

**Studies of JAK2 Mutations
in Myeloproliferative Disorders**

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SUMMARY

Myeloproliferative disorders (MPD) are diseases characterized by clonal hematopoiesis with overproduction of mature cells from erythroid, megakaryocytic and myeloid lineages. Polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) constitute classic MPDs. Activating somatic *JAK2* mutations are frequently found in patients with myeloproliferative disorders. These mutations lead to constitutive activation of the JAK-STAT signaling pathway, which plays essential roles in hematopoiesis. The *JAK2*-V617F mutation is involved in the pathogenesis of 95% of PV and about 50% of ET and PMF patients. *JAK2* exon 12 mutations surrounding amino acids 539-545 are found in the majority of PV patients who are negative for the *JAK2*-V617F mutation. Most of PV patients with *JAK2*-V617F have homozygous erythroid colonies as a result of mitotic recombination, which is rare in ET patients and PV patients with *JAK2* exon 12 mutations. *JAK2* exon 16 mutant alleles affecting a highly conserved arginine residue at position 683 (R683) are found in 18%-28% of patients with Down's syndrome-associated acute lymphoblastic leukemia (DS-ALL). In addition to *JAK2*, *MPLW515* mutations are identified in about 5% of PMF patients and 1-9% of ET patients through screening other players in hematopoiesis, which could lead to activation of JAK-STAT signaling.

In the first part of my thesis, I compared *JAK2*-V617F positive PV patients with those carrying *JAK2* exon 12 mutations in regard to the lineage distribution of these mutations and the presence of the mutations in erythroid progenitors in these PV patients. *JAK2*-V617F and *JAK2* exon 12 mutations represent clonal markers useful for tracking the hematopoietic lineages involved in MPD. The results provided clues about the stage (such as hematopoietic stem cells or committed progenitors) at which the transformation of hematopoietic progenitors occurred, which may cause different phenotypes. I developed a novel and sensitive assay to quantitate the amount of *JAK2* exon 12 mutations in purified platelets, granulocytes, monocytes, B lymphocytes, T lymphocytes and natural killer cells (NK cells). The lineage distributions of *JAK2* exon 12 mutations and *JAK2*-V617F were similar in platelets, granulocytes, and monocytes, which always

carried the mutations, while the involvement of lymphoid cells showed large interindividual variations and T cells were rarely involved. This similarity does not explain why exon 12 mutations and *JAK2*-V617F result in divergent phenotypes. Analysis of erythroid progenitors indicated clonal heterogeneity in PV patients. One patient displayed erythroid colonies homozygous for the exon 12 mutation, which is very rare in patients with *JAK2* exon 12 mutations, with evidence for mitotic recombination on chromosome 9p. In several patients with exon 12 mutations or *JAK2*-V617F, a substantial proportion of erythroid endogenous colonies (EECs) were *JAK2* wild type. One patient carried two independent clones: one with an exon 12 mutation and another clone carrying *JAK2*-V617F. The finding of clonal heterogeneity is compatible with the hypothesis that additional clonal events are involved in the pathogenesis of PV.

From the first part of my work, we noticed that in some patients the frequency of *JAK2*-V617F mutation in peripheral blood is very low, and can only be detected with very sensitive methods such as allele-specific PCR. It has also been observed that in about half of PV patients with *JAK2*-V617F, the homozygous erythroid colonies only constituted a small proportion of the total number of BFU-Es, and more than half of patients with *JAK2* exon 12 mutations had only a small percentage of BFU-Es carrying the mutation. To answer how such a small proportion of mutant cells can lead to a substantial increase in red cell population, we hypothesized that *JAK2*-V617F homozygous BFU-Es or *JAK2* exon 12 mutant BFU-Es proliferate more efficiently and prevail over wild type BFU-Es during terminal erythroid differentiation. In the second part of my thesis work, I performed a pilot experiment by comparing the amount of *JAK2* mutation in BFU-Es with that in reticulocytes from the same patient sample to address this question. Preliminary data showed that in some PV patients who had a higher ratio of mutated *JAK2* in reticulocytes than in granulocytes, the frequency of mutant allele increased during terminal differentiation from BFU-Es to reticulocytes, indicating a substantial amplification occurred at this stage. However, this phenomenon cannot be solely attributed to the presence of homozygous *JAK2*-V617F colonies since some patients who did not have homozygous *JAK2*-V617F colonies also had the mutation amplified. Future directions including analysis of a larger cohort of samples and examination of the clonal

origin of reticulocytes using X-chromosome inactivation assays will further elucidate the impact of *JAK2* mutations on erythroid terminal differentiation.

To define the pathologic role of various *JAK2* mutations, and investigate the functional differences between different *JAK2* mutations, the third part of my thesis work was to generate transgenic mouse models with inducible expression of *JAK2* exon 12 or exon 16 mutations. The most frequent *JAK2* exon 12 mutations (N542-E543del and E543-D544del) and *JAK2* exon 16 R683G mutation were chosen as our candidates. Using a highly efficient recombination engineering technique with bacterial artificial chromosomes (BACs), we generated the *JAK2* exon 12 mutant transgene constructs with the exon 12 sequence placed in the inverse orientation and flanked by antiparallel *loxP* sites. Similarly, the *JAK2* transgene construct with R683G was made to have the sequences encoding the kinase domain placed in the inverse orientation and flanked by antiparallel *loxP* sites. The *JAK2* R683G transgene construct is ready for microinjection. The *JAK2* exon 12 mutant transgene constructs were microinjected into the pronucleus of zygotes from C57/BL6 mice and transferred to foster mice. Three transgenic founders with *JAK2* exon 12 N542-E543del and two transgenic founders with *JAK2* exon 12 E543-D544del have been obtained. These founders will be crossed with VavCre or MxCre transgenic mice in order to induce expression of mutant human *JAK2*. Detailed blood counts, pathological abnormality assessment and genotype-phenotype relationship analysis will be performed.

GENERAL INTRODUCTION

Hematopoiesis

Hematopoiesis is a highly orchestrated process of blood cell formation and homeostasis in both embryonic and adult life. Multiple anatomical sites are involved in embryonic hematopoiesis. In humans, primitive hematopoiesis starts in the yolk sac as early as in the first few weeks of embryonic development, which mainly produces primitive blood cells but also generates cells persisting as adult hematopoietic stem cells. Afterwards, definitive hematopoiesis starts in the intraembryonic aorta-gonad-mesonephros (AGM) region, which is a major site of the in situ generation of definitive hematopoietic stem cells (HSCs).¹ From 6 weeks until 6-7 months of gestation, the fetal liver and spleen become the major sites of hematopoiesis and this activity remains detectable until 2 weeks after birth. Later on, the bone marrow gradually becomes the primary blood-forming site and during childhood and adult life the bone marrow is the only source of normal hematopoiesis.²

Hematopoietic stem cells and progenitors

Adult hematopoietic stem cells (HSCs) are the cells in peripheral blood or bone marrow (BM), which can differentiate into all different lineages of blood cells, and simultaneously replicate themselves through self-renewal to prevent depletion of the stem cell pool in the BM. HSC self-renewal is a specialized cell division. It could be either symmetrical, producing two identical HSCs with the same pluripotent property as the parental cells, or asymmetrical, producing a HSC and a progenitor with limited self-renewal capacity but possessing the ability for clonal expansion and maintenance of the circulating blood cell population. HSCs are responsible for the constant renewal of blood cells everyday. In normal conditions, the majority of HSCs are kept in a quiescent state (G0 phase),³ thereby preserving their capacity to self-renew. In the event of a stress such as bleeding or infection, quiescent HSCs and progenitors are stimulated to proliferate and differentiate into mature blood cells. When the stress disappears, the kinetics of

hematopoiesis goes back to baseline level. The frequency of HSCs in bone marrow is quite low. About 1 in every 10,000 to 1,000,000 bone marrow cells is thought to be a stem cell.

Based on the ability of self-renewal, HSCs are divided into 2 populations: long-term HSCs, which are capable of self-renewal and give complete hematopoietic lineage recovery in secondary transplantation in mouse models, and short-term HSCs, which can regenerate all blood lineages but can not renew themselves over long term.

HSCs are able to generate every lineage found in the hematopoietic system. It is widely accepted that HSCs generate multiple lineages through a successive series of intermediate progenitors. During this process, the progeny from HSCs gradually lose their self-renewal ability, but keep their proliferation capacity. The long-term HSCs give rise to short-term HSCs and short-term HSCs further differentiate into common lymphoid progenitors (CLPs), which can generate all cells of the lymphoid lineages, and common myeloid progenitors (CMPs). CMPs then give rise to either granulocyte/macrophage progenitors (GMPs) or megakaryocyte/erythrocyte progenitor (MEPs)^{4,5} The progenitors finally commit to a certain lineage and give rise to mature blood cells (Figure 1). However, Iwasaki recently showed evidence that HSCs can form bipotent myeloerythroid and myelolymphoid progenitors before proceeding into the myeloid versus lymphoid pathway.⁶ Ultimately, terminally differentiated cells are produced and released from bone marrow into peripheral blood.

Regulation of hematopoiesis

Most of the mature blood cells are short-lived, and it is estimated that each day up to 10^{12} fresh blood cells need to be produced in normal adults to maintain the steady-state hematopoiesis. This highly orchestrated process of blood cell production from HSCs to committed progenitors and finally to terminally differentiated mature cells is mainly regulated by 3 mechanisms: the lineage specific transcription factors, hematopoietic growth factors (HGFs) and the interaction between hematopoietic cells and the bone marrow niches.⁷ The HGFs are a group of acidic glycoproteins that bind to type I cytokine receptor family members. Among them, the primary regulators of erythrocyte,

platelet, and neutrophil production are erythropoietin (EPO), thrombopoietin (TPO) and granulocyte-colony stimulating factor (G-CSF) respectively. Binding of ligand to receptor triggers receptor conformational changes and transduces extracellular signals inside the cell to instruct cell survival, proliferation, and differentiation. Dysregulation of hematopoietic growth factors underlies a number of disorders such as lymphomas, myeloproliferative disorders and leukemia.

Erythropoiesis

The normal life span of mature erythrocytes is around 120 days. Each day approximately 10^{12} new red blood cells are produced in the human body to maintain homeostasis. In human adults, normal erythropoiesis occurs in the bone marrow. The hematopoietic stem cells successively differentiate into colony-forming unit granulocyte, erythroid, monocyte, and megakaryocyte (CFU-GEMM), erythroid colony forming cells (BFU-E, CFU-E), and become proerythroblasts, the first morphologically recognizable cells of erythroid lineage. The proerythroblasts undergo a number of divisions, and give rise to basophilic normoblasts, polychromatic normoblasts, then to the orthochromatic normoblast. Finally, the orthochromatic normoblast extrudes the nuclei and develops into reticulocyte. The reticulocytes still contain some ribosomal RNA at this stage. After 1-2 days in the bone marrow, reticulocytes are released into peripheral blood where they become mature red blood cells after another 1-2 days.⁸

The production of red blood cells is regulated by erythropoietin (EPO), a cytokine produced mainly in the peritubular interstitial cells of the kidney. The production of EPO is controlled by oxygen tension in the tissue of the kidney. The response to EPO is related to the expression of EPO receptor on the cell surface. EPO receptor is expressed not only on erythroid progenitors but also on several non-hematopoietic tissues such as myocytes, cortical neurons and epithelia cells of prostate, ovary and breast. During erythroid lineage development, the requirement for EPO begins between the burst-forming unit (BFU-E) and colony-forming unit (CFU-E) until erythroblast.⁸

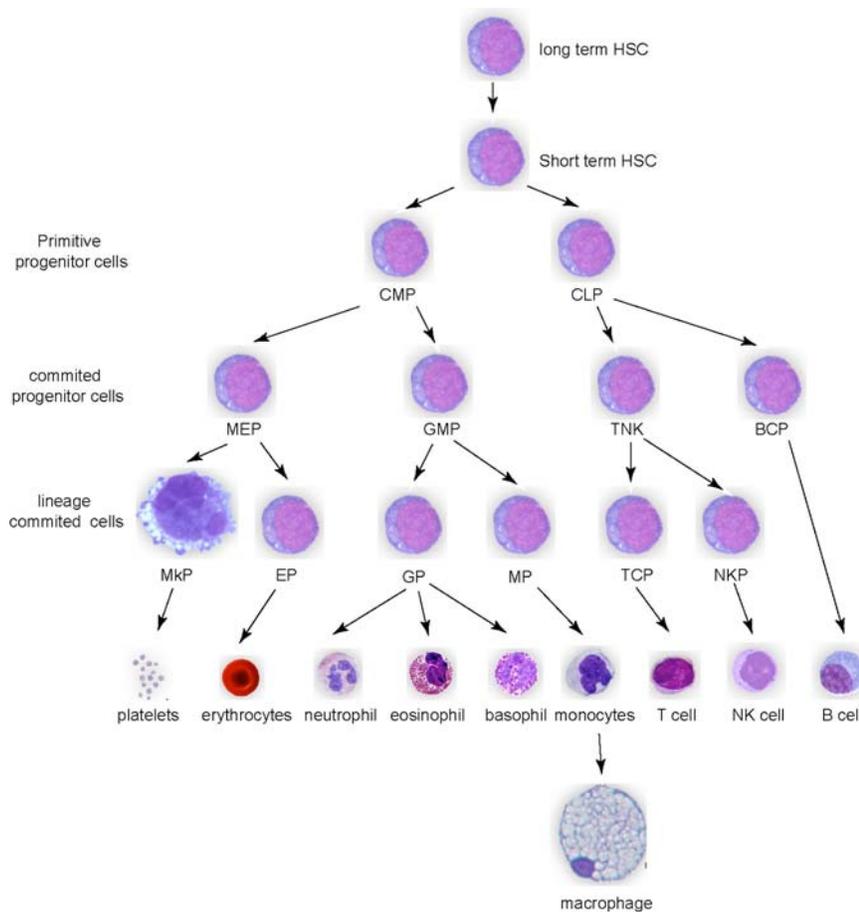


Figure 1. A common model of Hematopoiesis. HSC, hematopoietic stem cell; CMP, common myeloid progenitors; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythrocyte progenitor; GMP, granulocyte/macrophage progenitor; TNK, T cell natural killer cell progenitor; BCP, B cell progenitor; MkP, megakaryocyte progenitor; EP, erythroid progenitor; GP, granulocyte progenitor; MP, monocyte progenitor; TCP, T cell progenitor; NKP, natural killer cell progenitor.

Cytokines and hematopoietin receptor superfamily

Cytokines are a family of polypeptide growth factors that modulate gene expression in diverse cell types by binding to and activating members of the conserved cytokine-receptor superfamily. Common cytokines engaged in normal hematopoiesis include interleukins (ILs), interferons (IFNs), colony-stimulating factors (CSFs), erythropoietin (EPO), thrombopoietin (TPO) and leukemia inhibitory factor (LIF).⁹ All these cytokines have their cognate receptors on the cell surface, called cytokine receptors or hematopoietin receptors. This family of receptors also includes receptors for several structurally related factors and hormones, such as growth hormone (GH), prolactin (PRL) and ciliary neurotrophic factor (CNTF). Some cytokine receptors have different cytoplasmic domain due to alternative mRNA splicing, such as G-CSFR, GM-CSFR and MPL. Different signaling pathways could be activated via the different cytoplasmic domains.

Most hematopoietin receptors belong to type I and type II cytokine receptors. The type I cytokine receptor is either a homodimer of a receptor such as EPO receptor, TPO receptor (MPL), G-SCF receptor,¹⁰ or a heterodimer with a specific ligand-binding subunit and a common signal-transducing subunit which might be shared with other cytokine receptors. The common subunits include 1) common β chain, shared by GM-CSF receptor, IL-3R α , IL-5R α ; 2) common γ chain, shared by IL-2R α , IL-4R α , IL-7R α , IL-9R α , IL-13R α , IL-15R α and IL-17R α ; 3) gp130 subunit, shared by LIFR β , IL-11R α and IL6-R α . Each of these transmembrane proteins is comprised of an intracellular domain containing approximately 100 to 500 amino acids, a transmembrane domain of 20 to 25 residues, and one or two extracellular cytokine-binding domains with several common structural features.¹¹ The average extracellular domain is around 210 amino acids in size and it contains one to four conserved cysteine residues in the N terminal, a tryptophan-serine-x-serine-tryptophan (W-S-X-W-S) motif in the C terminal that is important for ligand-receptor interaction, and fibronectin type III domain. In the cytoplasmic domain, type I receptors are characterized by the presence of less conserved Box1 and Box2 domains, which are critical for the function of receptors and mediating mitogenic signals.^{12,13} Type II cytokine receptors mainly consist of receptors for interferons and IL-10, which are

similar to type I cytokine receptors except they do not possess the signature sequence W-S-X-W-S that is characteristic of type I receptors. The cytokine receptors are devoid of catalytic activity and they transmit their signals through cellular tyrosine kinases, in most cases via the family of Janus kinases (Jaks).

Jak-Stat signaling in hematopoiesis

Overview of the Jak-Stat activation mechanism

Janus tyrosine kinases play critical roles in cytokine signaling transduction of hematopoietic cells. They are involved in transducing signals from type I receptors (receptors for IL-2-IL-7, IL-13, GM-CSF, GH, PRL, EPO, and TPO) as well as type II cytokine receptors (receptors for IFN- α , β , γ).¹⁴ The Janus kinase-signal transducer and activator of transcription (Jak-Stat) pathway is widely exploited by members of cytokine receptor superfamily, including EPOR, MPL, G-CSFR, receptors for interferons and many kinds of interleukins.

Janus kinases

The Janus kinases are a family of large cytoplasmic tyrosine kinases with molecular weights in the range of 120–140 kDa (1130–1142 aa). In mammals, there are four members of the Jak family: Jak1, Jak2, Jak3, and Tyk2.¹⁵ From C-terminal to N-terminal, Jaks consist of seven conserved domains, termed Jak homology (JH) domains 1-7. (Figure 2) JH1 and JH2 domains exert the most important functions of Jaks. JH1 acts as a kinase domain, containing the ATP-binding region and the activation loop. The JH2 domain is the pseudokinase domain, which is highly homologous to tyrosine kinase domain, but lacks the catalytic activity due to the absence of necessary residues. The pseudokinase domain is believed to have autoinhibitory function and regulate both basal activity of the Jak kinases and cytokine-induced activation of the catalytic function.^{16,17} Expression of JH2-deficient Jak2 resulted in cytokine-independency in cytokine-dependent cell lines.¹⁶ JH3-JH4 region comprises the SH2-like domain, which is homologous to SH2 domain, but does not bind to phosphotyrosine residues.¹⁸ The JH5-JH7 region constitutes the FERM domain (Four-point-one, Ezrin, Radixin, Moesin), which mediates the association between Jaks and other proteins.¹⁹ Recently,

autophosphorylation of Y119 in the FERM domain has been shown to down regulate receptor-mediated kinase activation.²⁰

Jaks are vital for normal hematopoiesis. Jak1 is mainly involved in IL-6 signaling.²¹ *Jak1* deficient mice showed perinatal lethality and defective lymphoid development.²² Jak3 plays a non-redundant role in the function of all receptors utilizing the γ c chain.²³ *Jak3* knock out mice showed severe combined immunodeficiency, which affected both T cells and B cells.²⁴ Tyk2 is involved in interferon- α/β signaling and mediates activation of interferon-responsive genes.²⁵ Jak2 is activated in response to a variety of cytokines, including EPO²⁶, TPO, IL-5^{27,28}, IL-3, and GM-CSF.²⁹ Fetal liver cells from *Jak2*-deficient embryos fail to respond to EPO, IL-3, TPO, and mice deficient in *Jak2* are embryonic lethal due to the absence of definitive hematopoiesis. All these in vitro and in vivo experimental data demonstrate that Jak2 plays essential, non-redundant roles in signal transduction induced by these cytokines in hematopoiesis.³⁰

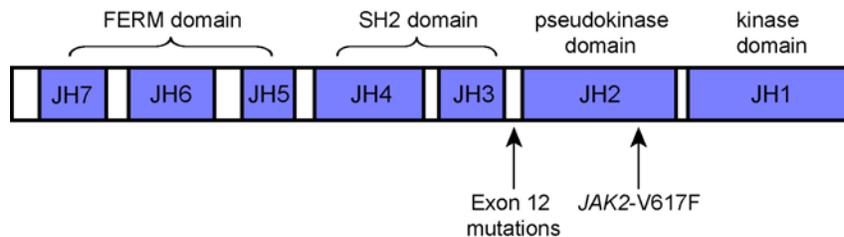


Figure 2. Janus homology domains of the Jak family of kinases. Jaks consist of seven conserved domains, termed Jak homology (JH) domains 1-7. The position of *JAK2*-V617F and *JAK2* exon 12 mutations of *JAK2* are indicated with arrows.

Stats

Stats are transcription factors with about 750-800 amino acids and latent in the cytoplasm. They are recruited to the receptor complex and get activated by phosphorylated Jaks. Subsequently, activated Stats dimerize, translocate to the nucleus, bind DNA and ultimately affect gene expression. The Stat proteins are comprised of amino terminal domain, coiled-coil domain, SH2 domain, linker, DNA binding domain,

and transcriptional activation domain. The amino terminus domain forms a hook-like structure, which may facilitate Stats binding to targets. The coiled-coil domain mediates the interaction of Stats with a variety of other factors, and the linker domain is involved in transcriptional control. The SH2 domain is essential for Stats functions and has several responsibilities: serving as the docking site to bind tyrosine phosphorylated receptor subunits, helping the association with the activated Jaks, and forming the hinge in the nutcracker-like structure when bound to DNA.³¹

There are 7 mammalian Stats, Stat 1 to 6, including Stat5a and Stat5b. Disturbance of *Stat* genes in mouse models showed defects on development and hematopoiesis. Stat1 plays important roles in IFN signaling. *Stat1* knockout mice had no developmental defects, but showed defective innate immune responses to viruses and bacteria infections and susceptible to tumor formation.³² due to impaired IFN responses. Stat2 is involved in IFN α/β signaling. *Stat2* knockout mice were viable but susceptible to viral infections.³³ *Stat3* deletion is embryonic lethal.³⁴ Stat4 gets activated mainly through IL-12 and IL-23. Disruption of IL-12 signaling due to absence of Stat4 resulted in defective formation of Th1 cells in *Stat4* knockout mice.^{35,36} *Stat5a* and *5b* double knockout mice showed defects in transducing signals from IL-2, IL-3, GM-CSF and G-CSF.^{37,38} Stat6 is involved in IL-4 signaling and *Stat6* knockout mice had a block in Th2 cell development and IgE class switching.^{39,40}

Jak-Stat signaling pathway

The Jak-Stat pathway consists of three families of genes: the Janus tyrosine kinases family, the Stat (signal transducers and activators of transcription) family and the CIS/SOCS family, which acts as negative regulators of the Jak-Stat pathway.

Engagement of cytokine receptors by hematopoietic growth factors induce conformational changes in the receptor, bringing the two cytoplasmic Jaks into close juxtaposition, which leads to activation of the kinases by transphosphorylation. Activated Jaks then phosphorylate the cytoplasmic domain of the receptor, creating docking sites for secondary signaling proteins such as Stats. Stats are then recruited to the cytokine

receptor and phosphorylated by Jaks. Phosphorylated Stats dimerize by interactions between the Src homology 2 (SH2) domains and translocate to the nucleus, where they bind specific regulatory sequences and activate transcription of target genes. Activation of Jak2 also entails activation of the phosphatidylinositol 3-kinase (PI3K)–Akt pathway⁴¹ and mitogen-activated protein kinase (MAPK) pathway,⁴² each of which activates an overlapping subgroup of downstream signaling molecules such as cell cycle activators, anti-apoptosis molecules and transcription factors (Figure 3). Combination of activated Jaks and Stats determine the specificity in cytokine signaling.

Negative regulation of Jak-Stat signaling pathway

Activation of Jak-Stat signaling is rapid and transient. Several mechanisms and molecules contribute to turn off the signaling pathway.

Protein tyrosine phosphatases (PTPs)

Three types of protein tyrosine phosphatases have been shown to negatively regulate Jak-Stat signaling pathway.

