Hematopoietic stem cells, progenitor cells and leukemic stem cells in adult myeloproliferative neoplasms

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Abstract

The understanding of myeloproliferative neoplasms has changed dramatically since Dameshek proposed his classification over fifty years ago. Our knowledge of the types of cells which comprise the hematopoietic system and of how they are regulated has also appreciated significantly over this time. This review will relate what is currently known about the acquired genetic mutations associated with adult myeloproliferative neoplasms to how they lead to the hematopoietic perturbations of myeloproliferative disease. There will be a particular focus on how stem and progenitor cell compartments are affected by BCR-ABL1 and JAK2V617F mutations, and the particular issue of resistance of leukemic stem cells to conventional and targeted therapies.
Introduction

Myeloproliferative neoplasms are a class of hematopoietic neoplasms characterised by an overproduction of cells of mature myeloid lineages which may lead to complications of rheostasis, thrombosis and hemostasis, splenomegaly, bone marrow failure and leukemic transformation in affected patients (DAMESHEK, 1951).

The Philadelphia chromosome with the BCR-ABL1 fusion gene arising from the 9;22 chromosomal translocation in chronic myeloid leukaemia (CML) was the first example of an acquired causative mutation that could be associated with a myeloproliferative disorder (Chandra et al., 2011). Several gene mutations were subsequently associated with Philadelphia/BCR-ABL1 negative (Ph-) myeloproliferative neoplasms (reviewed in (Tefferi, 2010) (Vainchenker et al., 2011)). Most notable of those is the valine to phenylalanine substitution in the JAK2 gene (JAK2V617F) that occurs in ~95% of polycythemias vera (PV), ~60% of essential thrombocythemia (ET) and ~50% of idiopathic myelofibrosis (IMF) (James et al., 2005) (Levine et al., 2005) (Delhommeau et al., 2006) (Barosi et al., 2012). These acquired genetic mutations have been incorporated into the diagnostic criteria of myeloproliferative neoplasms as proposed by the World Health Organisation (Swerdlow, 2008), integrating molecular and cytogenetic findings with traditional morphologic pathology in recognition of the importance of specific mutations which lead to constitutively activated kinases that drive disease phenotypes in most myeloproliferative neoplasms (Delhommeau et al., 2006) (Gupta et al., 2002) (Vardiman and Hyjek, 2011) (Anastasi, 2011).

The realisation that BCR-ABL1 and JAK2V617F mutations lead to specific tyrosine kinase activation resulted in the development of targeted therapies directed at kinase inhibition.
Kinases, such as ABL1 or JAK2, are the targets of choice for inhibition by small molecules, and were therefore attractive targets for pharmaceutical intervention with kinase inhibitors (Arora and Scholar, 2005). In the case of CML, this has resulted in the $BCR-ABL1$ kinase inhibitor, imatinib, becoming the standard of therapy for treatment of CML especially for those patients diagnosed in chronic phase (Hochhaus et al., 2009). This can be attributed to the reduction in the incidence of CML blast crisis, improvement in overall survival and the tolerability of imatinib in the vast majority of patients with CML (Hehlmann, 2012). The superiority of imatinib (O'Brien et al., 2003), and more recently, second generation tyrosine kinase inhibitors (TKIs) (reviewed (Cortes and Kantarjian, 2012)) over previous therapy for CML is evidenced and related to their dramatic ability to reduce the molecularly detectable $BCR-ABL1$ positive disease (Cross et al., 2012). JAK2 kinase inhibitors have also entered into clinical practice for patients with IMF. In recent seminal phase III trials, JAK1/2 inhibition was very successful at decreasing splenomegaly and disease related constitutional symptoms with significant improvement in quality of life scores (Verstovsek et al., 2012) (Harrison et al., 2012), but did not show survival benefits compared to best available therapy (Harrison et al., 2012). Inhibition of JAK2 as a therapeutic target is also is unlikely have the same magnitude of benefit in reducing disease burden as has been seen for $BCR-ABL1$ inhibition in CML, with dose limiting toxicity of endogenous JAK2 inhibition apparent in hemopoietic cells as well as off-target effects being noted (Tefferi, 2012).

The success of $BCR-ABL1$ kinase inhibitors in a significant majority of patients with CML raised the initial hope that these TKIs may provide potentially definitive curative therapy with complete eradication of $BCR-ABL1$ disease. Despite these advances it has been shown
that TKIs may not completely eradicate the disease clone in CML, even in patients who obtain an apparently complete molecular response (Branford et al., 2007) (Ross et al., 2010). This observation can be explained in significant part by the unique physiological characteristics of hematopoietic stem cells harboring the \textit{BCR-ABL1} mutation. Similarly, current approaches to treatment of Ph- myeloproliferative neoplasms, although successful at preventing and managing several complications of PV, ET and IMF, do not for the most part eradicate the underlying disease clone. Allogeneic transplantation is current the only strategy whereby this may occur for CML and Ph- myeloproliferative neoplasms. Allogeneic transplantation however, carries significant cost in terms of non-relapse and relapse related morbidity and mortality for both CML (Goldman et al., 2010) and the select group of patients with Ph-myeloproliferative neoplasms who have transformed to acute leukaemia or have IMF and who are suitable for the procedure and have appropriately matched donors (Barosi et al., 2012) (Gupta et al., 2012).

