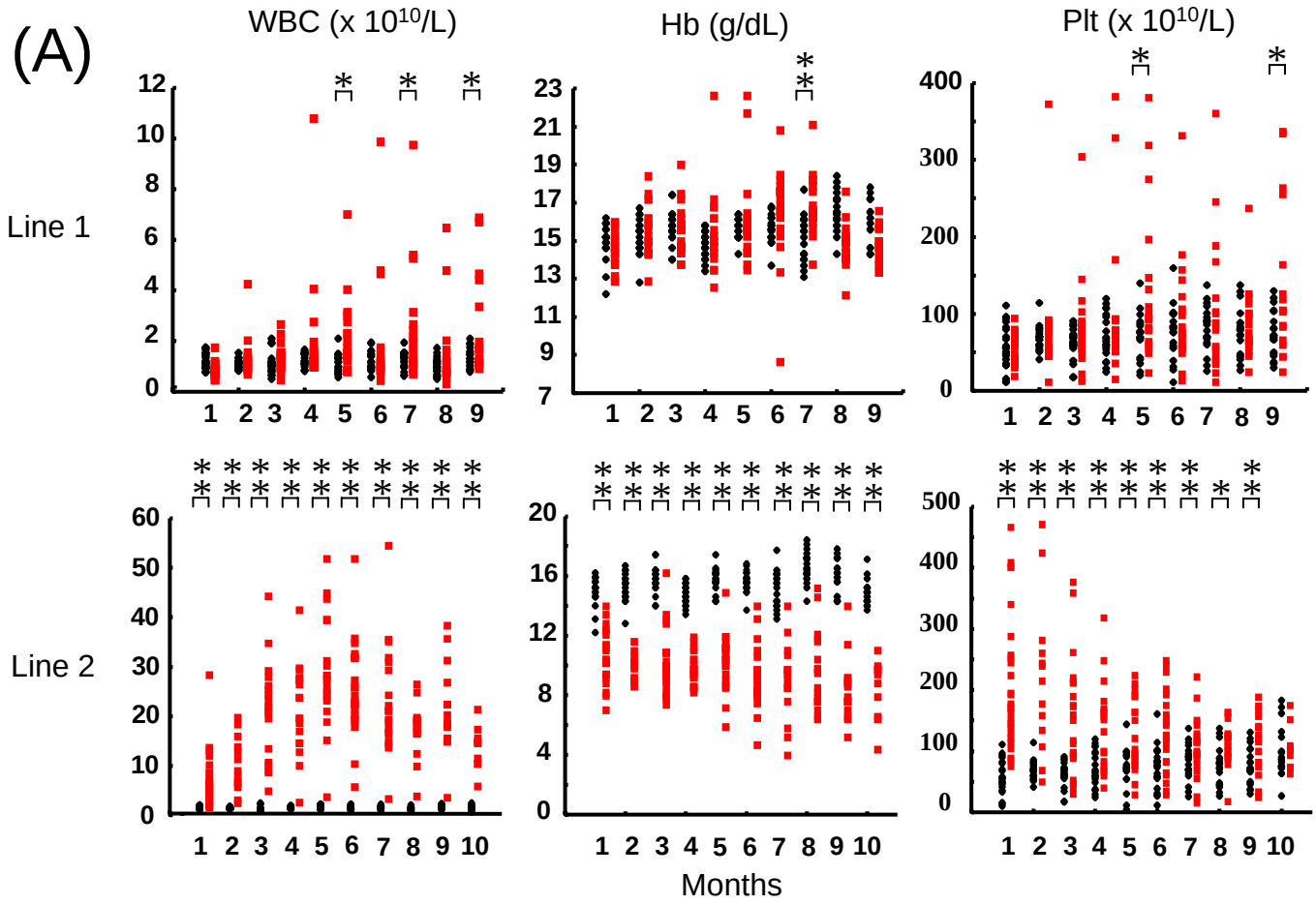
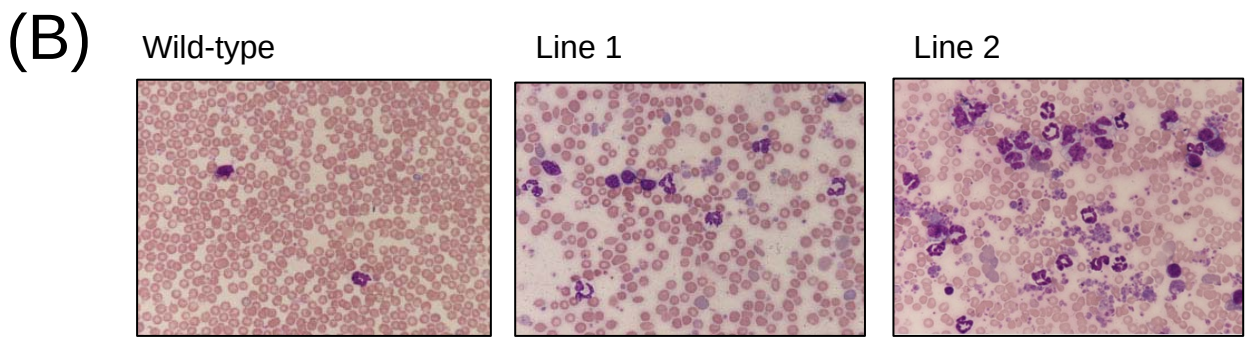

