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REVIEW

Molecular mechanisms for survival regulation of chronic myeloid leukemia stem cells

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ABSTRACT

Studies on chronic myeloid leukemia (CML) have served as a paradigm for cancer research and therapy. These studies involve the identification of the first cancer-associated chromosomal abnormality and the subsequent development of tyrosine kinase inhibitors (TKIs) that inhibit BCR-ABL kinase activity in CML. It becomes clear that leukemia stem cells (LSCs) in CML which are resistant to TKIs, and eradication of LSCs appears to be extremely difficult. Therefore, one of the major issues in current CML biology is to understand the biology of LSCs and to investigate why LSCs are insensitive to TKI monotherapy for developing curative therapeutic strategies. Studies from our group and others have revealed that CML LSCs form a hierarchy similar to that seen in normal hematopoiesis, in which a rare stem cell population with limitless self-renewal potential gives rise to progenies that lack such potential. LSCs also possess biological features that are different from those of normal hematopoietic stem cells (HSCs) and are critical for their malignant characteristics. In this review, we summarize the latest progress in CML field, and attempt to understand the molecular mechanisms of survival regulation of LSCs.

KEYWORDS molecular mechanisms, chronic myeloid leukemia, leukemia stem cell

INTRODUCTION

Chronic myeloid leukemia (CML) is a myeloproliferative disease that originates from an abnormal hematopoietic stem cell (HSC) harboring the Philadelphia (Ph) chromosome (Wong and Witte, 2004; Melo and Barnes, 2007; Savona and Talpaz, 2008). The discovery of the Philadelphia chromosome by Nowell and Hungerford in 1960 through analyzing leukemic cells from seven chronic phase CML patients (Nowell and Hungerford, 1960), provided a major clue to the pathogenesis of CML. This chromosomal abnormality is generated by a reciprocal translocation between chromosome 9 and 22 [t (9; 22) (q34; q11)], which results in the formation of the chimeric BCR-ABL protein that functions as a constitutively active tyrosine kinase (Wong and Witte, 2004; Ren, 2005). This aberrant BCR-ABL oncoprotein constitutes the molecular basis of CML, which has provided the foundation for designing therapeutic drugs and led to the development of the BCR-ABL tyrosine kinase inhibitor (TKI)—imatinib. Imatinib is the first and most successful TKI found to be highly effective in CML treatment (Druker et al., 1996, 2001a, 2001b). It binds to the ABL kinase domain through forming hydrogen bonds, and impairs ATP binding, subsequently blocking the substrate activation (Schindler et al., 2000). Targeted therapy with imatinib induces a complete hematologic and cytogenetic response in more than 90% of chronic phrase CML patients (Druker et al., 2006).

However, resistance to TKI has been frequently observed, especially in patients with advanced-stage disease (Gorre et al., 2001). One major reason is the occurrence of point mutations in the kinase domain of BCR-ABL (Gorre et al., 2002). Using a PCR strategy to determine the sequence of the ATP binding pocket and activation loop of the kinase domain of ABL obtained from relapsed patient samples, Gorre et al. identified a single nucleotide C to T change, which results in a threonine to isoleucine substitution at position 315 (Gorre et al., 2001). This substitution of theronine precludes the formation of a hydrogen bond with TKI but does not interfere with ATP binding (Gorre et al., 2002). To date, more than 50 point mutations have been identified, including mutations of contact residues, mutations in the ATP binding loop and the regulatory motifs (O'Hare et al., 2007). These mutations prevent ABL from adopting the specific conformation required for high-affinity imatinib binding (O'Hare et al., 2007).

To override this resistance, secondary-generation TKIs,

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such as dasatinib and nilotinib, have been developed. Dasatinib is a dual ABL and Src family kinases (SFKs) kinase inhibitor (Lombardo et al., 2004). Previous studies showed that dasatinib overcame imatinib resistance conferred by most ABL kinase mutations, as dasatinib is able to bind to BCR-ABL in its inactive and active states with higher affinity (Shah et al., 2004; Talpaz et al., 2006; Cortes et al., 2007; Guilhot et al., 2007). Similarly, nilotinib has a higher affinity for the inactive conformation of BCR-ABL by 20–30 fold (Weisberg et al., 2005), which makes it highly efficacious in patients with CML following failure of imatinib therapy (Kantarjian et al., 2006). However, both inhibitors cannot inhibit the gatekeeper residue mutant T315I. With the development of structural biology and computational biology, an alternative strategy to target other BCR-ABL motifs that are remote from the kinase domain has been developed (Adrian et al., 2006; Chan et al., 2011; Zhang et al., 2011). This strategy would be unaffected by kinase mutations that confer the ability of TKI-resistance. Through a high-throughput screen, GNF-2, a highly selective non-ATP competitive inhibitor of BCR-ABL, was identified (Adrian et al., 2006). A recent study using nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography confirmed that GNF-2 bound to the C-terminal myristoyl pocket of ABL, leading to changes in the structural dynamics of the ATP-binding site (Zhang et al., 2011). GNF-5 is an analogue of GNF-2 with an improved pharmacokinetic feature. It can act cooperatively with an ATP competitive inhibitor to inhibit both wild type and T315I BCR-ABL activity (Zhang et al., 2011). Additionally, using structure-based drug design, Chan et al developed an inhibitor, DCC-2036, which potently inhibits both unphosphorylated and phosphorylated forms of ABL1 by inducing an inactive conformation (Chan et al., 2011). This inhibitor exhibited efficacy against the T315I mutant *in vivo* (Chan et al., 2011). The development of these inhibitors provides the possibility of overcoming TKI resistance caused by kinase mutations, including the T315I.

