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

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Abstract

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 (HSCs) are the suspected disease initiating cells and these cells must acquire a clonal advantage relative to non-mutant HSCs in order to perpetuate disease. In 2005, several groups identified a single gain-of-function point mutation in 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 JAK2V617F homozygosity, individual patient heterogeneity and the differential regulation of downstream JAK2 signaling pathways. Several groups have made knock-in mouse models expressing JAK2V617F and have observed divergent phenotypes, each recapitulating some aspects of disease. Intriguingly, most of these models do not observe a strong HSC self-renewal advantage compared to WT littermate controls, raising the question of how a clonal advantage is established in MPN patients. This review summarizes the current molecular understanding of MPNs, the diversity of disease phenotypes and proposes that the increased proliferation induced by 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]. In order 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 will detail the process of stem cell subversion through the lens of the myeloproliferative neoplasms, a set of chronic, clonal disorders that are an excellent model system for studying cancer progression.

HSC 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 the 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 microenvironment [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 showed a relative deficiency in lymphoid cells and the latter displayed a balanced proportion of mature cell production. Importantly, secondary transplantation studies using clonally derived bone marrow suspensions or single purified HSCs showed 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 amongst cells from patients with both solid tumors and hematological malignancies (i.e., inter-tumor variation) [18-21]. Most recently, clonal heterogeneity has been also observed within a tumor from a single patient (i.e., intra-tumor 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 due to 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 only occur in primitive cells (or drive differentiated cells backwards 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, where a rare subpopulation of CSCs is present at the apex of the clonal hierarchy (reviewed in [25]). Since stem cells persist for a long period of time there is a greater opportunity for mutations to accumulate in these cells compared to 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 microenvironment 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-renew properties, thereby eliminating the hierarchical component of the tumor.

Hierarchical cancer stem cell model in hematological malignancies

The suggestion that malignant clones mimic normal cell biology was accompanied by the supposition that CSCs represented 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, since higher frequencies of cells that are capable to drive tumor growth have been found 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 relapse following therapy that removes the bulk tumor but spares the tumor initiating CSCs [36, 37]. While the CSC concept cannot be universally applied, a substantial amount 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 hematological diseases in which cells of the myelo-erythroid lineage are overproduced. They include both BCR-ABL positive chronic myelogenous leukemia (CML) as well as the three classical, BCR-ABL negative MPNs known as polycythemia vera (PV), essential thrombocythemia (ET) and myelofibrosis (MF) (reviewed in [38]). MPNs have been shown to derive from the outgrowth of a single HSC or early myeloid

progenitor that acquires 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 associated with the vast majority of PVs, ETs and MFs [42-45]. Subsequent discoveries in non-JAK2V617F MPNs identified somatic mutations in JAK2 exon 12 in PV patients [46, 47], activating mutations of the thrombopoietin receptor gene *MPL* in ET and MF patients [48, 49] and most recently, two studies identified that the majority of patients with non-mutated JAK2 carry a mutation in the endoplasmic reticulum chaperone calreticulin (CALR) [50, 51]. A summary of the most frequently mutated genes is presented in Table 1.

Despite sharing common genetic features, PV, ET and MF are classified as three distinct diseases with distinct clinical phenotypes (reviewed in [38]). Still they all share some clinical features, including a chronic course, the risk of major thrombotic events and the risk of transformation to acute leukemia. In particular, similar characteristics are observed in the early phase of disease, making them difficult to distinguish [52].

MPNs are a useful disease model to understand tumors since they arise form a single cell, readily permit clonal analysis and are chronic diseases, thus facilitating dissection of disease evolution. MPNs are not associated with a differentiation block and therefore permit studies of the earliest stages of malignancy that are inaccessible in other tissues. The discovery of JAK2V617F in 2005 [42-45] has permitted research on the role of gene dosage [53], the role of JAK2V617F in hematological transformation [54] and the specific effect on mutant HSCs compared to their downstream progenitors [55].

