Myeloproliferative Neoplasm Stem Cells

Adam J. Mead[1] and Ann Mullally[2]

[1]MRC Molecular Haematology Unit, MRC Weatherall Institute of Molecular Medicine and NIHR Biomedical Research Centre, University of Oxford, Oxford, UK

[2]Division of Hematology, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

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Correspondence:

amullally@partners.org

1, Blackfan Circle
Karp Building, Room 5.217
Boston, MA 02115

Phone: (617) 355-9002
Fax: (617) 355-9124

OR

adam.mead@imm.ox.ac.uk

MRC Weatherall Institute of Molecular Medicine
University of Oxford
John Radcliffe Hospital
Headington, Oxford. OX3 9DS

Phone: 00-44-1865-222425
Fax: 00-44-1865-222500

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Abstract

Myeloproliferative neoplasms (MPN) arise in the hematopoietic stem cell (HSC) compartment as a result of the acquisition of somatic mutations in a single HSC that provide a selective advantage to mutant HSC over normal HSC and promote myeloid differentiation to engender a myeloproliferative phenotype. This population of somatically mutated HSC, which initiate and sustain MPN, are termed MPN stem cells. In greater than 95% of cases, mutations that drive the development of an MPN phenotype occur in a mutually exclusive manner in one of three genes: *JAK2*, *CALR* or *MPL*. The thrombopoietin receptor, MPL is the key cytokine receptor in MPN development and these mutations all activate MPL-JAK-STAT signaling in MPN stem cells. Despite common biological features, MPN display diverse disease phenotypes as a result of both constitutional and acquired factors that influence MPN stem cells, and likely also as a result of heterogeneity in the HSC in which MPN-initiating mutations arise. As the MPN clone expands it exerts cell-extrinsic effects on components of the bone marrow niche that can favor the survival and expansion of MPN stem cells over normal HSC, further sustaining and driving malignant hematopoiesis. Although developed as targeted therapies for MPN, current JAK2 inhibitors do not preferentially target MPN stem cells and as a result rarely induce molecular remissions in MPN patients. As the understanding of the molecular mechanisms underlying the clonal dominance of MPN stem cells advances, this will help facilitate the development of therapies that preferentially target MPN stem cells over normal HSC.

Introduction

Myeloproliferative neoplasm (MPN) stem cells are defined as a clonal population of rare cells within the bone marrow that harbor an MPN-initiating somatic mutation, are capable of indefinite self-renewal, and that, through a combination of cell-intrinsic and cell-extrinsic effects, undergo clonal expansion. In this review, we will focus on BCR-ABL negative MPN and will highlight five main features of MPN stem cells: (i) MPN disease-initiating somatic mutations, (ii) the effects of MPN somatic mutations on HSC function, (iii) the role of MPN stem cells in the heterogeneity of disease phenotype in MPN, (iv) the MPN stem cell niche and (v) therapeutic targeting of MPN stem cells.

MPN disease-initiating somatic mutations

The molecular basis of MPN has been defined in almost all cases. In greater than 95% of cases of MPN the mutations that drive the development of an MPN phenotype are accounted for by somatic mutations in three genes: *JAK2*, *CALR* or *MPL*, and notably these mutations occur in a mutually exclusive manner[1]. Mutations in *JAK2* and *MPL* occur as gain-of-function point mutations (i.e. JAK2V617F and MPLW515L/K respectively), while the mutations in *CALR* occur as +1 base pair frameshifts in the last coding exon of *CALR*, which result in the generation of a novel C-terminus[1,2]. Recent work indicates that *CALR* mutations confer a neo-morphic function on mutant CALR that results in activation of MPL signaling[3-6]. Additional germ line and somatic mutations in *JAK2* or *MPL* were recently identified in approximately 19% of the so-called “triple negative” MPN cases[7]. Previously, negative regulators of the JAK-STAT signaling pathway, including LNK[8], c-CBL[9,10] and SOCS[11] were also shown to be somatically inactivated at low frequency in MPN, highlighting the primacy of the JAK-STAT signaling pathway in MPN pathogenesis.