BEYOND TYROSINE KINASE INHIBITORS—CANCER STEM CELLS IN CML

Although the majority of CML patients achieve a complete hematological response (Cortes et al., 2007; Guilhot et al., 2007; Hochhaus et al., 2007; Ottmann et al., 2007), clinical relapse eventually occurs in the majority of patients once treatment is interrupted (Cortes et al., 2004). Additionally, only a very low percentage of complete molecular response is achieved from therapy with imatinib, or new generation TKIs (Piazza et al., 2005; Kantarjian et al., 2006; Talpaz et al., 2006). This suggests that a population of residual malignant cells is unaffected by TKIs. Now, it is accepted that these TKI-refractory malignant cells are LSCs. The existence of LSCs in CML has been experimentally proved in a series of studies (Graham et al., 2002; Bhatia et al., 2003; Hu et al., 2006). Using carboxyfluorescein diacetate succinimidyl diester (CFSE) to track cell division, Graham et al showed that imatinib treatment eradicated almost all the dividing $CD34^+$ cells from patients with CML in the chronic phase, leaving behind the non-proliferating quiescent cells. These data imply that quiescent CML stem cells are insensitive to imatinib (Graham et al., 2002). Further study demonstrated that Ph^+ $CD34^+$ cells persisted in CML patients who achieved complete cytogenetic response with imatinib treatment (Bhatia et al., 2003). Analysis using colony forming cell assay (CFC) and long-term bone marrow culture-initiating cells (LTC-IC) confirmed that these cells retained primitive progenitor ability (Bhatia et al., 2003). In the retroviral transduction/transplantation CML mouse model, we found that treatment with imatinib and the secondary generation of TKI, dasatinib, dramatically prolonged the survival of CML mice; however, all treated CML mice eventually died of this disease (Hu et al., 2006). Fluorescence-activated cell sorting (FACS) analysis showed that TKIs reduced the numbers of BCR-ABL-expressing HSCs but failed to eliminate these cells completely in CML mice (Hu et al., 2006). Together these studies indicate that TKIs cannot eradicate LSCs, making it necessary to reassess the notion of LSCs and their role in CML development, to understand the molecular mechanisms of LSC maintenance and to design new strategies to eradicate them.

Cancer stem cell

Cancer stem cell is not a new concept (Reya et al., 2001), it has attracted broad interest recently due to its promising clinical implications in cancer therapy. Like normal stem cells, cancer stem cells can be defined as a specific cell population inside cancer that can self-renew and has the ability to initiate cancer development (Reya et al., 2001; Huntly and Gilliland, 2005; Wang and Dick, 2005; Visvader and Lindeman, 2008). Bonnet and Dick first identified and characterized LSCs from human AML samples (Bonnet and Dick, 1997). They isolated $CD34^+$ CD38- cells and transplanted them into NOD/SCID mice. They found that these cells could not only initiate AML in NOD/SCID mice but also differentiate *in vivo* into leukemic blasts (Bonnet and Dick, 1997). More importantly, serial transplantation demonstrated that these cells had a capacity to self-renew and transferred AML disease into secondary recipients (Bonnet and Dick, 1997). Therefore, this study showed for the first time that LSCs were exclusively $CD34^+$ $CD38^-$ cells, which have the same cell-surface phenotype as normal human primitive cells. Subsequently, similar approaches were applied to solid tumors. Cancer stem cells have been identified in many types of solid tumors, including breast (Al-Hajj et al., 2003), brain (Singh et al., 2003), pancreas (Li et al., 2007), colon (Ricci-Vitiani et al., 2007), lung (Kim et al., 2005), and prostate (Goldstein et al., 2010). These studies indicate that cancer stem cells are a rare phenotypically distinct subpopulation of cancer cells in most of cancers, and these rare cells represent the driving force of tumorigenesis. Thus, targeting cancer stem cells might be an attractive and potentially effective strategy for cancer treatment. These types of therapies would require an

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understanding of the molecular mechanisms of self-renewal and survival of cancer stem cells.

