Clonal heterogeneity as a driver of disease variability in the evolution of myeloproliferative neoplasms

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Myeloproliferative neoplasms (MPNs) are clonal hematological diseases in which cells of the myelo-erythroid lineage are overproduced and patients are predisposed to leukemic transformation. Hematopoietic stem cells are the suspected disease-initiating cells, and these cells must acquire a clonal advantage relative to nonmutant hematopoietic stem cells to perpetuate disease. In 2005, several groups identified a single gain-of-function point mutation in \textit{JAK2} that associated with the majority of MPNs, and subsequent studies have led to a comprehensive understanding of the mutational landscape in MPNs. However, confusion still exists as to how a single genetic aberration can be associated with multiple distinct disease entities. Many explanations have been proposed, including \textit{JAK2V617F} homozygosity, individual patient heterogeneity, and the differential regulation of downstream \textit{JAK2} signaling pathways. Several groups have made knock-in mouse models expressing \textit{JAK2V617F} and have observed divergent phenotypes, each recapitulating some aspects of disease. Intriguingly, most of these models do not observe a strong hematopoietic stem cell self-renewal advantage compared with wild-type littermate controls, raising the question of how a clonal advantage is established in patients with MPNs. This review summarizes the current molecular understanding of MPNs and the diversity of disease phenotypes and proposes that the increased proliferation induced by \textit{JAK2V617F} applies a selection pressure on the mutant clone that results in highly diverse clonal evolution in individuals.

Introduction

Multipotent hematopoietic stem cells (HSCs) are a rare subpopulation of cells in the bone marrow that sit atop a complex and tightly controlled hierarchical production process that eventually leads to the formation of all types of mature blood cells [1]. To maintain a homeostatic balance, a given HSC must on average produce one daughter cell that contributes to active hematopoiesis and another daughter cell that retains the HSC maintenance capacity of the parent cell [2]. Perturbations in a single HSC that drive an amplification or depletion of particular mature blood cell types can typically be tolerated unless that HSC also acquires a clonal advantage [3,4].

The concept of heterogeneity in HSCs, and indeed cancers in general, has emerged in the context of the enormous genomic knowledge gathered in recent years, and therefore, it is important to consider the molecular pathogenesis of disease from a cell biological perspective. This review details the process of stem cell subversion through the lens of myeloproliferative neoplasms, a set of chronic, clonal disorders that are an excellent model system for studying cancer progression.

Hematopoietic stem cell heterogeneity

Even in the earliest stages of studies in HSC biology, marked heterogeneity in self-renewal of pluripotent hematopoietic
cells was observed [5,6]. More recently, differences in proliferation, cell cycle status, self-renewal durability and types of mature cells produced have all been uncovered (reviewed in [7]). These aspects could be explained by diverse intrinsically determined, (epi-) genetic differentiation programs of individual stem cells [8], by variances in the stem cell micro-environment [9], or by an HSC organizational structure governed by stochastic fate choices [10]. Recently, single-cell transplantation studies of highly purified HSC populations revealed variability in white blood cell output and self-renewal durability, identifying at least four distinct HSC subtypes, only two of which displayed durable self-renewal properties [11]. The first of these exhibited a relative deficiency in lymphoid cells, and the latter, a balanced proportion of mature cell production. Importantly, secondary transplantation studies using clonally derived bone marrow suspensions or single purified HSCs revealed highly similar repopulation patterns, suggesting that distinct HSC characteristics are intrinsically determined [11,12]. These studies were further bolstered by the prospective enrichment of lineage-biased HSC subtypes using alternative flow cytometric isolation strategies [13–16]. Even more recently, a reclassification of HSC subtypes has been proposed based on reconstitution time periods [17].

