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Molecular Mechanisms in Philadelphia Negative Myeloproliferative Neoplasia

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1. Introduction

The myeloproliferative neoplasia (MPNs) are a spectrum of clonal disorders of the hematopoietic system. The distinct clinical manifestations are dictated by the primary cell type affected, and thus chronic myeloid leukemia (CML) is a proliferation of mature granulocytes, polycythemia vera (PV) is an expansion or red blood cells, essential thrombocythemia (ET) results in an increase of platelets, etc.. The natural history of MPNs is generally chronic in nature, and patients come to medical attention either by coincidence (abnormal blood findings during routine exam) or by signs and symptoms related to the expansion of the hematopoetic system (e.g., an enlarged spleen). Common to most MPNs is a small but finite risk of disease evolution to an acute leukemia, where hematopoetic development is blocked at an early stage of differentiation, leading to the accumulation of poorly functioning myeloid blasts at an expensive of critical depletion of normal white blood cells and platelets, leading to morbidity and mortality from infections and bleeding complications. If they do not progress to an acute leukemia, the natural history of MPNs often results in fibrosis of the bone marrow, migration of hematopoesis to other organs (spleen and liver), and eventual complications of this secondary organ involvement, as well as from decreased normal blood counts from marrow fibrosis. A unifying theme in the pathogenesis of MPNs is the activation of tyrosine kinases. The ''poster child'' is CML, where the BCR-ABL translocation is found in all cases; the fusion BCR-ABL activates proliferative and antiapoptotic pathways; and most importantly, inhibition by tyrosine kinase inhibitors (TKIs) can markedly reverse the natural history of the disease. The molecular lesions responsible for PV, ET, and myelofibrosis (MF) were unknown until relatively recently. From 2005, a flurry of reports found that a point mutation in JAK2, resulting in a valine for phenylalanine substitution at codon 617 (JAK2V617F), occurred at a high prevalence in these disorders. The mutation was found in roughly half of MF and ET cases and nearly all PV cases. Constitutive activation of JAK2 activates STAT and MAPK proliferative signaling pathways, leading to transformation of hematopoetic progenitors. Curiously, not all hematopoetic stem cells in cases with the JAK2V617F harbor the mutation. Moreover, the data suggested a differential dosage effect in the different diseases. Whereas in most cases the JAK2V617F is heterozygous with a normal JAK2 allele, in many cases of

PV the mutation is homozygous through the process of acquired uniparental disomy. Curiously, in vitro cultures of PV cases will often show homozygous JAK2V617F erythroid colonies, whereas similar colonies from ET patients are heterozygous for the mutation. There has been a substantial body of work attempting to study the effects of the JAK2V617F in mouse models. Early reports focused on a bone marrow transplantation model, where mouse bone marrow cells harboring exogenous JAK2-V617F were transplanted into irradiated mice. These models produced a syndrome of what appeared mostly like PV, but most failed to completely recapitulate the spectrum of leukocytosis, thrombocytosis, and myelofibrosis found in human disease. Transgeneic models followed, which again produced a spectrum of MPN disorders, with a suggestion of phenotype relating to the JAK2V617F expression levels. Very recently, several groups have created knockin systems placing a conditionally inducible JAK2V617F allele under control of the endogenous JAK2 promoter. This allows for control of the JAK2V617F expression in only hematopoetic tissues, getting one closer to replicating the disease experience of the human patient. Marty et al. found that the heterozygous expression of JAK2V617F produced a PV-like syndrome, not like human where heterozygousity is more often associated with ET. However, Akada et al. demonstrated that both heterozygous and homozygous JAK2V617F caused a PV syndrome, with a demonstration of a dose effect, as indicated by the fact that homozygous expressors had a greater manifestation of elevated blood counts and spleen size, compared to those mice with lower levels of JAK2V617F. In addition, Li et al. have produced a very provocative study in which a human JAK2V617F knockin was created. This model produced a transplantable disease with some features of both ET and PV. Of interest is the finding that affected mice had reduced numbers of primitive hematopoetic cells that had evidence of impaired normal function (cell cycling, apoptosis, and DNA damage). Moreover, competitive marrow transplantation showed impaired hematopoetic stem cell function. Recently, Mullally and colleagues used a conditional JAK2V617F expression model to yield physiological levels of the mutated allele. The phenotype in the mice resembled much of the cellular biology and clinical features of human PV, and it was serially transplantable with great efficiency. Separation of the bone marrow into immature Lineage– SCA-1+ c-Kit+ (LSK) and more mature myeloid erythroid progenitor (MEP) and granulocytic monocytic progenitor (GMP) subpopulations demonstrated that the ''MPN-initiating'' JAK2V617F cell capable of transplantation resided in the LSK population, but not in the committed myeloid MEP or GMP progenitors. Surprisingly, several studies showed that mutant cells in the LSK compartment were quite similar to wildtype cells in regard to cell cycle status, STAT signaling, and gene expression (though JAK2V617F cells showed enrichment of the erthyroid, myeloid, and megakaryocytic differentiation pathways). Similar to the Li et al. paper, competitive transplantation experiments showed that mutant cells had at best a minor competitive edge compared to wild-type, and a small number of mutated cells nonetheless causes a PV phenotype. Lastly, the authors demonstrated that the MPN initiating cell was not killed by JAK2 inhibition. Mice treated with the inhibitor had a dramatic decrease in spleen size and a reduction of erthyroid precursors in the marrow, but LSK cells from treated mice were able to cause the PV phenotype in subsequently transplanted mice. These studies in total offer an increased understanding of the MPN that may usher in a new era of therapy, much like what occurred in the study of CML. Similar to CML, these studies suggest the initiating cell resides in the primitive compartment but is

genetically and phenotypically quite similar to its normal complement. Like CML, mutated cells in the stem cell compartment appear resistant to kinase inhibition. However, as we move toward better therapies for MPN, these findings have implications in the feasibility of ''stem cell'' therapy, because there may not be a large therapeutic window to selectively kill MPN ''stem cells.'' In addition, a limitation of murine systems is that however eloquent, they are still only models of human disease. For example, human MPN may well have additional genetic lesions contributing to initiation and progression, and mouse models cannot easily recapitulate this complexity. In this regard it is interesting that several other mutations have recently been discovered in MPN (e.g., TET2, ASXL1, IDH1, and IDH2); TET2 mutations have been found in JAK2V617F-positive and -negative clones from the same patient, suggesting that TET2 mutations may be a relatively early event in MPN. Moreover, if tumor initiation and progression is influenced by interactions with a host's innate immunological system, then disease in the mouse model might be expected to be very different than in humans. Nonetheless, the work presented by Mullally and others are quite significant, and provide us with powerful tools to better understand disease and test new agents of therapy.However, it should be noted that some mutations might possess more than one mechanism of action, for example, *JAK2*V617F results in dysregulation of kinase signaling but might also have an epigenetic effect. Recently, Dawson et al.identified a novel nuclear role of JAK2 in the phosphorylation of Tyr 41 of histone H3 leading to chromatin displacement of HP1a. The authors suggested that the inability of HP1a to regulate chromatin could reduce the potential tumor suppressive functions of HP1a resulting in erratic mitotic recombination and transcription deregulation of several JAK2-regulated genes such as *LMO2*. These results were confirmed in hematopoietic cell lines and in the CD34+ cells collected from the peripheral blood of one PMF patient with JAK2V617F mutation. Our group defined the subcellular localization of JAK2 in total BM cells and in sorted cell populations collected from MPN (ET, PV, PMF) patients with the JAK2V617F mutation or from MPN patients with wild type (wt) JAK2. We find that in contrast to cells with normal JAK2 in which the protein is detected predominantly in the cytoplasm, JAK2 is mostly nuclear in V617F-positive CD34+ cells. However, this nuclear localization is no longer observed in V617F-positive differentiated cells. After expressing JAK2V617F in K562 cells, we observe a similar preferential accumulation of JAK2 in the nucleus in contrast to untrasfected- and wt JAK2-expressing cells in which the protein is found in the cytoplasm. The mutated-JAK2 nuclear translocation is mainly reverted by the addiction of the JAK2 inhibitor AG490.

1.1 Functional hallmarks of myeloid progenitors in myeloproliferative neoplasms

The BCR-ABL-negative myeloproliferative diseases, which include polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), were recently renamed myeloproliferative neoplasms (MPNs).[1] PV, ET and PMF are disorders of hematopoietic stem cells (HSCs) and early myeloid progenitors,[2, 3] where myeloid progenitors are hypersensitive and/or independent of cytokines for survival, proliferation and differentiation. For instance, the majority of PV patients harbor erythropoietin (Epo)-independent erythroid colonies.[4] Several intracellular anti-apoptotic pathways and molecules, such as STAT3, Akt or BclXL, are activated/induced constitutively in such MPN myeloid progenitors,[5, 6, 7] along with hypersensitivity to insulin-like growth factor 1 (IGF-1),

granulocyte macrophage colony-stimulating factor, interleukin 3 (IL-3), granulocyte colony-stimulating factor (G-CSF) or thrombopoietin (Tpo).[8,9, 10, 11] Unlike many malignancies, where the INK4a locus is inactivated, erythroid progenitors from PV patients exhibit increased expression of the INK4a/ARF locus.[12]

1.2 Mutations involved in PV, ET and PMF

The acquired somatic *JAK2* V617F mutation is harbored by the majority of PV patients and by more than 50% of ET and PMF patients.[13, 14, 15, 16, 17]Subsequent identification of exon 12 mutants of *JAK2* in a minority of PV patients gave a molecular lesion to virtually all PV cases.[18] Sequencing of the gene coding for the Tpo receptor (TpoR/c-Mpl) identified mutations in the juxtamembrane tryptophan residue W515 (W515L and W515K) in a low percentage of PMF and ET patients, the majority of which are *JAK2* V617F-negative.[19, 20] The W515 residue of TpoR is required to maintain the receptor inactive in the absence of ligand.[21] All these mutations lead to constitutively active JAK–STAT pathway, especially JAK2, STAT5, STAT3, MAP kinase ERK1,2 and Akt.[13, 16, 21]Advancement over the past 3 years in the MPN research field has raised several major questions, such as: (i) What are the molecular bases for the significant differences in the *in vivo* phenotypes induced by *JAK2* V617F and TpoR W515L? (ii) How can a unique somatic mutation, *JAK2* V617F, be involved in the induction of three different diseases ET, PV or PMF? (iii) What mechanisms are responsible for the evolution of MPNs toward acute myeloid leukemia? (iv) What are the effects of *JAK2* V617F and of the other JAK2 and TpoR mutants at the level of HSC? and (v) What preceding and subsequent (to *JAK2* V617F) genetic events contribute to myeloproliferative diseases?Answers to these questions have begun to emerge. Gene dosage, as initially suggested by genotype/phenotype studies in patient cells,[22] by retroviral bone marrow reconstitution studies[23] and most recently probed in transgenic mice,[24]could be critically involved in inducing one or the other of the MPN phenotypes. It is fascinating that progenitors homozygous for the *JAK2* V617F mutation occur in almost all PV patients, but very rarely in ET patients.[25, 26] Although this can be seen as an argument in favor of the gene dosage hypothesis, other preceding or subsequent genetic changes might have an important function. Interestingly, host-modifying influences might have a major part in establishing the disease phenotype.[27] A screen for genetic variation within the genes coding for EpoR, TpoR, G-CSF receptor (G-CSFR) and JAK2 led to the discovery of three JAK2 single nucleotide polymorphisms that were significantly but reciprocally associated with PV and ET, but not with PMF. Three additional JAK2 single nucleotide polymorphisms were uniquely associated with PV. Such single nucleotide polymorphisms, although not in the coding region of the genes, might affect the levels of gene transcription, regulation by other factors or possibly expression of other genes.

