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Concise Review: Chronic Myeloid Leukemia: Stem Cell Niche and Response to Pharmacologic Treatment

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ABSTRACT

Nowadays, more than 90% of patients affected by chronic myeloid leukemia (CML) survive with a good quality of life, thanks to the clinical efficacy of tyrosine kinase inhibitors (TKIs). Nevertheless, point mutations of the ABL1 pocket occurring during treatment may reduce binding of TKIs, being responsible of about 20% of cases of resistance among CML patients. In addition, the presence of leukemic stem cells (LSCs) represents the most important event in leukemia progression related to TKI resistance. LSCs express stem cell markers, including active efflux pumps and genetic and epigenetic alterations together with deregulated cell signaling pathways involved in self-renewal, such as Wnt/β-catenin, Notch, and Hedgehog. Moreover, the interaction with the bone marrow microenvironment, also known as hematopoietic niche, may influence the phenotype of surrounding cells, which evade mechanisms controlling cell proliferation and are less sensitive or frankly resistant to TKIs. This Review focuses on the role of LSCs and stem cell niche in relation to response to pharmacological treatments. A literature search from PubMed database was performed until April 30, 2017, and it has been analyzed according to keywords such as chronic myeloid leukemia, stem cell, leukemic stem cells, hematopoietic niche, tyrosine kinase inhibitors, and drug resistance.
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SIGNIFICANCE STATEMENT

This article deals with the revision of scientific literature on the role of leukemic stem cells (LSCs) and stem cell niche in relation to the response to pharmacological treatments. Nowadays, current research focuses on LSC specific targets and novel drugs able to eradicate most or all LSCs. Abnormal signaling pathways together with genetic and epigenetic alterations could contribute to LSCs deregulation, to the development and progression of chronic myeloid leukemia, and together contribute to the intrinsic resistance to tyrosine kinase inhibitors, supporting the development of new therapies capable of eradicating specifically the LSCs.

INTRODUCTION

Involvement of Leukemia Stem Cells in Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML), also known as chronic myelogenous leukemia, is a myeloproliferative disorder arising from myeloid CD34+/CD38–/CD90+ progenitors in the bone marrow (BM) [1]. CML originates because of an incomplete process of differentiation of the hematopoietic stem cells (HSCs) to adult cells and by an accumulation of their immature form into the BM and the peripheral blood (PB). CML disease is characterized by the translocation of the Abelson (*ABL1*) gene from chromosome 9 to the long arm of chromosome 22, the Breakpoint Cluster Region (*BCR*) [t(9;22)(q34;q11)], generating the so called "Philadelphia chromosome." This translocation allows the constitutive activation of the fusion oncoprotein BCR-ABL1, a tyrosine kinase (TK) activating different signaling pathways. The resulting phenotype is characterized by an uncontrolled proliferation, a reduced sensitivity to apoptotic signals and production of an expanded progenitor population known as cancer stem cells (CSCs) or leukemia stem cells (LSCs). Indeed, the generation of *BCR-ABL1* fusion gene in a specific multipotent HSC population may be sufficient to initiate the expansion of hematopoietic cell clones (HSCs-granulocytic cell lineage), representing the first step in the genesis of CML, followed by the acquisition of additional genetic mutations beyond *BCR-ABL1* [2].

The first evidence of LSCs was proposed 20 years ago by Bonnet and Dick [3], based on the idea that only a small subset of leukemic cells display self-renewal and long-term disease-propagating ability [3–6]. Nowadays, the presence of LSCs

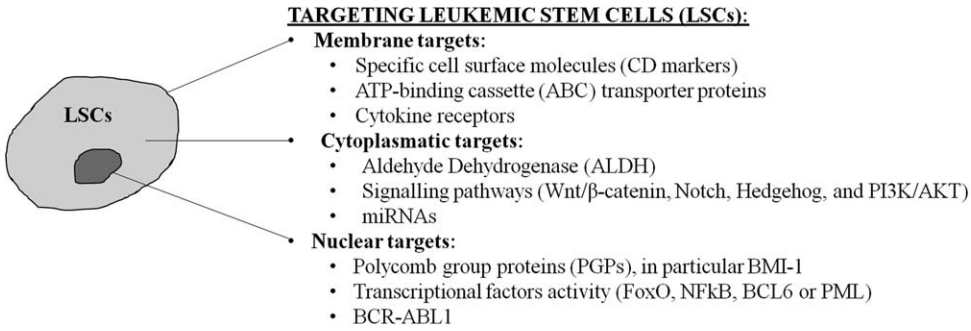

Figure 1. Leukemic stem cells (LSCs) and potential targets of drug therapy.

is partially associated with initiation, drug resistance and relapse of CML. Although tyrosine kinase inhibitors (TKIs), such as imatinib, nilotinib, dasatinib, bosutinib, and ponatinib, have revolutionized the treatment of CML, offering survival rates higher than 90% [7], at least 25% of patients show TKI resistance, more frequently due to the presence of leukemic clones expressing *ABL1* mutations [8–14]. However, it has been demonstrated that persistence of LSCs could be independent of BCR-ABL1 kinase activation [15, 16]. Overall, bilogical evidences suggest that curative approaches in CML must focus not only on kinase-dependent mechanisms but also on kinase-independent mechanisms of resistance [4], hence offering the possibility to reduce the risk of disease relapse. Nowadays, current researches are focused on specific targets of LSC and novel drugs are designed to eradicate the LSC population [17, 18]. These strategies include (a) targeting specific LSC surface markers (b) modulation of signaling pathways (i.e., Wnt or Hh) able to reverse LSC quiescence by interfering with the activity of transcriptional factors (i.e., FoxO, BCL6, or PML), (c) interaction with the hematopoietic niche, (d) inhibition of drug-efflux pumps (e.g., ABC transporters), (e) targeting differences in epigenetic regulation between normal and LSCs (Fig. 1).