SH2-containing phosphatases (SHPs) have 2 family members termed SHP1 and SHP2 sharing 55% homology of protein sequence. SHP1 is mainly expressed in hematopoietic cells, while SHP2 is more ubiquitously expressed. They are characterized by the presence of two SH2 domains and a phosphatase catalytic domain. The SH2 domain can associate with the phosphotyrosine residues on the activated receptors, on JAKs or on signaling molecules, which subsequently trigger the activation of the phosphatase domain to dephosphorylate the substrate.⁴³ Mice deficient for *Shp1* showed hematopoietic dysfunctions and displayed hyperphosphorylation of Jak1 and Jak2 following cytokine treatment. Silencing of *Shp-1* gene was detected in various hematologic malignancies, such as leukemia, lymphomas and myeloma.^{44,45,46}

The receptor-like tyrosine phosphatase, CD45, a hematopoietic-specific phosphatase, is the second type of tyrosine phosphatase that negatively regulates Jak-Stat signaling.⁴⁷ Absence of CD45 leads to augmented Jak and Stat phosphorylation in hematopoietic cells.⁴⁸

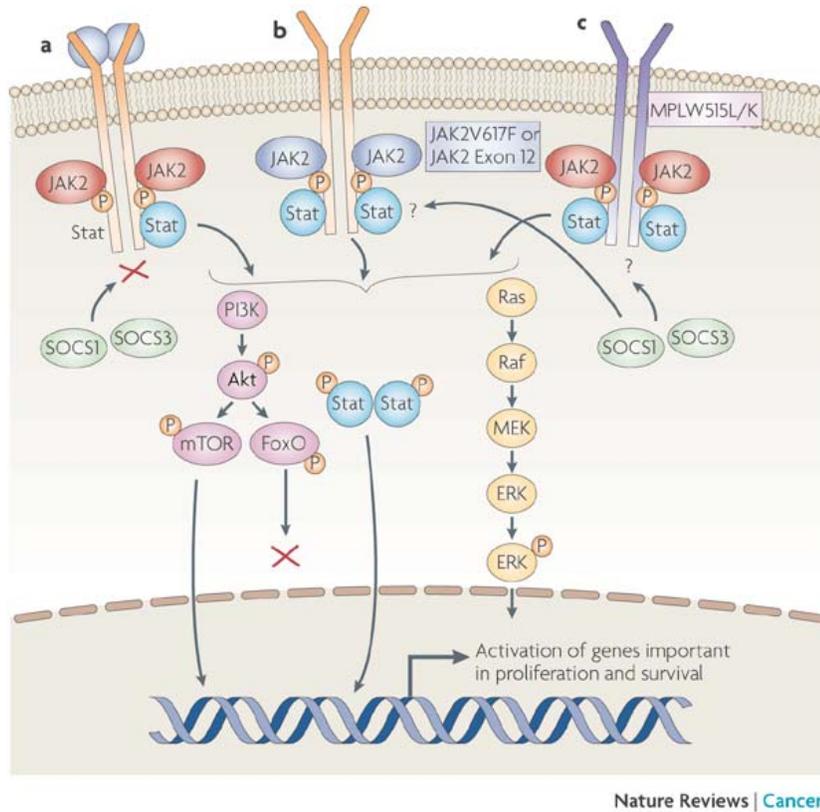
The third group of phosphotyrosine phosphatases includes phosphotyrosine phosphatase 1B (PTP1B) and T cell protein tyrosine phosphatase (TC-PTP), which have high similarities in their catalytic domains. They specifically recognize tyrosine residues in the Jak activation loop. PTP1B interacts with Jak2 and Tyk2 while TC-PTP dephosphorylates Jak1 and Jak3.⁴³ In contrast to PTP1B, which is expressed in many tissues, TC-PTP is mainly expressed in hematopoietic cells and comprised of two isoforms created by alternative splicing, the nuclear form TC45 and the cytoplasmic form TC48. The nuclear form of TC-PTP is also responsible for the deactivation of nuclear Stat1 and Stat3.⁴⁹

Suppressors of cytokine signaling (SOCS) proteins

SOCS proteins are small proteins that possess SH2 domains and conserved C-terminal SOCS/CIS boxes. This protein family has 8 members, cytokine-inducible SH2 containing protein (CIS) and SOCS1-7.⁵⁰ The suppression of Jak-Stat signaling is achieved mainly by 3 mechanisms. Firstly, SOCS can compete with STATs for phosphorylated docking sites on the receptors. For example, CIS can bind to STAT5 binding site of the receptor, prevent STAT5 binding and thus inhibit signaling.⁵¹ In addition, SOCS such as SOCS-1 associate with the activation loop of JAKs directly via its SH2 domain to inhibit the function of JAKs. Similarly the kinase inhibitory region of SOCS-1 and SOCS-3 can bind to the JAK catalytic pocket and block its catalytic activity. The third way is to target signaling proteins to the ubiquitin proteasome pathway through the SOCS box.⁵²

Protein inhibitor of activated STAT proteins (PIAS)

PIAS family is comprised of 5 members: PIAS1, PIASx, PIAS3- α , PIAS3- β and PIASy. They consist of a N-terminal LXXLL co-regulator domain, a zinc finger domain and a C-terminal acidic domain. PIAS proteins bind to activated STATs dimers and block transcription by either inhibiting DNA binding of STATs (PIAS1 and PIAS3) or mediating recruitment of transcriptional repressors (PIASx and PIASy).⁵³ Recent research showed that in addition to the inhibition of STAT proteins, PIAS actually interact with a variety of transcription factors.



Nature Reviews | Cancer

Levine RL, Nature Reviews Cancer 2007⁵⁴

Figure 3. Jak-Stat signaling pathway. a) Binding of cytokine receptors with ligands results in Jak2 phosphorylation, recruitment and phosphorylation of Stats and activation of downstream signaling pathways including Stat transcription factors, PI3K pathway, and MAP kinase pathway. b) The *JAK2V617F* and *JAK2* exon 12 mutant kinases bind receptors and get phosphorylated in the absence of cytokines, leading to ligand-independent activation of downstream signaling pathways. c) *MPLW515L/K* mutants are able to phosphorylate wild-type JAK2 in the absence of TPO, which results in the activation of signaling pathways downstream of JAK2. SOCS proteins, most notably SOCS-1 and SOCS-3, normally mediate negative regulation of JAK2 signaling.

Myeloproliferative disorders

The concept of Myeloproliferative disorders (MPD) was first proposed by Damshek in 1951, who considered chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) as closely interrelated disease entities.⁵⁵ The discovery of Philadelphia chromosome resulting from t(9;22)(q34;q11) and the *bcr/abl* transcript established CML as a separate disease entity.⁵⁶⁻⁵⁸ A specific kinase inhibitor, namely imatinib mesylate (Gleevec; Novartis, Switzerland) was subsequently developed and used successfully in the treatment of CML patients.^{59,60} PV is a disease with increased red blood cells because of clonal expansion. About half of PV patients have neutrophil leukocytosis or raised platelet counts. ET is characterized by thrombocytosis due to abnormal megakaryocyte proliferation and overproduction of platelets. PMF is a progressive fibrosis of the bone marrow and characterized by increased collagen in bone marrow with subsequent development of extramedullary hematopoiesis in spleen and liver. In summary, this heterogeneous group of diseases is characterized by increased hematopoiesis with normal differentiation, leading to elevated amount of mature non-lymphoid blood cells and/or platelets in the peripheral blood.

MPD clonal origin

X chromosome-linked markers have been successfully used to explore the clonal origin of MPDs.⁶¹ During development, somatic cells of females randomly inactivate one of the two X chromosomes. As a result, women heterozygous for a polymorphic X-linked allele have a mixture of cells expressing one or the other allele in normal tissues. If cells are clonal, i.e. they come from the same ancestor; all of the cells inactivate the same X chromosome, thus showing a homogeneous type of X-inactivation pattern. Based on this theory, Adamson et al showed that female PV patients had the same form of glucose-6-phosphate dehydrogenase (G6PD) in their erythrocytes, granulocytes, and platelets, demonstrating the clonal origin of PV.⁶² El Kassab N⁶³ and Tsukamoto N⁶⁴ studied the polymorphism of the human androgen receptor gene (*HUMARA*), as well as the restriction fragment length polymorphisms (RFLP) of the X-chromosome phosphoglycerate kinase (*PGK*) and hypoxanthine phosphoribosyltransferase (*HPRT*)

genes in female ET and PMF patients and found out that most of patients also had clonal hematopoiesis. All these data suggest that PV, ET and PMF originated from a multipotent hematopoietic progenitor or a stem cell, which acquired proliferative and/or survival advantage in the course of the disease.

Cytogenetic analysis provides genetic basis to study clonal hematopoiesis. The recurrent but non-specific cytogenetic abnormalities such as numerical gains or losses and chromosome translocations are frequent in Philadelphia-negative chronic myeloproliferative disorders. About 10-15% PV and 30-40% PMF patients had abnormal karyotype at diagnosis; while in ET patients, only 5-6% carried chromosomal aberrations. In PV patients, the most frequent abnormalities are del(20q), trisomies 8 and 9 as determined by fluorescence in situ hybridization (FISH)^{65,66} while del(13q), del(20q) and partial trisomy 1q are more commonly seen in PMF patients.⁶⁷

Loss of heterozygosity of chromosome 9p (9pLOH)

Loss of heterozygosity (LOH) is a kind of chromosomal alteration that is undetectable by cytogenetic analysis or fluorescent in situ hybridization (FISH) analysis. It is the result of mitotic recombination caused by exchange of chromosomal DNA between non-sister chromatids during mitosis. Using genome-wide microsatellite screening, Kralovics et al⁶⁸ identified loss of heterozygosity in three genomic regions on chromosomes 9p, 10q, and 11q and found out that 9pLOH is the most frequent chromosomal lesion in PV patients with a prevalence of about 30%.

JAK2 mutations in MPD patients

Although various cytogenetic abnormalities were reported in patients with MPD, no specific abnormality was found until 2005, in which year, our group together with several other groups independently discovered a somatically acquired *JAK2* mutation (*JAK2*-V617F) in MPD patients.⁶⁹⁻⁷² This discovery is an important advance in our understanding of the pathogenesis of myeloproliferative disorders. The mutation changes the guanine to thymine at position 1849, which results in the substitution of valine to phenylalanine at position 617 (*JAK2*-V617F) in the pseudokinase domain of the *jak2*

protein. According to these reports, *JAK2-V617F* can be detected in about 90-95% of PV patients and about 50% of ET and PMF patients. Many patients with PV or PMF are homozygous for the V617F mutation, as a result of mitotic recombination affecting chromosome 9p, but homozygosity is rare in patients with ET.^{69,73} Less frequently, *JAK2-V617F* has also been observed in patients with chronic myelomonocytic leukemia (CMML), myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), atypical myeloproliferative disorder, hypereosinophilic syndrome and systemic mastocytosis. It is worth to note that *JAK2-V617F* is exclusive to disorders of myeloid lineages, and has never been found in lymphoid malignancies.⁷⁴⁻⁷⁶ Analysis of different hematopoietic compartments in MPD patients by several groups shows that *JAK2-V617F* can be found in hematopoietic stem cells (HSCs) with a phenotype of CD34⁺CD38⁻CD90⁺Lin⁻, and myeloid progenitors, and occasionally B and T cells.⁷⁷⁻⁸¹

Careful analysis of other *JAK2* exons led to the discovery of *JAK2* exon 12 mutations in a subset of PV patients who are negative for *JAK2-V617F*. Interestingly, unlike *JAK2-V617F*, being the only mutation in exon 14, fourteen different *JAK2* exon 12 mutations (missense mutation, deletions and insertions) have been reported.⁸²⁻⁸⁸ These mutations mainly occur in the vicinity of codon 539 and 543 and all of them cause a phenotype closely related to isolated erythrocytosis, because they are not observed in any patients with ET or PMF. In addition, sequencing of receptors associated with *JAK2* signaling in *JAK2-V617F* negative MPD patients led to the discovery of two gain-of-function somatic mutations in the thrombopoietin receptor (*MPL*), *MPLW515L* and *MPLW515K*, which are located at the transmembrane-juxtamembrane junction of *MPL*.^{89,90} Three additional *MPL* mutations in the vicinity (*MPLS505N*, *MPLA506T* and *MPLA519T*) were subsequently identified. In total, about 5% PMF patients and 1-9% ET patients carried *MPL* mutations.⁸⁹⁻⁹³

Aberrant cell signaling in MPDs

It was observed more than 30 years ago that bone marrow cells from PV patients had the ability to differentiate into erythroid colonies in the absence of exogenous erythropoietin in vitro (endogenous erythroid colonies, EECs), whereas progenitor cells from normal

persons gave rise to erythroid colonies only in the presence of EPO.⁹⁴ Albeit EECs are present in almost all PV patients, they are not specific to PV, because a proportion of ET and PMF patients also have EECs.^{94,95} The presence of EECs has become one of the diagnostic criteria for PV as proposed by the World Health Organization (WHO).⁹⁶ Similarly, some studies showed megakaryocytic progenitor cells from the majority of ET patients, formed factor independent megakaryocytic colonies (EMC) under serum-containing conditions, which is not the case for reactive thrombocytosis. Interestingly, EMCs can also be detected in some PV and PMF patients.^{97,98} These abnormalities associated with in vitro growth of hematopoietic progenitors indicate the presence of altered cytokine receptor signaling, which is confirmed by the discovery of *JAK2* mutations in the majority of MPD patients.⁶⁹⁻⁷²

Functional studies of *JAK2*-V617F and *JAK2* exon 12 mutations showed that expression of either type of *JAK2* mutations, but not wild type *JAK2*, induces cytokine hypersensitivity and cytokine-independent growth in the presence of EPO or TPO receptors.^{69,70,72,82} Auto-phosphorylation assays showed that *JAK2*-V617F mutation constitutively activated the *JAK2* tyrosine kinase⁷² and that both *JAK2*-V617F and *JAK2* exon 12 mutations can lead to increased phosphorylation of *JAK2* and its downstream effector, STAT5.^{69,82} In addition to the activation of STAT5, MAP kinase and PI3K pathways are also activated as assessed by phosphorylation of ERK and AKT respectively (Figure 3), but their exact role in the pathogenesis of MPD is unclear.

JAK2-V617F is located in the JH2 domain of *JAK2*, which is the pseudokinase domain (Figure 2). As described above, the JH2 domain is important for autoinhibition of *JAK2*. This pseudokinase domain plays a critical role in regulating both basal activity of Jak kinases and cytokine induced activation of the catalytic function.^{16,17} It is predicted that *JAK2*-V617F leads to loss of autoinhibition on the JH1 domain, which results in constitutive activation of *JAK2* in the absence of ligands.^{99,100} Detailed structural analysis of interaction between JH1 and JH2 domains would provide more clues for understanding the functional aberrance of *JAK2*-V617F.

Various *JAK2* exon 12 mutations affect residues 537 through 543 and cause unregulated *JAK2* activity. This region is predicted to lie within the linking region between the SH2 and JH2 domains of *JAK2*. These affected residues are close to the loop carrying V617F.⁸² However, detailed structural and biochemical analysis is needed to verify this prediction.

Models to study JAK2 mutations in vivo

In vivo studies using retroviral bone marrow transplantation assays demonstrated that overexpression of *Jak2*-V617F in bone marrow cells results in a PV-like phenotype with increased hematocrit, leukocytosis, extramedullary hematopoiesis and subsequent myelofibrosis, but no thrombocytosis.^{70,101-104} These results indicate that *JAK2*-V617F is sufficient to cause PV although different genetic backgrounds of mice (Balb/C vs. C57/6) may have impact on the degree of leukocytosis and myelofibrosis. Interestingly, thrombocytosis, which is a common phenomenon in patients with PV and ET, was only observed in a group with low mutant *Jak2* expression in one study.¹⁰¹ Similar bone marrow transplantation studies using *Jak2*-K539L (exon 12 mutation) led to expansion of erythroid lineages and mild expansion granulocyte lineage as compared to *JAK2*-V617F, but no megakaryocytes expansion was observed.⁸²

Until now, three transgenic mouse models with *JAK2*-V617F have been established.¹⁰⁵⁻¹⁰⁷ Low level expression of *JAK2*-V617F induced a phenotype resembling ET with thrombocytosis, while high expression of the transgene developed a PV-like phenotype in mice, indicating the dosage of the mutant allele is important for the phenotype manifestation. Currently, it is still not completely clear why a single *JAK2*-V617F mutation can cause three different disease phenotypes.

Recombination-mediated genetic engineering (Recombineering)

Bacterial Artificial Chromosomes (BACs)

Bacterial artificial chromosome is a kind of cloning vector based on the *E. coli* fertility plasmid (F-factor). Compared to traditional high-copy plasmids, BACs can propagate up to 300 kb genomic DNA as 1-2 copy plasmids in a well characterized recombination

deficient *E. coli* host strain,¹⁰⁸ which makes it possible to include large upstream regulatory sequences of a gene in a BAC. Besides large accommodation, BACs have high cloning efficiency; they are easy to purify and remain intact because they exist as supercoiled circular DNA that are relatively resistant to shearing; they are highly stable and unlikely to undergo rearrangements. These advantages have made BACs an important tool to study gene functions in transgenic systems.

Recombinogenic Engineering

Conventional cloning methods that rely on the use of restriction enzymes and DNA ligases limit the engineering of large DNA molecules, such as BACs. In recent years, the development of homologous recombination-based strategies in *E. coli* allows a wide range of modifications of DNA molecules. Homologous recombination occurs through homology arms, the sequence of which is shared by the two DNA molecules that could recombine, thus providing a precise and specific way to exchange genetic information between two DNA molecules. Using phage recombination to carry out genetic engineering has been called recombinogenic engineering, or recombineering.¹⁰⁹

E. coli is inherently not ready for transformation by double stranded DNA (dsDNA), because RecBCD exonuclease in *E. coli* can rapidly digest exogenously introduced linear DNA.¹¹⁰ The introduction of a defective λ -prophage system, termed Red, into the *E. coli* chromosome can inhibit RecBCD and mediate homologous recombination. λ -Red system encodes three genes, *Gam*, *Exo* and *Bet*, which are important for recombination. *Gam* encodes an inhibitor of the *E. coli* RecBCD exonuclease activity and thereby protects the foreign linear DNA from degradation by RecBCD. Double strand breaks in DNA are the initiation sites for this recombination. *Exo* encodes a 5'-3' exonuclease that degrades nucleotides from 5' ends of the break and leaves 3' overhangs. *Bet* encodes a pairing protein that binds to the 3' single strand tails and mediates its annealing and homologous recombination with complementary DNA. The expression of these proteins is under the tight control of a temperature sensitive λ -*cI857* repressor. (Figure 4) At 32°C, the expression of these genes is suppressed by the repressor. Shifting bacteria to 42 °C for as short as 15 minutes will inactivate the repressor, and the three genes are transcribed from

the strong λ -pL promoter. Finally, the very efficient homologous recombination occurs through homologies of only 50 bp or less.¹¹¹

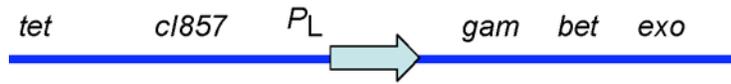


Figure 4. Defective λ -prophage is integrated into the *E. coli* chromosome. Expression of *gam*, *bet* and *exo* is under the control of *PL* promoter and temperature-sensitive *cI857* repressor. At 32°C, the repressor inhibits the transcription of *gam*, *bet*, and *exo*. At 42°C, the repressor is inactivated, and *gam*, *bet* and *exo* are expressed.

General steps to generate a BAC recombinant using recombineering

The following steps are generally used in recombineering in BACs (Figure 5).¹¹²

1. Amplifying a cassette by PCR with flanking regions of homology.
2. Introducing a BAC into a strain that carries recombination function.
3. Transforming the cassette into bacteria containing a BAC and recombination functions.
4. Generating a recombinant in vivo.
5. Detecting a recombinant by selection, counterselection or by direct screening.

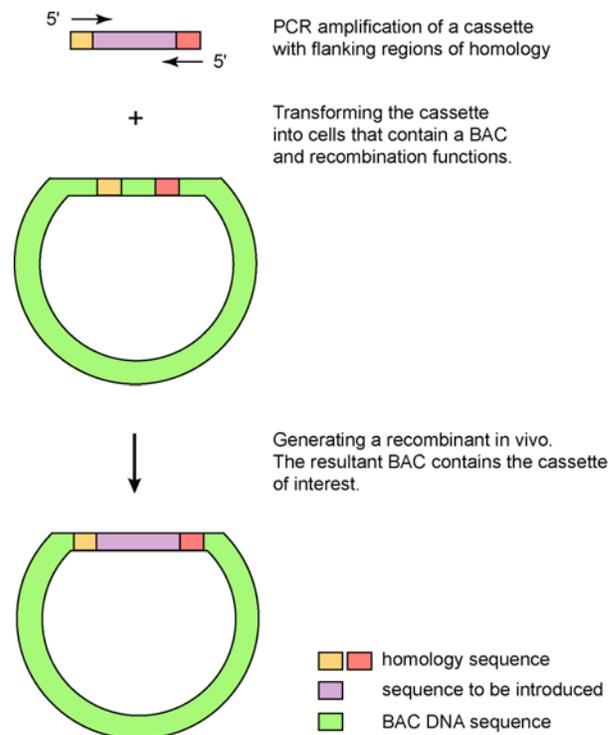


Figure 5. General steps of recombineering.

RESULTS I:

Clonal heterogeneity in polycythemia vera patients with *JAK2* exon12 and *JAK2-V617F* mutations

Abstract

We studied the lineage distribution of *JAK2* mutations in peripheral blood of 8 polycythemia vera (PV) patients with exon 12 mutations and in 21 PV patients with *JAK2-V617F*. Using a quantitative allele discrimination assay, we detected exon 12 mutations in purified granulocytes, monocytes and platelets of 8 patients studied, but lymphoid cells showed variable involvement and the mutation was absent in T cells. A similar distribution was observed in patients with *JAK2-V617F* and clonal analysis showed that the mutation is very rare in peripheral blood T cells. Endogenous erythroid colonies (EECs) grew in all patients analyzed. One patient displayed erythroid colonies homozygous for the exon 12 mutation with evidence for mitotic recombination on chromosome 9p. In several patients with exon 12 mutations or *JAK2-V617F*, a substantial proportion of EECs were negative for both *JAK2* mutations. One patient carried two independent clones: one with an exon 12 mutation and a second with *JAK2-V617F*. The lineage distributions of exon 12 mutations and *JAK2-V617F* are similar and do not explain why exon 12 mutations and *JAK2-V617F* can cause different phenotypes. The finding of clonal heterogeneity is compatible with the hypothesis that additional clonal events are involved in the pathogenesis of PV.

Introduction

An acquired activating mutation in exon 14 of the Janus kinase 2 (*JAK2-V617F*) is commonly found in patients with myeloproliferative disorders (MPD), with the highest incidence in polycythemia vera (PV).^{69-72,113} Mutations in exon 12 of *JAK2* are detected selectively in patients with PV that are negative for *JAK2-V617F* and in some patients with idiopathic erythrocytosis.⁸² Interestingly, different mutant *JAK2* exon 12 alleles surrounding amino acid 539-545 were identified (Figure 1). The *JAK2-V617F* and exon 12 mutations represent clonal markers useful to track the hematopoietic lineages involved in MPD. In patients with MPD, *JAK2-V617F* is present in purified hematopoietic stem cells, in myeloid lineages of the peripheral blood and in variable proportions of lymphoid cells.^{77-80,114} The presence of *JAK2-V617F* in T cells remains controversial.^{78-81,114} Using a sensitive novel assay, we quantitated the involvement of exon 12 mutations in purified peripheral blood lineages and in erythroid progenitor assays. In addition, we addressed the question of whether *JAK2-V617F* is present in T cells by clonal analysis.

Materials and Methods

Patients

The screening for *JAK2* exon 12 mutations in MPD patients was performed by DNA sequencing using primers 5'-CAAAGTTCAATGAGTTGACCCC-3', and 5'-TGCTAACATCTAACACAAGGTTGG-3', which are located in *JAK2* exon 12 neighbouring introns. Patients from whom frozen cells were available were included for further study. The diagnosis of MPD was made according to the World Health Organization.^{115,116} Two patients with *JAK2* exon 12 mutation (Vi064, Vi327) were from Vienna, Austria. All other patients were from Basel, Switzerland. The collection of patient samples was approved by the local ethics committees. Written consent was obtained from all patients.

Cells, DNA and RNA

Isolation of granulocytes, platelets, and peripheral blood mononuclear cells (PBMC) was performed as described.^{68,69,117} PBMCs were sorted into CD3⁺CD56⁻ (T cells), CD3⁻CD56⁺ (Natural Killer cells), CD14⁺ (monocytes) and CD19⁺ (B cells) fractions using a FACSVantage SE (Becton Dickinson, Franklin Lakes, USA). Gates were set for maximal purity of the sorted cell populations. Fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies against CD56, CD14 and isotype controls and phycoerythrin (PE) conjugated antibodies against CD3, CD19 and isotype controls were from Becton Dickinson (San Jose, CA). RNA isolation from platelets and cDNA synthesis were performed as described.¹¹⁷ DNA from single colonies was isolated using Chelex-100 Resin (Biorad, Hercules, CA).¹¹⁸

Quantification of *JAK2* exon 12 mutations

JAK2 exon 12 mutations were detected and quantitated using an allele discrimination assay (Figure 2). For genomic DNA we used the primers 5'-FAM-ACTTTCAGTGTATTTTGAAGTGAT-3', and 5'-GTTTCTTGAATGTAAATCAAGAAACAGA-3' and for RNA/cDNA the primers 5'-FAM-AAACTGTTCGCTCAGACAAT

-3' and 5'-GTTTCTTCTCTTCGTACGCCTTTA-3' were used. The PCR products were separated by capillary electrophoresis and the peak fluorescent intensities were measured on an ABI3130 Genetic Analyzer (Applied Biosciences, Carlsbad, CA). The calculation of the percentages of the *JAK2* exon 12 mutant allele were calculated using the formula: %mut=(height of mut-peak)/(height of mut-peak + wt-peak)x100. Microsatellite PCR and gene copy number analyses for chromosome 9p were described previously.^{69,119}

Quantification of *JAK2* exon 14 mutation by allele-specific PCR¹¹⁹

DNA standard for quantification were prepared by mixture different ratios of normal human PBMC genomic DNA (*JAK2* wild type) and DNA from DAMI cell lines (homozygous for *JAK2*-V617F) (Figure 3). The allele-specific PCR to detect *JAK2*-V617F was carried out using 20 ng of genomic DNA, 45 nM forward primer *JAK2*-F (5'-GTTTCTTAGTGCATCTTTATTATGGCAGA-3') and 22.5 nM each of the allele-specific reverse primers *JAK2*-R-T (5'-6Fam-AAATTACTCTCGTCTCCACAGAA-3') and *JAK2*-R-G (5'-6Fam-TTACTCTCGTCTCCACAGAC-3') in a buffer containing 50 mM KCl, 10 mM Tris pH 8.0 and 1.5 mM MgCl₂. Thirty PCR cycles with denaturing at 94°C for 30 seconds, annealing at 61°C for 30 seconds, and extension at 72°C for 30 seconds were applied. The PCR products were analyzed using the ABI3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA). The percentage of chromosomes carrying the G>T transversion representing the *JAK2*-V617F allele (%T) were calculated using the same formula as above.

Detection of the *JAK2*-V617F mutation by single nucleotide primer extension

(SNaPshot Assay)

The SNaPshot Multiplex Kit (Applied Biosystems, Carlsbad, CA) was used to detect *JAK2*-V617F mutation in platelet RNA samples following the manufacturer's protocol. Primers 5'-CGTACGAAGAGAAGTAGGAG-3' and 5'-CCCATGCCAACTGTTTAGC A-3' were used to amplify *JAK2* from platelet cDNA. The primer 5'-AAGCATTTGGTTTTAAATTATGGAGTATGT-3' was used for the SNaPshot primer extension reactions.

