New insights into the pathogenesis of chronic myeloid leukaemia: towards a path to cure

N. Thielen*, G.J. Ossenkoppele, G-J. Schuurhuis, J.J.W.M. Janssen

Department of Haematology, VU University Medical Center, Amsterdam, the Netherlands,
*corresponding author: tel.: +31(0)20-4442604, fax: +31 (0)20-4442601, e-mail: n.thielen@vumc.nl

KEYWORDS
Chronic myeloid leukaemia; leukaemic stem cells; molecular pathways; pathogenesis

INTRODUCTION

Chronic myeloid leukaemia (CML) is a myeloproliferative clonal disease arising at the level of a pluripotent stem cell. It is consistently associated with the presence of the Philadelphia (Ph) chromosome, which was first described by Peter Nowell and David Hungerford in 1960 and was named after the city where they discovered it.1 In 1973, it was Janet Rowley who discovered that the Ph chromosome results from a (9;22)(q34;q11) reciprocal translocation that juxtaposes the \textit{c-abl} (\textit{ABL}) oncogene on chromosome 9 with the \textit{breakpoint cluster region} (\textit{BCR}) on chromosome 22.2,3 The resulting mRNA molecules, encoded by this newly formed \textit{BCR-ABL} gene, results in the formation of the \textit{BCR-ABL} protein responsible for the disease entity CML. Depending on the breakpoint on the \textit{BCR} gene, either a p210 fusion protein (M-bcr breakpoint) or a p190 fusion protein (m-bcr breakpoint) is generated. The p210 fusion protein is most common in CML, while the p190 fusion protein is mostly generated in acute lymphoblastic leukaemia.4 The \textit{BCR-ABL} protein has constitutive kinase activity and is considered to be essential for the survival and growth of leukaemic cells.5 By triggering multiple downstream signalling pathways \textit{BCR-ABL} promotes cell proliferation and transformation, suppresses apoptosis, alters cell adhesion to bone marrow stroma and induces genetic instability.5,6 Patients with CML may present with night sweats, fatigue, abdominal fullness, gout, leucocytosis and splenomegaly, but half of the patients are accidentally diagnosed by blood testing for other reasons. A massive accumulation of immature and mature myeloid cells is present in peripheral blood, bone marrow and spleen. In most cases, the disease initially presents in a relatively well-tolerated chronic phase, when functionally normal mature blood cells are produced. However, if inadequately treated or therapy resistant, CML evolves into an accelerated phase and will eventually progress to a rapidly fatal blast crisis, in which cell differentiation is blocked.7 In this phase, the disease either resembles acute myeloid (two thirds of cases) or acute lymphoblastic leukaemia (one third of cases).5 Before the introduction of the tyrosine kinase inhibitor (TKI) imatinib mesylate (IM), conventional treatment consisted of spleen irradiation, hydroxycarbamide and busulfan or interferon-alpha (IFN-alpha). Of these, only IFN-alpha was able to induce cytogenetic responses in around 20% of patients. Allogenic stem cell transplantation was (and still is) the only potentially curative treatment of the disease but, due to age restrictions and donor availability, only a minority of patients were eligible for this potentially dangerous treatment.8 The introduction of IM in the early 21st century revolutionised the treatment of CML. In the vast majority of patients, IM treatment induces cytogenetic and even molecular responses with very low or undetectable \textit{BCR-ABL} transcript levels. These patients remain free from progression to blast crisis. However, IM does not cure the disease because it is unable to eradicate the leukaemic stem cells (LSCs), which therefore provides a potential reservoir for relapse.7 Likewise, the LSC is not affected by the recently introduced second-generation TKIs nilotinib and dasatinib.9 Because CML was the first malignant disease to be associated with a pathognomonic genetic abnormality, it is one of the most extensively investigated malignancies. Studying CML has not only unravelled the molecular pathogenesis of this disease but it also provided a framework for an increased understanding of molecular events involved in cancer initiation and progression of many other malignancies.7 In this review we present current insights into the pathogenesis of CML and discuss
novel molecular targets for therapy, with the emphasis on elimination of CML stem cells.

**PATHOGENESIS OF CHRONIC PHASE CML**

The first evidence that the Ph chromosome alone was sufficient to initiate chronic phase CML came from an experiment in which murine bone marrow was transplanted in lethally irradiated mice after infection with a retrovirus encoding \( BCR-ABL \). The transplanted mice showed several haematological malignancies including a CML-like myeloproliferative disease.\(^{15}\) This was underscored in studies introducing forced \( BCR-ABL \) expression by viral gene transduction in human haematopoietic cells after transplantation in mice.\(^{16}\)