Evidence for an MPN disease-initiating role for *JAK2*, *CALR* and *MPL* somatic mutations has been provided by retroviral bone marrow transplant (BMT) assays, where ectopic expression of each mutation *alone* is sufficient to engender MPN in mice[5,6,12], however it is important to note that the MPN is polyclonal in all these models. Recent work using a transgenic mouse model where human JAK2V617F is expressed form the endogenous human *JAK2* promoter, demonstrated that although MPN can be initiated by transplanting a single JAK2V617F-expressing long-term (LT) HSC into a lethally irradiated wild-type recipient mouse, MPN

developed in only a minority of recipients in whom long-term reconstitution occurred[13], supporting the concept that JAK2V617F is not always disease-initiating when modeled at the level of a single LT-HSC. The question of whether JAK2V617F is disease-initiating in MPN was also recently raised in the context of human hematopoiesis following a series of studies in which JAK2V617F mutations were detected in the peripheral blood of normal individuals who do not have any apparent hematological disease[14-16]. This phenomenon, where clonally-restricted somatic mutations in genes associated with hematological malignancies (including JAK2V617F) are found in normal individuals, has been termed clonal hematopoiesis of indeterminate potential (CHIP) and is strongly associated with increasing age[17]. JAK2V617F is among the most common CHIP-associated mutations and in most cases the *JAK2* mutations are isolated events that occur in the absence of other hematological malignancy-associated mutations, suggesting that JAK2V617F *alone* is sufficient to engender clonal hematopoiesis. However, intriguingly, the prevalence of JAK2V617F-positive MPN is significantly lower than that of JAK2V617F-positive CHIP, suggesting that in many cases JAK2V617F *alone* may be sufficient to engender clonal hematopoiesis but insufficient to induce MPN. This discrepancy may be explained in part by the observation from the Copenhagen General Population Study that higher JAK2V617F allele burden is associated with development of clinical MPN, and that a minimum threshold JAK2V617F allele burden is required for the development of overt disease[18]. However, since the presence of concomitant somatic mutations was not assessed in the Copenhagen study definitive conclusions regarding the sufficiency of JAK2V617F *alone* to cause MPN cannot be made.

It is also important to note that other somatic genetic alterations, for example *TET2* loss-of-function mutations, can precede the acquisition of JAK2V617F as reported in the original description of *TET2* mutations in myeloid malignancies[19] and later validated in subsequent studies[20,21]. Indeed, *TET2* is also a common CHIP gene, as are other epigenetic genes, such as *DNMT3A*[14-16]. Somatically mutated, clonally expanded HSC in which additional somatic mutations subsequently occur to drive the development of a disease phenotype have been termed “pre-leukemic” HSC and their role in the development of myeloid malignancies has now been clearly established, validating the original description of *TET2* mutations preceding JAK2V617F acquisition in MPN[19]. It has also been shown that *TET2* mutations can follow JAK2V617F and the order in which these mutations are acquired can impact both the age of onset and clinical features of MPN[21].

The acquisition of *TET2* mutations in established MPN has also been associated with leukemic transformation, as have mutations in other epigenetic genes such as *ASXL1*, in splicing genes such as *SRSF2*, and in additional genes including *IDH1/2*, *TP53*, *NRAS* and *RUNX1*[22,23]. Interestingly, AML that arises out of JAK2V617F-mutant MPN retains the JAK2V617F allele only approximately 50% of the time[24,25], suggesting that the acquisition of additional somatic mutations in MPN stem cells that harbor a phenotypic driver mutation (i.e. *JAK2*, *CALR* or *MPL*) is not the only route to AML. Leukemic transformation that emerges from a pre-MPN clone (lacking an MPN phenotypic driver mutation) may be promoted by inflammatory cytokines secreted by the MPN clone, particularly in the context of MF (see MPN stem cell niche section below).

What is the impact of MPN disease-initiating somatic mutations on the cell of origin in MPN?