Identification of the LSCs

The isolation of LSCs from their normal counterparts is one of the major challenges in CML. LSCs display cell surface markers similar to those expressed by HSCs, including the expression of CD34, Thy1/CD90, CD133, aldehyde dehydrogenase (ALDH) and several lineage markers that characterize different types of mature cells (Table 1) [5, 10, 11, 19, 20]. Despite the similarities between HSCs and LSCs, recent studies identified new surface markers for LSCs, such as the interleukin-1 receptor accessory protein (IL1RAP), and the differential expression of already known markers, such as Siglec-3 (CD33) and interleukin-3 receptor alpha chain (IL-3RA/CD123), overexpressed in LSCs rather than HSCs [21–23]. LSCs also express several surface markers relevant for LSC-niche interaction, such as CD44, CXCR4, and stroma cell-derived factor-1 (SDF-1) [24]. More recently, CD26, also known as dipeptidyl peptidase IV (DPPIV), has been identified as a specific marker for CD34+/CD38– LSCs, together with IL-2RA (CD25) and CD52 [8, 25]. Indeed, CD26 is known as the target enzyme disrupting the SDF-1-CXCR4-axis by cleaving SDF-1, thus facilitating the mobilization of LSCs from the BM niche. On the contrary, this biomarker was not detected in normal HSCs or in other hematopoietic malignancies [8, 25]. Another phenotypic marker used to distinguish HSCs from leukemic cells includes the ability to cause efflux of Rhodamine-123 (Rho) and Hoechst 3342, which defines the so-called Side Population (SP), characterized by the presence of different members of the ATP-binding cassette (ABC) family of transporters, including ABCB1/P-gp (P-glycoprotein) and ABCG2/BCRP1 (breast cancer resistance protein 1) [19]. In particular, the chemotherapy-resistant phenotype of LSCs is due to the presence of a higher expression of ABCB1/P-gp, ABCC1/MRP1 (multi-drug resistance related protein 1), LRP (lung-resistance protein), and ABCG2/BCRP1 [26]. Moreover, in addition to SP phenotype, ALDH, a cytosolic enzyme playing a key role in overall chemotherapy resistance, may be considered another LSC marker [27]. Finally, the high levels of *BCR-ABL1* transcript in LSCs were found to be linked to the autocrine secretion of IL-3 and G-CSF (granulocyte-colony stimulating factor) that may induce the proliferation and survival of CML progenitors, and contribute to their mobilization through an increased secretion of chemokines such as CXCR12, CCL2 (chemokine ligand 2) and CCL3/MIP-1α, compared with HSCs.

Mechanisms of LSCs Self-Renewal Deregulation

Signaling Pathways in CML. Self-renewal deregulation has been recognized as one of the most important events in leukemia progression [28]. The aberrant activation of this feature is often related to deregulated signaling pathways; indeed, their modulation is considered a useful tool in CSCs research (Fig. 2) [29].

Wnt/β-catenin, Notch, and Hedgehog (Hh) are the most important signaling pathways either in normal HSCs homeostasis or in maintaining CML stem cells [30]. As a matter of fact, the disruption of differentiation and the enhancement of self-renewal in CML progenitor cells is a critical component of disease progression [31].

The Wnt pathway is crucial for HSC self-renewal and interaction with BM niche, especially during the switch from chronic to blastic phase (BP), and its activation has been associated with the presence of the oncoprotein BCR-ABL1. When bound to its receptor, Wnt ligand induces the inhibition of β-catenin phosphorylation by glycogen synthase kinase 3 β (GSK3β), and its cytoplasmatic accumulation. The subsequent nuclear translocation of β-catenin activates several transcription factors, such as LEF/TCF, which expand the HSC pool [32]. On the contrary, LSCs have aberrant activation of β-catenin in BP-CML patients. Indeed, in both K562 cells and in a CML murine model, BCR-ABL1 could stimulate β-catenin through the inhibition of GSK3β mediated by phosphoinositide 3-kinase (PI3K)/Akt signaling in blastic crisis (BC) of CML, with an increase of leukemic burden and disease progression in mouse model [33].

Notch signaling pathway is involved in the interaction among leukemic, metastatic, and normal cells, and their native microenvironment. Nakahara and coworkers reported that the Notch

Table 1. Cell markers that differentiate HSCs and LSCs

	HSCs	LSCs
CD25 (IL-2RA)	–	+
CD26 (DPPIV)	–	+
CD33 (Siglec-3)	+/–	+
CD34	+	+
CD38 (cyclic ADP ribose hydrolase)	–	–
CD44 (LHR)	+	+
CD47	+/–	+
CD52	+/–	+
CD71 (TfR1)	–	–
CD90 (Thy-1)	+	+
CD117 (c-KIT)	+	+
CD123 (IL-3RA)	+/–	+
CD133 (Prominin-1)	+	+
Specific Lineage Markers (Lin)	–	–
Aldehyde dehydrogenase (ALDH)	+	+++
Side population (SP cells)	+	+++
BCR-ABL1 translocation	–	+

Abbreviations: DPPIV, dipeptidyl peptidase IV; HSCs, hematopoietic stem cells; LSCs, leukemic stem cells.