Although CML is considered to be a stem cell disease and the \( BCR-ABL \) translocation is presumed to be present in the leukaemic stem cell, CML cells differentiate down the myeloid lineages with almost all myeloid cells bearing the Philadelphia chromosome, while the lymphoid compartment is only partly affected. Only 50% of mature B cells are Ph\(^+\) while virtually no T or NK cells carry the Philadelphia chromosome.\(^{17}\) As an explanation, it was postulated that BCR-ABL induces a loss of differentiation beyond the T-lymphoid progenitor stage, while the Ph-negative progenitors produce the remaining lymphocytes.\(^{18}\)

The remarkable finding that very low \( BCR-ABL \) transcript levels are detected by ultrasensitive PCR techniques in the leucocytes of 30 to 75% of healthy adults and in several cell lines suggests that the generation of \( BCR-ABL \) translocations is a relatively frequent event, but also that additional genetic or epigenetic changes are required to generate clinical chronic phase CML. Other explanations for this phenomenon may either be that the chromosome translocation in healthy individuals occurs in more committed progenitor cells with long-term self-renewal capacity which are unable to form a clone with leukemic potential, or that the immune system in CML patients is unable to recognise and eliminate the BCR-ABL expressing cells while it is adequate in normal persons.\(^{14}\)

Why the \( BCR \) and \( ABL \) genes translocate is unknown but the relative closeness of non-homologous \( ABL \) and \( BCR \) genes in interphase nuclei of bone marrow cells could be an explanation.\(^{19}\)

**CML STEM CELLS**

Haematopoietic stem cells (HSCs) are defined by their two properties: they are capable to reproduce themselves, a property known as self-renewal, and they have the capability to give rise to all mature haematopoietic cell lineages throughout an individual’s lifetime. This means that one of the daughter cells retains its HSC identity, while the other daughter cell becomes a multipotent progenitor. Hereby, life-long haematopoiesis is provided.\(^{20}\)

More than half a century ago, in 1951, it was William Dameshek who first suggested that CML cells derive from an HSC\(^{21}\) and this concept is still considered to be correct. The \( BCR-ABL \) translocation alone is sufficient to initiate chronic phase CML as is evidenced by several mouse model experiments that either used transplantation with donor mice derived \( BCR-ABL \) transected bone marrow cells, or \( BCR-ABL \) transgenic animals. The BCR-ABL tyrosine kinase activity results in aberrant stem cell differentiation and survival with a subsequent expansion of the progenitor pool and their downstream progeny.\(^{10,18,19}\)

Although their progeny may be normally sensitive to both chemotherapy and TKIs, LSCs are quiescent, non-cycling cells that are inherently unsusceptible to chemotherapy and TKIs. Several mechanisms are responsible for this IM resistance. Firstly, a higher \( BCR-ABL \) mRNA and protein expression (up to 100 fold and 3-10 fold, respectively) was found in the most primitive compartment compared with more committed progenitor cells.\(^{22}\) This may lead to insufficient inhibition of its kinase activity by IM and thereby to reduced cell killing. In more advanced CML, there is an increase in the average expression of BCR-ABL, while the large difference between the most primitive stem cells and the more committed progenitor cells remains. This high BCR-ABL expression parallels the autocrine production of IL-3, G/GM-CSF which only occurs in the most primitive stem cells.\(^{23}\) Possibly, this autocrine loop offers additional protection of stem cells against IM. Secondly, IM influx into the most primitive stem cells is hampered by their very low expression of their influx pump Oct-1; levels of Oct-1 transcripts in more mature progenitor cells were more than 100-fold higher.\(^{24,25}\)

Thirdly, the mRNA levels of the \( ABCB1 \) (P-glycoprotein) and \( ABCC2 \) (BCRP) efflux pumps for which IM is a substrate were twofold higher in the most primitive cells compared with progenitors.\(^{26}\) Fourthly, it has been demonstrated that the LSC compartment may already harbour several resistant \( BCR-ABL \) mutated stem cells before initiation of TKI therapy, which may thus confer a growth advantage after TKI treatment is started.\(^{27}\) Lastly, in sharp contrast with the above-mentioned mechanisms, it was recently demonstrated by Corbin et al. that IM did abrogate tyrosine kinase activity in CML stem cells. However, CML stem cells could survive and proliferate even if BCR-ABL was inhibited, as long as cytokines were present. This implicates that LSCs may not be BCR-ABL independent and thus that non-TKI-based strategies for CML stem cell killing will be required.\(^{28}\)

Due to their persistence during and after therapy, LSCs form a potential reservoir for relapse, disease progression
and resistance. This is supported by the work of Mahon et al. showing that 60% of IM-treated patients, who had had undetectable BCR-ABL transcripts for more than two years and who discontinued IM, rapidly relapsed, mostly within six months. Remarkably, almost 40% of patients did not relapse within one year, as will be discussed further below.

Stem cells can be distinguished from more committed progenitor cells by using long-term culture assays in which stem cells are considered those that are still able to form colonies after at least five weeks of culture; these are called long-term culture initiating cells (LTCICs).