Wild-type
Line 1
Line 2
Cytokine stimulation
—
+
—
+
—
+
pY-ERK1/2
ERK1/2
pY-STAT5
STAT5
pY-AKT
AKT

Figure 2


(A)
WBC (x 10^10/L)
Hb (g/dL)
Plt (x 10^10/L)
Line 1
Line 2
Months


(B)
Wild-type
Line 1
Line 2

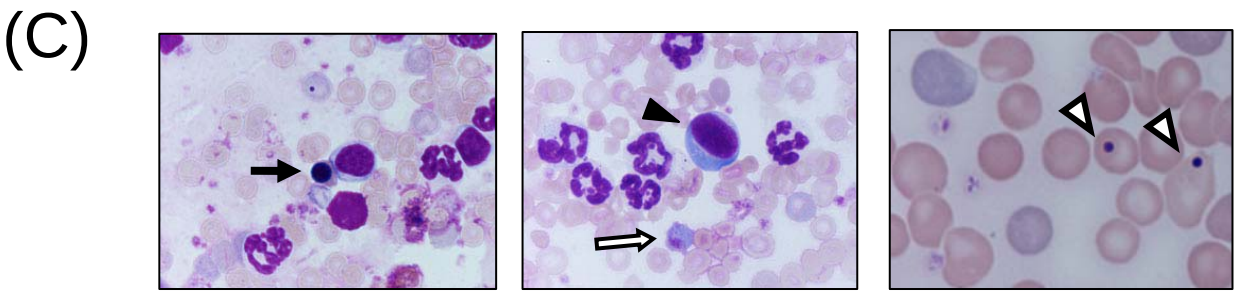
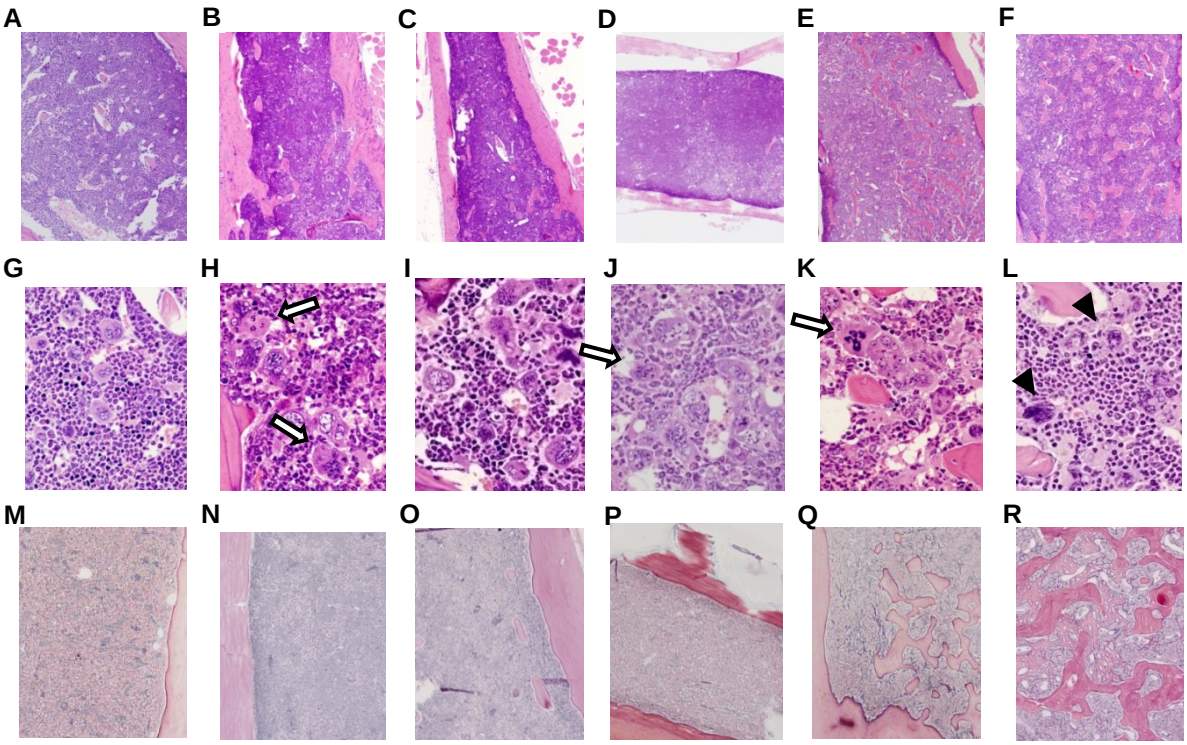

