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REVIEW

Chronic myeloid leukemia stem cells and developing therapies

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Abstract
Chronic myeloid leukemia therapy has remarkably improved with the use of frontline BCR–ABL kinase inhibitors such that newly diagnosed patients have minimal disease manifestations or progression. Effective control of disease may also set the stage for eventual 'cure' of this leukemia. However, the existence of Philadelphia chromosome-positive leukemic cells that are unaffected by BCR–ABL inhibition represents a major barrier that may delay or prevent curative therapy with the current approaches. The most commonly reported mechanism of resistance to tyrosine kinase inhibitor-based therapies involves BCR–ABL gene mutations and amplification, but these changes may not be solely responsible for disease relapse when inhibitor-based therapies are curtailed. Therefore new targets may need to be defined before significant advancement in curative therapies is possible. Emerging evidence suggests that persistence of chronic myeloid leukemia stem cells or acquisition of stem cell-like characteristics prevents complete elimination of chronic myeloid leukemia by tyrosine kinase inhibition alone. This review focuses on several recently emerging concepts regarding the existence and characteristics of chronic myeloid leukemia stem cells. Definitions based on human primary cells and animal model studies are highlighted as are the potential signaling pathways associated with disease repopulating cells. Finally, several recently defined therapeutic targets and active compounds that have emerged from stem cell studies are described. Our goal is to provide an unbiased report on the current state of discovery within the chronic myeloid leukemia stem cell field and to orient the reader to emerging therapeutic targets and strategies that may lead to elimination of this leukemia.

Keywords: *Chronic myeloid leukemia, stem cell, therapy, signaling pathways, inhibitors*

Background

Research into the biology, etiology, and response to targeted therapy of chronic myeloid leukemia (CML) has provided significant milestones in our understanding of the genetics of cancer, the cell transformation process, and the benefits and consequences of targeted therapy [1,2]. CML was the first tumor identified with a specific cytogenetic or chromosomal abnormality, later named the Philadelphia chromosome. Subsequent studies demonstrated that the Philadelphia chromosome was a genetic rearrangement that resulted in formation of a chimeric gene composed of portions of the c-Bcr and c-Abl genes. This gene fusion resulted in expression of the BCR–ABL protein with unregulated tyrosine kinase activity. BCR–ABL transduction into bone marrow from naive animals resulted in the emergence of a leukemic-like disorder and generated interest in the development of BCR–ABL inhibitors that might be useful for treating BCR–ABL-expressing tumors. Ciba-Geigy pharmaceuticals first described STI-571 with promising inhibitory activity against c-Abl and BCR–ABL in 1996 [3]. Several years later, Novartis developed STI-571 as imatinib and demonstrated early clinical success in treating patients with CML and some forms of BCR–ABL+ ALL [4]. The limited side-effects and effectiveness of imatinib in leukemias 'addicted to BCR–ABL' demonstrated that targeted therapy was achievable, and reinforced the search for additional tumor-addictive targets that may be inhibited by small molecules [5]. Continuing clinical evaluation of imatinib demonstrated that some patients developed resistance to imatinib, which was rendered ineffective through amplification or mutation of the BCR–ABL oncogene, as well as other mechanisms [6,7]. This observation prompted

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evaluation of additional molecular events that may limit the application of targeted therapy in other cancers as well. Imatinib resistance has led to a robust pipeline of second- and third-generation kinase inhibitors that bypass imatinib resistance through specific molecular modifications [8–10]. Some of these next-generation inhibitors are also being tested against other cancers. Overall, it is clear that CML research and the application of kinase-specific targeted therapy to treat this disease have provided a wealth of unanticipated information in cancer biology and therapy.

Similar insight may be derived from more detailed studies of CML relapse in patients with minimal residual or undetectable disease. Patients in chronic phase typically achieve a major reduction in their Philadelphia chromosome-positive (Ph+) cells, and many will achieve even greater suppression of disease such that BCR–ABL transcripts are not detectable (i.e. no evidence of leukemia). The troubling observation is that patients must maintain imatinib therapy to continue in disease remission, as discontinuation of imatinib often results in disease relapse, even in patients with no evidence of leukemia [11–14]. This has given rise to the description of a leukemia repopulating cell that is protected from elimination by BCR–ABL inhibition alone [15]. Several lines of evidence suggest that disease repopulation occurs through the preservation of CML stem cells [16]. A comprehensive assessment of that evidence is the initial focus of this review. Part of this evidence has been derived from establishment of animal models enlisting BCR–ABL as the transforming event. Animal models have also been employed to identify specific genes that function in CML 'stemness' and disease repopulation [17]. The function of specific genes identified in primary CML specimens representing CML cells with disease repopulating activity has also been functionally evaluated in various animal models, and the contribution of specific pathways to CML 'stemness' has been gleaned from a combination of both approaches [17–19]. The pathways defined in these models are discussed. Lastly, as more information accrues from various approaches in defining and functionally evaluating CML cells for disease repopulating activity, many new and potentially appropriate therapeutic targets have emerged [17,18,20]. The underlying evidence for their involvement in maintaining or supporting stem cell function is discussed.

The genetic and biologic basis for CML and the successful application of targeted therapy for this disease have played a major role in understanding tumor biology and the concept of oncogene addiction. This has provided a greater understanding of cancer and its therapy than was previously anticipated. Given the tools and agents currently available to dissect this disease, as well as an alignment with animal models to test emerging concepts, it is anticipated that unraveling the nature of the CML stem cell will likely increase our understanding of stem cell behavior and biology in other diseases as well. This review attempts to illustrate that potential.

Chronic myeloid leukemia stem cells and their origin

A widely accepted characteristic in cancer biology is that a small portion of cancer cells within a tumor retain properties to initiate tumors. This characteristic has been defined as the cancer stem cell, and the properties involved include asymmetric cell division important for self-renewal and a deregulated ability to differentiate [21,22]. Although no universal immunophenotypic marker(s) exist that will identify all cancer stem cells, for leukemia it was described in acute myeloid leukemia (AML) that the CD34+/CD38− population is able to transfer human AML in immune-compromised mice, similar to normal hematopoietic stem cells [23,24]. It was later shown that Ph+ CML CD34+/CD38− cells efficiently engraft in NOD/SCID (non-obese diabetes/severe combined immunodeficiency) [25] and NOD/SCID/interleukin 2γ (IL-2γ) [26] immune-compromised mice. The latter study further demonstrated that patient samples required prescreening in the long-term culture initiating-cell system, as Ph+ leukemic stem cells are accompanied by normal stem cells that cannot be efficiently separated from Ph+ Lin−/CD34+/CD38− [26], which tend to overtake the Ph+ CML stem cells in the engrafted population. Therefore it is accepted that the leukemia stem cell in CML resides in the primitive human CD34+/CD38− population.

The proposition that the appearance of the Ph chromosome is the first genomic event in CML is still an open debate [27]. Murine retroviral [28] and transgenic BCR–ABL [29,30] models argue that BCR–ABL is a sufficient single oncogenic event in hematopoietic stem cells (HSCs) to be able to elicit the leukemia phenotype. New technologies such as comparative genomic hybrization (CGH) and single nucleotide polymorphism (SNP) arrays have not identified, as yet, early genetic events that may precede the appearance of the Ph chromosome. However, they have identified copy number changes in CML and deletions in Ph+ ALL associated with disease progression [31,32]. Early studies by Gunsilius *et al.* [33] suggested the possibility of a hemangioblastic (an early hematopoietic/endothelial stem cell) origin of the BCR–ABL fusion gene, since the Ph chromosome was detected in outgrowth endothelial cells from patients with CML. This was later supported by

reports using different investigational techniques [34,35]. However, a report by Otten *et al.* [36] brings this hemangioblastic origin into question. The prerequisite expression of CD34 on the long-term human HSC is also debated, as a Lin−/CD34− population is proposed to exist that contributes to hematopoiesis (reviewed in [37]). Such a Lin−/CD34− population was recently described in CML, where ~30% of these cells contained the BCR–ABL oncogene, and gene expression profiling suggested that this population overexpress pro-angiogenic factors, which may contribute to an increased differentiation potential in immune-deficient mice to endothelial cells as compared to normal Lin−/CD34− cells [38]. In addition, similar to CML leukemia stem cells, these cells were refractory to the tyrosine kinase inhibitor imatinib, with no change in the BCR–ABL/ABL ratio following treatment. Although further independent confirmation will be needed to resolve this issue, these observations allow the debate to continue as to the existence of a Ph leukemia stem cell of hemangioblastic origin.