A number of lines of evidence support the concept that the original target cell that acquires an MPN-initiating mutation is a LT-HSC that resides at the apex of the hematopoietic hierarchy (Figure 1). In MPN patients, the JAK2V617F mutation is detectable in immunophenotypically defined ($CD34^+$ $CD38^-$) long-term (LT) HSC[26] and in all mature cell lineages[27,28], indicating that the mutation is acquired by a multipotent cell. By genotyping hematopoietic colonies from MPN patients, *CALR* mutations were also demonstrated to be present in the earliest phylogenetic node[1]. Additional evidence from genetic mouse models further support the idea that Jak2V617F disease-initiating cells reside exclusively in the immunophenotypically defined LT-HSC compartment, and that Jak2V617F multipotent progenitors lack disease-propagating potential[29,30]. Following the acquisition of an MPN-initiating mutation by a single HSC, the development of an overt MPN phenotype requires clonal expansion, conferred through a selective advantage of MPN stem cells over their normal counterparts (Figure 2). In contrast to acute leukemia, where myeloid progenitor cells can acquire aberrant self-renewal[31,32], disease initiation and propagation in MPN can only be sustained by cells residing within the immunophenotypically defined LT-HSC compartment[30,33].

The impact of MPN phenotypic driver mutations on the expansion of the HSC pool can be inferred from the somatic mutant allele burden in granulocytes at diagnosis. In this regard, it is informative to compare the JAK2V617F mutant allele burden in chronic phase MPN (i.e. polycythemia vera (PV) and essential

thrombocythemia (ET)) with that of more advanced phase disease MPN (i.e. myelofibrosis (MF)). In PV, the JAK2V617F-mutant allele burden is often low at diagnosis[34], and in ET, JAK2V617F heterozygous clones can remain stable over years[35] with recent evidence suggesting that homozygous JAK2V617F clones do not necessarily expand[36]. Concordant with this observation, in a study of PV, ET and MF patients, expansion of the $CD34^+$ $CD38^-$ HSC compartment was not present in PV and ET patients but was present in MF patients[37]. A separate study showed that JAK2V617F allele burden was higher in the $CD34^+$ cell compartment of patients with MF as compared to PV and ET[38]. Interestingly, this expansion of *JAK2*-mutant cells in the $CD34^+$ compartment of patients with MF was found to be independent of JAK2V617F homozygosity, suggesting that clonal expansion in the $CD34^+$ compartment in MF is driven by other somatic genetic alterations enriched in MF as compared to PV or ET (for example epigenetic loss-of-function mutations). In contrast, the mutant CALR allele fraction measured in granulocytes is typically 40-50% at the time of ET diagnosis suggesting that heterozygous mutant CALR HSC become clonally dominant quickly[39]. Consistent with this observation, the age of onset of MPN is younger in *CALR*-mutant patients as compared to *JAK2*-mutant patients[39,40].

The impact of MPN-initiating mutations on HSC function has been studied *in vivo* using both genetic mouse models and patient-derived xenograft (PDX) models. JAK2V617F in particular has been the subject of intensive study and Jak2V617F genetic mouse models have been reviewed in detail elsewhere[12,41]. Although these models have demonstrated that JAK2V617F alone is sufficient to engender MPN and have shown that oncogenic JAK2V617F signaling does not confer self-renewal capacity upon non self-renewing hematopoietic cells, the impact of JAK2V617F on HSC self-renewal (as assayed by competitive repopulation) has been highly variable, depending on the genetic targeting approach taken to generate the particular mouse model. Recently, using a transgenic JAK2V617F mouse model, the level of JAK2V617F expression in single LT-HSC was shown to influence reconstitution capacity, with high JAK2V617F-expressing single LT-HSC demonstrating an exhaustion phenotype in a serial transplantation assay, as compared with low JAK2V617F-expressing single LT-HSC, which retained their long-term repopulation capacity[13]. Both *Tet2* and *Ezh2* loss have been shown to enhance the repopulating activity of Jak2V617F disease-propagating MPN stem cells in genetic mouse models[42-46], in the case of Ezh2 loss, the enhanced repopulation was mediated through reactivation of a fetal lin28b programme[42-46].