1.3 Unknown effects of JAK2 V617F signaling in HSCs

JAK2 V617F mutation was detected at the HSC and the common myeloid/lymphoid progenitor levels, it skews the HSC differential potential toward the erythroid lineage and gives a selective proliferative advantage to myeloid lineages.[28, 29] The HSC compartment of PV and PMF patients was found to contain *JAK2* V617F-positive long-term, multipotent and self-renewing cells, with a much higher proportion of mutated HSCs in PMF than in PV.[30] It is not clear at this moment whether *JAK2* V617F profoundly affects the biology of HSCs[30] or

whether it only gives a strong selection advantage past the HSC stage. A certain degree of heterogeneity exists between HSC subsets.[31] HSCs exist in niches, some near osteoblasts and others near endothelial cells. Exactly where and in which HSC subset the *JAK2* V617F mutation initially occurs might have a major impact on the subsequent disease phenotype.The *JAK2* V617F mutation was present in 30–40% of splanchnic venous thrombosis patients (Budd–Chiari syndrome and portal vein thrombosis).[32, 33,34, 35] A 'special' stem cell with hematopoietic/endothelial potential was suggested to be at the origin of splanchnic venous thrombosis, and it might harbor *JAK2*V617F.[36] A recent case report described a human allogeneic transplantation with*JAK2* V617F-positive cells from such a splanchnic venous thrombosis donor (with one episode of *JAK2* V617F-positive splanchnic venous thrombosis), but no MPN, to her HLA-matched sister with high-risk myelodysplastic syndrome (RAEB2).[37]The recipient exhibited a *JAK2* V617F burden similar to the donor immediately after transplant, but this burden decreased over time, and 7 years later, the recipient continues to be in remission and to exhibit low levels of *JAK2* V617F positivity.[37] These data suggest that, indeed, the *JAK2* V617F mutation can occur in an HSC, but at least in the transplantation setting, this HSC has no proliferative advantage. A considerable amount of data suggests that in addition to the presence of the*JAK2* V617F mutation, preceding or subsequent genetic events might be necessary for developing the MPN disease. First, in certain MPN patients, the clonality of expanded myeloid progenitors is found to be larger than the *JAK2* V617F clone, with the acquisition of *JAK2* V617F being a late genetic event.[38] Second, acute myeloid leukemia cases developed in*JAK2* V617F-positive patients can occur with leukemic blasts not harboring *JAK2*V617F.[39] Third, Epo-independent colonies might not always harbor the *JAK2*mutation in patients with the *JAK2* V617F mutation.[40] Such preceding or subsequent events could be associated differently with the three diseases, namely ET, PV and PMF.

1.4 The signaling space

Molecular cell biology textbooks list eight major signaling pathways that control gene expression that are linked to eight classes of cell surface receptors: cytokine receptors, receptor tyrosine kinases, receptors for transforming growth factor (TGF)-β, Wnt, Hedgehog, tumor necrosis factor (TNF)-α, Notch (Delta) and G-protein-coupled receptors.[41] Nuclear and corticoid receptor pathways, as well as integrin signaling, complete the picture of intracellular cell signaling. In several paperit has been well described that aberrant signaling occurs in MPNs through some of the listed pathways, such as cytokine receptors, receptor tyrosine kinases, TGF-β and TNF-α. It is not clear whether aberrant signaling in MPNs is simply due to the constitutive nature of signaling induced by mutated JAK2 or TpoR, or whether specific cross-talk events occur to other pathways that might confer specificity to JAK2 V617F versus JAK2 signaling. It is important to recognize from the outset that the experimental systems used by signal transduction research, such as phosphorylation studies, co-immunoprecipitation, gene expression and determination of protein localization, may not be able to identify subtle relative changes, such as kinetics and amplitude differences, between signals engaging generic pathways that are redundantly triggered by many stimuli. Such subtle quantitative differences might, however, be crucial for the disease phenotypes *in vivo*. That is the reason why genetics, *in vivo* models, results obtained with inhibitors and data derived from primary cells, must be taken into account to draw a picture describing aberrant signaling in MPNs.

1.5 Constitutive signaling and kinase activity of JAK2 V617F

The mammalian genome codes for four Janus kinases (JAKs), JAK1, JAK2, JAK3 and Tyk2. On the basis of homology, JAKs share seven JAK homology domains (JH), denoted as JH1–JH7. From the C to the N terminus, JH1 represents the kinase domain, JH2 the pseudokinase domain, JH3 and JH4 contain an SH2-like domain and linker regions, whereas JH5–JH7 contain a FERM (band 4.1, ezrin, radixin, moesin) domain[42, 43] (Figure 1).

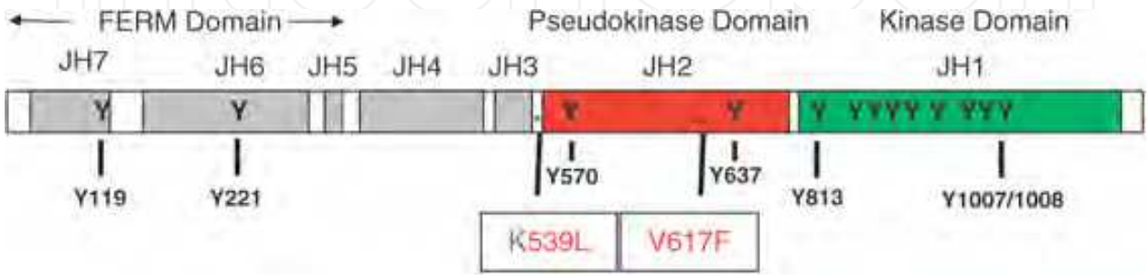


Fig. 1. Schematic illustration of Janus kinase (JAK)2 and the different JAK homology (JH) domains. The V617F mutation occurs in the pseudokinase domain rendering the kinase domain constitutively active. Exon 12 mutations, such as K539L, occur in the linker region between the JH3 and JH2 domains. Tyrosine residues that can be phosphorylated are depicted by their single letter. See text for details.

JAKs have been proposed to have a bipartite structure and the N terminus is required for binding to receptors, chaperoning and stabilizing them at the surface,[44, 45, 46, 47] whereas the kinase domain is absolutely crucial for signaling. The pseudokinase domain precedes the kinase domain, and because of sequence differences at key residues required for catalysis, it cannot transfer phosphate and thus is catalytically inactive.[42]Nevertheless, the pseudokinase domain is structurally required for the response of JAKs to cytokine receptor activation and for inhibiting the basal activity of the kinase domain.[48, 49] The V617F mutation occurs in the pseudokinase domain, leading to constitutive activation of the kinase domain (Figure 1). Although no X-ray crystal structure of full-length JAK2 exists, modeling has suggested that the pseudokinase domain of JAK2 maintains the kinase domain inactive in the basal state.[50] Thus, the V617F mutation is expected to relieve the inhibitory effect of JH2 on JH1 and to lead to basal kinase activity. The homologous V617F mutations in JAK1 and Tyk2 also lead to constitutive activation,[51] which strongly supports this model. Activating mutations in the pseudokinase domain of JAK1 at the homologous V658 position or at neighboring residues have been reported in 20% of patients with T-acute lymphoblastic leukemia.[52, 53] In transiently transfected JAK2-deficient cells, such as the γ-2A human fibrosarcoma cell line,[54] JAK2 V617F expression leads to constitutive activation of STAT5 and STAT3 signaling. In such transient transfection experiments, JAK2 V617F is constitutively tyrosine phosphorylated at the activation loop Y1007. Co-transfection of wild-type JAK2 reduces signaling by JAK2 V617F, presumably due to competition for an interaction partner, such as

a cytokine receptor,[13] but does not prevent constitutive phosphorylation of JAK2 V617F.[16]To investigate the catalytic activity of JAK2 V617F mutation, kinase assays have been performed on GST fusion proteins. In COS7 overexpression conditions, when compared with wild-type JAK2, the JAK2 V617F mutated protein exhibits enhanced basal kinase activity on a reporter GST fusion protein containing the sequence of the activation loop of JAK2 containing tyrosine (Y) 1007.[17, 55] The kinase activity was clearly increased, but it appeared to be weak. In stably transfected Ba/F3 cells, JAK2 V617F also exhibits enhanced kinase activity on the same Y1007-containing GST fusion protein, but the levels of activation were also small (C Pecquet *et al.*, unpublished results). This is very different from BCR-ABL or other fusion proteins such as TEL-JAK2, where the kinase domain alone is oligomerized and activated by a fused exogenous oligomeric domain. In contrast, the kinase domain of JAK2 V617F is expected to maintain most of the negative regulatory intramolecular interactions that normally limit kinase domain activation.

1.6 JAK2 V617F and cytokine receptors

The FERM domain of JAKs is responsible for appending JAKs to cytokine receptors. Cytokine receptors contain in the cytosolic juxtamembrane region a proline-rich sequence, usually PxxPxP, denoted as Box 1, located 10–15 amino acid residues downstream of the TM domain, and further down 50–60 amino acid residues downstream of the TM domain, a sequence composed of hydrophobic and charged residues denoted as Box 2.[56]JAK2 binds to the region of EpoR that encompasses cytosolic residues of Box 1, Box 2 and also most of the residues between these boxes.[44, 56]Interaction between JAK2 and EpoR or TpoR is disrupted by a point mutation (Y114A) in the FERM domain.[47] Expression of the double-mutant JAK2 V617F Y114A in Ba/F3-EpoR cells did not lead to constitutive signaling through STAT5 or to autonomous growth,[57] suggesting that the V617F mutation does not suffice for activation in the absence of the assembly between the JAK2 V617F and a cytokine receptor. It can be noted that members of the JAK family are localized to membranes through recruitment by cytokine receptors, whereas mutations such as Y114A lead to cytosolic localization.[58] Furthermore, a mutation in the pseudokinase domain of JAK2 was identified (Y613 to glutamic acid, Y613E), which promotes constitutive activation only when JAK2 is in complex with the EpoR.[59] This result suggests that in the absence of an association with a cytokine receptor, JAK2 is locked into an inactive state and that receptor binding through the FERM domain is important for activation.[59]Another argument supporting the notion that binding to a cytokine receptor is important for the activity of the V617F mutant arises from the lack of activation of JH2–JH1 fusion proteins where the V617F mutation was introduced in the JH2 sequence.[60] The low basal activity of JH1 was shown to be suppressed by fusion with JH2.[49, 61] However, the presence of the FERM–SH2 domains is required for the activation effect exerted by the V617F mutation. JAK2 is crucial for signaling by EpoR[62] and TpoR,[63, 64] participates in signaling by G-CSFR[65, 66] (Figure 2) and also mediates signaling by the IL-3/IL-5/granulocyte macrophage colony-stimulating factor family of cytokines, as well as by several type-II cytokine receptors, such as interferon-γ receptor 2[67]. Given that MPNs mainly affect the erythroid, the megakaryocytic and the granulocyte lineages, as stated before, complexes between JAK2 V617F and EpoR, TpoR and G-CSFR may explain cytokine hypersensitivity and independence in these diseases (Figure 2).

