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Cellular and Molecular Networks in Chronic Myeloid Leukemia: The Leukemic Stem, Progenitor and Stromal Cell Interplay

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Abstract

The use of imatinib, second and third generation ABL tyrosine kinase inhibitors (TKI) (*i.e.* dasatinib, nilotinib, bosutinib and ponatinib) made CML a clinically manageable and, in a small percentage of cases, a cured disease. TKI therapy also turned CML blastic transformation into a rare event; however, disease progression still occurs in those patients who are refractory, not compliant with TKI therapy or develop resistance to multiple TKIs. In the past few years, it became clear that the BCR-ABL1 oncogene does not operate alone to drive disease emergence, maintenance and progression. Indeed, it seems that bone marrow (BM) microenvironment-generated signals and cell autonomous BCR-ABL1 kinase-independent genetic and epigenetic alterations all contribute to: *i.* persistence of a quiescent leukemic stem cell (LSC) reservoir, *ii.* innate or acquired resistance to TKIs, and *iii.* progression into the fatal blast crisis stage. Herein, we review the intricate leukemic network in which aberrant, but finely tuned, survival, mitogenic and self-renewal signals are generated by leukemic progenitors, stromal cells, immune cells and metabolic microenvironmental conditions (*e.g.* hypoxia) to promote LSC maintenance and blastic transformation.

Keywords

Stem cells; microenvironment; chronic myeloid leukemia

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

1. INTRODUCTION

Exposure to harmful environmental conditions (*e.g.* ionizing radiation) and/or genetic predisposition seems to be the cause of chronic myeloid leukemia (CML), a biphasic hematopoietic stem cell (HSCs)-derived but progenitor-driven myeloproliferative disorder characterized by the accumulation of apparently normal myeloid cells [1, 2]. The hallmark of CML is the Philadelphia (Ph^+) chromosome that originates from the reciprocal translocation t(9;22)(q34;q11). This encodes the constitutively active *BCR/ABL1* oncogenic tyrosine kinase whose expression is essential for the onset, maintenance and progression of the disease and its response to TKI therapy although results from a *BCR-ABL1* knock-in animal model argue against the plethora of data supporting a role for BCR-ABL1 as the oncogene sufficient to induce CML [1-3].

The initial chronic phase (CML-CP), if left untreated naturally evolves into the fatal blast crisis stage (CML-BC) [1, 2]; however, this became a rare event after the advent of ABL TKIs [4, 5]. Three TKIs (imatinib, nilotinib, and dasatinib) are currently used as first-line treatment for patients with newly diagnosed CML-CP [6, 7]. Although the majority of CML-CP patients show a strong and persistent response to imatinib (IM), ~40% of IM-treated CML-CP patients develop BCR-ABL1 mutation-induced drug resistance [4-6, 8]. Likewise, ~35-50% of patients become resistant to second generation TKIs [6, 8]. For patients failing the frontline therapy, other second or third generation TKIs are available and their use depends on each patient's clinical characteristics and on the type of BCR-ABL1 mutations present (*e.g.* ponatinib for patients with BCR-ABL1 gatekeeper T315I mutation); however, only ~50% TKI-resistant CML-CP and ~35% -BC patients show a significant response [6, 8-10]. Furthermore, ponatinib induces severe adverse effects (*i.e.* vascular occlusion, heart failure and hepatotoxicity) in some patients [5, 8, 11, 12] and, as reported for first and second generation TKIs, CML-BC patients do not show long-term response to ponatinib treatment [11, 13]. Similarly, the selection of clones with BCR-ABL1 compound mutations in multiple TKI-treated patients may also confer resistance to ponatinib [14]. For those patients resistant to multiple TKIs, allogeneic BM transplantation remains the only therapeutic option [7, 15]. Other experimental drugs (*e.g.* omacetaxine, KPT-330) are also available but their impact on disease-free survival is not clear yet [13, 16, 17]. Furthermore, TKI therapy discontinuation of IM-treated CML-CP patients in complete cytogenetic (CCyR) and molecular (CMR) response for more than 2 years usually results in disease relapse [10, 18], whereas treatment-free remission (TFR) seems to be more durable and frequent in patients who achieved deeper molecular responses (≥4-log reductions in *BCR-ABL1* levels) upon treatment with imatinib or second generation TKIs [8, 18, 19]. Notably, sustained MMR, but not CMR, after TKI discontinuation was significantly associated with high (600 – 800 mg per day) dose imatinib or second-generation TKI treatment [20]. By contrast, prior history of suboptimal response or IM resistance correlated with disease relapse after second-generation TKI discontinuation [21].

Based on these considerations and on the evidence showing that disease relapse is the most common event after TKI discontinuation [8] and that BCR-ABL1 kinase-independent TKI resistance is commonly observed in CML patients in advanced phases (accelerated or blastic) or in CML-CP patients presenting additional cytogenetic alterations (~10% of CML)

[22, 23], it is becoming clear that cell autonomous genetic or epigenetic changes and/or microenvironmental factors might be responsible for the persistence of Ph^+ cells with stem cell behavior. These LSCs survive and self-renew regardless of BCR-ABL1 activity, present innate or acquired TKI resistance, and are capable of reinitiating CML-CP and/or promoting and maintaining CML-BC [1, 23].