This review will focus on hematopoietic stem cell and progenitor cell biology and relate what is currently known about acquired genetic mutations associated with adult myeloproliferative neoplasms to how they perturb hematopoiesis to generate myeloproliferative phenotypes. There will be a particular focus on how stem and progenitor cell compartments are affected by \textit{BCR-ABL1} and \textit{JAK2V617F} mutations and the issue of resistance of leukemic stem cells to conventional and targeted therapies.

\textbf{Hematopoietic Stem Cell Biology}
Mammalian hematopoiesis has been well characterised. Multiple specialised cellular compartments and their important molecular regulators have been identified. Hematopoietic stem cells (HSCs) are rare and specialised residents of the bone marrow and underpin the development and maintenance of hematopoiesis throughout the lifetime of an organism. Located in highly specialised and relatively hypoxic bone marrow micro-environments (Parmar et al., 2007) (reviewed in (Adams and Scadden, 2006)) adjacent to N-cadherin+ osteoblasts (Zhang et al., 2003) in endochondral ossified endosteal bone niches (Chan et al., 2009), HSCs are maintained in a relatively quiescent state where they retain the characteristics of pluripotency, the "potential" of a single cell to generate all the cell lineages of the hematopoietic system, and self-renewal, the ability to divide "asymmetrically" to sustain a pool of daughter stem cells which possess the pluripotency and self-renewal abilities of the parent HSC (Tajbakhsh et al., 2009) (Ting et al., 2012) (Figure 1).

HSC quiescence and self-renewal are maintained by several molecular regulators acting via specific cell signaling pathways (reviewed in (Blank et al., 2008)). Thrombopoietin/Mpl receptor (Qian et al., 2007) (Yoshihara et al., 2007), Wnt/β-catenin (Fleming et al., 2008), Notch1/Jagged1 (Stier et al., 2002) (Calvi et al., 2003), osteopontin (Nilsson et al., 2005), TGF-β/Smad4 (Karlsson et al., 2007), Tie2/Angiopoietin1 (Arai et al., 2004) and stem cell factor/cKit signaling (Kent et al., 2008), have all been demonstrated to be important in regulating HSC quiescence/self-renewal.

Importantly, quiescent HSCs have been shown to be resistant to conventional chemotherapeutic agents (Hodgson and Bradley, 1979) and γ radiation (Ploemacher et al., 1992). The resistance of quiescent HSCs to mechanisms which can trigger cell death by apoptosis is an im-
portant defining characteristic of this population and is mediated by specific molecular regulators, such as the prosurvival Bcl-2 family protein, Mcl-1 (Opferman et al., 2005).

Notably, HSCs may be stimulated into cell cycle by other regulators. Inflammatory mediators such as interferon alpha (Essers et al., 2009) and interferon gamma (Baldridge et al., 2010) may induce "quiescent" HSCs into cell cycle, whilst Prostaglandin E2 generated by the cyclooxygenase (COX) enzyme in HSCs, may modulate HSC self-renewal acting via EP2 and EP4 receptors (North et al., 2007) (Frisch et al., 2009) (Durand and Zon, 2010).

Hematopoietic progenitor cells are the progeny of HSCs. Progenitors comprise a significant proportion of adult bone marrow, coordinate the production of precise numbers of mature blood cells of diverse functional lineages, and expand in times of hematopoietic stress to attempt to meet physiologic requirements. The steady state production of mature functional cell lineages and the hematopoietic stress response is mediated in large part by hematopoietic cytokines, such as erythropoietin (EPO) for erythroid (Miyake et al., 1977) (Gasson et al., 1985), granulocyte colony stimulating factor (G-CSF) for granulocyte (Nicola et al., 1983) (Metcalf and Nicola, 1983), and thrombopoietin (TPO) for the megakaryocyte lineage and platelet production (Kaushansky et al., 1994) (Lok et al., 1994) (Ng et al., 2012a).

**Hematopoietic cytokines, cell signaling molecules and mutations of epigenetic regulators associated with myeloproliferative neoplasms**

The discovery of hematopoietic cytokines, their cognate receptors and the characterisation of the molecules which comprise the intracellular signaling pathways has led directly to significant therapeutic advances in supportive hematological care and peripheral stem cell mobilisation (reviewed in (Metcalf, 2010) (Robb, 2007)).
The use of genetically manipulated murine models has allowed the precise characterisation of the roles of specific signaling molecules in physiological hematopoiesis. For instance, during EPO and EPO receptor signaling (reviewed in (Constantinescu et al., 1999), Figure 2), JAK2, a member of the Janus (JAK) family of non-receptor protein tyrosine kinases (Wilks et al., 1991), was shown to be an essential down-stream mediator that was required for definitive fetal erythropoiesis (Parganas et al., 1998) (Neubauer et al., 1998). STAT5, which is recruited to the EPO receptor and is phosphorylated by JAK2 to induce its activation, nuclear translocation and transcription (Constantinescu et al., 1999), was shown to be important during fetal erythropoiesis through analysis of mice lacking both STAT5a and STAT5b redundant isoforms (Teglund et al., 1998). The role of STAT5 in fetal erythropoiesis was attributed in significant part, to its transcription of the pro-survival BCL-XL protein and inhibition of apoptosis of fetal erythrocytes (Socolovsky et al., 1999).

