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The Ph-positive and Ph-negative myeloproliferative neoplasms: some topical pre-clinical and clinical issues

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This review focuses on topical issues in the biology and treatment of the myeloproliferative neoplasms (MPNs). Studies in transgenic mice suggest that BCR-ABL1 reduces the fraction of self-renewing 'leukemic' stem cells in the bone marrow but that some of these cells survive treatment with imatinib. This also seems to operate in humans. Data from models also strongly support the notion that JAK2V617F can initiate and sustain MPNs in mice; relevance to disease in humans is less clear. These data also support the hypothesis that level of JAK2V617F expression influences the MPN phenotype: higher levels favor erythrocytosis whereas lower levels favor thrombocytosis. Although TET2-mutations were thought to precede JAK2V617F in some persons with MPNs, it now appears that TET2 mutations may occur after JAK2V617F. Further understanding of signal-transduction pathways activated in chronic myeloid leukemia suggests various possible targets for new therapies including the WNT/beta catenin, notch and hedgehog pathways. Finally, the clinical role of the new JAK2- and BCR- ABL1-inhibitors is considered. Much further progress is likely in several of these areas soon.

Key words: myeloproliferative neoplasm, JAK2, Ph-negative, Ph-positive.


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Introduction

The advent of tyrosine kinase inhibitors (TKI) has proved to be an extraordinarily important advance in the management of patients with early phase Ph-positive chronic myeloid leukemia and has also influenced the direction of research into the underlying biology of leukemia and other tumors. The identification of the JAK2V617F mutation in polycythemia vera and other Ph-negative myeloproliferative neoplasms (MPN) has made an extremely important contribution to our understanding of the basic biology of these disorders. There remain, however, many unanswered questions. This paper has three discrete but related themes. First, it reviews some of the murine systems that have been developed in recent years to model the JAK2V617F-positive MPNs and BCR-ABL1-positive chronic myeloid leukemia (CML) with a view to defining those questions that might be answerable with appropriate model systems. Second, it reviews the very recent data relevant to the issue of whether TET2 mutations predispose to development of an MPN or are ‘merely’ secondary events. Third, it summarizes briefly some of the recent results of using new agents to treat the Ph-negative MPN and describes some molecular pathways that could be exploited for therapy in the future.

(a) Transgenic models of JAK2-mutant MPNs

The MPNs and related conditions, many of which are characterized by dysregulated tyrosine kinase (TK) signaling,1 are good candidates for mouse models. These models are typically established by expressing the relevant mutant signaling molecules, e.g. BCR-ABL1 in CML or mutant PTPN11 (in juvenile myelomonocytic leukemia) in mouse hematopoietic cells. There are two common strategies: (1) gene transfer into hematopoietic cells by retro- or lenti-viruses followed by transplantation; and (2) expression via a chromosomal transgene (for review see 2). Each method has advantages and dis...
advantages for modeling and for pre-clinical evaluation of molecularly targeted therapies.

Several previous publications reported data on the retroviral strategy for modeling MPNs induced by Jak2V617F. However, the transgenic mouse field is rapidly catching up. Three new transgenic models of Jak2V617F-induced MPNs were presented at the 2009 ASH meeting. Here, we compare these new models with previously published models and consider what they can teach us about the pathophysiology of seemingly-corresponding MPNs (Table 1).

One of the major advantages of the transgene approach is the ability to express the mutant TK at near physiological levels. This contrasts with retroviral vector models in which relevant oncogenes are expressed at relatively high levels. This disparity might be particularly important for Jak2 as studies in cell lines indicate Jak2V617F must associate with a type-I cytokine receptor (such as EpoR or MPL) for signaling activity. Consequently, competition between the retrovirus-encoded mutant Jak2 and endogenous, wild-type Jak2 may influence disease phenotype. The first published transgenic model from Skoda and co-workers used a novel conditional inverted allele of human Jak2V617F under the control of the human Jak2–promoter. A constitutively expressed Vav-Cre transgene or the interferon-inducible Mx-Cre transgene was used to flip the transgene into the correct orientation. Depending on whether Cre was expressed at high/sustained levels (Vav-Cre) or at lower/transient levels (Mx-Cre), recombination took place predominantly within or between tandemly repeated transgenes yielding different copy numbers and expression of mutant versus wild-type Jak2 with mRNA ratios of ~0.6 for Vav-Cre and ~1.0 for Mx-Cre. The Vav-Cre;FF (flip-flop) mice had normal hemoglobin and WBC levels but increased platelets. In contrast, the Mx-Cre;FF mice had variable but significant increases in WBCs and platelets and increased hemoglobin (170-210 g/L) and low plasma Epo levels. Skoda and co-workers concluded that lower expression of Jak2V617F favoured an ET-like phenotype whereas higher expression favored a PV-like phenotype. Subsequently, reports of two transgenic Jak2V617F models were published where-in mouse or human Jak2V617F was expressed from an H-2Kb or Vav promoter. Here, there were significant differences in the phenotype and penetrance between founder mice (Table 1). However, in both models mice developed MPNs with variable degrees of polycythemia and thrombocytosis, extramedullary hematopoiesis, splenomegaly and Epo-independent erythroid colony (EEC) formation. Mice with lower relative levels of mutant Jak2 expression tended towards an ET-like phenotype with predominant thrombocytosis. These data support a correlation between level of Jak2V617F expression and MPN phenotype.

Founder transgenic mice with constitutive expression of dysregulated TKs (such as BCR-ABL1 and Jak2V617F) show marked phenotype variability. This may be related to different transgene insertion sites and/or to deleterious effects of transgene expression during embryogenesis, the consequence of which is to select for decreased transgene expression in survivors.

