Small-molecule inhibitors in myeloproliferative neoplasms: are we aiming for the right targets?

Stefan N. Constantinescu[1,2] and William Vainchenker[3-5]

[1]Ludwig Institute for Cancer Research, Brussels Branch, Brussels, Belgium; [2]Université Catholique de Louvain de Duve Institute, Brussels, Belgium; [3]Inserm, Unité Mixte de Recherche (UMR) 1009, Hematopoïèse Normale et Pathologique, Villejuif, France; [4]Institut Gustave Roussy, UMR 1009, Villejuif, France; and [5]Université Paris-Sud, UMR 1009, Villejuif, France

The ATP-binding pocket of the kinase domain of JAK2 is the major target of the present treatment of myeloproliferative neoplasms. Several inhibitors of JAK2 that are ATP competitive have been developed, but they do not discriminate between wild-type and mutant JAK2. These inhibitors have been used in myelofibrosis and, for the first time, treatment induced a reduction in spleen size and in constitutional symptoms. However, no dramatic effects on BM fibrosis, allele burden, or peripheral blast numbers were observed. These data indicate that other avenues should be explored that would either target mutant molecules (JAKs or receptors) more specifically and spare wild-type JAK2 or that would address other pathways that contribute to the malignant proliferation. Future success in treating myeloproliferative neoplasms will depend on advances of the understanding of JAK-STAT signaling and also on a better understanding of the disease pathogenesis, especially the role that mutants in spliceosome factors and epigenetic regulators play in the phenotype of the disease and the precise mechanism of fibrosis development.

Introduction

It is well established that the majority of human myeloproliferative neoplasms (MPNs) are driven by a constitutive or enhanced activation of the JAK-STAT pathway, especially JAK2, the thrombopoietin receptor (TpoR/MPL), and immediate downstream mediators such as STAT5, STAT3, MAPK, and PI3K.[1] Many laboratories are attempting inhibition of these signaling proteins and possibly of adaptors that couple them in different cell types (Figure 1). Investigators have made the most progress in developing ATP-competitive JAK inhibitors. Two phase 3 placebo-controlled clinical trials with one such inhibitor, ruxolitinib,[2,3] have led to US Food and Drug Administration (FDA) approval of this molecule for the treatment of myelofibrosis. In addition, data are being evaluated from these trials and previous phase 1/2 trials[4] and compared with historical controls to determine whether an effect of JAK2 inhibition could be detected on survival or leukemic transformation, although early scrutiny did not seem to indicate major effects.[5] Conversely, a statistically significant advantage in high-risk myelofibrosis patients compared with matched historical controls was reported recently.[6] Prospective data suggested a possible survival advantage in the COMFORT-I trial for myelofibrosis patients treated with ruxolitinib, but the post hoc analysis and the low number of patients limit this conclusion.[3] JAK2 inhibitors do not appear to be truly "disease modifying" in primary myelofibrosis (PMF), at least up to now, because of the low doses used to avoid toxicity of inhibiting wild-type JAK2, because drivers other than JAK2 are essential for PMF, or because secondary myelofibrosis might respond differently to JAK2 inhibitors than PMF and not enough data are available at the moment to make this distinction. Therefore, novel molecular targets are being sought for myelofibrosis and MPNs in general.

Possible new avenues could also include novel ways of targeting JAK2 V617F or exon12 mutants, either other regions of the kinase domain of JAK2 that might be allosterically regulated or the pseudokinase domain for JAK2 V617F (Figure 2), with the goal of developing mutant specific inhibitors. For example, favoring the degradation of activated JAKs would automatically achieve the goal of specific targeting JAK2-mutated cells. In addition, targeting cytokine receptors such as thrombopoietin receptor (TpoR/MPL), especially the mutant MPLW515L/K/A and its signaling via cytosolic tyrosine 626 (Figure 3), could be useful in myelofibrosis. It is possible that targeting other cytokine receptors or preventing their association with JAKs by disrupting the interactions between the NH2 terminus of JAKs and the juxtamembrane domain of receptors would achieve sufficient inhibition. Other avenues may be the inhibition of downstream pathways emanating from receptors in synergy with JAK2 inhibition, as well as inhibition of gene induction by constitutively active STATs (Figure 1) because of the hypothesis that MPN cells are addicted to some specific pathways or molecules. Finally, hypersensitivity of MPN progenitors to growth factors using tyrosine kinase receptors remains an unexplored avenue. The effectiveness of type I IFN in reducing the JAK2 V617F allele burden is provocative, and elucidation of the mechanisms of action of type I IFN in MPNs will generate novel useful targets. Another approach combined with signaling inhibition may concern the fibrosis itself and molecules such as TGF-β and their activation.

There is increasing evidence that JAK/STAT signaling plays a central role of the pathogenesis, but does not represent the entire pathogenesis, of MPNs. This has been underscored by the identification of mutations in other pathways such as epigenetic regulators or the splicing machinery. Furthermore, the constitutive activation of signaling pathways leads to chromatin effects and genomic instability, which themselves could be targeted eventually.

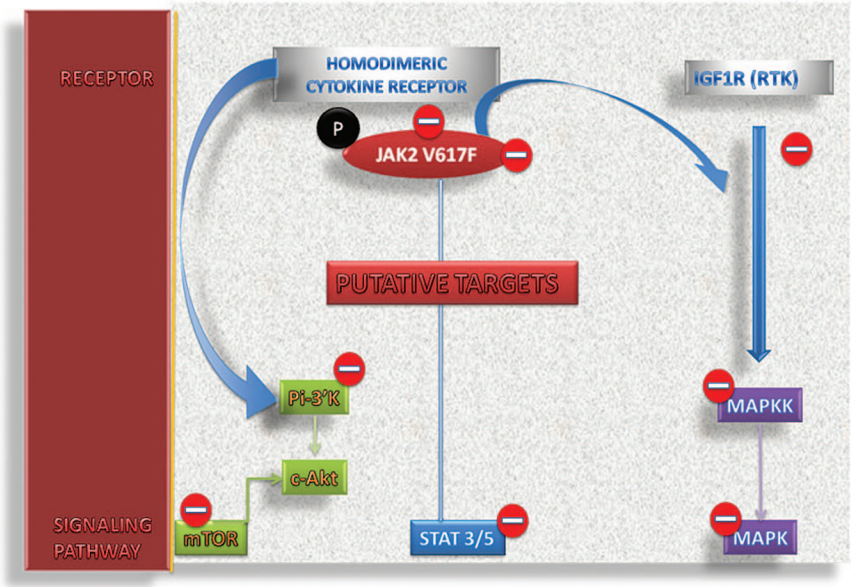

Figure 1. Targets of intervention by putative small-molecule inhibitors around the cytokine receptor, JAK-STAT, MAPK, and PI3K-mTOR pathways. Such targets include the interaction between JAK2 V617F and cytokine receptors, the pseudokinase domains of JAK2 V617F, and direct inhibition of STATs and the MAPK and PI3K pathways downstream of either cytokine receptors or tyrosine kinase receptors such as the IGF1 receptor, which could itself be targeted.