MPN studies in xenografts have found that JAK2V617F-mutant $CD34^+$ cells from patients with PV and ET engraft relatively poorly[47], while $CD34^+$ cells from either the peripheral blood or spleens of patients with MF demonstrate sustained engraftment[48,49] and in the case of splenic $CD34^+$ cells, serial transplantation into secondary recipients was achieved[49]. The fact that JAK2V617F SCID repopulating cells (SRC) do not gain a proliferative advantage over wild-type SRC over time in CD122 depleted NOD/SCID mice[50] suggests that JAK2V617F is not a strong driver of clonal expansion at the level of the HSC. However, one important caveat in the interpretation of these results is that incompatibilities between human cytokine receptors (expressed on transplanted $CD34^+$ cells) and murine cytokines (produced by the recipient murine bone marrow) may significantly impact JAK2V617F SRC activity in these studies. Co-mutation of *TET2* with JAK2V617F has been shown to increase the NOD-SCID re-populating capacity of $CD34^+$ cells, when compared to $CD34^+$ cells harboring JAK2V617F alone[19]. In an effort to overcome issues with species incompatibility in xenografts, a humanized bone marrow ossicle transplantation model was recently employed to study MPN stem cells[33]. In this study, the $CD34^+$ compartment was fractionated into $CD34^+CD38^+$ and $CD34^+CD38^{lo/-}$ sub fractions and engraftment was observed exclusively in ossicles transplanted with $CD34^+CD38^{lo/-}$ cells, confirming that the MPN-initiating population is contained solely in the immunophenotypically defined LT-HSC fraction[33].

Finally, the impact of cytokine receptor signaling in MPN stem cells has been assessed using various mouse models. In the HSC compartment, the thrombopoietin receptor, Mpl is a key growth factor receptor that is absolutely required for normal HSC function[51], and importantly, all MPN-initiating mutations activate MPL signaling (Figure 1). Using genetic mouse models, a critical role for Mpl in Jak2V617F-driven MPN-initiation has been demonstrated[52]. A similar requirement for Mpl in mutant CALR-driven MPN-initiation was recently demonstrated using a retroviral bone marrow transplant model[6]. Conversely, IL3Rb signaling has been shown to be expendable for MPN initiation[53], and in the case of erythropoietin signaling, restricting Jak2V617F expression to erythropoietin receptor (EpoR) expressing erythroid precursor cells has been found to result in a markedly attenuated MPN phenotype, supporting the absence of a role for the EpoR signaling in Jak2V617F-driven MPN initiation[30]. While these data firmly establish MPL as the crucial growth factor receptor for MPN

development, we still have an incomplete understanding of the molecular events downstream of activated MPL signaling that allow MPN stem cells to gain a clonal advantage in the bone marrow.

How do MPN stem cells contribute to heterogeneity of disease phenotype in MPN?

Although MPN have many clinical and biological features in common, including activation of JAK-STAT signaling, they also exhibit a range of distinct clinical phenotypes. Understanding the factors that influence the impact of an MPN disease-initiating mutation on the HSC that acquires it, and on its clonal progeny, is of fundamental importance in understanding heterogeneity of disease phenotype in MPN and these factors are summarized in Figure 3. Firstly, it is clear that MPN phenotype is partly determined by the specific phenotypic driver mutation that is acquired. For example, *CALR* and *MPL* mutations are almost always associated with an ET or MF phenotype, but not a PV phenotype. This partly relates to the mechanism by which these mutations induce MPN, namely through activating signaling through MPL[3-6,54], which is selectively expressed on HSC and cells in the megakaryocyte differentiation pathway (Figure 1). Activation of this pathway consequently promotes megakaryopoiesis and a thrombocytosis phenotype. Specific signaling events downstream of a specific driver mutation can also influence MPN phenotype, through selectively promoting specific lineage differentiation, for example, *JAK2* exon 12 mutations which activate JAK2 signaling and are associated with an isolated erythrocytosis phenotype both in MPN patients[55] and in mouse models[56]. Whether this lineage specificity occurs as a result of instructive signaling in multipotent progenitors, for example by differentially enhancing erythropoietin signaling[57], or as a consequence of selective signaling in committed erythroid precursor cells, remains unclear[58].