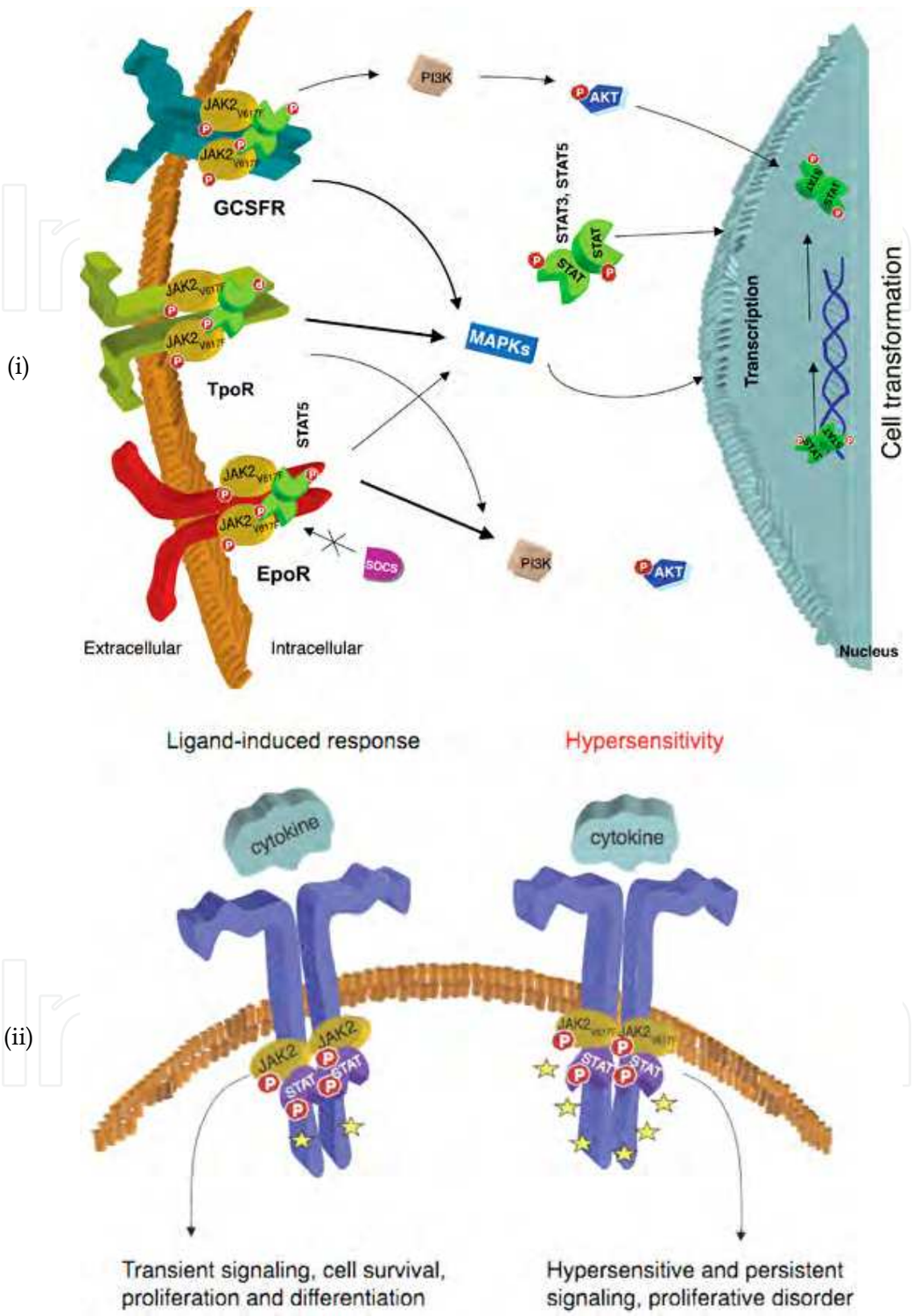


Fig. 2. Model of constitutive (ligand-independent) signaling induced by JAK2 V617F through erythropoietin receptor (EpoR), thrombopoietin receptor (TpoR) and granulocyte

colony-stimulating factor receptor (G-CSFR). (i) Janus kinase 2 (JAK2) is the main JAK used by EpoR and TpoR, but JAK1 is also used physiologically by G-CSFR. Primarily, EpoR and TpoR are expected to be bound by JAK2 V617F, whereas G-CSFR is expected to be in complex with JAK2 V617F at high JAK2 V617F levels, i.e., in homozygous *JAK2* V617F situations. Scaffolding of JAK2 V617F on the cytosolic tails of cytokine receptors leads to the enhanced activation of JAK2 V617F and downstream signaling through STATs, MAP kinase, PI-3-kinase (PI3K) and Akt. SOCS proteins are expected to engage both EpoR and activated wild-type JAK2, leading and down-modulation of JAK2 activity; the EpoR–JAK2 V617F complex appears to escape the down-modulation activity of SOCS3. (ii) Cytokine receptors that are in complex with JAK2 V617F are hypersensitive to their ligands for signaling. Cytokine binding to receptors coupled to wild-type JAK2 induce transient physiologic signals, leading to survival, proliferation and differentiation of myeloid progenitors. In contrast, receptors coupled to JAK2 V617F respond to lower levels of ligand, and are constitutively signaling after ligand withdrawal. It is not known whether dimeric receptor complexes, where one monomer is coupled to JAK2 V617F and the other to wild-type JAK2, are also hypersensitive to ligand or constitutively active.

1.7 EpoR and MPNs

EpoR functions as a preformed dimer on the cell surface, which upon cytokine binding undergoes a conformational change that triggers the activation of the receptor pre-bound JAK2.[68, 69] This involves a rotation of the receptor monomers within the dimer,[70] which is transmitted to JAK2 by switch residues, that is W258 in the juxtamembrane domain of EpoR.[69] Current data suggest that JAK2 V617F can scaffold on the cytosolic domain of EpoR and induce Epo-independent signaling, possibly by phosphorylating key cytosolic tyrosine residues on EpoR, which leads to strong STAT5 activation.[71]Early on after the identification of *JAK2* V617F, the need for a co-expressed type I dimeric cytokine receptor for constitutive signaling by JAK2 V617F provoked a controversy, which in the end led to a model of how dimeric receptors might actually promote JAK2 V617F activation. One study by Levine *et al*.[16] reported that JAK2 V617F readily induced autonomous growth in Ba/F3 cells engineered to express the EpoR (Ba/F3 EpoR cells), but not in parental Ba/F3 cells. In contrast, the study by James *et al*.[13] had shown that JAK2 V617F could induce autonomous growth in both Ba/F3 EpoR and parental Ba/F3 cells. This controversy (also described in Ihle and Gilliland[72]) was solved by carefully assaying the levels of JAK2 V617F transduction: at low levels, co-expression of a type I cytokine receptor was necessary for autonomous growth, whereas at higher levels JAK2 V617F alone induced autonomous growth, most likely by binding to an endogenous cytokine receptor, such as the IL-3-receptor β-subunit.[60] It is not clear whether other receptors—besides dimeric type I—could also promote signaling by low levels of transduced JAK2 V617F (Figure 2(ii)). Nevertheless, given that EpoR is a dimer in the absence of ligand, an insightful model was proposed by Harvey Lodish. In it, dimerization of JAK2 V617F by such a receptor is considered necessary for the activation of JAK2 V617F signaling, and the subtle V617F mutation promotes kinase activation when JAK2 is scaffolded on an inactive receptor dimer (Figure 2(i)).[71] Given that in MPNs the three lineages affected are controlled by the three type I dimeric cytokine receptors, EpoR, TpoR and G-CSFR, the model that JAK2 V617F mainly functions as a transforming kinase in association with these receptors is very plausible. EpoR signals mainly by JAK2–STAT5 and PI-3–kinase/Akt (Figure 2(i)) pathways. It is a weak activator

of MAP kinase and of STAT3,[73] as it does not contain a consensus site for STAT3 binding, whereas several phosphorylated tyrosine residues (Y343, Y401, Y429 and Y431) can bind STAT5 and are required for maximal STAT5 activation.[74, 75] A consequence of STAT5 activation is induction of the anti-apoptotic BclXL protein expression,[76] which is constitutively expressed in PV erythroid progenitors.[7] The connection of EpoR with the PI-3–kinase pathway is accomplished by specific tyrosine residues, that is Y479, which appears to bind the regulatory subunit p85.[77, 78] PI-3–kinase and Akt activations are critically involved in erythroid differentiation,[79] possibly by the involvement of the transcription factor Forkhead family, FKHRL1.[80] Another mechanism appears to be the phosphorylation of S310 of GATA1.[81] Thus, scaffolding of an activated JAK2 to EpoR is predicted to activate the JAK2–STAT5 and PI-3–kinase/Akt pathways and stimulate proliferation and differentiation of erythroid progenitors.

1.8 EpoR and exon 12 JAK2 mutations

Patients with exon 12 *JAK2* mutations, such as JAK2 K539L, exhibit an erythrocytosis phenotype, without pathology changes to megakaryocytes typical for MPNs.[18] Unlike the uniqueness of the point mutation that generates JAK2 V617F, several deletions and insertions were noted in the case of exon 12 mutations.[18, 82] An attractive hypothesis is that exon 12 mutants of JAK2 favor interaction with EpoR over TpoR or G-CSFR, although a mechanistic basis for such a preference has yet to be found. Modeling of JAK2 suggests that the K539L falls in a loop in the linker region between the SH2 and the JH2 domain (Figure 1), which would be placed in space quite close to the loop represented by β4–β5 where V617 is located.