Targeting JAKs

Targeting JAK2-interacting proteins that regulate its stability

JAK2 V617F was recently found to be a preferred client of HSP90 and a therapeutic target in MPNs.[7] HSP90 inhibition is effective in inhibiting JAK2 V617F and mutants of JAK2 that are resistant to ATP-competitive inhibitors.[8] HDAC6 inhibitors are predicted to reduce the chaperone function of HSP90 in stabilizing client JAK2 V617F, because acetylated HSP90 cannot interact and protect client proteins against degradation.[9]

Starting from the AG490 tyrphostin JAK2 inhibitor, related structures were screened for JAK2 inhibition and a novel molecule was obtained that potently blocks downstream JAK2 signaling, but is not a kinase domain inhibitor.[10] The new molecule, WP1130, induces a block in JAK2 deubiquitination, being a special type of K63 deubiquitination inhibitor; the ubiquitinated JAK2 then trafficks to a detergent-insoluble compartment represented by perinuclear aggresomes, where JAK2 is not degraded but is inactive for signaling.[10] Interestingly, aggresomes were reported to contain histone deacetylase 6 (HDAC6), 20S proteasome, and heat-shock protein (HSP) 90.[11]

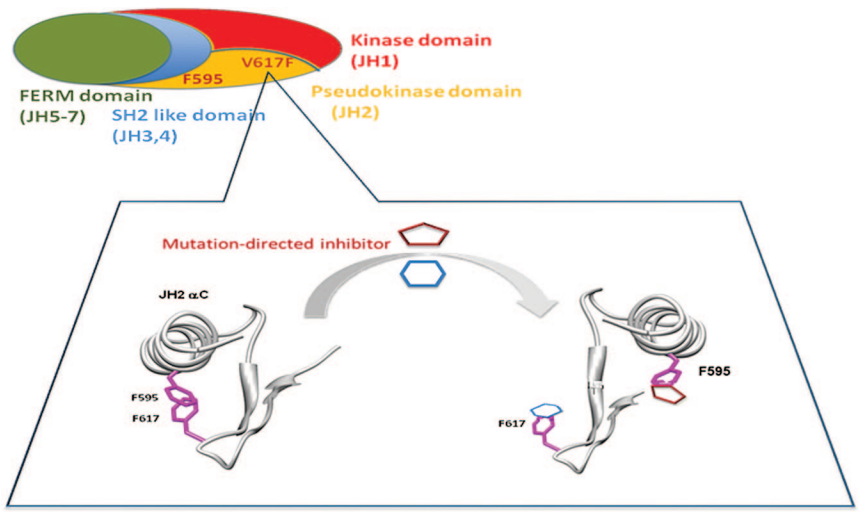

Figure 2. Searching for "mutant"-specific JAK2 inhibitors by targeting a predicted mechanism of activation of JAK2 kinase domain (JH1) by the V617F pseudokinase (JH2) mutation. A small molecule must interrupt the predicted F617-F595 interaction based on the demonstration that F595 (or an aromatic residue) is required for activation by the V617F mutation.

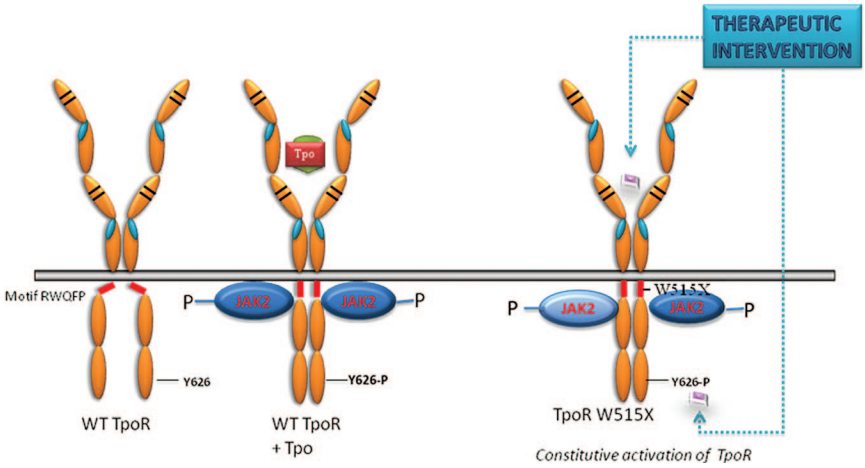

Figure 3. Targeting for inhibition the TpoR in MPNs. Sites of inhibition for TpoR could be the extracellular juxtamembrane region, the cytosolic juxtamembrane domain containing the W515 residue, and the cytosolic phosphorylated Y626, which is absolutely required for in vivo pathogenic effects of TpoR W515 mutants.

Targeting the pseudokinase domain (JH2)

The initial model of JAK function posited that the JH2 domain prevents activation of the JH1 domain.[12,13] However, the JH2 domain is also required for physiologic cytokine-dependent JAK activation.[14] Biochemical and mutagenesis experiments suggest that the pseudokinase domain binds ATP and might be a dual specific kinase that is capable of autophosphorylation at both S523 and Y570.[15] In theory, small molecules that would stabilize an inhibitory state of JH2 on JH1 could prevent activation by V617F, but there is also the risk that affecting ATP binding or conformation of JH2 might in itself activate JH1.[15]