Identification of chronic myeloid leukemia stem cell

Many efforts have been undertaken to identify cancer stem cells in CML. After retroviral transduction with BCR-ABL, myeloid progenitors, common myeloid progenitor (CMP) and granulocyte-macrophage progenitors (GMP) did not serially replate, indicating that these cells lack the ability to self-renew (Huntly et al., 2004). Using the SCLtTA/TRE-BCR-ABL transgenic mouse model, Reynaud et al confirmed that BCR-ABL-expressing progenitors including CMP, GMP and common lymphoid progenitor (CLP), failed to transfer CML disease into secondary recipients (Reynaud et al., 2011). Therefore, these data demonstrate that BCR-ABL cannot confer properties of self-renewal to committed myeloid progenitors. To identify CML stem cells, we tested whether BCR-ABL-expressing hematopoietic stem cells (HSCs) function as LSCs. Using a BCR-ABL induced CML mouse model, we sorted BCR-ABL expressing $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ (LSK) cells from primary CML mice and transplanted them into lethally irradiated secondary recipients. We found that these BCR-ABL-expressing LSK cells induced CML disease in the secondary recipients (Hu et al., 2006), demonstrating that BCR-ABL expressing LSK cells function as LSCs. To further characterize the ability of this stem cell population to transfer CML to secondary recipient mice, we separated BCR-ABL-expressing (GFP^+) Lin^- cells into four populations: $Lin^-Sca\text{-}1^+c\text{-}Kit^-$ (LSK^-), $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ (LSK), $Lin^-Sca\text{-}1^-c\text{-}Kit^-$ (LS^-K^-), and $Lin^-Sca\text{-}1^-c\text{-}Kit^+$ (LS^-K) (Fig. 1A). We serially diluted sorted GFP^+ LSK cells from CML mice and transplanted 1×10^4, 5×10^3, 1×10^3 or 1×10^2 cells respectively into lethally irradiated mice along with 2×10^5 normal bone marrow cells. FACS analysis showed that myeloid leukemia cells (GFP^+ $Gr\text{-}1^+$) were detected in the peripheral blood of mice receiving 1×10^4, 5×10^3 or 1×10^3 GFP^+ LSK cells at 10 days after bone marrow transplantation (BMT), whereas almost no leukemia cells were found in mice receiving 1×10^2 GFP^+ LSK cells (Fig. 1B). At day 15 after BMT, leukemia cells almost disappeared from mice receiving 1×10^3 GFP^+ LSK cells (Fig. 1B). Eventually, mice receiving 1×10^4 (100% of the mice) or 5×10^3 (50% of the mice) GFP^+ LSK cells developed CML, and lower cell doses (1×10^3 or 1×10^2) did not (Fig. 1B). We also tested the other GFP^+ cell populations (LS^-K, LSK^-, and LS^-K^- cells) for their ability to induce CML in secondary recipient mice. In this experiment, we transplanted 3×10^4 cells along with 2×10^5 normal bone marrow cells into lethally irradiated mice. We found that leukemia cells were barely detectable in peripheral blood of recipient mice (Fig. 1C), indicating these populations are incapable of inducing leukemia. Similarly, Naka et al. also confirmed that BCR-ABL-expressing LSK cells from CML mice could efficiently induce CML development in recipient mice, and no other CML cell populations expressing differentiation markers induced CML (Naka et al., 2010). The identification of CML LSCs allows us to further understand the mechanisms by which CML stem cells survive and self-renew, which will contribute to the design of new strategies aimed at targeting CML stem cells as a curative therapy for CML.

MOLECULAR PATHWAYS REGULATING THE SURVIVAL OF LSCs

The finding that CML LSCs are resistant to TKI treatment leads us to ask why LSCs evade BCR-ABL kinase inhibitor treatment. What is the nature of LSCs? How do LSCs survive whereas other CML cells are eliminated by TKI therapy? To answer these questions requires a full understanding of the biology of LSCs. As LSCs share functional properties (self-renewal, and pluripotency) with normal HSCs (Reya et al., 2001; Lessard and Sauvageau, 2003; Park et al., 2003), it is reasonable to think that signaling pathways involved in the regulation of normal stem cells should also be essential for LSCs. Studies have demonstrated that Wnt/β-catenin (Zhao et al., 2007), hedgehog (Zhao et al., 2009), and FOXO (Naka et al., 2010) signaling pathways are required for maintaining LSCs (reviewed in (Chen et al., 2010b)). In here, we focus on BCL6 (Hurtz et al., 2011), and HIF1α (Wang et al., 2011; Zhang et al., 2012b) signaling pathways that are functionally required for both normal HSC and CML LSCs.