pathway plays an important role in advanced stages of CML [34]; the Hairy enhancer of split 1 (*Hes1*), a Notch target gene, in combination with *BCR-ABL1*, was able to induce the BP in an in vivo mouse model, with the consequent rapid death of animals. In addition, Hes1 was highly expressed in 8 of 20 CML patients in advanced stages, suggesting that Hes1 may be a key molecule for the transition from chronic phase (CP) to BP [34]. Moreover, the aberrant activation of another self-renewal pathway critical for the maintenance of the LSCs during treatment with TKIs is Hedgehog (Hh), which is able to induce malignant expansion of LSCs in an in vivo murine model, through a crosstalk with other pathways [35, 36]. Hh signaling seems to be essential for CSC maintenance in myeloid leukemia and the loss of the transmembrane protein smoothened (SMO), an essential component of this pathway, impaired HSCs renewal and decreased the induction of CML by the BCR-ABL1 oncoprotein, whereas constitutively active SMO was found to augment LSC number and accelerate the disease [37]. In CML, *BCR-ABL1* induces also the activation of PI3K/AKT/mTOR pathway, resulting in a deregulated activity of several transcription factors, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and forkhead box class O (FoxO). Members of the FoxO transcription factors (i.e., FoxO1, 3a, 4, and 6) are targeted for proteolysis via Akt-mediated signaling [38], and play an important role in the maintenance of normal HSCs and LSCs [39, 40]. Interestingly, the effect of BCR-ABL1 on FoxO3a was also modulated via transforming growth factor-β (TGF-β) signaling. In a mouse model of a CML-like myeloproliferative disease, Naka and collaborators showed that the nuclear localization of FoxO3a, together with a decreased Akt phosphorylation in CML-CSCs, was associated with a decreased potential of CSCs to promote malignancies, despite the expression of BCR-ABL1 [40]. Several other proteins related to FoxOs, like the proto-oncogene B-cell lymphoma 6 protein (*BCL6*), represent important FoxO downstream effectors of self-renewal signaling in CML-initiating cells [41]. In addition, BCR-ABL1 may activate multiple down-stream pathways, including the Raf/MEK/ERK, SRC, and JAK/STAT pathways [42, 43]. In a recent review, it has been reported that Aurora kinase A (AURKA), through mechanisms of phosphorylation of a series of genes (i.e., RASSF1A tumor suppressor gene) and proteins (i.e., anti-apoptotic modulators Bcl-2 and MCL-1 and pro-apoptotic regulators Bax, Bim, and PUMA), is involved in the tumorigenesis of different hematological neoplasms (i.e., CML), and in the control of proliferation, epithelial-mesenchymal transition (EMT) and metastasis, as well as self-renewal ability of CSCs [44].

Many other genes are related to CML and stem cell maintenance and transformation. The tumor suppressor protein phosphatase 2A (PP2A) could reduce the survival and self-renewal of LSCs through BCR-ABL1 kinase-independent and PP2A-mediated inhibition of Jak2 and β-catenin [45], whereas arachidonate 5-lipoxygenase (5-LOX) gene (*Alox5*) and calcium-calmodulin-dependent protein kinase II (CaMKII), are critical regulators for LSCs in BCR-ABL-induced CML [46, 47]. Similarly, the tumor suppressor promyelocytic leukemia (*PML*) gene has been also identified as a regulator in the hematopoietic system and as an important controller for stem cell maintenance and differentiation [48].

Epigenetics in CML. Several studies showed that developmental pathways, including the polycomb-group protein (PGP), and in particular BMI-1, play a role as epigenetic repressors implicated in the regulation and expansion of LSCs during advanced phases and in therapy resistance of CML [49–51]. In vitro results demonstrated that the upregulation of BCR-ABL1 increased BMI-1 expression in CD34+ cells of CML patients, suggesting that BMI-1 activity was controlled by BCR-ABL1 [52], and that BMI-1 cooperated with BCR-ABL1 during disease progression [53]. In addition, our group recently reported that BMI-1 expression increased in all patients in the initial phases of treatment with imatinib, and that its downregulation was associated with better responses to TKIs and longer event-free survival (EFS) [54].

DNA methylation, histone modification, and miRNAs also play a significant role in leukemogenesis: aberrant DNA methylation could silence the expression of tumor suppressor genes in leukemia, whereas the overexpression of histone methyltransferases promotes oncogenesis (Table 2). Abnormal hypermethylation of the ABL1 promoter represents an important marker of CML pathogenesis associated with clonal evolution and disease progression [55]. For example, the methylation of additional genes, such as the transcription factor AP-2 α (*TFAP2α*), the early B-cell factor 2 (*EBF2*), the autophagy related 16-like 2 (*ATG16L2*), the death-associated protein kinase 1 (*DAPk1*), and the cell cycle regulating gene p15 (*CDKN2B*) was associated with progression, resistance to TKIs, and a low probability to achieve MR3 (BCR-ABL1/ABL1 ratio = 0.1% IS) at 18 months [56].

Histone modifications, including methylation and acetylation, are involved in progression of CML; several pro- and anti-apoptotic and cell cycle genes are targets of histone deacetylase (HDAC) and histone acetyltransferases (HATs) in cancer cells [57]. Sirtuin 1 deacetylase (SIRT1) regulates the acetylation of several transcriptional factors (i.e., TP53 and FoxOs) and consequently plays an important role in the maintenance of normal and leukemic SCs [58, 59]. Recently, it has been demonstrated that SIRT1 deacetylase is able to promote the acquisition of genetic mutations implicated in drug resistance and survival of CML LSCs