No high-resolution crystal structure exists for a full-length JAK or for a receptor-JAK complex. Based on the X-ray crystal structures of several kinases, such as Csk, c-Abl, c-Kit, or Flt3, we noted that the closest located residue to the homolog of F617 in those kinases would be a phenylalanine (F595) located in the middle of the helix C of JH2[16] (Figure 2). We showed that this residue F595 is indispensable for the constitutive activity of JAK2 V617F. Mutation of F595 to Ala, Lys, Val, or Ile significantly decreases the constitutive activity of JAK2 V617F, but F595W and F595Y were able to restore it, implying an aromaticity requirement at position 595.[16] In contrast, F595 JAK2 mutants are activated by erythropoietin (Epo)-bound EpoR.[16] To our surprise, substitution of F595 to Ala was also able not only to prevent JAK2 V617F constitutive activation, but also to decrease the constitutive activity of 2 other JAK2 mutants, T875N and R683G, as well as JAK2 K539L, albeit to a lower extent. Our data indicated that F595 is a functional hot spot by which conformational information is transmitted from the JH2 to JH1, leading to JH1 activation.[16] Independent computational approaches and studies of other receptor systems also predicted or concluded an important role for F595 in JAK2 mutant activation.[17,18] A small molecule able to disrupt the predicted π-π interaction between F617 and F595 might be useful as a specific JAK2 V617F inhibitor (Figure 2). A very recent X-ray crystal structure study of the wild-type and V617F mutated pseudokinase domain of JAK2 indeed showed that aromatic stacking interactions occur around V617F involving F595 and F594, leading to a more rigid conformation and longer helical structure for helix C of JH2 in the V617F mutant, which is predicted to trigger activation of JH1.[19]

Targeting other JAKs and the cytokine storm

It is well known that a cytokine storm occurs in MPNs, with several cytokines being produced, which amplifies cytokine production and might contribute to constitutional symptoms, cachexia, and progression to myelofibrosis. Many of these inflammatory cytokines act via other JAKs, such as JAK1. Conversely, several JAK2 inhibitors used in myelofibrosis treatment are actually also JAK1 inhibitors and they exert benefic effects on constitutional symptoms and spleen size. Because the inhibition of JAK2 leads to anemia and thrombocytopenia and because high levels of JAK2 inhibition are required to effectively block downstream pathways, the efficacy is often counteracted by side effects that lead to treatment interruption. A highly specific JAK1 inhibitor might be a useful agent that can be administered during times when JAK2 inhibitors are stopped, which will allow the continuation of beneficial effects on the constitutional symptoms and possibly on the spleen. Conversely, recent studies with JAK2 V617F cell lines maintained in the presence of JAK2 inhibitors indicated that resistance involving epigenetic up-regulation of JAK2 V617F is acquired, eventually allowing persistence of MPN cells despite JAK2 inhibitors.[20] In such cells, heterodimerization occurs between JAK2 V617F and JAK1 or TYK2, which leads to activation of JAK2 V617F in trans by these JAKs in the presence of effective inhibitory concentrations of JAK2 inhibitors[20]

Targeting cytokine receptors

Cytokine receptors play a central role in the pathogenesis of MPNs because they can be either mutated, leading to a constitutive activation, or nonmutated but behave as a scaffold molecules for mutated JAK2, allowing its dimerization and constitutive signaling

Active TpoR mutants

Five to 10% of essential thrombocythemia (ET) and PMF patients who do not harbor JAK2 V617F carry activating mutations in TpoR at the cytosolic juxtamembrane position W515, such as W515L/K/A/R.[21-25] These mutations are activating because W515 is the key residue of an amphipathic juxtamembrane motif (Figure 3) that is required to prevent TpoR self-activation.[26] The in vivo phenotype induced by TpoR W515 mutants is much more severe than that of JAK2 V617F when using a retroviral strategy[21,25] and rapidly leads

to myelofibrosis and spleen fibrosis. No X-ray crystal structure exists for the transmembrane and cytosolic domain of TpoR (or of any cytokine receptor), so it is not clear whether it would be possible to specifically target a mutant receptor and spare the wild-type TpoR. The in vivo phenotype induced by TpoR W515 mutants appears to depend on phosphorylation of one cytosolic tyrosine residue of TpoR, namely Y626 (Figure 3), which normally links the receptor to STATs, especially STAT3 and STAT5, shc, and MAPK ERK1/2.[25] A double W515A Y626F mutant was not pathogenic in adoptive BM transfer experiments in mice, and the single W515A mutant induced fatal MPN and fibrosis within 45-50 days. A small molecule or peptide that would prevent signaling by TpoR Y626 will likely be of great benefit. Signaling by this Y626 does not appear to be absolutely essential for the wild-type TpoR, because delta60 knock-in mice, in which this residue is deleted, have normal steady-state platelet numbers.[27]

Targeting the wild-type TpoR/MPL

High levels of Tpo induce myelofibrosis in mice,[28] and Tpo or Tpo mimetic treatment in humans also can induce a reversible myelofibrosis.[29] The mechanisms by which Tpo induce myelofibrosis remain unclear. Two main hypotheses can be invoked: (1) a large number of megakaryocytes would always lead to myelofibrosis development because one possible function of megakaryocytes in the BM is the regulation of the microenvironment through secretion of cytokines such as PDGF or TGF-β, and (2) increased signaling induces either a true dysmegakaryopoiesis, as observed in PMF, or an increased secretion of fibrotic cytokines by the megakaryocytes. Therefore, one possibility would be to inhibit TpoR signaling in MPNs (Figure 3). Preliminary experiments in our laboratory indicate that coexpression of wild-type TpoR and JAK2 V617F in BM transplantation experiments leads to a more severe phenotype than JAK2 V617F alone and that, again, this phenotype depends on Y626 of TpoR (Pecquet et al, unpublished observations). Therefore, inhibition of TpoR, possibly by an anti-TpoR mAb that blocks signaling and triggers internalization and degradation, could be envisaged to retard progression of polycythemia vera (PV) or ET to myelofibrosis or to reduce fibrosis in PMF. However Tpo does not only act on MK differentiation, but also on hematopoietic stem cells (HSCs) in the mouse, where it is major factor in regulating HSC quiescence.[30-32] It is unknown whether Tpo plays the same role in human HSC homeostasis, but the fact that constitutional loss-of-function mutations of MPL (congenital amegakaryocytic thrombocytopenia) leads to aplastic anemia strongly suggest that Tpo is also a key determinant of human HSC function. Therefore, targeting physiologic TPO/TPOR signaling may be deleterious.

Another possibility will be to target profibrotic cytokines such as TGF-β or its activation. Presently, the mechanisms of TGF-β activation in the BM in PMF are unknown. TGF-β itself could be targeted by neutralizing Abs and this approach has been efficient in cardiac fibrosis. However, there will be a need for long-term administration of a humanized anti–TGF-β Ab, which makes this strategy difficult. A more appealing approach would be to target the TGF-β receptors by small molecules.