As treatment of CML restores normal haematopoiesis in the vast majority of patients, residual normal stem cells must be present at the time of diagnosis. Frequencies of both Ph- and Ph+ LTCICs are lower than in normal bone marrow, but the residual normal stem cells in CML have higher self-renewal capacity than the leukaemic stem cells. This is evidenced by long-term marrow cultures where the Ph population outgrows their malignant counterparts in many cases. Diminished self-renewal capacity of CML stem cells correlates with autocrine IL-3 and G/GM-CSF induced increased cycling. This in turn leads to a vastly enlarged pool of progenitor cells, resulting in a massive accumulation of myeloid cells in chronic phase CML, despite the reduction in leukaemic stem cell numbers.

Apart from discrimination of either leukaemic or normal stem cells through long-term cultures, LSCs can be distinguished from normal HSCs by their immunophenotypic properties. Both LSCs and HSCs reside in the CD34+CD38-Lin- population as is evidenced by in vivo transplantation experiments with irradiated, severely immunocompromised mice. Recently, we demonstrated that LSCs have higher CD34 and CD45 expression than normal HSCs and have different forward/sideward light scatter properties. Moreover, we showed that LSCs may have aberrant expression of CD7, CD11b and CD56, while these markers are never expressed by normal HSCs. Furthermore, LSCs express higher levels of Thy-1 (CD90) compared to residual normal HSCs.

**Figure 1.** Schematic representation of signalling pathways involved in BCR-ABL mediated leukaemogenesis. Inhibitory pathways are shown as —, activating pathways as —. Dashed arrows represent translocation of proteins from cytoplasm to nucleus and vice versa. To improve readability of the figure, only the main intermediates of the signalling pathways are shown.
BCR-ABL ONCO PROTEIN AND ITS CRITICAL DOWNSTREAM MOLECULAR PATHWAYS

BCR-ABL has constitutive tyrosine kinase activity and drives several important downstream signalling pathways, necessitating a cooperative interplay to cause leukaemogenic potential. Eventually, most of them converge at the level of transcription factors, such as STAT proteins, c-Myc and Bcl-2 family, cooperating in leukaemogenesis and conferring a crucial role in the maintenance of LSCs (figure 1). In the next paragraphs we will highlight the most important pathways or proteins involved in CML and its transformation to blast crisis and describe the newest insights into the molecular pathology of CML.

Jak-STAT pathway
The Jak-STAT pathway is the principal signalling mechanism for a wide array of cytokines and growth factors, such as IL-3 and G/GM-CSF. Binding to their receptors results in Jak2 activation, leading to recruitment and activation of signal transducers and activators of transcription (STAT) factor families. Subsequently, STAT proteins migrate into the nucleus where they regulate transcription of genes that are involved in cell proliferation and survival, such as cyclin D1, cyclin D2, Bcl-X, c-Myc and NFkB (figure 1). In CML, Jak2 and STAT5 are constitutively activated. There are two ways in which BCR-ABL affects the Jak-STAT pathway. Firstly, although IL-3 and G/GM-CSF production is very low in the quiescent leukaemic progenitors, they may spontaneously enter the cell cycle and at that moment start producing autocrine active IL-3 and G/GM-CSF, leading to STAT activation via the normal route. Secondly, BCR-ABL is also able to directly activate STAT5, thereby bypassing Jak2. In turn, Jak2 is also able to activate BCR-ABL. In this context, it is important that in vitro Jak2 knockdown or Jak2 inhibition drastically reduced the BCR-ABL level and BCR-ABL activation, causing reduction of oncogenic signalling. In addition, inhibition of Jak2 can also overcome IM resistance in resistant cell lines, including T315I mutants (a highly resistant mutant) and blast crisis cells, paving the way for a role of Jak2 inhibitors in clinical CML.

Wnt/β-catenin
The Wnt/β-catenin signalling pathway is important for HSC self-renewal. Activation of this pathway is a hallmark for CML. Under normal conditions, β-catenin binds to the complex containing axin and the enzyme glycogen synthase kinase-3β (GSK3β). After serine/threonine phosphorylation it is degraded by the proteasome. Direct BCR-ABL mediated activation of β-catenin by phosphorylation of tyrosine residues Y86 and Y654 (Y phosphorylation) renders the free protein more stable and prevents its proteasomal degradation. Activated β-catenin then translocates to the nucleus where it interacts with lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factor, subsequently regulating the transcription of genes such as c-Myc and cyclin D1 (figure 1). In CML, especially in the accelerated phase and blast crisis, the granulocyte-macrophage progenitor pool has elevated levels of nuclear β-catenin compared with normal progenitors, for which a mutation in the β-catenin inactivating enzyme GSK3β seems to be responsible. Treatment with IM impairs Y phosphorylation and increases β-catenin binding affinity to the axin/GSK3β degradation machinery, resulting in degradation of β-catenin by the proteasome and thereby in normalisation of β-catenin levels. In vitro cell transduction with axin, a strong β-catenin antagonist, reduced the replating capacity of leukaemic cells. Altogether, β-catenin activation may play an important role in CML progression and IM resistance. Targeting β-catenin in synergy with IM might be of therapeutic value for CML patients, including IM-resistant patients.