The BCL6 pathway

B cell lymphoma 6 (*BCL6*) is a known proto-oncogene encoding a BTB/POZ-zinc-finger transcriptional repressor (Phan and Dalla-Favera, 2004; Duy et al., 2010). It is known that BCL6 is necessary for germinal-center formation and is involved in the pathogenesis of B-cell lymphoma (Duy et al., 2010). Therefore, the majority of previous studies about BCL6 focus on B cells and B lymphoma. BCL6 suppresses p53 transcription by binding the promoter of p53 and modulates DNA damage-induced apoptotic responses in germinal-center B cells (Phan and Dalla-Favera, 2004). Constitutive expression of BCL6 protects B cell lines from apoptosis induced by DNA damage, suggesting that deregulated BCL6 expression contributes to lymphomagenesis in part by functional inactivation of p53 (Phan and Dalla-Favera, 2004). Recent studies indicate that BCL6 plays a critical role in a protective feedback signaling mechanism by LSCs in response to TKI treatment (Duy et al., 2011; Hurtz et al., 2011). Duy et al. first demonstrated that TKI treatment induced BCL6 up-regulation that is mediated by decreased phosphorylation of STAT5 and enhanced FoxO4 expression in Ph^+ acute lymphoblastic leukemia (ALL) cells. The target genes of BCL6 in TKI-treated Ph^+ ALL cells, including *p53, Arf, p21, and p27,* were identified by a ChIP on chip and comparative gene expression assay (Duy et al., 2011). Furthermore, BCR-ABL-transduced $BCL6^{-/-}$ B lymphoblast failed to induce leukemia in immunodeficient mice, which was suggestive of a defect in the self-renewal of $BCL6^{-/-}$ LSCs (Duy et al., 2011). Interestingly, deletion of BCL6 restored the sensitivity of B-ALL cells to imatinib, in-

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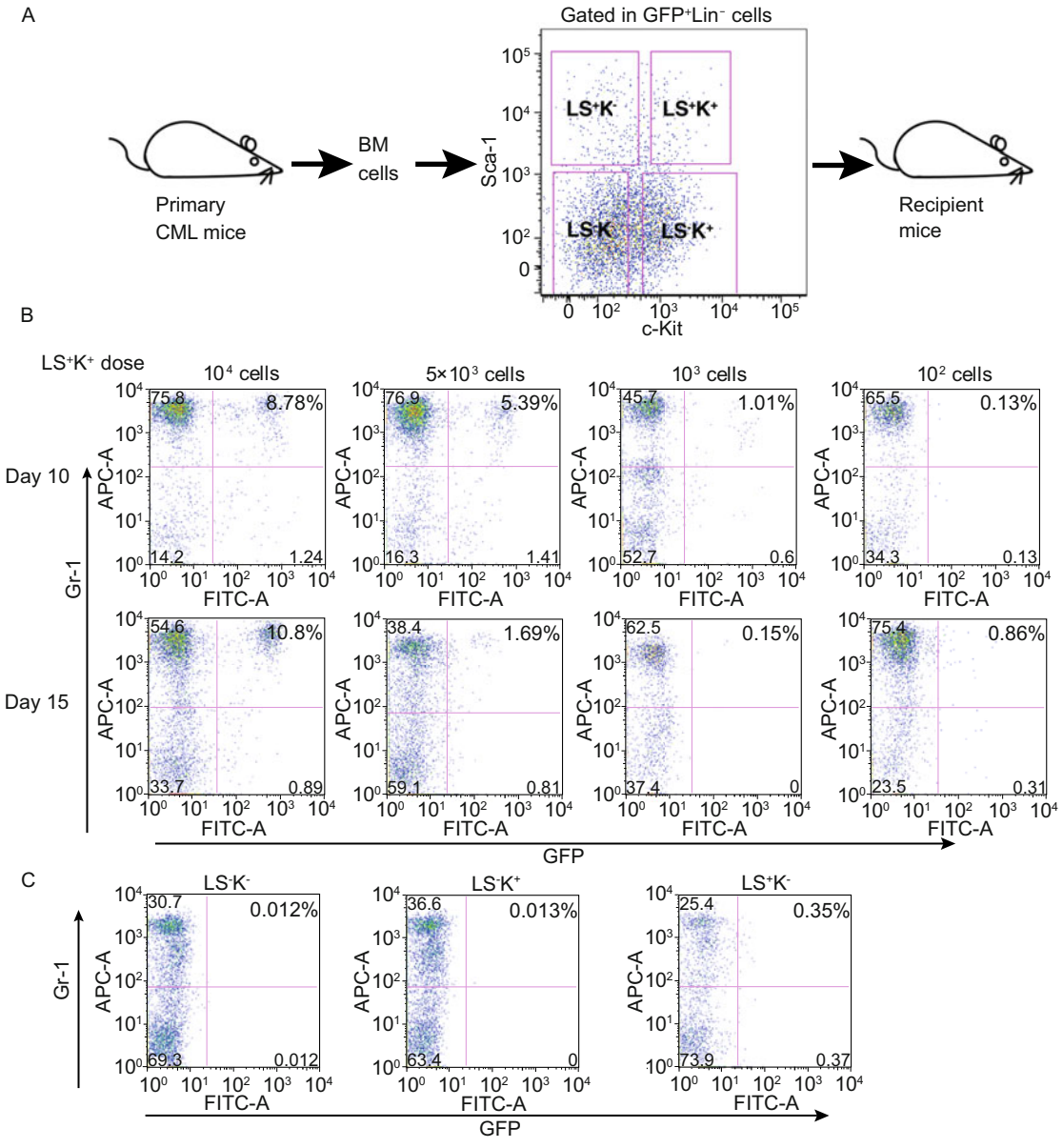

Figure 1. Identification of chronic myeloid leukemia stem cells. (A) Four populations of cells (LSK-; LSK; LS-K-; LS-K) were sorted from BCR-ABL induced CML mice and transplanted into lethally irradiated recipients at different doses. (B and C) Only LSK cells can function as LSCs and transfer CML disease into secondary recipients.