Molecular characterization of MPNs

The mutational landscape of MPNs is increasingly well understood. The 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 due to loss of the negative regulation of the pseudo-kinase (JH2) domain and leads to 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 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 (*SOCSs*) genes, and mutations in other negative regulators of cytokine signaling, including Casitas B-cell lymphoma (*CBL*) [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 shown to have roles in MPN pathogenesis. The final class of mutations, suspected for their role in driving the clonal advantage in MPNs are those involving epigenetic regulation. Such epigenetic modifiers include *TET2*, *IDH1/2*, *DNMT3A*, *ASXL1* and *EZH2* [62].

Disease progression, illustrated most clearly by transformation to AML, does not seem to be explained by individual genetic aberrations, although some mutated genes are frequently present in secondary AML (e.g., *IDH1/2*, *TET2*, *ASXL1*, *LNK*, *TP53*, *EZH2*, *IKZF1* and *RUNX1*). However, in MF patients, only a handful of these mutations are associated with a poor prognosis, including *IDH1/2*, *TET2, ASXL1*, and *TP53* [63-65]. These 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 *ASXL1* represents 22% of MF patients, but only 5% in PV and ET patients [66]. The frequencies of *TET2* and *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 lacked an identifiable mutation. Recently, however, two groups discovered a somatic mutation in the endoplasmic reticulum chaperone CALR in the majority of JAK2 mutation negative patients [50, 51] and it was shown to be mutually exclusive with JAK2V617F and MPL. CALR-mutant ET and MF patients had a milder phenotype compared with JAK2V617F-mutant ET and MF patients and showed a significantly longer overall survival [50]. 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 has mostly focused on JAK2-mutant compared to JAK2-non-mutant patients. Such studies had not accounted for CALR status and many “mutation negative” patients are likely to be CALR positive and these old datasets could hold substantial new information (as performed recently by Rampal *et al*. [69]).

Characterization of different MPN subtypes

Despite this extensive molecular knowledge, it remains puzzling why this collection of mutations result in three distinct disease entities. Current approaches to classify MPN subtypes have also met with confusion for several reasons (Figure 1): first, MPN subtypes are diagnosed mainly based on 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 further ET or MF characteristics, they can be diagnosed with neither ET nor MF, although the clinical features of an 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 undistinguishable from post-ET MF and that the frequency of JAK2V617F, CALR, and MPL mutations is similar in ET and PMF. Also, patients that are diagnosed with PMF might have had thrombocytosis for many years before diagnosis. This has major implications for MPN diagnoses, since 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 show a PV-like phenotype [74-76] with wide variations in hematocrit, platelet and white blood cell values whilst a human V617F knock-in mouse model shows an ET-like phenotype where mice progress to PV or MF [77]. The effect on blood cell types produced is different 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 show an increased primitive cell fraction [74, 78, 79] and other models show a decrease or no change in stem and progenitor cell fraction [55, 76, 77].