Murine models have also permitted dissection of intracellular signaling molecules required for manifestations of disease phenotypes associated with BCR-ABL1 and JAK2V617F mutations (Figure 3). Both BCR-ABL1 and JAK2V617F appear dependent on STAT5 signaling for features of myeloproliferation in murine models (Funakoshi-Tago et al., 2010) (Yan et al., 2012) (Walz et al., 2012). Endogenous JAK2 may serve as an important mediator of the disease phenotype associated with the FIP1L1-PDGFRα fusion identified in a proportion of patients with chronic eosinophilic leukaemia (Li et al., 2012a). Of particular note, although BCR-ABL1 appears dependent on STAT5 for the development of chronic myeloid leukaemia-like myeloproliferative disease, endogenous JAK2 may be required for initiation of transformation to B- acute lymphoblastic leukaemia in these models (Hantschel et al., 2012). This last finding highlights that not only the acquired genetic lesion, but specific interactions with
endogenous signaling molecules and the cell types in which this occurs may all play an important role in the specific manifestations of myeloproliferative neoplasms.

Similarly, other mutations identified in a minority of cases of Ph- myeloproliferative neoplasms, such as gain-of-function mutations in JAK2 exon 12 (Scott et al., 2007) and MPL (Pardanani et al., 2006), and loss-of-function mutations for negative regulators such as CBL (Aranaz et al., 2012) and LNK (Pardanani et al., 2010), may all directly perturb cell signaling pathways.

A subset of patients with Ph- myeloproliferative neoplasms and interestingly CML patients with advanced phases of disease (Makishima et al., 2011), have had mutations identified in epigenetic regulators such as ASXL1, DNMT3A, IDH1, IDH2, TET2, SUZ12 (Martinez-Aviles et al., 2012) and EZH2 (Guglielmelli et al., 2011), of which several have been associated with significant differences in disease outcome for Ph- myeloproliferative neoplasms. Recently, recurrent mutations in the serine/arginine-rich splicing factor 2 (SRSF2), relatively uncommon in patients with chronic myeloproliferative neoplasms (Yoshida et al., 2011), were identified in ~18.9% of patients with myeloproliferative neoplasms which had undergone leukemic transformation and conferred a worse overall survival in this group of transformed myeloid leukemia (tAML) by univariate analysis (Zhang et al., 2012). DNMT3A mutations have also been found to occur more frequently in IMF and transformed acute myeloid leukemia associated with the JAK2V617F mutation when compared to ET and PV cohorts (Stegelmann et al., 2011).

Although regulators such as the polycomb group proteins SUZ12 and EZH2 have been demonstrated to have direct roles in the regulation of HSCs and thrombopoiesis through their
involvement in the Polycomb Repressor Complex 2 (PRC2) (Majewski et al., 2008) (Majewski et al., 2010), and the TET2 DNA methylation enzyme has been shown to modulate HSC self-renewal (Moran-Crusio et al., 2011) (Ko et al., 2011) (Shide et al., 2012), it remains unclear as to whether this occurs via regulation of intracellular cell signaling molecule expression or by other mechanisms. Taken together however, these findings suggest that mutations in epigenetic regulators may modulate the emergence of specific phenotypes associated with Ph- myeloproliferative neoplasms, including those associated with the JAK2V617F mutation (reviewed (Vainchenker et al., 2011)). Of note, ectopic expression of the histone-methyltransferase EZH2 in hematopoietic stem cells was recently shown to induce a myeloproliferative disease (Herrera-Merchan et al., 2012). Interestingly, mutant JAK2V617F has also been shown to lead to aberrant phosphorylation and reduction of the methyl transferase activity of protein arginine methyltransferase 5 (PRMT5) and subsequent H2A and H4 histone arginine methylation. PRMT5 activity was demonstrated to have direct effects on colony formation and erythroid differentiation in vivo (Liu et al., 2011), suggesting perturbation of this epigenetic regulator may also be a mechanism by which the JAK2V617F mutation can lead to myeloproliferation. These findings also suggest that for a subset of patients, the efficacy of therapy targeted to specific signaling molecules, such as with JAK2 inhibitors for IMF or BCR-ABL1 TKIs for patients with advanced phases of CML, may be limited, especially if other mutations are present that may drive the disease in HSCs and progenitor cells.