Colony assays

Erythroid colony assays with peripheral blood cells were performed using commercial reagents as previously described.¹¹⁷ Media #04531 (without Epo) from Stem Cell Laboratories (Vancouver, BC, Canada) were used for EEC cultures, whereas media #04441 (with 3U/ml human recombinant Epo) were used to grow BFU-E. PBMCs from patients were plated at a density of 5×10^5 cells/ml methycellulose media and grown at 37°C. At day14, single erythroid colonies were picked and DNA from single colonies was isolated using Chelex-100 Resin (Biorad, Hercules, CA).¹¹⁸ Alternatively, DNA and RNA from single colonies were isolated using the peqGold-TriFast reagent (Peqlab Biotechnologie, Erlangen, Germany). Reverse transcription was carried out using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, USA). To confirm the erythroid lineage identity of EECs, expression of *glycophorin A* was used as a marker. Primers used were; human *glycophorin A* forward, 5'-CATCTCATCACAGACAAATGATACG-3' and reverse, 5'-TCAGAGAAATGATGGGCAAGT-3'. Expression of *β-actin* was used as a control for the quality of cDNA. The primers used were: forward, 5'-CTCTTCCAGCCTTCCTTCCT-3' and reverse, 5'-ATGCTATCACCTCCCCTGTG-3'.

T cell cloning

PBMCs of patients with *JAK2-V617F* were cloned as described.¹²⁰ Briefly, cells were stimulated with PHA (1μg/ml), IL-2 (100U/ml), and irradiated (35Gy) allogeneic PBMCs (5×10^5 cells/ml) in RPMI-1640 medium (GIBCO, Paisley, UK) supplemented with 5% human serum. DNA from individual clones was analyzed for the presence of *JAK2-V617F*. Flow cytometry analysis of clones positive for *JAK2-V617F* with antibodies against CD3 and CD56 was performed on a Cyan_{ADP} flow cytometer using Summit software (DakoCytomation, Fort Collins, USA).

Statistical analysis

We used linear and ordinal regression to analyze the correlations between disease duration and the percentage of mutant allele and between the percentage of mutant allele

and the number of lineages involved. SPSS version 15.0 (SPSS Inc., Chicago, IL) was used for the analysis.

Results

We screened 143 patients with myeloproliferative disorders and 10 patients with secondary erythrocytosis for the presence of *JAK2*-V617F and *JAK2* exon 12 mutations. Among these patients, 70 were PV, 54 were ET and 19 were PMF. In accordance with what have been reported by Scott LM ⁸², *JAK2* exon 12 mutations were only found in patients with PV, not in ET, PMF or secondary erythrocytosis. We found 5 out of 11 *JAK2*-V617F negative PV patients carried *JAK2* exon 12 mutations. Interestingly, one patient carries both *JAK2*-V617F and a *JAK2* exon 12 mutation. The screening result was summarized in Table 1. We found 5 different *JAK2* exon 12 mutations in 6 PV patients in our cohort (Figure 1). Until now, altogether 14 different *JAK2* exon 12 mutations including point mutation, 3 or 6 base pair deletion and insertion have been reported.⁸²⁻⁸⁸

Table 1. *JAK2* exon 12 mutations screening in MPD patients.

disease type	number of patients	V617F	number of patients	number of patients with 3-6 bp del in exon12
PV	70	+	59	1
		-	11	5
ET	54	+	35	0
		-	19	0
PMF	19	+	8	0
		-	11	0
SE	10	+	0	0
		-	10	0
normals	4	+	0	0
		-	4	0

PV, polycythemia vera; ET, essential thrombocythemia; PMF, primary myelofibrosis; SE, secondary erythrocytosis; del, deletion. Data from Basel in reference 83.

In our further study, we included 21 *JAK2*-V617F positive PV patients, all of the 6 PV patients carrying *JAK2* exon 12 mutations from our cohort and 2 patients with *JAK2* exon12 mutations from Vienna. The clinical data of patients involved in this study is listed in Table 2.

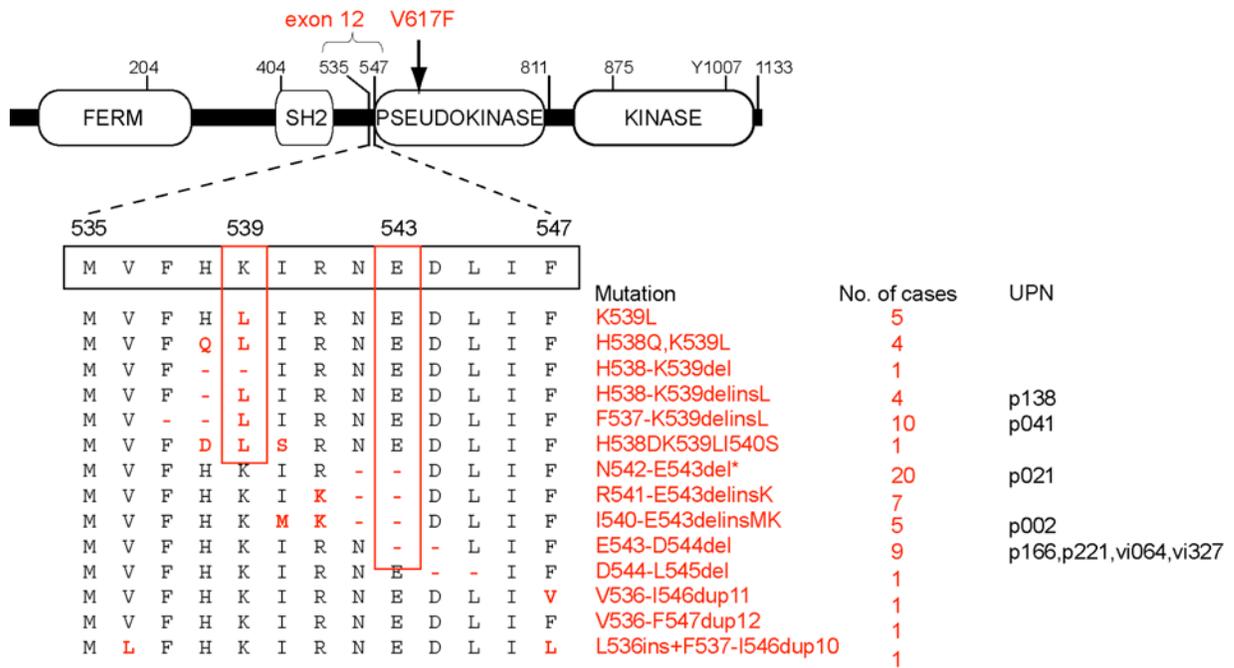


Figure 1. Schematic presentation of JAK2 exon 12 mutations and JAK2-V617F.

The location of exon 12 mutations and JAK2-V617F in the Jak2 protein is shown (top). The amino acid changes caused by the individual exon 12 mutations are shown below using the single letter code. The frequency of each mutation is expressed by the number of cases reported so far. All cases with mutation in JAK2 exon 12 published to date⁸²⁻⁸⁸ and own cases are shown. UPN, unique patient number.

Table 2. Clinical features at diagnosis of patients carrying *JAK2* exon12 mutations and *JAK2*-V617F mutation.

exon12 UPN	Sex/Age	Blood paramaters at diagnosis				Red cell mass	serum Epo level (U/l) (normal 12-23 U/L)	EEC	palpable splenomegaly	bone marrow trephine details
		Hb (g/L)	Hct (%)	WBC (10 ⁹ /L)	Plt (10 ⁹ /L)					
p002	F/52	226	0.66	6.1	215	ND	<2.0	yes	no	3
p041	M/57	214	0.68	7.5	297	40.8	17.2	yes	no	0
p138	F/71	194	0.64	8.6	102	ND	1	yes	no	3
p021	M/59	200	0.56	9.4	388	ND	5.2	yes	no	0
p166	F/55	170	0.54	9	956	ND	0.1	yes	no	3
p221	F/37	171	0.58	9.3	689	ND	3	yes	yes	3
Vi064	F/67	199	60	11.2	700	ND	<2.5	yes	no	0
Vi327	F/80	177	62	5.7	253	ND	ND	yes	no	0

exon14 UPN	Sex/Age	Blood paramaters at diagnosis				Red cell mass	serum Epo level (U/l) (normal 12-23 U/L)	EEC	palpable splenomegaly	bone marrow trephine details
		Hb (g/L)	Hct (%)	WBC (10 ⁹ /L)	Plt (10 ⁹ /L)					
p104	M/76	163	0.49	10.3	630	32.5 (25)	4.4	yes	no	3
p021	M/59	200	0.56	9.4	388	ND	5.2	yes	no	3
p023	M/71	209	0.65	11	645	ND	0	yes	no	ND
p115	F/91	170	0.52	11.5	764	ND	ND	yes	no	ND
p052	F/85	178	0.5	6.3	302	ND	ND	yes	no	ND
p026	M/73	194	0.57	11.3	447	ND	3.6	yes	no	2
p022	M/31	161	0.48	14.4	270	37.2 (25.5)	1.1	yes	no	2
p038	F/62	207	0.62	10.9	427	ND	1.7	yes	yes	3
p025	M/77	207	0.62	11.3	459	45.1 (26)	12.4	yes	no	ND
p046	F/72	202	0.63	9.5	284	ND	0.1	yes	no	ND
p033	M/77	191	0.6	12.6	726	ND	1.2	yes	no	ND
p103	F/82	182	0.49	11.3	382	ND	1.3	yes	no	3
p069	M/39	219	0.67	6.1	187	ND	3	yes	yes	2
p024	M/56	175	0.51	11	927	ND	2	yes	yes	2
p093	M/62	163	0.47	6.5	585	34.4 (24.5)	3.9	yes	no	3
p116	M/40	166	0.52	9.6	598	40 (28)	4.4	yes	yes	3
p053	M/70	225	0.71	8.6	238	ND	2.3	yes	no	3
p136	M/76	203	0.52	9.5	598	ND	2.9	yes	no	2
p070	M/73	217	0.68	9.6	289	ND	0.3	yes	yes	ND
p035	M/73	208	0.66	18.5	337	ND	9.7	yes	yes	ND
p016	M/74	187	0.6	12.2	1084	ND	ND	yes	yes	ND

UPN, unique patient number; Hb, hemaglobin; Hct, hematocrit; WBC, white blood cell count; Plt, platelet count; ND, not done. Numbers in bracket in the red cell mass column indicate expected normal red cell mass in each individual patient tested. Numbers in bone marrow trephine: 0 indicates not typical for PV, 1 indicates typical cytology, 2 indicates typical histology, and 3 indicates both were typical.

To quantitate the presence of *JAK2* exon 12 mutations in hematopoietic lineages, we developed a sensitive allele discrimination assay that exploits the frequent presence of deletions of 3 or 6 bases in patients with *JAK2* exon 12 mutation (Figure 2). And for quantification of *JAK2*-V617F, we had previously developed sensitive allele-specific PCR assay (AS-PCR)¹¹⁹ (Figure 3). Using these assays we could reliably detect 1% of mutant alleles.

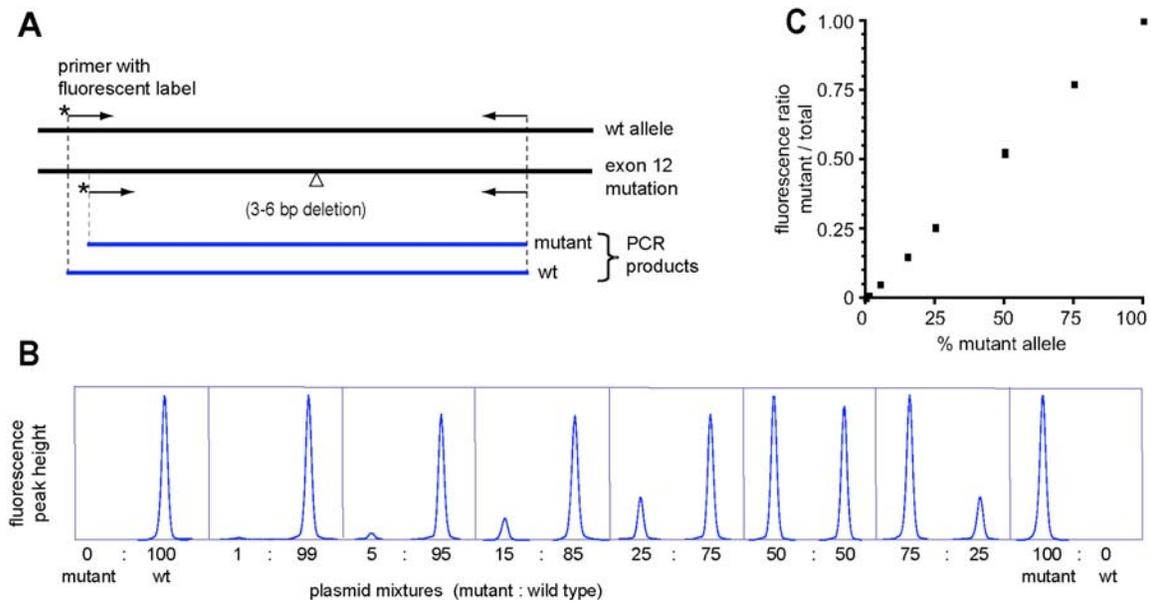


Figure 2. Allele discrimination assay to quantitate *JAK2* exon 12 mutations.

A) Design of the PCR assay. One of the primers was fluorescently labeled (asterisk). Exon 12 mutations with deletions of nucleotides will yield PCR products that differ in length from the wild type allele. B) Mixtures of plasmids containing wild type *JAK2* and *JAK2* exon 12 mutation were used as standard. The chromatograms of a dilution series for an exon 12 mutation with a 6 nucleotides deletion (I540-E543delinsMK) is shown. C) Standard curve. Quadruplicate reactions were performed and the percentages of exon 12 mutation in the DNA templates (x-axis) were plotted against the ratios of the fluorescent intensities (y-axis). Error bars indicate standard deviation. Separate standard curves were generated with cloned fragments for each of the other exon 12 mutations studied (not shown)

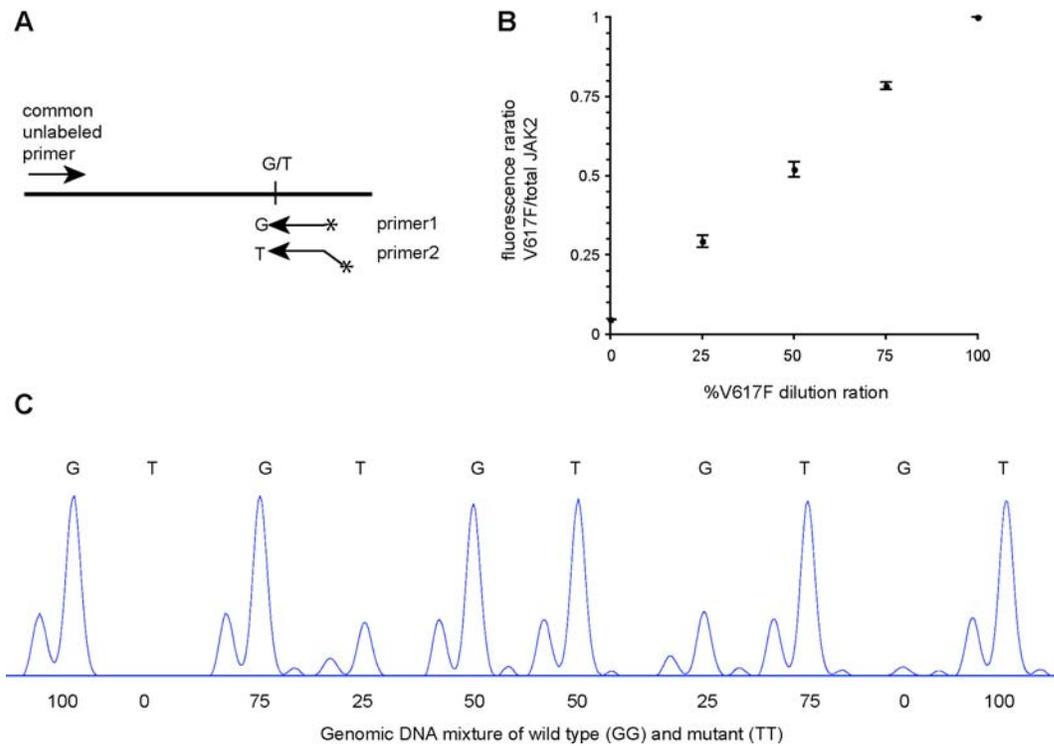


Figure 3. Allele-specific PCR to quantify *JAK2-V617F* mutation.

A) Strategy of allele-specific PCR. Arrows indicate primer positions: one common forward primer and two fluorescently labeled reverse primers (marked by asterisks). Note that one of the labeled primers is extended by 3 nonhomologous nucleotides to allow separation of the PCR products by size. B) Analysis of the linearity of the *JAK2-V617F* allele-specific PCR. The reactions were performed using homozygous wild-type (G) and homozygous mutant (T) genomic DNA dilutions with increasing proportion of the homozygous mutant DNA. Quadruplicate reactions were performed. The G and T peak fluorescence ratios were determined and plotted for each genomic DNA dilution. Error bars indicate standard deviation. C) The chromatograms of five DNA samples from panel B are shown.

We studied the lineage distribution of *JAK2* mutations in peripheral blood of 8 PV patients with mutations in exon 12 and in 21 PV patients with *JAK2-V617F* (Figure 4). Five different *JAK2* exon 12 mutations were observed by sequencing and all of them contained deletions of 3 or 6 bases (Figure 1). Peripheral blood cells were fractionated into granulocytes, platelets and mononuclear cells, which were further sorted into monocytes, Natural Killer (NK) cells, B and T cells. In all patients analyzed, exon 12 mutations were detectable in granulocytes, platelets and monocytes, with the highest allelic ratios in most cases present in platelets and the lowest in monocytes (Figure 4, upper panel). Similarly, the *JAK2-V617F* mutation was present in granulocytes, platelets and with the exception of p104 also in monocytes (Figure 4, lower panel). Interestingly, in patient p021 we detected two different *JAK2* mutations: N542-E543del (exon 12) and *JAK2-V617F* (exon 14). Both mutations were present in granulocytes, platelets and monocytes, with the exon 12 mutation showing higher allelic ratios than *JAK2-V617F*.

In contrast to the myeloid lineages, in which the exon 12 mutations or *JAK2-V617F* were always detectable, the lymphoid lineages showed large inter-individual differences (Figure 4). Only 3/8 patients (38%) with exon 12 mutations displayed detectable signal in lymphoid cells. In patients p221 and Vi064, a small subset of NK cells carried the mutation and only patient Vi327 showed an allelic ratio greater than 10% in NK and B cells. *JAK2-V617F* showed variable engagement of lymphoid lineages as well, with NK cells being most frequently involved (14/21, 67%) and in some cases showing very high (>70%) allelic ratios (p016, p033, p035, p103). B cells had low *JAK2-V617F* allelic ratios (<15%), except in one patient (p035). T cells of all patients were negative for exon 12 mutation and only 2/21 patients (p016 and p035) displayed *JAK2-V617F* in T cells, albeit with allelic ratios below 5%. To unambiguously determine the presence of *JAK2-V617F* in T cells, we established T and NK cell clones from peripheral blood of 10 PV patients with high *JAK2-V617F* allelic ratios. In 4/10 patients *JAK2-V617F* positive clones were obtained and these clones represented only 1% of the total clones analyzed (Figure 4). Surprisingly, only a single *JAK2-V617F* positive clone in patient p035 consisted of T cells, whereas *JAK2-V617F* positive clones in all other patients were NK

cells (Figure 5). Thus, T cells in peripheral blood only very rarely carry the *JAK2*-V617F mutation.

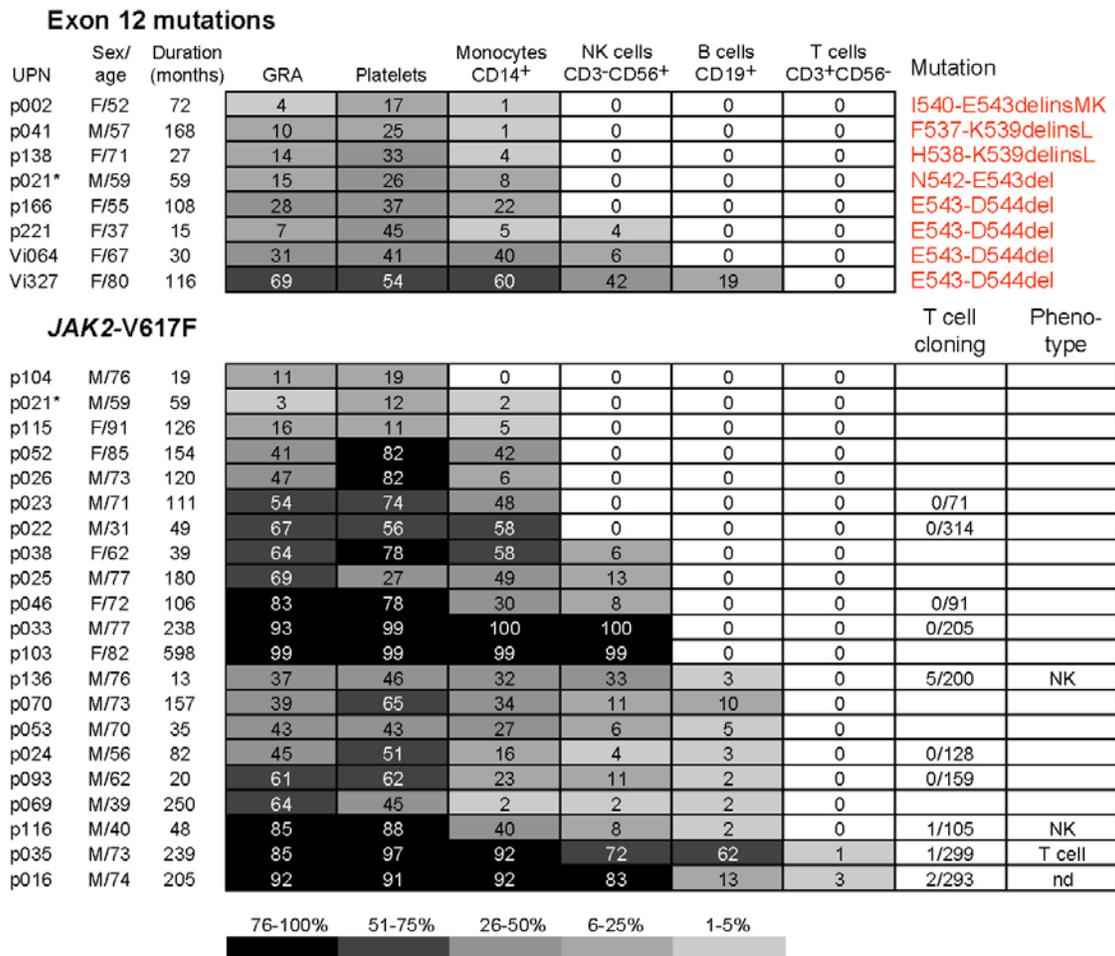


Figure 4. Lineage distribution of *JAK2* exon 12 mutations (top) and *JAK2*-V617F (lower part).

Numbers in boxes indicate the percentages of chromosomes 9 with exon 12 mutations and the shading of boxes corresponds to the ranges shown at the bottom. UPN, unique patient number; F, female; M, male; GRA, granulocytes; NK cells, natural killer cells; nd, not determined. Numbers in column for T cell cloning indicate *JAK2*-V617F positive clones/total clones analyzed. The phenotypes of *JAK2*-V617F positive clones were determined by flow cytometry. NK cell phenotype: CD3⁻CD56⁺; T cell phenotype: CD3⁺CD56⁻. *Note that patient p021 was positive for exon 12 mutation N542-E543del and for *JAK2*-V617F.

A correlation between the disease duration and the allelic ratios of *JAK2-V617F* in granulocytes, platelets, monocytes and NK cells was noted (Figure 6A), but no such correlation was found for exon 12 mutations (not shown). The percentages of mutant alleles also correlated with the number of lineages involved for both the exon 12 mutations and *JAK2-V617F* (Figure 6B).

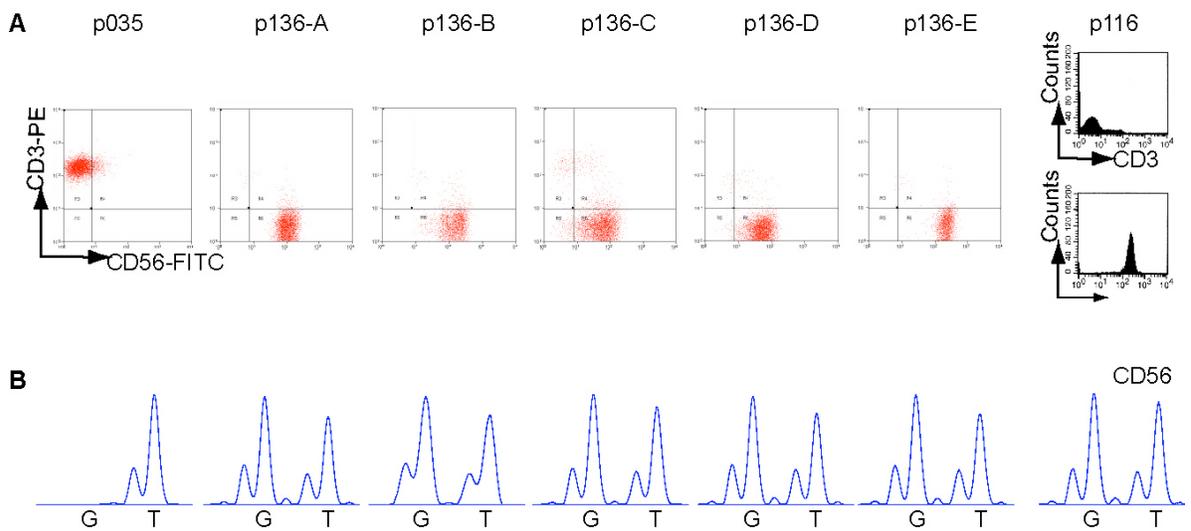


Figure 5. Phenotypic analysis of *JAK2-V617F* positive clones.