1.9 TpoR and MPNs

TpoR is coupled to and activates both JAK2 and Tyk2,[64, 83, 84] which appear to have comparable affinities for the receptor juxtamembrane domain and to promote cell surface traffic of the receptor to a similar extent.[47] However, JAK2 is much more effective than Tyk2 in transmitting the signals of the receptor.[47,64] TpoR activates JAK2, STAT5 and PI-3–kinase/Akt,[85] but in contrast to EpoR, it is a very strong activator of Shc, of the MAP kinase pathway and of STAT3 (Figure 2(i)).[83, 86, 87, 88, 89, 90] It is interesting that the first consequence of expressing the JAK2 V617F (at lower than physiologic levels in the transgenic model) mutation is to promote platelet formation.[24] Bipotential megakaryocyte–erythroid progenitors appear to be stimulated to engage on the platelet formation by STAT3 activation, whereas STAT5 activation favors erythroid differentiation programs.[91] STAT5 emerged as a critical factor for lineage commitment between erythroid and megakaryocytic cell fates. Depletion of STAT5 from CD34(+) cells in the presence of Tpo and stem cell factor favors megakaryocytic differentiation at the expense of erythroid differentiation.[91] Overexpression of an activated form of STAT5 impaired megakaryocyte development favoring erythroid differentiation at the expense of megakaryocyte differentiation.[91] Thus, at low levels of expression, JAK2 V617F might only activate STAT3, which might suffice for platelet formation. At higher expression levels, coupling to both TpoR and EpoR will lead to STAT5 activation, and this would favor the erythroid program. It is not clear at this point whether the PV phenotype is exclusively the result of EpoR activation or whether the pathologic activation of TpoR might also contribute to the PV phenotype. It is interesting to note that overexpression of TpoR in certain animal models led to an expansion of the

erythroid compartment.[92]The SH2 and PH (pleckstrin homology domain) adapter protein Lnk was shown to not only bind to phosphorylated tyrosine residues of both TpoR and EpoR but also to exert a negative role on signaling by these receptors.[93, 94] It is not known whether the defects in this negative regulatory mechanism are operating in MPNs. Co-expression of JAK2 V617F and TpoR in Ba/F3 cells leads to down-modulation of TpoR, most likely due to internalization and down-modulation seen after excessive activation of cytokine receptors (J Staerk, C Pecquet, C Diaconu and SN Constantinescu, unpublished observations). This is consistent with early studies that have identified a maturation defect and down-modulation of cell surface TpoR in platelets and megakaryocytes from MPN patients.[95] More recently, an inverse correlation was reported between the burden of *JAK2* V617F and the levels of cell surface TpoR on platelets.[96] Although these results suggest that JAK2 V617F may contribute to the down-modulation of TpoR, several patients with MPNs in the absence of JAK2 V617F also exhibited down-modulated TpoR. Such down-modulation is not seen for EpoR. TpoR is a long-lived receptor at the cell surface[47] and recycles,[97] which is not the case for EpoR. Further experiments are necessary to follow up on the original observation of TpoR down-modulation in MPNs, which may be due to traffic alterations, excessive internalization and degradation or decreased protein synthesis. Several mutations in Mpl induce myeloid malignancies. A mutation in the transmembrane domain of Mpl, S505N, constitutively activates the receptor[98]and has been discovered in familial ET.[99] The S505N mutation in the transmembrane domain is expected to promote constitutive activation due to polar interactions between the asparagines that replace the natural serine. As stated earlier, mutations in Mpl at W515 induce severe MPNs with myeloprofibrosis.[19, 20] W515 mutations activate constitutive signaling by the receptor because W515 belongs to an amphipathic juxtamembrane helix (RWQFP in the human receptor), which is required for maintaining the un-liganded receptor in the inactive state. Another activating mutation was recently described for TpoR, where a threonine residue in the extracellular juxtamembrane region (located symmetrically from the W515 mutation on the N-terminal side of the transmembrane domain) is mutated to alanine (T487A) in a non-Down's syndrome childhood acute megakaryocytic leukemia.[100] In bone marrow transplantation assays, this Mpl T487A also induces a severe myeloproliferative disease, close to the phenotype induced by TpoR W515L.[100] Juxtamembrane mutations such as W515L/K or T487A may not only promote active dimeric conformations, but they could also induce receptor conformational changes by changing crossing angles between receptor monomers, whereas the S505N highly polar mutation in the transmembrane domain is predicted to stabilize an active dimeric conformation of the receptor. It will be interesting to test side by side in bone marrow transplantation experiments the effects of S505N, W515L and T487A mutations and to assess whether indeed the phenotype of the TpoR S505N mutation would be milder.

1.10 G-CSFR and MPNs

Bone marrow transplanted mice with HSCs expressing JAK2 V617F present not only an MPN phenotype, with low Epo, as predicted, but also with low G-CSF serum levels, suggesting that constitutive activation of G-CSFR occurs in these mice.[101]G-CSFR uses both JAK1 and JAK2 for signaling.[65, 66] JAK2 V617F may affect G-CSFR signaling with less efficiency than for EpoR and TpoR, as JAK1 may be the key JAK for G-CSFR. This is perhaps the reason why the granulocytic lineage is affected to a lower extent in MPNs, when compared with the erythroid and megakaryocytic lineages, especially at low levels of JAK2

V617F. Activation of the G-CSFR JAK2 V617F complexes may lead to enhanced numbers of granulocytes, constitutive activation of granulocytes (with release of enzymes) as well as interactions with platelets, which would contribute to thrombotic complications. It is not clear whether leukocytosis, which is seen in certain MPN patients and which appears to be associated with certain complications or evolution toward leukemia,[102] may be due to the pathologic activation of G-CSFR by JAK2 V617F. Granulocytes from patients with MPNs presented altered gene expression promoted by JAK2 V617F expression and confirmed a recapitulation of cytokine receptor signaling, resembling profiles of granulocytes activated by G-CSF.[103]Similar to TpoR, G-CSFR activates STAT3 and MAP kinase pathways, in addition to the JAK2–STAT5 and PI-3–kinase/Akt pathways. It can be noted that for this receptor, a very delicate balance has been identified between the activation of STAT3, required for differentiation and inducing a stop in cell growth (necessary for differentiation), and STAT5, which promotes proliferation.[102] Binding of SOCS3 through its SH2 domain to a phosphorylated tyrosine residue in the receptor's cytosolic end specifically downregulates STAT5 signaling. Deletion of the cytosolic region, which contains the binding site for SOCS3, leads to enhanced STAT5-to-STAT3 signaling ratio,[102] and this is associated with evolution toward acute myeloid leukemia of patients with severe congenital neutropenia. G-CSFR activation might synergize with other mechanisms and promote the mobilization of CD34(+) stem cells and progenitors from the bone marrow to the periphery. Interestingly, an increased number of circulating CD34(+) cells in MPN patients has been observed, and they exhibit granulocyte activation patterns similar to those induced by the administration of G-CSF.[104] The release of CD34(+) cells is generally due to a combination of increased levels of proteases[105] and especially due to the downregulation of the CXCR4 receptor on CD34(+) cells.[106] An altered SDF-1/CXCR4 axis was demonstrated in PMF patients with CD34(+) cells in the periphery.[107] These findings are supported by the rapid mobilization of CD34(+) cells with AMD3100, a CXCR4 antagonist.[108]

1.11 Tyrosine phosphorylation pattern of JAK2 V617F

JAK2 V617F is constitutively tyrosine phosphorylated. However, besides Y1007 in the activation loop, which is crucial for activation,[109] it is not known whether other phosphorylated tyrosines overlap with those phosphorylated in the wild-type JAK2. It can be noted that, JAK2 contains multiple tyrosine residues, of which at least 14 can be phosphorylated[110] (Figure 1). Some of these tyrosine residues exert positive (Y221) and other negative (Y119, Y570) effects on signaling by JAK2.[111, 112, 113] Y813 is a recruitment site for SH2-containing proteins,[114] such as SH2B, which can promote homodimerization of JAK2.[115] In theory, the constitutive activation of JAK2 V617F might promote a different pattern of phosphorylated tyrosines from that of wild-type JAK2.

1.12 STAT activation and MPNs

A hallmark of MPNs is constitutive or hypersensitive activation of the STAT family of transcription factors in myeloid precursors. As mentioned, the expressions of JAK2 V617F, TpoR mutants or exon 12 JAK2 mutants lead to constitutive STAT5 and STAT3 activation in various systems.[18, 116] As a function of the MPN disease type, one or the other of the STATs was suggested to be predominantly activated by JAK2 V617F. For example, in myelofibrosis, JAK2 V617F expression in neutrophils is associated with the activation of STAT3 but

apparently not with that of STAT5.[117] In another study, in bone marrow biopsies and irrespective of JAK2 V617F, PV patients exhibited high STAT5 and STAT3 phosphorylation and ET patients exhibited high STAT3, but low STAT5 phosphorylation, whereas myelofibrosis patients exhibited low STAT5 and STAT3 phosphorylation.[118] Thus, constitutive activation of the STAT5/STAT3 signaling appears to be a major determinant of MPNs, irrespective of the particular JAK2 or receptor mutation. Furthermore, STAT3 activation by IL-6 has been shown in a murine model system to hold the potential to experimentally induce MPN. Mice homozygous for a knockin mutation in the IL-6 receptor gp130 (gp130(Y757F/Y757F)), which leads to gp130-dependent hyperactivation of STAT1 and STAT3 develop myeloproliferative diseases with splenomegaly, lymphadenopathy and thrombocytosis. gp130(Y757F/Y757F) is hyperactive owing to impaired recruitment of negative regulators such as SOCS3 and the SHP2 phosphatase.[119,120] The hematological phenotype disappeared when the knockin mice were crossed with heterozygous Stat3(+/-) mice.[121] Thus, the threshold of STAT3 signaling elicited by IL-6 family cytokines may have an important function in the myeloid lineage and may contribute to the development of MPN.

1.13 SOCS3 and JAK2 V617F

Suppressors of cytokine signaling (SOCS) proteins negatively regulate cytokine receptors and JAK–STAT signaling. There are eight members of the SOCS/CIS (cytokine-inducible SH2-domain-containing protein) family, namely SOCS1–SOCS7 and CIS. Each SOCS molecule contains a divergent N-terminal domain, a central SH2 domain, and a C-terminal 40 amino acid domain known as the SOCS box.[122] CIS/SOCS proteins are supposed to function as E3 ubiquitin ligases and target proteins bound to the SOCS N terminus, such as active JAKs, as well as themselves for proteasome-mediated degradation.[122] SOCS1 and SOCS3 can also inhibit the catalytic activity of JAK proteins directly, as they contain a kinase inhibitory region (KIR) that targets the activation loop of JAK proteins. SOCS proteins bind receptors and then target the activation loop of JAKs for inhibition by KIR and SH2 interactions.[122]SOCS3 is known to strongly down-modulate EpoR signaling.[123] JAK2 V617F appears not to be downregulated by SOCS3, possibly due to continuous phosphorylation of SOCS3, which can impair its E3 ligase activity.[124] Constitutive tyrosine phosphorylation of SOCS3 was also reported in peripheral blood mononuclear cells derived from patients homozygous for the JAK2 V617F mutant.[124] Taken together, a model was proposed in which JAK2 V617F may escape physiologic SOCS regulation by hyperphosphorylating SOCS3. It would be important to also determine whether exon 12 mutants of JAK2 are able to overcome down-modulation by SOCS3.Furthermore, one of the two tyrosine residues in the C terminus of SOCS3 that become phosphorylated upon ligand-activated cytokine receptors interacts with the Ras inhibitor p120 RasGAP.[125] This leads, in the case of IL-2 signaling, to sustained ERK activation, whereas the JAK–STAT pathway is down-modulated.[125]Whether part of the sustained ERK activation detected in cells transformed by JAK2 V617F may involve complexes of degradation-resistant SOCS3 and p120 RasGAP is yet to be determined.

1.14 Other JAK2 mutations activate JAK2

Substitution of pseudokinase domain residue V617 by large non-polar amino acids causes activation of JAK2.[126] Saturation mutagenesis at position 617 of JAK2 showed that, in addition to V617F, four other JAK2 mutants, V617W, V617M, V617I and V617L, were able to

induce cytokine independence and constitutive downstream signaling. However, only V617W induced a level of constitutive activation comparable with V617F, and like V617F it was able to stabilize tyrosine-phosphorylated SOCS3.[126] Also, the V617W mutant induced a myeloproliferative disease in bone-marrow-reconstituted mice, mainly characterized by erythrocytosis and megakaryocytic proliferation. Although JAK2 V617W would predictably be pathogenic in humans, the substitution of the Val codon, GTC, by TTG, the codon for Trp, would require three base pair changes, which makes it unlikely to occur. Therefore, codon usage and resistance to SOCS3-induced down-modulation are two mechanisms that might explain the uniqueness of JAK2 V617F in MPNs.