Recently different molecules, such as FT011, have been developed to inhibit TGF-β and have been tested in other fibrosis diseases with some success.[33]

TpoR down-modulation in MPNs

The mechanisms by which one acquired somatic JAK2 V617F mutation induces 3 diseases, ET, PV, and PMF, remain unclear. The most discussed hypothesis is that the level of JAK2 V617F activity is correlated with the phenotype, with low levels inducing ET, medium levels inducing PV, and very high levels inducing PMF.[34] Genetic engineering in mice elegantly showed that the ratio between JAK2 V617F and wild-type JAK2 is crucial for the MPN phenotype,[35] but it is not clear whether in humans, in which the entire disease stems from one HSC (unlike in mice, in which all cells carry one allele mutated in knock-in or transgenic contexts), this is the only factor driving phenotype. Interestingly, this hypothesis posits that the highest sensitivity to JAK2 V617F (or for its effects) is exhibited by megakaryocytes because they respond first. The prediction also would be that all PV patients should have ET. However, the duplication of JAK2 V617F may arise during the latent phase of the disease, thus explaining why PV does not derive from ET. However, many but not all PV patients have thrombocytosis. Part of the answer is that many PV and PMF patients actually exhibit marked down-modulation of TpoR levels in megakaryocytes and platelets,[36] which is not limited to JAK2 V617F patients, but with a reciprocal correlation between JAK2 V617F allele burden and TpoR expression levels.[37] TpoR down-modulation is mediated by constitutive ubiquitination and proteasome degradation of TpoR, along with inhibition of its recycling.[38] Unexpectedly, TpoR can also transduce negative signals[39] that arrest proliferation and induce senescence of megakaryocytes at advanced stages of differentiation.[39] Receptor down-modulation prevents such negative effects, and allows eventually late megakaryocytes to proliferate. Overall, an increasing body of evidence suggests that TpoR pathologic signaling is the key to MPN progression. Inhibition of JAK2 in vivo in patients with myelofibrosis and in JAK2 V617F knock-in mice leads to a restoration of platelet TpoR levels.[38] The same can be obtained in JAK2 V617F knock-in mice by inhibiting proteasomes with bortezomib (trade name Velcade).[38]

Because distinct dimeric orientations of TpoR were found to induce physiologic platelet formation, myeloproliferation, and myelodysplastic phenotypes in vivo in BM adoptive transfer experiments,[40] it is possible that pathologic signaling via TpoR might have more implications than previously thought.

Targeting other cytokine receptor complexes

In theory, inhibition of EpoR should decrease hematocrit in PV patients, but this will also rapidly lead to anemia. Whether the interaction between EpoR and JAK2 V617F could be disrupted more easily than that between EpoR and JAK2 is unknown. Furthermore, G-CSFR is using JAK2 for signaling in HSCs and granulocyte progenitors.[41] However, G-CSFR can also bind and activate JAK1.[42] The prediction is that JAK2 V617F activates signaling by G-CSFR late in the disease. Because G-CSFR activation leads to release of HSCs and $CD34^+$ cells from the BM to the periphery, it is tempting to speculate that constitutive G-CSFR activation might contribute to extramedullary hematopoiesis, so a strategy disrupting JAK2 V617F from G-CSFR might be useful.

JAK2 is also essential for signaling by other type I and type II receptors, such as IL3, and IFN-γ, respectively[43] and it was shown to be active in complexes with diverse receptors, such as IL27R,[44] or gp130 (our unpublished results). It remains to be established whether such JAK2 V617F-receptor complexes are pathogenic in MPNs and can be also targets for inhibition.

Myeloid hematopoiesis is also regulated by cytokines or growth factors that bind and activate tyrosine kinase receptors. Erythroid colonies from PV patients are hypersensitive to insulin-like growth

factor 1 (IGF1), and increased tyrosine phosphorylation of the IGF1 receptor beta subunit is detected in mononuclear cells from PV patients.[45] In Ba/F3 cells transformed to autonomous growth by JAK2 V617F, IGF1 induces further tyrosine phosphorylation of JAK2 and STAT5, whereas in JAK2 V617F–expressing cells before selection for autonomous growth, IGF1 induces cell proliferation, which is not the case for parental Ba/F3 cells.[46] This cross-talk between cytokine receptor-JAK and receptor tyrosine kinase pathways might be relevant for the early stages of MPN, when the clonal dominance occurs, selecting out mutated clones for proliferation. Interestingly, synergy between Epo and serum growth factors become essential when the distal part of EpoR is deleted. These truncated EpoR proteins are hypersensitive to Epo when cells are grown in serum, but are less responsive to Epo than wild-type EpoRs when cells are grown in defined medium without IGF1.[47] Therefore, activation of MAPK and PI3K is obligatory for erythroid cell proliferation and differentiation and can occur either from the distal end of the EpoR or via the parallel pathway of IGF1R signaling in normal conditions. In the case of PV progenitors, IGF1 hypersensitivity can be the result of the cross-talk between the JAK-STAT and IGF1R pathways. It is possible that JAK2 V617F, which is a weak kinase, only phosphorylates the proximal receptor tyrosines that are involved in STAT5 activation. This would not diminish the constitutive signal as long the MAPK and PI3K pathways are activated by IGF1, and progenitors will be hypersensitive because STAT5 is already activated. Targeting of IGF1 and its receptor, and possibly other tyrosine kinase receptors relevant for myeloid cell proliferation, could be one avenue for future attempts to induce a disadvantage for the mutated clone.

Targeting downstream molecules

Targeting serine/threonine kinase cascades emanating from the cytokine receptor-JAK complexes

Upon JAK activation, cytokine receptors become tyrosine phosphorylated and attract adaptors that link receptors to the 2 main serine threonine cascades, the MAPK ERK1/2 and PI3K-AKT-mTOR pathways.[1] Proliferation, survival, and differentiation of the 3 myeloid lineages require these pathways in different proportions and at different stages of differentiation. The PI3K pathway was suggested to contribute to the Epo independence of erythroid progenitors in PV.[48] The MAPK-ERK pathway was suggested to induce megakaryocyte senescence at high Tpo signaling levels[39] and to be involved in the myelofibrosis induced by TpoR W515 mutants.[25] The p38 MAPK pathway activated by FLT3 was shown to contribute to megakaryocyte abnormal proliferation and differentiation in myelofibrosis.[49] These pathways are known in the case of EGFR to be difficult to inhibit by inhibitors of the upstream tyrosine kinase (ie, EGFR kinase inhibitors), because a $> 90\%$ EGFR inhibition can allow almost intact MAPK ERK1/2 activation[50] given the incredible efficiency and amplification virtues of these cascades. Therefore, a better approach would be to test combinations of JAK2 inhibitors and serine threonine kinase inhibitors in MPN progenitors such as AKT inhibitors. This might allow the discovery of pathways to which such progenitors could be addicted. The dose of JAK2 inhibitor used would therefore be lower, thus avoiding the side effects of anemia and thrombocytopenia.