A) Flow cytometry analyses. One *JAK2-V617F* positive clone from patient p035 consisted of CD3⁺CD56⁻ T cells. All other positive clones from patients p136 and p116 were CD3⁻CD56⁺ Natural Killer cells. B) Allele-specific PCR for *JAK2-V617F*. The T cell clone from p035 was homozygous for *JAK2-V617F*, whereas the clones from patients p136 and p116 were heterozygous for *JAK2-V617F*.

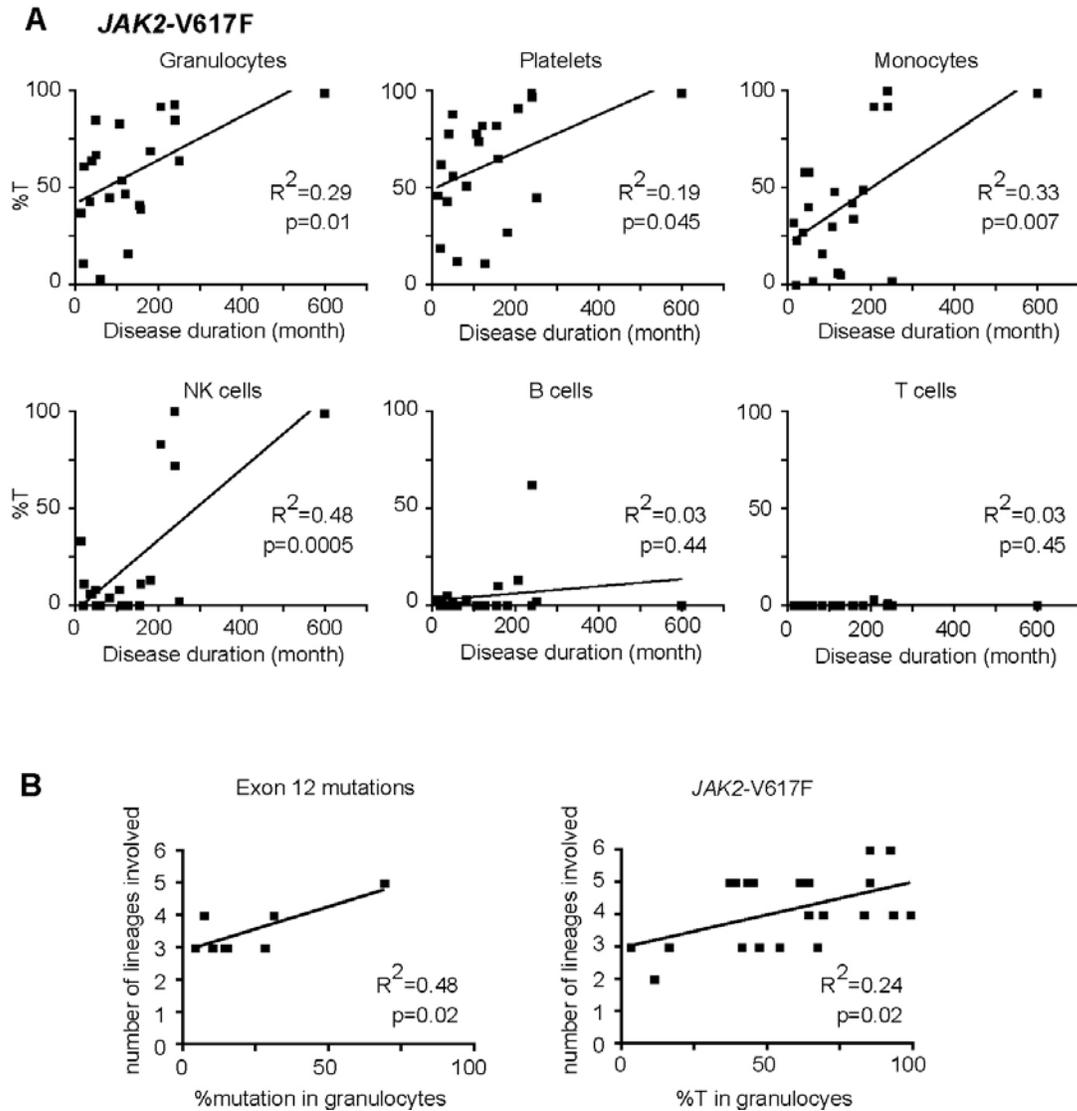


Figure 6. Statistical analysis.

A) Correlation between disease duration and the percentages of *JAK2* mutations. The R squared, and p values are shown in each plot. B) The correlations between the number of lineages involved and the percentages of *JAK2* exon 12 mutations (left panel) and the percentages of *JAK2*-V617F (right panel) in granulocytes are shown.

To examine the presence of the *JAK2* mutations in the erythroid lineage we performed colony assays in methylcellulose and determined the allelic ratios for each individual colony (Figure 7). Erythroid colonies that grew in the absence of Epo (EECs) were detected in all patients, although in some cases the total number of EECs was very small (Vi327, Vi064, p138, and p115). In 6 patients the exon 12 mutation was present in all EECs examined (Figure 7A). Among them, 4 patients carried the same exon 12 mutation (E543-D544del). The rest of patients with exon 12 mutations had some EECs with wild type *JAK2*. In 10/12 patients with *JAK2*-V617F, the mutation was found in all EECs, whereas in the remaining patients we detected variable proportions of EECs without *JAK2* mutations (Figure 7B). The erythroid phenotype of the colonies was confirmed by the presence of *glycophorin A* mRNA (Figure 8). Sequencing of the entire *JAK2* coding region in these colonies did not reveal any additional *JAK2* mutations. Interestingly, in the patient with two different *JAK2* mutations (p021) none of the erythroid colonies carried both mutations simultaneously, indicating that the exon 12 mutation and *JAK2*-V617F represent two separate clones. In patient Vi327 we found a homozygous exon 12 mutation in 4/32 colonies examined (Figure 7A). In this patient the allelic ratio of the exon 12 mutation was above 50% in granulocytes, platelets and monocytes (Figure 4). The homozygous colonies also exhibited loss of heterozygosity on chromosome 9p (9pLOH) (Figure 9). Copy number analysis of *JAK2* by real time PCR excluded gene amplification or deletion as the mechanism (not shown). In contrast, all PV patients with *JAK2*-V617F exhibited at least some homozygous colonies (Figure 7B). Only patient p021, who carries both *JAK2* mutations, did not display homozygous colonies.

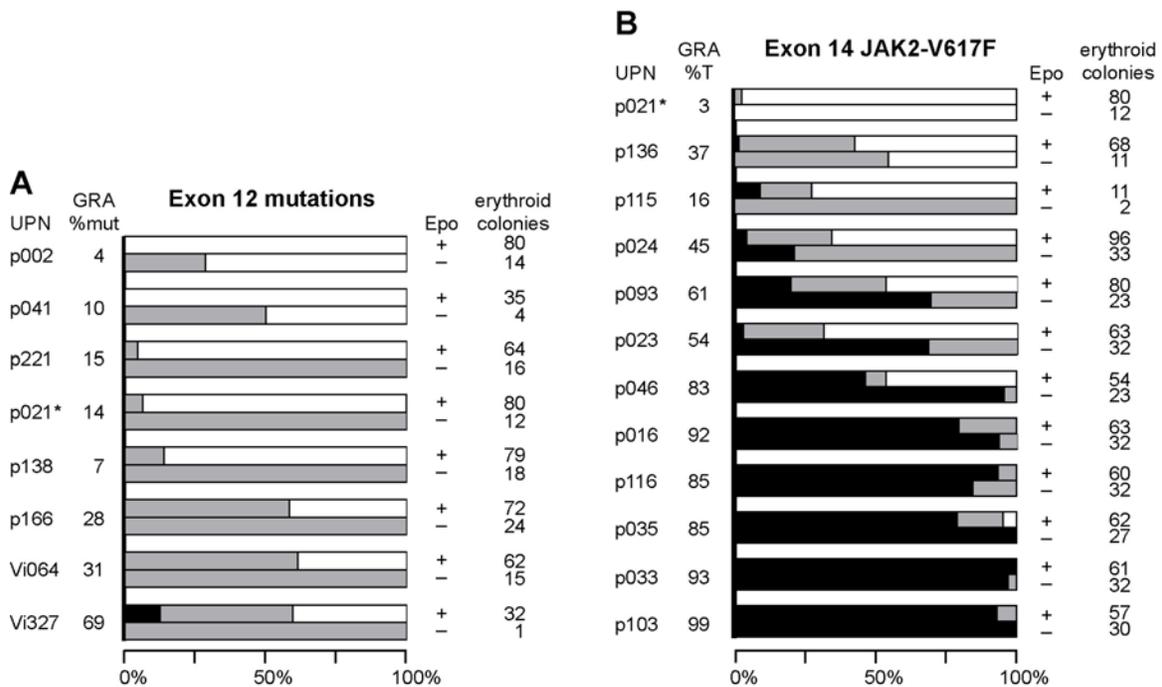


Figure 7. Distribution of *JAK2* mutations in erythroid progenitors.

The total number of erythroid colonies analyzed and the percentages of colonies with homozygous or heterozygous *JAK2* mutation or wild type *JAK2* are shown. Colony assays in methylcellulose were performed with peripheral blood cells of patients with *JAK2* exon 12 mutations (A) or *JAK2*-V617F mutation (B). Single erythroid colonies were picked and analyzed individually. Horizontal bars indicate the percentages of colonies with homozygous mutation (black boxes), heterozygous mutation (gray boxes) or wild type *JAK2* (white boxes). For each patient 2 bars are shown, the upper representing colonies grown in the presence of erythropoietin (Epo +), the lower representing colonies grown without erythropoietin (Epo -). The unique patient numbers (UPN) and the allelic ratios of the *JAK2* mutations (%mut or %T) in granulocytes (GRA) are shown in the two left columns and the total number of erythroid colonies analyzed is shown in the right column. *Note that in patient p021 colonies positive for exon 12 mutation and colonies with *JAK2*-V617F were found. None of these colonies carried both mutations simultaneously.

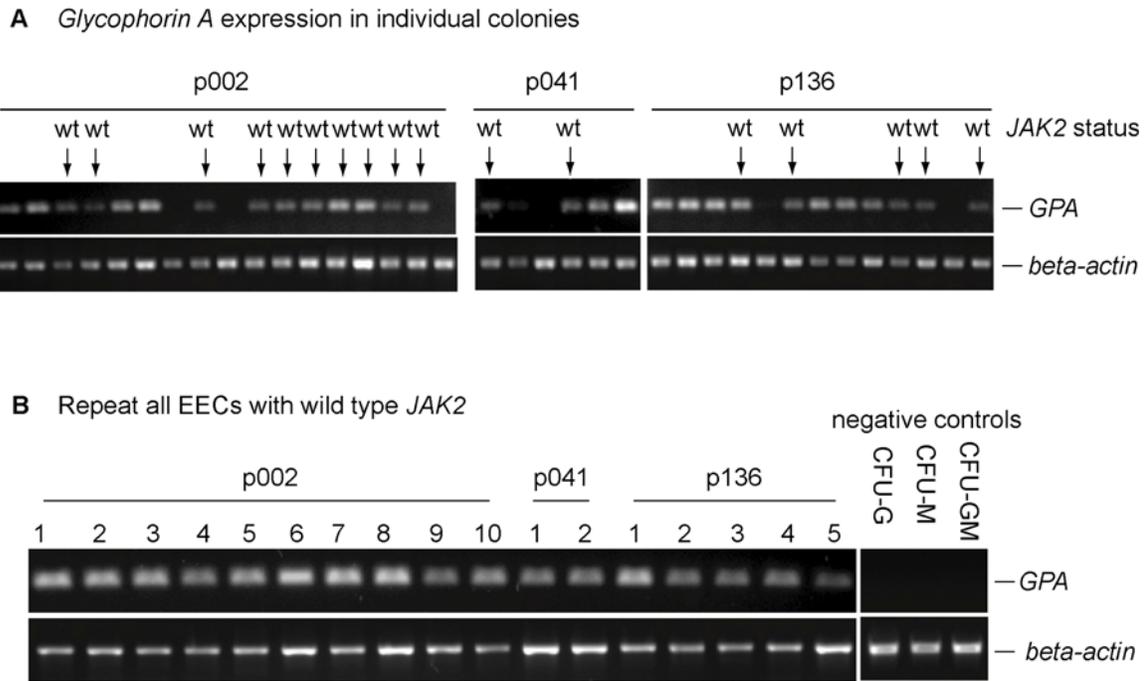


Figure 8. Expression of *glycophorin A* in single colonies.

Single colonies with erythroid morphology were picked and RT-PCR analysis was performed with primers specific for human *glycophorin A* (forward, 5'-CATCTCATCACAGACAAATGATACG-3' and reverse, 5'-TCAGAGAAATGATGGGCAAGT-3'). Beta actin (forward, 5'-CTCTTCCAGCCTTCCTTCCT-3' and reverse, 5'-ATGCTATCACCTCCCCTGTG-3') was used as a control for the quality of cDNA. A) Analysis in 3 patients is shown. wt, colonies with only wild type *JAK2*; *GPA*, *glycophorin A*. Only colonies expressing *glycophorin A* were included in the result presented in Figure 7. B) All colonies with wild type *JAK2* were re-analyzed for *glycophorin A*. Colonies with non-erythroid morphology (CFU-G, CFU-M and CFU-GM) are shown as negative controls.

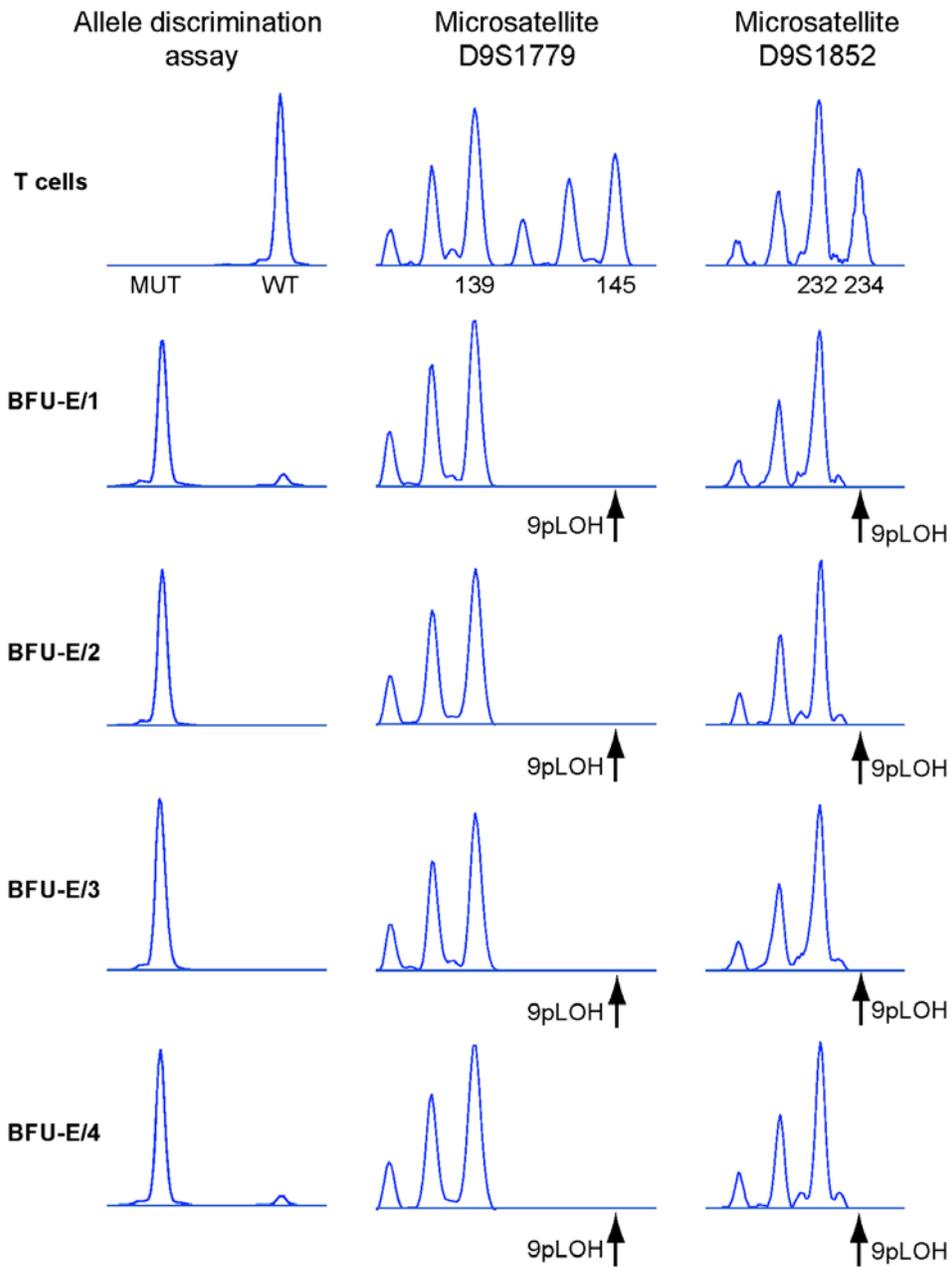


Figure 9. Molecular analysis of individual erythroid colonies of patient Vi327.

T cell DNA from patient Vi327 was used as control (top row). Allele discrimination assay shows presence of homozygous E543-D544del mutation in 4 burst forming unit-erythroid (BFU-E) colonies (left panel). Two microsatellite markers D9S1779 and D9S1852 demonstrate loss of heterozygosity on chromosome 9p (9pLOH) in the same colonies (middle and right panel). Numbers indicate allele sizes.

Discussion

Our findings show that the lineage distributions of exon 12 mutations and *JAK2*-V617F are similar (Figure 4). Thus, the lineage distribution does not explain why exon 12 mutations are associated solely with a PV phenotype,⁸² while *JAK2*-V617F can also be found in essential thrombocythemia and primary myelofibrosis. The reason for the large inter-individual variation in the allelic ratios of mutant *JAK2* in lymphoid lineages is unclear. A correlation between disease duration and the allelic ratios of *JAK2*-V617F was noted for the myeloid lineages and for NK cells, but not for B cells (Figure 6). In the original report, *JAK2* exon 12 mutations have not been observed in T cells,⁸² and were also absent in our study using a more sensitive detection assay. The presence of *JAK2*-V617F in T cells is controversial, since it has been described in T cells from most PV patients in one study,⁸⁰ but was rare in other reports.^{78,79,114} All these studies were performed using bulk cell populations. Our clonal analysis unambiguously shows that peripheral blood T cells only rarely carry the *JAK2*-V617F mutation (Figure 4B and 5). *JAK2*-V617F positive CD34⁺ progenitors from MPD patients were shown to be capable of differentiating into T cells in thymic organ cultures *in vitro*.⁷⁸ However, in patients this occurs very infrequently, possibly due to low frequency of *de novo* T cell genesis in adults.

Growth of EECs with only wild type *JAK2* has recently been reported in PV patients positive for *JAK2*-V617F¹²¹⁻¹²³. In contrast, no wild type EECs in PV patients were found by others.^{71,73,82,84} Our results confirm the finding of EECs with only wild type *JAK2* and indicate EECs can arise from additional as yet unknown mechanisms. These results suggest clonal heterogeneity of erythroid progenitors not only in patients with *JAK2*-V617F, but also in some patients with exon 12 mutations. Until now, exon 12 mutations were found exclusively in the heterozygous state. Here we show that progression to homozygosity occurred in a patient (Vi327) with exon 12 mutation and involved mitotic recombination resulting in 9pLOH (Figure 9), as commonly found in patients homozygous for *JAK2*-V617F.⁶⁹ A second case of homozygosity was found in another patient with exon 12 mutation by sequencing,⁸³ but material for clonal analysis was not

available in this patient. In contrast to the rare occurrence of homozygosity in patients with exon 12 mutations, the erythroid colonies of patients with *JAK2*-V617F invariably showed a proportion of homozygous colonies, as reported.⁷³ The only exception in our series was patient p021, in whom we found two different *JAK2* mutations (Figure 7). Analysis of single erythroid colonies in this patient demonstrated that two independent clones were present, one carrying an exon 12 mutation (N542-E543del) and the other with *JAK2*-V617F (Figure 7). Again, some EECs were negative for both *JAK2* mutations in this patient. Concurrent presence of *JAK2*-V617F with *MPL515* mutations was previously found in 3 patients with primary myelofibrosis and 3 patients with essential thrombocythemia.^{90,124} The allelic ratios for *MPL515* were higher than those for *JAK2*-V617F at all time points studied, but clonal analysis was not reported. Therefore, it remains to be determined if these cases represent sequential somatic mutations that occurred in the same clone. Thus, our patient p021 is the first case with a documented bi-clonal pattern.

In summary, we show that the distribution of exon 12 mutations and *JAK2*-V617F are similar and do not explain why exon 12 mutations and *JAK2*-V617F can cause different phenotypes. Both mutations are very rare or absent in T cells. The likelihood of multiple lineage involvement increased with higher allelic ratios of the *JAK2* exon 12 and *JAK2*-V617F mutations and the allelic ratios of *JAK2*-V617F correlated with disease duration. Absence of the exon 12 and *JAK2*-V617F mutations in EECs from several patients suggests the existence of additional mutation(s) responsible for Epo hypersensitivity or independence. The presence of two independent clones, one with an exon 12 mutation and another with *JAK2*-V617F, further demonstrates clonal heterogeneity. These results are compatible with a model in which a stem cell or progenitor carries an as yet unknown mutation that is sufficient to cause growth of EECs and increase the likelihood for acquiring somatic mutations in *JAK2*.

RESULTS II

Effect of *JAK2*-V617F and *JAK2* exon12 mutations on terminal erythroid differentiation in patients with polycythemia vera — a pilot study

Abstract

Somatic activating *JAK2* mutations are found in more than 99% of patients with polycythemia vera (PV). Among them, about 95% PV patients carry *JAK2*-V617F mutation and about 4% patients carry *JAK2* exon 12 mutations. Burst forming unit-erythroid (BFU-E) is the earliest progenitor committed to the erythroid lineage, and the presence of homozygous BFU-Es is a common feature of most PV patients with *JAK2*-V617F. However, in about half of PV patients with *JAK2*-V617F, the homozygous erythroid colonies only constitute a small proportion of the total number of BFU-Es, and more than half patients with *JAK2* exon 12 mutations have only very few BFU-Es carrying the mutations. To address the question how a small number of cells with *JAK2* mutations can cause overproduction of red blood cells, we simultaneously determined the amount of mutant *JAK2* in BFU-Es as well as in purified reticulocytes from peripheral blood of PV patients with *JAK2*-V617F or *JAK2* exon 12 mutations. Reticulocytes were isolated from peripheral blood by leukodepletion with high purity. Preliminary results showed that some PV patients had similar levels of *JAK2* mutation in granulocytes and reticulocytes while others had much higher *JAK2* mutation in reticulocytes than in granulocytes. In the latter subset of patients, the ratio of *JAK2* mutation in reticulocytes was significantly higher than that in BFU-Es, indicating the cells carrying *JAK2* mutation proliferated more efficiently during terminal differentiation from BFU-Es to reticulocytes. Future directions including analysis of a larger cohort of samples and examination of clonal origin of reticulocytes using X-chromosome inactivation assays will further elucidate the impact of *JAK2* mutations on erythroid terminal differentiation.

Introduction

Burst forming unit-erythroid (BFU-E), is the earliest progenitor committed to the erythroid lineage.¹²⁵ The number of BFU-E colonies grown in methylcellulose culture corresponds to the number of erythroid progenitors in the blood or bone marrow. BFU-Es undergo a number of differentiation and proliferation stages and give rise to enucleated reticulocytes. Reticulocytes retain in the bone marrow for 24-48 hours before released into peripheral circulation, where they gradually lose their residual RNA and become fully matured red blood cells.¹²⁶ Reticulocytes are the latest population of erythroid lineage that can be traced by the presence of RNA.

In patients with polycythemia vera (PV), more than 95% carry *JAK2-V617F* mutation and almost all *JAK2-V617F* negative PV carry *JAK2* exon 12 mutations.^{69-72,82} The distinguishing feature of PV is the excessive production of mature red blood cells, which results in increased hemoglobin and hematocrit. Bone marrow transplantation assays with *Jak2-V617F* or *Jak2* exon 12 mutations and transgenic mouse models with *JAK2-V617F* showed that *JAK2* mutations are sufficient to cause PV phenotype, suggesting mutant *JAK2* is a major factor driving over-expansion of erythrocytes.^{70,82,101-105}

Most PV patients with *JAK2-V617F* had homozygous erythroid colonies as a result of mitotic recombination, which is rare in ET patients and PV patients with *JAK2* exon 12 mutations.^{69,73,123} Presence of homozygous colonies is considered as one of the cellular parameters to distinguish between *JAK2-V617F* positive PV and ET. However, in our previous study¹²⁷ (See also Results I, Figure 7B), we observed in about half of PV patients with *JAK2-V617F*, the homozygous erythroid colonies only constitute a small proportion of the total number of BFU-Es, and at the same time, the percentage of *JAK2-V617F* in granulocytes is below 60%. Albeit there are almost no homozygous colonies in PV patients with *JAK2* exon 12 mutations, similar pattern that only a small percentage of BFU-Es carries the mutation is observed in more than half of patients in our cohort¹²⁷ (See also Result I, Figure 7A). To explain how such a small proportion of *JAK2-V617F* homozygous progenitors or progenitors with heterozygous *JAK2* exon 12 mutations can

lead to a substantial increase in red cell population, we hypothesized the homozygous BFU-Es with *JAK2-V617F* or heterozygous BFU-Es with *JAK2* exon 12 mutations proliferate and differentiate into mature erythroblasts in a more efficient manner and dominate red blood cell production in a cell autonomous pattern. Data from preliminary experiments provide evidence that during terminal differentiation from BFU-Es to reticulocytes, the frequency of mutant allele got increased, indicating a substantial amplification occurred.