(C)

Figure 3

	Wild-type	Line 1		Line 2		
	6M	3M	10M	3M	6M	9M
WBC (x 10^9/L)	8.4	21	31.2	234.5	518	223
Hb (g/dL)	12.4	14	14	8.7	9.3	5.2
Plt (x 10^{10}/L)	66.4	347.2	169.8	390	208	143

(A)



(B)

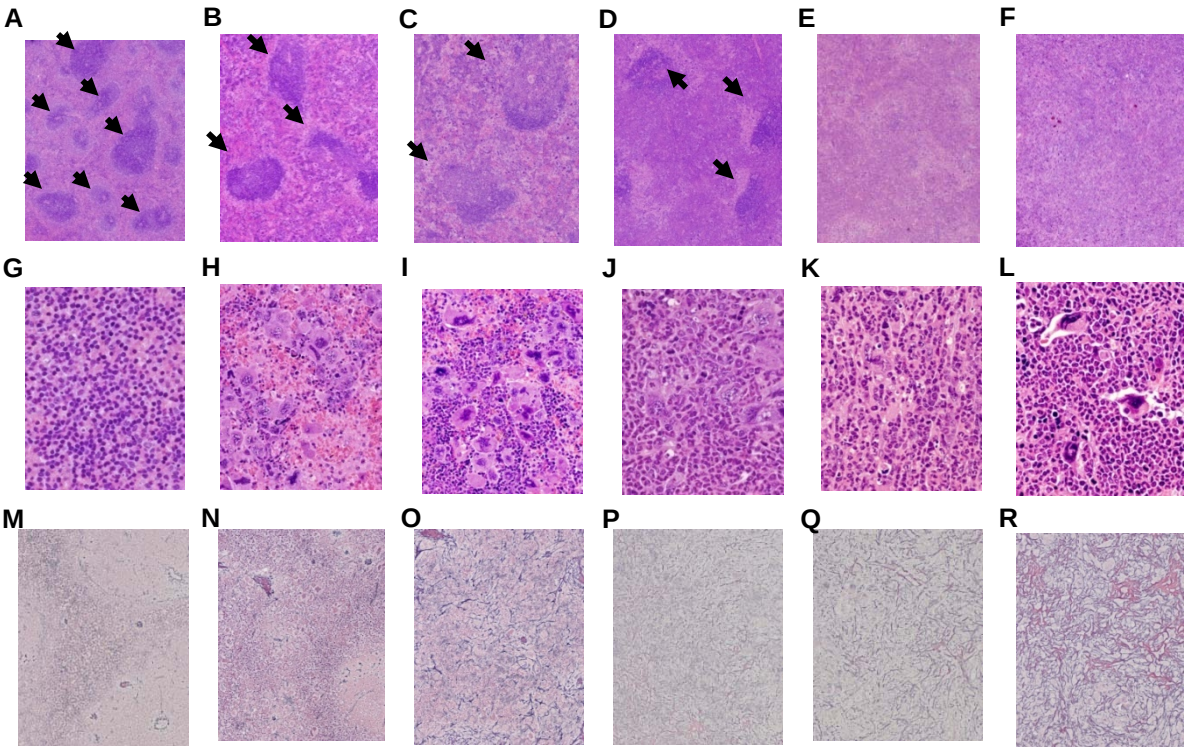


Figure 4

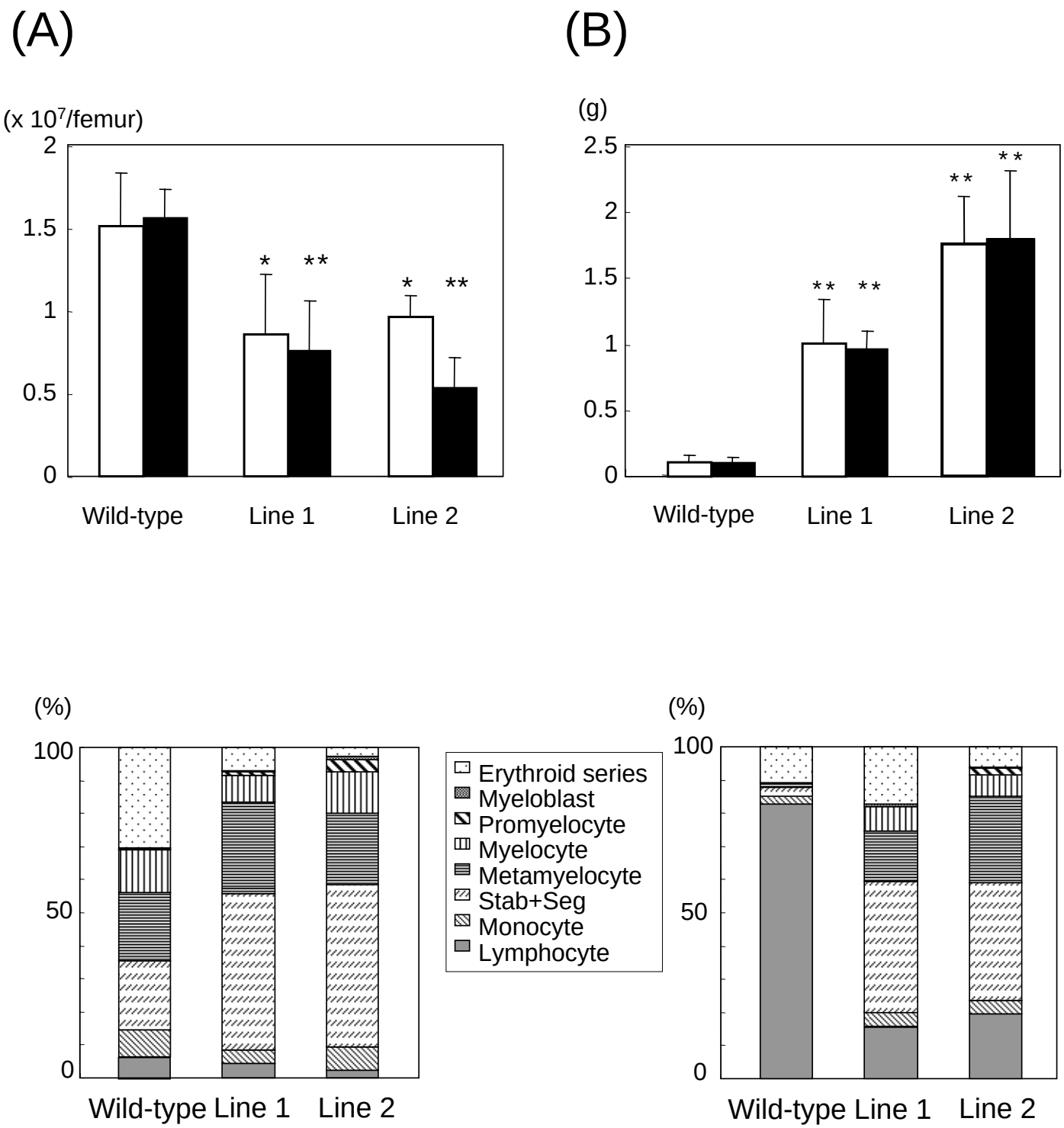

(A)
(x 10^7/femur)
2
1.5
1
0.5
0
*
**
*
**
Wild-type
Line 1
Line 2
(B)
(g)
2.5
2
1.5
1
0.5
0
**
**
**
**
Wild-type
Line 1
Line 2
(%)
100
50
0
Wild-type
Line 1
Line 2
Erythroid series
Myeloblast
Promyelocyte
Myelocyte
Metamyelocyte
Stab+Seg
Monocyte
Lymphocyte
(%)
100
50
0
Wild-type
Line 1
Line 2

