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Critical molecular pathways in cancer stem cells of chronic myeloid leukemia

Yaoyu Chen[1], **Cong Peng**[1], **Con Sullivan**[2], **Dongguang Li**[3], and **Shaoguang Li**[1,*]

[1]Division of Hematology/Oncology, Department of Medicine, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605, USA

[2]Department of Cancer Research, Maine Institute for Human Genetics and Health, 246 Sylvan Road, Bangor, ME 04401, USA

[3]School of Computer and Security Science, Edith Cowan University, 2 Bradford Street, Mount Lawley, WA 6050, Australia

Abstract

Inhibition of BCR-ABL with kinase inhibitors in the treatment of Philadelphia-positive (Ph^+) chronic myeloid leukemia (CML) is highly effective in controlling but not curing the disease. This is largely due to the inability of these kinase inhibitors to kill leukemia stem cells (LSCs) responsible for disease relapse. This stem cell resistance is not associated with the BCR-ABL kinase domain mutations resistant to kinase inhibitors. Development of curative therapies for CML requires the identification of critical molecular pathways responsible for the survival and self-renewal of LSCs. In this review, we will discuss our current understanding of these critical molecular pathways in LSCs and the available therapeutic strategies for targeting these stem cells in CML.

Keywords

BCR-ABL; leukemic stem cells; CML; therapeutic agents

Introduction

Philadelphia-positive (Ph^+) chronic myeloid leukemia (CML) is a myeloproliferative disease characterized by granulocytosis and splenomegaly. The disease course is triphasic, starting with a chronic phase, progressing to an accelerated phase, and ultimately ending in a terminal phase called blast crisis. The Ph chromosome occurs in over 90% of CML cases, and its presence in the setting of other symptoms is diagnostic of CML. Ten years ago, allogeneic bone marrow transplantation (BMT) was the recommended treatment for newly diagnosed patients with CML. Recipients of BMT had a high 5-year survival rate with a low chance of relapse (1). The majority of long-term survivors with BMT may be regarded as operationally ‘cured’, even if some patients still contain quiescent leukemia stem cells (LSCs) (2). The question is whether these patients are really cured, and the answer to this question should depend on whether the patients are still in danger of relapse. The fact is that CML patients receiving BMT can relapse (1), suggesting that LSCs are not eliminated. A real cure may require complete eradication of these stem cells. Although BMT is considered

*Correspondence: Shaoguang Li, Associate Professor of Medicine Shaoguang Li, University of Massachusetts Medical School, Department of Medicine, 364 Plantation Street, Worcester, MA 01605,; USA. Tel: 508-856-1691, Fax: 508-856-7969; shaoguang.li@umassmed.edu.

a ”curative” therapy for CML, the cure is apparently ”relative” due to the existence of residual LSCs. Another major issue in BMT is age restriction and availabilities of donors. Current standard therapy for CML is the BCR-ABL kinase inhibitors such as imatinib mesylate (Gleevec/Glivec, formerly STI571; Novartis) (3). After 5 years of treatment, the rate of complete cytogenetic response among patients receiving imatinib was 87%, with an estimated 7% of patients progressing to accelerated phase CML or blast crisis (4). Although it effectively prolongs the survival of CML patients, imatinib does not show any definite prospect of ”cure” (2), as these imatinib-treated patients in complete cytogenetic remission still contain malignant hematopoietic progenitor cells (5). The patients are likely required to take the drug for the rest of their lives(6). Is imatinib curative for CML? The fact that half of imatinib treated CML patients who achieved molecular remission and subsequently discontinued the treatment remained in molecular remission without imatinib suggests that these patients appear to be free of leukemia. However, all these patients who remained in molecular remission without imatinib had previously been treated with interferon-α (IFN-α) (6), raising a question as to whether IFN-α played a critical role in the induction of molecular remission by imatinib. Besides these facts, resistance to imatinib develops (4), and one of the major reasons for the resistance is the development of point mutations in the BCR-ABL kinase domain (7, 8). In addition, imatinib does not completely eradicate LSCs (5, 9), which does not appear to be related to the BCR-ABL kinase domain mutations (10, 11). This ”natural” resistance of LSCs to imatinib, as well as other kinase inhibitors, suggests that BCR-ABL somehow turns on unique molecular pathways in LSCs through both kinase-dependent and, more importantly, kinase-independent mechanisms. In this review we will focus on the pathways responsible for the inability of BCR-ABL kinase inhibitors to eradicate LSCs.