Blastic transformation likely results from acquired increase in BCR-ABL1 expression and kinase activity [24-26], which leads to altered mRNA metabolism [27], genetic instability [28] and alteration of pathways regulating survival, proliferation and differentiation of myeloid- or lymphoid-committed $CD34^+$ progenitors [1] (Fig. 1). Thus, BCR-ABL1 may represent the key factor responsible for the genetic heterogeneity of CML-BC. The molecular events leading to enhanced BCR-ABL1 expression/activity in CML-BC progenitors still remain to be fully understood, although *BCR-ABL1* gene amplification [29, 30], increased *BCR* promoter activity [31], impaired protein phosphatase 2A (PP2A) activity [32], and inhibition of SHP1 phosphatase [32, 33] might occur alone or in cooperation to increase *BCR-ABL1* expression and activity [25]. Among these events, loss of PP2A activity [32] and reactive oxygen species (ROS)-induced genomic instability [34-36] occur in a BCR-ABL1 dose- and kinase-dependent and kinase-independent manner in leukemic progenitors and LSCs, respectively, and significantly contribute to the epigenetic and genetic heterogeneity that characterizes CML-BC [1]. Thus, disease progression may result from the accumulation of a critical number or combination of different mutations [1, 2, 37-42] occurring at the stem and/or progenitor level, and from the pleiotropic effect of enhanced BCR-ABL1 activity, which starts to increase in accelerated phase [1, 38].

Hereafter, we will review the role of cell autonomous and microenvironmental events controlling TKI resistance, survival, proliferation/differentiation and/or self-renewal of CML-CP and BC LSCs.

2. LSC ORIGIN AND REGULATION IN CML-CP AND CML-BC

Genomic instability seems to be a key feature not only of Ph^+ leukemic progenitors, which rely on BCR-ABL1 kinase activity for survival and proliferation [1, 23, 43, 44], but also of CML (CP and BC) LSCs [1, 23, 45] in which BCR-ABL1 kinase but not expression seems to be dispensable for survival and self-renewal [46-48]. Accordingly, a discrepancy between BCR-ABL1 levels and activity has been reported in TKI-resistant quiescent LSCs compared to proliferating $CD34^+$ leukemic progenitors [48]. Indeed, BCR-BL1 expression *per se* is sufficient to recruit and activate JAK2/β-catenin-regulated self-renewal/survival signals in CML-CP and –BC quiescent LSCs [48]. In LSCs and progenitors from both disease stages, BCR-ABL1 also induces genomic instability in a dose-dependent manner by increasing DNA oxidative damage and unfaithful DNA repair [1, 45, 49]. TKI treatment of BCR-$ABL1^+$ cells revealed that BCR-ABL1 kinase mutations and other genetic aberrations associated with disease progression specifically occur in LSCs but not in leukemic progenitors [43]. This supports the idea that the origin and maintenance of Ph^+ LSCs requires BCR-ABL1 expression but not its kinase activity, and that other preexisting or BCR-ABL1 irreversibly-induced genetic or epigenetic changes contribute to LSC survival, self-renewal and innate resistance to TKIs.

Quiescent LSCs are not only resistant to TKIs but also increase upon TKI treatment [23, 50], suggesting that signals generated by TKIs can promote LSC survival. Furthermore, BCR-ABL1 kinase-dependent and –independent genetic (*e.g.* increased ROS, alterations of GSK3β and IKZF1) and epigenetic (*e.g.* C/EBPα downmodulation, SET and β-catenin overexpression) events contribute to the acquisition of LIC characteristics and TKI resistance by the CML-BC granulocytic-macrophage progenitors (GMPs) [1, 23, 51, 52]. Notably, *in vitro* and xenotransplant-based assays aimed at assessing the presence of LICs in different stem and progenitor cell subpopulations from CML-BC patients revealed that acquisition of self-renewal is not restricted to GMPs but can occur in HSCs and different progenitor cell fractions with variability between patients [53]. Thus, a better phenotypic characterization of LSCs in CML-CP and –BC, and a more in-depth analysis of the molecular pathways similarly altered in TKI-resistant quiescent LSCs and progenitors with LSC capability are necessary for eradicating CML (CP and BC) at leukemia-initiating cell (LIC) levels.

2.1. Phenotypic Characterization of LSCs

Targeting of leukemic HSCs in TKI-responsive CML-CP patients and in TKI-resistant CP and BC CML patients requires the correct identification of the leukemic cell pool harboring the Ph^+ LIC population capable of colony-forming cell (CFC), LTC-IC and NOD SCID-γ (NSG) repopulating activities. It is well accepted that the TKI-resistant LSCs in both CML-CP and –BC patients include lineage-negative $CD34^+/CD38^-/CD90^+/CD45RA^-/CD71^-/HLA\text{-}DR^{low}$ cells with innate drug-resistance but also $CD34^+$ cells that have been forced into the G_0 phase of the cell cycle by ABL TKI treatment [46, 50, 54-58]. In CML-BC, lineage-negative $CD34^+/CD38^+/CD45RA^+/CD123^+$ GMPs and, perhaps, other progenitors with acquired self-renewal serve as the LICs [51, 53]. Such a simplistic definition does not take into consideration that markers distinguishing between normal and leukemic HSCs are not well established yet. In this regard, CD123 has recently been proposed as a CML LSC marker because of its overexpression in the CML (CP and BC) stem cell-enriched $CD34^+/CD38^-$ cell fraction [59]. Similarly, Evi1 levels are increased in $CD34^+/CD38^-/CD90^+$ CML LSCs and correlate with enhanced self-renewal [60]. Interestingly, recent reports show that CD25 and CD26 expression appears to be restricted to leukemic $CD34^+$ cells [61-63]. In addition, their expression is higher in leukemic $CD34^+/CD38^-$ than $CD34^+/CD38^+$ cells and increases during blastic transformation (CD26 only in myeloid BC), suggesting the potential for targeting these surface markers in CML eradication and CML-BC clinical trials [61-63]. IL-1 receptor accessory protein (IL-1RAP) expression seems restricted to CML LSCs [58, 64] although it is controversial whether IL-1RAP antibody-mediated targeting may efficiently eradicate CML because its expression in LSCs might be downregulated by TKIs as shown for leukemic progenitors [58].