A more recent observation in patients with CML, utilizing an individual patient DNA polymerase chain reaction (PCR) strategy with peripheral blood to identify genomic BCR–ABL, showed that although a small number of patients were able to maintain complete molecular response following cessation of imatinib, they remained positive for the BCR–ABL translocation [39]. Such a phenomenon has previously been reported in patients who received interferon, which was frontline before the introduction of imatinib [40], who retained a 'disease-free' existence while having mRNA BCR–ABL-positive cells [41,42]. These observations suggest a poorly understood intrinsic and/or extrinsic mechanism(s) of therapy-free leukemia suppression. Mechanisms may include an immune-protective activity and/or promotion of dormant leukemia stem cells [43]. Although BCR–ABL is able to promote loss of the long-term HSC in mice [44], which is in line with the observed loss of human Ph+ cells in immune-compromised mice engrafted with chronic phase CML leukemia stem cells [26], the debate continues as to whether complete loss of the Ph chromosome is required for a disease-free existence [43]. Continuing studies will hopefully define the earliest stem/progenitor that has the Ph chromosome and clarify whether the appearance of the Ph chromosome genetic alteration is preempted by an unknown genetic and/or epigenetic event.

Microenvironment

The bone marrow microenvironment is an essential contributor to the maintenance of the HSC. The microenvironment is reported to consist of an osteoblastic (endosteal) and vascular niche, which contains a variety of cells including osteoblast, osteoclast, perivascular reticular, adipocyte, and endothelium in addition to mesenchymal stem cells. Studies with leukemia cells have shown that co-culture on bone marrow derived stromal cells or conditioned media of stromal cells promotes resistance to chemotherapy [45,46]. The importance of the bone marrow niche for Ph+ ALL survival is also highlighted by evidence acquired in ARF-deficient mice. Using retroviral transduction of the p190 isoform of BCR–ABL the authors demonstrated that the bone marrow microenvironment of ARF-deficient mice was able to promote a more aggressive disease and protection against imatinib therapy that relied on IL-7 production by the microenvironment [47]. In addition, recent reports highlight the role of the cytokine transforming growth factor β (TGFβ) in maintaining normal hematopoietic [48] and CML stem cells [49] in the bone marrow niche, promoting a dormant state. Furthermore, deletion studies of Rho-family members of guanosine triphosphatases (GTPases) that regulate HSC migration, survival, adhesion, and proliferation through various signaling modules such as β1-integrin and c-Kit are not only important for HSC interaction with the bone marrow niche (reviewed in [50]), but are involved in BCR–ABL transformation [51,52] and BCR–ABL leukemia stem cell maintenance [53]. Hematopoietic stem cell maintenance signaling entities such as the Wnt/β-catenin, Hedgehog, and Notch have all been show to affect CML leukemia development, leukemia stem cell maintenance, and transformation to blast crisis [28,54–59]. The soluble factors and ligands of these signaling cascades are produced by various cells in the hematopoietic bone marrow niche [60,61], implying that multiple stem cell regulatory signaling events are involved in Ph+ leukemia stem cell biology.

Importantly, the bone marrow microenvironment is hypoxic (~1–6% O_2), under which its elements regulate HSC maintenance (reviewed in [62] and [63]). Hypoxic culture conditions promote resistance to BCR–ABL tyrosine kinase inhibitors in CML cell lines that gain attributes of stem cell quiescence and self-renewal, and change their metabolic mitochondrial activity to the anaerobic glycolytic process [64]. These changes are associated with an increased enzyme activity and expression of the protein glyoxalase-I (Glo-I), involved in detoxifying products from the glycolytic metabolic pathway, making the cells sensitive to a Glo-I inhibitor [64]. Resistance to imatinib in CML cell lines was also linked to changes in metabolic characteristics [65] due to deregulated induction of hypoxia inducible factor 1α (Hif1α) [66], associated with a change in oxidative metabolism to non-oxidative glycolytic metabolism [67] (aerobic

glycolysis or Warburg effect), through Hif1α regulation of genes involved in glycolysis, such as transketolase [66]. Apoptosis was achieved in these imatinib-resistant cells by combined treatment with imatinib and the agent oxythiamine that targets transketolase [66]. This change in mitochondrial oxidative cycles to glycolysis was recently demonstrated in the long-term HSC compartment in mice [68]. This adaptation relied on the activity of the Hox family transcription factor Meis1 and its positive control of Hif1α, an important transcriptional regulator of hypoxia responses involved in cancer biology [69]. Using Hif1α-deficient mice, Takubo *et al.* [70] also showed the role of Hif1α in regulating HSC maintenance. Evidence that the HSCs reside in the most hypoxic bone marrow region [71,72] next to the connection between hypoxic niches, Hif1α, Notch, and Wnt signaling (reviewed in [73]) show that the hypoxic environment is important in normal HCS maintenance. In summary, these observations support the hypothesis that a hypoxic niche and the Warburg effect regulate cancer and leukemia stem cell maintenance and chemoresistance [67,74,75], expanding the possibilities for therapeutic intervention [45,74].

Alterations in components of the bone marrow niche itself elicit defective hematopoiesis that can lead to myeloproliferative disease and/or leukemia. For example, deregulated Notch signaling in stromal elements through conditional elimination of the ubiquitin E3 ligase Mind bomb-1, which regulates Notch ligand stability, promotes a myeloproliferative disorder [76]. Further, a myeloproliferative syndrome develops when the retinoic acid receptor γ (RARγ) is deleted that is completely reliant on the RARγ-deficient microenvironment [77]. Similar conditions exist in Mx1-Cre-PTEN conditional deficient mice, where both stromal and stem cell intrinsic loss of PTEN (phosphatase and tensin homolog) are required for leukemia development [78]. More recently, a murine model of microenvironmental deregulated osteoblast lineage development promoted myelodysplasia that progressed to AML with acquired genetic abnormalities [79]. In this model the regulator of microRNA processing, Dicer [80], was conditionally deleted in osteoblast progenitors that differentiate from mesenchymal stem cells. The loss of Dicer specifically led to a rearrangement of the microenvironment, affecting B-cell development, promoting leukopenia of all leukocytes, and slightly increasing proliferation of HSCs [79]. These observations support the hypothesis that elements of the bone marrow microenvironment can influence disease development and/or progression with acquired genetic alteration(s). The question is: can deregulated microenvironmental signaling promote Ph+ CML? New evidence of a non-random, but specific recombination and fusion of the androgen-regulated gene TMPRSS2 to the transcription factor ERG or ETV1 gene in prostate cancer following androgen signaling and genotoxic insult [81,82] support the possibility that extracellular signaling impacts the nuclear architecture, i.e. chromosome three-dimensional localization and orientation. These results demonstrate that genotoxic stress combined with specific signaling conditions can promote a specific translocation event. Such conditions may conspire to promote the formation of the Ph chromosome.

Recently, Mohrin *et al.* demonstrated that quiescent murine HSCs use the error-prone non-homologous end-joining DNA repair mechanism following irradiation, leading to acquisition of chromosomal abnormalities, including translocations [83]. They observed that the primitive HSCs, contrary to their progenitors, are more radio-resistant to low-dose irradiation. High expression of pro-survival factors may underlie relative distinctions in radio-resistance between these populations. In contrast, human cord blood-derived HSCs are more sensitive to genotoxic stress, undergoing apoptosis and being unable to repopulate immune-compromised mice, a condition that can be reversed by overexpression of the pro-survival factor Bcl-2 [84]. Human HSCs display slower kinetics of DNA double-strand break repair (as measured by γH2A staining) than progenitors, suggesting that accuracy of DNA damage repair is important in this population. These observations highlight differences in HSC response to DNA damaging genotoxic stresses that rely on differential mechanisms of regulating DNA damage repair, proliferation, and survival. Milyavsky *et al.* [84] did not detect hematologic transformation in irradiated human HSCs in the NOD/SCID mouse model even when functional p53 levels were suppressed or Bcl-2 was overexpressed. This suggests that human cord blood HSCs are very efficient in DNA repair, thereby protecting against malignancy. However, development of a human hematologic malignancy in animals may be influenced by the age of the CD34+/CD38− population and/or NOD/SCID model. The NOD/SCID mouse model is sensitive to genetic modification of the microenvironmental niche, and will result in expansion of differential human leukemia lineages after bone marrow (BM) transplant [85].

Tyrosine kinase inhibitors targeting BCR–ABL appear to influence the bone marrow microenvironment [86]. This is highlighted by reports that imatinib [87–89] and the second-generation tyrosine kinase inhibitor dasatinib [90] preferentially affect osteoblast lineage development over osteoclasts, increasing the trabecular bone volume in patients with CML [88]. This may enhance the osteoblastic

niche's ability to maintain normal and Ph+ leukemia stem cells. Imatinib creates an ideal niche environment by enhancing the Ph+ leukemia stem cells' ability to home to the bone marrow environment by: (1) up-regulating CXCR4 [91], an important regulator of HSC bone marrow homing (reviewed in [92]), and (2) restoration of the deregulated CXCR4/LYN/BCR–ABL signaling, which promotes mobilization of the leukemia stem cells from the niche [93]. Therefore, both features of the disease and its frontline standard of care may influence the BM microenvironment essential for stem cell survival.