Tumor heterogeneity

Genetic, morphologic, phenotypic, clinical, and cell surface marker heterogeneity has been observed among cells from patients with both solid tumors and hematologic malignancies (i.e., intertumor variation) [18–21]. Most recently, clonal heterogeneity has been also observed within a tumor from a single patient (i.e., intratumor variation) [18,20,22,23]. Traditionally, two main theories have been proposed to explain tumor heterogeneity. The first, the “stochastic model,” suggests that all cells in the clonal hierarchy are equally susceptible to malignant transformation. Such tumor cells would be biologically equivalent, but would behave variably because of further genetic, epigenetic, or environmental changes. Heterogeneity would thus be explained by the existence of multiple cells within a tumor possessing the ability to drive a malignant clone [24]. The second, the “hierarchical model,” suggests that tumorigenic mutations occur only in primitive cells (or drive differentiated cells backward to the primitive state) and cause malignancy through an aberrant differentiation cascade. In this model, cancer stem cells (CSCs) are biologically distinct from other tumor cells and are the only ones that can give rise to a new tumor (also referred to as a tumor-initiating cell). Evidence for this latter theory is strongest in acute leukemias, in which a rare subpopulation of CSCs is present at the apex of the clonal hierarchy (reviewed in [25]). Because stem cells persist for a long period, there would be a greater opportunity for mutations to accumulate in these cells compared with short-lived mature cells, and fewer mutations would be required to maintain self-renewal [26]. Recently, it has been suggested that genetic diversity, epigenetic modifications and the tumor micro-environment can, together or separately, influence stemness and thereby influence tumor progression [27]. As a result of acquiring advantageous mutations, several malignant subclones could be established. In some clones, there would be a strict hierarchy in which only a few CSCs would exist, whereas in other clones, the majority of cells would retain self-renewal properties, thereby eliminating the hierarchical component of the tumor.

Hierarchical cancer stem cell model in hematologic malignancies

The suggestion that malignant clones mimic normal cell biology was accompanied by the supposition that CSCs represent a rare subpopulation of stem cells. Subsequent evidence in melanoma [28] and glioblastoma [29] has challenged this theory’s applicability to all cancers. Moreover, no strong consensus exists about the rarity of tumor-initiating cells, because higher frequencies of cells that are capable of driving tumor growth have been observed in various lymphoma and leukemia transgenic mouse models [30]. However, many cancers have substantial evidence of a cancer stem cell model, including breast cancer [31], brain cancers [32,33], and colon cancer [34,35]. In myeloid malignancies, CSCs are suggested to be responsible for the relapse after therapy that removes the bulk tumor, but spares the tumor-initiating CSCs [36,37]. Although the CSC concept cannot be universally applied, a substantial number of tumor types are associated with a hierarchical organization (reviewed in [3]).

Myeloproliferative neoplasms as a model to track clonal evolution

Myeloproliferative neoplasms (MPNs) are clonal hematologic diseases in which cells of the myelo-erythroid lineage are overproduced. They include BCR-ABL-positive chronic myelogenous leukemia, as well as the three classic BCR-ABL-negative MPNs known as polycythemia vera (PV), essential thrombocytopenia (ET), and myelofibrosis (MF) (reviewed in [38]). MPNs have been found to derive from the outgrowth of a single HSC or early myeloid progenitor that acquires a somatic mutation(s) [39–41]. Mutant cells clonally expand as the result of developing a hypersensitivity to, or independence from, cytokines that regulate proliferation, differentiation, and/or survival. Formal evidence for an acquired genetic lesion in BCR-ABL-negative MPNs came in 2005, when several research groups discovered a single-point mutation in JAK2, called JAK2V617F, which is associated with the vast majority of cases of PV, ET, and MF [42–45]. Subsequent discoveries in non-JAK2V617F MPNs identified somatic mutations in JAK2 exon 12 in patients with PV [46,47] and activating mutations of the thrombopoietin receptor gene MPL in patients with ET and MF [48,49]; most recently, two studies reported that the majority of patients with nonmutated JAK2 carry a mutation in
Table 1. Most commonly affected genes in myeloproliferative neoplasms