To circumvent these problems four recent Jak2V617F models used “knock-in” approaches whereby the mutation was introduced into the normal Jak2 locus, so that the mutant Jak2 would be expressed physiologically. In two models, mutant Jak2 expression was further conditionally activated or regulated by Cre-lox recombination (Table 1). Mice in all four models developed MPN phenotypes. In the model from Golam Mohi and colleagues, a mouse Jak2V617F allele was expressed after Mx-Cre-mediated recombination. Heterozygous and homozygous transgenic mice developed an MPN with polycythemia and thrombocytosis with a more marked phenotype in homozygotes. In the model described by Tony Green and co-workers, mice expressing a conditional human Jak2V617F allele developed predominantly an ET-like phenotype with thrombocytosis and moderate polycythemia but not splenomegaly or myelofibrosis. The model reported by Jean-Luc Villeval and colleagues expressed a knock-in mouse Jak2V617F allele constitutively; these mice developed a severe MPN phenotype with polycythemia, thrombocytosis, splenomegaly and myelofibrosis.

Most recently, Ebert and colleagues reported the phenotype of a similar constitutively expressed murine Jak2V617F knock-in allele, where mice developed fatal MPN with polycythemia and splenomegaly but lacking thrombocytosis and myelofibrosis. The MPN was transferred to secondary recipients by transplantation of stem (Lin-/Sca-/Kit+, LSK) cells, but not by committed progenitors.

What can we learn from these diverse mouse models of aberrant Jak2-expression? First, although considerable data suggest that one or more mutation(s) may antedate the Jak2V617F mutation in persons with MPNs, the high prevalence of one or more MPN phenotypes in the knock-in transgenic models coupled with the polyclonal MPN observed in the retroviral models strongly suggests that Jak2V617F can initiate and sustain MPN in mice. Whether this conclusion applies to humans is unknown.

Second, one of the most interesting questions is how one genetic lesion, Jak2V617F, can cause diverse MPN phenotypes. Is dose the answer? For example, in humans homozygosity for Jak2V617F occurs exclusively in PV, and not in ET. In these models, there is support for the concept that expression of Jak2V617F at levels similar to or higher than endogenous Jak2 is associated with erythrocytosis whereas lower expression levels favor thrombocytosis. In the most recent and more “physiological” knock-in models, Jak2V617F expression is at levels equal to endogenous Jak2. In the models of Green and Villeval mice developed polycythemia, albeit to different extents. However, the model reported by Mohi does not fit this paradigm: Jak2V617F expression was (unexpectedly) only about half that of Jak2 allele yet mice developed polycythemia. Homozygosity increased platelets further (Table 1).

There are various possible reasons for these phenotype differences including divergence between human and mouse Jak2 and variability in mouse strains (which influences the MPN phenotype in retroviral models). Lastly, we should recall that these mice differ from humans with MPNs in that the mice lack a normal population of hematopoietic stem cells and are wholly dependent on Jak2V617F-associated hematopoiesis for blood cell production. As always, we can learn much from mouse models but they rarely replicate precisely the disease in humans. In fact, the diversity of MPNs in humans with a seemingly canonical Jak2 mutation far exceeds the aforementioned diversity of mouse models. It is well known that
familial MPNs with JAK2 mutation have diverse phenotypes.\(^2\) This must mean factors other than JAK2 operate.

**b) Mouse models of Ph-positive MPNs**

Mouse models of Ph-positive CML are critical for the understanding of functionally relevant cellular and molecular abnormalities and were developed using three approaches: (1) xenotransplants; (2) retroviral transplantation models; and (3) transgenic mice. Although xenotransplants of human CML cells to mice allow us to study and manipulate human cells in vivo, engraftment of human cells in NOD/SCID or similar mouse strains is generally less efficient than transplants using allogeneic or syngeneic murine cells. Moreover, although transplantation of human CML acute phase cells causes acute leukemia in NOD/SCID mice,\(^9\) chronic phase (CP) cells do not cause disease despite obvious engraftment.\(^{3,4}\) In contrast, retroviral expression of BCR-ABL1 in hematopoietic stem and progenitor cells results in versatile mouse models of CML facilitating functional analysis of BCR-ABL1 mutants, identification of critical target genes and cooperating oncogenes and assessment of new therapies.\(^{22-25}\) This topic was reviewed by van Etten.\(^{29}\) In addition, transgenic mouse models were generated that express BCR-ABL1 under the control of different promoters and enhancers. These models shed light on the nature of the CML-initiating cell and CML stem cell biology. Here we update only the transgenic mouse models (P210 BCR-ABL1).

Honda and co-workers crossed Tec promoter-driven BCR-ABL1 transgenic mice with BXH2 mice in a screen for common integration sites and identified a novel cooperating gene, Zfp423.

Coordinate overexpression of BCR-ABL1 and Zfp423 induced acute myeloid and lymphoid (B and T cell) leukemias in mice reminiscent of acute phase.\(^{30}\) These investigators reported a similar outcome by crossing Tec-BCR-ABL1 transgenic mice with Bcl11\(^{47}\) or H2AX\(^{50}\) mice.

Sánchez-García and co-workers generated transgenic mice expressing BCR-ABL1 under the control of a Sca-1 promoter to recapitulate CML as a stem cell disease in mice. Interestingly, embryonic lethality, commonly described previously in BCR-ABL1 transgenic mice, was not reported.\(^{31}\) Within 6-18 months, the transgenic mice developed a CML-like disease characterized by mildly increased WBC, increased neutrophils, splenomegaly and hepatomegaly and ultimately acute leukemia resembling the acute phase of CML in humans. The authors reported that cross-breeding of two founder lines shortened development of this phenotype to 1-2 months. BCR-ABL1 expression was confined to the Sca-1\(^{+}\) cell population including hematopoietic stem cells (HSCs). However, the fact that a significant proportion of mice developed solid cancers (10% lung adenocarcinoma, 5% liver cancer, 2% gastrointestinal stromal cancers and others) shows that Sca-1-driven oncogene expression in non-hematopoietic cells may hamper some aspects of transgenic mouse modeling.