2. Animal models of MPNs

2.1 JAK2 mutants

Mouse bone marrow reconstitution experiments with HSCs retrovirally transduced with JAK2 V617F resulted in strain-dependent myeloproliferative disease phenotypes. In these models, JAK2 V617F is expressed at ~10-fold higher than endogenous levels. In C57Bl6 mice, JAK2 V617F induces erythrocytosis, and in some animals myelofibrosis, although most reconstituted mice remain alive several months, and in some the erythrocytosis regresses; in Balb/c mice, the phenotype is more severe with erythrocytosis being followed by myelofibrosis.[13, 23, 127] Only very rarely, at low transduction levels, was a thrombocytosis phenotype observed, which, together with initial studies on primary patient cells,[22] led to the suggestion that gene dosage may be important for a particular phenotype to develop.[23] This hypothesis has been supported by studies in transgenic mice in which the expression of the JAK2 V617F was carefully regulated.[24] When JAK2 V617F levels were lower than endogenous JAK2 levels, an ET phenotype was obtained. When levels of JAK2 V617F were similar to those of endogenous wild-type JAK2, a PV-like phenotype was developed. Interestingly, upon development of the PV phenotype, a selection for higher levels of JAK2 V617F occurs, up to 5- to 6-fold higher than the endogenous JAK2 levels, which is not the case for the ET phenotype.[24] This indicates positive selection for JAK2 V617F expression in PV progenitors, a phenomenon that can be detected in stably transfected Ba/F3 cells.[51] In addition overexpression of several non-mutated JAK proteins, including JAK2, was shown to promote hematopoietic transformation to cytokine independence.[128]The phenotype induced by exon 12 mutants of JAK2 is more restricted to erythrocytosis, without the abnormal megakaryocyte clusters seen in the classical MPNs.[18] These *in vivo* data indicate that a difference must exist between signaling by JAK2 V617F and exon 12 JAK2 mutants.

2.2 Mpl (TpoR) mutants

The phenotype induced by TpoR W515L is different than that induced by JAK2 mutants, in that it is much more severe, with initial myeloproliferation, marked thrombocytosis, splenomegaly and myelofibrosis, and is established within 20–30 days after reconstitution.[19] At least one other mutation at W515 was identified in patients with PMF and ET, that is W515K.[20] W515 is located in an amphipathic motif (RWQFP) at the junction between the transmembrane and cytosolic domains. This motif is required to maintain the un-liganded TpoR in an inactive state. Given that a W515A mutation is also active,[21] it is not surprising that such different residues (Leu and Lys) are found in active TpoR mutants. We predict that other W515

mutations will be found in patients, as the loss of a Trp (W) residue is responsible for activation. The striking difference in severity and histopathology between the phenotypes induced by JAK2 V617F and Mpl 515 mutants is hard to understand when standard phosphorylation studies are performed on cell lines, such as Ba/F3 cells, where the same redundant pathways are activated by both JAK2 V617F and Mpl 515 mutants. Interestingly, deletion of the amphipathic motif (Δ5TpoR) that contains W515 or the mutation of either the lysine K514 (R514 in the human receptor) or the W515 in this motif to alanine leads to constitutive JAK2 and STAT activation and colony formation in primary cells and hypersensitivity to Tpo.[21] In hematopoietic cells transformed by Δ5TpoR (amphipathic motif deleted) or TpoR W515A, we noted enhanced STAT5 and MAP kinase activation in the absence of Tpo, and high levels of activation of STAT5, STAT3 and MAP kinase pathways on Tpo treatment.[21] Such TpoR mutants do not down-modulate cell surface levels of TpoR,[21] unlike TpoR in complex with JAK2 V617F, which is down-modulated (J Staerk *et al.*, unpublished observations). It is therefore tempting to speculate that prolonged activation of MAP kinase and STAT3 might be a distinct feature of TpoR W515 mutants, whereas JAK2 V617F, which couples not only to TpoR but also to other cytokine receptors expressed in myeloid progenitors, would generate a weaker or different signal. Taken together, these data suggest that some level of signaling specificity must exist, which would make the excessive activation of TpoR through JAK2 V617F qualitatively different from that induced by W515 mutations. Understanding this difference at the signaling level will be of utmost importance.

2.3 JAK2 inhibitors

Since 2005, mutations that directly or indirectly led to deregulated activation of non-receptor tyrosine kinase (TK) Janus activated kinase 2 (JAK2) have been implicated in the pathogenesis of MPN. These mutations activate the JAK2–signal transducer and activator of transcription (STAT) intracellular signaling pathways, which lead to increased cellular proliferation and resistance to apoptosis. These discoveries spurred the development of molecularly targeted agents (JAK2 inhibitors) as therapy for MPNs.

In clinical development, JAK2 inhibitors exhibit differential inhibitory activity against the JAK family members, and some exhibit effects on other receptor kinases and therefore are not selective forJAK2 TK. For example, INCB018424 inhibits JAK1, whereas CEP-701 and TG101348 inhibit FLT3. JAK2 inhibitors are small molecules that act by competing with adenosine triphosphate for the adenosine triphosphate–binding catalytic site in the TK domain. The V617F mutation locates outside the TK domain of JAK2. Therefore, the current JAK2 inhibitors target both wild-type and mutated JAK2 indiscriminately. This could explain why these drugs are active in patients with both wild-type and mutated JAK2. However, targeting the wild-type JAK2 is expected to lead to myelosuppression as a result of the exquisite signalling through JAK2 of thrombopoietin and erythropoietin receptors in normal hematopoiesis. This probably explains the reported therapy-related anaemia and thrombocytopenia observed with JAK2 inhibitors in clinical trials.

Previous research demonstrated that JAK2-deficiency is embryonically fatal in mice due to a lack of erythropoiesis. As a consequence, no in vivo JAK2 knock out animal model exists, which makes the biological characterization of JAK2 more difficult. siRNA knock down models may be useful for this purpose, but for the time being, they cannot be considered as

therapeutic agents. JAK2 antagonists, however, could be used efficiently for analyzing the possible therapeutic benefit of JAK2 inhibition in hematologic malignancies and myeloproliferative disorders.

On the other hand, only a small number of JAK2 inhibitors have been reported in the literature so far. AG490 possesses significant JAK2 inhibition, for that reason it has been used extensively as a research tool. AG490 blocks leukemic cell growth significantly both in vitro and in vivo.[16] On the other hand, this compound lacks sufficient target specificity, therefore the interpretation of results obtained with AG490 may not be limited to JAK2 inhibition. Several other non-specific JAK2 inhibitors have already been reported in the literature (LFM-A13,21 INCB20,22 WP106623 and SD-100824). Since off-target effects may cause serious immune-modulative or proliferative side effects, specific JAK2 inhibition is highly desirable.

One molecule, TG101209, was able to inhibit proliferation of both JAK2 V617F- and TpoR W515L/K-expressing hematopoietic cells.[130] It may be recalled that TpoR W515-mutated proteins are expected to constitutively activate the wild-type JAK2.

TG101348, a nano-molar JAK2 inhibitor, was highly specific for JAK2 as evidenced by a 300-fold selectivity over JAK3. TG101348, was effective in a murine model of MPN induced by JAK2 V617F131 and inhibited the engraftment of JAK2 V617F-positive HSCs and myeloid progenitors in a bioluminescent xenogeneic transplantation assay.[132] Importantly, the inhibitor decreased GATA1 expression and phosphorylation of GATA1 at S310 and, as expected, STAT5 activation. These signaling events might be associated with erythroid-skewing of JAK2 V617F-positive progenitor differentiation, as phosphorylation at GATA1 S310 was shown to be important for erythroid differentiation.[81] GATA1 is absolutely required for erythroid and megakaryocyte formation.[133, 134] Although both EpoR and GATA1 are crucial for erythroid differentiation, this phosphorylation event, which depends on PI-3-kinase and Akt,81 appears to be the only one described where a direct EpoR downstream signal affects GATA1.

An experimental JAK2 inhibitor has been shown to be well tolerated and to produce a significant reduction in disease burden and durable clinical benefit in patients with myelofibrosis reports a study. [161] Currently there are no FDA approved treatments for myelofibrosis. Common treatments of the malignancy, which is associated with anaemia and splenomegaly, are palliative and do not alter the natural course of the disease. Median survival ranges from less than two years to greater than 15 years. Patients with myelofibrosis frequently harbour JAK-STAT activating mutations that are sensitive to TG101348, a selective small-molecule JAK2 inhibitor. It is estimated that JAK2 mutations occur in approximately 50 % of patients. Last September researchers reported positive results from a trial testing the experimental agent INCBO18424, (which inhibits JAK1 as well as JAK2) in patients with advanced myelofibrosis. In the current phase 1 study, Ayalew Tefferi and colleagues from the Mayo Clinic enrolled 59 patients, including 28 who took part in the dose-escalating phase. A substantial portion of patients experienced improvements in primary symptoms including splenomegaly, leukocytosis, thrombocytosis, and constitutional symptoms. There was also evidence for significant reductions in genomic disease burden, indicating the potential for disease-modifying activity. Although the drug was generally well tolerated, it caused anaemia in some patients, especially at higher doses.

A follow-up study is planned to see whether adjusting the dose will allow patients to achieve the benefits without the anaemia. In an accompanying editorial Srdan Verstovsek from the University of Texas MD Anderson Cancer Center wrote, "The development of JAK2 inhibitors has ushered in a new era of targeted therapies for Philadelphia-negative MPNs. These drugs do not eradicate the malignant clone, but they provide significant clinical benefit. Given that the current clinical management of patients with MF is largely palliative and minimally effective, significant improvements in two of the three most important clinical manifestations of MF (splenomegaly and systemic symptoms) seen with JAK2 inhibitors is significant therapeutic progress." Long term results, he added, are required to determine the full potential of JAK2 inhibitors in myelofibrosis and to determine whether they will have an impact on survival.

3. Cross-talk: JAK2 V617F and other pathways

3.1 JAK2 V617F and other tyrosine kinases

Tyrosine kinase receptors have been suggested to contribute to the pathogenesis of PV. In patients with PV, circulating erythroid progenitor cells are hypersensitive to IGF-1 and this effect requires the IGF-1 receptor.[10, 135]Expression of JAK2 V617F in Ba/F3 cells renders the cells responsive to IGF-1 at doses where parental Ba/F3 cells are unresponsive.[51] After selection for autonomous growth, these Ba/F3 JAK2 V617F cells acquire the ability to respond to IGF-1 by further tyrosine phosphorylation of the mutant JAK2 and of STAT5 and STAT3. Which adaptor/signaling protein mediates this cross-talk is not clear, but it might be relevant for the hypersensitivity of PV erythroid progenitors to IGF-1.