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Frequency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAK2V617F</td>
<td>Cytokine signaling</td>
<td>&gt;95% PV, 50–60% ET/MF</td>
<td>[42–45]</td>
</tr>
<tr>
<td>JAK2 (exon 12)</td>
<td>Cytokine signaling</td>
<td>1–2% PV</td>
<td>[47]</td>
</tr>
<tr>
<td>CALR</td>
<td>Cytokine signaling</td>
<td>25–33% ET/MF</td>
<td>[50,51]</td>
</tr>
<tr>
<td>MPL</td>
<td>Cytokine signaling</td>
<td>3–5% ET, 8–11% MF</td>
<td>[48,66]</td>
</tr>
<tr>
<td>SH2B3 (LNK)*</td>
<td>Cytokine signaling</td>
<td>2–6%</td>
<td>[59,66]</td>
</tr>
<tr>
<td>CBL*</td>
<td>Cytokine signaling</td>
<td>5–10%, most common in MF</td>
<td>[60,62,66]</td>
</tr>
<tr>
<td>SF3B1</td>
<td>Splicing</td>
<td>3–5%</td>
<td>[61,66]</td>
</tr>
<tr>
<td>SRSF2</td>
<td>Splicing</td>
<td>3–5%</td>
<td>[61,66]</td>
</tr>
<tr>
<td>U2AF1</td>
<td>Splicing</td>
<td>3–5%</td>
<td>[61,66]</td>
</tr>
<tr>
<td>ZRSR2</td>
<td>Splicing</td>
<td>1–2%</td>
<td>[61]</td>
</tr>
<tr>
<td>TET2*</td>
<td>Epigenetic modifier</td>
<td>5–17%</td>
<td>[57,58,62,66]</td>
</tr>
<tr>
<td>DNMT3A</td>
<td>Epigenetic modifier</td>
<td>6–8%, most common in MF</td>
<td>[57,58,62,66]</td>
</tr>
<tr>
<td>IDH1/IDH2*</td>
<td>Epigenetic modifier</td>
<td>3–15%, most common in MF</td>
<td>[57,58,62,66]</td>
</tr>
<tr>
<td>ASXL1*</td>
<td>Epigenetic modifier</td>
<td>4–22%, most common in MF</td>
<td>[57,58,62,66]</td>
</tr>
<tr>
<td>EZH2*</td>
<td>Epigenetic modifier</td>
<td>8% most common in MF</td>
<td>[57,58,66]</td>
</tr>
<tr>
<td>p53*</td>
<td>Transcription factor</td>
<td>1–2%</td>
<td>[57,58,66]</td>
</tr>
<tr>
<td>IKZF1*</td>
<td>Transcription factor</td>
<td>Rare</td>
<td>[57,58,66]</td>
</tr>
<tr>
<td>FOXP1</td>
<td>Transcription factor</td>
<td>Rare</td>
<td>[57,58,66]</td>
</tr>
<tr>
<td>ETv6</td>
<td>Transcription factor</td>
<td>Rare</td>
<td>[57,58,66]</td>
</tr>
<tr>
<td>CUX1</td>
<td>Transcription factor</td>
<td>Rare</td>
<td>[57,58,66]</td>
</tr>
<tr>
<td>NF-E2</td>
<td>Transcription factor</td>
<td>Rare</td>
<td>[57,58,66]</td>
</tr>
<tr>
<td>RUNX1*</td>
<td>Transcription factor</td>
<td>Rare</td>
<td>[57,58,66]</td>
</tr>
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ET = essential thrombocythemia; MF = myelofibrosis; PV = polycythemia vera.
*Mutations associated with disease progression.

JAK2V617F mutation is the most prevalent mutation in MPNs and is present in the majority of PV patients (>95%) and in a significant proportion of ET and MF patients (50–60%) [42–45]. The kinase activity is dysregulated because of loss of the negative regulation of the pseudo-kinase (JH2) domain, resulting in independence and/or hypersensitivity of hematopoietic cells to growth factors and cytokines [56]. JAK2V617F is present in cells of the hematopoietic compartment, but not in germline DNA [42–45], and serves as a powerful diagnostic tool to discriminate PV, ET, and MF from reactive causes of erythrocytosis or thrombocytosis.

Several additional mutations have been subsequently identified in MPNs, including genes involved in cytokine signaling, splicing machinery, transcription factors, and epigenetic modifiers (reviewed in [57,58]). Mutated genes that also target the JAK-STAT cytokine signaling pathway have been found in exon 12 of JAK2 and in the myeloproliferative leukemia virus oncogene (MPL), and these are mutually exclusive with JAK2V617F. Co-occurring mutations involved in the JAK-STAT pathway have also been identified, including loss-of-function mutations in the adaptor protein Sh2b3 (LNK), somatic mutations in suppressors of cytokine signaling genes, and mutations in other negative regulators of cytokine signaling, including CBL (Casitas B-cell lymphoma) [59,60]. RNA splicing mutations (including SF3B1, SRSF2, and U2AF1) [61] and transcription factor mutations (including IKZF1, FOXP1, ETv6, CUX1, NF-E2, RUNX1, and p53) have also been found to have roles in MPN pathogenesis. The final class of mutations, suspected of having a role in driving the clonal advantage in MPNs, comprises those involving epigenetic regulation. Such epigenetic modifiers include TET2, IDH1/2, DNMT3A, ASXL1, and EZH2 [62].
Disease progression, illustrated most clearly by transformation to acute myeloid leukemia (AML), does not seem to be explained by individual genetic aberrations, although some mutated genes are frequently present in secondary AML (e.g., \textit{IDH1/2}, \textit{TET2}, \textit{ASXL1}, \textit{LNK}, \textit{TP53}, \textit{EZH2}, \textit{IKZF1}, and \textit{RUNX1}). However, in MF patients, only a handful of these mutations are associated with a poor prognosis, including \textit{IDH1/2}, \textit{TET2}, \textit{ASXL1}, and \textit{TP53} [63–65]. The latter mutations are almost always detected in both the primary MPN and the secondary AML, with a prevalence of around 20%. Interestingly, the prevalence of \textit{ASXL1} represents 22% of MF patients, but only 5% of PV and ET patients [66]. The frequencies of \textit{TET2} and \textit{IDH1/2} mutations in secondary AML are around 25% and 10%, respectively [63] and an even higher frequency (45.5%) of TP53 mutations has been observed in post-MPN AML [67].