ICSBP/Gas2
Another connection of CML with the Wnt-β-catenin pathway is via interferon consensus sequence binding protein (ICSBP), an interferon regulatory transcription factor. In CML, decreased expression of ICSBP is associated with poor prognosis, drug resistance and progression to blast crisis. Recently, the growth arrest specific 2 (Gas2) gene was identified as an ICSBP target. Gas2 expression, induced by ICSBP downregulation, inhibited calpain protease activity (a cysteine protease which modulates p53 levels), subsequently increasing stabilisation and activation of β-catenin (figure 1). This pathway may cooperate with the β-catenin inducing pathways described in the previous paragraph and suggests a still undetermined pro-leukaemic role of Gas2. Remarkably, we found high Gas2 levels in primary CD34+ cells of bone marrow and peripheral blood in chronic phase CML compared with normal CD34+ cells (unpublished data).

PP2A
Protein phosphatase 2A (PP2A) is a serine/threonine phosphatase with tumour suppressor activity. It acts by reversing kinase-induced phosphorylation of several key proteins involved in signal transduction pathways regulation, cell cycle progression, DNA replication, gene transcription and protein translation. BCR-ABL indirectly down-regulates PP2A via induction of the SET protein which is a physiological PP2A inhibitor (figure 1). IM treatment restores PP2A levels. The mutual antagonists
BCR-ABL and PP2A share several downstream targets essential for leukaemogenesis, among which are Rb, c-Myc, STAT3, ERK1/2, Akt, MAPK, BAD and JAK2. An essential part of PP2A tumour suppressor activity is the dephosphorylation of the serine-62 (S62) residue of the oncogenic transcription factor c-Myc by PP2A, resulting in an unstable c-Myc form. In this respect, it is quite interesting that fingolimod (FTY720), a PP2A activator, induced apoptosis and impaired clonogenicity of IM/dasatinib sensitive and resistant myeloid and lymphoid cell lines and in CML blast crisis and Ph+ALL progenitors, while normal CD34+ haematopoietic progenitors were not affected. Another recently described endogenous inhibitor of PP2A, cancerous inhibitor of PP2A (CIP2A), protects this c-Myc S62 residue from dephosphorylation by PP2A, thereby increasing c-Myc protein stability (figure 1). This appears relevant for CML, as was recently established by Lucas et al. They showed that CIP2A is a prognostic biomarker for CML progression under IM treatment: in patients who progressed to BC, CIP2A protein level at diagnosis was significantly higher than in good responders. Indeed, the probability of disease progression in these patients was 100% at 21 months. The above-mentioned studies suggest that CIP2A and PP2A qualify as therapeutic targets in CML.

C-Myc

The upregulation of the proto-oncogene product c-Myc plays a central role in perturbing CML signalling. Although c-Myc activity sensitises cells towards apoptosis induction, it stimulates proliferation by induction of several cell cycle activation enzymes. Due to disabled apoptotic pathways, mainly caused by concomitant upregulation of several antiapoptotic proteins, as described below, the net effect in CML is pro-oncogenic. Next to the role of PP2A, β-catenin and the Jak/STAT pathway on c-Myc stability in CML, several other pathways affect c-Myc expression. Firstly, BCR-ABL binds to adaptor proteins, such as GRB2, SHC, CRKL and CBL, after which this complex recruits the nucleotide exchange factor SOS. SOS in turn activates RAS which further signals through RAF, MEK and MAPK, eventually leading to increased c-Myc expression. Secondly, BCR-ABL forms a multimeric complex with Jak2, Gab2, PI3K and Akt. This so-called BCR-ABL network downregulates the kinase activity of GSK3β eventually resulting in NFκB activation and subsequent enhancement of c-Myc expression (figure 1). Lastly, BCR-ABL inhibits the expression of thanatoss-associated protein 11 (THAP11). THAP11 is a c-Myc transcription factor which mediates downregulation of c-Myc (figure 1). It was recently demonstrated that BCR-ABL suppresses THAP11 expression in CML cells, thereby promoting CML cell proliferation via upregulation of c-Myc. Silencing of BCR-ABL by kinase inhibitors or siRNA induced THAP11 expression and in turn repressed c-Myc expression.