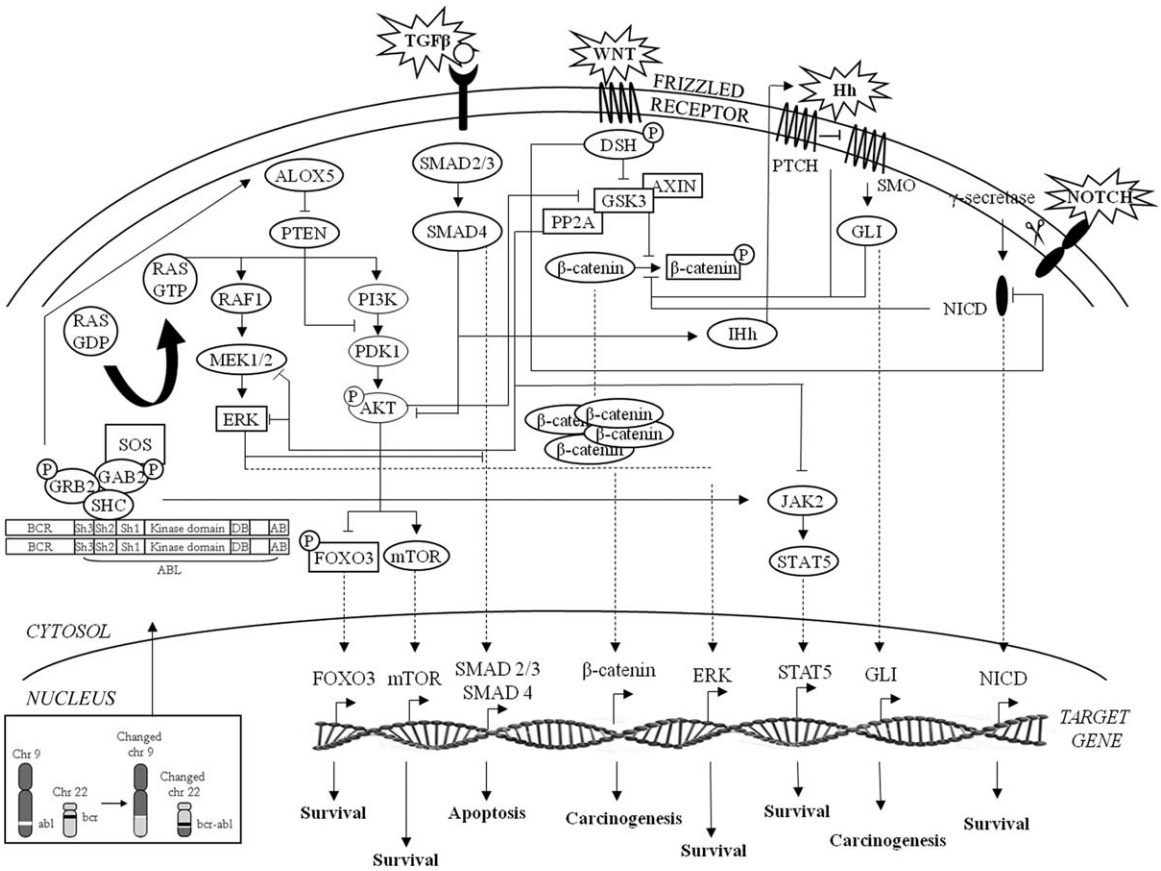

Figure 2. The BCR-ABL pathway involves an intricate signaling networks including RAS/RAF/MEK/ERK, PI3K/AKT/mTOR, TGF-β, WNT/β-catenin, Hedgehog, and NOTCH. In the nucleus, the translocation from the normal chromosomes 9 to 22 generates the BCR-ABL hybrid gene, producing the constitutively active tyrosine kinase oncoprotein. In the cytosol, the BCR-ABL protein, following recruitment of GRB2/GAB2/SOS complex, stimulates the conversion of GDP-bound form of RAS in its active GTP-bound state. Therefore, the permanently activated RAS/RAF/MEK/ERK pathway leads to an abnormal cell proliferation. In addition, the ignition of the PI3K/AKT/mTOR pathway causes both the activation of mTOR and the inhibition of FOXO3, resulting in LSCs survival. This result is also promoted by the activation of TGF-β/SMAD4 way. Simultaneously, BCR-ABL supports the novel ALOX5 signaling pathway, suppressing the tumor suppressor gene *PTEN*. The inhibition of GSK3 maintained through the deregulation of both PI3K/AKT/mTOR and WNT/β-catenin pathways results in increased cytoplasmatic β-catenin levels and, therefore, carcinogenesis. The progress of the disease in also correlated to Hh pathway, through the release of GLI transcription factor, and NOTCH signaling through either the suppression of β-catenin phosphorylation by NICD or NICD inhibition by DSH. Abbreviations: ABL, Abelson; AKT, v-Akt murine thymoma viral oncogene homolog 1; ALOX5, arachidonate 5-lipoxygenase; BCR, break point cluster region; DSH, Disheveled; GSK3, glycogen synthase kinase 3; ERK, extracellular signal-related kinase; FOXO3, forkhead box O3; GAB2, GRB2-associated-binding protein 2; GLI, glioma-associated oncogene; GRB2, growth factor receptor-bound protein 2; Hh, Hedgehog; IHh, Indian Hedgehog; JAK2, Janus kinase; MEK1/2, mitogen-activated protein kinase 1/2; mTOR, mammalian target of rapamycin; NICD, Notch receptor intracellular domain; PDK1, pyruvate dehydrogenase kinase 1; PI3K, phosphoinositide-3-kinase; PP2A, protein phosphatase 2A; PTCH, Protein patched homolog 1; PTEN, phosphatase and tensin homolog; RAF1, rapidly accelerated fibrosarcoma 1; RAS-GDP, RAS-guanosine diphosphate; RAS-GTP: RAS-guanosine triphosphate; SHC, SHC-transforming protein; SMAD2/3/4, small mother against decapentaplegic 2/3/4; SMO, smoothened; SOS, son of sevenless homolog; STAT5, signal transducer and activator of transcription; TGF-β, transforming growth factor beta.