Koschmieder and co-workers generated BCR-ABL1 expressing mouse strains using the murine stem cell leukemia gene (SCL)\(^{5}\) enhancer.\(^{35}\) After inter-crossing SCLtTA and TRE-BCR-ABL1 mice to generate double-transgenic SCLtTA/BCR-ABL1 mice, BCR-ABL1 expression was induced by tetracycline withdrawal. These mice developed a CML-like disease within 30 to 120 days (increased WBC and neutrophils, splenomegaly and granulocyte infiltration of the liver, gut and lung); these features were reversible and re-inducible. These data suggest that developing a CML phenotype requires sustained BCR-ABL1 expression. Furthermore, Schemionek and co-workers from the same group showed that this CP-CML is transplantable into congenic mice.\(^{34}\) The LSK population, but not the Lin\(-/\)Sca1\(^{-}\)/Kit\(^{+}\) progenitor or the granulocyte compartments, contains the CML-initiating cell. BCR-ABL1 reduces long-term repopulating (LT-) HSC numbers in the bone marrow and induces their differentiation. Spleen LT-HSC numbers are not reduced. Further experiments showed that the leukemia-inducing potential of BCR-ABL1 positive bone marrow is compromised by serial transplantation. These data suggest that BCR-ABL1 decreases the fraction of self-renewing stem cells in the bone marrow. Interestingly, this is very similar to a recently developed knock-in mouse model for JAK2\(^{6,7}\) which showed reduced leukemia stem cell (LSK) cell numbers and impaired repopulation potential in the JAK2\(^{6,7}\)-positive mice.\(^{36}\) This parallel reinforces important similarities of the JAK2\(^{6,7}\) and BCR-ABL1 oncogenes in vivo. The CML-like disease in transplant recipients is reversible by stopping BCR-ABL1 expression and by giving imatinib. However, CML-initiating cells survive independently of BCR-ABL1 expression and give rise to recurrent CML when the BCR-ABL1 is re-expressed or imatinib is discontinued.

Zhang and colleagues\(^{36}\) were able to confirm these results. Using quantitative competitive repopulation assays they showed that the frequency of functional HSCs was reduced substantially in BCR-ABL1-positive mice compared to controls. Moreover, although one in 6 Fli3\(^{CD150/CDS48}\) LSK cells possessed repopulating activity, only one in 80 cells caused leukemia in transplanted mice. This may reflect the presence of BCR-ABL1-negative stem cells and thus allows the study of BCR-ABL1- positive and -negative hematopoiesis in the same mouse.

This group provided evidence for significant in vivo activity of the combination of imatinib and the nuclear histone deacetylase (HDAC) inhibitors LAQ824 or LBH589.\(^{3}\) BCR-ABL1-transgenic mice treated with imatinib and LBH589 had prolonged leukemia-free survival after discontinuing treatment compared with mice treated with either drug alone. Expression of one of the putative targets, MCL-1, was strongly inhibited by combination therapy, suggesting a role for MCL-1-inhibition in controlling or killing CML stem cells. Transgenic mice may be valuable to identify molecular target that may prove clinically useful. For example, Perrotti and co-workers showed that RNA binding proteins hRNA-A1, K, and –E2 are increased in BCR-ABL1 positive transgenic mice and that this results in SET activation and suppression of protein phosphatase 2A (PP2A).\(^{36,38}\) These authors reported that the PP2A activator, FTY-720, induces apoptosis of BCR-ABL1 positive LSK cells in SCLtTA/BCR-ABL1 mice.\(^{39}\) This work is described in greater detail later in this paper.

Finally, Sengupta and co-workers showed that BCR-ABL1-positive spleen cells can transplant CML-like disease. These cells have decreased adhesion and increased migration compared to BCR-ABL1 negative cells.\(^{30}\) By crossing BCR-ABL1 transgenic mice with Rac2\(^{2}\) mice these investigators showed that Rac2 is critical for BCR-ABL1-induced disease and for the proliferation and survival of leukemia stem cells (LSC).\(^{40}\) Together with retroviral transplantation and as xeno-
Table 1. Comparison of transgenic JAK2<sup>V617F</sup> mouse models of MPN.