In summary, the bone marrow environment is influenced by many environmental and genetic elements as well as the nature of the stem cell population itself. It is not completely clear whether the normal and leukemia stem cells in Ph+ leukemia occupy the same niche or rely on the same signaling events required for survival and replication. Our interest in targeting CML stem cells may be complicated by these factors, but may benefit from further investigation of deregulated signaling, mimicry of the hypoxic hematopoietic niche, simulation of the environmental/genotoxic stress and/or inflammatory responses, and other conditions that can impact nuclear architecture and DNA repair mechanisms available in the stem cell niche. Greater understanding and simulation in appropriate animal and *in vitro* models will be essential in determining the cellular and niche targets and conditions that provide the best opportunity for eliminating CML stem cells.

Pathways activated in chronic myeloid leukemia stem cells

Multiple genes and enzymes have been described as potential regulators of CML stem cell self-renewal, differentiation, and apoptosis, and suggest a complex array of proteins and conditions that are essential to maintaining some degree of CML disease repopulating activity or stem cell activity [17,18,20]. Some of the CML-specific pathways associated with their ability to function in regulating stem cells may be relevant in only a subset of conditions and dependent upon the model used to generate BCR–ABL+ disease or its assessment of activity. For example, BCR–ABL has been used to initiate the transformation of naive bone marrow cells by a variety of transfer and expression techniques (reviewed in [17]). The characteristics of disease in recipient mice often resemble CML, although the stages of disease progression and mortality do not fully match clinical observations. Nonetheless, BCR–ABL+ cells from these animals are used to isolate cells with surface protein expression patterns that are associated with stem cell characteristics [15,94]. However, it is reported that CML 'stemness' may be derived from acquisition of stem cell function in more mature progenitors as well [28]. This observation suggests that animal models may reproduce only a subset of conditions that lead to disease repopulating activity. For this reason, the exclusivity of a gene's role in disease repopulation must factor in the conditions used to initiate, propagate, or recapitulate the disease. In addition, as disease propagation assays are conducted in immunocompromised animals, only cells with a specific level of aggressiveness capable of evading immunosurveillance may be scored as positive for disease repopulating activity. This appears to be one important consideration, as very different conclusions can be drawn when cells are analyzed in various immunocompromised backgrounds [95]. To fortify conclusions from animal models, primary CML specimens are often analyzed to confirm expression and function of a gene in tumor repopulating or stem cell activity assays. The other mechanism employed to assess a gene or pathway function in CML stem cell activity (often used to interrogate the activity of a drug or inhibitor) is to purify cells from patients with CML using cell surface markers that define BCR–ABL+ primitive or early progenitor populations [20]. Cells are cultured in a defined media in the presence or absence of a compound or gene silencing/knockdown condition. Recovery of disease repopulating activity is then assessed. The advantage of this model is that natural disease features are retained, but the conditions used to extract, purify, and analyze these cells may introduce a bias, as cell culture and growth factor conditions can only partially reproduce the conditions and niche normally occupied by this subfraction of CML cells. The important feature from this growing body of work is that pluses and minuses exist in each system of disease generation and assessment. For this reason the emergence of pathways that appear to play a role in CML repopulating activity may vary and lead to incomplete resolution of the precise role of each pathway in this process. The increasing number of pathways with potential involvement in CML stem cell activity and behavior needs to be placed in context of the model system employed to initiate disease, the means of analyzing function, and the alignment with clinical observations. This section attempts to analyze each pathway defined in CML stem cell activity in this context. The salient features of each assessment are also summarized in Table I.

Protein kinases

BCR–ABL. CML is associated with BCR–ABL expression, and its targeted inhibition provides effective therapy for CML, but disease relapse cannot be

Table I. Signaling pathways, targets, and inhibitors for chronic myeloid keukemia stem cells.

Pathway	Target(s)	Evidence for stem cell role	Inhibitor	Impact of inhibitor	Stage of inhibitor development
BCR–ABL	BCR–ABL	Primary specimens, animal models	1. Imatinib 2. Nilotinib 3. AP25534	Reduces early and late CML progenitors	1. Clinically approved 2. Clinically approved 3. Clinical studies
Src-family kinases	Lyn, Hck, Fgr	Primary specimens, animal models	1. Dasatinib 2. Bosutinib 3. INNO-406	Reduces early and late CML progenitors	1. Clinically approved 2. Clinical studies 3. Clinical studies
PKCβ	PKCβ	Primary specimens	BMS-214662	Cell growth arrest	Pre-clinical studies
PP2A	Sphingosine-1 receptor, adenylate cyclase	Primary specimens, cell models	1. FTY720 2. Forskolin	Cell growth arrest	1. Clinical studies 2. Clinical studies
PTEN	mTOR, TORC1, TORC2, PI3K, Akt	Cell lines, animal models, patient samples	1. Rapamycin 2. RAD-001 3. CCI-079 4. OSI-027 5. PI-103 6. PP242	Cell growth arrest	1. Clinically approved 2. Clinical studies 3. Clinical studies 4. Pre-clinical studies 5. Pre-clinical studies 6. Pre-clinical studies
Wnt/β-catenin	1. GSK3β kinase 2. Casein kinase 1 3. Tankyrase	Patient samples, cell lines	1. Differentiation-inducing factor (DIF) 2. Pyrvinium 3. XAV939	Cell growth arrest	1. Pre-clinical studies 2. Clinically approved 3. Pre-clinical studies
Hedgehog	Smoothened	Animal models, patient specimens	1. Cyclopamine 2. NVP-LDE225 3. IPI926 4.PF-04449913	Cell growth arrest	1. Pre-clinical studies 2. Clinical studies 3. Pre-clinical studies 4. Pre-clinical studies
Alox5	5-LO	Animal models, patient specimens	Zileuton	Cell growth arrest	Clinically approved
PML	PML	Animal models, patient specimens	Arsenic trioxide	Cell apoptosis and growth arrest	Clinically approved
Autophagy	Lysosomes	Patient specimens, cell lines	Chloroquine, 3-methyladenine	Potentiates imatinib activity	Pre-clinical studies
Interferon-α	IFN-α receptor	Patient specimens, animal models	Pegylated IFN	Growth arrest, apoptosis, immunomodulation	Clinically approved
Histone deacetylase	Histones, other chromatin complexes	Patient specimens, cell lines	1. SAHA 2. LBH589 3. LAQ824	Growth arrest, apoptosis	1. Clinically approved 2. Clinical studies 3. Clinical studies
Bcl-2 family	Bcl-2, BH3 domains	Patient specimens, cell lines	1. Genasense 2. ABT-263 3. GX15-070MS	Apoptosis	1. Clinical studies 2. Clinical studies 3. Clinical studies
Protesome and ubiquitin cycle	20S proteasome, Hsp90, DUB	Patient specimens, animal models, cell lines	1. Bortezomib 2. IPI-504 3. WP1130	Apoptosis	1. Clinically approved 2. Clinical studies 3. Pre-clinical studies
Rac2 GTPase	GTPase	Patient specimens, animal models	NSC23766	Growth arrest, apoptosis	Pre-clinical studies

PKCβ, protein kinase Cβ; PTEN, phosphatase and tensin homolog; PML, promyelocytic leukemia protein; GTPase, guanosine triphosphatase; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; 5-LO, 5-lipoxygenase; IFN-α, interferon-α; Hsp90, heat shock protein 90; DUB, deubiquitinase.

completely prevented by imatinib therapy alone [96,97]. This is proposed to be due to the insensitivity of the CML repopulating cells to imatinib. Animal models generated by retroviral transfer of BCR–ABL have shown that BCR–ABL kinase inhibition with imatinib (as well as with more potent and broad-spectrum kinase inhibitors, e.g. dasatinib) reduced BCR–ABL-expressing cells but a small tumor repopulating fraction was retained in the marrow of treated mice [98]. This fraction was insensitive to kinase inhibitor treatment *ex vivo*, and was similar to the result obtained when primary CD34+/CD38− CML specimens were treated with BCR–ABL kinase inhibitors [99,100]. These cells did not harbor mutations in the BCR–ABL gene, which would predict insensitivity to imatinib, and were primarily more quiescent than the more mature cell population. However, imatinib did appear to

quench BCR–ABL signal transduction, as demonstrated by assessing the phospho-CrkL status as a surrogate of BCR–ABL kinase activity [99,100]. These observations provided supporting evidence for the existence of a primitive and quiescent BCR–ABL+ cell that was apoptotically insensitive to imatinib and therefore refractory to BCR–ABL inhibition. These observations, as well as a growing body of clinical data, suggest that disease relapse is associated with the incomplete eradication of CML cells by BCR–ABL inhibition alone, and have led to the search for targets within that unresponsive cell fraction that may distinguish the CML stem cell from normal HSCs. This search has led to a growing list of potential targets.