Pathways leading to apoptosis resistance

Deregulation of apoptosis allows LSCs to propagate. In CML decreased sensitivity towards apoptosis is a final consequence of BCR-ABL tyrosine kinase activity, and mainly involves the aberrant expression of the Bcl-2 family of apoptosis regulator proteins, such as the antiapoptotic members Bcl-2, Bcl-XL and Mcl-1 and the pro-apoptotic members Bad and Bim (figure 1). The antiapoptotic family members block translocation of cytochrome-c from mitochondria to the cytosol, thereby preventing execution of apoptosis via caspase activation. An important regulator of Bcl-2 superfamily-induced apoptosis in CML is the antiapoptotic PI3K/Akt pathway (see next sections). BCR-ABL-induced PI3K/Akt activation results in Bad phosphorylation. Bad then dissociates from Bcl-2 and binds to the 14-3-3 adaptor protein, leaving less free Bad available for heterodimer formation with the antiapoptotic protein Bcl-XL (figure 1). Consequently, more antiapoptotic Bcl-2 and Bcl-XL remains in the cytoplasm, preventing cytochrome-c efflux from the mitochondria and subsequent apoptosis induction. Next, activation of STAT3 by BCR-ABL induces Bcl-XL expression, in turn contributing to apoptosis resistance of BCR-ABL expressing cells. Furthermore, the Bcl-2 inhibitor and antagonist Bim, an important downstream target supporting cell survival, is downregulated by BCR-ABL, which in turn upregulates Bcl-2, again preventing execution of apoptosis (figure 1).

Pathways related to adhesion

Normal adhesion of HSCs and progenitor cells to the bone marrow microenvironment is regulated by interaction of integrins and other cell surface receptors with protein components of the extracellular matrix (ECM), such as fibronectin. This interaction not only aims to retain the cells in the bone marrow until they are mature, but also allows different intracellular signal pathways to modulate cellular functions, such as proliferation, migration and apoptosis. In CML, BCR-ABL induces altered adhesion to the ECM, resulting in release of immature cells into the peripheral blood, which is a characteristic feature. This relates to downregulation by BCR-ABL of L-selectin, ICAM-1 and CCR7, proteins that are implicated in cell adhesion and motility. Moreover, BCR-ABL, in a multimeric complex with adaptor proteins, binds several key proteins involved in β-integrin signalling, such as focal adhesion kinase, Pyk-2, vinculin, talin and paxillin, thereby making these proteins unavailable for normal β-integrin mediated signalling transduction. As signalling through β-integrin is essential for the reorganisation of F-actin fibres in the cytoskeleton and...
their linkage to the ECM, perturbation of this signalling path results in diminished adhesion of CML cells to the bone marrow microenvironment and in increased cellular motility.66,67

Perturbation of this process may also be important for another reason: normal β1-integrin signalling upregulates the cyclin dependent kinase inhibitor P27KIP1, which in turn inhibits cyclin-dependent kinase-2 (CDK-2), preventing cells from entering the cell cycle. Disrupted β1-integrin signalling thereby provides an additional mechanism by which BCR-ABL stimulates entry into the cell cycle (figure 1).62

Pathways involved in stem cell maintenance
Hedgehog, BMI-1 and Notch

Since the introduction of TKIs and the knowledge that LSCs can persist during treatment, efforts towards determining the molecular pathways that are critical for stem cell maintenance have been intensified. Recent studies showed that the developmental Hedgehog (Hh) pathway, known for its role in embryonic development, tissue regeneration and repair, plays a crucial role in governing the maintenance of leukaemia-initiating cells.63,64 Hh proteins mediate signal transduction in nearby and distant tissues by binding to their specific receptor Patched (PTC). PTC negatively regulates Smo, an Hh intermediate. After binding of Hh proteins to PTC, Smo is released from the inhibition of PTC, and is now able to activate the pathway, resulting in Gli transcription factor mediated transcription of target genes, such as Ptch1, cyclin-D1 and Bcl-2 (figure 1).63,65 Hh signalling is regulated by GSK3β, which may be important in view of recent data on this enzyme in advanced-phase CML.66 Noteworthy, the investigators found no significant effect of treatment with IM on the expression levels of the diverse proteins involved in the Hh pathway.63 A recent study showed that the expression of Hh ligand Sonic hedgehog (Shh), Smo and the transcription factor Gli1 were significantly higher in CML stem cells than in normal controls, with even higher levels in advanced stages of the disease, suggesting a role of the Hh pathway in disease progression. Interestingly, deleting Smo in a CML murine model resulted in depletion of LSCs, but not of normal HSCs.64 Combination therapy of nilotinib and the Smo-inhibitor cyclopamine reduced human and mice LSCs and prolonged time to relapse threefold after ending treatment compared with nilotinib monotherapy. This indicates that Smo inhibition might reduce the LSC pool.64,67