Expression of JAK2V617F in HSCs and Progenitors

Knowledge of the biology of HSC and progenitor cells would suggest that maintenance of a myeloproliferative phenotype requires acquired mutations to be harboured in cell populations with the ability to self-renew in order to allow ongoing generation of progeny containing the
disease-associated mutations. Importantly, expression of the \textit{JAK2V617F} mutation been demonstrated in bone marrow cell populations expressing surface markers associated with HSCs in patients with PV (Jamieson et al., 2006) (Butcher et al., 2007). Interestingly however, X-chromosome inactivation analysis in females affected by PV (Adamson et al., 1976) (Briere and el-Kassar, 1998) (Mitterbauer et al., 1999) (Zamora et al., 2005) and molecular profiling of \textit{JAK2V617F} mutations in peripheral blood leukocyte populations, noted this mutation was detected in lymphoid cells only in a minority of patients (Ishii et al., 2006) (Butcher et al., 2007). One interpretation of these data is that the \textit{JAK2V617} mutation does not lead to a proliferative advantage to lymphocytes. Indeed, global gene expression profiling of HSC and progenitor cells of patients carrying \textit{JAK2V617F} demonstrated a relative reduction in genes associated with B- and T- cells (Berkofsky-Fessler et al., 2010). This suggests that rather than conferring an abnormal self-renewal ability on early myeloid progenitor populations, the \textit{JAK2V617F} mutation may lead to a passive selectivity against lymphoid lineage formation from HSCs resulting from no lymphoid acquired growth advantage, with the circulating lymphocytes in the majority of patients with PV representing pre-existing lymphoid cells prior to acquisition of the mutation.

Supporting this contention are murine models of PV in which HSC and progenitor populations expressing \textit{JAK2V617F} were transplanted into lethally irradiated recipients. These experiments demonstrated that the HSC population, but not the progenitor cell population, was capable of inducing and maintaining a PV-like disease in transplanted recipients (Mullally et al., 2010) (Mullally et al., 2012). These findings argue against \textit{JAK2V617F} having a fully transformative effect on progenitor cells unlike some other leukaemia oncogenes (Zuber et al., 2009). Rather, it appears progenitor cell populations with cytokine signaling hypersensi-
tivity still require derivation from HSCs with the \textit{JAK2V617F} mutation in order for the myeloproliferative phenotype to develop (Dupont et al., 2007).

The physiologic effect of the \textit{JAK2V617F} mutation on HSC function remains unresolved. Interpretation of studies of xenotransplantation of human stem and progenitor cells harbouring the \textit{JAK2V617F} mutation into immunodeficient mice has been limited by the poor rates of cross-species engraftment (Ishii et al., 2007) (James et al., 2008). Analysis of one murine model in which the human \textit{JAK2V617F} mutation is expressed at endogenous levels demonstrated that this mutation did not confer functional advantages and even impaired HSCs containing the mutation when compared to HSCs expressing the wild-type JAK2 (Li et al., 2010). This finding, however, is in contrast with other murine models in which the stem and early progenitor compartment is relatively expanded in mice carrying a \textit{JAK2V617F} mutation expressed from the endogenous JAK2 promoter (Akada et al., 2010) (Yan et al., 2012), with HSCs with the \textit{JAK2V617F} mutation in one model demonstrating a weak competitive advantage relative to wild-type HSCs (Mullally et al., 2012).

Both human and murine \textit{JAK2V617F} expression can lead to erythroid expansion in several mouse models (reviewed in (Li et al., 2011)), with an increase of erythroid progenitors (Mullally et al., 2010) (Akada et al., 2010) (Akada et al., 2012) and cytokine-independent erythroid progenitor colony growth (Akada et al., 2010) (Yan et al., 2012) (Akada et al., 2012). These findings phenocopy erythroid progenitors in human disease which demonstrated enhanced sensitivity to cytokine signaling especially to EPO (Dai et al., 1991) (Dai et al., 1992) (Dupont et al., 2007), as well as EPO independent colony growth (Prchal and Axelrad, 1974). These data demonstrate a causal link between the \textit{JAK2V617F} mutation and the PV phenotype \textit{in vivo}. Not all murine models expressing human \textit{JAK2V617F} however lead to
erythroid expansion. A human JAK2V617F transgenic model demonstrated a dose-dependent JAK2V617F to murine wild-type JAK2 ratio may underlie expression of a PV or ET phenotype, with the ET phenotype associated with a lower JAK2V617F to wild-type JAK2 ratio of < 0.4 (Tiedt et al., 2008). Interestingly, this finding was not observed in a model using murine JAK2V617F which also had lower expression of JAK2V617F when compared to wild-type JAK2 (Mullally et al., 2010). These findings suggest that factors such as the JAK2V617F to wild-type JAK2 ratio, JAK2V617F species specific differences and mouse genetic background may all play a role in the manifestation of disease phenotype in these models.