Two examples of pathways linked to the PI3K have been suggested to play an important role in MPNs. The first is represented by mTOR. A phase 1/2 study with everolimus, an mTOR inhibitor, in 39 high- or intermediate-risk primary or post-PV/post-ET myelofibrosis subjects reported benefic effects in myelofibrosis, with reductions in spleen size but without a decrease in JAK2 V617F allele burden.[51] The response rate was between 23% and 60% depending on the criteria used. It will be interesting to determine whether such mTOR inhibitors exert synergic effects with JAK2 inhibitors.

Downstream of AKT is inhibition of the Forkhead Box O3 (FoxO3) transcription factor by phosphorylation and sequestration in the cytosol. FoxO3 is crucial for the maintenance of HSCs, and possibly for cancer/leukemia stem cells, and the absence of FoxO3 leads to high levels of reactive oxygen species (ROS) in progenitors and amplification of the AKT/mTOR pathway, leading to some myeloproliferation.[52] It would be desirable to eliminate FoxO3 from cancer cell stem cells and restore it to MPN progenitors, so agents that prevent or eliminate ROS would be predicted to be useful in the treatment of MPNs.

Targeting the PI3K/AKT/mTOR pathway might be effective for at least 2 main reasons. The genetic instability induced by a kinase such as JAK2 V617F or activated JAK2 is dependent on ROS accumulation related to degradation and production. ROS degradation is regulated by enzymes such as catalase or superoxide dismutase, direct targets of FoxO3, which is inhibited by phosphorylation by AKT, and the production might be dependent on NADPH oxidase and the Rac pathway. This last pathway seems to depend on STAT and PI3K activation. ROS accumulation leads to oxidative DNA damage, as known for 8-oxoguanine, which induces DNA double-strand breaks and subsequently mutations. The PI3K pathway may contribute significantly to the acquisition of new mutations and disease progression. Therefore, inhibition of this pathway might be important to decrease evolution of the disease toward myelofibrosis and leukemia. In addition, there is some evidence that JAK2 V617F inhibits p53 stabilization and activation[53] through an increase level of mdm2, the ubiquitin ligase that degrades p53. This increase in mdm2 is mediated by an increased translation induced by the mTOR pathway.[53] This functional inhibition of p53 plays an important role in genetic instability, and p53 seems to be central in leukemic development because, in contrast to sporadic acute myeloid leukemia (AML), mutations of p53 are frequent in post-MPN AML. Inhibition of the PI3K pathway, including mTOR, is predicted to restore normal p53 function in the chronic phase of MPNs. In addition, treatments targeting the interaction of p53 with mdm2, such as Nutlin-3, might also be an approach to restore p53 function and to have an effect not only on genetic instability, but also on myeloproliferation. This type of drug is presently in development.

Targeting STATs

It has been suggested that PV is mainly associated with increased STAT5 and STAT3 activation, whereas ET involves mainly STAT3 activation, with PMF being associated with lower levels of STAT5 and STAT3 activation.[54] More recently, STAT1 activation was reported in ET.[55] STAT5A/B double-knockout mice are resistant to the JAK2 V617F–induced MPN,[56,57] thus confirming that STAT5 is essential for MPNs.

Inhibitors of STAT5, such as pimozide,[58] and inhibitors of STAT3 and STAT1 could be useful alone or in association with JAK inhibitors or inhibitors of MAPK and PI3K. Conversely, all of these STAT proteins play major roles in the immune response and epithelial regeneration, so side effects are to be expected.

Targeting of some STAT5 targets could also be useful. It has been shown that spontaneous erythroid colony formation can be obtained by overexpressing BCL-XL, which is a direct target of STAT5. It has been shown that a BH3-mimetic, ABT-737, can induce apoptosis of JAK2 V617F cells alone or in association with IFN-α.[59] This therapy might be associated with JAK2 inhibitors, but carries the risk of markedly inducing thrombocytopenia. Another downstream molecule of STAT activation could be the PIM kinases.

When STATs are constitutively activated, they are persistently present in the nucleus and have an increased chance to bind to low-affinity sites or to recruit adaptors not normally recruited by transiently activated STATs. Constitutively activated STAT molecules could regulate genes not normally targeted by cytokine-activated STATs. These genes (eg, the LIM-domain lipoma preferred partner [LPP], which hosts miR-28, a negative regulator of TpoR mRNA translation and of megakaryocyte differentiation) can be markers of disease or could be involved in driving disease.[60] Genomics studies may unravel such novel targets that might be involved in further genetic instability or progression and could become novel targets in MPNs.

Avenues for targeting epigenetic regulators in MPNs

The constitutive nature of JAK2 V617F activation is predicted to amplify previously unrecognized roles for JAK2, which in the context of cytokine signaling are transient or not detectable by current assays. JAK2 was shown to phosphorylate Y41 of histone 3 and to exclude heterochromatin protein 1a alpha from chromatin, leading to gene induction, such as induction of Lmo2.[61] Such nuclear JAK2 signaling is predicted to be important for the persistently activated JAK2 V617F. Furthermore, mouse embryonic stem (ES) cells carrying JAK2 V617F exhibited increased levels of H3Y41 phosphorylation and could replace leukemia inhibitory factor for ES cell renewal, which was inhibited by JAK2 inhibitors.[62] Therefore, chromatin signaling by JAK2 can play a role in ES cell renewal.

JAK2 V617F and the exon 12 K539L mutant were also reported to induce enhanced binding to and pathological phosphorylation of protein arginine methyltransferase 5 (PRMT5), inhibiting its arginine methyltransferase activity and favoring myeloproliferation.[63] Given these results, 2 prediction can be made: (1) several novel preferred substrates might be identified for mutated JAK2 proteins that could be important as targets in MPN treatment, and (2) downstream signaling partners of those preferred interacting partners might also play a major role in MPN and could be targets for inhibition.

One of the reasons of the egress of $CD34^+$ cells from the BM in PMF might be that the CXCR4 chemokine receptor for SDF1 is not expressed on PMF $CD34^+$ cells.[64] This was shown to be related to promoter methylation. Treatment with 5-azacytidine or sequential treatment with 5-azacytidine and trichostatin A led to an increase of membrane expression of CXCR4.[65] Whether inhibitors of JAK2 reduce spleen size due to an effect on chemokine secretion or chemokine receptor function is not clear, but elucidation of the molecular targets of these effects would greatly improve searches for other molecules that would have similar effects without inducing the anemia and thrombocytopenia that are side effects of JAK2 inhibitors.