3. ROLE OF MICROENVIRONMENT IN LSC MAINTENANCE

The inability to find markers to selectively purge LSCs while sparing normal cells suggests that leukemic stem and progenitor cells promote morphological (*e.g.* BM fibrosis) and functional (*e.g.* cytokine/chemokine production; altered stromal-hematopoietic cell interaction) changes in the BM microenvironment that, in turn, provide a protective niche for

LSCs thereby contributing to their quiescence, self-renewal and TKI-resistance [65-68] (Fig. 2). Additionally, TKIs (*i.e.* imatinib) may promote survival of quiescent CML cells by enhancing the expression of CXCR4 that, in turn, favors LSC retention in the BM and their interaction with the mesenchymal stroma [69, 70]. Conversely, G-CSF production by leukemic progenitors decreases CXCL12 expression thereby resulting in decreased LSC homing and BM retention [67]. However, the importance of CXCR4 in LSC maintenance is still unclear as TKI treatment combined with inhibition of CXCR4 mobilized $CXCR4^+$ cells without exerting an effect on leukemia burden [71]. Nonetheless, there is strong evidence indicating that the direct interaction of stromal cells with LSCs plays a pivotal role in LSC survival and TKI resistance. Indeed, N-cadherin-mediated LSC and progenitor cell adhesion to mesenchymal stromal cells enhances LSC self-renewal and survival and protects CML stem/progenitor cells from TKI-induced apoptosis through stabilization/activation of β-catenin signaling [68]. In addition, microenvironment-generated signals also increase ROS-dependent genomic instability in CML-BC GMPs [72-75].

CML stem/progenitor cells remodel the BM niche to skew hematopoiesis towards the expansion of leukemic over normal cells [67, 76]. This is achieved through the aberrant reprogramming of mesenchymal stromal cells that are conditioned to produce cytokine and chemokine (*e.g.* MIP1α, MIP1β, IL-1β, IL-4, IL-6, TNFα, CXCL12, LIF, BMP2/4) [67, 76, 77]. Of note, BMP2/BMP4 proteins control BM myelofibrosis, promote BMP4-dependent amplification and maintenance of LSCs, and induce the BMP2-dependent expansion of committed myeloid progenitors [77]. Furthermore, the presence of IL-2 in the microenvironment increases CD25 levels in LSK mouse cells, consistent with the high CD25 expression observed in Ph^+ $CD34^+/CD38^-$ cells [62]. In addition, proliferating leukemic progenitors secrete CCL3 and TPO that, upon inducing BM myelofibrosis through an expansion of osteoblastic progenitors, confer a selective growth advantage to LSCs over normal HSCs [76], an event likely essential for the pathogenesis of CML [78]. Interestingly, specific activation of the pituitary hormone (PTH) receptor increases TGFβ production by the remodeled osteoblast compartment resulting in TGFβ-induced inhibition of LSC engraftment and survival in immunocompromised mice [65]. Unexpectedly, TGFβ, by inhibiting Akt, induces FOXO3a nuclear localization with consequent increase in the LIC population [79].

The BM niche exerts its LSC protective role not only by releasing soluble factors but also through the presence of an oxygen gradient, which progressively decreases from 6% to 1% in the sinusoidal cavity and endosteal niche, respectively [80]. Culturing leukemic $CD34^+/CD38^-$ cells in hypoxic conditions protects cells from TKIs [81] and increases their survival and self-renewal in a BCR-ABL1 kinase-independent manner [81], suggesting that hypoxia may contribute to LSC quiescence [81, 82]. This likely occurs through modulation of hypoxia-inducible factor-1α (HIF1α). In fact, loss of HIF1α causes cell cycle arrest, impaired engraftment, increased LSC apoptosis, and results in a less severe and non-transplantable CML-BC-like phenotype in immunocompromised mice [83]. Hypoxia also seems to be involved in the modulation of E-cadherin and CXCR4 thus also contributing to LSC homing and retention in the BM niche [84]. Finally, hypoxia increases ROS levels thus contributing to genomic instability and therefore clonal evolution and, perhaps, blastic transformation [85]. Accordingly, hypoxia enhances the capacity of $CD34^+$ CML

progenitors to behave as LSCs in a BCR-ABL1 kinase-independent manner [81, 82]. These HSC-like CML progenitors cultured in hypoxic conditions have a gene expression profile similar to normal $CD34^+$ cells [81] while in normoxic conditions, quiescent CML HSCs are more similar to dividing early progenitors than quiescent normal HSCs are to normal progenitors [86, 87].