Materials and Methods

Patient samples

Eight PV patients with *JAK2*-V617F, two PV patients with *JAK2* exon 12 mutations, one PV patient with no known *JAK2* mutation and three ET patients with *JAK2*-V617F were included in this study. All patients were from Basel, Switzerland. The diagnosis of myeloproliferative disorders (MPD) was assigned using World Health Organization (WHO) criteria. The collection of patient samples was approved by the local ethics committees. Written consent was obtained from all patients. EDTA anticoagulated peripheral blood was collected and processed within 4 hours after collection.

Isolation of reticulocytes

Reticulocytes were isolated from 20 ml of human EDTA peripheral blood following a process of leukodepletion.^{128,129} Briefly, after centrifugation the blood at 700 g for 10 min, the plasma and the buffy coat layers were removed by aspiration. The packed red blood cells were washed 3 times with 50 ml 0.9% NaCl and centrifuged at 1200 rpm for 10 min to remove platelets. At the third wash, red blood cells were resuspended in 2 ml 0.9% NaCl and layered on top of a column consisting of 2 ml 1:1 mixture (by weight) of α -cellulose (C-8002, Sigma) and sigmalcell type 50 microcrystalline cellulose (S-5504, Sigma). After the blood went into the column completely, the column was eluted with 2 ml 0.9% NaCl (Figure 1). The reticulocyte-enriched eluate was washed twice with 0.9% NaCl and pelleted by centrifugation.

Purity control of reticulocytes

To check the purification efficiency, complete blood counts including reticulocytes before and after the purification process were performed using an Advia 120 Hematology Analyzer (Bayer, Leverkusen, Germany).

RNA isolation from reticulocytes

The reticulocytes-enriched pellet was lysed with peqGold-TriFast reagent (Peqlab Biotechnologie, Erlangen, Germany), mixed thoroughly and stored in -80°C prior to

processing. The RNA isolation was performed as recommended by the manufacturer's protocol. Reverse transcription was carried out using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, USA).

Erythroid colony assay

Erythroid colony assay was performed using the same sample as the one in reticulocyte isolation. Media #04441 (with 3U/ml human recombinant Epo) (Stem Cell Technologies, Vancouver, Canada) were used to grow BFU-Es. PBMCs from patients were plated at a density of 5×10^5 cells/ml methycellulose media and grown at 37°C. At day14, single erythroid colonies were picked and DNA from single colonies was isolated using Chelex-100 Resin (Biorad, Hercules, USA).¹¹⁸

Quantification of *JAK2-V617F* in reticulocytes

Allele-specific PCR was used to quantify the presence of *JAK2-V617F* in BFU-E colonies and allele discrimination assay was used to quantify *JAK2* exon 12 mutations.¹²⁷ The expression level of *JAK2-V617F* was quantified by Real-Time PCR (RT-PCR) as described¹³⁰ using Taqman® Universal PCR Master Mix (Applied Biosystems, Branchburg, USA) on a 7500 Fast Machine (Applied Biosystems, Foster City, USA). Two forward primers specific to *JAK2* wild type and *JAK2-V617F* respectively, one common reverse primer and a FAM-labeled probe specifically recognizing the amplicon are used. The sequences are as follows:

JAK2-WT forward: 5' -GCGCGGTTTTAAATTATGGAGTATGTG-3'; *JAK2-V617F* forward: 5'-GCGCGGTTTTAAATTATGGAGTATGTT-3', the common reverse primer 5'-CCGCTTTTTCAGATATGTATCTAGTGATCC-3' and 6-FAM probes 5'-TGGAGACGAGAATATTCTGGTTCAGGAGTTTG-3'.

Results and Discussion

Several methods are used to purify reticulocytes or isolate reticulocytes RNA. We tried and compared a couple of methods and selected the one based on leukodepletion. The first method is based on the observation that mature red blood cells as well as reticulocytes are lysed by red cell lysis buffer (8.3g/L NH_4Cl , 1.0g/L KHCO_3 , 0.1 mM EDTA). After pelleting other type of cells by centrifugation, the reticulocytes RNA is present in the supernatant, and can be precipitated by acid. Because the mature red blood cells do not contain DNA and RNA, and reticulocytes do not contain DNA, the resultant precipitation is RNA from reticulocytes. However, it is impossible to monitor the purity of RNA. Theoretically, only red cells are lysed by the buffer, but during blood processing and lysis, some cells are dead and release their content (DNA and RNA) into the lysis solution, contaminating the reticulocyte RNA, which is hard to control. A second method is based on Dynabeads-mediated positive selection. Briefly, beads coupled with CD71 antibody are mixed with blood cells and the CD71 positive cells are bound to the beads, and thus separated from other types of cells. It is also difficult to judge the purity of reticulocytes, because the beads are not easy to get rid of thus makes immunostaining or FACS analysis of purity difficult. The third method is the one we finally decided to use in this study. It is based on the capacity of the special cellulose to retain leukocytes in the matrix of a column, while the red blood cells including reticulocytes can pass through (Figure 1).^{128,129} We modified the original method in the following ways. First we centrifuge the blood to remove platelet rich plasma and buffy coat. Second, to remove the remaining platelets, we washed the red cell pellet before and after passing the column. We can routinely do the blood count before and after purification process to infer the purity of the reticulocytes. Using this method, we normally get rid of more than 99% of leukocytes and platelets (Table 1). After removal of platelet rich plasma and buffy coat, a main part of leukocytes and the majority of platelets were removed. The rest of leukocytes were further removed by passing the cellulose column. Thus following this processing procedure, RNA isolated from reticulocytes-enriched pellet well represents reticulocytes RNA with high purity.

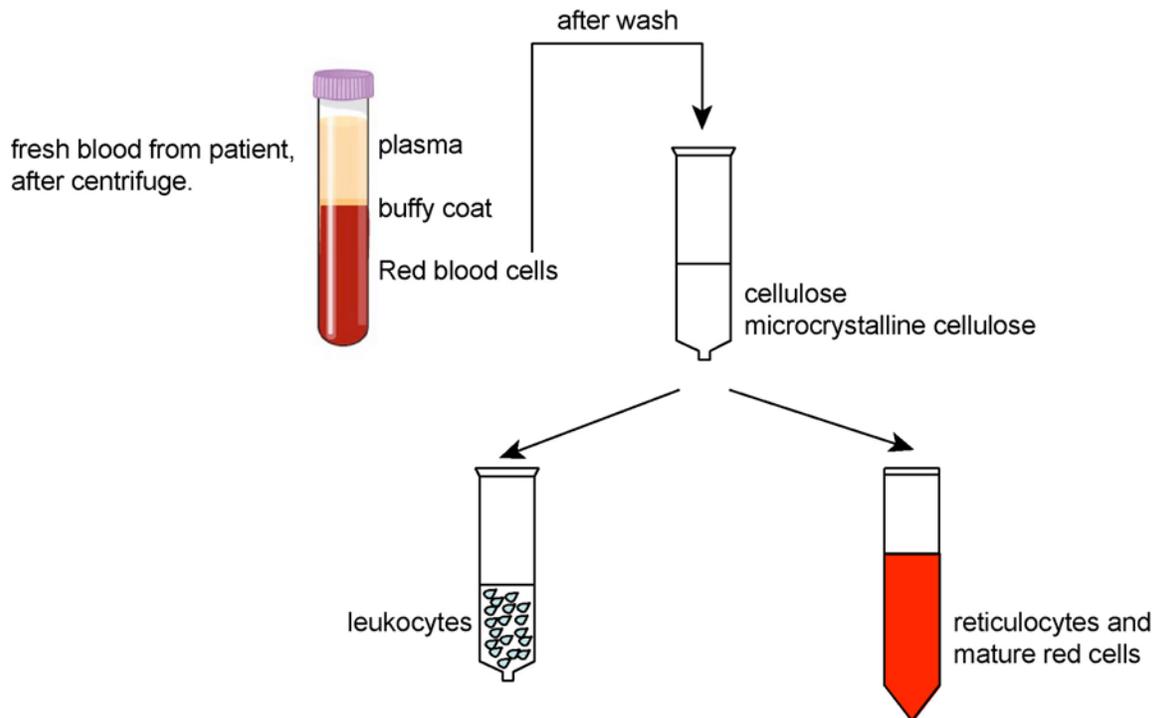


Figure 1. Work flow of reticulocytes isolation based on leukodepletion. Fresh peripheral blood was first centrifuged to separate platelet rich plasma, buffy coat and red blood cells. The red blood cells were washed 3 times with 0.9% NaCl and loaded on top of a column made of 1:1 (by weight) α -cellulose and microcrystalline cellulose. The leukocytes were kept in the column (left) and the red blood cells including reticulocytes passed through the column (right).

Table 1. WBC, platelet, and reticulocyte count from whole blood, before and after purification.

diagnosis and unique patient number	WBC (10 ⁹ /l)			PLT (10 ⁹ /l)			RETIC (10 ⁹ /l)		
	whole blood	Pre	Post	whole blood	Pre	Post	whole blood	Pre	Post
PV <i>JAK2</i> wild type									
p039G	6.07	0.68	0.01	200	0	0	47	33.5	19.7
PV <i>JAK2</i> -V617F									
p019D	7.53	2.15	0.09	1230	2	0	NA	74.5	33
p022I	11.68	3.61	0.2	411	1	1	129	55.3	42.9
p116M	14.73	1.55	0.03	474	4	1	132	37.1	27
p024N	10.03	2.17	0.09	930	1	1	102	39.5	28.5
p214C	6.86	1.16	0.12	273	1	1	25	23.6	13.7
p038H	6.51	2.84	0.03	375	2	1	70	43.3	45.4
p023S	10.58	0.52	0.03	405	0	0	142	29.5	22.1
p204B	6.09	0.37	0.03	460	2	2	70	18.1	8.3
PV <i>JAK2</i> exon 12 mut									
p138K	14.28	1.97	0.01	71	3	1	188	31.9	25.8
p166E	11.59	4.96	0.14	1102	0	0	126	63.4	42.5
ET <i>JAK2</i> -V617F									
p018K	12.7	5.76	0.4	92	0	1	44	104.4	81.5
p192C	10.1	3.87	0.25	787	1	1	88	43	38.9
p199C	6.3	1.59	0.04	507	1	1	64	53.5	22.9

Abbreviations: WBC, white blood cell; PLT, platelet; RETIC, reticulocyte; NA, not available. Pre, before passing the column; post, after passing the column.

Depending on the availability of patient samples during the period of study, and the percentage of mutant allele in the granulocytes, we conducted experiment with samples from 8 PV patients with *JAK2*-V617F, 2 PV with *JAK2* exon 12 mutations, 1 PV with no known *JAK2* mutation and 3 ET patients with *JAK2*-V617F. Most patients had a heterozygous pattern in their granulocytes except one patient (p116), who had a homozygous pattern in his granulocytes. The clinical data of these patients were listed in Table 2. RNA from reticulocytes was prepared from all patients included in this study. As a pilot experiment, we performed erythroid progenitor assay in 5 PV patients with *JAK2*-V617F and one PV patients with *JAK2* exon 12 mutation using the same sample for reticulocyte isolation. We can analyze in parallel the mutation status in erythroid progenitors as well as terminally differentiated reticulocytes from the same time point. Because reticulocytes do not have nuclei, thus no DNA, we measured the expression of mutant *JAK2* at RNA level. Our previous finding showed linear correlation between the percentage of *JAK2*-V617F mRNA and the allelic ratio of *JAK2*-V617F in genomic DNA,¹⁰⁵ and therefore we believe that mRNA level would reflect the ratio of mutant

JAK2 positive cells. We then analyzed the expression of mutant *JAK2* in reticulocytes to infer the amount of cells carrying the mutations.

Table 2. Clinical data from patients included in this study.

diagnosis and unique patient number	Sex/age	JAK2-V617F GRA	Hb (g/l)	HCT (l/l)	WBC (x10 ⁹ /l)	PLT (x10 ⁹ /l)	Retic (x10 ⁹ /l)	treatment
PV <i>JAK2</i> wild type								
p039G	M/62	0	133	0.4	6.07	200	47	Hydroxyurea
PV <i>JAK2</i> -V617F								
p019D	F/60	49	113	0.34	7.53	1230	NA	Lanvis
p022I	M/34	55	143	0.45	11.68	411	129	phlebotomy
p116M	M/43	92	142	0.43	14.73	474	132	phlebotomy
p024N	M/59	68	146	0.46	10.03	930	102	phlebotomy
p214C	F/78	4	147	0.43	6.86	273	25	hydroxyurea
p038H	F/65	74	135	0.43	6.51	375	70	phlebotomy
p023S	M/74	39	168	0.46	10.58	405	142	hydroxyurea
p204B	M/78	60	120	0.35	6.09	460	70	hydroxyurea
PV <i>JAK2</i> exon 12 mut								
p138K	F/73	36	115	0.42	14.28	71	188	phlebotomy
p166E	F/49	29	144	0.47	11.59	1102	126	none
ET <i>JAK2</i> -V617F								
p018K	F/81	47	94	0.27	12.7	92	44	not known
p192C	M/44	17	129	0.41	10.1	787	88	not known
p199C	F/52	2	100	0.29	6.3	507	64	hydroxyurea

Abbreviations: Hb, hemoglobin, normal range 120-160 g/l for female and 140-180 g/l for male; HCT, hematocrit, normal range 0.36-0.46 l/l; WBC, white blood cells, normal range 3.5-10.0×10⁹/l; PLT, platelet, normal range 150-450×10⁹/l; retic, reticulocyte, normal range 40-140×10⁹/l.

We first measured the expression of mutant *JAK2* in reticulocytes by quantitative Real-Time PCR (qRT-PCR) and identified two subgroups of PV patients with *JAK2*-V617F mutation. One group of patients had similar level of *JAK2* mutation in their granulocytes and reticulocytes (p039G, p019D, p022I, p116M, named group A). The 3 patients with ET (p018K, p192C, p199C) had the similar pattern (Table 3, upper panel and Figure 2A). In contrast, the other group of PV (p024N, p214C, p038H, p023S, P204B) showed higher level of mutations in their reticulocytes than in the granulocytes. The two patients with *JAK2* exon 12 mutations (p138K, p166E) belong to this group (Table 3, lower panel and Figure 2B). Patient p039 do not have *JAK2*-V617F or exon 12 mutations in his granulocytes, although it is possible that the low amount of cells carrying the mutation

might not be detected in the bulk of granulocytes. If the *JAK2* mutation is the causative mutation in this patient, the mutation should be present in a proportion of erythroid progenitors and reticulocytes. However, we failed to detect known *JAK2* mutations either from erythroid progenitors or reticulocytes. This indicates another yet unknown mutation might contribute to the PV phenotype in this patient. (Table 3)

Table 3. *JAK2* mutation analysis in reticulocytes and erythroid progenitors.

diagnosis and unique patient number	Sex/age	GRA <i>JAK2</i> mut%	RETIC <i>JAK2</i> mut%	BFU-E heter%	BFU-E homo%	mutant allele ratio in BFU-E
PV <i>JAK2</i> wild type						
p039G	M/62	0	0	0	0	0
PV <i>JAK2</i> -V617F						
p019D	F/60	49	48	ND	ND	NA
p022I	M/34	55	57	ND	ND	NA
p116M	M/43	92	99	6.7	93.3	96.7
ET <i>JAK2</i> -V617F						
p018K	F/81	47	47	ND	ND	NA
p192C	M/44	17	26	ND	ND	NA
p199C	F/52	2	9	ND	ND	NA
PV <i>JAK2</i> -V617F						
p024N	M/59	68	83	31.3	10	25.7
p214C	F/78	4	24	9	0	4.5
p038H	F/65	74	90	1.4	40.8	41.5
p023S	M/74	39	74	12.5	15	21.2
p204B	M/78	60	81	ND	ND	NA
PV <i>JAK2</i> exon 12 mut						
p138K	F/73	36	53	23.7	0	11.9
p166E	F/49	29	55	ND	ND	NA

Abbreviations: GRA, granulocyte; RETIC, reticulocyte; heter, heterozygous; homo, homozygous; BFU-E, burst forming unit-erythroid; ND, not done; NA, not available. The *JAK2* mutant allele ratio in BFU-Es was calculated as follows: Heterozygous colony has one mutant allele and homozygous colony has two mutant alleles. So the mutant allele ratio = (number of heterozygous colony+2× number of homozygous colony)/2×number of total colonies analyzed.

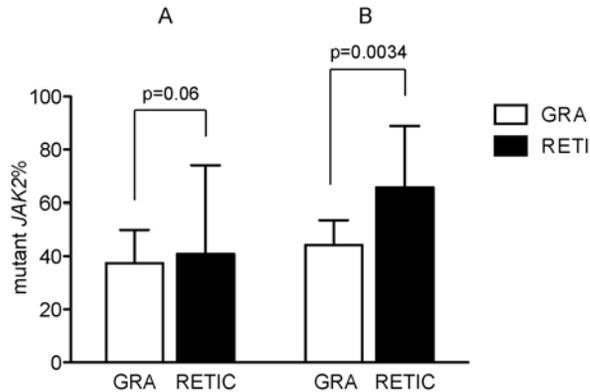


Figure 2. Comparison of the percentage of mutant *JAK2* in granulocytes and reticulocytes defined two subgroups. Patients of group A had similar amount of mutant *JAK2* in granulocytes to that in reticulocytes. Patients of group B had mutant *JAK2* in reticulocytes significantly higher than that in granulocytes. Paired student t-test was used. GRA, granulocyte; RETI, reticulocyte.

For pilot experiment, five PV patients from group B (4 with *JAK2*-V617F and 1 with *JAK2* exon 12 mutation) and one PV patient homozygous for *JAK2*-V617F from group A were chosen for erythroid progenitor assay in methylcellulose in the presence of EPO. The percentage of heterozygous and homozygous colonies is listed in Table 3. As reported, most PV patients with *JAK2*-V617F carried some homozygous colonies.⁷³ The distribution of heterozygous and homozygous colonies varies among patients, though the number of patients is limited. In PV patients with *JAK2*-V617F, one patient (p038H) predominantly yielded wild type and homozygous colonies, but very few heterozygous colonies. Another patient (p214C) had only a small number of heterozygous colonies, and all other erythroid colonies were wild type.

Based on the percentage of heterozygous and homozygous colonies, we calculated the percentage of mutant *JAK2* in the total erythroid progenitors, and compared it with the mutant *JAK2* in reticulocytes (Figure 3). Heterozygous colony has one mutant allele, and homozygous colony presents two mutant alleles. So the mutant allele ratio was calculated as (number of heterozygous colony+2× number of homozygous colony) /2×number of total colonies analyzed. The result showed that the relative ratio of mutant *JAK2* in

reticulocytes was more than two fold higher than that in BFU-Es. The increase in the percentage of mutant *JAK2* in a cell population reflected a selective advantage of cells carrying mutations. *JAK2*-V617F occurs in hematopoietic stem cells (HSCs), but in PV patients, the mutant allele frequency was similar between HSC and more differentiated progeny, including common myeloid progenitor (CMP), granulocyte-macrophage progenitor (GMP), and megakaryocyte-erythroid progenitor (MEP).⁷⁷ Thus our data suggested that cells with *JAK2* mutations were selectively amplified in these patients during terminal erythroid differentiation from BFU-Es to erythroblasts. Amplification of erythroid cells with *JAK2* mutation has been observed by in vitro culture bone marrow CD34⁺CD38⁺ progenitors in the presence of EPO, showing increased *JAK2* mutant alleles after 7 days.¹²³ Instead of using in vitro culture system to grow erythroblasts, primary reticulocytes from patients were directly investigated in our study, which is more representative and less affected by culture conditions.

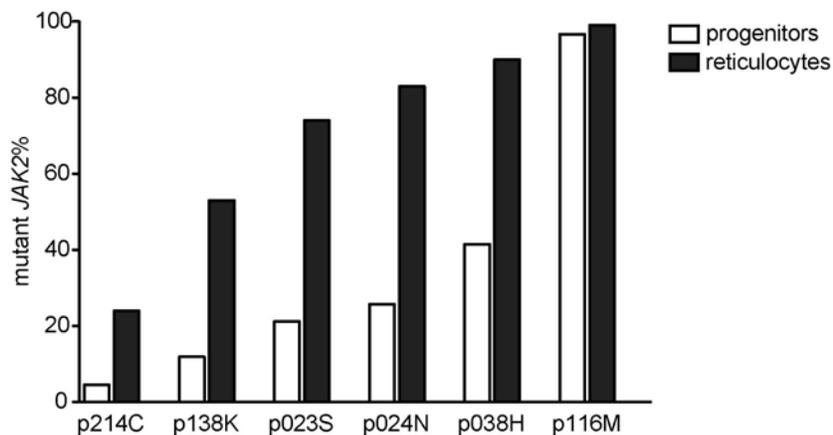


Figure 3. Comparison of the ratio of mutant *JAK2* in erythroid progenitors and reticulocytes. The amount of mutant *JAK2* was much higher in reticulocytes than in erythroid progenitors except p116M, who had almost all colonies carrying the mutation.

We provided evidence that in a proportion of PV patients, the amplification of cells carrying *JAK2* mutations occurred at the late stage of erythropoiesis, but this cannot be solely attributed to the presence of homozygous *JAK2*-V617F colonies since some patients who did not have homozygous *JAK2*-V617F colonies also had the mutation amplified (p214C). In vitro liquid culture of erythroid progenitors from PV patients showed more cells from PV patients were in proliferation state as compared with normal controls, possibly due to increased phosphorylation of PI3-Akt/PKB pathway, which is involved in cell proliferation and cell cycle regulation.¹³¹ Activation of PI3-Akt pathway by *JAK2*-V617F might confer potent proliferation ability on the cells at the late stage of erythropoiesis. Further experimental data will be needed to support this hypothesis.

For further information, analysis of a larger cohort of samples, especially of those who have clear PV phenotype but with low mutant *JAK2* percentage in their granulocytes (such as below 20%) will be required. On the other hand, more patients who have similar amount of mutant *JAK2* in their granulocytes and reticulocytes (group A) will also be included to clarify if there is any dosage change of mutant *JAK2* during the differentiation of erythroid progenitors into reticulocytes in this group. In addition, X-chromosome inactivation based clonality assays on reticulocytes from female patients will be in favor of illustrating if the expanded reticulocytes are of clonal origin or not. The accomplishment of these coming works would advance our understanding of the role of *JAK2* mutations in extensively producing mature red blood cells in PV patients.

RESULTS III

**Transgenic mouse models to study the function of *JAK2*
exon 12 mutations and *JAK2* exon 16 mutations in
myeloproliferative disorders**

Abstract

JAK2-V617F, *JAK2* exon 12 mutations and *JAK2* exon 16 mutations are acquired activating mutations involved in the pathogenesis of a series of blood disorders. *JAK2-V617F* is associated with polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF); *JAK2* exon 12 mutations only lead to PV with isolated erythropoiesis, while exon 16 mutations define a subgroup of acute lymphoblastic leukemia (ALL) that are associated with trisomy 21. The mechanisms by which different myeloproliferative phenotypes associate with the same *JAK2-V617F* mutation and mutations in the same *JAK2* gene cause different phenotypes are currently unclear. To study the molecular pathogenesis of different *JAK2* mutations, we chose the most frequent *JAK2* exon 12 (N542-E543del and E543-D544del) and exon 16 (R683G) mutations and generated transgenic mouse models with inducible expression of these mutations. Using highly efficient recombination engineering technique and bacterial artificial chromosome (BAC), we generated *JAK2* exon 12 mutant transgene constructs with the exon 12 sequence placed in the inverse orientation and flanked by antiparallel *loxP* sites. Similarly, the *JAK2* transgene construct with R683G was made to have the sequences encoding the kinase domain placed in the inverse orientation and flanked by antiparallel *loxP* sites. Three transgenic founders with *JAK2* N542-E543del and two transgenic founders with *JAK2* E543-D544del have been obtained. Crossing of these founders with *VavCre* or *MxCre* transgenic mice is expected to induce expression of mutant human *JAK2*. Detailed blood count, pathological abnormalities and genotype-phenotype relationship analysis will be performed. Microinjection of the *JAK2-R683G* construct is currently ongoing.

Introduction

Activating somatic *JAK2* mutations are frequently found in patients with myeloproliferative disorders (MPD). *JAK2*-V617F is the first *JAK2* mutation discovered that is involved in the pathogenesis of 95% PV, and about 50% of ET and PMF patients.⁶⁹⁻⁷² Gain-of-function *JAK2* exon 12 mutations were subsequently found in the majority of PV patients who are negative for *JAK2*-V617F. *JAK2* exon 12 mutations induce clinical phenotype with isolated erythrocytosis, which is different from PV patients with *JAK2*-V617F.⁸² Recently, somatically acquired *JAK2* exon 16 mutant alleles affecting a highly conserved arginine residue at 683 (R683) have been described in 18%-28% of patients with Down's syndrome-associated acute lymphoblastic leukemia (DS-ALL).^{132,133} The occurrence of exon 16 mutations is associated with trisomy 21. It has been shown that the cooperation of *GATA1* mutation with trisomy 21 associated overexpression of several genes resulted in Down's syndrome related acute megakaryocyte leukemia.^{134,135} Although the pathogenesis of *JAK2* exon 16 mutations is presently unknown, a similar mechanism might exist for ALL in Down's syndrome. However, its elucidation will need development of experimental models of the disorder.^{132,133}

The most common *JAK2* exon 12 mutation is N542-E543del followed by E543-D544del, while the most frequent exon 16 mutation is R683G. Each of these *JAK2* mutations has the ability to transform cytokine-dependent cell lines to cytokine independence and cause constitutive JAK-STAT activation. These observations raise the question of whether mutations in different positions of *JAK2* have intrinsically different kinase activities leading to different signaling in hematopoietic cells, which results in different phenotypes.