dicating a protective role for BCL6 in LSCs of Ph^+ ALL treated with TKIs (Duy et al., 2011). Muschen and colleagues also extended their findings to LSCs in CML (Hurtz et al., 2011). They demonstrated that human primary $CD34^+$ CML cells exhibited enhanced BCL6 expression upon TKI treatment and that the Pten-Akt-FoxO pathway was responsible for this up-regulation of BCL6 (Hurtz et al., 2011), which is validated by our recent study. We found that *Pten* was down-regulated by BCR-ABL, and that conditional deletion of Pten accelerated CML development, and overexpression of Pten delayed CML development because of the impaired function of LSCs (Peng et al., 2010). Additionally, deletion of *BCL6* impaired the colony-forming potential in vitro of LSCs and failed to induce CML (Hurtz et al., 2011), suggesting BCL6 is required for the maintenance of LSCs in CML. Furthermore, p53 was identified as a key transcriptional target of BCL6 in CML (Hurtz et al., 2011). Most importantly, both studies showed that a combination of TKIs and inhibition of BCL6 by the retro-inverso peptide inhibitor RI-BPI, which blocks the repressor activity of BCL6, effectively eradicated LSCs in CML and Ph^+ ALL. These findings regarding BCL6 up-regulation after TKI treatment provide one possible explanation for why and how LSCs persist in patients despite long-term TKI treatment, and make it a potential target in TKI resistant LSCs in both CML and Ph^+ ALL.

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The hypoxia inducible factor pathway

Hypoxia inducible factor 1 (HIF1) belongs to the family of basic helix-loop-helix (bHLH) transcription factors (Semenza, 2003). It forms a heterodimer that consists of a constitutively expressed HIF1β subunit and a HIF1α subunit, and mediates cellular adaptation to hypoxia (Semenza, 2003). The expression of HIF1α is regulated in multiple ways. Under normoxic conditions, HIF1α is degraded rapidly, which is triggered by the oxygen-hydroxylation of proline residues 402 and 564 by a proline hydroxylase (PHD) (Simon and Keith, 2008). The hydroxylated HIF1α is recognized by the Von Hippel-Lindau protein (pVHL), which is the recognition component of an E3 ubiquitin-protein ligase (Keith and Simon, 2007; Simon and Keith, 2008). HIF1α expression is also regulated by growth factors, cytokines and other signaling pathways such as PI3K and MAPK pathways (Zhong et al., 2000). It is widely accepted that HIF1α plays an important role in cancer progression by activating transcriptional programs for self-renewal and multipotency of cancer stem cells (Soeda et al., 2009; Mendez et al., 2010). Recent studies from our group and another group have added HIF1a as one of the key pathways in LSCs of hematological malignancies (Wang et al., 2011). Wang et al. demonstrated that cancer stem cells in lymphoma from transgenic mice with an insertional mutation of the *Epm2a* gene had a high level of HIF1α under normoxia (Wang et al., 2011). Both knockdown of HIF1α by shRNA and inhibiting HIF1α activity by the inhibitor, echinomycin, which blocks HIF1α DNA binding activity, reduced the colony forming ability *in vitro* and abrogated lymphoma development *in vivo* (Wang et al., 2011), suggesting HIF1α is essential for maintenance of CSCs of lymphoma. Furthermore, similar to the findings in the lymphoma CSCs, higher HIF1α activity was only observed in $CD34^+$ $CD38^-$ LSCs of human AML (Wang et al., 2011). Using a xenogeneic mouse model, they showed that echinomycin treatment effectively prevented the engraftment of LSCs from AML patients in a serial transplantation assay (Wang et al., 2011). To our interest, BCR-ABL induces *HIF1*α expression at the transcriptional level in leukemia cell lines, which is mediated by PI3K and mTOR but not MAPK pathways, as this up-regulation was only inhibited by the addition of either PI3K inhibitor LY294002 or mTOR inhibitor rapamycin (Mayerhofer et al., 2002). These findings suggest that HIF1α might contribute to the pathogenesis of CML. Using the retroviral transduction/transplantation CML mouse model, we found that an active HIF1α signaling pathway was maintained in LSCs of CML comparing to normal HSCs. $HIF1a^{-/-}$ LSCs gave rise to less colonies in vitro and failed to induce CML in the secondary recipients (Zhang et al., 2012b), suggesting that HIF1α is required for self-renewal of LSCs in CML. Further, we showed that $HIF1\alpha^{-/-}$ LSCs have enhanced levels of $p16^{Ink4a}$ and $p19^{Arf}$ and that deletion of $p16^{Ink4a}$ and $p19^{Arf}$ by lentivirus-mediated shRNA rescued the defective colony-forming potential of LSCs due to the absence of HIF1α(Zhang et al., 2012b). Importantly, microarray analysis showed significant changes of HIF1α target genes in $HIF1\alpha^{-/-}$ LSCs, compared to $HIF1\alpha^{-/-}$ HSCs, suggesting that LSCs of CML are more dependent on HIF1α activity than HSCs (Zhang et al., 2012b). Given the higher specificity and sensitivity of CSCs in lymphoma and human AML to HIF1α inhibitor echinomycin (Wang et al., 2011), it is reasonable to hypothesize that it is possible to eradicate LSCs in CML by targeting HIF1α, although the efficiency of echinomycin or other HIF1α inhibitors on LSCs of CML need to be further validated.