Treatment with imatinib, an inhibitor of the Abl, c-KIT and PDGF receptor kinase, leads to minimal responses in PV, but nevertheless some rare patients achieve remission and a decrease in the *JAK2* V617F allele burden.[136] Imatinib exerts a dose-dependent growth inhibitory effect on factor dependent cell paterson (FDCP) cells expressing JAK2 V617F, most likely by interrupting the cross-talk between JAK2 V617F and c-KIT.[137] Thus, this study predicts that in PV patients where imatinib exerts benefic effects, pathologic signaling occurs through c-KIT.Src tyrosine kinases have also been suggested to contribute to signaling by EpoR.[138] However, Src kinases were dispensable for the polycythemia phenotype induced by JAK2 V617F, as shown by bone marrow reconstitution studies in mice deficient for Lyn, Fyn or Fgr kinases.[139]

3.2 JAK2 V617F and TNF-α

In BALB/c mice reconstituted with HSCs transduced for the expression of JAK2 V617F, the PV-like phenotype is associated with increased serum levels of TNF-α.[101] TNF-α might be required for suppressing normal hematopoiesis, and in this manner it might favor the mutated clone. Reconstitution experiments in TNF-α knockout mice supported the notion that TNF-α might be required for the establishment of the MPN phenotype and for clonal dominance (Bumm TGP, VanDyke J, Loriaux M, Gendron C, Wood LG, Druker BJ, Deininger MW. TNF-α plays a crucial role in the JAK2-V617F-induced myeloproliferative disorder. *Blood*2007; 110. American Society of Hematology 2007 Abstract 675). Further studies will be required to firmly establish whether TNF signaling may contribute to clonal

dominance. Erythroblasts from JAK2 V617F-positive PV patients show increased death receptor resistance, which may give them a proliferative advantage over the non-mutated erythroblasts.[101, 140] This effect was mediated by incomplete caspase-mediated cleavage of the erythroid transcription factor GATA-1, which in normal erythroblasts is completely degraded on CD95 stimulation.

3.3 MPNs and TGF-β

An increase of TGF-β expression in circulating megakaryocytic cells and platelets was demonstrated in PMF.[141] Fibroblasts participating in myelofibrosis were shown to be polyclonal, as opposed to the hematopoietic progenitors, thus suggesting that myelofibrosis is a reactive process.[141]In myelofibrosis induced by excessive levels of Tpo,[142] severe spleen fibrosis was seen only in wild-type mice but not in homozygous TGF-β1 null (TGF-β1 (-/-)) mice.[143] Studies using peripheral CD34(+) cells cultured in medium with Tpo and stem cell factor concluded that PMF is a consequence of an increased ability of PMF CD34(+) progenitor cells to generate megakaryocytes and a decreased rate of megakaryocyte apoptosis, which lead to high levels of megakaryocyte-produced TGF-β.[144] In other models with hyperactive JAK–STAT signaling, such as knock-in with a mutant hyperactive gp130 receptor, the activation of the JAK–STAT pathway led to the expression of the inhibitory Smad7, which prevents the anti-proliferative effect of TGF-β.[145] A pathway linking Tpo, GATA-1 and TGF-βin the development of myelofibrosis was invoked, given that mice expressing low levels of the transcription factor GATA-1 also develop myelofibrosis.[146]

3.4 JAK2 V617F and chromatin

Studies in *Drosophila melanogaster* show that persistent activation of D-STAT by mutated D-JAK leads to chromatin effects and gene induction other than the normal targets of D-STAT, with counteracting heterochromatic gene silencing.[147]A genome-wide survey of genes required for JAK/STAT activity identified a WD40/bromodomain protein *Drosophila* homolog of BRWD3,[148] a gene that is disrupted in human B-cell leukemia patients. Whether any histone acetylase, deacetylase, methyl-transferase or other proteins containing bromo-, chromo- or chromoshadow domains are direct targets of JAK2 V617F is not clear. Recently, encouraging results were obtained with an HDAC (histone deacetylase) inhibitor, which seems to target only *JAK2* V617F-positive cells among primary myeloid progenitors from PV patients.[149] Thus, like other cancers,[150] MPNs might also show restriction of fate options through hypermethylation. This notion is supported by the different effects of sequential treatment with the DNA methyltransferase inhibitor, decitabine, followed by the histone deacetylase inhibitor, trichostatin A (TSA), on normal CD34(+) versus PMF CD34(+) cells. In the former, the treatment led to the expansion of cells, whereas in the latter the total number of CD34(+) cells and hematopoietic cells was reduced.[151]Furthermore, promoter de-methylation appears to be at least partially at the basis of the dysregulated expression of the polycythemia rubra vera-1 (PRV-1) protein,[152] which is a key marker of PV.[153] Finally, hypermethylation of the SOCS1 promoter was reported approximately in 15% of MPN patients irrespective of *JAK2* V617F positivity.[154] SOCS1 promoter methylation may contribute to growth factor hypersensitivity, as SOCS1 appears to maintain the ability to down-modulate JAK2 V617F signaling.[124] Recently, Dawson et al.[155]identified a novel nuclear role of JAK2 in the phosphorylation of Tyr 41 of histone H3 leading to chromatin

displacement of HP1a. The authors suggested that the inability of HP1a to regulate chromatin could reduce the potential tumor suppressive functions of HP1a resulting in erratic mitotic recombination and transcription deregulation of several JAK2-regulated genes such as *LMO2*[155,156]. These results were confirmed in hematopoietic cell lines and in the CD34+ cells collected from the peripheral blood of one PMF patient with JAK2V617F mutation. In this work we define the subcellular localization of JAK2 in total BM cells and in sorted cell populations collected from MPN (ET, PV, PMF) patients with the JAK2V617F mutation or from MPN patients with wild type (wt) JAK2. We find that in contrast to cells with normal JAK2 in which the protein is detected predominantly in the cytoplasm, JAK2 is mostly nuclear in V617F-positive CD34+ cells. However, this nuclear localization is no longer observed in V617F-positive differentiated cells. After expressing JAK2V617F in K562 cells, we observe a similar preferential accumulation of JAK2 in the nucleus in contrast to untrasfected- and wt JAK2-expressing cells in which the protein is found in the cytoplasm. The mutated-JAK2 nuclear translocation is mainly reverted by the addiction of the JAK2 inhibitor AG490.

4. Methods

4.1 Cell cultures

K562 were grown in RPMI 1640 medium (Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% glutamine. The cells were maintained at 37 C and 5% CO2. Transfection was performed by AMAXA electroporation in accordance with the manufacturer's instructions. Stable cell lines were obtained by puromycin selection (3μg/ml). Leptomycine B was added to a final concentration of 1 uM and the cells were harvested after 24 h of incubation. The differentiation of K562 cells was obtained by culturing the cells with 10 nMol PMA.

4.2 Plasmids

pMSCV-puro-JAK2 and pMSCV-puro-JAK2V617F were kindly provided by Dr. J. Cools.

4.3 Confocal immunofluorescence (CFI) microscopy

The BM fractions of CD34+, CD15+, CD41+, CD71+ cells and the K562 cells were washed once in PBS before cytocentrifugation onto polylysine-coated microscope slides. The cells were fixed for 5 min in methanol at room temperature and 5 min in acetone at -20 C. After stepwise incubation with a primary antibody and a secondary fluorescent antibody, the cells were stained with DAPI and mounted with glycerol. Confocal laser images were captured with a laser scanning microscope LSM 510 META microscope equipped with a 403 oil-immersion lens.

4.4 Patients

BM aspirates and PB samples were obtained from 15 patients newly diagnosed with MPN according to the WHO criteria. Ten of the patients (ET n=4, PV n=3, PMF n=3) had the V617F mutation. The remaining 5 patients (PMF n= 2, ET n=3) had a normal JAK2. Informed consent for the study was obtained from all patients in accordance with the Declaration of

Helsinki. The study was conducted according to the guidelines of the Italian ethics committee.

4.5 Cell sorting

Mononucleated cells were isolated by Ficoll centrifugation. After erythrocyte lyses, 10 million BM (or PB) cells were labelled by incubation with the antibodies CD15-APC, CD41-FITC, CD71-PerCP, CD34-Pe (BD Biosciences, San Jose, CA), analyzed, and sorted with a fluorescence-activated cell sorter FACSAria (BD Biosciences) using FACS Diva software (BD Biosciences) according to the manufacturer's recommendations.

4.6 ASO-PCR and RT-PCR

The JAK2V617F mutation was identified by ASO-PCR as previously described[13-15]. Briefly, total RNA was isolated with the TRIzol reagent (Invitrogen) and cDNA was synthesised with the First Strand cDNA Synthesis kit (MBI Fermentas). Syber Green RQ-PCR was performed as previously described[155]. Quantitative values were obtained from the threshold cycle number (Ct) by subtracting the average Ct of the target gene from that of GAPDH and expressed as $2^{-\Delta Ct}$.

4.7 Cell fractionation and immunoblotting

Cytoplasmic and nuclear fractions were prepared using nuclei isolation KIT by Sigma-Aldrich according to the manufacturer's instructions. Equal amounts of cytoplasmic or nuclear fractions or total cell extracts were separated by SDS–PAGE, transferred to nitrocellulose and probed with relevant antibodies.

4.8 JAK2 inhibitor and antibodies

AG490 was provided by Invivogen. To establish the best experimental condition, the cells were cultured for 3 hours with 2 different concentrations of the inhibitor (12.5 uM and 25 uM). The following antibodies were used at the stated dilutions: anti-JAK2 antibodies (D2E12 no. 3230; Cell Signaling Technology), p-JAK2 (tyr1007/tyr1008)-R and p-JAK2 (tyr1007/tyr1008) (Santa Cruz), anti-b-tubulin (T5201; Sigma-Aldrich), anti-laminin A (Sigma-Aldrich); western blot 1:1000. Alex Fluor-488-conjugated IgG (Invitrogen) immunofluorescence 1:250.

4.9 Apoptotic rate

K562 cells were stained with propidium iodide and annexin V before and after 3 h incubation with AG490. Cell cycle parameters were assessed by FACS analysis using FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA, USA).

4.10 Statistical analysis

Student *t* test was used to evaluate individual differences between means. $P \leq 0.05$ was considered significant.

5. Results and discussion

5.1 V617FJAK2 goes into the nucleus of JAK2 mutated K562

To determine whether the V617F mutation affects the sub-cellular localization of JAK2, we used CIF microscopy to analyze K562 cells stably transfected with pMSCV-puroJAK2V617F or pMSCV-puroJAK2. The results (Figure 3A) confirm the nuclear and cytoplasmic localization of JAK2 in K562 as reported by Dawson et al[155]. However, we consistently observed a much stronger nuclear signal in the cells expressing JAK2V617F than in those carrying wt JAK2, suggesting that the mutation leads to a nuclear accumulation of JAK2V617F. By CIF, this accumulation can be seen as a diffused nuclear pattern (Figure 3A top, upper and middle panels) or as nuclear spots (Figure 3A top, lower panel). This altered sub-cellular distribution was not affected by the addition of the nuclear export inhibitor leptomycin B (data not shown) and was confirmed by Western blot analysis of K562 cells (Figure 3A, bottom panels).