<table>
<thead>
<tr>
<th>Lab</th>
<th>JAK2 source/Expression</th>
<th>Strategy</th>
<th>Phenotype and Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skoda</td>
<td>Human/Conditional Tg</td>
<td>Human JAK2&lt;sup&gt;V617F&lt;/sup&gt; cDNA Tg containing 5’ promoter and exons 1-12, with JAK2&lt;sup&gt;V617F&lt;/sup&gt; mutation in an inverted cDNA (exons 13-25) flanked by loxP sites. Recombination by Vav-Cre (sustained/high level Cre) or Mx-Cre + plpC (transient/moderate Cre). Vav-Cre flipped ~80% of cDNAs into sense orientation with Tg copy # of ~2.0, while Mx-Cre flipped ~70% with ~5.7 Tg copies.</td>
<td>By QRT-PCR using species-specific primers, Tg: endogenous JAK2 was ~0.8 (Vav-Cre) and 1.0 (Mx-Cre). At 20 wks, Vav-Cre/Ff (flip-flop) mice had normal Hgb and leukocytes but predominant thrombocytosis (plts 2000-4000 x 10&lt;sup&gt;9&lt;/sup&gt;/L), while at 20 wks post-plpC, Mx-Cre/Ff mice had variable but significant increases in WBC and plts, accompanied by an increased Hgb (17-21 g/dL) and suppressed Epo levels. Both strains developed EMH, splenomegaly, and MF, but neither had EEC. Conclusion: lower expression of JAK2&lt;sup&gt;V617F&lt;/sup&gt; favors an ET-like phenotype, while higher levels favors a PV-like phenotype with expansion of erythroid cell mass. MPN phenotype from Mx-Cre/Ff transferred by BMIT. (Two founders with a non-flxed BAC Tg died before breeding, while a second conditional FF Tg founder had no phenotype upon Mx-Cre induction.)</td>
<td>(12)</td>
</tr>
<tr>
<td>Shimoda</td>
<td>Mouse/Constitutive Tg</td>
<td>Mouse JAK2&lt;sup&gt;V617F&lt;/sup&gt; cDNA Tg driven by the H-2Kb promoter. Two founders (lines 1 and 2), both born at expected Mendelian ratio. Background: mixed B6;DBA→B6.</td>
<td>By QRT-PCR, line 1 expressed 0.45x as much mutant JAK2 as endogenous (diploid) wt JAK2, and line 2 expressed ~1.35x as much as endogenous. Line 1 (incomplete penetrance): 19% had modest polycythemia (Hgb 18-20g/dL), 35% had thrombocytosis (1400-3000 x 10&lt;sup&gt;9&lt;/sup&gt;/L), and a correlation between higher Tg expression and PV-like phenotype. Line 2: uniform and extreme neutrophilia (WBC 100-400 x 10&lt;sup&gt;9&lt;/sup&gt;/L) and thrombocytosis (2000-5000 x 10&lt;sup&gt;9&lt;/sup&gt;/L) from one month, with anemia (Hgb 9-10 g/dL) unaccompanied by MF. Leukocytosis and thrombocytosis declined w/ time while anemia persisted, coincident w/increased MF. Both lines had EMH, splenomegaly, and EEC.</td>
<td>(13)</td>
</tr>
<tr>
<td>Zhao</td>
<td>Human/Constitutive Tg</td>
<td>Human JAK2&lt;sup&gt;V617F&lt;/sup&gt; cDNA Tg driven by the Vav promoter. Three founders generated, two Tg lines maintained (A and B). Background: mixed B6;DBA→B6, F0-F6. Tg copy number ~13 for A and ~2 for B, single chromosome integration site for each.</td>
<td>By QRT-PCR with species-specific primers, mutant JAK2 transcript level was ≤1/16th that of endogenous/wt mouse JAK2, even for line A. Line A developed prominent thrombocytosis (plts 1200-5000 x 10&lt;sup&gt;9&lt;/sup&gt;/L) and modest leukocytosis and erythrocytosis (mean Hgb 18.1), with EMH, splenomegaly, EEC, and moderate MF. Line B had a much milder phenotype, with modest thrombocytosis (mean 1300 x 10&lt;sup&gt;9&lt;/sup&gt;/L) and splenomegaly and no EEC or MF.</td>
<td>(14)</td>
</tr>
<tr>
<td>Mohi</td>
<td>Mouse/Conditional KI</td>
<td>Targeting vector with a loxP-flanked cassette containing mouse Jak2 cDNA (exons 13-24)-polyA-transcription stop, followed by genomic Jak2 exons 15-17 (with V617F mutation in e15), was knocked into the Jak2 locus between exons 12 and 17. Background: mixed B6;129Sv.</td>
<td>Hetero- or homozygous Mx-Cre;KI mice were induced w/plpC. By direct sequencing of QRT-PCR products (TG ratio), mutant allele expressed at ~0.53x the level of wt Jak2. Phenotype at 12 wks post-plpC was 100% penetrant; both heterozygous and homozygous Mx-Cre;Jak2&lt;sup&gt;V617F&lt;/sup&gt; mice had polycythemia (mean Hgb 22 and ~18 g/dL, respectively), but leukocytosis (mean 28 and ~57 x 10&lt;sup&gt;9&lt;/sup&gt;/L) and thrombocytosis (mean 1700 and 3000 x 10&lt;sup&gt;9&lt;/sup&gt;/L) were more marked in homozygotes, as was splenomegaly, EEC frequency, and degree of MF. Polycythemia phenotype was transplantable by BMIT.</td>
<td>(15)</td>
</tr>
<tr>
<td>Green</td>
<td>Human/Conditional KI</td>
<td>Knocked in a human JAK2&lt;sup&gt;V617F&lt;/sup&gt; cDNA Tg, proceeded by a floxed pGK neo pA cassette, into the murine Jak2 exon 2 (containing AUG) locus. Background: mixed B6;129Sv/SvEvBrd.</td>
<td>6 week-old Mx-Cre;Jak2&lt;sup&gt;V617F&lt;/sup&gt; /+ mice were induced with plpC. Species-specific QRT-PCR for human and mouse Jak2 found equivalent expression. plpC-treated mice developed a chronic MPN characterized by moderately increased hematocrit (~18 g/dL es. 16 control) and platelet counts (~1700 x 10&lt;sup&gt;9&lt;/sup&gt;/L es. 1300 control), and EECs, but no splenomegaly or MF. Plasma Epo levels normal. MPN phenotype transferred by BMIT.</td>
<td>(10,35)</td>
</tr>
<tr>
<td>Vileval</td>
<td>Mouse/Constitutive KI</td>
<td>Knocked in a mouse Jak2 exon13 containing the V617F mutation into the Jak2 locus, removed the FRT-flanked neo cassette by crossing to FLP transgenic mice. Background: mixed B6;129Sv.</td>
<td>Mice developed MPN at 5 months of age, characterized by marked polycythemia (Hct 71±3.6%), leukocytosis (WBC 79±11 x 10&lt;sup&gt;9&lt;/sup&gt;/L) and thrombocytosis (4400±700 x 10&lt;sup&gt;9&lt;/sup&gt;/L). By allele-specific QRT-PCR, there were equal amounts of wild-type and mutant Jak2 mRNA expressed. Mice also exhibited splenomegaly, EECs, age-related MF. MPN phenotype was transferred to recipients by BMIT.</td>
<td>(11)</td>
</tr>
<tr>
<td>Ebert</td>
<td>Mouse/Constitutive KI</td>
<td>Knocked in an inverted mouse Jak2 exon 13 containing the V617F mutation into the Jak2 locus and recombined and inverted the exon in the germline. The parental allele was null and could not be homoygosed. Background: B6 backcrossed.</td>
<td>Mice developed fatal MPN by 8 weeks of age that was 100% penetrant and fatal with median survival of 146 days, characterized by polycythemia and splenomegaly but without thrombocytosis or EEC. Level of expression of mutant Jak2 allele not determined. MPN phenotype transferred by transplantation of stem (LSK) cells.</td>
<td>(16)</td>
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</table>

BAC, bacterial artificial chromosome; BMIT, bone marrow transplantation; EEC, endogenous (Epo-independent) erythroid colonies; EMH, extramedullary hematopoiesis; KI, knock-in; MF, myelofibrosis; QRT-PCR, quantitative real-time polymerase chain reaction; Tg, transgene
transplant models, transgenic mouse models can be exploited in studies of the CML phenotype, of CML stem cell biology, and of new therapeutic strategies.