Figure 5

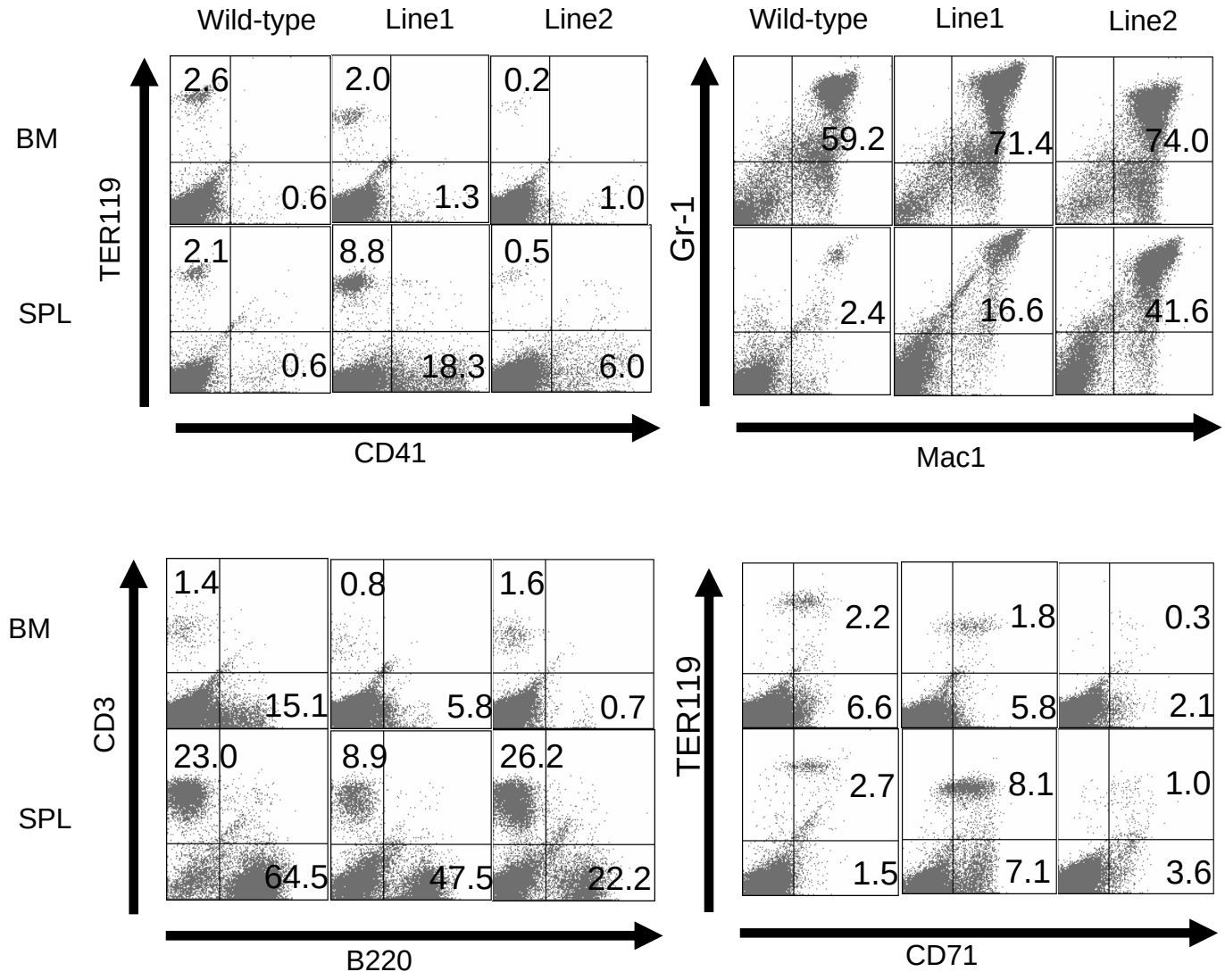

Wild-type
Line1
Line2
BM
SPL
TER119
CD41
2.6
0.6
2.0
1.3
0.2
1.0
2.1
0.6
8.8
18.3
0.5
6.0
Wild-type
Line1
Line2
Gr-1
Mac1
59.2
71.4
74.0
2.4
16.6
41.6
BM
SPL
CD3
B220
1.4
15.1
0.8
5.8
1.6
0.7
23.0
64.5
8.9
47.5
26.2
22.2
TER119
CD71
2.2
6.6
1.8
5.8
0.3
2.1
2.7
1.5
8.1
7.1
1.0
3.6