SURVIVAL OF LSCs ARE INDEPENDENT OF BCR-ABL KINASE ACTIVITY

Resistance of CML LSCs to TKIs has been observed (Graham et al., 2002; Hu et al., 2006). As mentioned earlier, primitive Ph^+ $CD34^+$ cells from CML patients exhibit insensitivity to TKIs (Graham et al., 2002). Understanding the mechanisms by which LSCs survive under treatment with TKIs will be critical to designing strategies for targeting LSCs. However, it is still unclear why and how LSCs in CML evade TKIs. The development of BCR-ABL mutations renders resistance, but resistance to TKIs is frequently present in the absence of a detectable mutation in BCR-ABL (Corbin et al., 2011).

It is widely accepted that Ph^+ leukemia cells are addicted to BCR-ABL kinase activity, and BCR-ABL activates numerous downstream signaling pathways which are critical in BCR-ABL induced leukemogenesis through regulating cell proliferation, survival and differentiation (Puil et al., 1994; Frank and Varticovski, 1996; Neshat et al., 2000). However, recent studies indicate that CML LSCs are independent of BCR-ABL kinase activity (Corbin et al., 2011; Hamilton et al., 2012). Using multiple complementary methods, including immunocytochemistry, immunoblot and LTC-IC assay, Druker and colleagues confirmed this idea in cultured cells (Corbin et al., 2011). Intracellular phosphotyrosine levels determined by FACS analysis and immunoblot showed similar degrees of BCR-ABL inhibition by TKIs in different immature cell subtypes, including $CD34^+$ $CD38^+$ and $CD34^+$ $CD38^-$ populations from newly diagnosed CML patients (Corbin et al., 2011). Further, phospho-CRKL immunoblots of sorted quiescent ($Ki67^-$) and cycling ($Ki67^+$) cells showed equal inhibition of BCR-ABL activity in both fractions (Corbin et al., 2011). These data demonstrated that imatinib is equally efficient in inhibiting BCR-ABL kinase activity in CML stem cells, progenitor cells, and quiescent cells. This inhibition is continuous and does not select a subpopulation with innate resistance, as a similar distribution of cell types in untreated versus imatinib treated cultures was observed (Corbin et al., 2011). Finally, when cytokines were added, these cells remained capable of *in vitro* proliferation despite continuous imatinib treatment, and an LIT-IC assay showed that quiescent primitive cells were only slightly reduced by imatinib treatment (Corbin et al., 2011). These restults suggested that the survival of primitive CML cells was independent of BCR-ABL kinase activity. This study also excluded the possibility that drug efflux/influx affects TKI sensitivity of CML stem cells, as comparable inhibition was observed in CML stem cells and progenitors

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(Corbin et al., 2011). Consistently, a study from another group also showed that CML stem cells are not dependent on BCR-ABL kinase activity for their survival (Hamilton et al., 2012). Human CML $CD34^+$ cells were cultured in serum-free SFM media and treated with dasatinib. Immunoblot analysis showed the complete inhibition of phosphor-CRKL by dasatinib treatment (Hamilton et al., 2012), suggesting the inhibition of BCR-ABL kinase activity. However, although fewer cells (10% of input) were achieved after 12-days of treatment, these cells displayed similar proliferation abilities compared to untreated cells when cytokines were supplied (Hamilton et al., 2012). They also retained their self-renewal ability (Hamilton et al., 2012). Together, these results suggest that CML stem cells utilize signaling pathways that are independent of BCR-ABL kinase activity for their maintenance and survival.

This notion raises various questions related to what the BCR-ABL kinase independent pathways are and how they regulate the survival of CML stem cells. In our lab, we have found several pathways that are independent of BCR-ABL kinase activity (Hu et al., 2004; Chen et al., 2009). One of these pathways is BCR-ABL/Alox5 pathway [reviewed in (Chen et al., 2010a)]. Below, we describe another pathway we discovered recently.