The BM also functions as a primary site for immune response towards leukemic stem/progenitor cells. Considering the immunogenic nature of CML and the effects of interferon alpha (IFNα) therapy [88], it is not surprising that IFNα treatment prior to TKI discontinuation may reduce relapse and maintain CCyR [89]. As IFNα is capable of activating natural killer (NK) cells, its protective effect may depend on NK cytotoxic activity [90]. Accordingly, it has been reported that BCR-ABL1 also promotes dendritic cell-mediated NK cell activation [91, 92]. Activated NK cells ($IFN\gamma^+/CD3^-/CD56^+$) are higher in patients with durable TFR and in IM-treated patients in CMR compared to fluctuating CMR patients and healthy controls, suggesting a role for these immune cells in the maintenance of CMR [93]. Similarly, dasatinib induces an expansion of large granular lymphocytes, T and NK cells, and such an effect seems to correlate with long-lasting response to TKI therapy [94, 95]. However, the lack of NK cell differentiation in the presence of CML progenitor-produced IL-15 [96], the reduced NK cell number, and their functional impairment in CML [97] argue against an immune surveillance role for NK cells in CML even if autologous human NK cells prevent CML development [98] and trigger antibody dependent cell cytotoxity (ADCC) against $CD34^+$ CML progenitors in response to anti-IL-3R (CD123) antibody therapy [59]. CML patients carrying BCR-ABL1 mutations display immunogenic T-cell epitopes [99], suggesting that cytotoxic T lymphocytes (CTL) have the potential to target CML stem/progenitor cells. Nevertheless, CTLs induce proliferation of CML LSCs by secreting IFNγ that, in turn, activates the ADAR1 p150-mediated β-catenin survival/self-renewal pathway [100, 101]. Likewise, CTLs augment β-catenin-mediated CML stem/progenitor cell survival also by CD70-mediated triggering of CD27 [102].

4. CELL AUTONOMOUS EVENTS REGULATING LSC MAINTENANCE IN CML (CP AND BC) AND THEIR ROLE AS THERAPEUTIC TARGETS

The modulation of survival and self-renewal signals by the BM microenvironment would not promote the expansion of the leukemic cell clone, drug resistance, and maintenance of the leukemic stem cell pool if those signals were not aberrantly exacerbated through cell autonomous events occurring in leukemic but not normal HSCs (Fig. 2). However, the correct analysis of the cell autonomous events occurring in TKI-resistant quiescent LSCs is polluted by a series of data generated in proliferating stem cell-enriched $CD34^+/CD38^-$ BM cells, and in $CD34^+$ CML-CP progenitors which show sensitivity to TKIs and are BCR-ABL1 oncogene-addicted [1, 23]. However, some signaling pathways are similarly activated in both quiescent LSCs and leukemic progenitors with the only difference that in the latter, oncogenic signals mostly rely on BCR-ABL1 kinase activity [1, 23]. While BCR-ABL1 expression but not its activity represents a key factor allowing the acquisition and maintenance of the leukemic $CD34^+/CD38^-$ LSC phenotype [46-48], it is unclear whether

BCR-ABL1 kinase activity is dispensable for the generation of the lineage-negative $CD34^+/CD38^+/CD123^+/CD45RA^+$ GMP subpopulation with self-renewal ability that exits cell cycle and becomes dormant [103, 104]. The use of a kinase-deficient BCR-ABL1 in a CML animal model argues against the BCR-ABL1 kinase-independence of GMP expansion and acquisition of self-renewal [105], unless homing of these cells in the hypoxic BM niche negatively influences BCR-ABL1 expression and kinase activity as reported for CML-BC cells shifted from normoxic to hypoxic culture and *vice versa* [106, 107]. Thus, downregulation of BCR-ABL1 kinase activity in quiescent LSCs residing in the hypoxic BM niche seems necessary for their self-renewal and survival. This is consistent with the reported ability of BCR-ABL1 to induce differentiation of mouse leukemic LSCs [108].

In the past few years, genetic and epigenetic cell autonomous events leading to the activation of oncoproteins or inhibition of tumor suppressors have been found to be necessary for CML (CP and BC) LSC survival/self-renewal and, likely, disease progression [1, 23, 35, 45, 109] (Fig. 1). Indeed, LSC behavior depends on aberrant, albeit finely tuned, signals that alter, for example, kinase/phosphatase balance, mRNA metabolism (including mRNA processing and miRNA expression), nucleocytoplasmic trafficking, self-renewal, proliferation/differentiation, and response to apoptotic stimuli (*e.g.* TKI-induced) [109].

4.1. Role of JAK2, PP2A and β-catenin

Among the signaling pathways operational in both CML-CP and –BC, the BCR-ABL1/JAK2/SET-PP2A/GSK3β/β-catenin network has an essential role in the regulation of LSC survival and self-renewal [1, 23, 48, 51, 109]. Indeed, the regulation of β-catenin represents a key factor controlling survival and self-renewal of CML LSCs [23, 54, 103, 110-113]. In quiescent $CD34^+/CD38^-$ LSCs from both CML-CP and –BC patients, β-catenin-dependent regulation of survival and self-renewal signals requires BCR-ABL1 expression but not its activity [48, 114]. Conversely, in CML-BC GMPs, β-catenin activation is not solely dependent on BCR-ABL1 kinase activity [51, 105] but relies on the cooperation of both BCR-ABL1-independent and –dependent mechanisms [51, 115, 116]. Several lines of evidence support a key role for β-catenin in the regulation of the CML-BC LSCs, including: BCR-ABL1-dependent and imatinib-sensitive β-catenin tyrosine phosphorylation inhibits β-catenin binding to the destruction complex axin/GSK3β/PP2A, thereby activating TCF4-dependent transcription [115]; axin overexpression prevents *in vitro* replating of CML-BC progenitors; genetic loss of β-catenin impairs self-renewal of normal HSCs and BCR-ABL1-expressing GMPs [112]; GSK3β downregulation increases CML progenitor self-renewal by activating β-catenin and elevating levels of sonic hedgehog pathway mediators such as GLI 1/2 [116, 117]; activation of PP2A impairs self-renewal and survival of leukemic HSCs and CMPs/GMPs in part by inducing BCR-ABL1-independent, JAK2- and GSK3β-dependent degradation of β-catenin [48]. This pathway is further dysregulated in CML-BC as alternative splicing of GSK3β occurs, resulting in a dominant negative form that allows for increased β-catenin activity and GMP self-renewal [116]. This is consistent with previous studies demonstrating that loss of β-catenin prevents LSC self-renewal [112, 114].