In contrast to the specific clinical phenotypes associated with *CALR*, *MPL* and *JAK2* exon 12 mutations, an intriguing aspect of MPN biology relates to the striking heterogeneity in clinical phenotype associated with the more common JAK2V617F mutation, which is associated with PV, ET and MF phenotypes in humans and also in mouse models[12,41]. An unresolved question is why a JAK2V617F mutation acquired by a single HSC can result in such a variety of clinical phenotypes. Furthermore, recent studies identifying the presence of CHIP in normal individuals[14-16] indicate that most individuals who acquire a JAK2V617F mutation in an HSC will never develop MPN. A number of possible explanations have been proposed for JAK2V617F-associated clinical

heterogeneity. First, evidence from MPN patients and mouse models indicates that phenotypic diversity in JAK2V617F-driven MPN relates to both quantitative differences in JAK2V617F allele burden[36,59] and qualitative differences in signaling downstream of JAK2V617F, including levels of STAT1 activation[60]. Second, other modifier genes, either through effects on HSC or on the bone marrow microenvironment, could influence disease phenotype, for example, constitutional differences in MYB expression[61]. It is interesting to note, however, that the same germ line predisposition loci are associated with both JAK2V617F-positive CHIP and JAK2V617F-positive MPN[62]. Third, presence of other somatic mutations, for example in *TET2*, and the order in which the mutations are acquired in HSC, have been shown to influence the resulting disease phenotype[21]. Fourth, cell-extrinsic environmental factors could influence eventual MPN phenotype. And finally, phenotypic heterogeneity could relate, at least in part, to the specific cell-of-origin of MPN.

A number of lines of evidence suggest that HSC are highly heterogeneous in their self-renewal capacity and their intrinsic lineage-bias[63]. For example, platelet-biased HSC that reside at the apex of the hematopoietic hierarchy have been characterized[64]. Furthermore, single cell transplantation studies in mice have identified myeloid lineage-restricted, self-renewing progenitors[65] and a similar cell population has also been described in the context of native non-transplant hematopoiesis[66]. Thus, characteristics of the specific individual HSC cell-of-origin that acquires a JAK2V617F mutation could also contribute to the phenotypic heterogeneity of JAKV617F-positive MPN and may provide an explanation, at least in part, for the development of JAKV617F-positive CHIP without progression to MPN. With respect to phenotypic heterogeneity, a JAK2V617F mutation arising in a platelet-biased HSC might result in an ET phenotype (Figure 4A), whereas the same mutation arising in a myeloid lineage-balanced HSC might promote a PV phenotype (Figure 4B). A recent study using single cell transplantation approaches to analyze a JAK2V617F transgenic mouse model provides some evidence in support of this hypothesis[13]. Single stem/progenitor cells from genetically identical donor mice were found to be capable of generating MPN *in vivo* with markedly variable phenotypes. There is also evidence to indicate that some HSC possess more limited self-renewal capability than others[63], and it may be that a JAK2V617F mutation arising in such a cell could result in CHIP (Figure 4C), with a clinical phenotype emerging only upon acquisition of a second mutation, for example in *TET2*, that enhances the self-renewal of the antecedent JAK2V617F-mutant clone[42] (Figure 4D). Acquisition of a JAK2V617F mutation in an HSC

already harboring a *TET2* mutation could influence the clinical phenotype[21,67], in addition to the age of MPN onset[21] (Figure 4E). Given the increased prevalence of CHIP in older individuals, it is interesting to note that aging has significant effects on HSC heterogeneity, including an increase in myeloid-biased differentiation, a decreased output of differentiated cells, and a reduced capacity for self-renewal in long-term secondary transplantation assays[68,69].

The MPN Stem Cell Niche

The bone marrow niche provides the microenvironmental signals that are essential for HSC function[70] and progress in understanding the cellular composition of the HSC niche has facilitated investigation of how these different components are perturbed during the development of myeloid neoplasms[71]. Indeed, a number of lines of evidence indicate that targeted disruption of the bone marrow niche *alone* is sufficient to induce an MPN phenotype *in vivo* [72-75].