Src kinases. Src family kinases are unregulated as CML cells undergo progression or become increasing insensitive to imatinib [101–104]. However, their role in CML stem cells has not been fully investigated. Animal models used to assess the impact of tyrosine kinase inhibitors (TKIs) on the persistence of stem cell function suggested that more potent inhibition of BCR–ABL, combined with Src kinase inhibition (using dasatinib), had greater effects on stem cell recovery than imatinib, but was unable to fully oblate residual BCR–ABL+ stem cells [98]. Further analysis of primary CML specimens also showed that dasatinib alone had only marginal activity in reducing stem cell activity in a long-term initiating culture system, which provides a culture-based assessment of potential stem cell activity [99]. These results suggest that Src family kinases play a limited role in CML stem cell function. However, we have noted increased levels of Lyn kinase in the nucleus of imatinib-resistant CML cells (unpublished observation), and control of stem-cell essential survival genes may be regulated by Lyn [105–107]. These observations suggest that individual kinases within the Src family can regulate gene expression in a lineage and context specific manner and will require more analysis for potential involvement in stem cell activity [108].

GSK3β. Glycogen synthase kinase 3β (GSK3β) is a major signaling component of the Wnt/β-catenin cascade [109]. GSK3β kinase activity is negatively controlled by upstream phosphorylation through BCR–ABL and BCR–ABL-independent mechanisms [54,55,110]. GSK3β-mediated phosphorylation of β-catenin increases its ubiquitination and proteasomal turnover, thereby reducing expression of β-catenin regulated genes controlling cell cycle and survival [111]. The significance of this kinase in CML stem cell activity was recently highlighted by the observation of its abnormal splicing, resulting in its loss of function, and increased β-catenin in the nucleus of blast crisis CML cells [109]. This cell population resembles more mature progenitors, which acquire self-renewal capacity. Given the deregulated function of this kinase in some CML specimens and the potential for BCR–ABL to stimulate phosphorylation to suppress GSK3β activity, it appears that reactivation of this kinase or suppression of β-catenin transcriptional activity in CML may have anti-stem cell activity in some forms of the disease.

PKCβ. During screens of novel pathway inhibitors for activity on CML stem cells, farnesyl transferase inhibitor (FTI) BMS-214662 emerged as a preferential mediator of apoptosis in this population [112]. These results suggested a role for modulation of Ras and other FTI-sensitive pathways as regulators of stem cell apoptosis. More recent studies of BMS-214662 provide further insight into its mechanism of action. BMS-214662 activated protein kinase Cβ (PKCβ) during induction of stem cell apoptosis, while inhibition of PKC activation in BMS-214662-treated cells blocked apoptosis [113]. BMS-214662 activity was further associated with increased phosphorylation of key cell cycle and survival regulators. Importantly, other FTIs that did not activate PKCβ were ineffective in the induction of CML selective stem cell apoptosis, suggesting that a combination of effectors underlies the stem cell activity of BMS-214662. Since BMS-214662 has undergone early clinical assessment for safety and impact in other cancers, patients with CML who have achieved a complete molecular response on imatinib therapy may have a reduced risk of relapse with BMS-214662.

Phosphatases

PP2A. PP2A is a serine/threonine phosphatase that plays a very critical role in the regulation of multiple pathways controlling cell growth, survival, and differentiation, with biologic properties of a tumor suppressor [114]. PP2A levels can be negatively regulated by BCR–ABL through the induction of SET (SE translocation) protein [115]. SET deregulation can also occur through other mechanisms and signal transduction pathways [105]. Importantly, reactivation of PP2A suppresses many of the activities of BCR–ABL and may prevent blastic transformation [116,117]. PP2A activity may also be important in early progenitor (CD34+/CD38−) CML cells as its activation is reported to suppress many CML stem cell-like activities *in vitro*, possibly through interruption of a BCR–ABL independent pathway (β-catenin) [114]. Clinical studies of PP2A

activators are planned, and the impact of this pathway on stem cell survival and CML relapse will need to be evaluated.

PTEN. PTEN is a lipid and protein phosphatase that controls multiple downstream pathways linked to cell survival and stem cell renewal [118]. BCR–ABL activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway which is antagonized by PTEN [119]. Akt-mediated phosphorylation of Forkhead box O (FOXO) transcription factors prevents their nuclear compartmentalization, resulting in their loss of function [120]. FOXO-knockout mice demonstrate that these proteins regulate the self-renewal capacity of HSCs [121]. Activation of the Akt pathway through BCR–ABL or TGFβ may therefore stimulate replication and survival of the leukemic stem cells (LSCs), which may be attenuated by functional PTEN [122]. Importantly, PTEN-knockout animals developed CML more rapidly when assessed by retroviral transduction of BCR–ABL [123]. Further, PTEN replacement delayed the onset of disease. Control of Akt activation or FOXO protein nuclear distribution may therefore be a useful strategy for suppressing the CML stem cell population, as demonstrated by studies of mice receiving a combination of imatinib with a TGFβ signaling inhibitor [122].

PTEN deficiency and activation of the PI3K cascade as well as imatinib treatment may also promote activation of the mammalian target of rapamycin (mTOR) pathway to regulate translation of proteins essential for CML and stem cell survival [124,125]. mTOR inhibitors such as rapamycin and second-generation inhibitors targeting two effectors of the mTOR cascade may be useful in suppressing stem cell survival and replication [125,126]. Indeed, mTOR inhibitors demonstrate promising activity when used in conjunction with TKIs [125]. Activation of multiple pathways regulated by PTEN may be important signaling modules that will need to be coordinately inhibited to affect CML stem cells in association with BCR–ABL inhibition.

Wnt/β-catenin

Animal models suggest that the Wnt/β-catenin pathway plays an essential role in renewal of both normal and CML stem cells [54]. However, in patients with CML, the role of Wnt/β-catenin signaling in stem cell-like activity may be dependent upon the stage of the disease and the progenitor compartment [28,55,109,127]. In chronic phase disease, β-catenin activation was detectable in the hematopoietic stem cell fraction, whereas patients in blast crisis had reacquired β-catenin activation within the granulocyte-macrophage progenitor (GMP) cell fraction [28]. β-Catenin activation was associated with defects in expression of GSK3β in some patients [109]. Other modulators of this pathway may also be involved, but currently there is limited additional information. The potential for effecting CML stem cell activity in chronic phase disease may ultimately be addressed through the use of inhibitors targeting various effectors of this pathway.

Hedgehog

The Hedgehog (Hh) pathway is another essential regulator of stem cell function in multiple tissues, and has been shown to be activated in a number of tumors, including leukemia [56,57,128]. The role of Hh in CML stem cell activity is implied with the use of animal models of BCR–ABL-initiated disease. The absence of Hh pathway activators or the presence of Hh inhibitors increases survival in BCR–ABL-expressing mice [56,57]. However, these effects were not restricted to BCR–ABL-expressing cells, as similar effects were noted in normal hematopoietic stem cells as well [56,57]. In additional animal studies, activation of the Hh cascade increased the CML stem cell compartment and reduced survival in animals expressing BCR–ABL [56,57]. Selective small molecule Hh pathway inhibitors have been used to support a role for this pathway in CML stem cell activity, as cyclopamine and tomatidine suppressed colony formation in specimens from patients with CML or BCR–ABL-expressing cells. Clinical studies of Hh pathway inhibitors are anticipated, and may provide more information regarding the benefit of Hh antagonism in reducing CML progression and risk of relapse.

Alox5

The potential role for mediators of inflammation in CML stem cell activity was recently demonstrated in an unbiased assessment of gene expression in stem cells remaining after imatinib therapy for BCR–ABL-induced leukemia in mice [129]. The *Alox5* gene encodes 5-lipoxygenase (5-LO), essential for leukotriene production and involved in many disorders, including cancer [130]. Inhibitors of 5-LO were reported several years ago to induce apoptosis in leukemic cells, but this response was presumed to be due to effects on cellular targets other than 5-LO [131,132]. Analysis of CD34+ CML cells from the bone marrow of patients suggested that *Alox5* is upregulated in CML stem cells [133,134]. In mouse models of BCR–ABL-transduced disease, imatinib reduced but did not fully eliminate BCR–ABL+ cells, and gene expression profiling of this residual

population demonstrated increased *Alox5* expression when compared to control animals [129]. *Alox5*$^{-/-}$ animals did not develop CML upon BM transduction with BCR–ABL, and treatment of *Alox5*$^{+/+}$ animals with BCR–ABL-induced CML with 5-LO inhibitor and imatinib reduced the lethality of the disease to a greater extent than imatinib alone. Interestingly, *Alox5* deficiency prevented myeloid disease, but animals still developed BCR–ABL+ acute lymphoblastic leukemia (ALL). These results suggest that *Alox5* is an important contributor to development of BCR–ABL-dependent CML, and 5-LO inhibitors may be an important strategy in the treatment of CML and elimination of myeloid progenitors with stem cell-like repopulating activity.