Regulation of GSK3β-dependent β-catenin activation in CML (CP and BC) LICs is mediated by induction/activation of JAK2 and SET-dependent inhibition of the PP2A tumor

suppressor [32, 48, 118-121]. Although activation of JAK2 in CML-BC progenitors depends on BCR-ABL1 activity [122-124], there is evidence that BCR-ABL1, regardless of its kinase activity, is able to recruit/activate JAK2 that, in turn, leads to PP2A inhibition/β-catenin activation in mouse $LSK^{BCR-ABL1+}$ and/or quiescent LSCs from CML patients [48]. Furthermore, the notion that JAK2 signaling (*e.g.* STAT3 and its downstream targets Bcl-X_L and Mcl-1) can be activated by cytokines (*e.g.*, IL-6, G-CSF, and GM-CSF) in the microenvironment and/or other extrinsic factors in TKI-treated CML cells [125, 126] suggests that JAK2 can be activated independently of BCR-ABL1 activity. Supporting a critical role for JAK2 in LSC survival it has been reported that combination of BCR-ABL1 TKIs with either pharmacologic suppression of JAK2 or activation of PP2A eliminates both BCR-ABL1-dependent and –independent survival signals, thereby resulting in efficient killing of CML (CP and BC) LSCs and progenitors [48, 127-129]. This led to the development of clinical trials (*e.g.* NCT01751425 NCT01914484; NCT01702064) with nilotinib and JAK2 inhibitors (*e.g.* ruxolitinib). However, drawing any conclusion on whether JAK2 inhibition either improves the therapeutic effects of nilotinib or leads to eradication of CML stem cells is still premature.

Inhibition of PP2A also has a major role in the regulation of LSC survival and self-renewal. Indeed, BCR-ABL1 recruits JAK2 that, in turn, stabilizes BCR-ABL1 through direct phosphorylation and induces/activates SET that, in turn, binds and inhibits PP2A in CML (CP and BC)-derived LSCs and progenitors [32, 48, 121-124, 130, 131]. Notably, SET-dependent suppression of PP2A activity requires BCR-ABL1 activity in oncogene-addicted CML progenitors [32, 120] but not TKI-resistant quiescent LSCs, in which BCR-ABL1 expression alone is necessary to recruit JAK2 [48]. This might represent an essential step for survival and self-renewal of CML LICs. Indeed, PP2A directly activates GSK3β and inactivates β-catenin, and indirectly induces inactivation/degradation of BCR-ABL1 and JAK2, likely through SHP1 recruitment/activation [32, 122, 132-134]. The important role played by PP2A and its inhibitors SET and CIP2A is also elegantly supported by *a)* clinical correlative data showing that levels of inactive PP2A in BM of CML patients at diagnosis directly correlate with blastic transformation [135], and *b)* preclinical data showing that enhanced expression of SET binding protein 1 (SETBP1), a SET stabilizing factor frequently mutated in hematologic malignancies, confers self-renewal to CML-BC-like mouse progenitors [136].

JAK2 activation might also occur in CML-BC progenitors independently of perturbation of the SET/PP2A interplay [32, 48]. Abelson helper integration site 1 (AHI-1) has been suggested to have a role in the BCR-ABL1 and JAK2 interplay [137]. AHI-1 interacts with JAK2 and BCR-ABL1 to confer TKI resistance [129]. Similar conclusions were drawn using JAK2 and β-catenin inhibitors [128, 138-141], although a recent study with JAK2 null mice [142] argues against JAK2 requirement for BCR-ABL1 leukemogenesis.

4.2. Other Therapeutic Targets

Sonic hedgehog (Shh) signaling also provides survival signals through activation of β-catenin in CML [143]. In fact, levels of the Shh signaling protein Smoothened (Smo1) seem critical for development of TKI resistance and self-renewal of LSCs and CML-BC