A synthetic class I histone deacetylase inhibitor, ITF2357, was surprisingly shown to target cells carrying the JAK2 V617F mutation, as well as the HEL cell line, which only expresses JAK2 V617F ITF2357-induced JAK2 V617F degradation without changing mRNA levels for JAK2 V617F.[66] These effects might be due to inhibition of deacetylation inhibition linked to preventing HSP90 interactions. This compound exerts proapoptotic effects in AML and multiple myeloma cells, and was shown to down-modulate secretion of several cytokines, IL6, IFN-γ, and VEGF, at doses similar to those inducing the proapoptotic effects.[67] A phase 2A study explored the effects of a HDAC inhibitor, givinostat, with a median duration of 20 weeks. The effects were 1 complete remission, 6 partial remissions, 4 nonresponses, and 2 trial withdrawals of 13 ET/PV patients.[68]

MPNs share with myelodysplastic syndrome and AML several genetic lesions, such as biallelic inactivation or mutations in TET2, ASXL1, DNMT3A, and EZH2, and more recently in genes of the spliceosome such as SRSF2 or SF3B1.[69,70] These mutations can occur before or after acquisition of JAK2 V617F. Mutations in epigenetic regulators appear to induce enhanced renewal to HSCs and/or extent proliferation of progenitors and defects in maturation, whereas mutations in SF3B1 may induce defects in maturation such as sideroblasts. However, mutations of these genes alone do not induce a malignant hematological disease, but participate in the development of the clonal dominance. The type of hematological disease will depend on associated oncogenic mutations such as JAK2 V617F for MPNs, whereas acquisition of both transcription factor/differentiation and proliferative mutations will lead to AML.

Mutations in components of the PRC2 complex appear to be very important and frequently associated with leukemic transformation of chronic myeloid disorders. The JARID2 member of the PRC2 is a gene frequently deleted in AML after MPN or myelodysplastic syndrome.[71] Another member of the PRC2, EZH2 is also mutated in MPNs and AML and, interestingly, EZH2 mutations seem to be mutually exclusive with TpoR W515 mutations.[69] Future directions would be to define ways to monitor function of PRC2 and to identify the signals and the basis of the genetic instability that leads to JARID2 deletions and dysfunction of PRC2. One difficulty in this approach is related to the fact that most mutations are loss-of-function mutations and are therefore more difficult to target.

Acknowledgments

The authors thank Dr Ana Enciu for help with the figures.

This research was supported in part by the FRS-FNRS Belgium, Fondation contre le cancer, The Salus Sanguinis Foundation, the ARC Program of the University Catholic of Louvain, the IAP Program Belgium, the Atlantic Philanthropies New York, and the Ludwig Institute for Cancer Research.

Disclosures

Conflict-of-interest disclosure: S.C. has been a member of scientific advisory boards for Novartis, Amgen, Dafra Pharma R&D, and Shire and has delivered lectures supported by Shire and Amgen. W.V. is on the board of directors or an advisory committee for and has received research funding from Celgen, Novartis, and Sanofi. Off-label drug use: None disclosed.

Correspondence

Dr Stefan N. Constantinescu, Ludwig Institute for Cancer Research and Université Catholique de Louvain de Duve Institute, Av Hippocrate 74, UCL 75-4, Brussels B1200, Belgium; Phone: 32-27-64-75-40; Fax:

32-27-64-65-66; e-mail: stefan.constantinescu@bru.licr.org; Dr William Vainchenker, Inserm U 1009, Institut Gustave Roussy PR 1, 39 rue Camille Desmoulins, 94805 Villejuif Cedex, France; Phone: 33-14-21-15-363; Fax: 33-01-42-11-52-401; e-mail: verpre@igr.fr.