To circumvent issues related to the drug resistance of LSCs, it is essential to fully understand how BCR-ABL signals through both kinase-dependent and -independent pathways in LSCs. It is particularly critical to fully understand the biology of BCR-ABL-expressing LSCs and to identify key genes that play significant roles in their survival and self-renewal. Recently, several DNA microarray assays based on $CD34^+$ cells derived from CML patients or $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells from CML mouse were performed, and different strategies were taken to analysis these DNA profiles(12–14), providing valuable information for identifying critical molecular pathways in LSCs. Recent studies in CML LSCs have been reviewed thoroughly by Goldman and colleagues (15). Therefore, in this review we will not only summarize recent progress in the delineation of novel mechanisms and treatment strategies for LSCs in CML, but also extensively summarize our understanding of the biology of LSCs through the identification of target genes for the development of potential anti-stem cell therapies using CML mouse model. In doing so, we will describe in detail why and how the experiments were done to help to explain the challenges and solutions to them in current research on CML LSCs.

Mouse models of CML

Three types of mouse models for CML are widely used: the BCR-ABL transgenic model, the retroviral bone marrow transduction/transplantation model and the human xenograft NOD/SCID mouse model (Fig. 1). Of these, the most frequently used CML mouse model is the retroviral bone marrow transduction/transplantation model, which was initially described in 1990s (16–18). The efficiency of CML induction was later improved to 100%(19–21), which allows the use of the model to study *in vivo* BCR-ABL signaling and disease response to new therapeutic strategies. In this model, donor mice were treated with 5-fluorouracil (5-FU), transduced with BCR-ABL retrovirus, and then transplanted into lethally irradiated syngeneic recipient mice (21). Similar to human CML, the induced myeloproliferative disease in mice shows increased number of BCR-ABL-expressing mature granulocytes in

peripheral blood, splenomegaly and bone marrow invasion of LSCs and progenitor cells(10). This CML model has become an useful tool for identifying novel genes involved in BCR-ABL leukemogenesis and for testing new therapeutic targets in LSCs(10, 11, 22).

The BCR-ABL transgenic mouse model was first developed in 1990 using P190 form of BCR-ABL. These transgenic mice are either moribund with or die of acute leukemia (myeloid or lymphoid) 10–58 days after birth (23). This model was refined to be driven by the metallothionein promoter. In this system, when the P210 isoform of BCR-ABL was expressed, mice showed excessive proliferation of lymphoblasts shortly after birth, resembling acute lymphoblastic leukemia (ALL) (24). Later, BCR-ABL inducible transgenic mice using a tet-off system were developed, with these mice developing rapid ALL (25). Although these transgenic mice indicate that BCR-ABL is the cause for leukemia, they did not develop typical CML. Recently, inducible CML was observed when BCR-ABL transgene was driven by tTA placed under the control of the murine stem cell leukemia (*SCL*) gene 3' enhancer(26).

The most physiologically-relevant mouse model of CML requires the transfer of human CML cells into immunodeficient NOD/SCID recipient mice. In doing so, bone marrow or peripheral blood cells from CML patients were purified for CD34+ cells, and the cells were cultured in serum-free medium containing a serum substitute, comprised of the Flt3-ligand, stem cell factor, IL-3, IL-6 and granulocyte-colony stimulating factor (27, 28). These CD34+ leukemic cells had an *in vitro* progenitor activity and were capable of engrafting immunodeficient NOD/SCID mice (28). This system had been used to test the inhibitory effects of potential drugs on human CML cells (29).

Cancer stem cells

Cancer stem cells (CSCs) constitute a subpopulation of malignant cells capable of self-renewal and differentiation(30–36). In the mid-1990s, seminal work by Dick and colleagues identified a stem cell-like population from a human acute myeloid leukemia (AML). They demonstrated that these cells were capable of transferring AML into an immunodeficient mouse host. The isolated cells were $CD34^{+}CD38^{-}$, which were similar to the cell-surface phenotype of normal SCID-repopulating cells. They showed that these stem-like cells were capable of initiating human AML in NOD/SCID mice. In addition, the data they collected suggested that normal primitive cells, rather than committed progenitor cells, are targets for leukemic transformation(37, 38). These cells homed to the bone marrow and proliferated extensively in response to *in vivo* cytokine treatment, resulting in a pattern of dissemination and leukemic cell morphology similar to that seen in the original patients. The frequency of $CD34^{+}CD38^{-}$ cells in the peripheral blood of AML patients was one engraftment unit in 250,000 cells (37, 38). Recently, CSCs have been defined by their ability to recapitulate the generation of a continuously growing tumor. Weissman and colleagues proposed that a candidate CSC population should exhibit the following properties: 1) The unique ability to engraft; 2) The ability to recapitulate the tumor of origin both morphologically and immunophenotypically in xenografts; and 3) The ability to be serially transplanted (39).