(c) Role of TET2 in the Ph-negative MPNs

Why is it difficult to identify pre-JAK2V617F events in MPNs? It is quite likely that even if these events are important they have subtle or no clinical consequence by themselves. Shortly after the JAK2V617F mutation was described studies of clonality of hematopoietic cells suggested the existence of pre-JAK2V617F mutations, at least in some people.41-43

The first pre-JAK2V617F event described was deletion of the long arm of chromosome 20 (del[20q]). In some patients with MPNs del(20q) seemed to precede the JAK2V617F mutation. In others this sequence was reversed.20,44 Thus del(20q) appeared independent and was unlikely to predispose to JAK2V617F-mutation. Other data from persons with MPNs and JAK2V617F mutation whose disease transformed to blastic phase (BP) gave an intriguing picture: leukemia cells from one-half of the cases were JAK2V617F-negative.45,46 These data argue for the existence of molecular lesions that might predispose to JAK2V617F mutations in MPN and/or to other JAK2V617F independent acute myeloid leukemias.

In 2009, mutations in TET2 and ASXL1 were identified in various myeloid neoplasias, including in around 10% of patients with MPNs.46-49 TET2, like other members of the TET family enzymes, presumably has an important function in DNA demethylation. It has recently been shown that the murine Tet proteins catalyze the conversion of 5-methylcytosine to 5-hydroxymethylcytosine on DNA.50 Thus TET2 mutations are thought to alter hematopoietic stem cell (HSC) functions and myeloid development via epigenetic modifications. These mutations were ideal pre-JAK2V617F candidate events. Indeed, studies of clonality in appropriate patients with typical MPNs showed that most cells had both mutations but that some cells had a TET2 mutation without a JAK2 mutation44-47 suggesting that the TET2 mutation was antecedent. In one case, the TET2 mutant clone acquired JAK2 and MPL mutations in distinct sub-clones.51 Is this the whole story? Unfortunately, no. Data from other patients with MPNs show a JAK2 mutation preceding the TET2 mutation.44,52,53 Other patients with MPNs had clones with either a JAK2 or TET2 mutation but not both.44 Similar studies of ASXL1 mutations have not been published but the same complexity is expected. What if people with these mutations develop transformation to blastic phase?5 In some instances, blast phase cells with TET2 defects had no TET2 mutation detected before transformation.51,54

In contrast, in 2 of 3 cases of MPN with ASXL1 mutations after transformation the mutation was also detected before transformation.51,54 This seeming discordance with TET2 should be tempered as TET2 mutations were detected in 10-20% of de novo cases of acute myelogenous leukemia (AML) and MPNs undergoing transformation to blastic phase.51

It is, therefore, possible that TET2 mutations, but also ASXL1 and del(20q), have different roles in initiating MPNs and causing AML alone or are associated with other molecular abnormalities.51,54

What of familial MPNs? The first studies of TET2 mutations in familial cases reported no inherited mutations.52 However, a recent report described sisters with a germline mutation of TET2.44 One presented with a JAK2V617F-positive PV; the second was normal. This is the first evidence that TET2 mutations can be present in hematopoietic cells with no clinical consequence for decades. These data suggest no or only a very subtle effect on hematopoiesis of TET2 mutations. It could give a slight proliferative advan-

Table 2. Current trials of targeted therapies for Ph-negative MPNs.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
<th>Phase</th>
<th>Disease</th>
<th>Efficacy</th>
<th>Toxicity</th>
<th>Ref</th>
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<tbody>
<tr>
<td>INCBI8424</td>
<td>JAK2</td>
<td>III</td>
<td>MF</td>
<td>Spleenoregal Symptoms</td>
<td>Anemia</td>
<td>Thrombocytopenia</td>
</tr>
<tr>
<td>TG101348</td>
<td>JAK2</td>
<td>II</td>
<td>MF</td>
<td>Spleenoregal Symptoms</td>
<td>Anemia</td>
<td>Thrombocytopenia</td>
</tr>
<tr>
<td>SB1518</td>
<td>JAK2</td>
<td>II</td>
<td>MF</td>
<td>Spleenoregal Symptoms</td>
<td>Gastrointestinal</td>
<td>(117)</td>
</tr>
<tr>
<td>CEP701</td>
<td>JAK2</td>
<td>II</td>
<td>MF</td>
<td>Spleenoregal Symptoms</td>
<td>Gastrointestinal</td>
<td>(118,119)</td>
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<tr>
<td>RAD-001</td>
<td>mTOR</td>
<td>II</td>
<td>MF</td>
<td>Spleenoregal Symptoms</td>
<td>Anemia</td>
<td>Thrombocytopenia</td>
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<tr>
<td>Pomalidomide</td>
<td>IMID</td>
<td>III</td>
<td>MF</td>
<td>Anemia</td>
<td>Minimal</td>
<td>(122)</td>
</tr>
<tr>
<td>Pegylated Interferon Alpha-2a</td>
<td>Biological</td>
<td>III</td>
<td>PV/ET</td>
<td>Erythrocytosis Thrombocytopenia Symptoms</td>
<td>Myelosuppression Depression</td>
<td>(123,124)</td>
</tr>
</tbody>
</table>
tage to HSCs by causing a few “extra” mitoses, which could prepare the way for subsequent oncogenic mutations. This is reminiscent of the “fertile ground” hypothesis proposed for the JAK2 46/1 haplotype to explain the predisposition to acquire a JAK2(V617F) MPN.31-35 Intriguingly, this haplotype is the only marker that we can reasonably call a “pre-JAK2(V617F) condition” to date, although most individuals who carry it will never develop any JAK2(V617F) MPN.