Another gene that is upregulated in CML is BMI-1, a member of the Polycomb group (PcG) of genes. BMI-1 acts as an inhibitor of cyclin D1.63 Hh signalling is regulated by GSK3β, which may be important in view of recent data on this enzyme in advanced-phase CML.66 Noteworthy, the investigators found no significant effect of treatment with IM on the expression levels of the diverse proteins involved in the Hh pathway.63 A recent study showed that the expression of Hh ligand Sonic hedgehog (Shh), Smo and the transcription factor Gli1 were significantly higher in CML stem cells than in normal controls, with even higher levels in advanced stages of the disease, suggesting a role of the Hh pathway in disease progression. Interestingly, deleting Smo in a CML murine model resulted in depletion of LSCs, but not of normal HSCs.64 Combination therapy of nilotinib and the Smo-inhibitor cyclopamine reduced human and mice LSCs and prolonged time to relapse threefold after ending treatment compared with nilotinib monotherapy. This indicates that Smo inhibition might reduce the LSC pool.64,67

Another gene that is upregulated in CML is BMI-1, a member of the Polycomb group (PcG) of genes. BMI-1 is a gene implicated in stem cell renewal and proliferative activity of normal and leukaemic stem cells and a repressor of the tumour suppressor complex p16INK4a/p14ARF (figure 1). In CML CD34+ cells, the level of expression of BMI-1, correlated with transformation to blast crisis in the non-transplanted patient and thereby affected prognosis.68 The opposite was observed in CML patients treated with an allogenic stem cell transplantation. High BMI-1 expression prior to transplantation was associated with better overall survival, without a significant association with relapse: the high BMI-1 expression was shown to be inversely associated with increased non-relapse mortality, especially due to diminished incidence of acute graft versus host disease. This suggests that PcG genes are involved in immune regulation.69-72

Activation of Notch, a transmembrane receptor that is also involved in stem cell maintenance, has been described in human acute leukaemias. As with Hedgehog and wnt signalling, Notch signalling is regulated by GSK3β.66,70 Upon binding of its ligands, Notch releases its intracellular domain NIC, which in turn enters the nucleus and associates with transcription factors (figure 1).71 Notch not only has a contradictory role in differentiation of normal haematopoietic cells: in different malignancies, both oncogenic and tumour suppressor roles have been described.71 The role of Notch in CML is also controversial, since in one study, activated Notch signalling inhibited cell proliferation and reduced the ability of colony forming, suggesting an inhibitory effect on LSCs, while in another study activated Notch participated in the evolution from chronic phase CML to blast crisis.72,73 Clearly, further research is needed to establish the role of BMI-1 and Notch in CML.

Promyelocytic leukaemia tumour suppressor protein

Recently, Ito et al. showed for the first time that promyelocytic leukaemia tumour suppressor protein (PML) expression was high in CML CD34+ stem cells and was inversely associated with clinical outcome. PML acts as a repressor of the mammalian target of rapamycin (mTOR), which in turn plays a role in HSC maintenance and leukaemogenesis (figure 1). In vitro and in vivo data regarding inhibition with the PML inhibitor As2O3 showed disruption of LSC maintenance, resulting in impaired quiescence and sensitisation of LSCs to pro-apoptotic stimuli, probably making As2O3 a potential beneficial therapeutic agent in CML.74

Pten pathway

A gene recently identified by microarray analysis of LSCs in CML is phosphatase and tensin homologue (Pten). Compared with HSCs from healthy donors, decreased transcriptional activity was found in LSCs in CML patients.75 Pten dephosphorylates phosphatidylinositol
in pancreatic cancer cell lines it was shown that Pten PI3K activity and activation of Akt, eventually promoting inactivation decreases its phosphatase activity, favouring to suppress development of several tumours. Thus, Pten inhibited more differentiated leukaemia cells. Remarkably, of CML in this model. Zileuton targeted the LSC while IM and IM. The combination proved to be better than treatment of CML mice with the Alox-5 inhibitor zileuton effect on LSCs. 77 This led to the use of combination Most importantly, Alox5 deficiency had a specific inhibitory role on myelopoiesis and modulation of proliferation and pathological processes, such as oxidative stress response, production of leukotrienes, such as leukotriene C4 5-LO, which is encoded by the Alox5 alox5 pathway and leukotrienes and is believed to stimulate cell growth. 75

**Alox5 pathway**

5-LO, which is encoded by the Alox5 gene, induces production of leukotrienes, such as leukotriene C4 (LTC4). Next to their role in numerous physiological and pathological processes, such as oxidative stress response, inflammation and cancer, they also have a stimulating role on myelopoiesis and modulation of proliferation and apoptosis in haematopoietic cells.77-78 Alox5 is upregulated by BCR-ABL (figure 1). It is questionable if this is a tyrosine kinase mediated mechanism, as IM does not abolish this phenomenon. Possibly, this effect provides a further explanation why LSCs are insensitive to TKIs (see above). Anyhow, Alox5 seems pivotal in CML leukaemogenesis as BCR-ABL transduced Alox5−/− mice failed to develop CML. Most importantly, Alox5 deficiency had a specific inhibitory effect on LSCs.77 This led to the use of combination treatment of CML mice with the Alox-5 inhibitor zileuton and IM. The combination proved to be better than treatment with either drug alone in prolonging survival of CML in this model. Zileuton targeted the LSC while IM inhibited more differentiated leukaemia cells. Remarkably, loss of Alox5 also caused downregulation of β-catenin expression in LSCs (see above). These data indicate that targeting the Alox5 pathway might be a rational approach for optimising CML treatment.79 Phase 1 studies with the combination of imatinib and zileuton are already planned.