Leukemia stem cells in CML

CML is a stem cell disease that results in the clonal expansion of BCR-ABL-expressing cells. In CML patients, a leukemic clone typically includes cells belonging to all of the myeloid lineages and also frequently includes some B cells. BCR-ABL occurs in a pluripotent hematopoietic stem cell, and LSCs in CML could be defined as part of properties of normal hematopoietic stem cells (HSCs). Eaves and colleagues isolated various subpopulations of $CD34^{+}$ cells from CML patients, and cells in each of the $CD34^{+}$ subpopulations were examined for the presence of BCR-ABL mRNA. BCR-ABL mRNA

could be found in $CD34^+CD38^-$ and $CD34^+CD38^+$ cells (40). Furthermore, Dick, Eaves and colleague reported that enriched $CD34^+$ cells from patients with CML could be transplanted into NOD/SCID mice (27), although the mice did not develop lethal CML-like disease. The failure of BCR-ABL to induce typical CML in NOD/SCID mice does not necessarily indicate that this model is not suitable for examining LSCs, as human leukemia cells were indeed transplanted and survived in the recipient mice. It is obvious that the use of a NOD/SCID strain that allows more efficient engraftment of donor cells would likely improve the engraftment of human CML cells (41). A full understanding of the biology of LSCs requires the development of a good animal model that allows analysis of CML LSCs in the future.

Identification of BCR-ABL expressing HSCs as LSCs in chronic phase CML in mice

Unsuccessful induction of CML in NOD/SCID mice by engrafting human CML cells calls for the establishment of a more efficient CML mouse model for studying the biology of LSCs. In this regard, the BCR-ABL retroviral BMT mouse model has been used frequently. An initial step is to identify LSCs in CML mice. Toward this goal, bone marrow cells from wild type mice were transduced with a retrovirus expressing BCR-ABL and sorted by FACS into two separate populations, Sca-1^- or Sca-1^+, and then these two populations of cells were transferred into respective wild type recipient mice. Only the mice receiving BCR-ABL-transduced Sca-1^+ cells developed and died of CML (10), indicating that the Sca-1^+ population contains LSCs. To further narrow down the specific cell lineages that function as LSCs in the Sca-1^+ population, BCR-ABL-expressing HSCs were sorted and transplanted into syngeneic recipient mice. These recipient mice developed and died of CML. This observation suggests that LSCs reside in BCR-ABL-expressing HSC population. To confirm definitively that BCR-ABL-expressing HSCs are LSCs of chronic phase CML, bone marrow cells were isolated from primary CML mice, and BCR-ABL-expressing HSCs were FACS-sorted and transplanted into secondary recipient mice. These mice also developed and died of CML, providing conclusive evidence that BCR-ABL-expressing Lin^-c-Kit^+Sca-1^+ cells function as LSCs in chronic phase CML (10).

Identification of granulocyte macrophage progenitors (GMP) as LSCs in blast crisis CML

CML chronic phase is pathologically different from CML in blast crisis. This difference may be reflected in the difference of LSCs between these two disease stages. The expansion of the progenitor pool ($CD34^+Lin^-$) was found in bone marrow from patients with CML in blast crisis, whereas the population of HSCs ($CD34^+CD38^-$ $CD90^+Lin^-$) did not show an expansion (42), suggesting that LSCs for blast crisis CML reside in more differentiated progenitor cells. In support of this idea, real-time PCR showed that BCR-ABL transcripts were more abundant in myeloid progenitors than HSCs. Furthermore, a mouse model which expressed BCR-ABL in an established line of *E2A*-knockout mouse bone marrow cells also showed that BCR-ABL transformed GMPs function as LSCs (43). Although Wnt/β-catenin pathway is normally active in HSCs but not in GMPs, a striking increase in activated β–catenin was found in GMPs from CML patients in blast crisis and from mice with blast crisis (42, 43). These results demonstrate that GMPs function as LSCs in CML blast crisis, and these stem cells are similar to LSCs in AML (36).

Different properties of LSCs in chronic phase and blast crisis CML may be the cause of the difference between these two disease phases (Table. 1). Identification of LSCs in CML provides a powerful assay system for studying the effect of BCR-ABL kinase inhibitors on LSCs and for identifying critical genes/pathways in these stem cells.

LSCs are resistant to BCR-ABL kinase inhibitors

The BCR-ABL kinase inhibitor imatinib was developed to treat CML and now serves as the frontline therapy for the patients with chronic phase CML (3). Despite its ability to control

CML, imatinib does not appear to cure the disease, as LSCs evade treatment. In one report, Holyoke and colleagues isolated $Lin^{-}CD34^{+}$ stem cells from the peripheral blood of patients with CML in chronic phase, and cultured the cells with and without growth factors, and in the presence or absence of imatinib. They observed that imatinib killed almost all dividing cells; however, a significant population of viable $CD34^{+}$ cells were unaffected by the treatment and were confirmed to be leukemic in nature (9). The fact that imatinib could not target the quiescent BCR-ABL-expressing LSCs made it apparent that imatinib treatment alone could not cure CML (5, 9). Both *in vitro* and *in vivo* studies revealed that $CD34^{+}$ cells derived from the bone marrow of CML patients could not be effectively killed by imatinib treatment. $CD34^{+}$ CML stem cells, especially the non-dividing $CD34^{+}$ cell population, were not sensitive to imatinib-inhibition *in vitro*, and this was further confirmed by the detection of BCR-ABL mRNA transcripts in $CD34^{+}$ bone marrow cells from CML patients after a long-term treatment with imatinib (5, 9). The minimal effect of BCR-ABL kinase inhibitor on LSCs was also observed in the CML mouse model (10).