Despite the discovery of this large range of mutations, about one-third of ET and MF patients lack an identifiable mutation. Recently, however, two groups discovered a somatic mutation in the endoplasmic reticulum chaperone \textit{CALR} in the majority of \textit{JAK2} mutation-negative patients [50,51] that was found to be mutually exclusive with \textit{JAK2V617F} and \textit{MPL}. \textit{CALR}-mutant ET and MF patients had a milder phenotype compared with \textit{JAK2V617F}-mutant ET and MF patients and had significantly longer overall survival [50]. \textit{CALR} can now be used as an additional diagnostic tool to discriminate several MPN subtypes [68], providing a diagnosis in patients previously difficult to classify.

Such a finding has major implications for previous MPN research that focused mostly on \textit{JAK2}-mutant compared with \textit{JAK2}-nonmutant patients. Such studies had not accounted for \textit{CALR} status, and many “mutation-negative” patients are likely to be \textit{CALR} positive; thus, these old data sets could hold substantial new information (as performed recently by Rampal et al. [69]).

**Characterization of different myeloproliferative neoplasm subtypes**

Despite this extensive molecular knowledge, it remains puzzling why this collection of mutations results in three distinct disease entities. Current approaches to classification of MPN subtypes have also met with confusion for several reasons (Fig. 1): First, MPN subtypes are diagnosed mainly on the basis of binary decisions regarding values that are continuous (e.g., hemoglobin, hematocrit, or red cell mass). This makes patients with borderline values difficult to diagnose, as the disease progresses and is further complicated by the fact that a raised hematocrit does not always predict an increased red cell mass and vice versa. This confusion is illustrated particularly well in patients who present with isolated thrombocytosis and increased bone marrow reticulin fibrosis. If these patients lack other ET or MF characteristics, they can be diagnosed with neither ET nor MF, although the clinical features of a MPN are clearly present [70].

Second, the existence of three distinct disease entities could itself be questioned: Is primary myelofibrosis (PMF) a separate disease when it could be viewed as an accelerated phase of previously undiagnosed ET or PV [38,70,71]? This concept is supported by the fact that PMF is clinically distinguishable from post-ET MF and that the frequencies of \textit{JAK2V617F}, \textit{CALR}, and \textit{MPL} mutations are similar in ET and PMF. Also, patients who are diagnosed with PMF might...
have had thrombocytosis for many years before diagnosis. This has major implications for MPN diagnoses, because PV and ET could then be considered as chronic phase MPNs and MF as accelerated phase MPNs, preceded by another (undiagnosed) MPN [38].

A third reason for confusion in our understanding of MPN classification is the large variability observed in disease phenotypes in mouse knock-in models (reviewed in [72,73]). Heterozygous mouse JAK2V617F models have a PV-like phenotype [74–76] with wide variations in hematocrit, platelet, and white blood cell values, whereas a human V617F knock-in mouse model has an ET-like phenotype, where mice progress to PV or MF [77]. The effect on blood cell types produced differs among the four models, varying from a mild increase in some lineages [76,77] to a more than 80-fold increase in erythroid and myeloid precursors [75]. Also, the effect on stem and progenitor cells is not consistent among these mouse models; some models have an increased primitive cell fraction [74,78,79], and other models have a decrease or no change in stem and progenitor cell fraction [55,76,77].