Retroviral-mediated expression of *JAK2*-V617F in mouse bone marrow transplantation models demonstrated that the expression of mouse *Jak2*-V617F is sufficient to induce a PV-like phenotype with increased hematocrit, leukocytosis, extramedullary hematopoiesis and subsequent myelofibrosis.^{70,101-104} Similar bone marrow transplantation

assays with *JAK2*-K539L (an exon 12 mutation) resulted in expansion of erythroid lineages and mild expansion of the granulocyte lineage as compared to *JAK2*-V617F, but no megakaryocytes expansion was observed.⁸² A pilot bone marrow transplantation study using I682-D686del (an exon 16 mutation) caused myeloproliferative disorders similar to that caused by V617F.¹³⁶

Three groups have established transgenic mouse models expressing human *JAK2*-V617F in the hematopoietic systems.^{105,106,107} The expression of *JAK2*-V617F results in an MPD phenotype in these mouse models suggesting the presence of *JAK2*-V617F is sufficient to cause the disease. Mice expressing higher level of *JAK2*-V617F develop a PV-like phenotype, while mice with lower expression of *JAK2*-V617F display an ET-like phenotype, which indicates the dosage of mutant gene might contribute to the manifestation of disease phenotype.¹⁰⁵

Currently, no transgenic mouse model for *JAK2* exon 12 or exon 16 mutations is available. Using a bacteria artificial chromosome (BAC) as transgene carrier and a homology-mediated recombination (recombineering) strategy, we are generating inducible transgenic mouse models in which mutant *JAK2* can be expressed in the blood system in the presence of VavCre or MxCre. BACs usually contain important regulatory sequences required for normal gene expression, so it is relatively easy to get dose-dependent and integration site independent transgene expression, which makes BACs a good choice for making transgenic mice.^{137,138} Recombineering strategies with a *galK*-based selection system take advantage of phage recombination functions that generate recombinant relying on homologies of 50 bp or less, which allows genomic DNA in BACs to be modified easily.

The functional differences of different *JAK2* mutations and molecular mechanisms underlying diverse disease phenotypes caused by *JAK2* mutations will be studied in vivo by comparison of transgenic mice containing *JAK2*-V617F, *JAK2* exon 12 mutations and *JAK2* exon 16 mutations.

Material and Methods

Bacterial strains

All strains used for BAC recombination were maintained at 32°C because of the temperature-inducible prophage. Bacterial strain SW102 (SW101 $\Delta galK$),¹³⁷ was used for the construction of BACs containing *JAK2* exon12 mutations. Bacterial strain EL250 (DY380 (cro-bioA) $\langle \rangle$ araC-P_{BAD} *Flpe*)¹³⁹, which possesses *araC* and the arabinose promoter-driven *flpe* recombinase gene, was used for the construction of the BAC containing *JAK2*-R683G. The genotype of DY380 is DH10B [λ c1857 (cro-bioA) $\langle \rangle$ Tet] *galK*⁺ gal490, and the genotype of SW101 is DY380 *gal*⁺.¹³⁷

BAC constructs for *JAK2* exon 12 mutations

The human *JAK2* N542-E543del and human *JAK2* E543-D544del transgenes were constructed based on the already existing human *JAK2* WT BAC construct.¹⁰⁵ This 192 kb long construct, which is modified from BAC CTD2025A15 (CalTech D human BAC library), contains 96 kb 5'-upstream sequence, *JAK2* genomic sequence from exon 1 to intron 12, *JAK2* cDNA sequence from exon 13 to 25, a polyadenylation signal from *SV40* and an FRT site.

BAC and plasmids for *JAK2*-R683G mutation

The human *JAK2* exon16-R683G transgene was constructed using BAC CTD2025A15 (CalTech D human BAC library), which is approximately 190 kb long and contains part of the *JAK2* gene reaching from 96 kb upstream of exon 1 to the first 1 kb of intron 12. The BAC integration construct flip-flop (FF) was a gift from Dr. R Tiedt.¹⁰⁵ It was assembled from the rest of intron 12 with 100 bp overlap, a *lox66* site in intron12, cDNA encoding *JAK2* exons 13-25, a polyadenylation signal from *SV40*, a *lox71* site after pA site, an ampicillin resistance cassette flanked by Frt sites and 100 bp of sequence homologous to the BAC vector pBeloBAC11. The segment between the *loxP* sites was in an inverted orientation. The bacteria strain EL250, which can be transiently induced to express *Flpe* recombinase was used to propagate the BAC and for subsequent homologous recombination.

Procedures to generate BAC with *JAK2* exon 12 mutations

Minimal media and indicator plates

The preparation of minimal media and indicator plates were done following standard protocols.¹³⁷

Gal positive selection: M63 + agar (15 g/l; Roth, Karlsruhe, Germany) + D-galactose (0.2%; Sigma, Stienheim, Germany) + D-biotin (1 mg/l; Supelco, Bellefonte, USA) + L-leucine (45 mg/l; Sigma, Stienheim, Germany) and chloramphenicol (12.5 mg/ml; Sigma, Stienheim, Germany).

Gal counterselection: M63 + agar + glycerol (0.2%; Sigma, Stienheim, Germany) + D-biotin (1 mg/l) + L-leucine (45 mg/l) + DOG (0.2%; Sigma, Stienheim, Germany) and chloramphenicol (12.5 mg/ml).

Gal indicator plates: MacConkey agar (Difco, BD Biosciences) + D-galactose (1%) and chloramphenicol (12.5 mg/ml).

Washing solution: 1× M9 medium (6 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 1 g/L NH₄Cl, 0.5 g/L NaCl)

*PCR amplification of the *galK* targeting cassette*

The *galK* targeting cassette for *JAK2* exon 12 was PCR amplified using 1 ng of *pgalK* plasmid as template.¹³⁷ The plasmid contains wild type *galK* open reading frame and an em7 promoter. The following primers were used:

JAK2-galK-F 5'-*TAAGGAAGTGATTATAATTTTGAATGTATGAAGTAACTAAGAAGAAATATCCTGTTGACAATTAATCATCGGCA*-3' and *JAK2-galK-R* 5'-*CAAAAAGAAAATTAAGATTCCATTCTTAGGATAATTAAGATAATTAATTTTCAGCACTGTCCTGCTCCTT*-3'.

The sequence homologous to *JAK2* intron 11 and 12 is in italics and the sequence recognizing *galK* is underlined. PCR conditions are as follows, 94°C 2min, followed by 30 cycles of 94°C 15 sec, 60°C 30 sec, 72°C 1 min, and a final extension for 15 min. 2 µl DpnI was added per 25 µl reaction, and incubated at 37°C for 1 hour to remove remaining plasmid template. The DpnI-digested PCR product was gel-purified, and 100 ng was used for recombineering.

Preparation of electrocompetent cells and electroporation

500 μ l overnight culture of SW102 was diluted in 25 ml Luria–Bertani (LB) medium in a 50 ml conical flask and grown at 32°C until OD600 value reached 0.6. Then, 10 ml of culture was transferred to another 50 ml flask and induced at 42°C for exactly 15 min in a waterbath. The remaining culture was left at 32°C as uninduced control. After 15 min the two samples were cooled in ice water slurry for 15 min and centrifuged for 5 min at 5000g at 0°C. The pellet was washed twice with 10 ml ice-cold ddH₂O. After the second washing, the supernatant was removed completely, and the pellet (50 μ l each) was kept on ice until electroporated with BACs or PCR products. An aliquot of 25 μ l was taken for each electroporation in a 0.1 cm cuvette (BioRad) using a Bio-Rad gene pulser at 25 μ F, 1.75 kV with a pulse controller set at 200 Ω .

GalK positive selection

The *JAK2* WT BAC was introduced into empty SW102 bacteria using electroporation. Electrocompetent SW102 cells containing *JAK2* WT BAC was prepared as described above, and electroporated with *galK* targeting fragments. The bacteria were recovered in 1 ml LB for 1 h in a 32°C shaking block. Then, 1 ml of the bacteria were pelleted and washed twice with 1 ml of 1 \times M9 salts. After the second wash, the pellet was resuspended in 1 ml of 1 \times M9 salts and plating 100 μ l or 100 μ l after 1:10 dilution on M63 minimal medium plates. The plates were cultured at 32°C for 3 days. Two colonies from the plate were streaked on indicator plate and a single dark red Gal⁺ colony (*JAK2-galK* BAC) was used for future *galK* negative selection step.

Construction of inducible flip-floped human *JAK2* exon12 N542-E543del and human *JAK2* exon12 E543-D544del fragments for recombineering

A 465 bp fragment containing either *JAK2* exon12 N542-E543del or *JAK2* exon12 E543-D544del was PCR amplified from granulocyte DNA of PV patients using primer pair 2075 (forward), 5'-CAAAGTTCAATGAGTTGACCCC-3' and 2076 (reverse), 5'-TGCTAACATCTAACACAAGGTTGG-3', which are located in intron 11 and intron 12, respectively. The PCR product was cloned into the TOPO TA cloning vector (Invitrogen

Carlsbad, USA) and sequenced to make sure no other mutation is present. These plasmids were used as templates for the next PCR step.

To make the inducible flip-floped transgene, a *lox71* site was introduced between intron 11 and primer 2076 while a *lox66* site was introduced between primer 2075 and intron12. The following primers were used, with underline showing the homology arm in intron 11 and 12, italics showing sequence of *lox71* (forward) and *lox66* (reverse), and bold showing the same sequence as primer 2076 and 2075 respectively.

lox71 intron forward: 5'-TAAGGAAGTGATTATAATTTTGAATGTATGAAGTAAC
TAAGAAGAAATATTACCGTTCGTATAGCATAATTATAACGAAGTTATTGCTAACA
TCTAACACAAGGTTGG-3'; *lox66* intron reverse: 5'-CAAAAAGAAAATTAAGAT
TCCATTCTTAGGATAATTAAGATAATTAATTTATAACTTCGTATAGCATAATTAT
ACGAACGGTACAAAGTTCAATGAGTTGACCCC-3'

The PCR conditions were 94°C for 2 min, 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, and final extension for 15 min. The PCR product was digested with DpnI and gel purified to serve as the targeting fragment for recombineering. This PCR product has the inverted sequence of mutant *JAK2* exon 12.

galK counterselection

300 ng purified PCR fragment containing inverted mutant *JAK2* exon 12 was electroporated into competent SW102 bacteria containing *JAK2-galK* BAC. The bacteria were then recovered in 1ml LB at 32°C for 4.5 hours, washed twice with 1 ml of 1×M9 salts, plated onto Gal counter selection plates, and grown at 32°C for 3 days. 20 colonies were picked and screened for successful recombination and correct sequence.

Verification of positive recombinants

The selected Gal negative clones were analyzed in the following steps. First, PCR screening to detect successful recombination based on the size of PCR product. The primers 2435, 5'-TGGAAATTCCTTGAAATAATGAGC-3' and 2436, 5'-TCTTGAGA ACTTGGGAGTTGC-3' were used, which were located outside *lox* intron primers. PCR conditions were: initial denature at 94°C for 5 min, followed by 30 cycles of 94°C 30 sec,

60°C 30 sec, 72°C 40 sec and a final elongation for 15 min. Second, the clones with successful recombination were sequenced with BigDye® Terminator v3.1 Cycle Sequencing Kit and AB 3130 genetic analyzer (Applied Biosystems, Darmstadt, Germany) using the same primer pair as above, which covered the region of homologous recombination. Finally, a clone with correct sequence was chosen and analyzed by XhoI, PacI and SallI digestion of BAC DNA using unmodified *JAK2*-WT BAC DNA as a control. Digested DNA was run in a pulse field gel (CHEF-DRII, Biorad, Hercules, USA). The conditions were: 6V/s, 1 to 25s linear ramping for 16 hours at 14 °C. The clone with correct sequence and restriction pattern was selected for microinjection.

Cloning strategy for *JAK2* flip-floped exon16-R683G BAC construct

Overlap extension PCR¹⁴⁰ was used to introduce the point mutation into *JAK2* exon16. The wild type human *JAK2* cDNA was used as template to introduce the *JAK2* exon16 R683G mutation. The unique BsiWI restriction site in primer 2575 and HpaI restriction sites in primer 2581 were present naturally in the sequence of human *JAK2* exon 13 and exon 22 respectively.

Firstly, two separate PCR reactions using primer pair 2575/2577 and 2576/2581 were set up to get two PCR products with overlapping ends. The sequences of the primers are: 2575, 5'-TTAAAGGCGTACGAAGAGAAGTAGGAGAC-3'; 2577, 5'-TTTCCTGTCTTCCTGTCTTCTTCTCCGAT-3'; 2576, 5'-TGTGCCAAAAATATTCTGCTTATCGGAGA-3' and 2581, 5'-TCTTGTGGCAAGACTTTGGTTAACCCAAA-3'. Primers 2577 and 2576 are mutagenic primers divergently oriented but overlapping at their 5' ends. PCR conditions were: initial denature at 94°C for 5 min, followed by 30 cycles of 94°C 30 sec, 60°C 30 sec, 72°C 40 sec and a final elongation for 15 min. The two intermediate PCR products with overlapping ends were mixed and 1:500 diluted to serve as the template in the second round of PCR using primers 2575 and 2581. PCR conditions were the same as above except elongation for 1 min instead of 40 sec. The final PCR product was gel purified and cloned into the TOPO TA cloning vector (Invitrogen, Carlsbad, USA) for sequencing. A clone containing *JAK2*-R683G and without other mutation was selected. The plasmid was digested with BsiWI and HpaI. The BsiWI/HpaI fragment was

gel purified and ligated into BsiWI and HpaI digested BAC integration construct FF, thus giving rise to BAC *JAK2* R683G integration construct FF. The BAC integration fragment was obtained by digesting BAC *JAK2*-R683G integration construct FF with EcoRI and AgeI.

The resultant BAC integration fragment was subsequently transformed into electrocompetent EL250 cells containing BAC CTD2025A15 and recovered at 32°C for 1 h. The transformants were spread on an LB plate with both Amp (100 µg/ml) and chloramphenicol (12.5 µg/ml) and grown at 32°C overnight. A clone with double resistance was selected and the ampicillin cassette was removed by the transiently expressed Flpe recombinase.¹³⁹ Briefly, a 5 ml overnight culture was added into 20 ml of LB with chloramphenicol and grown until OD₆₀₀ 0.4. 0.2 ml of sterile 10% L-arabinose was added to induce Flp expression for 1 hour. The culture was diluted and plated on LB with chloramphenicol, then grown at 32°C overnight. The colonies were subjected to sequencing and restriction enzyme digestion for verification. The correct BAC with *JAK2*-R683G was ready for microinjection.

Pronuclear microinjection

For oocyte injection, BAC DNA was isolated from 200 ml overnight culture with the Nucleobond BAC 100 DNA purification kit (Macherey-Nagel, Dueren, Germany). 50 µg BAC DNA was digested with SalI overnight at 37°C to remove the vector backbone. Five milliliter Sepharose CL4b column (GE healthcare, Uppsala, Sweden) was equilibrated with 30 ml injection buffer (10 mM Tris HCl pH7.5, 0.1 mM EDTA). Digested DNA was run through the column and eluted with 10 ml injection buffer. Ten 0.5 ml fractions were collected and 10 µl from each fraction was used to run pulse field gel electrophoresis for testing the quality of purified BAC fragments. The fraction with OD_{260/280} ratio higher than 1.8 was chosen for pronuclear injection. Purified BAC DNA was microinjected into pronucleus of C57BL/6 zygotes and transferred to foster mice. The microinjection was done at the Transgenic Mouse Core Facility at Biozentrum, University of Basel.

Genotyping and copy number analysis

For genotyping the presence of human *JAK2* transgene, DNA was isolated from toe sections of newborn mice using Wizard® SV 96 Genomic DNA Purification System (Promega, Madison, USA). PCR was performed using human *JAK2* specific primers 1403, 5'-GAGCAAGCTTTCTCACAAGC-3' and 1404, 5'-AATTCTGCCCCACTTTGGTGC-3' that amplify a 530 bp fragment. The number of integrated transgene copies was determined by real-time polymerase chain reaction (Real-time PCR) with Power SYBR Green PCR Master mix on a 7500 Fast Machine (Applied Biosystems, Foster City, USA). The primers 1748, 5'-GTGGCAGCAACAGAGCCTATC-3', 1749, 5'-GGAGCTTCAGCACCTCGAGAT-3' for human *JAK2*, and 1950, 5'-TGGCAGCAGCAGAACCTACA-3' and 1951, 5'-GGAGCTTCAGCCCCACG-3' for mouse *Jak2* were used. The threshold cycles (CT) for human *JAK2* and mouse *Jak2* were simultaneously measured for each sample in duplicates.

Results

Generation of BAC constructs with *JAK2* exon 12 N542-E543del or *JAK2* exon 12 E543-D544del.

To make an inducible BAC construct with *JAK2* exon 12 N542-E543del and *JAK2* exon 12 E543-D544del, we modified our previous BAC construct with wild type human *JAK2*. We used a highly efficient phage-based *E. coli* homologous recombination system with a selectable *galK* marker, allowing genomic DNA in bacterial artificial chromosomes to be modified without using restriction enzymes or DNA ligases. Using as short as 50 bp of homology, recombination is accomplished efficiently.

We started with *JAK2* wild type BAC¹⁰⁵ containing 96 kb of *JAK2* upstream genomic sequence, *JAK2* genomic sequence from exon 1 to intron 12, *JAK2* cDNA sequence from exon 13-25, and a polyadenylation signal from *SV40* (Figure 1A). *JAK2* wild type BAC was electroporated into SW102 cells, which were unable to grow in galactose minimal medium because of lacking the *galK* cassette. A constitutively active *galK* expression cassette was amplified from *pgalK* plasmid with arms homologous to *JAK2* intron 11 and intron 12 respectively (Figure 1A). SW102 cells containing *JAK2* WT BAC were heat-induced and made electrocompetent, then electroporated with *galK* cassette. SW102 cell-mediated homologous recombination enabled the *galK* cassette to replace wild type *JAK2* exon 12. Gal⁺ recombinant colonies (*JAK2-galK* BAC) were selected on galactose minimal plates at 32°C for 3 days with chloramphenicol to maintain the BAC. To get a pure Gal⁺ clone, two colonies were streaked on MacConkey galactose indicator plates. One single bright red Gal⁺ colony was picked and used in the subsequent recombineering step. (Figure 1B)

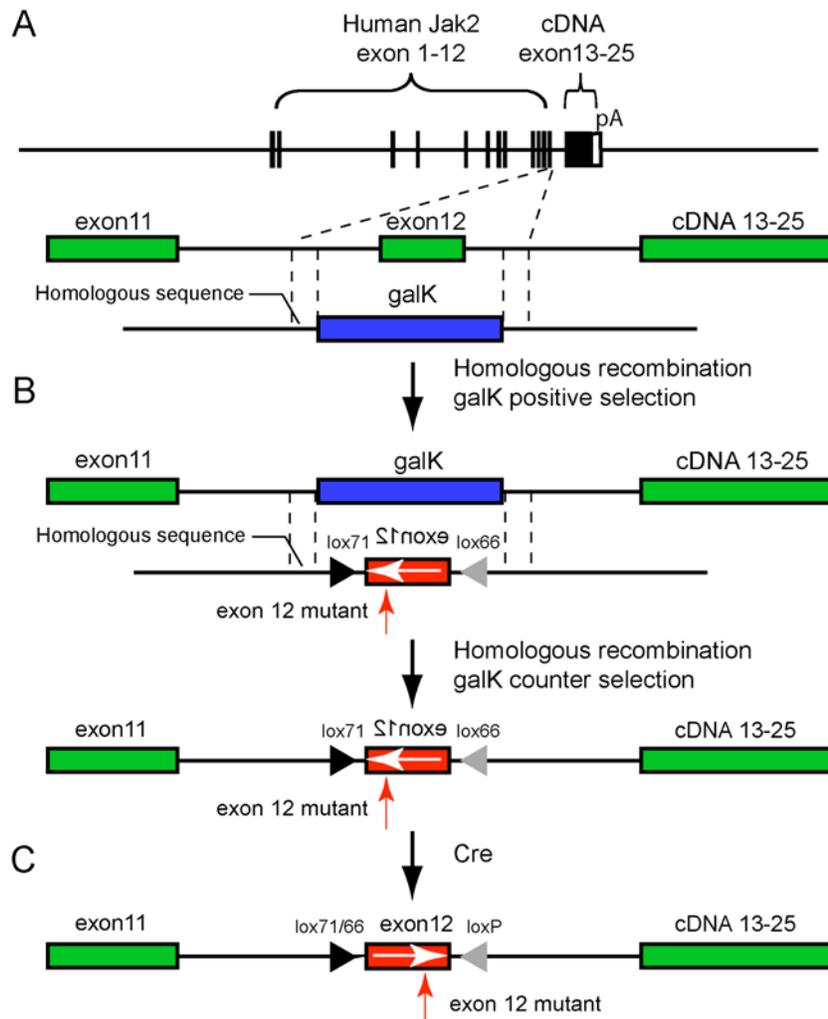


Figure 1. Generation of BAC constructs with *JAK2* exon 12 mutations by recombineering. A) *galK* positive selection. Wild type *JAK2* exon12 in the *JAK2* WT BAC (up) was replaced by a *galK* expression cassette (low) by selection on M63 minimal plates with galactose and chloramphenical. B) *galK* negative selection. The *galK* cassette is replaced by a PCR fragment with inverted mutant *JAK2* exon 12 (N542-E543del or E543-D544del, indicated by red arrow) flanked by *loxP* sites (black and gray triangle) and 50 bp homology. This was achieved by negative selection using minimal medium containing 2-deoxy-galactose (DOG) with glycerol as the sole carbon source. C) Inducible transgene construct. Cre-mediated recombination will flip the orientation of exon 12 into the correct position to allow normal transcription of *JAK2* mRNA. Recombination will give rise to one wild type *loxP* and one double mutant *lox71/66* site, which disables further recombination by Cre-recombinase.

A 465 bp fragment containing either N542-E543del or E543-D544del was cloned from granulocyte DNA of PV patients. Clones with the desired mutations but no other mutations based on sequencing analysis were selected. These clones were used as template for the next PCR amplification. A PCR fragment with desired mutations and inverted *JAK2* exon 12 sequence was amplified using a primer pair containing 50 bp homologous sequence with *JAK2* intron 11 and intron 12 respectively. To make inducible flip-floped *JAK2* exon 12 N542-E543del or exon 12 E543-D544del transgenes, *loxP* sites were inserted just adjacent to the homologous arm (Figure 1B). In this configuration, no full length human *JAK2* protein can be made since splicing from exon 11 to exon 13 will result in a frame shift creating a stop codon. Transgenic founder mice will be crossed with VavCre and MxCre transgenic mice to induce expression of Cre recombinase. Recombination of anti-parallel *loxP* sites by Cre will result in flipping the orientation of the exon 12 sequence, restoring a functionally active transgene (Figure 1C). To ensure no additional recombination, mutant *loxP* sequences were used (*lox66* in the forward primer and *lox71* in the reverse primer), so that after one recombination a double mutant *loxP* site is produced, which is unable to respond to Cre recombinase.

200 ng of DpnI-digested, gel-purified *JAK2* exon 12-inverted fragment was electroporated into heat-induced electrocompetent SW102 Gal⁺ cells containing *JAK2-galK* BAC. Upon recombination, the *galK* expression cassette was replaced with inverted mutant *JAK2* exon 12 in the BAC (Figure 1B). The cells were washed with M9 salts to remove any remaining rich medium and selected against *galK* on plates with minimal media containing glycerol, 2-deoxy-galactose (DOG) and chloramphenicol for 3 days. Galactokinase, the product of the *galK* gene, phosphorylates DOG and produces toxic 2-deoxy-galactose-1-phosphate, so only cells without the *galK* cassette can survive. Twenty *galK* negative colonies from each construct were picked and subjected to PCR screening using primer pairs located outside the homologous region. Correct recombination gave rise to a product of 860 bp, while no recombination or rearrangement gave products with wrong sizes (data not shown). 18 out of 20 clones for N542-E543del, and 12 out of 20 clones for E543-D544del underwent correct recombination, showing high efficiency of this method. 8 clones from each construct were selected for sequencing using primers

covering the entire region of homologous recombination. Figure 2 shows that the correct *JAK2* exon 12 mutations were introduced after recombination. To determine whether any unexpected deletions, insertions or other recombination were generated during this procedure, two clones with correct sequence from each exon 12 mutation and the original wild type clone were selected for restriction analysis by XhoI and PacI and run on a pulse field gel, (Figure 3A). All of the four analyzed *JAK2* exon 12 mutant BAC clones had the same restriction pattern as the original unmodified *JAK2* WT BAC, suggesting no additional events had occurred. Thus the correct BAC constructs with *JAK2* N542-E543del or E543-D544del were generated and ready for pronuclear injection.

Purification of the linear BAC DNA for pronuclear injection. *JAK2* N542-E543del BAC clone 8 and *JAK2* E543-D544del BAC clone 5 were used for oocyte injection. 50 µg BAC DNA was digested with SalI to remove the vector backbone and purified over a Sepharose CL-4B column. Ten fractions were collected and 10 µl from fractions 4 to 10 were run on a pulse field gel to detect the integrity of the linear BAC and separation from BAC backbone (Figure 3B).

Production of the BAC transgenic mice. Two different concentrations of purified BAC DNA (1 ng/µl and 0.25 ng/µl) were used for pronuclear injection into fertilized C57BL/6 mouse zygotes. Newborns were obtained with both concentrations. For the *JAK2* N542-E543del BAC construct, 22 newborn mice were obtained, and three of them (N1, N2 and N3) contained the transgene as confirmed by genotyping using human *JAK2* specific primers (Figure 4A). For the *JAK2* E543-D544del BAC construct, 23 newborn mice were obtained, and two of them (D1 and D2) contained the transgene (Figure 4B). Transgene copy number in all founder mice was measured using quantitative Real-time PCR by comparing the CT value of human *JAK2* with mouse *Jak2* (Table 1). N3 has around 7 copies, D2 has around 2 copies, and all other founders have one copy. These mice will be crossed with VavCre and MxCre transgenic mice. The double transgenic mice will flip the inverted *JAK2* exon 12 sequence leading to transcription of mutant *JAK2* and detailed characterization will be carried out.