The second generation CML drug dasatinib, a dual BCR-ABL/SRC kinase inhibitor, effectively controls CML in patients. In CML mouse model, mice treated with dasatinib lived significantly longer than those treated with imatinib (10). These survival data correlated with significantly lower numbers of BCR-ABL-expressing leukemic cells in peripheral blood compared with placebo- and imatinib-treated mice. However, all dasatinib-treated CML mice eventually died of this disease, indicating that like imatinib, this drug does not completely eradicate LSCs in CML mice. This conclusion on the failure of dasatinib to eradicate LSCs is supported by the observation that quiescent human $CD34^{+}CD38^{-}$ CML cells are resistant to dasatinib treatment (44). As mentioned above, human CML cells did not induce typical CML in NOD/SCID mice, questioning whether these quiescent human $CD34^{+}CD38^{-}$ CML cells are true LSCs in CML, which needs to be further addressed in the future. However, BCR-ABL-expressing HSCs ($GFP^{+}CD34^{-}c\text{-}Kit^{+}Hoe^{-}$) exists in the side population of bone marrow cells from the imatinib- or dasatinib-treated CML mice, and this cell population can efficiently transfer CML to recipient mice (10). This observation indicates that neither imatinib nor dasatinib completely eradicates BCR-ABL-expressing HSCs, suggesting that neither drug alone will cure CML and targeting of multiple pathways in LSCs is required to cure the disease. When LSCs in dasatinib-treated CML mice were further analyzed by identifying the $GFP^{+}Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ population, compared with placebo-treated mice, dasatinib-treated mice exhibited fewer LSCs, which are likely dividing rather than quiescent. Despite this, dasatinib treatment failed to eradicate these cells completely in CML mice (10).

In mice that were treated with BCR-ABL kinase inhibitors and eventually succumbed to CML, there was a continuous accumulation of imatinib- or dasatinib-insensitive LSCs in the bone marrow, which might ultimately lead to an accumulation of proliferating leukemic cells in the bone marrow and lungs. LSCs in bone marrow of imatinib-treated CML mice were further measured at multiple time points in the course of the disease (11). BCR-ABL promoted self-renewal of LSCs and increased the number of LSCs in placebo-treated CML mice. Although this BCR-ABL-driven increase in the number of LSCs could be inhibited to a lower level by imatinib treatment, total numbers and percentages of LSCs in bone marrow of the treated mice gradually increased during imatinib treatment. Undefined pathways that could not be inhibited by imatinib exist, and contribute to the maintenance, survival, and self-renewal of LSCs. To further demonstrate the minimal effect of imatinib on LSCs, $Sca1^{+}$ cells derived from the bone marrow of primary CML were transplanted into secondary recipient mice to induce secondary CML. Cohorts of these secondary recipients were then treated for varying durations of time. Then the same number of bone marrow cells from these secondary CML mice was transferred into syngeneic tertiary recipients. A comparison of disease latencies among these different groups of recipient mice revealed that the longer

the duration of imatinib treatment in the secondary CML mice, the more rapid the induction of tertiary CML(11), further demonstrating that imatinib is unable to suppress LSCs, and with time, LSCs continue to accumulate and cause rapid death of CML mice.

While it is still unclear why imatinib and dasatinib do not completely eradicate LSCs, several reasons should be excluded. The first, the notion that the drug cannot access stem cells is incorrect, as inhibition of intracellular BCR-ABL phosphorylation by dasatinib in the stem cells were detected (10). The second, the inability of dasatinib to cure CML mice is not attributable to the appearance of BCR-ABL-T315I resistant clone in the mice because CML mice treated with dasatinib for about 3 months contained >40% of $GFP^+Gr\text{-}1^+$ cells, among which there were large numbers of LSCs. Sequencing analysis of isolated genomic DNA from bone marrow cells of these mice did not show the T315I mutation in the BCR-ABL kinase domain (10). In addition, the failure of imatinib to eradicate LSCs is not related to the c-*kit* function, because both imatinib and dasatinib inhibit c-*kit* (45). These results suggest that inhibition of BCR-ABL kinase activity alone is insufficient to eradicate LSCs (11).