**FoxO pathway**

Lastly, a novel player in the field is the Forkhead-O (FoxO) subfamily of transcription factors. They play an important role in haematopoiesis and regulate diverse physiological processes, such as cell-cycle arrest, stress resistance, apoptosis and self-renewal capacity of HSCs. All four FoxO members (FoxO1, FoxO3, FoxO4 and FoxO6) act downstream of the PI3K/Akt pathway.80 They are phosphorylated by Akt upon growth factor stimulation or insulin, resulting in nuclear export and as a consequence FoxO is degraded in the cytoplasm (figure 1). In the absence of growth factors or insulin, unphosphorylated FoxO members reside in the nucleus and act as transcription factors, resulting in pro-apoptotic signalling.81 In mice, individual FoxO1 and FoxO4 knockout mouse did not show an overt haematopoietic phenotype, but triple loss of FoxO members (FoxO1, FoxO3, FoxO4) caused defective long-term repopulation activity, correlating with increased cell cycling and apoptosis of HSC.82 In CML, BCR-ABL activates the PI3K/Akt pathway and this in turn suppresses the FoxOs, thereby supporting the proliferative and antiapoptotic properties of CML cells. Using mouse models, Naka et al. showed that particularly FoxO3a has a role in the maintenance of CML LSCs. Cells with nuclear FoxO3a and decreased Akt phosphorylation were enriched in the stem cell compartment. In addition, transforming growth factor-β (TGF-β) acts as a crucial regulator of Akt activation and controls FoxO3a localisation in CML LSCs. The combination of TGF-β inhibition and IM efficiently depleted LSCs and attenuated CML development.83 Also, degradation of FoxO3A by the proteasome plays an important role in suppression of FoxO. In line with that, treatment with bortezomib, a proteasome inhibitor, resulted in increased levels of FoxO and led to a complete molecular remission in a case of Ph+ acute lymphoblastic leukaemia.51

**MOLECULAR AND CELLULAR EVENTS INVOLVED IN TRANSFORMATION TO BLAST CRISIS**

In the eight-year follow-up of the IRIS trial, around one third of the patients discontinued IM due to primary or secondary resistance or IM intolerance. These patients are at risk for disease progression to accelerated phase or blast crisis.84 The processes responsible for this transformation

Thielen, et al. Pathogenesis of CML.
are still not fully understood although it is generally believed that unrestrained and increasing BCR-ABL activity causes genetic instability and ultimately promotes clonal evolution. BCR-ABL overexpression is considered to be the result of a multistep, time-dependent process, characterised by a multiplicity of genetic and epigenetic events. Genetic abnormalities include the presence of additional chromosomes, gene deletions and insertions, point mutations of which doubling of the Philadelphia chromosome, loss of 17p and trisomy 8 are the most common. It has been hypothesised that accelerated shortening of telomeres, particularly in the context of increased telomerase activity, might facilitate acquisition of these genetic aberrancies. On the molecular level, mutations at the cyclin-dependent kinase inhibitor 2A/2B (CDKN2A/B) and Ikaros transcription factor (IKZF1) are most common in lymphoid blast crisis. Relevant data concerning transformation of stem cells were demonstrated by Jamieson et al. Progression to blast crisis is associated with expansion of the myeloid progenitor compartment, which aberrantly requires self-renewal resulting in LSC generation and blast crisis transformation. This correlated with increased β-catenin levels and upregulation of β-catenin target genes expression, due to inactivating mutations in the GSK3β protein, as described above. Furthermore, progression to blast crisis was avoided in a β-catenin knockout mouse model of CML. Changes similar to those seen with β-catenin are seen for BCR-ABL; while increases of BCR-ABL levels due to amplification are seen in granulocyte-macrophage progenitors, they stay relatively constant in LSCs during progression. Epigenetic changes, such as increased methylation of several genes, have also been reported. Apparently, genomic instability is also a feature of the transformation process which may be related to the BCR-ABL induced increased production of reactive oxygen species and abnormal DNA repair mechanisms.