A

Exon12 WT	CAAATGGTGTTCACAAAATCAGAAATGAAGATTTGATATTT
	Q M V F H K I R N E D L I F
N542-E543 del	CAAATGGTGTTCACAAAATCAGA-----GATTTGATATTT
	Q M V F H K I R - - D L I F
N543-E544 del	CAAATGGTGTTCACAAAATCAGAAAT-----TTGATATTT
	Q M V F H K I R N - - L I F
Exon16 WT	TGTGCCAAAAATATTCTGCTTATCAGAGAAGAAGACAGGAAG
	C A K N I L L I R E E D R K
R683G	TGTGCCAAAAATATTCTGCTTATC G GAGAAGAAGACAGGAAG
	C A K N I L L I G E E D R K



Figure 2. Sequence of BAC constructs for exon 12 and 16 mutations.

A) Alignment of wild type and mutated *JAK2* exon 12 and exon 16 sequence with corresponding amino acids. The red characters indicate mutations. B) Sequencing for exon 12 and exon 16 mutations in the BAC constructs. Upper panel, *JAK2* N542-E543del compared with wild type sequence; middle panel, *JAK2* E543-D544del compared with wild type sequence; lower panel, *JAK2*-R683G compared with wild type sequence. The deleted nucleotides are shown within the red boxes. Arrows show the positions where mutations occur.

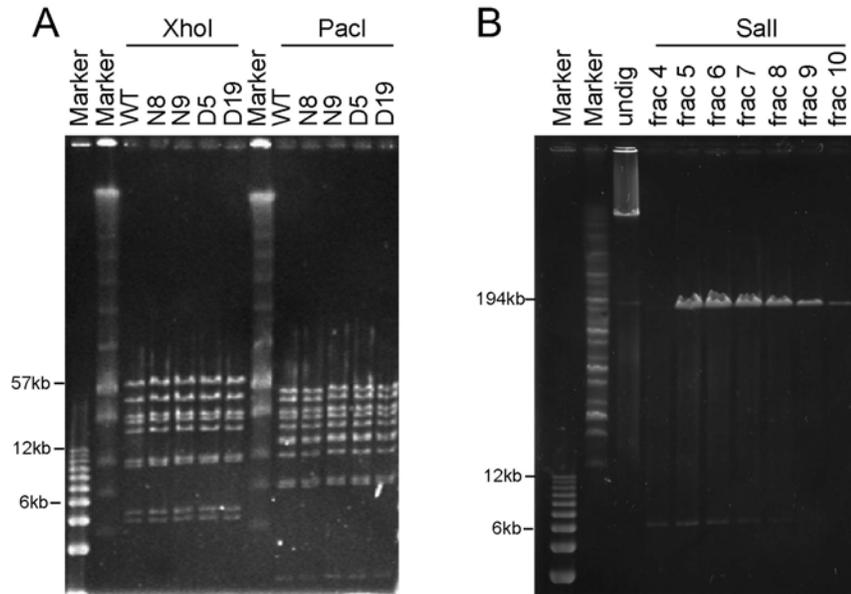


Figure 3. Restriction analysis and purification of BAC clones. A) XhoI and PacI restriction analysis of BAC DNA by pulse field gel electrophoresis. Unmodified wild type construct and two of each *JAK2* exon 12 mutant constructs after *galK* counterselection were digested with XhoI and PacI. N, *JAK2* N542-E543del; D, *JAK2* E543-D544del, numbers indicate the clone numbers. B) Purification of linearized BAC using Sepharose CL-4B column. *JAK2* N542-E543del BAC is shown. SalI digestion removes most part of the BAC backbone. Fractions from 4 to 10 were run on a pulse field gel. The 6 kb fragment is the backbone, while the 186kb fragment contains the *JAK2* transgene.

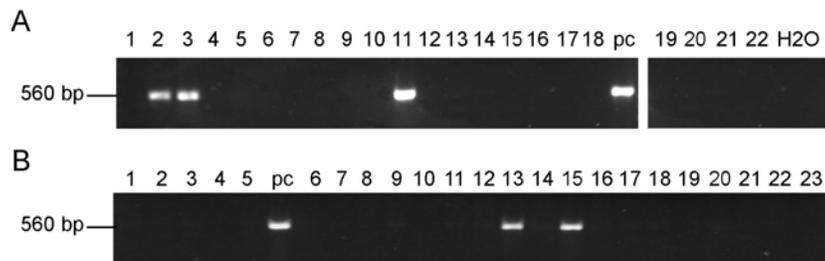


Figure 4. Genotyping for the presence of transgene in newborn mice. Human *JAK2* specific primers were used. DNA from human *JAK2*-V617F transgenic mice was used as positive control (pc). A) Genotyping for *JAK2* N542-E543del transgene. Three transgenic founders were obtained. B) Genotyping for *JAK2* E543-D544del transgene. Two transgenic founders were obtained.

Generation of BAC construct with *JAK2* exon 16 R683G mutation.

We modified BAC integration construct FF (Figure 5B) to introduce *JAK2* R683G in the sequence and recombined it with BAC CTD2025A15 (Figure 5A). By site-directed mutagenic overlap extension PCR, we introduced *JAK2*-R683G into a PCR-amplified fragment from wild type human *JAK2* cDNA, containing *JAK2* sequence from exon 13 to exon 22, and flanked by naturally present BsiWI and HpaI restriction sites (Figure 6A). The PCR fragment was cloned into the TOPO TA cloning vector and checked for correct sequence. A clone with *JAK2*-R683G, named *JAK2*-R683G intermediate, was selected for the next step. The BsiWI/HpaI fragment was cut out from the *JAK2*-R683G intermediate plasmid and subcloned into the BAC integration construct FF, replacing the counterpart in the construct, and we named it BAC integration construct FF R683G (Figure 6B, step 1). The BAC integration fragment was cut out by EcoRI and AgeI from BAC integration construct FF R683G (Figure 6B, step 2), and used for recombination with BAC CTD2025A15 in EL250 cells. Clones with resistance to both Amp and Chloramphenicol were selected and expression of Flpe recombinase was induced by L-arabinose to remove the Amp cassette (Figure 6B, step 3, Figure 5C). We then performed sequencing analysis of *JAK2* exon 16 and restriction pattern analysis to confirm the right BAC clone (Figure 2B lower panel and Figure 7). The digestion pattern was the same as predicted by vector NTI software, indicating no other recombination occurred. This final *JAK2* R683G BAC construct now contained 96 kb 5'-upstream sequence of *JAK2*, *JAK2* genomic sequence from exon 1 to the first 1 kb of intron 12, and inverted *JAK2* cDNA exon 13-25 (with R683G) plus a polyA site, flanked by anti-parallel *lox71* and *lox66* sites. No full-length *JAK2* protein can be made in this configuration. As with the BAC constructs with exon 12 mutations, recombination of the antiparallel *loxP* sites will flip the orientation of the cDNA fragment from exon 13 to the polyA site, restoring the functionally active *JAK2* transgene. The construct now is waiting for oocytes injection to generate transgenic mice.

Table 1. Copy number analysis of founders for *JAK2* transgene by real-time PCR.

mouse number	human jak2			mouse jak2			delta CT	copy number $2^{2^{-\Delta\Delta CT}}$
	CT1	CT2	average	CT1	CT2	average		
N1	26.20	26.17	26.19	25.05	24.95	25.00	1.19	0.9
N2	26.90	26.77	26.83	24.76	24.82	24.79	2.04	0.5
N3	23.68	23.45	23.57	25.30	25.35	25.33	-1.76	6.8
D1	26.13	26.15	26.14	24.31	24.34	24.33	1.81	0.6
D2	24.18	24.13	24.15	24.14	24.29	24.22	-0.07	2.1
negative ctrl	35.70	35.84	35.77	24.10	24.03	24.06	11.71	0.0
10 copy ctrl	23.05	23.04	23.04	25.36	25.36	25.36	-2.31	9.9
20 copy ctrl	23.12	23.20	23.16	26.62	26.45	26.53	-3.37	20.7
1 copy ctrl	27.11	27.22	27.16	26.16	26.25	26.20	0.96	1.0

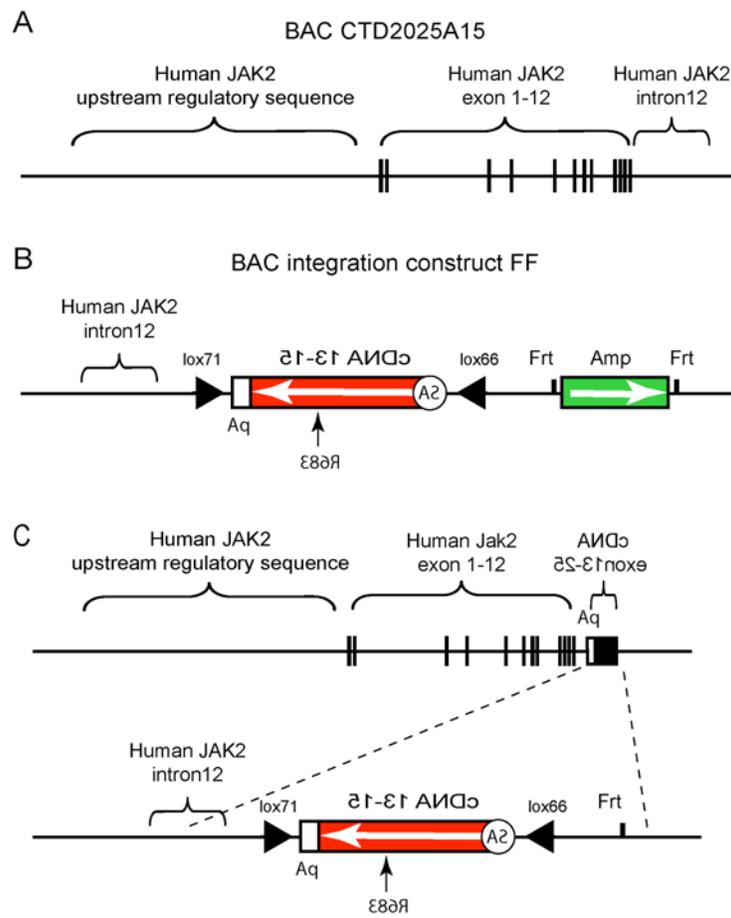


Figure 5. Structure of BAC CTD2025A15 and BAC integration construct FF.

A) BAC CTD2025A15 contains 96 kb of *JAK2* 5' upstream sequence, *JAK2* genomic sequence from exon 1 to the first 1kb of intron 12. B) BAC integration construct FF contains the remaining sequence of *JAK2* intron 12, cDNA of exons 13-25 in an inverted orientation, and an *Amp* cassette flanked by *Frt* sites. C) Final construct with *JAK2* R683G. pA, polyadenylation signal; SA, splice acceptor.

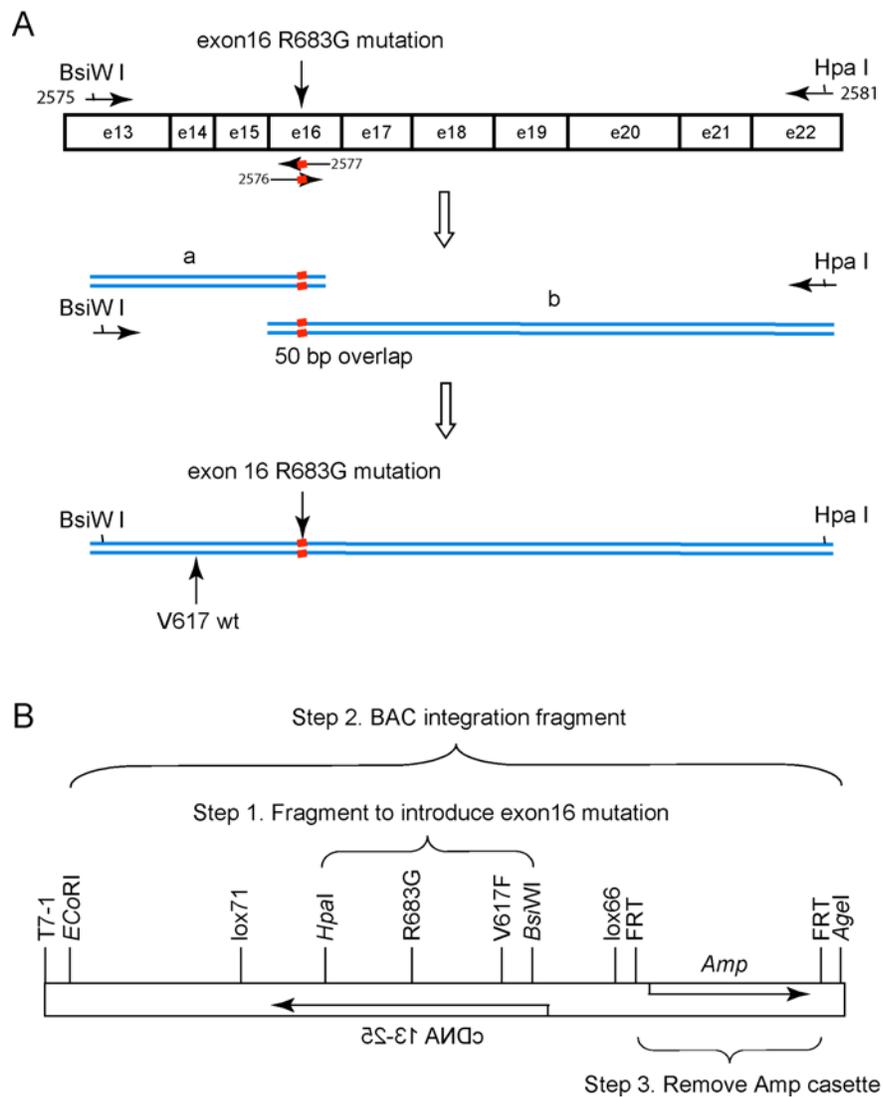


Figure 6. Strategy to make BAC integration fragment containing *JAK2* R683G mutation. A) Schematic diagram of site-directed mutagenesis by overlap extension PCR. Block represents template DNA containing wild type human *JAK2* cDNA sequence from exon 13 to exon 22. Arrows represent primers. The site to introduce the R683G mutation is indicated by red dots. First, two separate PCR reactions were set up using primer pairs 2575/2577 or 2576/2581. PCR products using primer pair 2575/2577 gave rise to product a, while primer pair 2576/2581 gave rise to product b, which have overlap in their ends. In the second step, the two intermediate PCR products were mixed and diluted as the template for the second round of PCR using primer pair 2575/2581. The unique BsiWI and HpaI restriction sites in primer 2575 and 2581 were present naturally in the sequence

of human *JAK2* exon 13 and exon 22, respectively. B) Strategy of generating the BAC integration fragment. Step 1: the final PCR product from A was digested with *BsiWI* and *HpaI* and inserted to the BAC integration construct FF in an inverted orientation. Step 2: the integration fragment was cut out using *EcoRI* and *AgeI* and recombined with BAC CTD2025A15 by homologous recombination. Step 3: the Amp selection marker was removed by arabinose-induced expression of *Flpe* recombinase.

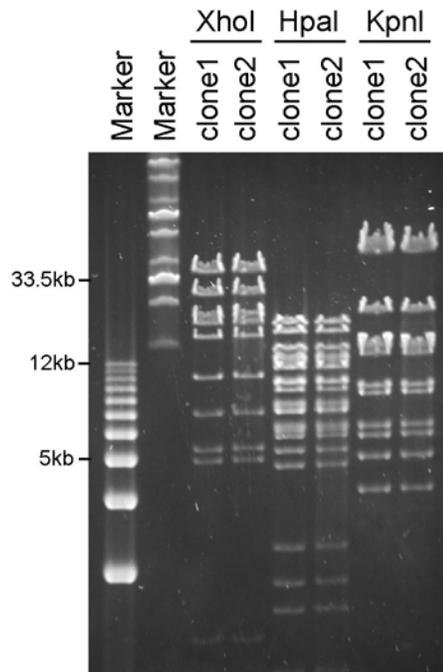


Figure 7. Restriction analysis of two final BAC clones with *JAK2*-R683G. The digestion pattern is the same as predicted by vector NTI software.

Discussion

MPD includes a heterogeneous group of diseases characterized by excessive production of various myeloid lineages of blood cells. Various *JAK2* mutations are found to be responsible for the pathogenesis of MPD. *JAK2-V617F* is found to be present in the majority of PV and a large proportion of ET and PMF patients.⁶⁹⁻⁷² While PV patients have a tendency to progress from heterozygous to homozygous *JAK2-V617F* by means of homologous recombination, ET patients usually stay at heterozygous state stably.^{69,73} *JAK2* exon 12 mutations occur in PV patients who are negative for *JAK2-V617F* with clinical manifestation of isolated erythrocytosis. Newly identified *JAK2* exon 16 mutations involving R683 define a subgroup of acute lymphoblastic leukemia that is associated with trisomy 21. The occurrence of homozygosity is rare in PV patients with *JAK2* exon 12 mutations and DS-ALL patients with exon 16 mutations.^{82,127,132}

Transgenic mouse model is a very useful tool for studying human gene function and regulation in the whole organism, which could recapitulate human diseases that involves deregulation of a particular protein. It is not completely clear why *JAK2-V617F* is associated with a wide spectrum of phenotypes, while *JAK2* exon 12 mutations can only lead to PV and *JAK2* exon 16 mutations are related to DS-ALL. Three research groups have established transgenic mouse models expressing human *JAK2-V617F*. The results showed evidence that low levels of *JAK2-V617F* favor megakaryopoiesis, while higher levels of *JAK2-V617F* promote erythropoiesis and granulopoiesis, suggesting the mutant gene dosage might contribute to the onset of phenotype.¹⁰⁵⁻¹⁰⁷

To further define the pathologic role of various *JAK2* mutations, and study the functional differences between different *JAK2* mutations, we are establishing several transgenic lines inducibly expressing *JAK2* exon 12 and *JAK2* exon 16 mutations. Fourteen different *JAK2* exon 12 mutant alleles and five *JAK2* exon 16 mutant alleles have been reported so far.^{82-84,86-88,132} We chose two of the most frequent exon 12 mutations (N542-E543del and E543-D544del) and one of the most frequent exon 16 mutations (R683G) to develop transgenic mouse lines.

Bacterial artificial chromosomes (BACs) can accommodate large genomic DNA fragments, which make it possible to include large upstream regulatory sequences of a gene in a BAC. Using highly efficient prophage-based *Escherichia coli* homologous recombination systems, we have generated three BAC constructs containing human *JAK2* exon 12 or exon 16 mutations. Constitutive expression of *JAK2*-V617F transgene in mice has led to death before establishment of transgenic lines,¹⁰⁵ so we used the *Cre-loxP* system to realize inducible expression of *JAK2* mutants by inverting the sequence of exon 12 (for exon 12 constructs) or cDNA13-25 (for the R683G construct) and flanking them with *loxP* sites. The expression of Cre recombinase will be obtained by crossing the mice with VavCre or MxCre transgenic mice, and the transcription of mutant human *JAK2* mRNA is naturally under the control of *JAK2* promoter. So far, we have successfully obtained 3 transgenic founder mice with *JAK2* N542-E542del (N1, N2 and N3) and 2 transgenic founder mice with *JAK2* E543-D544del (D1 and D2). The BAC construct with *JAK2* R683G is ready for pronuclear injection. To induce activation of the transgene, we will cross the mice with the VavCre and MxCre transgenic mice^{141,142} to get double transgenic mice. In VavCre mice, expression of Cre recombinase is directed by the *Vav* promoter, which has been shown to be restricted to hematopoietic systems, so the double transgenic mice should have expression of mutant *JAK2* exon 12 only in their blood systems. In MxCre mice, the expression of Cre is under the control of interferon inducible *Mx1* promoter, which can be induced by injection of polyinosine-polycytosine (pIpC). In these mice, DNA recombination is mediated in bone marrow at high efficiency, so it is widely used in studies of hematopoiesis.¹⁴²

In the five transgenic founders, N3 and D2 have more than one copy (7 and 2 respectively) as determined by real-time PCR, the others have around one copy or less. Low copy numbers of transgenes are normal for introducing BAC DNA into the zygotes because of the large size of BAC DNA compared to plasmid DNA, although higher copy numbers can be observed occasionally.

As we did for the *JAK2*-V627F transgenic mice, the integration site of the transgene will be determined by fluorescence in situ hybridization (FISH) and spectral karyotyping

(SKY) analysis. The exact integration site can be identified by restriction digest and circular ligation followed by PCR and sequencing. Clarifying the integration sites will tell us how the multicopies are arranged in the genome and if they interrupt functions of flanking genes, which could also contribute to pathogenesis.

In the first set of experiments, all transgenic founder mice will be crossed with MxCre mice and the offspring positive for double transgenes will be injected with pIpC to induce Cre expression. We will observe the presence of abnormalities in blood counts and whether there is an MPD-like phenotype developing following Cre-mediated recombination. *JAK2* exon 12 mutations only occur in PV patients at low percentage and rarely transform to homozygosity. Therefore, we expect that the transgenic mice with exon 12 mutations will exhibit predominantly increased erythropoiesis.

If we can get at least one transgenic line of each construct with phenotype, we will follow the blood counts of each line and make a series of comparisons between VavCre N strain mice, and MxCre N strain mice, between VavCre D strain mice, and MxCre D strain mice, and between N strain and D strain mice. The phenotype of mice with human *JAK2*-V617F transgene was shown to be correlated with the ratios between the expression levels of mutant *JAK2* and mouse wild type *Jak2*. MxCre FF1 mice, which had a higher *JAK2*-V617F expression, developed a PV-like phenotype, while the VavCre FF1 mice having a lower *JAK2*-V617F expression resulted in ET-like phenotype.¹⁰⁵ The influence of expression levels of the exon 12 mutations on the phenotypic outcome will be determined. Our N3 mouse has around 7 copies, so it is possible to get MxCre N3 mice with different number of active transgene copies by adjusting Cre expression level through altering the number of pIpC injections as shown in *JAK2*-V617F transgenic mice¹⁰⁵. It will be interesting to see how different mutant gene dosages affect the presentation of phenotype, and if the two different *JAK2* exon 12 mutations will cause the same phenotype.

The BAC construct with *JAK2*-R683G is ready for pronuclear injection. After the mice are born and genotyped, the analysis procedure will be similar to that of *JAK2* exon 12

mutant transgenic mice in regard to copy number measurement, integration site analysis, induction of Cre expression by crossing with VavCre and MxCre mice and blood count analysis, etc. The simultaneous occurrence of *JAK2* exon 16 mutations and trisomy 21 in DS-ALL indicates multigene involvement in chromosome 21 might contribute to the development of ALL in Down's syndrome patients, since recipient mice transplanted with bone marrow cells expressing *Jak2* exon 16 I682-D686del only displayed a MPD-like phenotype.¹³⁶ It is interesting to see what kind of phenotype developed in the transgenic mice with *JAK2*-R683G. This mouse model will be a useful tool to discover the yet unknown genes that interact with *JAK2*-R683G to drive the phenotype to B lymphoid specific leukemia.

The further analysis using these transgenic models will include the comparison of the expression profiles of *JAK2*-V617F and *JAK2* exon 12 as well as *JAK2* exon 16 transgenic mice. It will elucidate downstream targets regulated by different *JAK2* mutations, which might contribute to distinct phenotypes caused by different mutations that are all constitutively activated. Furthermore, these mouse models could also be used to test the effects of Jak2 inhibitors on the MPD phenotype. The diverse inhibitors could exert different efficiency on blocking activity of different *JAK2* mutations, which might reflect structural dissimilarity behind interaction of compounds with different mutants and shed light on how configurational differences lead to divergent phenotypes.

PERSPECTIVES

Myeloproliferative disorders (MPD) are a group of related diseases characterized by excessive production of one or more terminally differentiated non-lymphoid blood cell lineages. A somatically acquired *JAK2-V617F* mutation is the most frequent genetic lesion found in PV, ET and PMF. More than 95% of PV and about 50% of ET and PMF patients carry this mutation.⁶⁹⁻⁷² Besides *JAK2-V617F*, *JAK2* exon 12 mutations contribute to the pathogenesis of most PV patients who are negative for *JAK2-V617F*.⁸² The mutations in *JAK2* lead to constitutive activation of the JAK-STAT signaling pathway resulting in unregulated proliferation of blood cells. *JAK2* plays a central role in mediating signaling from cytokine receptors required for normal hematopoiesis such as receptors for erythropoietin (EPOR), thrombopoietin (MPL), and granulocyte colony-stimulating factor (GCSFR).³⁰ Sequencing of these candidate receptors led to the identification of *MPL* mutations, which are present in about 5% of PMF patients and 1-9% of ET patients.⁸⁹⁻⁹³ These findings provided crucial insights into the genetic basis of MPD, but many questions are still open regarding the molecular pathogenesis of MPD.

Identification of disease-causing mutations in *JAK2* and *MPL* mutation negative patients

Although *JAK2-V617F* can be found in almost all PV and half of ET and PMF patients, the disease-causing mutations in the rest of MPD patients remain mysterious. Analysis of the differences in experimental and clinical features of *JAK2-V617F* positive and *JAK2-V617F* negative MPD cannot separate them into distinct subsets based on the presence of *JAK2-V617F*.¹⁴³ The subsequent identification of *JAK2* exon 12 mutations in PV and *MPL* mutations in ET and PMF patients indicates that in *JAK2-V617F* negative MPD, other somatic mutations responsible for aberrant signaling may act in a similar fashion as *JAK2-V617F*. Considering the important role of JAK-STAT signaling in hematopoiesis, maybe partially through the crosstalk with MAPK and PI3K pathways, high throughput sequencing of all components in these pathways including positive and negative regulators will possibly lead to identification of novel mutations participating in the pathogenesis of MPD.