Src family kinases in leukemogenesis

The Src family kinases (SFKs) are non-receptor intracellular tyrosine kinases and are comprised of nine members: Src, Fyn, Yes, Blk, Yrk, Fgr, Hck, Lck and Lyn (Quintas-Cardama et al., 2007). SFKs display diverse roles in leukemogenesis (Warmuth et al., 1997; Donato et al., 2003; Hu et al., 2004; Wu et al., 2008). Enhanced phosphorylation levels of Src have been found in primitive and committed progenitor cells isolated from CML patients (Konig et al., 2008), and BCR-ABL interacts with and activates Hck and Lyn in myeloid cells through multiple binding domains (Warmuth et al., 1997). Our previous data also show that BCR-ABL can activate the SFKs Lyn, Hck and Fgr in B-lymphoid cells (Hu et al., 2004). These results suggest that SFKs are involved in the proliferation of BCR-ABL induced leukemia. $Lyn^{-/-}$ mice are hyperresponsive to myeloid growth factors and develop a myeloproliferative disorder, showing splenomegaly and increased numbers of myeloid progenitors (Harder et al., 2001). Loss of two SFKs, Lyn and Hck, results in more HSCs with increased proliferation, reduced apoptosis and skewed differentiation to macrophages. In addition, $Lyn^{-/-}$ $Hck^{-/-}$ mice exhibit clinical signs indicative of a myeloproliferative disease by 2 months of age (Xiao et al., 2008). However, deletion of three SFKs, Lyn, Hck and Fgr, does not affect hematopoiesis, and these mutant mice show no signs of myeloproliferative disease (Meng and Lowell, 1997).

Regulation of Blk by BCR-ABL is independent of BCR-ABL kinase activity

The role of SFKs in leukemogenesis has been challenged by recent findings. Our previous data showed that deletion of Lyn, Hck and Fgr is dispensable for BCR-ABL induced CML but is required for B-cell acute lymphoblastic leukemia (B-ALL). This finding indicates that SFKs might play a different role in CML and B-ALL. The studies on another SFK, Blk, mainly focus on B cell development, and its role in leukemogenesis remains unknown. The absence of Blk modestly affects B cell development (Texido et al., 2000); however, combined deficiency of the three Src kinase, Blk, Fyn, and Lyn, impairs pre-B cell receptor mediated NF-kB activation, and results in blocking of B cell development (Saijo et al., 2003). Recently, we found that Blk plays an inhibitory role in BCR-ABL-induced CML (Zhang et al., 2012c). Deletion of Blk accelerates CML development; conversely, overexpression of Blk suppresses the development of CML through its inhibitory effects on the function of LSCs. Blk suppresses LSC function through a pathway involving an upstream regulator, Pax5, and a downstream effector, p27. Inhibition of this Blk pathway accelerates CML development, whereas increased activity of the Blk pathway causes the delay of CML development. Importantly, we showed that Blk is dispensable for normal hematopoiesis (Zhang et al., 2012c). Our data demonstrated that Blk regulation in LSCs of CML is independent of BCR-ABL kinase activity, and this pathway plays a critical role in regulating the survival of CML LSCs. Therefore, these findings of BCR-ABL kinase independent pathways provide an alternative explanation for the resistance of LSCs to TKIs. These findings also demonstrate that completely different strategies, such as targeting the pathways of stem cell self renewal or targeting the BCR-ABL kinase independent pathways, are needed to eliminate LSCs.

LIPID METABOLISM AND LEUKEMIA STEM CELLS

Tumor metabolism is not a new field. However, in recent years, it becomes a very hot topic and interest has been inspired, because it becomes clear that many signaling pathways affected by genetic mutations have a profound effect on metabolism. This makes this topic once again one of the most intense research areas. Similarly, some studies have been initiated to understand the functional relationship between HSC or leukemia stem cell and metabolism. For example, IDH1/2 mutations have been found in human AML patients, resulting in increased ROS level and higher expression of HIF1a (Dang et al., 2009; Ward et al., 2010). However, these studies have focused largely on glycolysis and energy homeostasis. Our recent study begins to uncover the important role of lipid catabolism in CML development and LSC maintenance.

Stearoyl-Coenzyme A desaturase (Scd) is an endoplasmic reticulum enzyme that belongs to a family of Δ9-fatty acid desaturase isoforms. Four mouse Scd isoforms (Scd1–4) and two human Scd isoforms (hScd1 and 5) have been identified (Ntambi and Miyazaki, 2003; Sampath and Ntambi, 2011). Scd1 is involved in regulating metabolic pathways related to preadipocyte differentiation, insulin sensitivity, metabolism,