Whilst the initiating MPN mutation acquired by a single HSC is likely to occur in the context of an unperturbed niche, as the MPN progresses, the expanding malignant clone exerts cell-extrinsic effects on components of the bone marrow niche, and evidence from mouse models suggests that this niche disruption can favor the survival and expansion of MPN stem cells over normal HSC, resulting in a so called malignant “self-reinforcing” niche[76]. For example, JAK2V617F-positive HSC have been shown to induce bone marrow neural damage through aberrant production of interleukin-1b, which in turn leads to a reduction in mesenchymal stem cell numbers and acceleration of MPN progression[77], prompting plans to develop a clinical trial to test the efficacy of β3-adrenergic agonists in MPN. Indeed, more advanced MPN is characterized by the development of bone marrow fibrosis that is promoted by pro-inflammatory cytokines, including transforming growth factor beta (TGFB) and tumor necrosis factor alpha (TNFA), which are produced by abnormal populations of myelomonocytic cells and megakaryocytes[76,78]. Both TGFB and TNFA are also known negative regulators of normal HSC[79,80] and in a retroviral JAK2V617F model, TNFA was demonstrated to facilitate the preferential expansion of JAK2V617F-mutant hematopoietic cells[81]. The bone marrow niche is also perturbed in MPN as a result of enhanced angiogenesis as evidenced by increased bone marrow microvessel density in MPN patients[82], possibly occurring as a consequence of increased VEGF expression[83]. The disrupted niche can also

lead to mobilization of HSC into peripheral blood and spleen resulting in splenic extramedullary hematopoiesis, which is a cardinal feature of more advanced MPN[49]. Furthermore, the microenvironmental disruption of the bone marrow in MPN might also promote leukemic transformation as demonstrated by a MF-xenograft study in which a high frequency of AML of *mouse origin* was noted. In this study, murine AML development was confined to the MF-xenograft group (and was not seen in xenografts of normal bone marrow or cord blood cells) suggesting that paracrine signaling arising from the MF cells drove leukemogenesis in normal mouse cells *in vivo*[84]. Recent work has highlighted lipocalin as an important paracrine factor in promoting DNA damage and disease evolution in MPN[85,86].

Finally, a number of pathways mediating bone marrow fibrosis, inflammatory signals and niche dysregulation are potentially amenable to therapeutic targeting. New approaches to modeling niche disruption such as those using humanized bone marrow ossicles in xenograft assays will be helpful in further exploring the underlying mechanisms of niche disruption in MPN and in testing novel therapeutic interventions[33].

Therapeutic targeting of MPN stem cells

The finding that MPN originate from and are propagated by rare self-renewing stem cells that closely resemble normal LT-HSC phenotypically, represents both a challenge and a therapeutic opportunity. The opportunity lies in the fact that effectively targeting MPN disease-propagating stem cells is not only required, but is also likely to be sufficient to eradicate the disease and achieve a cure. However, in myeloid malignancies, stem cell populations have been demonstrated to be selectively resistant to therapies that are able to induce cytogenetic remissions[87] and even the most successful targeted therapies in chronic myelogenous leukemia (CML) fail to eradicate leukemia stem cells in the majority of patients[88]. This selective therapeutic resistance of leukemia stem cell populations is, at least in part, related to their marked quiescence in comparison with progenitor populations[89,90]. Nevertheless, as somatic mutations confer a competitive advantage to MPN stem cells, the pathways that mediate this selective advantage should, in principle, be amenable to therapeutic targeting.

The only current curative therapy for MPN, and therefore the only treatment that is able to eradicate MPN stem cells, is allogeneic hematopoietic stem cell transplant. However, even following allogeneic transplant, relapse

is unfortunately not an infrequent occurrence[91], indicating that the MPN stem cell populations can be resistant even to high dose conditioning chemotherapy and allo-immunity mediated clearance. Furthermore, due to the toxicity associated with the transplantation procedure, this approach is currently restricted to a minority of younger patients with advanced MPN[92]. Recently, CRISPR/Cas9 gene editing has emerged as a promising new approach with the potential to treat human disease and although an enormous amount of work will be required to demonstrate the safety and feasibility of gene-editing as a therapeutic approach in patients, the recent demonstration that the causative mutation for sickle cell disease (SCD) could be repaired in HSC from SCD patients[93] is an important advance. However, whether such a strategy could be used to treat clonal disorders such as MPN by repairing JAK2V617F or MPLW515L/K mutations in HSC is uncertain at this point. Clearly the challenges of applying this approach to a clonal disorder are considerably greater, as disease eradication cannot be achieved unless all MPN stem cells are successfully repaired.