Table 2. Epigenetic mechanisms inducing leukemic stem cells (LSCs) deregulation

Epigenetics	Role
Polycomb group proteins (PGPs)	Involved in the regulation and expansion of LSCs during the CML advanced phases
DNA methylations	Significant role in leukemogenesis Methylation of several genes (i.e., *ABL1*) are associated with progression of CML and resistance to TKIs
Histone modifications (methylation and acetylation)	Significant role in leukemogenesis and in CML progression (i.e., *SIRT1*)
Micro RNAs	Significant role in leukemogenesis miRNAs expression in CML patients reflect the disease progression from the chronic to advanced phases

Abbreviations: CML, chronic myeloid leukemia; SIRT1, sirtuin 1 deacetylase; TKIs, tyrosine kinase inhibitors.

[60–62]. In an in vitro model, Wang and colleagues showed that the inhibition of SIRT1 deacetylase by small molecule inhibitors or gene knockdown blocks the acquisition of *BCR-ABL1* mutations and progression during treatment with TKIs [61]. The same authors also demonstrated that *SIRT1* silencing reduced and compromised the maintenance of LSCs in the BALB/c CML model through an increased expression of cyclin-dependent kinase CDK6 and a reduction of TP53 expression [63].

Finally, miRNAs play a relevant role in CML. Machova Polakova and colleagues identified differential expression profiles of several miRNAs (miR-150, -20a, -17, -19a, -103, -144, -155, -181a, -221 and -222) in CML patients at different stages of disease, reflecting the transformation from the chronic to advances phases of disease [64]. Interestingly, the target of miRNAs are proteins involved in cell cycle, growth regulation, and signaling pathways related to CML [64]. Analyzing these data, Ferreira and coworkers found that TKIs (imatinib, nilotinib, and dasatinib) modulated the expression of a series of different miRNAs in *BCR-ABL1*-positive cells obtained from patients in different phases of CML including miR-let-7d, -let-7e, -15a, -16, -21, -130a, -142-3p, and -145, with different levels with respect to healthy subjects and according to the phase of the disease [65]. In vitro, the long-term exposure of CML K562 cells to nilotinib and dasatinib induced drug-resistance in association with increased levels of DNA methyltransferase and downregulation of miR-217 [66]. On the contrary, miR-30e induced apoptosis and sensitized CML K562 cells to imatinib via regulation of the BCR-ABL1 protein [67]. In a recent work, the miRNA expression profile was assessed in different population of LSCs (Lin- CD34+ CD38- and Lin- CD34- CD38-), and showed that the upregulation of miR-29a-3p and miR-660-5p in Lin- CD34+ CD38- CML LSCs, and downregulation of their respective targets TET2 and EPAS1, induces TKI-resistance. At the same time, miR-494-3p downregulation was observed, leading to c-MYC upregulation, and consequent TKI-induced apoptosis [68].

Taken together, these data demonstrate that several epigenetic modulations of important genes play a critical role in the pathogenesis of CML and in drug resistance to therapy. Since epigenetic changes have the potential to be modulated, they may represent potential molecular tools to detect and treat cancer. Nowadays, new drugs targeting different epigenetic mechanisms are in development in several clinical trials or approved for specific cancer types, including DNA methylation inhibitors, HADC inhibitors, miRNA antisense oligonucleotides and miRNA mimics.

LSCs Interaction with the Hematopoietic Niche

In addition to the *ABL1* mutations, poor response to treatments is often associated with quiescent LSCs located within the endosteal region of the BM [69, 70]. Indeed, LSCs are capable of interacting with the BM microenvironment, hence influencing the phenotypes of surrounding cells, which acquire an increased proliferation potential and a reduced sensitivity or clear resistance toward TKIs [71]. Indeed, there is evidence indicate that leukemic cells can exploit physiological niche signals and overcome the control by the normal microenvironment and/or can remodel BM niches, supporting disease progression at the expense of the normal hematopoiesis [72]. Generally, during normal hematopoiesis, a bidirectional interaction between the niche and HSCs is required for the maintenance of quiescence of normal BM stem cell. It is well known that the BM microenvironment supports and regulates the self-renewal, proliferation and mobilization of HSCs, through cytokines, chemokines, and adhesion molecules [73]. These BM cytokine networks are also important for HSC maturation, pluripotency, and contact with immune cells. Generally, the BM niches contribute positively or negatively to HSCs status. Several factors, including the osteoblastic signaling molecule Jagged-1 [74], osteopontin, and the chemokine (CXC motif) receptor 4 (CXCR4) and ligand 12 (CXCL12 or SDF-1) are important for the proliferation, apoptosis, trafficking, and mobilization of HSCs to the BM, respectively, [75–77]. Current evidence suggests that the HSC mobilization by G-CSF is mediated by induction of BM proteases, attenuation of functions of adhesion molecules, such as integrin $\alpha4\beta1$ (very late antigen-4, VLA-4), vascular cell adhesion molecule-1 (VCAM-1), and disruption of CXCL12/CXCR4 signaling in the BM [78]. In relation to CML, altered adhesion of leukemic cells to the stroma was shown; indeed, Jin and colleagues demonstrated that in CML, BCR-ABL1 activity is critical for the downregulation of CXCR4 expression, resulting in a defective adhesion of CML cells to BM stroma [79]. Moreover, in the in vitro systems of KBM-5 cells derived from a BC-CML patient cocultured with BM-derived stromal cells (BMSCs), the restoration of CXCR4 expression by TKIs was associated with the increased migration of CML cells [79]. It has also been found that CML cells may be able to reduce functional CXCL12 expression in the surrounding cells, to facilitate their egress from BM to other sites including spleen and PB. Reduced CXCL12 expression in CML cells can also arise from an additional mechanism. BM CML cells may exhibit increased levels of several cytokines and chemokines including IL-1α, IL-1β, IL-6, G-CSF, TNF-α, CCL3, and CCL4 [80]. Moreover, in addition to chemokine receptors, integrins and other components of the extracellular matrix have been implicated in the stroma-mediated chemoresistance and hematopoietic cell homing [81]. Other factors, such as the metallo-proteinase 9 (MPP9), angiopoietin-1, and stem cell factor (SCF, also known as KIT ligand) are instead involved in hematopoiesis and HSCs maintenance [82].