Importantly, selective expression of the JAK2V617F in progenitor cells expressing the EPO receptor, thus restricting JAK2V617F expression to predominantly erythroid progenitor cells, was shown to be sufficient for producing a PV-like phenotype which in addition to the pathological features of PV, was able to recapitulate erythroid progenitor expansion and EPO independent progenitor colony growth as had been seen in murine models in which JAK2V617F was expressed in HSCs (Akada et al., 2012) (Mullally et al., 2012). These findings highlight both the importance of EPO signaling as well as EPO receptor-expressing progenitor cells for the manifestation of the PV phenotype associated with the JAK2V617F mutation.

Expression of BCR-ABL1 in HSCs and Progenitors

Characterisation of patients with CML has demonstrated BCR-ABL1 expression in bone marrow cell populations enriched for HSCs and progenitor cells (Jamieson et al., 2004) (Hope et al., 2003) (Warner et al., 2004). In CML patients, it was noted there was an expansion of the progenitor pool particularly in patients with accelerated phase or blast crisis, with notable
amplification of *BCR-ABL1* transcripts in myeloid progenitors in the latter phase. The granulocyte-macrophage progenitors from CML patients also appeared to demonstrate an *in vitro* self-renewal capacity not seen in controls, which was related to increased expression of beta-catenin in CML progenitor cells relative to control progenitor cells (Jamieson et al., 2004). This latter finding was not replicated in committed murine hematopoietic progenitors in a murine model of *BCR-ABL1*-transduced CML (Huntly et al., 2004). Notably however, progenitors from CML patients and murine models in which *BCR-ABL1* expression had been transduced into hematopoietic stem and progenitor cells, demonstrated growth factor-independent colony formation which was not seen in normal controls (Graham et al., 2002) (Yan et al., 2012).

Global gene expression profiling of CML CD34+ bone marrow cells enriched for HSC and progenitor cells has demonstrated an up-regulation of cell cycle-associated genes when compared to control populations (Graham et al., 2007) (Bruns et al., 2009) (Benito et al., 2012). These findings suggest that *BCR-ABL1* induces proliferation within the HSC and progenitor cell compartment. These changes were shown to resolve with imatinib therapy (Benito et al., 2012). This last finding may be interpreted as either imatinib inhibition of *BCR-ABL1*-induced cellular proliferation, or a restoration of the composition of the HSC/progenitor cell compartment with *BCR-ABL1* tyrosine kinase inhibition (Jamieson et al., 2004) resulting in imatinib-induced apoptosis of proliferating *BCR-ABL1*-expressing progenitor cells (Kuroda et al., 2006).

**The leukemic stem cell and CML**
A population of highly quiescent HSCs expressing \textit{BCR-ABL1} can be isolated from untreated CML patients (Holyoake et al., 1999) and from CML patients treated with imatinib (Hamilton et al., 2012). These quiescent, non-proliferating CD34+ CML cells have been shown to be resistant to a range of pro-apoptotic stimuli including chemotherapy and tyrosine kinase inhibition with imatinib (Graham et al., 2002) (Holtz et al., 2005) (Figure 4). The quiescent \textit{BCR-ABL1}-expressing HSCs can be regarded as leukemic stem cells (LSCs). By way of comparison, proliferating CD34+ progenitors in CML are sensitive to imatinib-induced apoptosis that is mediated in significant part by the BCL-2 family pro-apoptotic proteins Bim and Bad (Kuroda et al., 2006). Interestingly, a BIM deletion polymorphism was recently shown to confer intrinsic imatinib resistance and inferior responses in patients with CML treated with imatinib (Ng et al., 2012b). It has also been suggested that BCL-2 family pro-survival proteins may play a role in resistance and survival of CML LSCs, although these data remains unpublished (Crews and Jamieson, 2012).

Several signaling pathways may allow survival of the CML LSCs in spite of \textit{BCR-ABL1} tyrosine kinase inhibition, such as Hedgehog pathway signaling (Dierks et al., 2008), and Wnt/beta-catenin signaling, the latter which may in part be regulated via Prostaglandin E2 (Heidel et al., 2012). Recently, induced pluripotent stem cells derived from human CML patients by transfection with factors Oct3/4, Sox2, KLF4 and c-myc (CML-iPSCs) were shown to express \textit{BCR-ABL1} and demonstrated a decrease in STAT5 and CRKL phosphorylation upon treatment with imatinib. Notably however, phosphorylation of ERK1/2, AKT and JNK were unchanged after imatinib treatment suggesting CML-iPSCs were capable of utilizing alternative signaling pathways other than STAT5 for survival. This was in contrast to imatinib-sensitive hematopoietic cells derived from CML-iPSCs which were shown to be \textit{BCR-ABL1} and...
STAT5 dependent (Kumano et al., 2012). Similar conclusions have been reached using primary CML CD34+ bone marrow cells and transgenic murine models of CML LSCs (Hamilton et al., 2012).