PML

Promyelocytic leukemia protein (PML) is a tumor suppressor that predominantly localizes to nuclear bodies and is essential to maintain stability of HSCs [135]. Chronic phase CML cells express PML, and lower expression predicts better prognosis [136]. Animal models predict that PML expression regulates CML stem cell cycle timing and diminished PML expression results in exhaustion of the stem cell fraction. Importantly, arsenic trioxide and other agents currently used for therapy of other leukemias induce the degradation of PML. In animal models, down-regulation of PML with arsenic trioxide combined with other apoptosis-inducing agents (cytarabine; Ara-C) eliminated leukemic cells [136]. Regulation of PML levels with arsenic trioxide combined with BCR–ABL inhibition may impact CML stem cell viability and reduce the chance of relapse in patients with CML by destabilizing CML-repopulating activity. However, the effects of arsenic trioxide in combination with imatinib treatment were not described in this study.

Autophagy

Cellular stress can induce autophagy, a cellular pathway resulting in lysosomal catabolism of intracellular components to provide reserves of energy to sustain survival [137]. Cancer cells can utilize autophagy as a means of protection from nutrient withdrawal and some forms of chemotherapy [137]. Autophagy can also initiate or effect tumor cell death under some circumstances, but induction of autophagy is primarily associated with evasion of cell death. Imatinib activates autophagy in CML cell lines and CD34+ CML cells (with stem cell activities) in a BCR–ABL kinase inhibition-dependent fashion [138]. Chemical inhibition or silencing of the autophagy cascade increases imatinib anti-CML activity [138]. This suggests that cellular stress induced in CML cells by BCR–ABL inhibition provides some protection from death, and supports the use of autophagic inhibitors to amplify imatinib activity, particularly in unresponsive CML stem cells. This may be an appropriate strategy, since BCR–ABL kinase inhibition is essential for initiation of autophagy and will only be manifested in CML cells [139]. However, loss of imatinib sensitivity (through mutations) or ineffective inhibition (as in stem cells) may restrict the use of this strategy to specific cases.

Interferon-α

Prior to the use of imatinib for CML, interferon-α (IFN-α) was considered an effective therapy in patients with chronic phase CML, resulting in longstanding cytogenetic remission in a subset of patients [140]. Interest in the use of IFN-α as a stem cell regulator in CML has grown as recent studies have shown that complete molecular responses can be improved with the use of imatinib and IFN-α [141–143]. IFN-α has a complex mechanism of action that involves receptor-mediated activation of several kinases and transcription factors capable of regulating the cell cycle, survival, immunomodulatory genes, and antiviral defenses [143–145]. Animal studies predict that IFN-α can affect stem cell quiescence or the environment in which CML cells are sensitive to imatinib [146]. IFN-α mediated immuno-recognition/regulation may also play a role in the observed clinical activity of this cytokine [143]. In some cases of interruption of imatinib therapy, disease relapse was noted to be less likely in patients who received prior IFN-α therapy [147–149]. These observations have led to an increasing interest in the clinical use of IFN-α in combination with tyrosine kinase inhibitors to suppress CML relapse and/or reduce CML repopulating activity. Interestingly, it is likely that the mechanism of action of IFN-α in CML and stem cells will continue to be poorly understood, as animal models may only partially resemble patients with CML and their immunomodulation by IFN-α. Nonetheless, the growing interest in clinical combination studies of IFN-α with several CML-directed therapies will likely provide very important information regarding the role of IFN-α in stem cell activity and elimination of CML.

Histone deacetylases

Although the targets are not completely understood, histone acetylation regulates gene expression through modification of chromatin complexes and other proteins [150]. Inhibitors of histone deacetylases

(HDACi) have been shown to reprogram gene expression and engage apoptosis in quiescent leukemic cells [151]. This has lead to an analysis of their impact on CML stem cells and disease repopulating activity, alone and in combination with imatinib in animal models [152]. Further reduction of CML stem cell-like activity was noted when HDACi were used in combination with imatinib [152]. HDACi-mediated apoptotic sensitization was associated with a complex change in gene expression, which suggests that a multiplicity of pathway changes underlie the anti-CML stem cell activity. Since HDACi are currently undergoing clinical trials in several cancers [150], these promising effects on CML stem cell activity are being explored in patients with cytogenetic remission following imatinib therapy [152]. Trials will assess whether HDACi will provide longstanding protection of patients with CML from disease relapse following discontinuation of imatinib. This approach may also uncover essential gene expression changes necessary for stem cell activity.

Mitochondrial regulators of apoptosis

BCR–ABL up-regulates multiple pathways to effect cell survival, many of which can be controlled with imatinib or other TKIs [153,154]. However, due to the quiescent nature of the CML stem cell and its protection from apoptosis provided by the niche, it is unclear whether BCR–ABL kinase inhibition alone provides an adequate reversal of apoptotic protection to impact these cells. Ultimately, mitochondrial proteins of the Bcl-2 family determine the apoptotic threshold and are therefore of interest in CML cells with stem cell function [155–157]. BH3 mimetics are small molecules that compete for partnered Bcl-2 family complexes within the mitochondria to reduce their anti-apoptotic function [158]. BCR–ABL up-regulates Bcl-X_L and Mcl-1, and both can be suppressed by imatinib [153]. However, recent studies suggest a particularly important role for Mcl-1 in stem cell survival [159]. BH3 mimetics can suppress the pro-survival function of most Bcl-2 homologs with a resulting induction of apoptosis in CML cells when used in combination with TKIs [160,161]. However, due to a structural distinction in Mcl-1, its function is not affected by BH3 mimetics [161]. Compounds with a broader spectrum of targets may overcome this restriction and have clinical activity in patients with hematopoietic malignancies [162–164]. However, their impact on CML stem cell apoptosis has not been reported. Mcl-1 is the only pro-survival member of the Bcl-2 family with a short half-life, controlled by ubiquitination and proteasomal destruction (see next section) [165]. Analysis of Mcl-1 binding partners demonstrated that a deubiquitinase (DUB), Usp9x, associates with Mcl-1 and controls its ubiquitination and destruction (Figure 1) [166]. Importantly, Mcl-1 stability was associated with increased expression of Usp9x, which occurs in a wide variety of tumors, including leukemias and lymphomas [166]. Silencing Usp9x expression induced apoptosis or enhanced apoptosis mediated by BH3 mimetics in many tumor models. Thus, Usp9x expression may stabilize Mcl-1 through control of its ubiquitination and proteasomal degradation. Interestingly, we have noted that the activity of several DUBs, including Usp9x, was elevated in BCR–ABL-transformed BaF3 cells (Figure 2). Further, TKIs had no affect on Usp9x activity in CML cells or BCR–ABL transformed cells (Figure 3). Therefore, Mcl-1 gene expression is subject to transcriptional up-regulation through BCR–ABL signal transduction, while Mcl-1 protein levels appear to be stabilized through deubiquitination by increased Usp9x activity in CML cells [153,166]. We previously described the small molecule WP1130 as a regulator of BCR–ABL protein levels and CML cell survival [167]. In more recent studies we demonstrate that WP1130 acts through partially selective inhibition of DUBs [168]. WP1130 suppresses deubiquitination of BCR–ABL, resulting in an increase in BCR–ABL ubiquitination and changes in its cellular solubility, subcellular distribution, and signaling activity (Sun *et al.*, unpublished data). Further, WP1130 effectively inhibits Usp9x activity, resulting in the rapid destruction of Mcl-1 and tumor cell apoptosis [168]. WP1130 treatment of CD34+/CD38− CML primary cells led to a rapid reduction of Mcl-1 protein (Figure 3), while imatinib did not affect Mcl-1 levels. Further, we have demonstrated that Usp9x silencing reduces Mcl-1 levels and sensitizes CML cells to imatinib or BH3 mimetics [169] (ABT-263) (Sun *et al.*, unpublished data). Previously, we demonstrated that WP1130 reduced CML tumor burden in mice, suggesting that it could be administered safely [167]. Together, these results suggest that agents that modulate mitochondrial regulators of apoptosis through disruption of their protein complex or alteration of their stability may be effective in suppressing stem cell survival alone and in combination with TKIs.