Gene dosage

Alongside the discovery of JAK2V617F, it was noted that while 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 (UPD). UPD 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]. UPD 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 suggested that gene dosage was an important distinguishing factor in different MPNs, whereby JAK2V617F homozygosity results in a PV phenotype. However, a recent study shows that homozygous clones are detectable in approximately 50% of the 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 analyze distinct MPN phenotypes associated with JAK2V617F is to use clonally-derived cells from MPN patients and genotype for their individual JAK2 mutational status. This approach readily permits the identification of clones with distinct genotypes from within the same patient, thereby avoiding inter-individual variations. Using this approach, Chen *et al*. showed 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 showed that an ET-like phenotype was accompanied by an enhanced STAT1 activity that resulted in an increase of megakaryocytic differentiation and a repression of erythroid differentiation. Furthermore, inhibition of STAT1 activity with a dominant negative form of STAT1 (STAT1DN) in progenitor cells resulted in a switch to a PV-like phenotype with an increase of 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 EPO-receptor lacked a STAT5 binding site and showed decreased malignant cell transformation and tumorigenesis [85] although the role of STAT5 in MPNs could not be totally clarified by this study. Subsequently, by crossing a JAK2V617F knock-in mouse strain with a STAT5 knock-out mouse strain, it was demonstrated 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 a redevelopment of a PV phenotype and blood counts that were comparable to the JAK2V617F knock-in mouse with wild-type levels of STAT5. These results concluded that STAT5 is required for the pathogenesis of PV induced by JAK2V617F [86]. This was corroborated by work that showed JAK2V617F mice did not result 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-alpha (IFN-α) which been used to treat MPNs for many years [88, 89] without a clear idea of the mechanism by which IFN-α operates. Recently, it was shown that in addition to achieving normalization of blood counts, IFN-α also reduced JAK2V617F allelic burden [90, 91] and activated the cell cycle machinery in quiescent mutant HSC populations [92, 93]. Work in JAK2V617F knock-in mouse models also supports the therapeutic potential of IFN-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 MPN 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 due to 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, JAK2V617F, MPL W515L/K and TET2 inactivation are the most recurrent somatic mutations, and are 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 "hypermutability theory" suggests that 46/1 is genetically more unstable and leads to a faster mutation acquisition while 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 amongst different MPN subtypes focuses on inherited modifiers in individual patients. Single nucleotide polymorphisms, or SNPs, have been analyzed in gene regions that are involved in the JAK-STAT pathway, including the genes *JAK2*, *MPL*, EPO-receptor (*EPOR*) and G-CSF-receptor (*GCSFR*), in large patient cohorts. These studies have identified specific SNPs in JAK2 and a single SNP 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 MPNs

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 hematological 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 shows 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 only observed 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 showing 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 out-compete 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 the 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 to 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 impacted 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 compared to a small minority of primitive cells, especially in PV and ET patients [109-112]. HSCs from MF patients, on the other hand, show a higher JAK2V617F mutation frequency [110]. This is mirrored in patients with CML, where HSC self-renewal ability is compromised [113] and BCR-ABL is only is expressed in a small proportion of primitive cells [114, 115]. This suggests that chronic phase disease is the result of progenitor cells that are benefitting from the proliferation advantage (and causing disease), while HSCs themselves are not benefitting.

Moving forward, therefore, it is critical to undertake studies of stem and progenitor cells in purified fractions. Studies which assess $lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells in mouse models or $CD34^{+}CD38^{-}$ cells in patients must be recognized as assays of progenitor cells (and not HSCs) since these populations are dominated by progenitor cells. In order 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 HSC biology

Clonal expansion can only occur when a clone has a survival and/or proliferation advantage compared to 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, since the balance of self-renewal and differentiation could be used as a therapeutic target similar to successful studies using all-trans-retinoic acid (ATRA) in acute promyelocytic leukemia (reviewed in [116])

Along these lines, treatment with IFN-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 since 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 non-mutant HSCs in MPN patients), secondary transplantation experiments must

be carried out. One knock-in model [74] shows a PV-like phenotype with expansion of phenotypically defined stem and progenitor cells in the bone marrow and spleen. In primary transplantation experiments using a 75:25 ratio of heterozygous mutant:WT cells, this same model did not display a strong HSC advantage since 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 was no HSC advantage observed [76]. Similarly, another knock-in model [94] described an increased production of mutant HSCs in primary transplantations that was not mirrored in secondary transplantations. 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 transplantations [55, 77] and a severe defect in JAK2V617F homozygous mice in primary and secondary transplantations [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 amongst 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 (Figure 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 due to 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 as well as the combinatorial effect of double or triple mutant stem and progenitor cells.

Conclusion and future directions

Much work has been undertaken to further understand the molecular mechanisms that drive MPN pathogenesis. Characterization 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" 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, since a substantial portion of "negative" patients would high burden CALR mutant patients. This should allow categorization 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 disease entities arise as the result of a single genetic aberration. Moreover, it is unclear how the recently discovered mutations in CALR result in similar disease phenotypes as 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 characterization of MPN subtypes will lead to a more appropriate and more personalized treatment of MPN patients.