Critical molecular pathways in LSCs

BCR-ABL appears to play a critical role in the maintenance of survival of LSCs, as all critical molecular pathways identified so far can be activated by BCR-ABL. It is reasonable to think that the stemness of BCR-ABL-expressing HSCs is maintained by a complex molecular network involving BCR-ABL and its interaction with other downstream signaling pathways. If this were the case, these pathways would be specifically involved in the survival regulation of LSCs but not normal stem cell counterparts. In other words, it is possible to identify genes that play critical role in the regulation of LSC function. This idea is supported by the identification of the *Alox5* gene as a key regulatory gene for LSCs but not normal hematopoietic stem cells (22). So far, there have been no data that do not support a role of BCR-ABL in the maintenance of the stemness of LSCs. However, a critical question to ask is why BCR-ABL kinase inhibitors such as imatinib and dasatinib are incapable of eradicating LSCs, if BCR-ABL kinase activity is inhibited? It has been shown that the inhibition of BCR-ABL kinase activity by imatinib in LSCs does not completely compromise BCR-ABL function (10), indicating the kinase-independent function of BCR-ABL. A complete removal of BCR-ABL protein would have a much stronger inhibitory effect on LSCs. Besides targeting BCR-ABL, the identification and inhibition of key BCR-ABL downstream signaling molecules/pathways will offer effective therapeutic strategies aiming to eradicate LSCs. Below are some examples of the key pathways activated by BCR-ABL in LSCs.

Wnt/β-catenin pathway

Wnt/β-*catenin* signalling plays an important role in HSC development (46). β-*catenin* is a key factor in this pathway, and its activation upon the Wnt ligand binding to the receptor and its stability after activation are highly regulated by a destruction complex involving the tumor suppressor adenomatous polyposis coli (APC), the scaffolding protein that binds newly synthesized β-*catenin*, and two kinases choline kinase (CKI) and glycogen synthase kinase 3β (GSK3β) which phosphorylate a couple of Ser and Thr residues in the amino terminus of β-*catenin*(47). The phosphorylated β-catenin recruits an E3 ubiquitin ligase, which targets β-*catenin* for proteasomal degradation (48). In blast crisis CML patient, β-*catenin* is activated in myeloid progenitors and the activated β-*catenin* translocates to the nucleus (42). In a CML mouse model, loss of β-*catenin* delays the development of CML (11, 49). The delayed CML development in the absence of β-*catenin* is due to a decreased ability of BCR-ABL to support long-term renewal of LSCs, as shown in the serial replating and transplantation assays. The inhibitory effect of β-*catenin* deletion on LSCs is associated with the reduction of the levels of p-Stat5α instead of *CEBPα, Id1, Pax5, cyclinD1,*

cyclinD2 or c-Myb (49). Recently, *Wnt*/β-*catenin* signaling pathway has also been shown to be required for the development of LSCs in AML, which derived from either HSC or more differentiated GMPs (50).

Hedgehog pathway

The Hedgehog (Hh) genes have been identified for their roles in *Drosophila* development, as mutations in the Hh genes alter the segmental pattern of the larva and cause embryonic lethality (51). Three homologs of Hh genes (Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh)) have been identified in mammalian system (52–54). The Hh proteins are secreted proteins and can mediate signal transduction in nearby and distant tissues by binding to their specific receptor, Patched (PTC). PTC is a transmembrane protein that negatively regulates another transmemberane protein, smoothened (SMO). When SMO is released from the inhibition of PTC, it eventually activates its downstream Gli transcription factor; Gli in turn regulates expression of its target genes, including *Gli1* and *Ptch* (55–57).

Hh signaling has also been indicated during primitive hematopoiesis based on mouse embryonic stem cell studies. Ihh is a primitive endoderm-secreted signal, and is sufficient to activate embryonic hematopoiesis and vasculogenesis in pre- or early-gastrulation-stage epiblasts(58). Moreover, study from zebrafish showed that the mutations of the Hh pathway members or inhibition of Hh pathway with the Hh inhibitor cyclopamine can cause a developmental defect in adult HSCs (59). However, likely due to the functional redundancy of the three Hh members, the individual Hh knockout mice do not have a significant defect in adult hematopoiesis (60, 61). In additon, the activation of Hh pathway has been observed in different human cancers. Activating point mutations of *Smo* or inactivating point mutations of *Ptch* have been detected in medulloblastoma, rhabdomyosarcoma and sporadic basal cell carcinoma (62–64). In CML patients, more than 4-fold induction of transcript levels for *Gli1* and *Ptch1* was observed in CD34+ cells in both chronic phase and blast crisis (65). In two studies using a CML mouse model, recipients of BCR-ABL transduced bone marrow cells from $Smo^{-/-}$ donor mice developed CML significantly slower than recipients of BCR-ABL transduced bone marrow cells from wild type donor mice (65, 66). When the frequency and function of LSCs were further examined, Smo deletion caused a significant reduction of the percentage of LSCs. In contrast, overexpression of *Smo* led to an increased percentage of LSCs and accelerated the progression of CML (65, 66). The underlying mechanism for the inhibitory effect of the *Smo* deletion on LSCs is likely through regulating the cell fate determinant *Numb*, because the *Smo* deletion causes an upregulation of *Numb* expression in LSCs and overexpression of *Numb* inhibits the propagation of LSCs *in vitro* (66).