Proteasome/ubiquitin cycle

Recent studies suggest that modulation of the stability and destruction of key proteins expressed in CML cells may be an effective approach to target the CML stem cell fraction. As noted above, Mcl-1 ubiquitination can be controlled by Usp9x, and inhibition leads to the proteasomal destruction of Mcl-1 and sensitization to apoptosis in CD34+

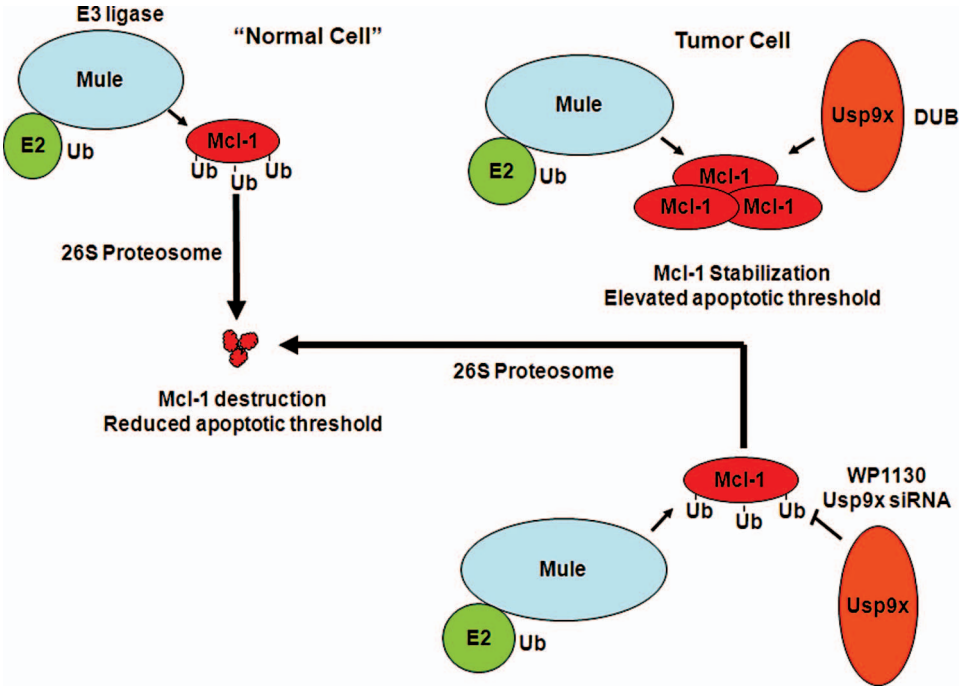

Figure 1. Model for Mcl-1 regulation by ubiquitination. Top left: In untransformed cells, Mcl-1 undergoes regulated ubiquitination by E3 ligase (Mule) to increase its destruction through the 26S proteasome. In the absence of the Usp9x deubiquitinase, Mcl-1 protein has a short half-life and is maintained at a low steady state level. Top right: Tumor cells induce or activate Usp9x to suppress the impact of E3 ligase-mediated Mcl-1 ubiquitination, resulting in an increased half-life and accumulation of Mcl-1, elevating the apoptotic threshold. Bottom right: Down-regulation of Usp9x (siRNA) or inhibition of its activity (by WP1130) in tumor cells results in recovery of Mcl-1 ubiquitination and its destruction by the proteasome to reduce the apoptotic threshold and decrease tumor cell survival.

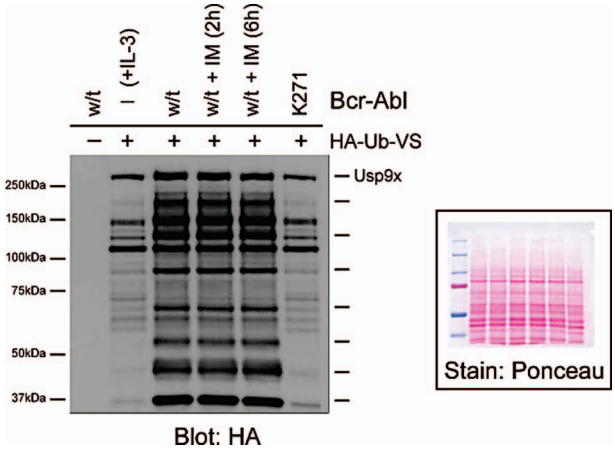

Figure 2. BCR–ABL activates DUB activity in BaF3 cells. Left: IL-3-dependent BaF3 cells were maintained in IL-3 or transformed with wild-type (w/t) or a kinase-dead mutant (K271) BCR–ABL. Cells were left untreated or treated with 5 μM imatinib (IM) for 2–6 h (as indicated) before cell lysates were prepared and assayed for DUB activity using the suicide substrate HA-labeling reagent, HA-Ub-VS, as recently described [168]. As a control, equal amounts of lysate from BCR–ABL-transformed BaF3 cells were not subjected to HA-Ub-VS labeling (lane 1). DUB activity was assessed by HA immunoblotting. MW standards are shown on the left and DUBs elevated in BCR–ABL transformed cells (compared to IL-3-maintained cells; lane 2) are denoted by tick marks on the right. The upper band was determined to represent Usp9x. Expression of mutant K271 (kinase-dead) BCR–ABL failed to increase DUB activity in BaF3 cells. Right: The HA blotted membrane was Ponceau stained to demonstrate equal protein load and transfer.

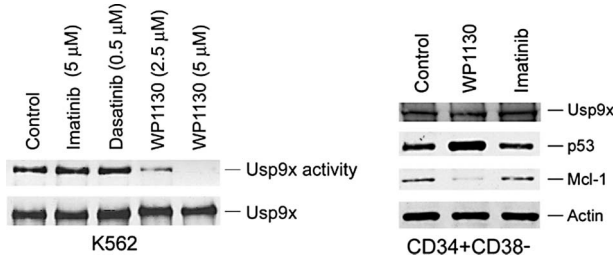

Figure 3. WP1130 suppresses Usp9x activity and induces Mcl-1 down-regulation in CML cells. Left: K562 cells were treated as indicated for 4 h before cell lysates were assayed for DUB activity using HA-Ub-VS labeling as described in Figure 2. The HA blot representing Usp9x activity is shown at the top. The blot was reprobed for Usp9x detection (bottom). WP1130 dose-dependently suppressed Usp9x activity. Right: CD34+/CD38− cells were isolated from a patient with CML and treated with 5 μM imatinib or WP1130 for 4 h before cell lysates were immunoblotted for Usp9x, p53, Mcl-1, and actin, as a protein loading control. WP1130 induced p53 accumulation and Mcl-1 down-regulation. Imatinib did not affect Mcl-1 or p53 levels in this cell population.

CML cells. Further, CD34+ CML cells were more sensitive to WP1130-induced apoptosis than CD34+ cells from normal donors (Sun *et al.*, unpublished data). Differential sensitivity to WP1130 may be due to expression of BCR–ABL and activation of Usp9x in these tumors, which are both inhibited by WP1130

through unique mechanisms. Other modulators of BCR–ABL protein stability or modulation of the stem cell proteasome may also be useful in suppressing CML stem cell survival. Since BCR–ABL forms an obligate association with heat shock protein 90 (Hsp90) to mediate its appropriate folding, disruption of this chaperone or inhibition of its activity makes BCR–ABL susceptible to misfolding, ubiquitination, and destruction through the proteasome [170]. Other proteins important in BCR–ABL signal transduction may also be affected by Hsp90 inhibition [170,171]. Recently, Hsp90 inhibition was shown to reduce CML stem cell activity in animals [172,173]. These effects were not blocked by BCR–ABL mutations that affect TKI activity, and Hsp90 inhibition was less effective in suppressing normal hematopoiesis, suggesting that this approach may be tumor selective and active in imatinib-resistant cells. More recently, the proteasome inhibitor was shown to reduce CML stem cell activity without directly affecting BCR–ABL protein or its activity [174]. However, bortezomib did not demonstrate CML selectivity, as CD34+ cells from normal donors were equally sensitive to bortezomib [174]. These studies provide evidence for an emerging strategy of manipulating protein stability through proteasome, Hsp90, or DUB inhibition to effect survival of CML stem cells. However, concerns remain regarding the safety and efficacy of this approach in patients with CML who have established long-term control of their disease. It is anticipated that new agents will emerge that preferentially affect CML stem cells through refinement of lead molecules and greater understanding of the role of individual targets in normal and leukemic stem cells.