The clinical development of JAK2 inhibitor therapies heralded a new era of molecular targeted therapy for BCR-ABL negative MPN. Ruxolitinib, the first clinically approved JAK2 inhibitor, has considerable efficacy in patients with PV and MF, with improvements in blood counts, spleen size and symptoms. Despite this success, JAK2 inhibitors have been disappointing in their ability to induce molecular remissions in MPN patients, indicating that JAK2 inhibitors do not preferentially target MPN cells over normal cells, as evidenced by a lack of significant reduction in the JAK2V617F allele burden in patients undergoing treatment. Recently, the four-year data from the COMFORT-I study, a phase 3 trial in MF, was reported. Only 12% of patients showed a >50% decrease in JAK2V617F allele burden and <2% patients achieved complete molecular remission (CMR)[94]. A retrospective analysis of *CALR*-mutant patients treated with ruxolitinib on the COMFORT-II study (another phase 3 trial in MF) was recently reported[95]. Although, *CALR*-mutant patients demonstrated comparable clinical responses to *JAK2*-mutant patients, there was no significant change in the *CALR*-mutant allele burden after a median of 60 weeks of treatment with ruxolitinib (n = 18). Underlying the failure of JAK2 inhibitors to induce molecular remission in BCR-ABL negative MPN is the lack of efficacy of these agents to target MPN stem cells, which is supported by evidence from both mouse models[29] and MF patient samples[96]. Encouragingly, complete inhibition of JAK2 in a retroviral MPLW515L MPN mouse model using a genetic knockout approach resulted in a superior therapeutic response than treatment with a JAK2 inhibitor[97]. A new

generation of type II JAK2 inhibitors are able to induce more effective JAK2 inhibition[98] and if developed for clinical use such agents may be more successful at targeting MPN stem cells. However, JAK2 signaling is also essential for normal HSC function, as evidenced by several studies that have shown that hematopoietic-specific conditional genetic deletion of Jak2 in adult mice results in severe cell-intrinsic defects in HSC function, impaired hematopoiesis, and reduced survival[99-101]. Although total loss of JAK2 protein as occurred in the case of genetic knockout mouse models may be different than more potent JAK2 kinase inhibition, these murine studies have raised concerns regarding the potential for on-target hematological toxicity from more potent JAK2 inhibitors. Recent work focused on the crystal structure and biochemical properties of the pseudokinase domain of JAK2, which is the domain in which the V617F mutation is found, may help to overcome this issue by advancing the development of JAK2V617F-mutant specific inhibitors[102,103].

Interferon has been used in the treatment of MPN for decades[104], and more recently it has been recognized that a minority of interferon-treated patients achieve molecular remissions with interferon treatment, with re-emergence of polyclonal hematopoiesis in some[105,106]. Interferon treatment is not a "molecularly targeted" therapy and its ability to induce molecular responses applies across a range of different hematological malignancies[107], including patients with CML and MPN patients with *JAK2V617F*[105,106] and *CALR* mutations[108]. While acknowledging that interferon treatment likely has multiple effects in MPN patients, potentially including immunological effects and an imapct on the bone marrow microenvironment, one possible mechanism by which it achieves molecular remissions in MPN may relate to its ability to stimulate normally quiescent stem cell populations into cycle. As this cycling is more marked in JAK2V617F-positive HSC, interferon leads to preferential depletion of MPN stem cells in Jak2V617F mouse models[109,110], a finding which has also been corroborated in primary MPN samples[111]. Long-term molecular remissions after discontinuation of interferon therapy have been reported suggesting that JAK2V617F-mutant HSC can be eradicated by interferon[112]. However, molecular relapses after cessation of interferon have also been observed[113] supporting the concept that in some patients, MPN stem cells persist and are able to mediate relapse. It should also be remembered that conventional chemotherapy agents such as busulfan have also been shown to induce molecular remissions in a small number of patients with PV[114], suggesting that the potential of conventional treatments to eradicate MPN stem cells in rare patients may not be restricted to interferon.