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

Much work remains to fully understand disease differences and the mechanisms that are involved in hematological transformation (e.g., to secondary MF or AML). While 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 identified. The exact role of these genetic aberrations in relation to disease phenotypes is still unclear, although *ASXL1* mutations have been associated with MF [63, 120]. With the vast majority of these mutations now identified, future efforts should be focused on relating these mutations to pathogenic 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.

In conclusion, while the search for distinguishing factors between 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 in order to identify the key regulators of disease development and progression.

Table 1: Most commonly affected genes in myeloproliferative neoplasms

Gene	Function	Frequency	Reference
JAK2^{V617F}	Cytokine Signalling	>95% PV, 50-60% ET/MF	[42-45]
JAK2 (Exon 12)	Cytokine Signalling	1-2% PV	[47]
CALR	Cytokine Signalling	25-33% ET/MF	[50,51]
MPL	Cytokine Signalling	3-5% ET, 8-11% MF	[48, 66]
SH2B3 (LNK)*	Cytokine Signalling	2-6%	[59, 66]
CBL*	Cytokine Signalling	5-10%, most common in MF	[60, 62, 66]
SF3B1	Splicing	3-5%	[61, 66]
SRSF2	Splicing	3-5%	[61, 66]
U2AF1	Splicing	3-5%	[61, 66]
ZRSR2	Splicing	1-2%	[61]
TET2*	Epigenetic Modifier	5-17%	[57-58, 62-63, 66]
DNMT3A	Epigenetic Modifier	6-8%, most common in MF	[57-58, 62, 66]
IDH1/IDH2*	Epigenetic Modifier	3-15%, most common in MF	[57-58, 62-63, 66]
ASXL1*	Epigenetic Modifier	4-22%, most common in MF	[57-58, 62-63, 66]
EZH2*	Epigenetic Modifier	8% most common in MF	[57-58, 66]
P53*	Transcription Factor	1-2%	[57-58, 66]
IKZF1*	Transcription Factor	rare	[57-58, 66]
FOXP1	Transcription Factor	rare	[57-58, 66]
ETV6	Transcription Factor	rare	[57-58, 66]
CUX1	Transcription Factor	rare	[57-58, 66]
NF-E2	Transcription Factor	rare	[57-58, 66]
RUNX1*	Transcription Factor	rare	[57-58, 66]