Rac2 GTPase

Rac2 is a GTPase with an integrative role in hematopoietic cell signal transduction and bone marrow engraftment/mobilization [52]. Using a prolonged latency, hematopoietic stem cell-specific animal model, $Rac2^{-/-}$ mice had significantly greater survival in BCR–ABL-initiated disease models than their wild-type $Rac2^{+/+}$ counterparts [175]. Increased survival in $Rac^{-/-}$ BCR–ABL-expressing mice was associated with exhaustion of the leukemic stem cell pool due to their increased apoptosis and decreased proliferation. These activities were not due to impaired interaction of the stem cells with the microenvironment, suggesting greater involvement of Rac2 in stem cell survival and signal transduction than adhesion. These characteristics define Rac2 GTPase as a potential therapeutic stem cell target. Previously, a Rac1/2 GTPase inhibitor was described with activity against many tumor cell types [176]. Whether or not this inhibitor is active within the stem cell fraction is unknown. It appears that more Rac2 selective inhibition may be essential as Rac1 deficiency predicts that it plays a more direct role in mobilization of stem/progenitor cells and less direct impact on stem cell survival [177].

Emerging therapeutic targets for chronic myeloid leukemia stem cells

Based on the increasing focus on identifying, understanding, and targeting CML stem cells, a tremendous number of potential therapeutic targets have emerged. Some targets are defined regulators of key enzymes or proteins that provide an essential or specific function in CML stem cells, while others target pathways with impact on stem as well as more differentiated cells. Many of the targets described below are summarized in Table I with regard to their discovery, potential/probable target, mechanism of action, and activity in animal or clinical studies.

Kinase inhibitors

A significant number of kinase inhibitors are effective against targets in CML cells (reviewed in [147]). The sufficiency of BCR–ABL kinase inhibitors alone to reduce CML stem cell activity and survival is still not fully known. However, both animal models and clinical experience suggest that CML stem cells are less sensitive to kinase inhibitors than more mature progenitors. These conclusions may be subject to change, as some mathematical models and clinical observations predict longstanding remission in patients achieving a 5-log reduction in BCR–ABL transcripts [148,149,178,179]. For this reason, more potent BCR–ABL kinase inhibitors may change the incidence of relapsed disease without completely eliminating the CML stem cell. Thus, it is appropriate to describe the new second- and third-generation TKIs that have been approved for CML or are being tested in imatinib-resistant disease, including inhibitors affecting T315I-mutant BCR–ABL. These are reviewed in reference [147].

Kinase activators

Control of the Wnt/β-catenin pathway appears to be critical in CML stem cell self-renewal and lineage selection decisions. Its importance in CML is further illustrated by the association between nuclear β-catenin content and defects in the Wnt/β-catenin/GSK3β cascade in patients with CML blast crisis. Reactivation of the β-catenin degradation pathway via activation of upstream kinases may represent one approach with potential to reestablish control over

CML stem cell function. However, this approach was perceived to be more difficult to establish, as reactivation of an attenuated kinase activity is less likely to be successful with small, drug-like molecules than specific target kinase inhibition. Very recent studies suggest that small molecules can induce activation of casein kinase 1 (CK1), with a resultant increase in β-catenin phosphorylation, leading to promotion of β-catenin turnover [180]. Increased turnover of β-catenin via CK1 activation was associated with a reduction in β-catenin-mediated gene expression and proliferation in colon cancer cells, with disrupted control of their Wnt/β-catenin protein complex. The compound identified in this screen, pyrvinium, was derived from a Food and Drug Adminstration (FDA)-approved drug library, which was previously used to treat pinworm and other protist infections. This approach may be applicable in CML and other stem cell disorders involving β-catenin. Other approaches in targeted activation of GSK3 and control of regulators of β-catenin turnover by small molecules are also being explored [181,182]. If these new chemical modulators of this cascade can be safely administered to patients, one likely focus will be on their potential to regulate CML stem cell survival and disease repopulating activity.

Studies of the effect of a FTI on CML cells also suggest an association between activation of an additional kinase and CML stem cell survival. BMS-214662 was originally described as a selective apoptosis-inducing agent in CML stem cells [112]. More recent assessment suggests that activation of PKCβ is an essential mediator of BMS-214662 anti-stem cell activity [113]. The role of FTI inhibition in this response is unclear, since other FTIs that did not activate PKCβ were ineffective in CML stem cells. Phosphorylation of specific targets in CML cells may underlie the observed effects of this compound on CML cells. It will be interesting to determine whether BMS-214662 also effects activation of other kinases with an identified role in stem cell signaling. Clinical studies are under way with BMS-214662 in other leukemias.

PP2A activators

FTY720 is a natural product-derived activator of a sphingosine-1-phosphate sensitive G-coupled protein receptor [183]. This compound is undergoing clinical analysis in multiple sclerosis. In CML cells, FYT720 causes reactivation of PP2A and affects BCR–ABL levels through kinase dephosphorylation (via SHP-1 phosphatase) and down-regulation [117]. These activities engage apoptosis in CD34+ CML and ALL cells without affecting normal CD34+. These results suggest that modulation of this pathway with FTY720 or other compounds with similar activity will provide an additional approach in safely targeting CML stem cells [184]. Clinical studies of FTY720 with imatinib or more potent TKIs are anticipated.

mTOR inhibitors

PTEN inactivation in CML animal models suggests that activation of the PI3K/Akt module by BCR–ABL is associated with CML stem cell proliferation and survival [123,124,185,186]. Many inhibitors of PI3K and Akt have been described and are in various stages of clinical assessment and evaluation [187,188]. In normal hematopoietic stem cell replication and differentiation, integrating signaling downstream of the PI3K/Akt module may be a critical regulator of the impact of kinase inhibition [124]. Through feedback loops from PI3K/Akt and or BCR–ABL inhibition, mTOR, a downstream mediator of protein translation and cell growth, may be an important determinant of cellular response to BCR–ABL-targeted therapy. mTOR inhibition with compounds such as rapamycin and CCI-779 primarily affects only one of two mTOR activated pathways, resulting in minimal impact on tumor cell survival [124]. To overcome this restriction, inhibitors that affect both the TORC1 and TORC2 signal complexes may provide greater impact on CML cells and the stem cell fraction [126,189]. Animal models of BCR–ABL-initiated disease and primary CML specimens appear to support that conclusion [124]. It will be important to track the clinical development of these inhibitors and their potential for impact on CML stem cell activity.

Hedgehog inhibitors

Hh is activated in CML cell and animal models through up-regulation of the Hh receptor, Smoothened (Smo) [56,57]. In the absence of Smo, CML stem cell expansion was suppressed, and repopulation of BCR–ABL disease in mice was diminished [57]. Hh gene targets are up-regulated in CML stem cells, and small-molecule antagonists of this pathway are undergoing early clinical studies in basal cell cancer [190]. Hh antagonism in CML cells results in up-regulation of Numb, a cell fate determinant regulator that was recently shown to be down-regulated by Musashi-2, an RNA-binding protein up-regulated in CML blast crisis [57,191,192]. Interestingly, Numb expression is linked to many developmentally regulated pathways, several of which are described in CML cells and may be regulated

through cross-talk between pathways [193]. Hh inhibitors will likely provide some additional clues of determinants that regulate CML stem cell activity and fate.

5-LO inhibitor

Alox5 is up-regulated in BCR–ABL-induced tumors in mice, and its expression is not sensitive to BCR–ABL kinase inhibition [129]. *Alox5* encodes 5-LO, which regulates leukotriene production. Studies with $Alox5^{-/-}$ mice support a role for 5-LO activity as a regulator of CML stem cell survival. A 5-LO inhibitor, zileuton, was able to prolong survival of BCR–ABL-transduced disease in mice and reduce BCR–ABL-positive cells in the periphery. Importantly, zileuton did not affect normal hematopoiesis, suggesting that the 5-LO inhibitor serves as a tumor-specific regulator of CML stem cell survival. Interestingly, zileuton did not prevent BCR–ABL-mediated ALL, suggesting that the activity sensitive to this inhibitor resides in the myeloid lineage [129]. Since zileuton was previously approved for use in humans, a phase 1 study of this drug in combination with imatinib in patients with CML is under way [194,195]. Based on animal studies, it will be interesting to assess the effects of this combination on myeloid and lymphoid disease.

PML

PML is expressed in chronic phase CML cells, and animal models predict that PML expression regulates CML stem cell cycle timing and its down-regulation exhausts the stem cell fraction [136]. Arsenic trioxide induces proteolytic degradation of PML and, in the presence of other apoptosis-inducing agents, eliminates leukemic cells in animal models [136]. Since arsenic trioxide is approved for clinical use, a combination study with TKIs is expected [196]. PML down-regulation combined with BCR–ABL inhibition may reduce the chance of relapse in patients with CML by destabilizing CML-repopulating activity.