progenitors [144, 145] but dispensable for normal HSC function [146]. However, it is still unclear whether Shh inhibition leads to CML eradication without harming normal HSC survival and self-renewal [147-152]. Several strategies to target CML LSCs and/or CML-BC GMPs with HDAC [153], Bcl-xL/BCL2/MCL1 [104, 154, 155], mTOR [156], PML [157, 158], Alox-5 [159, 160], autophagy and proteasome inhibitors [161, 162] showed promising results in preclinical settings and are currently under clinical investigation [23]. Moreover, the regulation of LSC behavior seems to also involve other druggable factors (*e.g.* TGFβ, AKT, FOXO3a, BCL6 and Rac2) that can be tested for their efficacy against CML (CP and BC) LSCs [47, 79, 163-167]. Altogether, these studies have also provided insight into the mechanisms of BCR-ABL1 kinase-independent LSC self-renewal/survival. In some cases, targeting LSC-related pathways does not induce killing but restores TKI sensitivity in LSCs [112, 144, 153, 157, 161, 162, 167-169]. Conversely, the therapeutic efficacy of PP2A-activating drugs [PADs; *e.g.* FTY720 (Fingolimod; Gilenya), OP449] is still underestimated [121, 170]. PADs are a novel and powerful class of anti-leukemic agents that, upon SET sequestration, reactivate PP2A which, in turn, inhibits BCR-ABL1 and JAK2 and their kinase-dependent and –independent oncogenic signals, suppress *in vitro* and *in vivo* BCR-ABL1-driven leukemia regardless of BCR-ABL1 mutation status, and potentiate the pro-apoptotic effect of TKIs [48, 120, 121, 170]. Importantly, PADs target quiescent TKI-resistant CML (CP and BC) LSCs without harming normal hematopoiesis [48]. Finally, targeting the RAS-related nuclear protein RAN and the karyopherin β family member XPO1 (exportin-1, CRM1), two interacting proteins with key functions in nucleocytoplasmic transport may represents a novel strategy to overcome BCR-ABL1 kinase-independent TKI resistance [171], induce apoptosis of CML-BC progenitors [17] and, perhaps, impair their self-renewal activity. In fact, treatment of leukemic progenitors with the clinically relevant XPO1 inhibitor KPT-330 leads to apoptosis and suppression of their clonogenic potential, significantly increased survival of leukemic mice, and restored sensitivity to TKI-induced apoptosis [17, 171]. Interestingly, altered XPO1-dependent subcellular localization of hnRNP-A1 and SET and, subsequently, reactivation of PP2A and inhibition of BCR-ABL1, significantly contribute to KPT-330-induced apoptosis in CML-BC [17].

4.3. Altered mRNA Metabolism in CML (CP and BC)

While there is substantial evidence that BCR-ABL1 kinase-dependent deregulation of mRNA metabolism significantly contributes to the phenotype of CML-BC progenitors through *i.* differential pre-mRNA splicing, editing, nuclear export and translation; *ii.* increased mTOR activity and autophagy; *iii.* altered RNA binding protein (*e.g.* hnRNPs, La, Msi2) expression; and *iv.* deregulated miRNA expression (reviewed in [27]), much less is known about the role of BCR-ABL1 kinase-independent mechanisms regulating mRNA metabolism in LSCs. In this regard, altered mRNA splicing and editing, which result in splice variants of BCL2 gene family and misspliced GSK3β, respectively, appear involved in the acquisition of LSC phenotype by CML-BC myeloid progenitors [100, 104, 116]. Likewise, induction of the KPT-330-sensitive [17] hnRNP-A1, which controls JAK2-dependent SET expression [32], occurs in mouse $LSK^{BCR-ABL1+}$ cells and may contribute to LSC survival/self-renewal [172]. Expression of a series of miRNAs was found to be dysregulated in CML (Fig. 3). miR-486-5p and miR-300 regulation in CML occurs through BCR-ABL1 kinase-dependent and –independent mechanisms and is potentially involved in

acquisition of LSC self-renewal/survival and TKI resistance [173, 174]. In fact, miR-486-5p expression increases in CML progenitors and its inhibition potentiates TKI-induced apoptosis likely through Akt signaling (PTEN and FoxO1) modulation, whereas miR-300 appears involved in the regulation of the JAK2/SET/β-catenin self-renewal/survival pathway [173, 174]. In addition, miR-326 is down-regulated in CML-BC progenitors and its restoration results in apoptosis of $CD34^+$ CML cells through suppression of Smo expression [175] whereas TKI-induced downregulation of miR-30a promotes autophagy [176], suggesting a potential role for these miRNAs in β-catenin-dependent regulation of LSC survival and TKI resistance. Other microRNAs (*e.g.* miR-130a/b, miR-138, miR30e, miR30a, miR29a, miR29b and miR23a), long non-coding RNA (lncRNA)-BGL3, and miRNA-regulating factors (*e.g.* Lin28/28b) may determine the CML-BC LSC phenotype including acquisition of TKI resistance [177-185] and regulation of BCR-ABL1 levels [186-192].

Finally, acquisition of self-renewal ability by GMPs requires suppression of differentiation. In most myeloid CML-BC cases, suppression of granulocytic differentiation is a BCR-ABL1 dose- and kinase-dependent effect that relies on inhibition of miR-328 expression [193-195]. Restoration of miR-328 expression in $CD34^+$ CML-BC progenitors negatively regulates PIM1-dependent survival, and exhibits decoy activity by interacting with the RNA binding protein hnRNP-E2, thereby preventing the inhibitory effects of hnRNP-E2 on the translation of C/EBPα [195], a transcription factor essential for normal and leukemic myeloid differentiation [194, 196, 197]. Likewise, downregulation of miR-150 in CML-BC myeloid progenitors appears to be important for Myb expression and differentiation arrest [198, 199].