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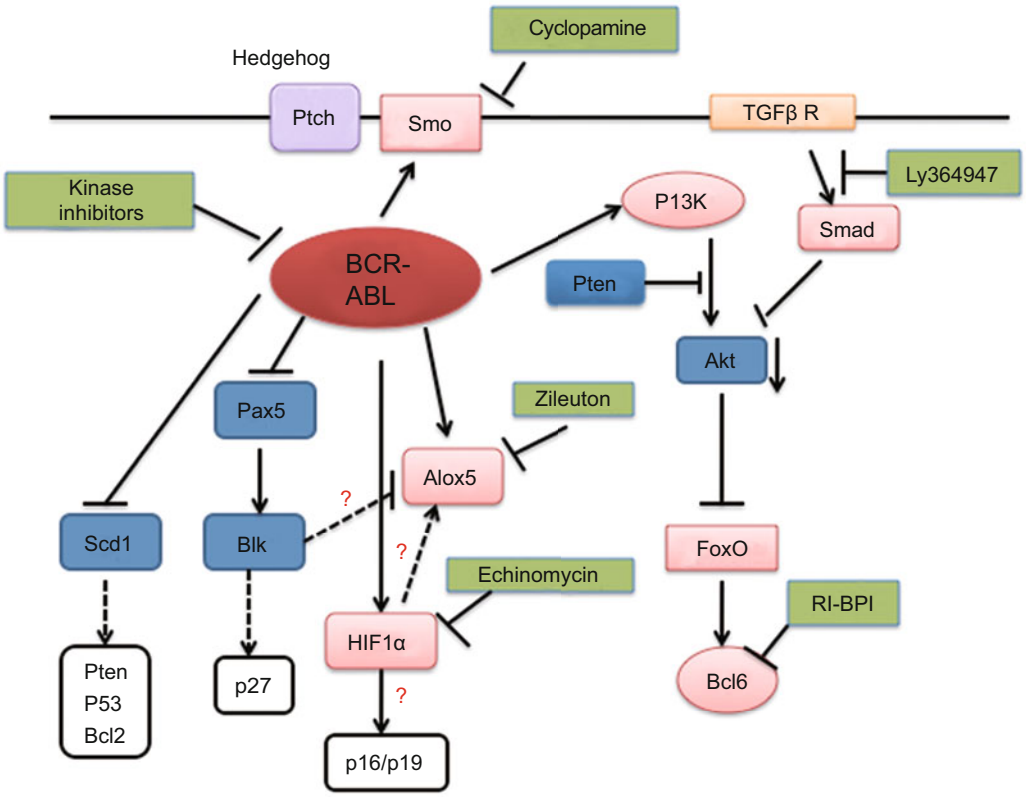

Figure 2. The combination of inhibiting BCR-ABL by TKIs and targeting key regulators of LSCs in CML treatment. TKI treatment plus inhibition of positive pathways, or restoration the expression of negative regulators could effectively target LSCs for curing CML.

and tumorigenesis (Kim and Ntambi, 1999; Falvella et al., 2002; Scaglia and Igal, 2005; Scaglia et al., 2009; Hess et al., 2010). Deletion of Scd1 leads to decreased synthesis of lipids, especially triglycerides, and causes the resistance to leptin-deficient-induced and diet-induced obesity via increased insulin sensitivity (Flowers et al., 2007). *Scd1*$^{-/-}$ mice have decreased expression of genes of lipogenesis and increased expression of lipid oxidative genes. However, the role of Scd1 in leukemogenesis remains unknown, although some studies suggest that fatty acid metabolism plays a role in hematopoiesis (Kinder et al., 2010; Ito et al., 2012). Recently, we found that deletion of Scd1 accelerates CML development through affecting the function of LSCs but not normal HSCs and that enforced expression of Scd1 dramatically delays the development of CML (Zhang et al., 2012a). These findings indicated that Scd1 plays a tumor suppressor role in BCR-ABL leukemogenesis. It is known that Scd1 affects insulin signaling. Rahman et al. found that the phosphorylation levels of Akt, insulin receptor (IR) and insulin receptor substrates 1 and 2 (IRS1/2) were elevated in the absence of Scd1(Rahman et al., 2003). Conversely, stable overexpression of Scd1 in muscle cells decreased the phosphorylation of IRS1 and Akt and was sufficient to impair glucose uptake and insulin signaling. We found that deletion of Scd1 results in higher Akt phosphorylation in CML leukemia cells, and Scd1$^{-/-}$ LSCs display dysregulated Pten, p53 and Bcl2 expression (Zhang et al., 2012a), suggesting these molecular changes downstream of Scd1 are be involved in the regulation of CML development by Scd1.

SUMMARY AND PERSPECTIVES

Targeting of leukemia stem cells has become an attractive strategy for curing CML. Available findings support a combination therapy that requires the use of a TKI in combination with a drug that targets a key regulator of LSCs (Fig. 2). For example, pharmacological blockade of Hedgehog signaling by cyclopamine (Dierks et al., 2008; Zhao et al., 2009), inhibition of the TGFβ-FoxO pathway by Ly364947 (Naka et al., 2010), inactivation of Bcl6 by the retro-inverso Bcl6 peptide inhibitor RI-BPI (Hurtz et al., 2011), or inhibition of Alox5 by zileuton (Chen et al., 2009), have been shown to inhibit CML development by suppressing LSCs. Inhibition of the HIF1α pathway by echinomycin may also be effective in eliminating LSCs. On the other hand, given the specific roles of Blk and Scd1 in CML LSCs (Zhang et al., 2012a, 2012c), restoration of Blk expression or enhancement of Scd1 activity provides an effective strategy for specifically targeting LSCs in CML.

ABBREVIATIONS

CFC, colony forming cell assay; CLP, common lymphoid progenitor; CML, chronic myeloid leukemia; FACS, fluorescence-activated cell sorting; GMP, granulocyte-macrophage progenitor; HSC, hematopoi-

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etic stem cell; LSC, leukemia stem cell; LTC-IC, long-term bone marrow culture-initiating cell; Ph, Philadelphia; PHD, proline hydroxylase; pVHL, Von Hippel-Lindau protein; NMR, nuclear magnetic resonance; SFK, Src family kinase; TKI, tyrosine kinase inhibitor;

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