**Gene dosage**

Along with the discovery of JAK2V617F, it was noted that although most MPN patients had cells with a heterozygous mutation, a small proportion, mostly PV patients, also possessed cells that were homozygous for JAK2V617F. Classically, loss of the wild-type allele in tumor suppressors is a common mechanism of developing homozygosity; however, for JAK2V617F, it typically results from acquired uniparental disomy. Uniparental disomy is a mitotic recombination and duplication of the mutant allele, and the progression from a heterozygous to a homozygous state in MPNs occurs in the distal part of chromosome 9p (UPD9p) [44]. Uniparental disomy was originally reported in 30% of PV patients, but was rare in ET [42–45], and homozygous JAK2V617F erythroid clones were observed in PV patients, but rarely in ET patients [80]. These data suggest that gene dosage is an important distinguishing factor in different MPNs and that although most MPN patients had cells with a heterozygous JAK2V617F mutation, a small proportion, mostly PV patients, also possessed cells that were homozygous for JAK2V617F. Classically, loss of the wild-type allele in tumor suppressors is a common mechanism of developing homozygosity; however, for JAK2V617F, it typically results from acquired uniparental disomy. Uniparental disomy is a mitotic recombination and duplication of the mutant allele, and the progression from a heterozygous to a homozygous state in MPNs occurs in the distal part of chromosome 9p (UPD9p) [44]. Uniparental disomy was originally reported in 30% of PV patients, but was rare in ET [42–45], and homozygous JAK2V617F erythroid clones were observed in PV patients, but rarely in ET patients [80]. These data suggest that gene dosage is an important distinguishing factor in different MPNs, whereby JAK2V617F homozygosity results in a PV phenotype. However, a recent study reported that homozygous clones are detectable in approximately 50% of ET patients and are undetectable in some PV patients [53]. These discordant observations make it difficult to draw conclusions about the causal role of homozygosity of JAK2V617F in PV patients. Nevertheless, the expression of mutant JAK2 at a specific level in various cell types seems to play a role in the different disease phenotypes [81], although gene dosage cannot be the sole contributor to the distinct disease phenotypes.

**Downstream JAK2 signaling**

The dysregulated kinase activity caused by the JAK2V617F mutation leads to independence and/or hypersensitivity of hematopoietic cells to growth factors and cytokines [56]. Several signaling cascades are activated as a result of mutated JAK2, including the STAT5, MAPK, RAS, and PI3K pathways [43,44,82,83]. In addition to gene dosage, these different signaling pathways could be another important player in driving phenotypically distinct disorders.

A powerful approach to analysis of distinct MPN phenotypes associated with JAK2V617F involves the use of cells clonally derived from MPN patients that are genotyped for their individual JAK2 mutational status. This approach readily permits the identification of clones with distinct genotypes from within the same patient, thereby avoiding interindividual variations. Using this approach, Chen et al. found that heterozygous mutant erythroid colonies of PV and ET patients exhibit differences in their signaling pathway despite bearing the same single genetic lesion and specifically pointed toward a differential response to STAT1. This work indicated that an ET-like phenotype was accompanied by enhanced STAT1 activity that resulted in an increase in megakaryocytic differentiation and repression of erythroid differentiation. Furthermore, inhibition of STAT1 activity with a dominant negative form of STAT1 in progenitor cells resulted in a switch to a PV-like phenotype with an increase in erythroid colonies. These results suggest that the disease phenotype of individual patients depends on a certain degree of intracellular STAT1 signaling [84].

The role of other signaling moieties downstream of JAK2 has also been examined in relation to JAK2V617F. One study demonstrated that JAK2V617F mutant cells co-expressing the erythropoietin receptor lacked a STAT5-binding site and exhibited decreased malignant cell transformation and tumorigenesis [85], although the role of STAT5 in MPNs could not be totally clarified by this study. Subsequently, crossing a JAK2V617F knock-in mouse strain with a STAT5 knock-out mouse strain revealed that deletion of STAT5 could rescue the PV phenotype induced by JAK2V617F expression [86]. Replacing STAT5 expression by retroviral transduction and transplantation in a STAT5-deficient mouse resulted in re-development of a PV phenotype and blood counts that were comparable to those of the JAK2V617F knock-in mouse with wild-type levels of STAT5. These studies concluded that STAT5 is required for the pathogenesis of PV induced by JAK2V617F [86]. This was corroborated by work that indicated that JAK2V617F mice did not have a PV phenotype after deletion of STAT5 [87]. Together these data suggest that STAT5 might be an interesting drug target, although the impact on normal STAT5 signaling would be a concern.

A final area of intense scrutiny is the role of interferon-α which has been used to treat MPNs for many years [88,89] without a clear idea of the mechanism by which interferon-α operates. Recently, it was reported that in addition to achieving normalization of blood counts, interferon-α also reduces JAK2V617F allelic burden [90,91] and activates the cell cycle machinery in quiescent mutant HSC populations [92,93]. Work in JAK2V617F knock-in mouse models also supports the therapeutic potential of
interferon-α, where the mutant stem and progenitor cells are killed more effectively than their wild-type counterparts [94,95]. This will certainly be an area of great interest in years to come.