References

1. Vainchenker W, Dusa A, Constantinescu SN. JAKs in pathology: role of Janus kinases in hematopoietic malignancies and immunodeficiencies. *Semin Cell Dev Biol*. 2008;19:385-393.
2. Verstovsek S, Mesa RA, Gotlib J, et al. A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. *N Engl J Med*. 2012;366:799-807.
3. Harrison C, Kiladjian JJ, Al-Ali HK, et al. JAK inhibition with ruxolitinib versus best available therapy for myelofibrosis. *N Engl J Med*. 2012;366:787-798.
4. Verstovsek S, Kantarjian H, Mesa RA, et al. Safety and efficacy of INCB018424, a JAK1 and JAK2 inhibitor, in myelofibrosis. *N Engl J Med*. 2010;363:1117-1127.
5. Tefferi A. JAK inhibitors for myeloproliferative neoplasms: clarifying facts from myths. *Blood.* 2012;119:2721-2730.
6. Verstovsek S, Kantarjian HM, Estrov Z, et al. Long term outcomes of 107 patients with myelofibrosis receiving JAK1/JAK2 inhibitor ruxolitinib: survival advantage in comparison to matched historical controls. *Blood.* 2012;120(6):1202-1209.
7. Marubayashi S, Koppikar P, Taldone T, et al. HSP90 is a therapeutic target in JAK2-dependent myeloproliferative neoplasms in mice and humans. *J Clin Invest*. 2010;120:3578-3593.
8. Weigert O, Lane AA, Bird L, et al. Genetic resistance to JAK2 enzymatic inhibitors is overcome by HSP90 inhibition. *J Exp Med*. 2012;209:259-273.
9. Kovacs JJ, Murphy PJ, Gaillard S, et al. HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. *Mol Cell*. 2005;18:601-607.
10. Kapuria V, Levitzki A, Bornmann WG, et al. A novel small molecule deubiquitinase inhibitor blocks Jak2 signaling through Jak2 ubiquitination. *Cell Signal*. 2011;23:2076-2085.
11. Kopito RR. Aggresomes, inclusion bodies and protein aggregation. *Trends Cell Biol*. 2000;10:524-530.
12. Saharinen P, Takaluoma K, Silvennoinen O. Regulation of the Jak2 tyrosine kinase by its pseudokinase domain. *Mol Cell Biol*. 2000;20:3387-3395.
13. Saharinen P, Silvennoinen O. The pseudokinase domain is required for suppression of basal activity of Jak2 and Jak3 tyrosine kinases and for cytokine-inducible activation of signal transduction. *J Biol Chem*. 2002;277:47954-47963.
14. Velazquez L, Mogensen KE, Barbieri G, Fellous M, Uze G, Pellegrini S. Distinct domains of the protein tyrosine kinase tyk2 required for binding of interferon-alpha/beta and for signal transduction. *J Biol Chem*. 1995;270:3327-3334.
15. Ungureanu D, Wu J, Pekkala T, et al. The pseudokinase domain of JAK2 is a dual-specificity protein kinase that negatively regulates cytokine signaling. *Nat Struct Mol Biol*. 2011;18:971-976.
16. Dusa A, Mouton C, Pecquet C, Herman M, Constantinescu SN. JAK2 V617F constitutive activation requires JH2 residue F595: a pseudokinase domain target for specific inhibitors. *PLoS One*. 2010;5:e11157.
17. Gnanasambandan K, Sayeski PP. A structure-function perspective of Jak2 mutations and implications for alternate drug design strategies: the road not taken. *Curr Med Chem*. 2011;18:4659-4673.
18. Lee TS, Ma W, Zhang X, Giles F, Kantarjian H, Albitar M. Mechanisms of constitutive activation of Janus kinase 2-V617F revealed at the atomic level through molecular dynamics simulations. *Cancer.* 2009;115:1692-1700.
19. Bandaranayake RM, Ungureanu D, Shan Y, Shaw DE, Silvennoinen O, Hubbard SR. Crystal structures of the JAK2 pseudokinase domain and the pathogenic mutant V617F. *Nat Struct Mol Biol*. 2012;19(8):754-759.
20. Koppikar P, Bhagwat N, Kilpivaara O, et al. Heterodimeric JAK-STAT activation as a mechanism of persistence to JAK2 inhibitor therapy [published online ahead of print July 22, 2012]. *Nature.* doi:10.138/nature11303.
21. Pikman Y, Lee BH, Mercher T, et al. MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. *PLoS Med*. 2006;3:e270.
22. Pardanani AD, Levine RL, Lasho T, et al. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. *Blood.* 2006;108:3472-3476.
23. Beer PA, Campbell PJ, Scott LM, et al. MPL mutations in myeloproliferative disorders: analysis of the PT-1 cohort. *Blood.* 2008;112:141-149.
24. Boyd EM, Bench AJ, Goday-Fernandez A, et al. Clinical utility of routine MPL exon 10 analysis in the diagnosis of essential thrombocythaemia and primary myelofibrosis. *Br J Haematol*. 2010;149:250-257.
25. Pecquet C, Staerk J, Chaligne R, et al. Induction of myeloproliferative disorder and myelofibrosis by thrombopoietin receptor W515 mutants is mediated by cytosolic tyrosine 112 of the receptor. *Blood.* 2010;115:1037-1048.
26. Staerk J, Lacout C, Sato T, Smith SO, Vainchenker W, Constantinescu SN. An amphipathic motif at the transmembrane-cytoplasmic junction prevents autonomous activation of the thrombopoietin receptor. *Blood.* 2006;107:1864-1871.
27. Luoh SM, Stefanich E, Solar G, et al. Role of the distal half of the c-Mpl intracellular domain in control of platelet production by thrombopoietin in vivo. *Mol Cell Biol*. 2000;20:507-515.
28. Villeval JL, Cohen-Solal K, Tulliez M, et al. High thrombopoietin production by hematopoietic cells induces a fatal myeloproliferative syndrome in mice. *Blood.* 1997;90:4369-4383.
29. Kuter DJ, Mufti GJ, Bain BJ, Hasserjian RP, Davis W, Rutstein M. Evaluation of bone marrow reticulin formation in chronic immune thrombocytopenia patients treated with romiplostim. *Blood.* 2009;114:3748-3756.
30. Yoshihara H, Arai F, Hosokawa K, et al. Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. *Cell Stem Cell*. 2007;1:685-697.
31. Qian H, Buza-Vidas N, Hyland CD, et al. Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. *Cell Stem Cell*. 2007;1:671-684.
32. de Graaf CA, Kauppi M, Baldwin T, et al. Regulation of hematopoietic stem cells by their mature progeny. *Proc Natl Acad Sci U S A*. 2010;107(50):21689-21694.
33. Zhang Y, Edgley AJ, Cox AJ, et al. FT011, a new anti-fibrotic drug, attenuates fibrosis and chronic heart failure in experimental diabetic cardiomyopathy. *Eur J Heart Fail*. 2012;14:549-562.
34. Lacout C, Pisani DF, Tulliez M, Gachelin FM, Vainchenker W, Villeval JL. JAK2V617F expression in murine hematopoietic cells leads to MPD mimicking human PV with secondary myelofibrosis. *Blood.* 2006;108:1652-1660.
35. Tiedt R, Hao-Shen H, Sobas MA, et al. Ratio of mutant JAK2-V617F to wild-type Jak2 determines the MPD phenotypes in transgenic mice. *Blood.* 2008;111:3931-3940.
36. Moliterno AR, Hankins WD, Spivak JL. Impaired expression of