*** Mutations associated with disease progression**

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Figure

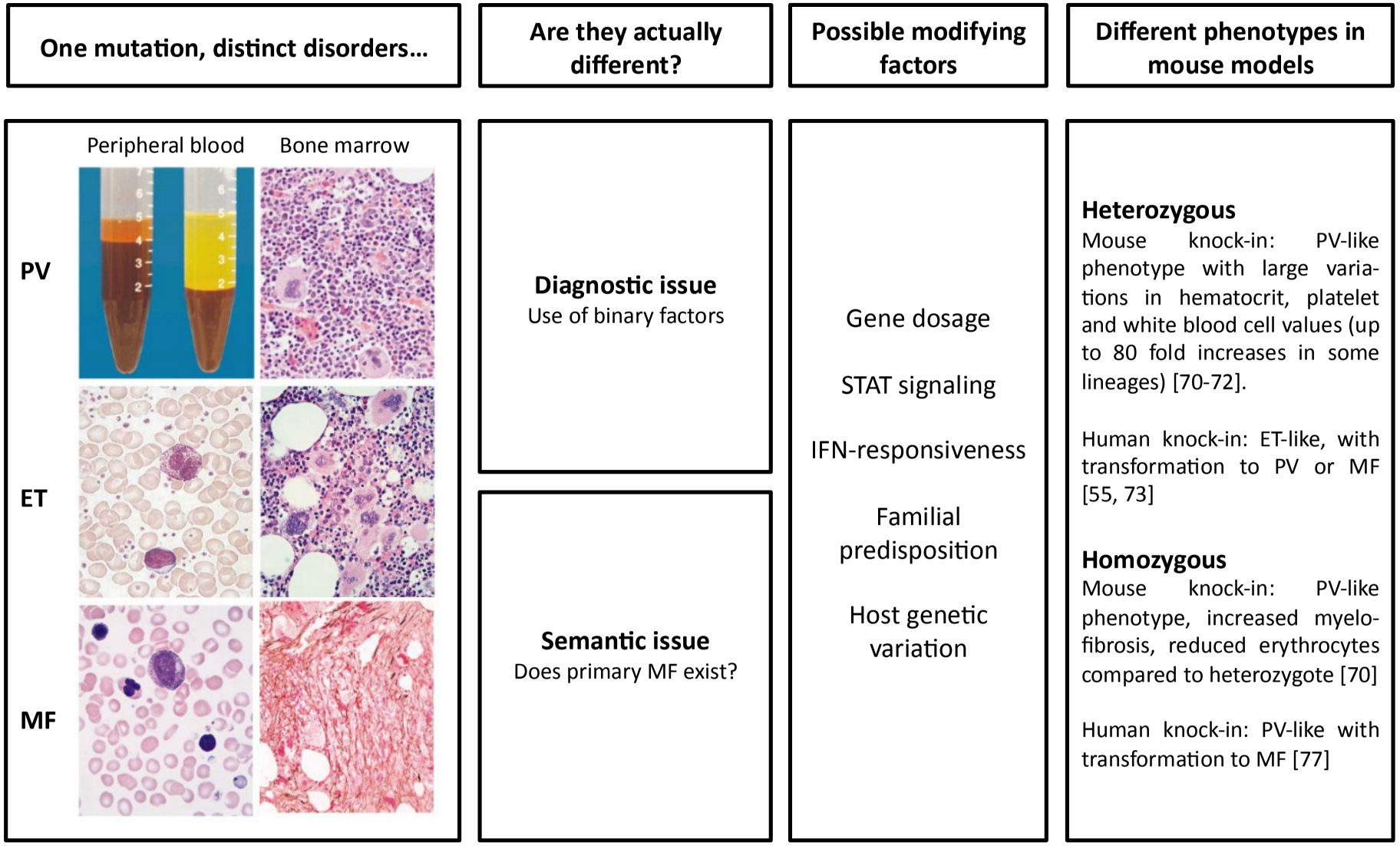

Figure 1. Classification of MPN subtypes. It remains unclear why one mutation is associated with three distinct disease entities. Since diagnosing MPN patients is based on binary decisions of continuous variables (e.g., platelet count) at single time points, patients with borderline values are difficult to classify. Moreover, primary MF may in fact represent the presentation of the accelerated phase of PV or ET. Decisions are further complicated by additional factors that could alter presentation (e.g., gene dosage, STAT signaling, the role of IFN-α, familial predisposition and host genetic variation). This variability is further illustrated by the diverse phenotypes observed in different mouse models of JAK2V617F..