However, LSCs and normal HSCs appear to be differently regulated by the microenvironment [80]. CML progenitors show an altered interaction with the BM microenvironment and are in a continuous state of proliferation due to deregulated functions of important intercellular adhesion molecules on the LSCs surface [83, 84]. It has been demonstrated that LSCs may alter the niche secreting various costimulatory molecules and suppressive cytokines that target metabolic pathways and create an anti-apoptotic environment, thus facilitating the escape from immune surveillance and inducing chemo-resistance. These cytokines include CXCL12, SCF-1, interleukin-6 (IL-6), several growth factors such as TGF-β, basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) [75]. During leukemogenesis, malignant clones become progressively independent from the physiological control of the niche. For example, the reduction of JunB expression in LSCs diminishes the responsiveness of these cells to Notch and TGF-β signals in the BM niche [85]. Moreover, the quiescence and self-renewal of LSCs are strongly affected by the leukemic niche. In particular, in a recent in vitro study, the use of a conditioned medium obtained from a leukemic (REH) cell line, enriched with IL-6 and IL-8, induced the loss of quiescence in HSCs, increasing the proliferative stimulus. This phenomenon could be related to several causes: (a) an increased expression of cell proliferation-associated markers (i.e., Ki-67 and c-Myc), (b) an upregulation of c-Kit, and (c) a downregulation of GATA2 and p53 proteins [86]. The effect of IL-6, together with TGF-α, has been also demonstrated in newly diagnosed chronic phase (CP) CML patients who subsequently received imatinib, suggesting that

these cytokines may play a role as novel biomarkers. Indeed, their high plasma levels were strongly predictive of subsequent failure to achieve early and deep molecular response, transformation to BP-CML and event-free survival [87].

The microenvironment can also contribute to leukemic survival and drug resistance through the release of exosomes, microvescicles specialized to transfer a number of signals, including immunosuppressive molecules and miRNAs in specific cells [88, 89]. Boyiadzis and coworkers suggested that tumor-derived exosomes can reprogram the BM environment, suppressing the anti-leukemia immunity, and mediating drug resistance [88].

Cancer cells exploit several immunological mechanisms, such as downregulation of target antigens, targeting regulatory T-cell functions, or secretion of immune suppressive mediators. It has been recently demonstrated that exosomes released by leukemic cells may contain membrane-associated TGF-β1, which is able to reduce the ability of natural killer (NK) cells to destroy the LSCs, with the following induction of an immunosuppressive effect that helps cancer cells to evade the immune response [89]. Raimondo and colleagues found that CML-derived exosomes promote the proliferation and survival of tumor cells, both in vitro and in vivo, by activating anti-apoptotic pathways by a ligand-receptor interaction between TGF-β1, present in CML-derived exosomes, and the TGF-β1 receptor in CML cells [90]. Similarly, recent work demonstrates that exosomes released from CML cells could stimulate BM stromal cells to produce IL-8 that, in turn, is able to positively modulate the malignant phenotype and survival of leukemia cells both in vitro and in vivo [91]. Interestingly, increased IL-8 serum levels have been found in hematologic malignancies compared with healthy controls [92]. Moreover, an increased expression of IL-8 and/or its receptors has been detected in cancer and stromal cells suggesting the ability of IL-8 to modulate the tumor microenvironment. Taverna and colleagues found that miR-126 was overexpressed in exosomes compared with parental cells. miR-126 induced a reduction of CXCL12 and VCAM1 expression in human umbilical vein endothelial cells (HUVEC), and negatively modulated the motility and adhesion of LAMA84 cells, suggesting that exosomal miRNAs have an important role in tumor-endothelial crosstalk occurring in the BM microenvironment, potentially affecting disease progression [93]. Exosomes derived from both LAMA84 cell lines and the plasma of CML patients contain amphiregulin (AREG), a member of the epidermal growth factor (EGF) family. AREG has several functions, among which are (a) activation of the EGF receptor signaling in stromal cells, (b) increased expression of SNAIL and its target MPP9 and IL-8, and (c) overexpression of annexin A2, promoting the adhesion, growth, and invasiveness of leukemic cells [94].

Hypoxia induces the expression of multiple pro-angiogenic factors (i.e., VEGF, bFGF, and IL-8) in the tumor microenvironment; it represents a common feature of myeloid malignancies, and is able to influence LSCs cycling, quiescence, differentiation, metabolism, and therapy resistance. Hypoxia-induced VEGF production in a mouse model of CML was found to be correlated with increased clonogenicity, maintenance, repopulation capacity, and resistance of *BCR-ABL1* positive cells [95]. Moreover, when LSCs hide themselves in the hypoxic BM niche, the BCR-ABL1 protein is not synthesized despite the high levels of transcript, thus facilitating the resistance to TKIs.