Finally, recent data show an important role of the RNA binding protein Musashi2 (Ms2) in CML progression. The expression of Ms2 is highly upregulated during human CML progression to blast crisis and is an indicator for poor prognosis. Ms2 represses Numb expression, a protein which drives commitment and differentiation and impairs development and propagation of blast crisis. Inhibition of Ms2 expression in mice restored Numb expression and prolonged survival.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Inhibitor</th>
<th>Activator</th>
<th>Substrate</th>
<th>Possible combinations</th>
<th>LSC/HSC specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAK-STAT</td>
<td>Diverse Jak2 inhibitors</td>
<td>Jak2</td>
<td>IL-3</td>
<td>DTT388I3 and imatinib or dasatinib</td>
<td>LSC &gt; HSC</td>
<td>111</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>Cycloamnpe</td>
<td>FTY720</td>
<td>PP2A</td>
<td>Proteasome inhibitor and imatinib</td>
<td>LSC</td>
<td>85</td>
</tr>
<tr>
<td>HH</td>
<td>Bortezomib</td>
<td>STAT5/Bcl-2</td>
<td>Smo</td>
<td>Cycloamnpe and nilotinib</td>
<td>LSC &gt; HSC</td>
<td>62</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>Bortezomib/</td>
<td>Bortezomib/</td>
<td>PP2A</td>
<td>Proteasome inhibitor and imatinib</td>
<td>LSC</td>
<td>85</td>
</tr>
<tr>
<td>PP2A</td>
<td>Cycloamnpe</td>
<td>FTY720</td>
<td>PP2A</td>
<td>Proteasome inhibitor and imatinib</td>
<td>LSC</td>
<td>85</td>
</tr>
<tr>
<td>PML</td>
<td>Arsenic trioxide</td>
<td>PML</td>
<td>mTOR</td>
<td>Arsenic trioxide and cytarabine</td>
<td>LSC</td>
<td>60</td>
</tr>
<tr>
<td>Alox-5</td>
<td>Zileuton</td>
<td>5-LO</td>
<td>Zileuton and IM</td>
<td>LSC</td>
<td>64,65</td>
<td></td>
</tr>
<tr>
<td>Alox-5</td>
<td>ETYA (AA analogue)</td>
<td>5-LO</td>
<td>5-LO</td>
<td>LY364947 and TGF-β</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>PI3K/Akt</td>
<td>Rapamycin</td>
<td>mTOR</td>
<td>COX2</td>
<td>Arachidonic acid</td>
<td>LSC</td>
<td>67</td>
</tr>
<tr>
<td>FoxO</td>
<td>Bortezomib</td>
<td>Proteasome</td>
<td>TGF-β</td>
<td>TGF-β-inhibitor and imatinib</td>
<td>LSC</td>
<td>68</td>
</tr>
</tbody>
</table>

PML = promyelocytic leukaemia tumour suppressor protein; LSC = leukaemic stem cells; HSC = haematopoietic stem cells; IM = imatinib mesylate.
CONCLUDING REMARKS

After the introduction of IM, CML has turned from an often fatal disease into a generally easily treatable chronic condition for most patients. Despite this great success, there are still problems that need to be addressed: around 45% of patients discontinue IM due to toxicity or unresponsiveness and approximately 8% of patients suffer from disease progression to the accelerated phase/blast crisis. The second-generation TKIs nilotinib and dasatinib are able to resolve side effects and to overcome resistance seen with some BCR-ABL mutations, but, like IM, are unable to eradicate the stem cell pool either.

CML LSCs are thought to be responsible for this TKI resistance and disease progression. They remain untouched by IM and other chemotherapy due to several mechanisms, described above. This allows LSCs to propagate and renders CML currently incurable. Searching for strategies to kill these LSCs and thereby achieving a cure of CML is the main focus of the current CML research.

A straightforward strategy for targeting LSC is through inhibition or activation of key molecules which lead to inhibition or restoration of signalling pathways, which play a role in regulation of both normal HSCs and LSCs. Examples of key molecules against which promising inhibitors or activators have already been developed are Jak2, PP2A, Hedgehog/Smo, 5-LO, PML, mTOR and FoxO (table 1). Combining several of these agents, possibly in combination with TKIs, may prove to be synergistic against CML stem cells as already suggested by in vitro and in vivo data. However, a potential problem of these strategies is that normal HSCs and LSCs share many common properties which means that signalling pathways determining the cell fate of LSCs also govern maintenance of normal HSCs. Therefore, normal HSCs may simultaneously be affected, leading to severe side effects. Accordingly, the next goal is to target genes that may simultaneously be affected, leading to severe side effects. Consequently, the complex pathogenesis of CML is slowly but steadily being unravelled. Although the introduction of TKIs has dramatically improved the prognosis of CML patients, many problems remain to be solved. Cure can still not be attained because TKIs are unable to eradicate the LSC compartment. Efforts towards determining the molecular pathways critical for the maintenance of these cells, as well as to develop better and faster techniques to differentiate the LSC from the normal HSC, have intensified the last decade. Using this knowledge will aid in development of new strategies to recognise and kill LSCs, hopefully progressing towards cure of CML.

REFERENCES


Thielen, et al. Pathogenesis of CML.