Alox5 pathway

The *Alox5* gene encoding arachidonate 5-lipoxygenase (5-LO) is involved in numerous physiological and pathological processes, including oxidative stress response, inflammation and cancer (67–74). 5-LO is responsible for producing leukotrienes, a group of inflammatory substances that cause human asthma (75). Altered arachidonate metabolism by leukocytes and platelets was reported in association with myeloproliferative disorders almost 30 years ago (76). Several selective 5-LO inhibitors were found to reduce proliferation of and induce apoptosis of CML cells *in vitro* (77, 78), although potential off-target effects of these inhibitors were not excluded in these studies. Recently, human CML microarray studies have shown that *Alox5* is differentially expressed in $CD34^+$ CML cells, suggesting a role of *Alox5* in human CML stem cells (12, 13). However, the function of *Alox5* in LSCs needs to be tested. A microarray analysis of gene expression in LSCs in CML mice showed that the *Alox5* gene was upregulated by BCR-ABL and that this upregulation

was not inhibited by imatinib treatment (22), providing a possible explanation why LSCs are not sensitive to inhibition by BCR-ABL kinase inhibitors (10). Furthermore, recipients of BCR-ABL transduced bone marrow cells from *Alox5*$^{-/-}$ donor mice were resistant to the induction of CML by BCR-ABL (22), demonstrating that *Alox5* is essential for CML development. FACS analysis of CML cells from the peripheral blood and bone marrow of recipients receiving BCR-ABL-transduced *Alox5*$^{-/-}$ donor bone marrow cells showed that myeloid leukemia cells proliferated initially, peaking around 2 weeks, then started to decline, and eventually disappeared after 7 weeks. *Alox5* deficiency mainly affected growth of BCR-ABL-expressing but not non-BCR-ABL-expressing donor bone marrow cells, suggesting that *Alox5* signaling is much more critical for the function of LSCs than for normal HSCs. The effect of *Alox5* on LSC function was further demonstrated and supported by the failure of BCR-ABL-expressing *Alox5*$^{-/-}$ bone marrow cells to induce CML in secondary recipient mice (22). Together, these results suggest that *Alox5* could be a specific target gene in CML LSCs. This idea was tested by treating CML mice with Zileuton, an inhibitor of 5-LO (see below).

Pten pathway

Besides *Alox5*, another important gene identified from the microarray analysis of LSCs of CML mice is *phosphatase and tensin homolog* (*Pten*). Recently, gene expression profiling in HSCs of chronic phase CML patients was found to be similar to that seen in normal myeloid progenitor cells, and *Pten* was found to be differentially expressed (14). *Pten* is often deleted or inactivated in many tumor types, including glioblastoma, endometrial carcinoma, and lymphoid malignancies (79–81). PTEN is a phosphatase that dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate(PIP3) (82). PIP3 is a direct product of PI3K and plays a critical role in the regulation of cell survival and growth through the activation the serine/threonine protein kinase PDK1 and its downstream target AKT (83). A study shows that PTEN is oxidized and inactivated in pancreatic cell lines, and that treatment with a 5-LO inhibitor prevented PTEN degradation (84), suggesting that 5-LO reduces the stability of PTEN and that *Pten* is functionally related to *Alox5*. To reveal the role of PTEN in LSCs, *Pten* were deleted from bone marrow cells of *Pten*$^{flox/flox}$ mice through the retroviral transduction of cells with a tricistronic cassette expressing BCR-ABL, iCre, and GFP (BCR-ABL-iCre-GFP) (85). Bone marrow cells from *Pten*$^{flox/flox}$ mice were transduced with BCR-ABL-iCre-GFP or BCR-ABL-GFP retrovirus, followed by transplantation of the transduced cells into recipient mice. Without *Pten*, CML developed much faster, which correlated with an increased percentage of GFP^{+}Gr-1^{+} myeloid leukemia cells and an increased number of leukemia cells in peripheral blood of the mice (85). The effect of *Pten* on the function of LSCs was tested by comparing the ability to induce CML between LSCs that expressed BCR-ABL-PTEN-GFP and those that expressed BCR-ABL-GFP. The same number of LSCs cells sorted from CML mice receiving either BCR-ABL-GFP or BCR-ABL-PTEN-GFP transduced bone marrow cells were transplanted into recipient mice. The number of leukemia cells in CML mice receiving BCR-ABL-PTEN-GFP transduced LSCs was 4-fold lower than that in CML mice receiving BCR-ABL-GFP transduced LSCs. Consistent with the less severe CML phenotype, survival of mice receiving LSCs transduced with BCR-ABL-PTEN-GFP was significantly longer than that of mice receiving LSCs transduced with BCR-ABL-GFP (85), indicating that PTEN suppresses the function of LSCs. An attractive idea to test in the future is whether *Alox5* deficiency leads to an upregulation of *Pten* in CML mice and whether the lack of CML development in the absence of *Alox5* is at least partially related to the upregulation of *Pten*.