A number of agents in clinical development are showing promise with regards to their ability to target MPN stem cells. For example, *in vitro* treatment of JAK2V617F-positive $CD34^+$ cells with MDM2 inhibitors (alone or in combination with interferon), has been found to reduce the degree of donor-derived chimerism and the JAK2V617F allele burden following injection of the treated $CD34^+$ cells into xenografts, suggesting that the treatment targets MPN stem cells[115]. Pre-clinical genetic mouse models have also identified selective sensitivity of MPN progenitors to estrogen-induced apoptosis[116], prompting initiation of a clinical trial (ISRCTN65011803) to test the efficacy of tamoxifen in MPN, with a primary endpoint focused on molecular response. As research studies continue to advance the understanding of the mechanisms by which MPN stem cells become clonally dominant, the potential to exploit the biological insights gained in order to selectively target MPN stem cells in patients will become more feasible.

Future directions

Although tremendous progress has been made in the field of MPN, particularly with regard to our understanding of the genetic basis of MPN and to the development of molecularly targeted therapies with JAK2 inhibitors, significant gaps remain in our understanding of the mechanisms by which MPN stem cells become clonally dominant in the bone marrow. Furthermore, recent studies on CHIP have highlighted additional deficits in our understanding of the factors that constrain and promote the transition from JAK2V617F-driven clonal hematopoiesis to JAK2V617F-induced MPN. This incomplete understanding of MPN stem cell biology underlies, at least in the part, the failure of current therapies to effectively target MPN stem cells, an essential step towards achieving cure. Progress in this area is further impeded by the failure of most clinical trials to incorporate appropriate biological sample banking that would allow future analysis of disease stem cells. Incorporation into clinical studies of biological secondary endpoints that focus on analysis of the stem cell compartment, such as assessment of molecular response in HSC, will provide an invaluable resource for future studies. Ultimately, effective eradication of MPN stem cells may require a multi-targeted approach, tackling both the cell-intrinsic and cell-extrinsic mechanisms that support MPN stem cell propagation.

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Figure Legends

Figure 1: Key somatic mutations and growth factor receptors important for MPN development

(A) Simplified "roadmap" of hematopoietic development. (B) Distribution of key growth factor receptors in different stem, progenitor and precursor cell populations. For each population, potential impact of *JAK2V617F*, *CALR* or *MPL* mutation is indicated.

Figure 2: Key steps during MPN development from normal hematopoiesis to acquisition of an MPN-initiating mutation in a single hematopoietic stem cell (HSC). The mutant HSC acquires a selective advantage over normal HSC and also promotes myeloid differentiation, eventually leading to a myeloproliferative phenotype. The expanded, abnormal myeloid clone disrupts the BM microenvironment, promoting a self-reinforcing malignant niche that favors MPN stem cells over normal HSC and leads to eventual mobilisation of MPN HSC into the peripheral blood (PB).

Figure 3: Summary of the factors that influence phenotypic heterogeneity in MPN.

Figure 4. Disease heterogeneity following acquisition of the JAK2V617F mutation in a single HSC.

X-axis represents time following acquisition of JAK2V617F indicated by the arrow. Y-axis represents relative contribution from this HSC clone to each lineage, indicated by color. (A) JAK2V617F occurs in a platelet-biased HSC resulting in essential thrombocythemia (ET), (B) JAK2V617F occurs in a lineage-balanced HSC resulting in polycythemia vera (PV) with tri-lineage myeloproliferation, (C) JAK2V617F occurs in an HSC with limited self-renewal capability resulting in clonal hematopoiesis of indeterminate potential (CHIP) (D) JAK2V617F precedes acqusition of a TET2 mutation resulting in a PV phenotype, (E) TET2 precedes acqusition of a JAK2V617F mutation resulting in an ET phenotype.

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