CONCLUSION

The use of first, second and third generation TKIs has made CML a clinically manageable disease. Despite this major improvement in the standard of care for CML, the complexity of CML-BC physiopathology, together with the failure of TKIs as therapeutic agents in CML-BC and their inability to eradicate CML at the stem cell level, fully justify the ongoing CML research. However, the complexity of such BCR-ABL1 kinase-dependent and/or -independent molecular networks controlling LSC phenotype in both disease stages makes it safe to conclude that the key to a successful CML eradication rests on understanding the bidirectional network of signals between leukemic HSC/progenitors and BM microenvironment. Thus, a better understanding of the events governing LSC behavior might lead to the “biological” cure of CML and effective treatment of CML-BC and, perhaps, other acute leukemias originating at the HSC level.

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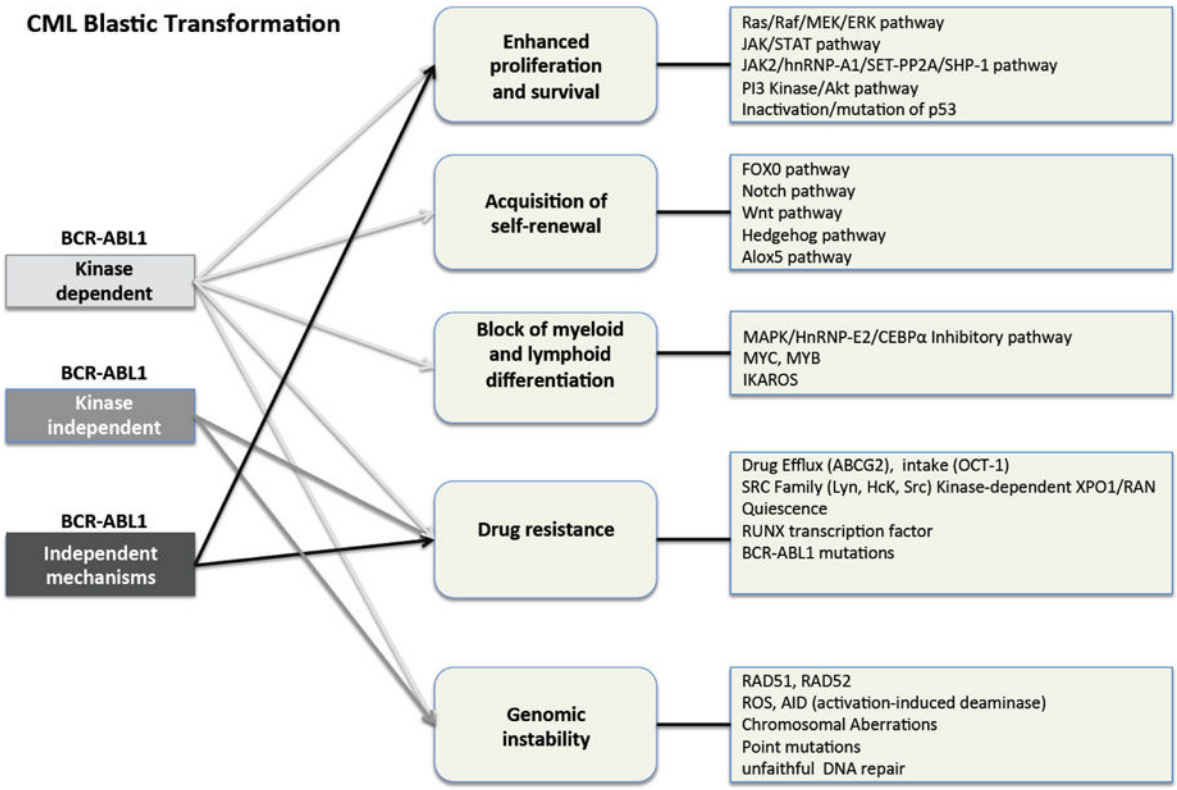

Fig. 1. Mechanisms of CML blastic transformation

Schematic representation of the main BCR-ABL1 kinase-dependent, kinase-independent and BCR-ABL1 independent molecular pathways that may account for enhanced survival/proliferation, acquired self-renewal, differentiation arrest, TKI resistance and genomic instability of leukemic $CD34^{+}$ progenitors.