BMSCs are an essential component of the HSC niche but they are deregulated in a series of myeloid malignancies, including CML. Recent studies, based on mice models of CML, demonstrate that the specific interaction between BMSCs and leukemic cells is important for leukemogenesis. BMSCs in CML failed to maintain normal HSCs due to a reduction of CXCL12 expression and expansion of LSCs. In addition, BMSCs secrete the placental growth factor (PlGF), which stimulate angiogenesis and promotes CML proliferation and metabolism, increasing the disease aggressiveness in a manner partially independent from BCR-ABL1 signaling [96].

Based on the above considerations, leukemia treatment should target the microenvironment, and new biomarkers predictive of treatment outcome should be developed to broaden treatment opportunities.

Resistance to TKIs Mediated by LSCs and Hematopoietic Niche

The introduction of TKIs in clinical practice has significantly improved the outcome of patients affected by CML. Nevertheless, for several individuals, treatment must be discontinued or changed due to the development of adverse drug reactions or resistance. In CML patients, resistance may essentially develop due to secondary *BCR-ABL* mutations, and/or *BCR-ABL*-independent mechanisms, including the activation of signaling pathways able to sustain the growth of progenitor LSCs (i.e., Wnt/β-catenin, Notch, PI3K, Hh), epigenetic control (i.e., polycomb genes), and the activity of transmembrane transporters (i.e., influx/efflux transporters).

One of the major causes of drug resistance is the altered function of the transmembrane transporters. Several members of the ATP-binding cassette (ABC) transporters family are upregulated in tumor cells [97] through the release of several factors by microenvironment. Indeed, the expression of ABC transporters is regulated by a complex network of signals (i.e., transcription factors, exosomes and epigenetic mechanisms), which allow the acquisition of the multi-drug resistant (MDR) phenotype [97–100].

Leukemic cells have an increased ability to pump drugs out of the cells, due to the over-expression of transmembrane transporters. The most widely studied ABC transporter members include ABCB1/P-gp, ABCC1/MRP1 and ABCG2/BCRP. In normal HSCs, these transporters play a relevant role in protection from genetic damage exerted by toxic substances, and participate also in HSC quiescence, differentiation and self-renewal, through the regulation of cellular signaling effectors [101, 102]. Resistance to imatinib may be mediated by the over-expression of ABCB1 in K562 cells with loss of the inhibitory effect on cellular proliferation and apoptosis [103], even if some contrasting results have been published [104]. Studies of AP-CML patients treated with imatinib have shown variations in the number of ABCB1-positive cells during treatment, suggesting that TKI treatment causes selection of LSCs characterized by the overexpression of transmembrane transporters, together with other ABC members, such as ABCC1 and ABCG2 [104, 105].

The MDR phenotype may arise not only through the efflux of ABC transporters, but also through several other phenomena such as the reduction of drug influx and imbalance in cell growth, survival, and death signaling. In addition to drug efflux, the uptake of some TKIs into cells is dependent on membrane transporters, such as the organic cation transporter 1 (OCT1) and the organic cation/carnitine transporter 2 (OCTN2). Low expression of these transporters induced a reduction of TKIs cytosolic concentrations, playing an important role in therapeutic failure [106]. The expression profile of influx carriers was significantly decreased in

Table 3. Signaling pathways and transcriptional factors deregulated in response to BCR-ABL1 activation

Pathway	Role	Effectors	Inhibitors
Wnt/β-catenin	• HSCs self-renewal • Interaction with BM niche • CML progression	Wnt β-catenin GS3K	Av65 Indometacin Av65 SB216763
Notch	• Interaction between leukemic, HSCs, and BM niche • CML advanced stages	γsecretase	RO4929097 MK0752
Hedgehog (Hh)	• Pathogenesis of CML	SMO	LDE225 (NCT01456676) BMS833923 (NCT01218477; NCT01357655) PF04449913 (NCT00953758) Cyclopamine GDC0449
PI3K/AKT/mTOR	• Normal hemopoiesis	PI3K AKT mTOR1/2	NVP-BEZ235 (NCT01756118) GDC0941 KU-0063794 Triciribine (NCT00642031) NVP-BEZ235 (NCT01756118) Rapamicin KU-0063794
FoxO/TGF-β	• Expression of genes involved in cell growth, proliferation and differentiation • Involvement in BCR-ABL activated PI3K/AKT pathway	TGF-β	Ly364947 SB431542
JAK/STAT	• Normal hemopoiesis • Key player in a variety of myeloproliferative disorders	JAK 1/2	Ruxolitinib (NCT01914484; NCT01751425) AG490 TG101209 CYT387 ONO44580
ALOX5	• Initiation and progression	ALOX5	Zileuton (NCT02047149; NCT01130688)
PML	• Critical role in hemopoiesis • Deregulated in CML • LSC maintenance	PML	Aresnic trioxide (NCT01397734; NCT00006091; NCT00250042; NCT00053248) Arsenic sulfide
PP2A	• Tumor suppressor protein downregulated in CML LSCs in comparison to normal HSCs	PP2A	LB100

Abbreviations: CML, chronic myeloid leukemia; HSC, hematopoietic stem cells; PI3K, phosphoinositide 3-kinase; PML, promyelocytic leukemia; PP2A, protein phosphatase 2A; SMO, smoothened; TGF-β, transforming growth factor-β.

imatinib-resistant cell lines (i.e., K562-RC) with respect to the parental K562 cell line [107]. Although those data strengthened the role of transporters in response or resistance to TKIs, the correlation among transporters, LSCs and TKIs resistance has not yet been described.