**Alternative theories for distinct myeloproliferative neoplasm phenotypes**

In addition to gene dosage and downstream JAK2 signaling differences, other mechanisms have been suggested to play a role in distinct MPN phenotypes. One theory suggests a familial predisposition for the development of MPNs because of additional inherited alleles. Although most MPNs appear to be sporadic, familial MPNs have been widely described and are clinically similar to the sporadic type. In familial cases, inactivation of JAK2V617F, MPL W515L/K, and TET2 is the most recurrent somatic mutation and is present in combination with inherited MPN-predisposition alleles [96–98]. It has been demonstrated that JAK2V617F preferentially appears on a specific JAK2 haplotype, called “46/1” [99], and this appears to be the most important common risk factor for the acquisition of JAK2V617F, resulting in the development of MPNs. Two theories have been proposed to explain this observation: the “hypermutable theory” suggests that 46/1 is genetically more unstable and leads to a faster mutation acquisition, whereas the “fertile ground theory” suggests that there is no difference in acquisition of JAK2V617F, but additional factors on the 46/1 haplotype provide a selective advantage to the JAK2 mutant clone [96].

Another possible explanation for the phenotypic diversity observed among different MPN subtypes focuses on inherited modifiers in individual patients. Single-nucleotide polymorphisms have been analyzed in gene regions that are involved in the JAK-STAT pathway, including the genes JAK2, MPL, EPOR (erythropoietin receptor) and G-CSFR (granulocyte colony-stimulating factor receptor), in large patient cohorts. These studies have identified specific single-nucleotide polymorphisms in JAK2 and only one specific-nucleotide polymorphism in EPOR that are associated with PV, but not with ET or MF, suggesting that host genetic variation might lead to a specific disease phenotype [100].

**Clonal evolution of myeloproliferative neoplasms**

The relationship between the JAK2V617F mutation and the clonal expansion of mutant cells that eventually results in an MPN is poorly understood. One certain requirement for all MPNs is that the initial clone must expand relative to other clones within a patient, and generally, this is thought to occur through the acquisition of a mutation or series of mutations in HSCs that give the mutant clone a self-renewal advantage over time. This process can eventually lead to monoclonal hematopoiesis, which is a well-documented phenomenon in hematologic malignancies [66,101]. The reduced clonal diversity with age may alter the competitive ability of endogenous HSCs, thereby permitting (or restricting) the expansion capabilities of a clone that acquires a JAK2 mutation. Importantly, the clonal evolution of MPNs sometimes involves the progression to a more advanced disease (e.g., JAK2V617F homozygosity, secondary MF, or AML).

**Expansion of homozygous clone**

Typically, JAK2V617F homozygosity has been regarded as a feature of PV, but recent evidence indicates that JAK2V617F homozygosity occurs with similar frequencies in both PV and ET [53]. The prevalence and clonal relationship of homozygous mutant erythroid colonies can be determined by genotyping colonies from JAK2V617F-positive PV and ET patients. When this was performed, homozygous erythroid colonies could be identified in both ET and PV patient samples, but the overt expansion of a dominant homozygous clone was observed only in PV. These data suggest the need for additional non-JAK2 mutations to drive disease progression, a hypothesis that is further supported by the observation in mouse models [55,77,81] and normal individuals [102,103] that JAK2V617F alone is insufficient to cause clonal expansion and disease progression. In humans, this is illustrated by transplantation experiments revealing that human cell engraftment in immunodeficient mice could not be achieved by primitive cells with a low burden of JAK2V617F. Furthermore, primitive cells from high-burden PV patients that were capable of repopulating in xenograft experiments were not able to outcompete normal cells [104].

Several lines of evidence suggest the presence of a pre-JAK2 event (reviewed in [98]). Clonogenic cytogenetic abnormalities have been observed in JAK2V617F-positive MPNs, including deletion of 20q, trisomy 8, and trisomy 9. Additionally, JAK2V617F-positive patients have developed JAK2V617F-negative AML [105,106], which supposes the existence of a pre-existing clone that diverges into a JAK2 mutant and a JAK2 wild-type subclone, the latter of which acquires the leukemogenic event. However, no consensus exists about the molecular identity of the “pre-JAK2-phase,” and this is further confounded by inaccurate JAK2 quantification and X-chromosome inactivation patterns [105].

**Different roles in different cells**

Evidence is mounting that the JAK2V617F mutation affects HSCs differently than progenitors and mature cells [55,79,107]. This is perhaps best understood by asking what the impact of increasing proliferation would be on a cell population that often divides (progenitor cells) compared with its impact on a cell population that is largely quiescent (HSCs). In the mouse, long-term HSCs are estimated to divide just five times throughout adult life, and their cell biology (and maintenance of self-renewal) is adapted to this frequency of cell division [108]. Indeed, recent evidence in single-cell assays suggests that HSC self-renewal capacity is negatively affected by JAK2V617F, but progenitor cells are given an enormous boost in their proliferation capacity [55].
In MPN patients, the JAK2V617F mutation is more prevalent in granulocytes than in a small minority of primitive cells, especially in PV and ET patients [109–112]. HSCs from MF patients, on the other hand, exhibit a higher JAK2V617F mutation frequency [110]. This is mirrored in patients with chronic myeloid leukemia, where HSC self-renewal ability is compromised [113] and BCR-ABL is expressed in only a small proportion of primitive cells [114,115]. This suggests that chronic phase disease is the result of progenitor cells that are benefiting from the proliferative advantage (and causing disease), whereas HSCs themselves are not benefiting.