In summary, HSCs and progenitor cells are distinct components of the hematopoietic system, each with unique biological properties. Acquired genetic mutations associated with myeloproliferative neoplasms may affect HSCs and progenitors differently and may perturb different cellular processes. Examination of BCR-ABL1 and JAK2V617F gene mutations in human and mouse studies highlight the importance of deregulation of cytokine and intracellular cell signaling pathways in specific progenitor cell populations in order to produce the myeloproliferative phenotypes associated with these mutations, and for which STAT5 has emerged as a critical player in both BCR-ABL1- and JAK2V617F- associated myeloid disease. Analysis of human samples and murine models have demonstrated that although BCR-ABL1 and JAK2V617F mutations significantly perturb the hematopoietic progenitor composition, as single mutations, they do not appear to fully transform progenitor cells into LSCs that can initiate and sustain disease in vivo. In addition, several other mutations in signaling molecules and epigenetic regulators have been characterized in myeloproliferative neoplasms which may play a role in the emergence of specific disease phenotypes.

**Persistence of the leukemic stem cell in patients with CML treated with TKIs**

Studies of human CML have demonstrated the presence of chemotherapy- radiotherapy- and targeted TKI therapy-resistant quiescent HSCs which harbour the causative mutation and which can maintain the de novo disease. These HSCs are the LSCs in CML, the unique prop-
erties of which reflect properties of HSCs in general, namely cell cycle quiescence and resistance to a variety of apoptotic stimuli.

Collectively, experimental data suggests CML LSCs are detectable by highly sensitive PCR in patients who have apparently achieved a stable complete molecular response (CMR) (Ross et al., 2011b) to the TKIs imatinib (Ross et al., 2010) and dasatinib (Ross et al., 2011a). Two prospective clinical trials, the French intergroup STIM study (Mahon et al., 2010) and Australasian Leukemia and Lymphoma Group CML8 study (Ross et al., 2010) have enrolled patients who had achieved stable CMR with undetectable peripheral blood BCR-ABL1 transcripts for at least 2 years while receiving imatinib, to assess the incidence and types of relapse upon cessation of the TKI. Eight of 18 patients on the CML8 trial maintained undetectable BCR-ABL1 after a median follow up of 2 years (Ross et al., 2010), while interim 12 month analysis for the STIM study demonstrated 41% molecular relapse free survival (Mahon et al., 2010). The latest update of the STIM study showed the probability of maintaining of CMR at 24 and 36 months in the STIM study remained ~ 39% (Mahon et al., 2011). Interestingly, 5/39 patients below a defined threshold for confirmed molecular relapse demonstrated fluctuating BCR-ABL1 transcript levels over a median follow up of 22 months, suggesting molecular detection of transcript by highly sensitive PCR may not predict imminent cytogenetic or hematologic relapse when remaining at less than 1 log rise in BCR-ABL1 transcript level. This observation may have basis in HSC biology. Observation of hematopoietic reconstitution in murine models (Capel et al., 1990) (Drize et al., 2001) (Cao et al., 2004) and non-human primates (Laukkanen et al., 2005) have demonstrated complex recruitment and extinction of individual HSC clones when reconstitution was observed closely over time. These observations suggest the fluctuating low level BCR-ABL1 transcript that are detected may re-
flect this phenomenon of clonal HSC recruitment and extinction. These findings also recall the concept a "functional cure" proposed for patients with CML who were assessed more than 18 months after allogeneic bone marrow transplant in whom low levels of transcript were detected, but remained in cytogenetic and hematologic remission despite not having definitive eradication of the leukemic clone (Radich et al., 2001).

Given the data related to the allogeneic transplant experience in CML and the quiescent nature of imatinib-resistant CML LSCs, close monitoring and longer term follow up will be required for patients enrolled in the CML8 and STIM trials to further elucidate the patterns of relapse over the coming years. Evidence from long term follow up of allogeneic transplantation for CML clearly demonstrates the occurrence of late CML relapse beyond five years (Radich et al., 2001) (Goldman et al., 2010). *BCR-ABL1* is also believed to be the driving mutation for CML blast transformation, such that patients may still be at risk of this event in the absence of definitive eradication of CML clone. Although the incidence of blast crisis has been markedly reduced in the imatinib era (Hehlmann, 2012), this has been in the context where therapy has been intended to be uninterrupted. Patients with molecular relapse enrolled in CML8 study have to date remained sensitive to reintroduction of imatinib (Ross et al., 2011b), although in the latest update of the STIM study, not all patients were able to return to an imatinib induced CMR. This included one patient who experienced a loss of complete cytogenetic response (Mahon et al., 2011).

**Interferon in CML and Ph- myeloproliferative neoplasms**

Ultimately, despite the success of BCR-ABL1 TKI treatment for the majority of patients with CML, elimination of LSCs in CML will be needed to be able to establish a definitive cure of
the disease which would permit the cessation of therapy. This is likely to require targeting signaling pathways and molecular regulators other than the BCR-ABL1 tyrosine kinase, either through inhibiting non BCR-ABL1 molecular regulators implicated in CML LSC survival, or through the use of therapeutic agents which may sensitise or synergise with imatinib to induce apoptosis in the LSC. Synergistic action of combination therapy had been suggested in a retrospective analysis of patients undergoing induction treatment with interferon alpha and imatinib (Palandri et al., 2010). In prospective studies, the use of interferon in combination with imatinib was suggested to improve the rate of molecular response when compared to imatinib alone (Simonsson et al., 2011) (Preudhomme et al., 2010). Improvements in molecular response rates however were not observed in the German CML IV study (Hehlmann et al., 2011). Importantly, these prospective studies did not show benefit in event free survival or overall survival with combination therapy over imatinib alone. Putative mechanisms by which interferon may synergise with imatinib are via interferon alpha-induced Fas-mediated apoptosis (Selleri et al., 1997), interferon-induced immune modulation (Burchert et al., 2010), or induction of LSC clones into cell cycle whereupon they may become sensitive to imatinib (Essers et al., 2009).