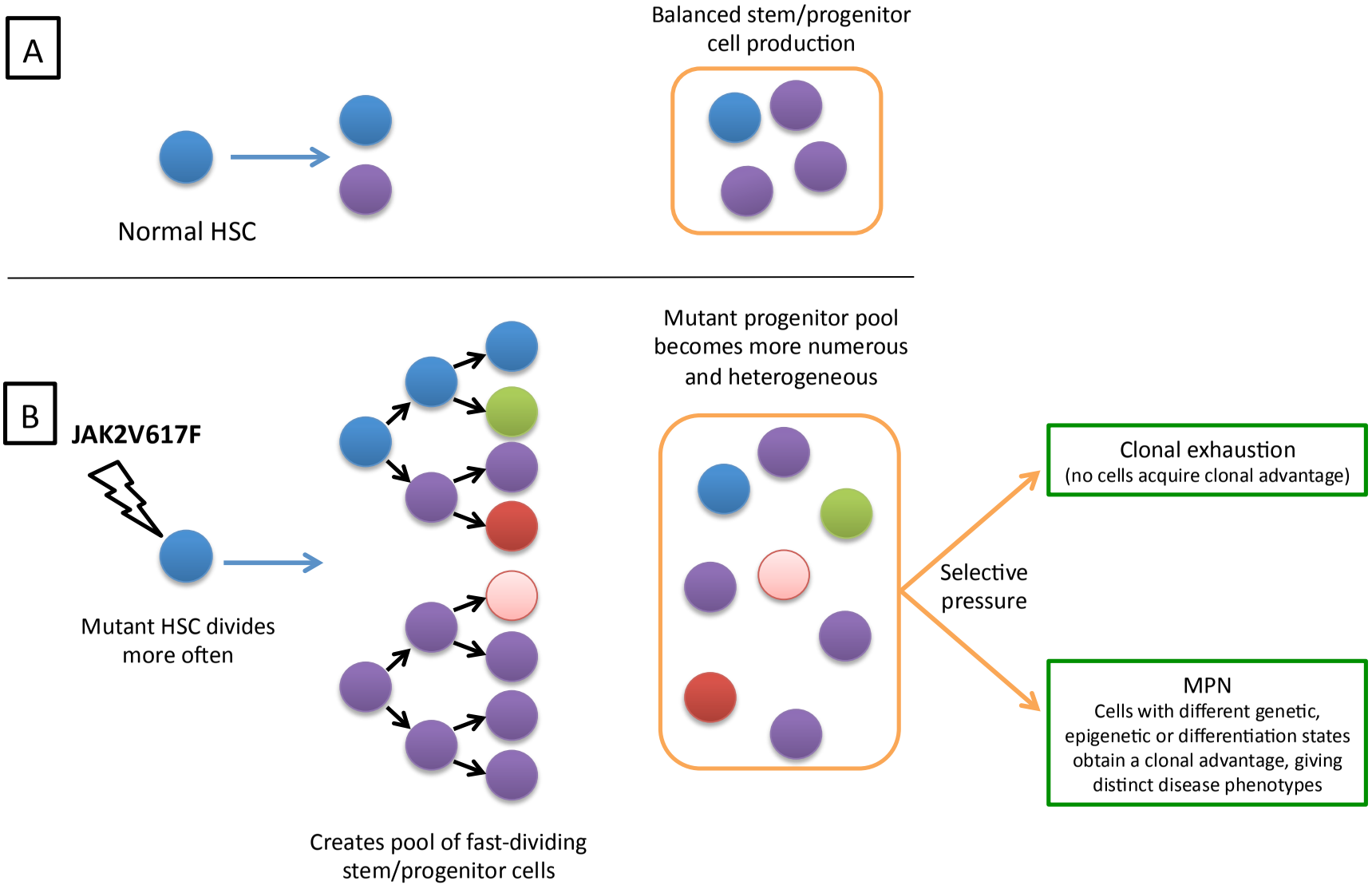

Figure 2. Clonal evolution in MPNs. A) Normal steady state: On average, for every non-mutant HSC which divides, one daughter cell retains HSC potential. Throughout the life of the organism, the HSC number remains approximately constant B) A JAK2V617F mutant HSC divides more frequently. Since the V617F mutation does not confer increased HSC self-renewal, HSC numbers will not increase, however, each HSC will generate more progenitors which will also divide more frequently. Both HSCs and progenitors, therefore will undergo additional replicative stress. Consequent selection pressure to acquire a clonal advantage results in one of the heterogeneous population of cells acquiring an additional mutation that drives higher survival and/or self-renewal. The cell that acquires this mutation need not be an HSC, but rather could be any number of JAK2V617F mutant stem/progenitor cells and the target cell of the second mutation could therefore determine the disease phenotype.