Moving forward, therefore, it is critical to undertake studies of stem and progenitor cells in purified fractions. Studies that assess lin⁻Sca-1⁺c-Kit⁺ cells in mouse models or CD34⁺CD38⁻ cells in patients must be recognized as assays of progenitor cells (and not HSCs) because these populations are dominated by progenitor cells. To study the impact of individual mutations on HSCs and the implications for establishment, maintenance, and expansion of a clonal population, it is therefore crucial to assess this in highly purified populations.

**JAK2V617F and hematopoietic stem cell biology**

Clonal expansion can occur only when a clone has a survival and/or proliferation advantage compared with other clones in the organism. In malignancies, acquisition of such a clonal advantage is critical for tumor establishment and disease progression. In MPNs, clonal expansion (or lack thereof) is especially interesting at the HSC level, because the balance of self-renewal and differentiation could be used as a therapeutic target similar to successful studies using all-trans-retinoic acid in acute promyelocytic leukemia (reviewed in [116]).

Along these lines, treatment with interferon-α in knock-in mouse models has recently been demonstrated to specifically deplete JAK2V617F-propagating cells, leading to cell cycle activation of long-term mutant HSCs [94,95]. Also, recent evidence has suggested that the MPN-associated adaptor molecule LNK modulates the homeostatic regulation of HSCs [117]. A complete understanding of how the clonal advantage is established and maintained could reveal additional information about the key mechanisms driving the clonal evolution of MPNs. If the establishment of an advantageous clone can be mapped out step by step, therapies could be tested at each stage of the process.

Mouse models are useful to examine relative self-renewal capacity in mutant HSCs because these cells can be isolated at high purities and are from a defined genetic background. Several JAK2V617F knock-in mouse models have been developed, although the effect on stem and progenitor cells is not entirely consistent among these mouse models (reviewed in [72,73]). Serial competitive transplantation experiments are the gold standard for testing relative stem cell activity, and these experiments are ideally carried out using age- and sex-matched wild-type littermate controls. To formally demonstrate a self-renewal advantage (and thereby infer a clonal advantage over nonmutant HSCs in MPN patients), secondary transplantation experiments must be carried out. One knock-in model [74] exhibits a PV-like phenotype with expansion of phenotypically defined stem and progenitor cells in the bone marrow and spleen. In primary transplantation experiments with a 75:25 heterozygous mutant:WT cell ratio, this same model did not display a strong HSC advantage, because donor chimerism in recipients was observed at a 70:30 ratio [118]. Another mouse knock-in model [76,79] demonstrated that JAK2V617F disease-initiating cells are predominantly long-term HSCs and achieve clonal advantage during disease progression. Intriguingly though, when secondary transplantation experiments were performed in this model there, no HSC advantage was observed [76]. Similarly, another knock-in model [94] described an increased production of mutant HSCs in primary transplantsations that was not mirrored in secondary transplantsations. The knock-in models produced by Li et al., using a human JAK2V617F, displayed a mild HSC defect in the heterozygous mouse that was more marked in secondary transplantsations [55,77] and a severe defect in JAK2V617F homozygous mice in primary and secondary transplantsations [55,77,81].

**A proposed model of selective pressure**

Most evidence suggests that JAK2V617F does not, on its own, confer a strong HSC self-renewal advantage, and disease heterogeneity among JAK2-mutant patients is substantial. The easiest explanation for such differences would be the acquisition of specific mutations for specific disease etiologies; however, the cataloguing exercise in several large genomic studies has yet to identify ET-specific or PV-specific collaborators alongside JAK2V617F. We therefore propose that selective pressure to obtain a self-renewal advantage that is imposed on the initiating JAK2V617F-positive clone results in stem and progenitor cell heterogeneity that eventually manifests as disease heterogeneity (Fig. 2). In this model, a single cell acquires the JAK2V617F mutation, and its consequent proliferation advantage creates a clone that grows rapidly, but lacks a long-term self-renewal advantage. Each and every cell of the clone would then be subjected to selective pressure to acquire a self-renewal advantage because of the increased replicative stress. The clone of cells bearing the JAK2V617F mutation would proliferate and differentiate, creating a large population of heterogeneous target cells for additional mutations. The lineage biases (e.g., platelets or erythrocytes) displayed by the distinct disease subtypes would then be derived from the intrinsic bias of the cell that acquires the self-renewal advantage (i.e., the target cell defines the disease). In this manner, it would be possible to develop diverse phenotypes from similar starting cell populations that evolve differently prior to acquiring a clonal advantage. These possibilities...
should be testable in mouse knock-in models, assessing both
the impact of single mutations and the combinatorial effect
doing double or triple mutant stem and progenitor cells.