the thrombopoietin receptor by platelets from patients with polycythemia vera. *N Engl J Med*. 1998;338:572-580.
37. Moliterno AR, Williams DM, Rogers O, Isaacs MA, Spivak JL. Phenotypic variability within the JAK2 V617F-positive MPD: roles of progenitor cell and neutrophil allele burdens. *Exp Hematol*. 2008;36:1480-1486.
38. Pecquet C, Diaconu CC, Staerk J, et al. Thrombopoietin receptor down-modulation by JAK2 V617F: restoration of receptor levels by inhibitors of pathologic JAK2 signaling and of proteasomes. *Blood.* 2012;119:4625-4635.
39. Besancenot R, Chaligne R, Tonetti C, et al. A senescence-like cell-cycle arrest occurs during megakaryocytic maturation: implications for physiological and pathological megakaryocytic proliferation. *PLoS Biol*. 2010;8(9). pii:e1000476.
40. Staerk J, Defour JP, Pecquet C, et al. Orientation-specific signalling by thrombopoietin receptor dimers. *EMBO J*. 2011; 30:4398-4413.
41. Touw IP, van de Geijn GJ. Granulocyte colony-stimulating factor and its receptor in normal myeloid cell development, leukemia and related blood cell disorders. *Front Biosci*. 2007; 12:800-815.
42. Shimoda K, Feng J, Murakami H, et al. Jak1 plays an essential role for receptor phosphorylation and Stat activation in response to granulocyte colony-stimulating factor. *Blood.* 1997; 90:597-604.
43. Watowich SS, Wu H, Socolovsky M, Klingmuller U, Constantinescu SN, Lodish HF. Cytokine receptor signal transduction and the control of hematopoietic cell development. *Annu Rev Cell Dev Biol*. 1996;12:91-128.
44. Pradhan A, Lambert QT, Reuther GW. Transformation of hematopoietic cells and activation of JAK2-V617F by IL-27R, a component of a heterodimeric type I cytokine receptor. *Proc Natl Acad Sci U S A*. 2007;104:18502-18507.
45. Mirza AM, Correa PN, Axelrad AA. Increased basal and induced tyrosine phosphorylation of the insulin-like growth factor I receptor beta subunit in circulating mononuclear cells of patients with polycythemia vera. *Blood.* 1995;86:877-882.
46. Staerk J, Kallin A, Demoulin JB, Vainchenker W, Constantinescu SN. JAK1 and Tyk2 activation by the homologous polycythemia vera JAK2 V617F mutation: cross-talk with IGF1 receptor. *J Biol Chem*. 2005;280:41893-41899.
47. Damen JE, Krosl J, Morrison D, Pelech S, Krystal G. The hyperresponsiveness of cells expressing truncated erythropoietin receptors is contingent on insulin-like growth factor-1 in fetal calf serum. *Blood.* 1998;92:425-433.
48. Ugo V, Marzac C, Teyssandier I, et al. Multiple signaling pathways are involved in erythropoietin-independent differentiation of erythroid progenitors in polycythemia vera. *Exp Hematol*. 2004;32:179-187.
49. Desterke C, Bilhou-Nabera C, Guerton B, et al. FLT3-mediated p38-MAPK activation participates in the control of megakaryopoiesis in primary myelofibrosis. *Cancer Res*. 2011;71:2901-2915.
50. Sevecka M, MacBeath G. State-based discovery: a multidimensional screen for small-molecule modulators of EGF signaling. *Nat Methods*. 2006;3:825-831.
51. Guglielmelli P, Barosi G, Rambaldi A, et al. Safety and efficacy of everolimus, a mTOR inhibitor, as single agent in a phase 1/2 study in patients with myelofibrosis. *Blood.* 2011;118:2069-2076.
52. Yalcin S, Marinkovic D, Mungamuri SK, et al. ROS-mediated amplification of AKT/mTOR signalling pathway leads to myeloproliferative syndrome in Foxo3(−/−) mice. *EMBO J*. 2010;29:4118-4131.
53. Nakatake M, Monte-Mor B, Debili N, et al. JAK2(V617F) negatively regulates p53 stabilization by enhancing MDM2 via La expression in myeloproliferative neoplasms. *Oncogene*. 2012;31:1323-1333.
54. Teofili L, Martini M, Cenci T, et al. Different STAT-3 and STAT-5 phosphorylation discriminates among Ph-negative chronic myeloproliferative diseases and is independent of the V617F JAK-2 mutation. *Blood.* 2007;110:354-359.
55. Chen E, Beer PA, Godfrey AL, et al. Distinct clinical phenotypes associated with JAK2V617F reflect differential STAT1 signaling. *Cancer Cell*. 2010;18:524-535.
56. Walz C, Ahmed W, Lazarides K, et al. Essential role for Stat5a/b in myeloproliferative neoplasms induced by BCR-ABL1 and Jak2V617F in mice. *Blood.* 2012;119(15):3550-3560.
57. Yan D, Hutchison RE, Mohi G. Critical requirement for Stat5 in a mouse model of polycythemia vera. *Blood.* 2012;119(15): 3539-3549.
58. Nelson EA, Walker SR, Weisberg E, et al. The STAT5 inhibitor pimozide decreases survival of chronic myelogenous leukemia cells resistant to kinase inhibitors. *Blood.* 2011;117:3421-3429.
59. Lu M, Zhang W, Li Y, et al. Interferon-alpha targets JAK2V617F-positive hematopoietic progenitor cells and acts through the p38 MAPK pathway. *Exp Hematol*. 2010;38:472-480.
60. Girardot M, Pecquet C, Boukour S, et al. miR-28 is a thrombopoietin receptor targeting microRNA detected in a fraction of myeloproliferative neoplasm patient platelets. *Blood.* 2010;116:437-445.
61. Dawson MA, Bannister AJ, Gottgens B, et al. JAK2 phosphorylates histone H3Y41 and excludes HP1alpha from chromatin. *Nature.* 2009;461:819-822.
62. Griffiths DS, Li J, Dawson MA, et al. LIF-independent JAK signalling to chromatin in embryonic stem cells uncovered from an adult stem cell disease. *Nat Cell Biol*. 2011;13:13-21.
63. Liu F, Zhao X, Perna F, et al. JAK2V617F-Mediated Phosphorylation of PRMT5 Downregulates Its Methyltransferase Activity and Promotes Myeloproliferation. *Cancer Cell*. 2011;19:283-294.
64. Bogani C, Ponziani V, Guglielmelli P, et al. Hypermethylation of CXCR4 promoter in CD34+ cells from patients with primary myelofibrosis. *Stem Cells.* 2008;26:1920-1930.
65. Wang X, Zhang W, Ishii T, et al. Correction of the abnormal trafficking of primary myelofibrosis CD34+ cells by treatment with chromatin-modifying agents. *Cancer Res*. 2009;69:7612-7618.
66. Guerini V, Barbui V, Spinelli O, et al. The histone deacetylase inhibitor ITF2357 selectively targets cells bearing mutated JAK2(V617F). *Leukemia.* 2008;22:740-747.
67. Leoni F, Zaliani A, Bertolini G, et al. The antitumor histone deacetylase inhibitor suberoylanilide hydroxamic acid exhibits antiinflammatory properties via suppression of cytokines. *Proc Natl Acad Sci U S A*. 2002;99:2995-3000.
68. Rambaldi A, Dellacasa CM, Finazzi G, et al. A pilot study of the Histone-Deacetylase inhibitor Givinostat in patients with JAK2V617F positive chronic myeloproliferative neoplasms. *Br J Haematol*. 2010;150:446-455.
69. Guglielmelli P, Biamonte F, Score J, et al. EZH2 mutational status predicts poor survival in myelofibrosis. *Blood.* 2011;118: 5227-5234.
70. Vainchenker W, Delhommeau F, Constantinescu SN, Bernard OA. New mutations and pathogenesis of myeloproliferative neoplasms. *Blood.* 2011;118:1723-1735.
71. Puda A, Milosevic JD, Berg T, et al. Frequent deletions of JARID2 in leukemic transformation of chronic myeloid malignancies. *Am J Hematol*. 2012;87(3):245-250.