For Ph- myeloproliferative neoplasms, Pegylated interferon alfa-2a (PEG-IFN-α-2a) in particular, has been shown to have significant activity in two phase II trials in patients with PV, or PV and ET, when used at relatively low and well tolerated doses. Significant hematological responses and progressive molecular responses for JAK2V617 assessable disease were noted in both studies. Interestingly, a small cohort of patients in both studies were shown to remain in sustained complete molecular response with undetectable JAK2V617F even after cessation of interferon therapy (Kiladjian et al., 2008) (Quintas-Cardama et al., 2009). Activi-
ty of low dose recombinant interferon alpha has additionally been shown for IMF, with clear bone marrow responses demonstrated for patients with early primary IMF (Silver et al., 2011). Taken together, these studies suggest Ph- myeloproliferative neoplasms demonstrate particular sensitivity to IFN alpha signaling. IFN alpha was shown to have anti-proliferative effects especially on human cell lines and patient derived CD34+ progenitors harboring the JAK2V617F mutation (Pasquier et al., 2009). PEG-IFN-α-2a has also been shown to induce apoptosis of hematopoietic progenitor cells derived from normal bone marrow and JAK2V617F positive CD34+ cells from patients with PV in vitro (Lu et al., 2010b). In this latter study, this effect was partially inhibited by a p38 MAP kinase inhibitor suggesting interferon signaling may in part act via this signaling pathway to increase p53 transcription. Consequently, combination treatment with low dose interferon alpha with the Bcl-xL inhibitor ABT-737 (Lu et al., 2010a) or the MDM2 antagonist Nutlin-3 which promotes apoptosis by retarding p53 degradation (Lu et al., 2012), was demonstrated to have synergistic and selective activity against JAK2V617F positive progenitors compared to controls in vitro. Relative selectivity of interferon alpha in vivo against amplifying hematopoietic stem and progenitor cells with the JAK2V617F mutation has been reported in a chimeric mouse model, including activity against disease-initiating LSC (Marty et al., 2011). To build on these findings, a phase III trial of PEG-IFN-α-2a in Ph- myeloproliferative neoplasms is required.

**Other potential drug targets against the leukemic stem cell in CML and JAK2V617F driven myeloproliferative neoplasms**

Pre-clinical data continue to suggest that modulation of CML LSCs may be feasible with other agents, such as indomethacin which in combination with imatinib, may aid in targeting CML LSCs (Heidel et al., 2012). Similarly, pan histone deacetylase inhibitors (Zhang et al.,
2010) and more specific inhibitors of NAD-dependent histone deacetylase SIRT1 (Li et al., 2012b) in combination with imatinib, have shown promising activity in the ability to potentially target the CML LSC. Whether these approaches will provide additional benefit to patients with CML in chronic phase or the more advanced accelerated phase or blast crisis disease in which mutations in genes involved in cell signaling pathways other than BCR-ABL1 or epigenetic regulators can be found, remain to be seen. Importantly, these additional non BCR-ABL1 mutations may account for the relative resistance to TKIs in these advanced stages of CML (Makishima et al., 2011).

Similarly, conventional chemotherapeutic approaches to Ph- myeloproliferative neoplasms are not curative and targeted therapy with JAK2 inhibitors are unlikely to drive a paradigm shift in the treatment of these neoplasms in the way that BCR-ABL1 TKIs have done for CML. Dose-limiting toxicity of JAK2 inhibitor therapy can be directly related to inhibition of wild-type JAK2. The maximal tolerated dose of the JAK1/2 inhibitor Ruxolitinib (Verstovsek et al., 2010) and selective JAK2 inhibitor TG101348 (Pardanani et al., 2011) was defined by hematologic toxicity and could be predicted given the importance of JAK2 signaling in mediating cytokine response in normal hematopoiesis (Khwaja, 2006). At selected therapeutic doses, thrombocytopenia and anemia are frequent side effects of the JAK1/2 inhibitor Ruxolitinib (INCB018424) (Harrison et al., 2012) and the selective JAK2 inhibitor TG101348 (Pardanani et al., 2011) with no clinically meaningful reduction in allele burden for JAK2V617F positive disease (Verstovsek et al., 2010) (Pardanani et al., 2011), induction of disease remission, or survival benefits over best supportive therapies observed (Harrison et al., 2012) (Tefferi, 2012). In contrast, ABL1 kinase does not appear to be required for normal myelopoiesis (Tybulewicz et al., 1991) (Schwartzberg et al., 1991) with the majority of patients with CML
in chronic phase treated with \textit{BCR-ABL1} inhibitors not experiencing dose-limiting hematologic toxicity at therapeutic doses capable of significant activity against \textit{BCR-ABL1} leukemic cells (Kantarjian et al., 2002) (Jabbour et al., 2011) (Kantarjian et al., 2012). It is therefore likely conventional therapies will remain as the mainstay of treatment for the majority of patients with Ph- myeloproliferative neoplasms, with allogeneic transplantation for select and suitable patients currently the only therapy that has the potential to eliminate the LSC clone, although with significant potential cost in terms of treatment-related, graft versus host disease-related, and disease-related morbidity and mortality.