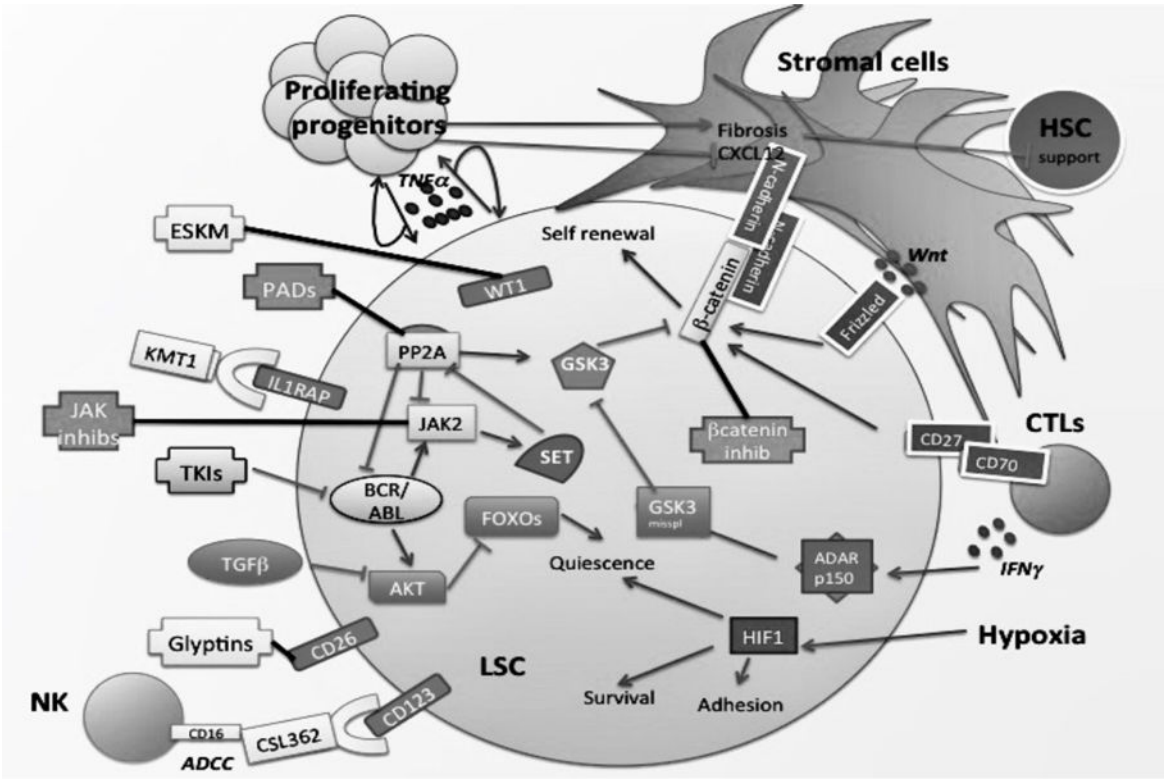

Fig. 2. Cell autonomous and microenvironmental signals regulating LSC behavior

LSCs display both cell autonomous and BM microenvironmentally-driven aberrant signals allowing them to survive, self-renew and escape TKI-induced killing. Potential therapeutic approaches targeting LSC markers (*e.g.* IL1-RAP, CD26)) and BCR-ABL1 kinase-independent pathways (*e.g.* SET/PP2A, β-Catenin, Jak2). New therapeutic avenues may also arise from the modulation of cell survival mechanisms triggered by hypoxia and the microenvironmental crosstalk between LSCs and stromal (N-cadherin-mediated) or immune cells (*e.g.* NK cells).

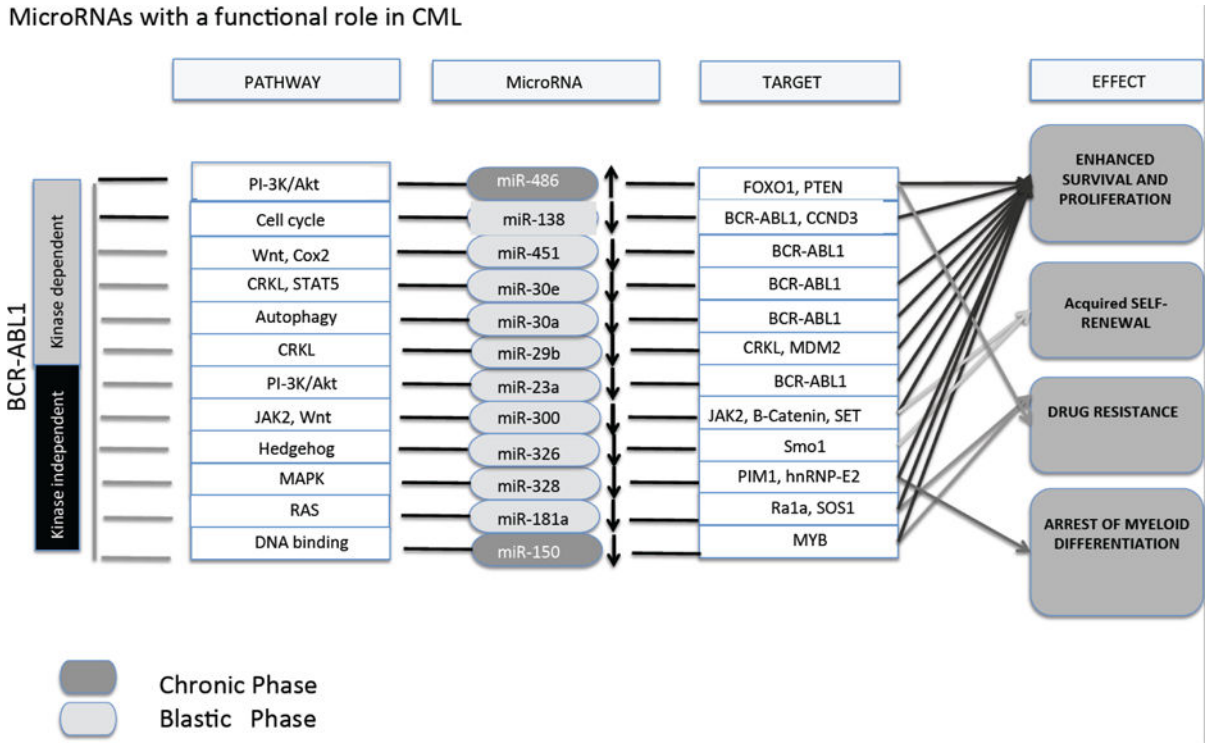

Fig. 3. MicroRNAs with potential roles in CML

BCR-ABL1 kinase-dependent and -independent pathways modulating miRNAs expression in chronic (dark gray) and blastic phase CML (light grey). These miRNAs control proliferation/survival enhancement, drug resistance, myeloid differentiation and self-renewal capability through modulation of the expression of specific mRNA targets.