Autophagy

Imatinib, through induction of cell stress, activates autophagy in CML cells [197]. Inhibition of this pathway results in greater sensitivity to imatinib in CD34+ CML cells and cell lines. This suggests that BCR–ABL kinase inhibition in the presence of an autophagic inhibitor will result in greater therapeutic activity. However, exploiting this potential in CML stem cell therapy may be difficult due to the complex interplay between cell stress pathways, the multiple regulatory mechanisms of autophagy, and the requirement for BCR–ABL kinase inhibition that may be suboptimal in this cell fraction [139]. Nonetheless, as more details and compounds with clinical potential to regulate autophagy emerge, clinical studies may be forthcoming. The use of mTOR inhibitors to block autophagy in combination with novel tyrosine kinase inhibitors affecting even the T315I-mutant BCR–ABL may be the most clinically available means of testing the role of combined inhibition of BCR–ABL and autophagy in stem cell responsiveness and patient relapse [10,139].

Interferon-α

Interest in exploring IFN-α activity in CML stem cells has increased due to early observations of limited disease relapse in patients who discontinued imatinib but had prior IFN-α therapy for their disease [149]. Recent studies suggest that disease responses are improved with combined BCR–ABL inhibition and IFN-α therapy [141,143]. Several lines of evidence suggest that IFN-α modifies stem cell behavior [146]. However, the underlying mechanism of IFN-α action in CML is not completely resolved, and is likely to be multifactorial. Many combination studies are under way, and, with the improved capacity to measure CML cells by genomic BCR–ABL sequencing [198], these studies may help to resolve the basis and benefit of imatinib/IFN-α therapy in disease control and reduced relapse through effects on CML stem cells.

Histone deacetylases

HDACi have recently been shown to improve imatinib therapy by affecting both primitive CML stem and progenitor cells [152]. HDACi alter gene expression patterns alone and in combination with imatinib to affect stem cell replication and survival genes. As several HDACi are showing antitumor activity and safety in clinical studies [150], HDACi with imatinib therapy may have greater impact on CML remission through effects on CML stem cells. Several clinical trials assessing that potential are anticipated.

Mitochondrial regulators of apoptosis

BCR–ABL-independent control of survival may underlie the apoptotic resistance of CML stem cells. Therefore, targeting the downstream effectors of apoptosis with small molecules may have therapeutic benefit in combination with kinase inhibition. Compounds that disrupt the apoptosis-suppressing Bcl-2

family proteins have been shown to induce apoptosis in CML cells in conjunction with BCR–ABL kinase inhibition [156]. Down-regulation of Bcl-2 itself may also have activity in CML cells [199]. However, animal models and clinical sample assessment suggest that Mcl-1 levels are critically important to stem cell survival [155,159]. BH3 mimetics can disrupt the anti-apoptotic activity of most apoptotic regulators but are ineffective against Mcl-1 [161,200]. Compounds affecting Mcl-1 function as well as other Bcl-2 family members have been described, but their clinical use may be limited [162–164]. Mcl-1 is the only Bcl-2 family protein that is regulated by ubiquitination, and recent studies demonstrate that a DUB capable of regulating Mcl-1 ubiquitination and proteasomal destruction (Usp9x) is up-regulated in leukemic stem cells and lymphoid malignancies [166]. Suppressing Usp9x expression facilitates the induction of apoptosis in cells with elevated expression of Usp9x and is associated with down-regulation of Mcl-1 [166]. We have detected elevated Usp9x activity (as well as other DUB activities) in BCR–ABL-transformed BaF3 cells (Figure 2). Usp9x DUB activity was not reduced by BCR–ABL kinase inhibition, suggesting a transformation-specific change that is not subject to short-term BCR–ABL kinase inhibition (Figure 2). We previously described WP1130, a small molecule with activity against BCR–ABL and CML cells *in vitro* and *in vivo* [167]. We have subsequently reported that this compound inhibits Usp9x and other specific DUB activities [168] to cause rapid BCR–ABL ubiquitination (Sun *et al.*, unpublished data), loss of its signal transduction activity, and a reduction in Mcl-1 protein levels. To determine whether Mcl-1 could be down-regulated by WP1130 in CML cells, primary CML cells from a patient undergoing leukapheresis were purified, and the CD34+/CD38− cell population was incubated with WP1130 or imatinib for 4 h before Usp9x and Mcl-1 levels were assessed by immunoblotting. As shown in Figure 3, imatinib did not reduce the Mcl-1 content, while WP1130 caused a marked reduction in the level of this stem cell survival protein. WP1130 treatment also increased the level of p53. These results suggest that elevated DUB activity in CML stem cells may control the stability of key survival and signaling proteins. Further assessment of the activity and safety of WP1130 in animal models is required to determine whether DUB inhibition provides an alternative approach to control of CML stem and progenitor cell survival. Together, these results suggest that modulation of key survival proteins in CML cells may be exploited, with new agents affecting mitochondrial protein function and stability.

Proteasome/ubiquitin cycle

The enzymes controlling the ubiquitin/proteasome cycle have emerged as potential therapeutic targets in many diseases, including B-cell tumors. Recent studies suggest that bortezomib, an inhibitor of the chymotryptic-like activity of the 20S proteasome, may be useful against CML stem cells [174]. Bortezomib induces apoptosis in CD34+ CML cells, but its cytotoxic effects are not restricted to leukemic cells, as normal CD34+ cells are equally sensitive. Bortezomib does not affect BCR–ABL proteolysis, which may account for its limited effects against CML versus normal progenitors. Many new compounds targeting the proteasome and its associated proteins have emerged [201], and it will be interesting to see whether CML-specific effects can be obtained by affecting different proteins and activities within the proteasome.

Other ubiquitin/proteasome cycle modulators may demonstrate CML stem cell activity with some selectivity. Hsp90 inhibition targets BCR–ABL and other proteins essential for CML cell survival. Geldanamycin and its derivatives block Hsp90 activity, affecting the stability and ubiquitination of many clients, including BCR–ABL [202]. The multiplicity of target effects in many cancers is suggested to underlie its potential to treat heterogeneous tumors. However, clinical studies suggest that geldanamycin and its derivatives may have a narrow therapeutic index, limiting their clinical use [203]. However, new derivatives with activity in animal models of BCR–ABL-transduced CML suggest selective activity against CML stem cells [173]. This derivative may provide the clinical safety profile needed to target CML stem cells through Hsp90 inhibition.

DUB inhibition may also introduce a novel approach in affecting BCR–ABL-expressing cells. We previously reported that WP1130 induced down-regulation of BCR–ABL and its signal transduction through an unknown mechanism [167]. We recently reported that WP1130 inhibits DUB activity [168] to affect BCR–ABL ubiquitination and other targets controlling cell survival (p53) and apoptosis (Mcl-1). Through its effects on DUB activity, WP1130 induces BCR–ABL ubiquitination (with K63-linked polymers) to sequester it into the aggresome, which blocks BCR–ABL signaling without inducing its proteolysis. In addition, through direct inhibition of Usp9x and Usp5 activity, WP1130 induces Mcl-1 down-regulation and p53 up-regulation, respectively. Importantly, WP1130 affects these proteins in CD34+ CML cells, and is 2–5-fold less toxic against CD34+ from normal donors. Previous animal models demonstrate activity against

BCR–ABL-expressing tumors, and its effects on stem cells in animal models of CML are planned. These results suggest that WP1130 or its derivatives may be useful in modulating CML cell signal transduction and survival through inhibition of specific DUB activities.

Rac2 GTPase

Rac2 GTPase activity controls CML stem cell proliferation and survival and may represent another novel therapeutic target [175]. A Rac1/2 GTPase inhibitor was previously described, but its specific activity in CML stem cells has not been reported [52]. However, since Rac1 inhibition has impact on other cell functions [177], a more specific Rac2 inhibitor may be essential to provide CML stem cell-directed activity.

Other agents

Omacetaxine (homoharringtonine). Omacetaxine is a natural product with clinical activity in patients with CML [204]. *In vitro* and *in vivo* studies demonstrate activity against BCR–ABL and T315I-BCR–ABL, the latter expressing greater sensitivity to omacetaxine [205]. Importantly, animals with BCR–ABL-transduced disease treated with omacteaxine had increased survival and a markedly diminished number of BCR–ABL+ stem cells. The mechanism of action may be related to suppression of BCR–ABL, Hsp90, and Mcl-1 protein translation. Since clinical activity of this compound has been previously described, the effect on the stem cell compartment and disease relapse is being examined.

Triptolide. Triptolide is another natural product with activity against CML and other tumors [206,207]. Recent studies suggest that it also has activity against quiescent CD34+ CML cells, with less toxicity against normal CD34+ cells [206]. Its anti-tumor activity appears to be related to its suppression of BCR–ABL, XIAP, and Mcl-1 gene and protein expression. Triptolide derivatives have been described, and are demonstrating anti-tumor activity in animals [208]. More encouraging, complete remission has been reported in patients with leukemia treated with a triptolide derivative [208]. These results are likely to promote an examination of triptolide for activity in patients with CML and its clinical impact on the CML stem cell fraction.

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