5.2 Preferential nuclear JAK2 in V617FJAK2 positive CD34+ but not in differentiated erythroid, granulocytic or megakaryocytic cells

To determine whether there is a preferential nuclear translocation of JAK2V617F *in vivo*, we analyzed by CIF microscopy the BM cells of 10 JAK2V617F-positive MPN patients (ET n=4, PV n=3, PMF n=3, allele burden median: 56%, 70%, 72% respectively) and of 5 MPN patients with wt JAK2 (PMF n= 2, ET n=3). We did not observe a significant signal in the nucleus of cells with wt JAK2 (Figure 3B). In contrast, we found a strong nuclear signal in 3%-5% of total BM mononucleated cells in 10 of 10 JAK2V617F-positive patients, suggesting that, unlike the wt JAK2, JAK2V617F has a predominantly nuclear homing. To identify the phenotype of these cells, we used fluorescence activated cell sorting (FACS) to isolate CD34+, CD15+, CD41+ and CD71+ fractions from the BM of three JAK2V617F-positive MPN patients (1 ET, 1 PV, 1 early PMF). We found nuclear JAK2 only in the fraction containing the CD34+ positive cells (Figure 3C, left panels). It should be noted that in these patients the CD34+ cells correspond to approximately 3% to 5% of total BM mononucleated cells. The nuclear localization of JAK2V617F was confirmed by WB analysis (Figure 3C, right panels). However, no predominant nuclear signal was detected in differentiated granulocytic, megakaryocytic and erythroid cells obtained from the patients (n=15) (Figure 3D). Similar results were obtained with PB cells (data not shown). The relocation of the mutated JAK2 to the cytoplasm was confirmed in K562 cells after their differentiation with PMA (Figure 4A). However, the relocation was not complete as observed for the primary BM cells and nuclear JAK2 was still observed. We believe that this is due to the nature of K562 cells, which are BCR-ABL-positive CML cells, and to the difficulty to obtain their terminal differentiation in vitro.

5.3 The JAK2 inhibitor AG490 relocate JAK2 in cytoplasm

To determine whether an alteration of JAK2 activity could interfere with the nuclear localization of JAK2, we incubated K562 cells expressing JAK2V617F or JAK2 with the selective JAK2 inhibitor AG490[158-160]. After 3 h of incubation at the IC50 dose of 25 uM, CIF images showed a relocalization of JAK2V617F to the cytoplasm in the vast majority of K562 cells (Figure 4B). Analyses with annexin V and propidium iodide did not reveal any significant change in the apoptotic rate of V617F-positive and wt-K562 cells.

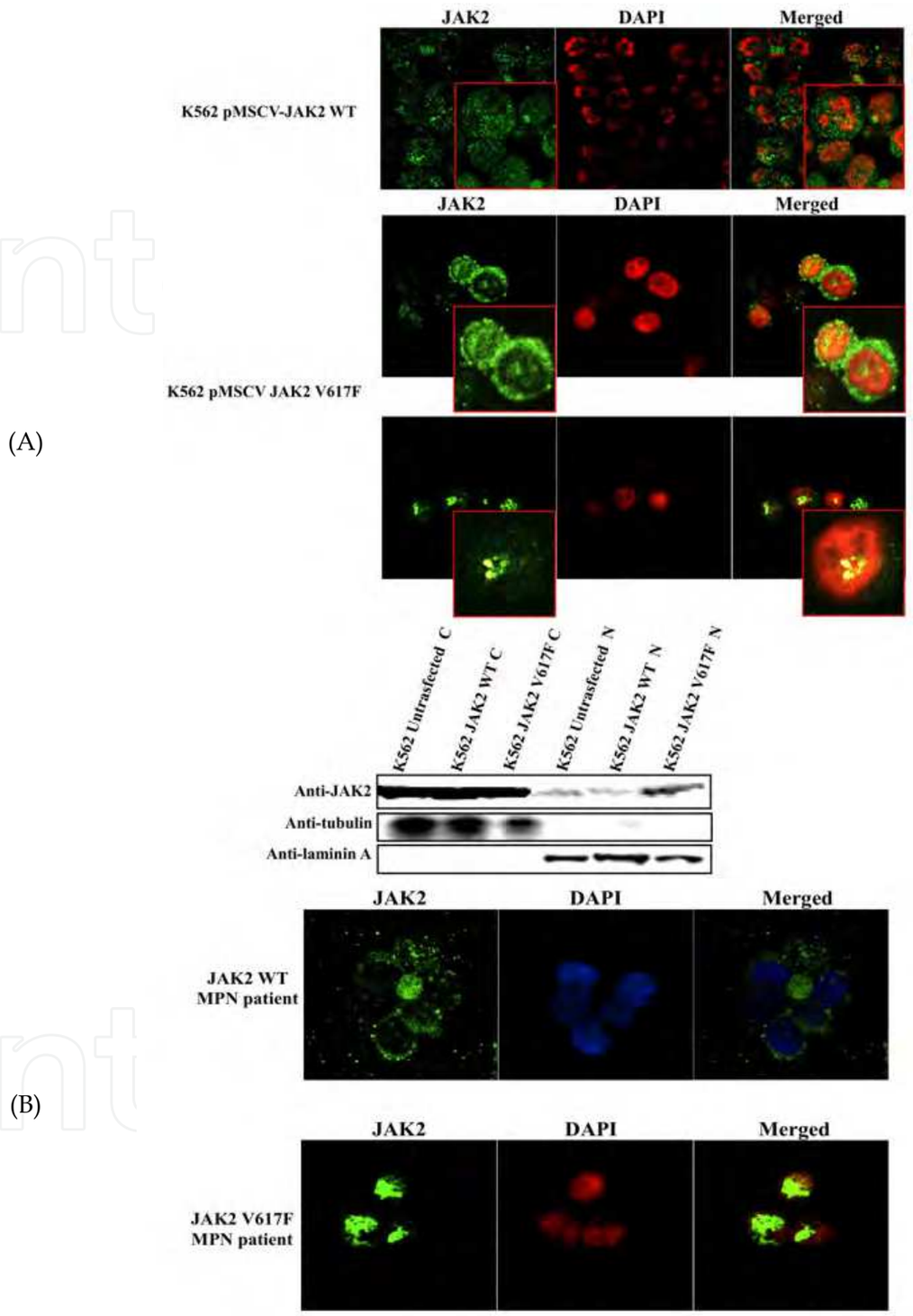


Fig. 3. V617F mutation favors nuclear translocation of JAK2 in K562 and early CD34+ progenitors isolated from BM of MPN patients. This translocation is not observed in differentiated cells.(A) CIF microscopy images of K562 cells stably transfected with pMSCV-JAK2 (upper panels) or pMSCV-JAK2V617F (middle and lower panels) and Western

blotting of cytoplasmic (C) and nuclear (N) fractions confirm that JAK2V617F is more abundant than JAK2 in K562 nuclei. (B)CIF images of BM cells from an MPN patient with wt JAK2 (upper panels) and an MPN patient with JAK2V617F (lower panels) confirm a nuclear increase of the mutated protein.

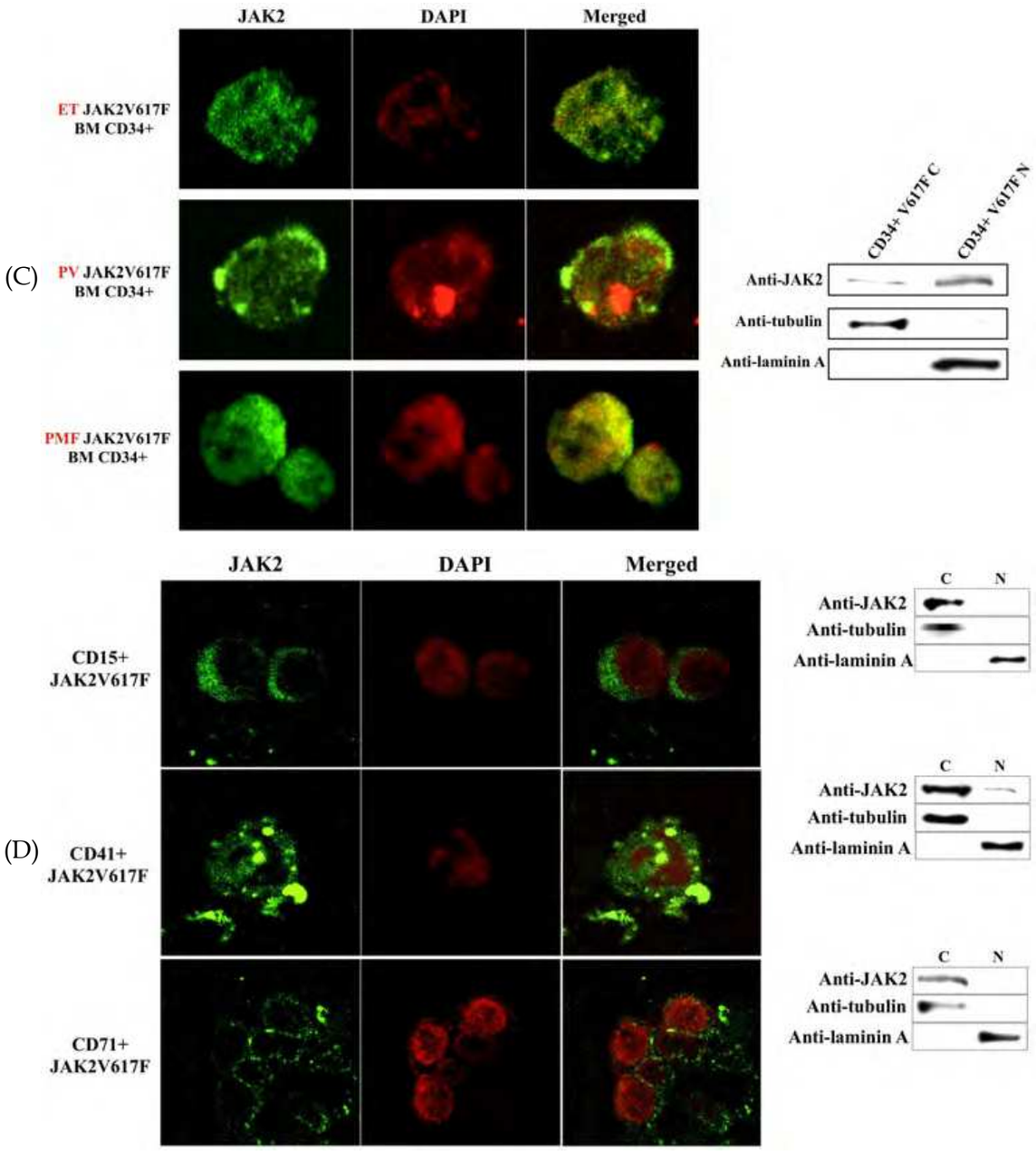


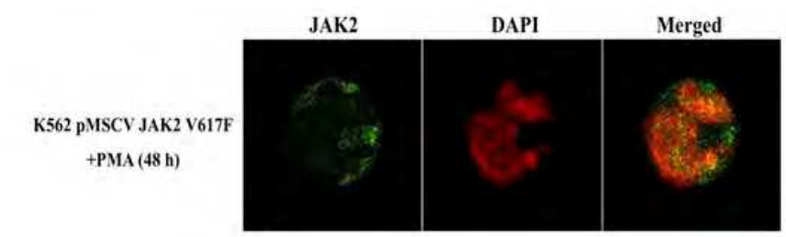
Fig. 3. V617F mutation favors nuclear translocation of JAK2 in K562 and early CD34+ progenitors isolated from BM of MPN patients. This translocation is not observed in differentiated cells.(C) Confocal IF demonstrates a predominantly nuclear accumulation of JAK2V617F in CD34+ cells isolated from BM of 1 ET, 1 PV and 1 earlyPMF (left panels) and Western blotting of cytoplasmic (C) and nuclear (N) extracts (right panel) confirm the data.