As discussed in the above paragraphs, several factors may contribute to the enhanced survival and growth of LSCs [36]. These cells have high BCR-ABL1 expression, and resistance may arise from the inability of the drug to fully inhibit the BCR-ABL1 kinase activity. Indeed, TKIs have been described to inhibit the oncogenic function in LSCs, without a complete eradication of the disease due to the presence of intrinsic and acquired drug resistance [9, 10]. Indeed, imatinib, nilotinib, and dasatinib efficiently kill most CML cells, but cannot eliminate quiescent LSCs (CD34+ CD38– CD45RA– CD71– HLA–), which are responsible for the progression of CML in patients who are diagnosed in advanced stages of disease. Unfortunately, the relationship between the interaction of LSCs with hematopoietic niche and resistance to TKIs is still an unsolved issue.

Novel Chemotherapeutic Approaches for CML

The search for strategies to specifically target the LSC populations are in development. New drugs such as DNA methylation inhibitors (DNMTi, i.e., 5-aza-2-deoxycitidine or decitabine, hydralazine and valproate) and HADC inhibitors (i.e., phenylbutyrate, romidepsin, entinostat, and vorinostat), are studied. In a phase I clinical trial the effect of vorinostat and decitabine has been evaluated in CML patients (NCT00275080). In addition, an open-label, phase I/II study investigated the combined treatment of dasatinib and decitabine in the control of CML disease (NCT01498445). Moreover, the efficacy of panobinostat (LBH589) was evaluated in combination with imatinib in inducing apoptosis in BC-CML CD34+ cells, and in downregulating BCR-ABL1 levels (NCT00686218).

Similarly, the use of microRNA antisense oligonucleotides and microRNA mimics have been also considered as potentially important approaches to use in CML [108, 109].

In relation to new drugs targeting the signaling pathways, an overview of the principal clinical trials is reported in Table 3. Targeting components of these survival pathways, alone or in

combination with TKIs, represents an attractive potential therapeutic approach against LSCs. However, many pathways are also active also in normal stem cells.

The targeting of the Hh pathway, known to be activated in CD34+ CP-CML, through the inhibition of SMO using LDE225 alone or in association with nilotinib, was effective in reducing the number and the self-renewal capacity of CML LSCs in vitro, without effects on normal HSCs [110]. A phase I clinical trial investigated the feasibility of administration of LDE225 with nilotinib in patients with CML who have failed other TKI treatments (NCT01456676).

Zileuton is an ALOX5 inhibitor, and in combination with imatinib showed an additive effect with prolonged survival in an in vivo mouse model [46]. Based on these positive data, the safety of the combined treatment zileuton plus imatinib has been evaluated in a phase I study enrolling CML patients (NCT01130688). Clinical phase 1/2 trials have been carried out in relation to arsenic trioxide (As_2O_3), a PML inhibitor. A phase 1/2 study has been developed to test the combined activity of As_2O_3 and imatinib in treating patients who have CP-CML (NCT00053248). Similarly, a more recent phase I clinical trial addresses the combination of As_2O_3 with imatinib, dasatinib or nilotinib in CML patients (NCT01397734). Starting from preliminary results, the approach seems to be effective, but the toxicity and the effects on normal HSCs needs to be monitored.

Several other drugs able to selectively inhibit signaling pathways are now in evaluation in preclinical models (Table 3) to assess their capabilities to effectively eradicate CML stem cells while sparing normal counterparts.

Conclusion

Unfortunately, we are still far away from the eradication of LSCs, despite the long survival with a good quality of life in more than 90% of patients affected by CML. Indeed, gatekeeper mutations of ABL1 may occur during the treatment with TKIs, thus interfering with drug efficacy, and LSC survival can be associated with about 20% of treatment failures. At the same time, the activation of the oncogene *BCR-ABL1* is responsible of the modulation of different signaling pathways, which allow stem/progenitor CML cells to evade cell death. Interestingly, epigenetic control could be relevant to the survival of LSCs. BMI-1 is over-expressed in the advanced phases of disease and correlates well with the clinical outcome of CML patients. Furthermore, the hypoxic microenvironment blocks the synthesis of the BCR-ABL1 protein, thus making the LSCs resistant to TKIs. On the other hand, the BM niche is able to hide the LSCs to the immune system through several mechanisms. At the onset of disease, high levels of T-regs and suppressive myeloid-derived suppressor cells, low numbers of NK, and the activation of the immune check point system, prevent the immune-dependent elimination of the LSCs. TKIs may ameliorate the effect but they are not able to overcome it. However, the persistence of LSCs is also independent of BCR-ABL1 kinase activation. Transmembrane transporters may play a relevant role for both the excretion and uptake of drugs. LSCs may activate the efflux pumps (i.e., ABC transporters), hence eliminating the TKIs, while low-activity uptake transporters (i.e., hOCT1) may be associated with lower intracellular concentrations of imatinib and, consequently, with a poor response to therapy.

Therefore, we know at least some fundamental mechanisms that support LSC survival, and we are able to identify the CD34+/CD38–/CD90+/CD26+ CML LSCs also in the PB, even if recent data have demonstrated the ability of CSCs to undergo dynamic changes at the level of cell surface markers followed by epigenetic regulations by the microenvironment.

Thanks to the huge steps forward in the treatment of CML, several pathogenetic mechanisms responsible for the initiation and survival of LSCs have been discovered, while others will be investigated in the next years and will likely nourish the future therapy of CML.

Author Contributions

E.A., M.D.R., and A.D.P.: conceived, edited, and performed a comprehensive review of the literature, and manuscript writing; E.A. and E.R.: have created figures and tables. M.D.R., S.G., R.D., and A.D.P.: reviewed the manuscript. E.A., M.D.R., S.G., G.R., E.R., S.C., B.C., M.P., R.D., and A.D.P.: contributed to the final revision and approval of the manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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