Figure 6

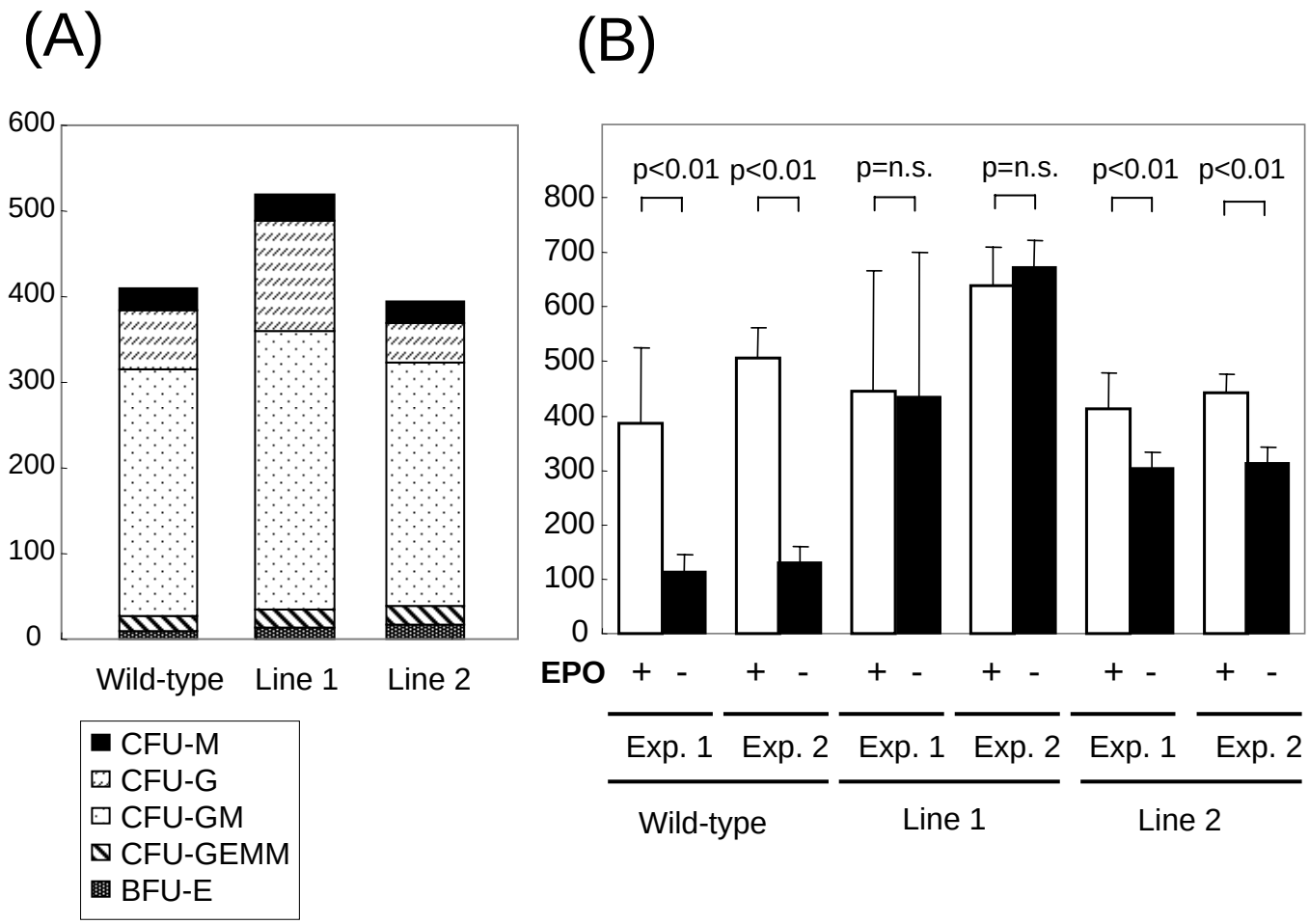

(A)
600
500
400
300
200
100
0
Wild-type
Line 1
Line 2
CFU-M
CFU-G
CFU-GM
CFU-GEMM
BFU-E
(B)
p<0.01
p<0.01
p=n.s.
p=n.s.
p<0.01
p<0.01
800
700
600
500
400
300
200
100
0
EPO + - + - + - + - + - + -
Exp. 1
Exp. 2
Exp. 1
Exp. 2
Exp. 1
Exp. 2
Wild-type
Line 1
Line 2

Figure 7