FoxO pathway

The FOXO (Forkhead O) subfamily of transcription factors plays critical roles in cell cycle arrest, stress resistance and apoptosis (86). There are four members (FOXO1, FOXO3,

FOXO4 and FOXO6), and they are functionally downstream of the PI3K-AKT pathway (87, 88). AKT directly phosphorylates FOXOs members, resulting in the exclusion of FOXOs from the nucleus and degradation in cytoplasma (89). FOXOs localize to nucleus and regulate apoptosis, cell cycle progression and oxidative stress responses (89–92). Although individual knockout mice of FoxO1 and FoxO4 did not demonstrate overt hematopoietic phenotype, FoxO1/3/4 triple conditional knockout mice showed the essential role of FoxOs in maintaining self-renewal capacity of HSCs (87, 88), shown by a marked decrease of the HSC compartment (including the short- and long-term HSC populations) in FoxO-deficient mice. The defective long-term repopulating capability of HSCs in FoxO-deficient mice was shown to be correlated with increased cell division and apoptosis of HSCs (87). FoxO transcription factors have also been shown to play essential roles in the maintenance of CML LSCs. FOXO3a localizes to the nucleus of cells and causes decreased Akt phosphorylation in LSC population (93). In addition, serial CML transplantation showed that the FoxO3a deficiency severely impairs the ability of LSCs to induce CML (93). Furthermore, TGF-β is a critical regulator of Akt activation and controls FoxO3a localization in LSCs of CML. A combination strategy of TGF-β inhibition, FoxO3a deficiency and BCR-ABL kinase inhibition results in an efficient LSC depletion and suppresses CML development (93).

Therapeutic strategies for eradicating LSCs in CML

Changing cellular properties of LSCs

Because BCR-ABL kinase inhibitors only kill proliferating leukemia cells, one potential strategy is to stimulate the proliferation of LSCs by stimulating primitive quiescent CML cells into cell cycle. Taking advantage of the role of G-CSF (granulocyte colony stimulating factor) in promoting cell cycle entry (94), treatment with G-CSF, in combination with imatinib, provides a novel method for treating CML, although current efforts did not result in expected outcome (95). Another attractive approach is to inhibit autophagy of LSCs using inhibitory compounds such as chloroquine (96), because inhibition of autophagy may promote cell death induced by imatinib (96). Finally, Pandolfi and colleagues discovered that non-proliferating LSCs lacking the tumor suppressor promyelocytic (PML) leukemia protein tends to enter cell cycle quickly, leading to LSC exhaustion (97).

Targeting critical signaling pathways in LSCs

Zileuton—Novel approaches aimed at inhibiting LSCs may more efficiently curtail leukemogenesis and hold real potential for curative therapy. Recently, this strategy has been applied to the development of an effective drug to eradicate LSCs in AML using an *in silico* screen of public gene expression database (98). As described above, using CML mice, a genetic study showed the essential role of *Alox5* in survival of LSCs but not normal hematopoietic stem cells (22). 5-LO as a potential target in LSCs for treating CML was further tested by treating CML mice with a placebo, the 5-LO inhibitor Zileuton, Imatinib alone, or both Zileuton and Imatinib in combination. As expected, Imatinib treatment was effective in treating CML, but Zileuton treatment was even more effective than Imatinib. Treatment of CML mice with both Zileuton and Imatinib had a better therapeutic effect than with either drug alone in prolonging survival of the mice. Prolonged survival of Zileuton-treated CML mice correlated with less severe leukemia cell infiltration to the lungs and the spleens. In peripheral blood of CML mice treated with Zileuton and Imatinib, myeloid leukemic cells gradually decreased with treatment, and importantly, Zileuton treatment did not have an inhibitory effect on normal myeloid cells in peripheral blood of the same animals, as the number of these non-leukemic cells increased during the treatment. In the bone marrow of Zileuton-treated CML mice, myeloid leukemia cells were also significantly reduced during the treatment. Prolonged survival of CML mice by Zileuton treatment is

consistent with the targeted inhibitory effect of Zileuton on LSCs. During the treatment, the ratio between the percentage of LT (long-term)-LSCs and that of ST (short-term)-LSCs/MPP cells increased, suggesting a possible blockade of differentiation of LT-LSCs. Zileuton treatment did not affect differentiation of GFP^{-}LT-HSCs in the same animals, demonstrating that inhibition of 5-LO did not suppress normal HSCs. These results clearly demonstrate that *Alox5* is a promising target gene for eradicating LSCs in CML. A Phase I/II study at University of Massachusetts Medical School (Worcester, Massachusetts, USA) to evaluate the safety of Zileuton in combination with imatinib in chronic phase CML patients has been approved by FDA, and the effectiveness of this novel anti-LSC therapy remains to be seen in near future.