Conclusions and future directions Much work has been undertaken to further understand the
molecular mechanisms that drive MPN pathogenesis. Character-
ization of the distinct disease subtypes, genetic events
leading to malignant transformation, and the individual
behavior of mutant HSCs are areas of current interest. The
mutational landscape has essentially been established, but
the task of understanding the individual and combinatorial
contributions of each mutation is in its infancy. Existing
data on MPNs should be reconsidered based on the complete
genetic landscape to re-assess questions about different MPN
subtypes and to understand “low-burden” versus “high-
burden” disease. Individual mutations should be related
to disease phenotype, prognosis, and therapeutic effects. Of
particular importance is for old studies of JAK2V617F-negative
MPNs to be viewed through the lens of CALR mutations,
as a substantial portion of “negative” patients would be high-
burden CALR-mutant patients. This should allow categoriza-
tion of disease into low- and high-clonal-burden disease and
give insight into the biology driving clonal expansion.

Despite the discovery of JAK2V617F almost a decade
ago, there exists no good explanation for how distinct dis-
ease entities arise as the result of a single genetic aberration.
Moreover, it is unclear how the recently discovered muta-
tions in CALR result in disease phenotypes similar to those
of MPNs bearing the JAK2V617F mutation. As a result,
diagnosing MPN patients can be difficult, especially in the
case of patients with borderline blood values. The accurate
categorization of MPN subtypes will lead to more appro-
priate and more personalized treatment of MPN patients.

Another difficulty in treating MPN patients is that
although current therapies can slow down the extensive
cell production, the disease itself remains uncured. One the-
ory suggests that mutant tumor stem cells are not suscepti-
able to current therapies, and as a result, the disease-causing
cells remain capable of forming a new advantageous mutant
clonal, leading to eventual relapse. The composition of these
newly formed clones could differ from that of the original
clonal and result in therapy resistance [119].

Much work remains to fully understand disease differ-
ences and the mechanisms that are involved in hematologic
transformation (e.g., to secondary MF or AML). Although
numerous mutations in the JAK-STAT signaling pathway
are mutually exclusive and most common in chronic phase
MPNs, co-occurring mutations (ASXL1 and TET2) with
suspected roles in clonal expansion have also been identi-
cified. The exact role of these genetic aberrations in relation
to disease phenotypes is still unclear, although ASXL1 muta-
tions have been associated with MF [63,120]. With the
vast majority of these mutations now identified, future ef-
forts should be focused on relating these mutations to path-
ogenic mechanisms, clinical outcome, and prognosis.
A final field of interest in MPN research is the cellular process underlying disease progression. Obtaining a specific proliferation and/or survival advantage is critical for tumor establishment and disease progression. The mechanism of acquiring a clonal advantage at the HSC level, however, is still poorly understood. Furthermore, once such an advantage is obtained, HSCs and their progeny are differently affected. For example, activating tyrosine kinase mutations on HSCs appears to negatively affect the self-renewal machinery, but improve the differentiation and proliferation potential of HSCs [55,113,121,122]. The effect of such mutations in progenitor and mature cells, on the other hand, is largely beneficial, as exemplified by JAK2V617F. This has major implications for disease initiation, especially if the proposed model of selective pressure is correct, whereby the heterogeneous cell population created as a result of the first mutation will determine eventual disease subtype based on the lineage biases and differentiation state of the cell that eventually acquires the self-renewal advantage.

Although the search for factors distinguishing PV, ET, and MF should be continued, this should be largely achievable by focusing on the relationship of currently discovered mutations and the clinical outcome and prognosis of patients bearing these genetic lesions. Understanding the evolution of disease and the individual burden of each mutation will help to expand our capacity to diagnose MPNs more correctly and, eventually, to identify more specialized therapies. A good starting point would be to reconsider the vast amount of existing data in the context of the complete molecular landscape of disease with ultimate goal to track clonal evolution in MPNs to identify the key regulators of disease development and progression.

References