(d) What molecular changes in the CML stem cells are critical for leukemia initiation or progression?

First and second generation TKI given to chronic phase (CP) CML patients substantially reduce the number of leukemia cells in the blood and bone marrow with improved survival. However, the story of the rationally-designed TKIs (imatinib, nilotinib, dasatinib and bosutinib) seems to have reached an ‘impasse’.31 For example, TKI-based therapies are largely ineffective in blast phase (BP) CML. More importantly, they are apparently unable to eradicate CML leukemia stem cells (LSCs). This raises several important questions; (1) Is BCR-ABL1 expression and/or activity essential to maintain the LSC reservoir in CP and BP? (2) Does BP result from genetic or epigenetic events that occur in LSCs or in more committed cells like granulocyte-macrophage progenitor (GMP) cells; and (3) what role, if any, does BCR-ABL1 play in blastic transformation?

Molecular events critical for the CML LSC

Considerable data indicate that TKI do not kill quiescent Ph-positive LSCs (defined as lineage-negative CD34+/CD38−/CD90+ cells capable of self-renewal and of inducing transplantable leukemia in mice). Consequently, there is agreement that BCR-ABL1 expression and/or activity is not essential for self-renewal and survival of the CML LSC.82

Several observations in this context are of interest: (1) increased genomic instability correlating with levels of reactive oxygen species (ROS) in quiescent and proliferating CML LSCs and progenitors is associated with BCR-ABL1 expression;31 (2) enhanced activation of the β-catenin and hedgehog pathways46;48 is important for CML LSC survival and self-renewal;31;40;42 (3) treatment with the PP2A activator FT7720 decreases the number of Ph-positive but not of normal quiescent HSCs;43 (4) activity of FoxO transcription factors also appears important for maintaining CML LSC quiescence;71 (5) ALOX5-regulated MSR1 gene might be critical for maintaining CML LSCs; (6) BM1-1 expression decreases bone marrow homing and increases proliferation and maturation of leukemia Lin−/Sca1−/Kit− HSCs;72 (7) deficient RAC2 expression decreases survival of CML LSCs;71 (8) loss of BCL6 expression results in decreased self-renewal of BCR-ABL1-transduced bone marrow cells;74 and (9) oxygen levels in the bone marrow microenvironment may also be responsible for the quiescence and TKI-resistance of CML LSCs.75;76 These diverse data suggest that CML LSC behavior is controlled by different factors. The various pieces of this puzzle need to be molecularly and functionally inter-connected and could then form the basis for new therapeutic interventions.

Molecular events critical for the CML blastic phase granulocyte/macrophage progenitor

Lymphoblastic transformation may be characterized by non-random genetic aberrations (involving CDKN2A/B and IKZF1) and by expression of the activation induced deaminase (AID) mutator enzyme.77;78 In contrast, myeloid blastic transformation seems correlated with increased BCR-ABL1 expression and kinase activity in CD34+ BP progenitors.82 BCR-ABL1 dose and kinase-dependent loss of PP2A activity and ROS induced genomic instability appear critical for the epigenetic and genetic heterogeneity characteristic of myeloid transformation.82;83 Increased BCR-ABL1 activity also appears essential for the differentiation-arrested phenotype of granulocyte/macrophage progenitors (GMP) in BP through alteration of the hnrNP-E2-CEBPα-miR-S28 network.82 There is a concomitant decrease in PTEN that is BCR-ABL1-dependent which accelerates development of a BP phenotype in mice.82 Interestingly, it seems that higher levels of BCR-ABL1 are necessary for the non-hypoxic induction of the hypoxia-inducible factor-1α (HIF-1α) that, in turn, seems to be required by BP progenitors to tolerate enhanced BCR-ABL1 signaling and to exhibit TKI-resistance in vivo.82

The real role of BCR-ABL1 is controversial both in the regulation of survival and in the acquisition of a stem cell phenotype (e.g. self-renewal) by the GMPs. It has been reported that BP GMPs act as LSCs and that acquisition of self-renewal is dependent, at least in part, on BCR-ABL1-dependent induction of β-catenin expression/activity.84;85 However, some recent data suggest that BCR-ABL1 activity is actually dispensable for the β-catenin-dependent self-renewal of BP GMPs86;87;88 and that aberrant modulation of other pathways (e.g. sonic hedgehog, HES1) may also contribute but do not, per se, confer “stemness” to BCR-ABL1-positive GMPs.89;90 Likewise, recent reports indicate that survival of BP progenitors might occur in a BCR-ABL1-independent manner and involve aberrant expression of pro-survival factors (e.g. BCL2, BCL-XL, MCL1,88;91 despite the fact that a plethora of evidence accumulated during the past two decades showed a strict dependence between BCR-ABL1 activity and proliferation/survival signals in CD34+ BP progenitors.81 It was recently reported that targeting BCR-ABL1 expression in the lineage-negative Sca1− cell compartment induces a CML-like MPD that in 70% of mice progresses to BP-like disease with lymphoid or myeloid features.81

These apparent incongruities might depend on the existence in BP of BCR-ABL1-positive progenitors which are capable of originating or maintaining a BP phenotype and that, for still unknown reasons, are not “oncogene addicted” and, therefore, intrinsically resistant both in vitro and in vivo to TKI treatment. Other data support this hypothesis and perhaps explain the paradoxical short- but not long-term BCR-ABL1-dependence of BP CD34+ progenitors in

Table 3. Potential strategies to target CML LSC.