Additional genetic events leading to clonal hematopoiesis

Retroviral expression of mutant *Jak2* in mouse bone marrow transplantation assays demonstrated that mouse *Jak2*-V617F or *Jak2* exon 12 mutations were sufficient to induce a PV-like phenotype, and mice transduced with *Jak2*-V617F developed myelofibrosis later on.^{70,101-104} *JAK2*-V617F transgenic mice displayed a PV or ET phenotype, depending on the expression levels of mutant human *JAK2* versus wild type mouse *Jak2*, which suggested that the presence of *JAK2*-V617F is sufficient to cause the disease.¹⁰⁵ However, accumulating evidence indicates that there are probably additional genetic events contributing to the development of MPD. Our previous data¹¹⁹ showed that in some MPD patients, the percentage of granulocytes and platelets with *JAK2*-V617F was often markedly lower than the percentage of clonal granulocytes determined by X-chromosome inactivation assays in female patients, suggesting that in a proportion of MPD patients *JAK2*-V617F occurred on the background of clonal hematopoiesis caused by other genetic events. In addition, studies of families with myeloproliferative disorders demonstrate that *JAK2*-V617F was not transmitted via germ line, but that family members rather inherited genetic predisposition for acquisition of *JAK2* mutations.¹⁴⁴ Moreover, AML that occurred in *JAK2*-V617F MPD patients frequently became *JAK2*-V617F negative during the transformation, and clonality analysis results supported the hypothesis that the MPD clone and AML clone might originate from a common *JAK2*-V617F negative ancestor.¹⁴⁵ *JAK2* mutation-negative erythroid endogenous colonies (EEC) were found in a number of PV patients, which hint at the existence of unknown mutation(s) in addition to *JAK2* mutations.^{121,122,127} Furthermore, the coexistence of *JAK2*-V617F and *JAK2* exon 12 mutations in different clones and the presence of *JAK2* wild type EECs from the same patient provided additional evidence that the acquisition of *JAK2* mutations has occurred within an already abnormal clone, which harbored unknown mutation(s) contributing to cytokine hypersensitivity and increase the likelihood for acquisition of *JAK2* mutations.¹²⁷ The inheritable or acquired alleles cooperating with *JAK2* mutations remain to be identified, and whether these alleles contribute to the manifestation of MPD phenotype needs to be elucidated. The ongoing screening based on SNP array analysis and gene expression profiling of *JAK2* mutation-negative EECs is expected to help in addressing these questions.

One mutation, three types of diseases

The *JAK2-V617F* mutation is associated with PV, ET and PMF.^{69,72} The question why the same mutation causes three related but clinically and pathologically distinct phenotypes in patients remains unclear. Patients with PV can progress from *JAK2-V617F* heterozygosity to homozygosity as a result of mitotic recombination, which is rare in ET patients, suggesting that there are important genetic differences between PV and ET.⁷³ Frequent presence of homozygous erythroid colonies in PV patients and data from bone marrow transplantation and transgenic mouse models are consistent with the notion that *JAK2-V617F* gene dosage influences the MPD phenotype. Low expression of *JAK2-V617F* transgene favors expansion of the megakaryocyte lineage, mimicking ET; intermediate expression was accompanied by trilineage expansion with increased erythropoiesis, granulopoiesis and thrombopoiesis, whereas high expression from retroviral vectors resulted in erythroid expansion, but normal granulopoiesis and megakaryopoiesis.¹⁰⁵ Moreover, the host genetic background may also contribute to phenotypic diversity among MPDs, as different strains of mice (Balb/c or C57BL6) have variations in the degree of leukocytosis and myelofibrosis.^{103,104} Analysis of single nucleotide polymorphisms (SNPs) on *EPOR*, *MPL*, *GCSFR* and *JAK2* locus in MPD patients identified unique SNPs in *JAK2* that were specifically associated with PV or ET.¹⁴⁶ These results need to be confirmed in a larger or different cohort. High resolution SNP array analysis within and surrounding the *JAK2* region may facilitate identification of specific alleles involved in MPD pathogenesis.

Different mutations in the same gene – different phenotype

While we are still wondering how a single *JAK2-V617F* is implicated in three apparently different MPD phenotypes, we are also curious about how mutations in different regions of the same gene (*JAK2-V617F*, *JAK2* exon 12 mutations and *JAK2* exon 16 mutations) cause different phenotypes. All of these mutations confer a similar cytokine independent growth advantage on cytokine-dependent cell lines such as BF3/EPOR cells in vitro. But in vivo, *JAK2-V617F* could be found in PV, ET and PMF patients; *JAK2* exon 12 mutations only cause PV with distinct clinical features from those of *JAK2-V617F*; *JAK2* exon 16 mutations involving R683 define a subgroup of acute lymphoblastic leukemia

that arises in the context of trisomy 21. Given that the in vitro cytokine independence was obtained only with coexpression of mutant *JAK2* and type I cytokine receptors, such as EPOR,¹⁴⁷ it is hypothesized that differential interaction of different mutant *JAK2* with different cytokine receptors might lead to transmission of divergent downstream signals. More detailed studies will lead to illustration of functional and structural differences among different *JAK2* mutations. We are now establishing inducible transgenic mouse models with *JAK2* exon 12 mutations or with *JAK2* R683G. The phenotype and pathological analysis of those mice will help us to understand the in vivo functions and the molecular pathogenesis of these mutations.

Development of JAK2 inhibitors for therapeutic treatment of PV, ET and PMF

Most of the current therapies of MPD are confined to alleviating symptoms and preventing thrombohaemorrhagic events, rather than eliminating malignant cells. Like the *BCL/ABL* fusion gene found in CML, the landmark discovery of *JAK2* mutations in the majority of MPD patients designates a critical therapeutic target for treatment. Since the constitutive activation of *JAK2* caused by mutations results in oncogenic transformation in vitro and in vivo, inhibition of *JAK2* activity would be an essential step to diminish excessive expansion of mature cells and block development of MPD. However, *JAK2* acts as a central regulator of hematopoiesis, so the treatment should be tuned to suppress aberrant activation but avoid destroying normal roles of *JAK2*. The application of large-scale small molecule library screening will identify more and more pharmaceutical compounds targeting *JAK2*. Our transgenic mouse models with *JAK2* mutations will provide a valuable tool for assessing effectiveness and safety of specific inhibitors in a preclinical scenario.

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APPENDIX

Brief report

Clonal heterogeneity in polycythemia vera patients with *JAK2* exon12 and *JAK2-V617F* mutations

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We studied the lineage distribution of *JAK2* mutations in peripheral blood of 8 polycythemia vera (PV) patients with exon 12 mutations and in 21 PV patients with *JAK2-V617F*. Using a quantitative allele discrimination assay, we detected exon 12 mutations in purified granulocytes, monocytes, and platelets of 8 patients studied, but lymphoid cells showed variable involvement and the mutation

was absent in T cells. Endogenous erythroid colonies grew in all patients analyzed. One patient displayed erythroid colonies homozygous for the exon 12 mutation with evidence for mitotic recombination on chromosome 9p. In some patients with exon 12 mutations or *JAK2-V617F*, a proportion of endogenous erythroid colonies were negative for both *JAK2* mutations. One patient carried

2 independent clones: one with an exon 12 mutation and a second with *JAK2-V617F*. The finding of clonal heterogeneity is compatible with the hypothesis that additional clonal events are involved in the pathogenesis of PV. (Blood. 2008;111:3863-3866)

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Introduction

Mutations in exon 12 of *JAK2* are detected selectively in patients with polycythemia vera (PV) that are negative for *JAK2-V617F* and in some patients with idiopathic erythrocytosis.¹ The *JAK2-V617F* and exon 12 mutations represent clonal markers useful to track the hematopoietic lineages involved in myeloproliferative disorder (MPD).²⁻⁶ In patients with MPD, *JAK2-V617F* is present in purified hematopoietic stem cells, in myeloid lineages of the peripheral blood, and in variable proportions of lymphoid cells.⁷⁻¹¹ Using a novel sensitive assay, we quantitated the involvement of exon 12 mutations in purified peripheral blood lineages and in erythroid progenitor assays. In addition, we addressed the question of whether *JAK2-V617F* is present in T cells by clonal analysis.

Cells, DNA, and RNA analyses

Isolation of granulocytes, platelets, and peripheral blood mononuclear cells was performed as described.^{5,15} Sorting of peripheral blood mononuclear cells, colony assays in methylcellulose, T-cell cloning,¹⁶ and the SNaPshot assay for RNA samples are described in Document S1. The allele discrimination assay for detection and quantification of *JAK2* exon 12 mutations is described in Figure S1. Allele-specific polymerase chain reaction (PCR) for the detection of *JAK2-V617F* and microsatellite PCR for chromosome 9p were performed as reported.^{5,17}

Statistical analysis

We used SPSS version 15.0 (Chicago, IL) to calculate linear and ordinal regression for the correlations between disease duration and the percentage of mutant allele and between the percentage of mutant allele and the number of lineages involved.

Methods

Patients

The screening for *JAK2* exon 12 mutation in MPD patients was performed by DNA sequencing.¹² All patients except p024 fulfilled the diagnostic criteria of PV according to the World Health Organization (Table S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).^{13,14} Patient p024 was initially diagnosed with essential thrombocythemia, and several months later phlebotomies were started because of rising hemoglobin (175 g/L). Two patients with *JAK2* exon 12 mutations (Vi064, Vi327) were from Vienna, Austria. All other patients were from Basel, Switzerland. The collection of patient samples was approved by the "Ethik Kommission Beider Basel" and the "Ethik Kommission der Universität Wien und des Allgemeinen Krankenhauses der Stadt Wien-AK." Written consent was obtained from all patients in accordance with the Declaration of Helsinki.

Results and discussion

We studied the lineage distribution of *JAK2* mutations in peripheral blood of 8 PV patients with mutations in exon 12 and in 21 PV patients with *JAK2-V617F* (Figure 1). Five different *JAK2* exon 12 mutations were observed by sequencing, and all of them contained deletions of 3 or 6 bases (Figure 1A). We devised a novel assay to quantitate *JAK2* exon 12 mutations with a sensitivity of 1% mutant alleles (Figure S1). In all patients analyzed, exon 12 mutations were detectable in granulocytes, platelets, and monocytes, with the highest allelic ratios in most cases present in platelets and the lowest in monocytes (Figure 1B top panel).

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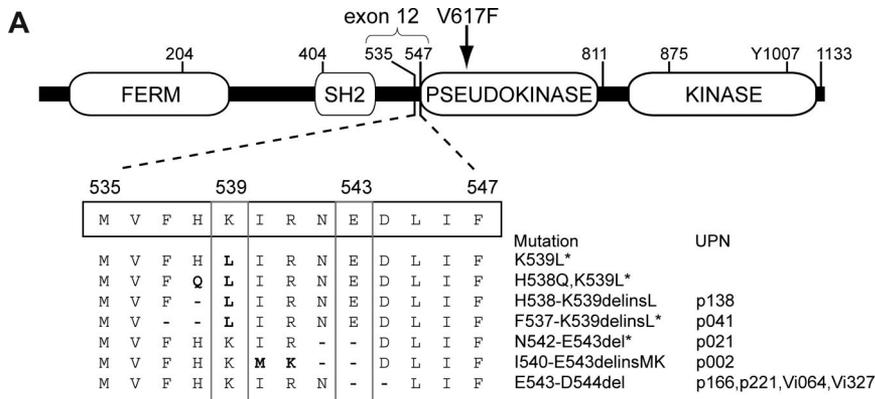


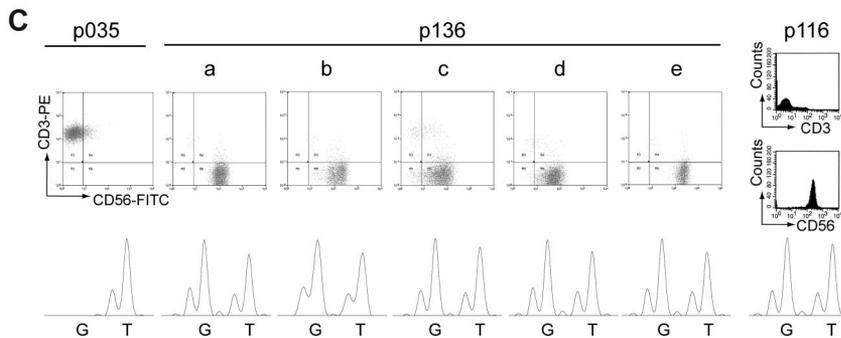
Figure 1. Distribution of *JAK2* mutations in peripheral blood lineages. (A) The location of exon 12 mutations and *JAK2*-V617F in the *Jak2* protein is shown (top). The amino acid changes caused by the individual exon 12 mutations are shown below using the single letter code. Previously described mutations (asterisk) and newly found mutations (no asterisk) are shown with the unique patient numbers (UPN) of the patients included in this study. (B) Lineage distribution of *JAK2* exon 12 mutations (top) and *JAK2*-V617F (bottom part). Numbers in boxes indicate the percentages of chromosomes 9 with exon 12 mutations, and the shading of boxes corresponds to the ranges shown at the bottom. UPN indicates unique patient number; F, female; M, male; GRA, granulocytes; NK cells, natural killer cells; nd, not determined. Numbers in column for T-cell cloning indicate *JAK2*-V617F positive clones/total clones analyzed. The phenotypes of *JAK2*-V617F positive clones were determined by flow cytometry. NK cell phenotype: CD3⁻CD56⁺; T-cell phenotype: CD3⁺CD56⁻. * Note that patient p021 was positive for exon 12 mutation N542-E543del and for *JAK2*-V617F. (C) Phenotypic analysis of *JAK2*-V617F positive clones. Flow cytometric analyses. One *JAK2*-V617F positive clone from patient p035 consisted of CD3⁺CD56⁻ T cells. All other positive clones from patients p136 and p116 were CD3⁻CD56⁺ NK cells (top panel). Allele-specific PCR for *JAK2*-V617F showing T-cell clone from p035 was homozygous for *JAK2*-V617F, whereas the clones from patients p136 and p116 were heterozygous for *JAK2*-V617F (bottom panel).

B Exon 12 mutations

UPN	Sex/ Duration age (months)	GRA	Platelets	Monocytes CD14 ⁺	NK cells CD3 ⁻ CD56 ⁺	B cells CD19 ⁺	T cells CD3 ⁺ CD56 ⁻	Mutation
p002	F/52 72	4	17	1	0	0	0	I540-E543delinsMK
p041	M/57 168	10	25	1	0	0	0	F537-K539delinsL
p138	F/71 27	14	33	4	0	0	0	H538-K539delinsL
p021*	M/59 59	15	26	8	0	0	0	N542-E543del
p166	F/55 108	28	37	22	0	0	0	E543-D544del
p221	F/37 15	7	45	5	4	0	0	E543-D544del
Vi064	F/67 30	31	41	40	6	0	0	E543-D544del
Vi327	F/80 116	69	54	60	42	19	0	E543-D544del

UPN	Sex/ Duration age (months)	GRA	Platelets	Monocytes CD14 ⁺	NK cells CD3 ⁻ CD56 ⁺	B cells CD19 ⁺	T cells CD3 ⁺ CD56 ⁻	T cell cloning	Pheno-type
p104	M/76 19	11	19	0	0	0	0		
p021*	M/59 59	3	12	2	0	0	0		
p115	F/91 126	16	11	5	0	0	0		
p052	F/85 154	41	82	42	0	0	0		
p026	M/73 120	47	82	6	0	0	0		
p023	M/71 111	54	74	48	0	0	0	0/71	
p022	M/31 49	67	56	58	0	0	0	0/314	
p038	F/62 39	64	78	58	6	0	0		
p025	M/77 180	69	27	49	13	0	0		
p046	F/72 106	83	78	30	8	0	0	0/91	
p033	M/77 238	93	99	100	100	0	0	0/205	
p103	F/82 598	99	99	99	99	0	0		
p136	M/76 13	37	46	32	33	3	0	5/200	NK
p070	M/73 157	39	65	34	11	10	0		
p053	M/70 35	43	43	27	6	5	0		
p024	M/56 82	45	51	16	4	3	0	0/128	
p093	M/62 20	61	62	23	11	2	0	0/159	
p069	M/39 250	64	45	2	2	2	0		
p116	M/40 48	85	88	40	8	2	0	1/105	NK
p035	M/73 239	85	97	92	72	62	1	1/299	T cell
p016	M/74 205	92	91	92	83	13	3	2/293	nd

76-100% 51-75% 26-50% 6-25% 1-5%



Similarly, the *JAK2*-V617F mutation was present in granulocytes, platelets, and with the exception of p104, also in monocytes (Figure 1B bottom panel). Interestingly, in patient p021, we detected 2 different *JAK2* mutations: N542-E543del (exon 12) and *JAK2*-V617F (exon 14). In granulocytes, platelets, and monocytes of patient p021, the exon 12 mutation was present in higher allelic ratios than *JAK2*-V617F.

Only 3 of 8 patients (38%) with exon 12 mutations displayed detectable signal in lymphoid cells. In patients p221 and Vi064, a

small subset of natural killer (NK) cells carried the mutation, and only patient Vi327 showed an allelic ratio greater than 10% in NK and B cells. *JAK2*-V617F also showed variable engagement of lymphoid lineages, with NK cells being most frequently involved (14 of 21, 67%) and in some cases showing very high (> 70%) allelic ratios (p016, p033, p035, and p103). B cells had low *JAK2*-V617F allelic ratios (< 15%), except in one patient (p035). *JAK2* exon 12 mutations have not been observed in T cells by DNA sequencing,¹ and were also absent in our study using a more

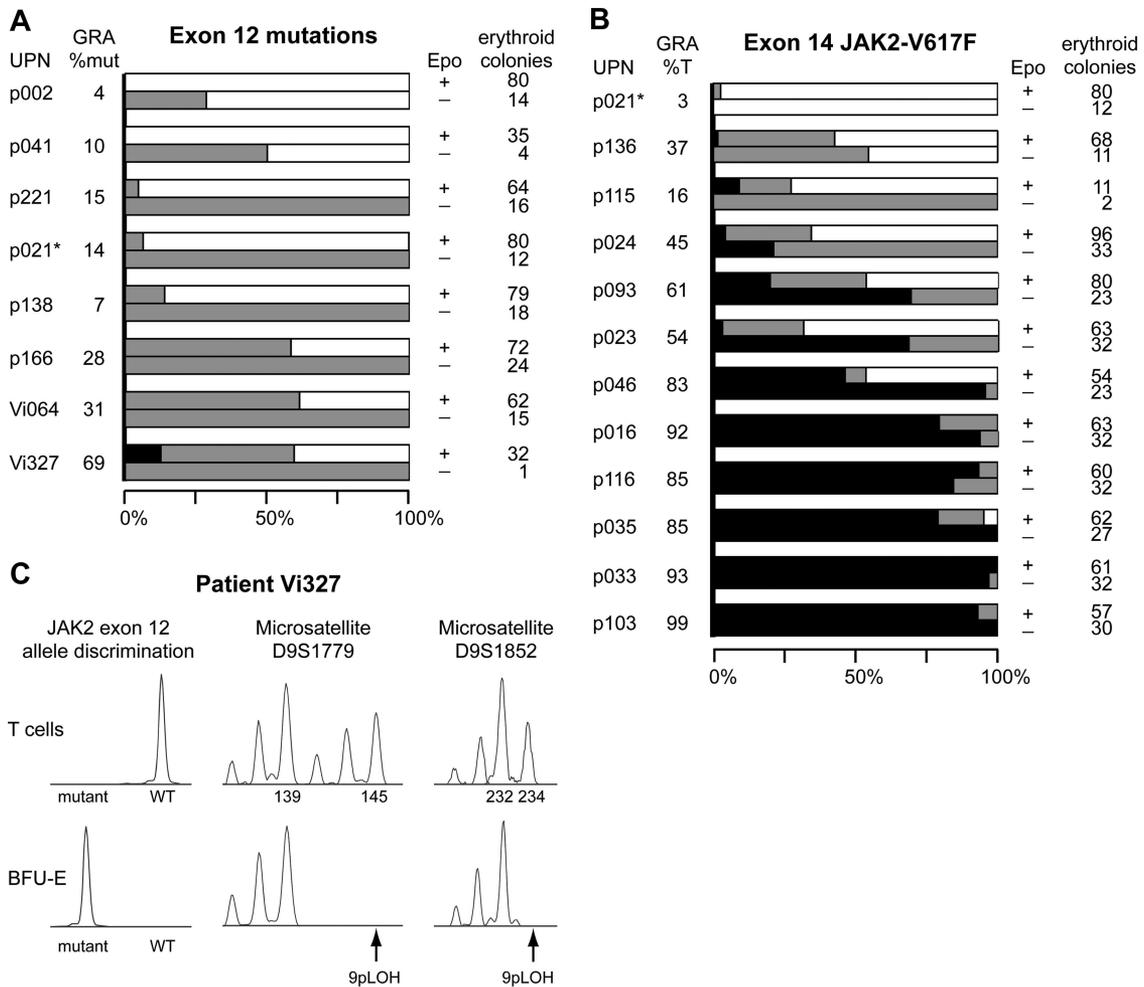


Figure 2. Distribution of *JAK2* mutations in erythroid progenitors and loss of heterozygosity on chromosome 9p (9pLOH) analysis in patient Vi327. The total number of erythroid colonies analyzed and the percentages of colonies with homozygous or heterozygous *JAK2* mutation or wild-type *JAK2* are shown. Colony assays in methylcellulose were performed with peripheral blood cells of patients with *JAK2* exon 12 mutations (A) or *JAK2*-V617F mutation (B). Single erythroid colonies were picked and analyzed individually. Horizontal bars indicate the percentages of colonies with homozygous mutation (■), heterozygous mutation (▨), or wild-type *JAK2* (□). For each patient, 2 bars are shown: the upper representing colonies grown in the presence of erythropoietin (Epo⁺) and the lower representing colonies grown without erythropoietin (Epo⁻). The unique patient numbers (UPN) and the allelic ratios of the *JAK2* mutations (%mut or %T) in granulocytes (GRA) are shown in the 2 left columns, and the total number of erythroid colonies analyzed is shown in the right column. *Note that in patient p021 colonies positive for exon 12 mutation and colonies with *JAK2*-V617F were found. None of these colonies carried both mutations simultaneously. (C) Molecular analysis of individual erythroid colonies of patient Vi327. Data from 1 of 4 BFU-E homozygous for the E543-D544del mutation is shown. T-cell DNA from patient Vi327 was used as control (top row). Allele discrimination assay shows the presence of a homozygous E543-D544del mutation (left panel). Two microsatellite markers, D9S1779 and D9S1852, demonstrate loss of heterozygosity on chromosome 9p (9pLOH) in the same colony (middle and right panels). Numbers indicate allele sizes.

sensitive detection assay. Only 2 of 21 patients (p016 and p035) displayed *JAK2*-V617F in T cells. The presence of *JAK2*-V617F in T cells from PV patients has been described in one study¹⁰ but was rare in other reports.^{8,9,18-22} All these studies were performed using bulk cell populations. We established T- and NK-cell clones from peripheral blood of 10 PV patients with high *JAK2*-V617F allelic ratios. In 4 of 10 patients, less than 1% of the total clones were *JAK2*-V617F positive (Figure 1B). Surprisingly, only a single *JAK2*-V617F positive clone in patient p035 consisted of T cells, whereas *JAK2*-V617F positive clones in 2 of 3 patients examined were NK cells (Figure 1B,C). *JAK2*-V617F positive CD34⁺ progenitors from MPD patients were shown to be capable of differentiating into T cells in thymic organ cultures in vitro.⁹ However, in patients, maturation of *JAK2*-V617F positive T cells occurs very infrequently, possibly because of low frequency of de novo T-cell genesis in adults. A correlation between the disease duration and the allelic ratios of *JAK2*-V617F in granulocytes, platelets, monocytes, and NK cells was noted (Figure S2), but no such correlation was found for exon 12 mutations (not shown). The

percentages of mutant alleles also correlated with the number of lineages involved for both the exon 12 mutations and *JAK2*-V617F (Figure S2).

Erythroid colonies that grew in the absence of Epo (EECs) were detected in all patients (Figure 2), although in some cases the total number of EECs was small (p041, Vi327, and p115). In 2 patients with exon 12 mutations (p002 and p041) and one patient with *JAK2*-V617F (p136), we found EECs with only wild-type *JAK2* sequences in 2 independent experiments. The erythroid phenotype of the colonies was confirmed by the presence of glycophorin A mRNA (Figure S3). Sequencing of the entire *JAK2* coding region in these colonies did not reveal any additional *JAK2* mutations. Growth of EECs with only wild-type *JAK2* has recently been reported in PV patients positive for *JAK2*-V617F.^{21,23,24} In contrast, no wild-type EECs in PV patients were found by others.^{1,3,25,26} Our results confirm the finding of EECs with only wild-type *JAK2* and suggest clonal heterogeneity of erythroid progenitors, not only in patients with *JAK2*-V617F but also in some patients with exon 12 mutations. Interestingly, in the patient with 2 different *JAK2*

mutations (p021), none of the erythroid colonies carried both mutations simultaneously (Figure 2), indicating that the exon 12 mutation and *JAK2-V617F* represent 2 separate clones. Concurrent presence of *JAK2-V617F* with *MPLW515* mutations was previously found in 3 patients with primary myelofibrosis and 3 patients with essential thrombocythemia.^{27,28} The allelic ratios for *MPLW515* were higher than those for *JAK2-V617F*, but clonal analysis was not reported. Therefore, it remains to be determined whether these cases represent sequential somatic mutations that occurred in the same clone. Thus, our patient p021 is the first case with a documented biclonal pattern. The finding of clonal heterogeneity is compatible with a model in which a stem cell or progenitor carries a predisposing mutation that increases the likelihood for acquiring somatic mutations in *JAK2* or other as yet unknown gene(s).

In patient Vi327, we found a homozygous exon 12 mutation in 4 of 32 colonies examined (Figure 2A). In this patient, the allelic ratio of the exon 12 mutation was more than 50% in granulocytes, platelets, and monocytes (Figure 1B). The homozygous colonies exhibited loss of heterozygosity on chromosome 9p (9pLOH; Figure 2C). Copy number analysis of *JAK2* by real-time PCR excluded gene amplification or deletion as the mechanism (not shown). Two other cases of homozygous exon 12 mutations were recently found by sequencing, but the mechanism has not been studied.^{12,26} All PV patients with *JAK2-V617F* exhibited at least some homozygous colonies (Figure 2B), as reported.²⁵ The only exception in our series was patient p021, in whom we found 2 different *JAK2* mutations. The reason why exon 12 mutations are

more invariably associated with increased erythropoiesis than *JAK2-V617F* remains to be determined.

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Authorship

Contribution: S.L. performed research, analyzed data, and wrote the paper; R.K. performed research and analyzed data; G.D., A.T., and H.G. analyzed data; R.C.S. designed research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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