(D) Confocal IF images of CD15+, CD41+, CD71+ cells isolated from a JAK2V617F–positive MPN patient (PV, JAK2 allele burden 71%) and Western blotting of cytoplasmic (C) and nuclear (N) extracts (right panel). DAPI, 4,6-diamidino-2-phenylindole; Anti-JAK2, Cell Signaling monoclonal antibody; Anti-Tubulin and Anti-Laminin A, Sigma-Aldrich monoclonal antibodies.

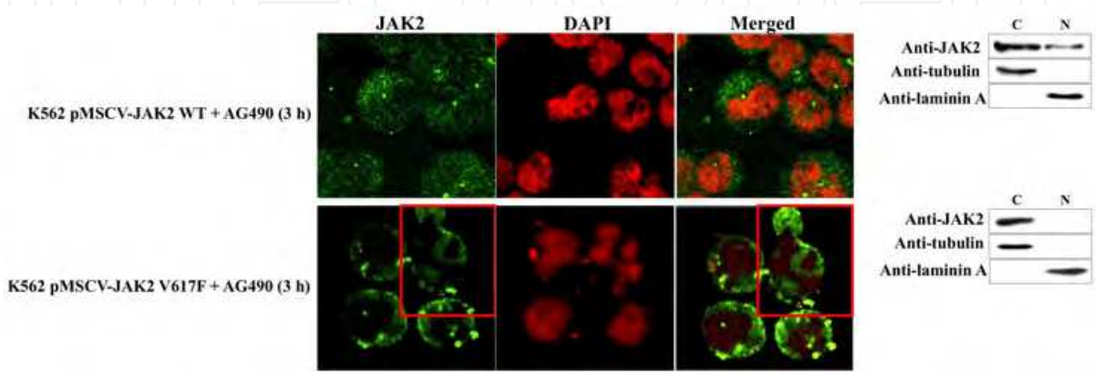
5.4 V617F JAK2 up-regulates LMO2 and AG490 restores its level.

By QRT-PCR we show that the V617F mutation strongly up-regulates the expression of *LMO2* in K562 and in CD34+ cells (Figure 4C, left panels). The link between *LMO2* expression and JAK2 inhibition has been reported previously[156,157]. In our assay, the addition of AG490 progressively and completely restore*LMO2* levels in V617F expressing K562 (Figure 4C, right panels).

(A)



(B)



(C)

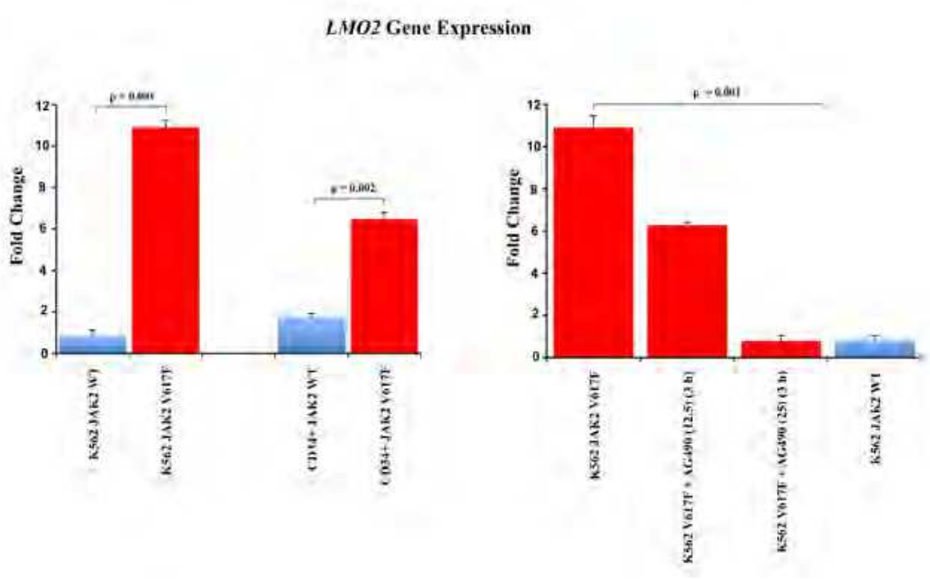


Fig. 4. V617F mutation causes up-regulation of *LMO2*. The JAK2 inhibitor AG490 replaces JAK2 into cytoplasm and restores LMO2 levels.(A) Confocal IF images show the redistribution of JAK2 and the replacement in the cytoplasm in V617F expressing K562 after PMA differentiation (B) Confocal IF images show the redistribution of JAK2 and the replacement in the cytoplasm in the vast majority of V617F expressing K562 (bottom panels) but not in wt cells (top panels) after AG490 incubation. (C) Quantitative RT-PCR revels that V617F mutation strongly up-regulates *LMO2* expression in K562 and in CD34+ cells (left panels). The addiction of AG490 progressively and completely restore*LMO2* levels in V617F expressing K562 (right panels). DAPI, 4,6-diamidino-2-phenylindole; Anti-JAK2, Cell Signaling monoclonal antibody.

6. Conclusions

Where do the major advances towards understanding the precise molecular bases of MPNs lead us? One striking observation is the key role played by various tyrosine kinases, constitutively activated either by balanced translocations or deletions generating fusion oncoproteins, or by activating point mutations. These mechanisms seem to be the molecular hallmark of MPNs, although there are probably alternative mechanisms directly involving cytokine receptors, adaptor proteins or transcription factors. All the molecular defects identified in MPNs to date confer proliferation and survival advantages on transformed cells, which retain the capacity to differentiate into mature cells. Differentiation may be disrupted by additional events, such as transcription factor deregulation, as frequently observed in acute myeloid leukaemia. *KIT* mutations and *FLT3* abnormalities are frequently found in cases of acute myeloid leukaemia. However, these molecular defects are now considered to be secondary events.

Strikingly, the diverse mutants and fusion proteins with constitutive tyrosine kinase activity each appear to stimulate a specific lineage. For example, PDGFR fusion proteins induce eosinophil differentiation, FGFR fusion proteins induce lymphoid malignancies, and

JAK2V617F mostly expands the erythroid compartment, whereas translocations involving the JAK2 kinase domain promote lymphoid proliferation as well. Thus, constitutive signalling via these different kinases is likely to result in effects on specific differentiation programs. Uncovering the molecular details of this specificity remains a major challenge, particularly as similar signalling molecules (*i.e.* STAT5, STAT3, RAS/MAPK, PI3K/AKT and others) are constitutively activated by all oncogenic fusion proteins.

Although the discovery of the JAK2V617F allele and the subsequent discovery of JAK2 exon 12 mutations and MPLW515L/K alleles have provided crucial insights into the genetic basis of PV, ET and PMF, many questions remain regarding the molecular pathogenesis of these MPNs. The activating mutations that cause JAK2 and MPL-negative MPN are not known, and the inherited and acquired alleles that can cooperate with JAK2V617F remain to be identified. In addition, the predominance of the JAK2V617F allele is surprising given that JAK2 exon 12 mutations, as well as activating JAK2 alleles identified in AML (JAK2T875N and JAK2ΔIREED), have similar in vitro and in vivo effects as JAK2V617F. The different JAK2 alleles might differentially interact with different cytokine receptor scaffolds, activate different signalling pathways, and/or be differentially affected by negative-feedback mechanisms; structural insight and additional in vitro and in vivo studies are needed to elucidate differences between JAK2V617F and the other activating JAK2 alleles. Another important question relates to the effects of JAK2V617F gene dosage on signalling and on phenotype. In vitro studies do not conclusively show whether the co-expression of wildtype JAK2 with JAK2V617F alters the signalling and/or transforming properties of the JAK2V617F kinase, and although retroviral models allow for an assessment of the in vivo effects of JAK2V617F expression, they do not provide the appropriate genetic context to investigate the importance of JAK2V617F gene dosage. Subsequent studies using more accurate genetic models will enable the delineation of the differential effects of JAK2V617F heterozygosity and homozygosity on signalling and on phenotype. Moreover, the role of the JAK2V617F allele in three distinct disorders of the myeloid lineage is not known, and the ability of different activated tyrosine kinases (for example, BCR-ABL and FIP1L1–PDGFRA) to cause distinct MPN remains to be delineated.

Dawson et al. identified a previously unrecognized nuclear role for JAK2 in the phosphorylation of the tyrosine 41 of the histone H3 with the exclusion of HP1a from chromatin and resulting in a disregulation of several JAK2-regulated genes such as LMO2 in haematopoietic cell lines and in one case on peripheral CD34+ cells from a JAK2V617F mutated PMF patient.

Our data corroborate recently published results of a nuclear localization of JAK2 in hematopoietic cells and they also extend these findings by showing that in all subtypes of MPN patients JAK2V617F accumulates in the nucleus of progenitor CD34+ cells while it remains mostly in the cytoplasm of their differentiated progeny. The chromatin alterations due to the preferential accumulation of JAK2V617F in the nucleus correlates with a significant increase in LMO2 expression in cell lines and in sorted CD34+ cells. The selective JAK2 inhibitor AG490 is able to revert nuclear JAK2 and normalize LMO2 levels in vitro, suggesting how the block in JAK2 nuclear translocation could be a new treatment strategy for JAK2 mutated patients. A question that remains to be answered is why mutated JAK2 is found only in the cytoplasm of the differentiated cells. MPN are clonal disorders arising in a pluripotent hematopoietic stem cell and it is well known that the constitutive activation of

JAK2 provides a sustained growth and survival advantage to the hematopoietic mutated stem cell clones. Signaling by the mutated kinase utilizes normal pathways, and normal or mutated JAK2 regulate EPO, TPO, and GCS-F response during differentiation, though they are probably not necessary to the differentiated cell. The signals that are required for the translocation of normal and mutated JAK2 to the nucleus are unknown. It is possible that the activation of the kinase by phosphorylation could be the first one of a number of modifications that control nuclear translocation, similarly to what happens to the STAT proteins. If this is true, then it is also possible that as the cell undergoes differentiaition these modifications are shut off, leaving mutated JAK2 predominantly in the cytoplasm.

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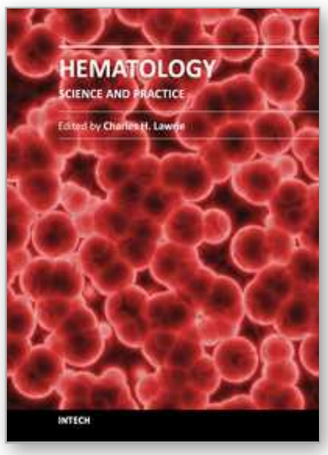

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Hematology encompasses the physiology and pathology of blood and of the blood-forming organs. In common with other areas of medicine, the pace of change in hematology has been breathtaking over recent years. There are now many treatment options available to the modern hematologist and, happily, a greatly improved outlook for the vast majority of patients with blood disorders and malignancies. Improvements in the clinic reflect, and in many respects are driven by, advances in our scientific understanding of hematological processes under both normal and disease conditions. Hematology - Science and Practice consists of a selection of essays which aim to inform both specialist and non-specialist readers about some of the latest advances in hematology, in both laboratory and clinic.

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