<table>
<thead>
<tr>
<th>LSC apoptosis</th>
<th>Microenvironment/self-renewal/quiescence/differentiation</th>
<th>Immune therapy</th>
</tr>
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<tbody>
<tr>
<td>BMS-214662</td>
<td>CXCR4 inhibitors</td>
<td>RISCT (127,128)</td>
</tr>
<tr>
<td>HDAC inhibitors*</td>
<td>Hedgehog pathway inhibitors80</td>
<td>Vaccines82;86</td>
</tr>
<tr>
<td>Proteasome inhibitors*</td>
<td>WNT pathway inhibition</td>
<td></td>
</tr>
<tr>
<td>Autophagy inhibitors*</td>
<td>Cytokines e.g. G-CSF126-128</td>
<td></td>
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Ph-negative and Ph-positive MPNs

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TKI-treated BP patients. For example, in vitro clonogenic or survival assays performed with CD34+ BP progenitors exposed to different TKIs never show complete inhibition of leukemic cell growth/survival, and other oncogenic tyrosine kinases (e.g. JAK2, LYN) may regulate and/or overcome BCR-ABL1 expression or activity.\(^{91,92}\) Interestingly, inhibition of the activity of the tumor suppressor PP2A plays a pivotal role in controlling BCR-ABL1 positive stem and progenitor cell survival because it impairs not only BCR-ABL1 but also JAK2, LYN and β-catenin activities when pharmacologically or molecularly reactivated.\(^{93}\)

(f) What is the role of the second generation TKI in front-line management of patients with CML in first chronic phase?

For patients with CML in CP the introduction of imatinib mesylate in 1998 was an important therapeutic milestone. About 65% of patients treated with imatinib achieve and maintain for seven or more years a complete cytogenetic response (CCyR) and their survival is substantially longer than that achievable with any previous therapies. The adverse events attributable to imatinib are relatively mild and usually manageable.\(^{94,96}\)

Conversely this means that one-third of CP patients cannot tolerate imatinib or have a leukemia that proves to be resistant to the drug.\(^{97}\) The best characterized mechanism of resistance is the acquisition of mutations in the BCR-ABL1 kinase domain (KD), of which the T315I mutation is the leading example,\(^ {98}\) although the majority of CP patients resistant to imatinib have no identifiable KD mutation.

Efforts have, therefore, been made to improve the results achieved with the original TKI. Dasatinib and nilotinib may now replace imatinib as first-line therapy on the basis of their efficacy in patients with CML in CP who had failed imatinib. The third new TKI, bosutinib, is not yet licensed but is now in a phase III clinical trial, initial results of which will be reported shortly. In the short term, all three drugs seem valuable for patients who have failed imatinib; about 40-50% of these patients achieve a CCyR.\(^ {99,100}\)

The initial results of the ENESTnd trial in which nilotinib was compared prospectively with standard dose imatinib, and of the DASISION study comparing dasatinib with imatinib, were reported recently.\(^ {101,102}\) Both studies were designed for patients with previously untreated CML in CP. Dasatinib and nilotinib both proved better than imatinib. The principal efficacy observations were a higher rate of incidence of CCyR at 12 months, which was achieved at a faster rate, and a reduced rate of progression to advanced phase following 18 months of follow up for the nilotinib study and 12 months for the dasatinib study.

All three drugs of the newer TKI have specific side effects. Thus dasatinib has been associated with pleural effusions, nilotinib with biochemical changes in liver function and bosutinib with gastrointestinal effects, particularly diarrhea. None of these drugs is active for patients in whom the Ph-positive clone consists predominantly of cells with a T315I mutation. Preliminary data suggest that a new multi-kinase inhibitor, ponatinib (previously AP24534), may be able to overcome resistance attributable to the T315I.\(^ {103,104}\)

There are still a number of unresolved issues in the management of CML in CP. For example, (a) it is not yet clear whether the clinician should leave treatment unchanged in a patient who achieves a complete cytogenetic response but no major molecular response; (b) it is not yet clear whether the second generation TKIs should be used for all newly diagnosed patients or whether it would be expedient to retain imatinib as initial therapy in some cases; (c) it would be very valuable if one could reliably predict responses in individual patients and design appropriate therapy for those who develop resistance to existing TKIs; and most importantly, (d) we need a therapeutic strategy (other than allogeneic stem cell transplantation) designed to ‘cure’ CML (see below).

(f) How can we target CML stem cells in responders to TKIs?

CML is recognized as a paradigm for cancer stem cells. Although TKIs have dramatically altered the history of chronic phase CML these drugs are expensive and unavailable to most people with CML who live outside North America or Western Europe. Side effects are substantial and drug-adherence studies suggest overall compliance levels of about 70 percent at two years, with levels of compliance strongly correlated to disease response.\(^ {105,106}\)

CML cells that originate from a CML stem cell follow a near normal differentiation hierarchy despite the fact that they originate from a leukemia stem cell (LSC). Therapy with TKIs targets the more mature BCR-ABL+ and results in and causes a rapid decline in the numbers of leukemia cells in the blood and bone marrow.\(^ {106,107}\) This is followed by a much slower reduction in CML progenitor cell numbers over several years. Thereafter, it is unclear whether the slope continues very slowly downwards with cure predicted by 17-20 years\(^ {107}\) or whether disease levels reach a plateau, presumably because at least some CML LSCs are resistant to killing by TKIs. Experimental evidence shows that CML LSCs survive despite continued exposure to TKIs was shown in vitro and in vivo.\(^ {108,109}\)

Major efforts are underway to apply systems biology approaches to compare normal HSC with CML LSC with the goal of finding novel targets that will allow selective eradication of LSCs without harming normal HSCs.\(^ {110,111}\) Many investigators have already begun pre-clinical work to target LSCs (Table 2). These approaches can be broadly categorized in 5 ways (Table 3): (1) drugs/strategies that induce apoptosis of quiescent LSCs in a selective or non-selective manner; (2) drugs/strategies that address micro-environmental LSC interactions and affect quiescence, self-renewal and/or differentiation; and (3) drugs/strategies focused on immune therapy.