Other pharmacological agents continue to be evaluated to address this clinical need for Ph-myeloproliferative neoplasms with the JAK2V617F mutation. Inhibitors of the HSP90 chaperone which associates and stabilizes JAK2 (Marubayashi et al., 2010), have been shown to promote degradation of wild-type and mutant JAK2V617F (Bareng et al., 2007) (Wang et al., 2009) (Proia et al., 2011) and inhibit JAK/STAT signaling \textit{in vitro} and \textit{in vivo}. Interestingly, the HSP90 inhibitor PU-H71 may have potentially greater selectivity for JAK2V617F expressing cells (Marubayashi et al., 2010). This selectivity also been suggested for parbinostat, a histone deacetylase/HSP90 inhibitor when used in combination with a JAK2 inhibitor (Wang et al., 2009). Taken together, these preliminary findings suggest there may be strategies in the future with which to be able to selectively target JAKV617F disease, with an improved therapeutic index over current JAK2 inhibitor therapy.

\textbf{Conclusion}

As further insights are gained into the biological consequences of mutations associated with myeloproliferation and therapies are developed which aim to target critical molecules such as
JAK2V617F in Ph- myeloproliferative neoplasms, BCR-ABL1 in CML, and other strategies against Ph- myeloproliferative neoplasms and the LSCs in CML emerge, we can continue to address the challenges that remain in the treatment of patients with myeloproliferative neoplasms. This is especially important for those patients in whom conventional and currently available targeted therapies are inadequate.

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Conflicts of Interest

There are no conflicts of interest.
References


kinase activity for their survival. Blood 119, 1501-1510.


Program 2011, 250-256.


Figure Legends

Figure 1. Schematic diagram of hematopoietic stem cell regulation by signaling pathways. Several ligands (blue box) and their cognate receptors (white box) implicated in the regulation of hematopoietic stem cell (HSC) quiescence and self-renewal within the HSC osteoblastic niche are shown. Interferon alpha and interferon gamma have been demonstrated to induce quiescent HSC into cell cycle.

Figure 2. Schematic representation of cytokine signal transduction. Ligand binding by erythropoietin (EPO) to its cognate EPO receptor results in receptor dimerisation, cross phosphorylation and activation of Jak2 molecules associated with the cytoplasmic region of the EPO receptor. Phosphorylation of Stat5 molecules by activated Jak2 kinases, leads to nuclear translocation of Stat5 molecules and binding to gene promoter regions with initiation of gene transcription. RNA Pol; RNA polymerase.

Figure 3. Schematic representation of intracellular signaling molecules important in the development of myeloproliferative disorder phenotypes. Stat5 appears to be required for erythroid and granulocytic myeloproliferation associated with JAK2V617F and BCR-ABL1 mutations, respectively. Endogenous Jak2 appears to be important for initiation of B lineage acute lymphoblastic leukaemia (B-ALL) associated with BCR-ABL1 mutations.

Figure 4. Schematic diagram of cell populations in chronic myeloid leukaemia (CML), and their sensitivity to the tyrosine kinase inhibitor, imatinib. Dotted arrows indicate strategies proposed to target quiescent leukaemic stem cells (LSC), such as inducing quiescent LSC into cell cycle or targeting non-BCR-ABL1 signaling pathways in the LSC.
Osteoblast HSC niche

<table>
<thead>
<tr>
<th>Stem cell Factor</th>
<th>cKit</th>
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<tr>
<td>Thrombopoietin</td>
<td>Mpl</td>
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<tr>
<td>TGF-beta</td>
<td>TGF receptor</td>
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<td>Wnt ligands</td>
<td>Beta-Catenin</td>
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<td>Prostaglandin E2</td>
<td>E2/E4 receptor</td>
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<td>Notch1/Jagged</td>
<td>Notch receptor</td>
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<td>Angiopoietin</td>
<td>Tie2</td>
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<td>Osteopontin</td>
<td>Beta1 integrin</td>
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- HSC
- Interferon alpha
- Interferon gamma
- Cell Cycle Entry & Differentiation
- Early Hematopoietic Progenitors

Quiescent Self-Renewing HSC population
Hematopoietic stem cells, progenitor cells and leukemic stem cells in adult myeloproliferative neoplasms

2012


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