In this rapidly developing field it is difficult to see where the most important advances will occur. Whilst others are focusing on optimizing vaccinations designed to eliminate LSC, the groups of Tessa Holyoake and Ravi Bhatia have studied approaches that target quiescent LSCs directly or aim to drive LSCs into cell-cycle to sensitize them to TKIs. Some of these approaches have been or will be in clinical trials.\(^ {112,113}\)

Autophagy is a cellular lysosomal degradation pathway essential for the regulation of cell survival and death. One of the key regulators of autophagy is mTOR. In normal HSCs, nutrients and growth factors signal via PI3K/AKT/mTOR and keep autophagy suppressed. This pathway is mimicked by BCR-ABL1 signaling in CML LSCs. In normal HSCs, growth factor deprivation results in inhibition of mTOR and induces autophagy. Similarly, when CML LSCs are exposed to TKIs there is potent inhibition of BCR-ABL1 signaling, which induces autophagy. We recently reported that
in CML LSCs this induced autophagy provides a survival mechanism that, at least in part, explains resistance to TKIs. Autophagy inhibitors are being developed but none is currently available for clinical trials. Chloroquine is a potent inhibitor of autophagy. We hypothesized that combining a TKI with chloroquine would shift the balance between apoptosis and induced autophagy in favor of enhanced apoptosis of CML LSCs. This was shown in vitro and in animal models and is the basis of a randomized clinical trial comparing imatinib with or without hydroxychloroquine in chronic phase CML patients achieving a major cytogenetic response.

(e) New therapies for Ph-negative MPNs?

The evolving understanding of the molecular pathogenesis of Ph-negative MPNs has led to an unprecedented variety of different therapies. JAK2-inhibitor testing began testing in 2007. These trials focused on persons with MPN-associated myelofibrosis (post-PV MF, post-ET MF and fibrotic MF). These drugs are now being studied with persons with PV and ET unresponsive to other therapies. Alternative therapeutic strategies against different targets are also being developed.

MPN-associated myelofibrosis

There is no FDA-approved therapy for MPN-associated myelofibrosis. Current therapies are off-label indications palliative for anemia and/or reduction in splenomegaly. Allotransplants cure some persons with MPN-associated myelofibrosis but are used in fewer than 5% of affected persons.

JAK2 Inhibitors

Patterns of efficacy and toxicity of JAK2-inhibitors are increasingly overlapping. Drugs in the most advanced phases of testing include INCB18424, TG101348, SB1518 and CEP-701 (Table 2). All rapidly reduce splenomegaly and improve myelofibrosis-associated constitutional symptoms, without significantly changing molecular or histological features of the disease.

Toxicities include anemia and thrombocytopenia. Gastrointestinal toxicity is common amongst drugs inhibiting FLT3.

Alternative targets

The immune-modulatory drug pomalidomide produces durable anemia responses without bone marrow suppression or neuropathy. The nuclear histone deacetylase inhibitor LBH589 (Novartis) has promising activity in myelofibrosis with responses in splenomegaly and anemia. A phase II study is ongoing in the USA. The mTOR (mammalian target of rapamycin)-inhibitor RAD001 has activity similar to JAK2-inhibitors (decreased splenomegaly and improved constitutional symptoms).

Polycythemia vera and essential thrombocytosis

Therapy of PV and ET relies on short-term anti-platelet drugs and selected cytoreduction for persons at high risk of vascular events (hydroxycarbamide, anagrelide and alkylating agents). In PV/ET we still need new drugs (1) for persons resistant or intolerant of current drugs to prevent vascular events; and (2) to delay progression to myelofibrosis and blastic transformation.

JAK-inhibitors

Data on therapy with INCB18424 were reported in 73 PV/ET patients failing hydroxy carbamide. There was a complete response in 94% of patients with PV and 61% of patients with ET. No patients had a vascular event on-study. Splenomegaly and constitutional symptoms improved similar to data in MPN-associated myelofibrosis. Results from a phase II trial with CEP701 in 59 subjects were less impressive with several on-study vascular events and substantial gastrointestinal toxicity.

Pegylated interferon alpha-2a

Conventional interferon alpha has been used in persons with ET and PV but it is not well-tolerated. Two recent trials show better tolerance, efficacy in preventing vascular events and the potential to produce molecular and histological responses in persons with high-risk ET and PV. This might decrease the likelihood of progression to myelofibrosis and blastic transformation. An international phase III trial comparing hydroxyurea and pegylated interferon alpha-2a is planned.

Participants

The paper follows a small meeting in Natchez, Mississippi, USA immediately after the 2009 American Society of Hematology myeloproliferative neoplasms (MPNs). The organisers invited some discussants to briefly review selected topics addressed at the Natchez meeting and add relevant new data presented or published in 2010. The following persons participated in the meeting: Ralph Arlinghaus (USA), Tiziano Barbui (Italy), Francois Delhommee (Paris), Connie Eves (Canada), Alan Gewirtz (USA), John Goldman (UK), Anthony Green (UK), Oliver Hantschel (Austria), Rudiger Hehlmann (Germany), Tessa Holyoke (UK), Brian Huntly (UK), Caterina Jameson (USA), Steffen Koschmieder (Germany), Robert Krailand (Austria), Ruben Mesa (US), Tanja Mughal (UK), Vivian Oehler (USA), Heike Pahl (Germany), Amimesh Pardanani (USA), Emmanuel Passegue (USA), Davide Perotti (USA), Josef Pechal (USA), Giuseppe Saggio (Italy), Richard Silver (USA), Tomasz Skorski (USA), Simonetta Sovrani (Italy), Moshe Talpaz (USA), Richard van Etten (USA), Alessandro Vannucchi (Italy), Jean Luc Villeval (France).

Authorship and Disclosures

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