IPI-504—Inhibition of the chaperone protein HSP90 may prove effective in disrupting LSC function, as treatment of BCR-ABL-expressing cells with the HSP90 inhibitor 17-AAG suppresses cell growth and induces apoptosis (99). Recently, a newly developed HSP90 inhibitor IPI-504 was used to investigate whether HSP90 is an effective target for inhibiting LSCs and for the treatment of CML *in vivo* in CML mouse model (100). The results showed that HSP90 stabilizes the BCR-ABL and mutant (T315I) BCR-ABL oncoproteins and that treatment with IPI-504 significantly prolonged survival of mice with wild type BCR-ABL induced CML but even more markedly prolonged survival of mice with the T315I BCR-ABL-induced CML. Impairment of LSC function by IPI-504 was also investigated by culturing CML bone marrow cells under the conditions that support survival and growth of HSCs (100). FACS analysis showed that compared with the untreated group, imatinib treatment did not reduce the percentage or number of LSCs, whereas IPI-504 treatment had a dramatic inhibitory effect on LSCs. The inhibitory effect of IPI-504 on LSCs was also observed *in vivo* by FACS analysis of LSCs, and IPI-504 treatment reduced the percentage and number of LSCs in bone marrow. When normal mice were treated with IPI-504 or placebo for 4 weeks, and analysis of bone marrow from these mice showed that there were no changes in levels of normal HSCs from any treatment groups, indicating that IPI-504 treatment did not inhibit survival of normal HSCs.

Omacetaxine—Omacetaxine (formerly known as homoharringtonine) is a cephalotaxine ester derived from the evergreen tree, *Cephalotaxus harringtonia*, and native to China. Omacetaxine has shown clinical activity alone and in combination with imatinib in CML patients resistant to imatinib or other tyrosine kinase inhibitors (101). Recently, omacetaxine has been shown to have the capability to kill LSCs effectively *in vitro* and in CML mice (102). When mice with BCR-ABL-induced CML were treated with omacetaxine, a significant reduction in total LSCs and overall leukemic cells was observed. Because its mechanism differs from tyrosine kinase inhibitors, omacetaxine also showed an inhibitory effect on LSCs expressing imatinib-resistant BCR-ABL-T315I. Interestingly, omacetaxine is more effective in treating BCR-ABL-T315I induced CML than treating wild type BCR-ABL-induced CML, which is consistent with the result from an earlier *in vitro* study (103). The underlying mechanism for the inhibitory effect of omacetaxine on LSCs is still unknown, although several potential pathways are thought to be involved, including HSP90, BCL-2 and MCL-1(102). The clinical efficacy of omacetaxine on CML could be related to the inhibitory activity of omacetaxine on LSCs (102).

Cyclopamine—Cyclopamine belongs to the jervine family of alkaloids derived from the plants of genus *Veratrum*, and is a specific Hedgehog inhibitor (104). Cyclopamine inhibits the Hh pathway by binding directly to Smo and affecting its protein conformation (105). When BCR-ABL induced CML mice were treated with cyclopamine, the untreated animals died of CML within 4 weeks, but 60% of the treated mice were still alive after 7 weeks (105). In addition, cyclopamine-treated mice had up to a 14-fold reduction in LSC

population. Furthermore, cyclopamine was effective in treating mice with BCR-ABL-T315I induced CML. These results indicate that the Hh pathway is required for the functional regulation of LSCs, and cyclopamine may be an effective drug for treating human CML, although its effect on normal HSCs needs to be evaluated further (66).

BMS-214662—BMS-214662 is a cytotoxic farnesyltransferase inhibitor capable of targeting and killing non-proliferating tumor cells (106). BMS-214662, alone or in combination with imatinib or dasatinib, induces apoptosis of both proliferating and quiescent primitive $CD34^+CD38^-$ CML stem cells with much less effect on normal $CD34^+$ HSCs (29). The selective inhibitory effect of BMS-214662 on CML stem cells may not be related to its anti-farnesyltransferase activity, as BMS-225975, a structurally similar farnesyltransferase inhibitor, did not inhibit CML stem cells. The apoptotic pathways involved in the inhibitory effect of BMS-214662 include Bax, reactive oxygen species, Cytochrome c, and Caspase-9/3. These pathways were coupled with protein kinase Cβ (PKCβ), E2F1 and Cyclin A-associated Cyclin-dependent kinase 2. Cotreatment of CML $CD34^+$ and $CD34^+CD38^-$ cells with the PKC modulators, bryostatin-1 or hispidin, markedly decreased these early events and the subsequent apoptosis. These results indicate that BMS-214662 may provide a molecular framework for the development of novel therapeutic strategies (29, 107).

FTY720—Protein phosphatase 2A (PP2A) is a tumor suppressor. The phosphatase activity of PP2A is inhibited by the BCR/ABL-induced expression of the PP2A inhibitor SET (108). FTY720 is a water-soluble, nontoxic PP2A inhibitor with a high oral bioavailability, and has been used as an immunomodulator in Phase III trials for patients with multiple sclerosis (109, 110). Recently, the therapeutic effect of FTY720 was tested in CML, and FTY720 was shown to activate PP2A and suppress BCR/ABL in myeloid and lymphoid cell lines. In human LSCs, PP2A activity was reduced by 90% in CML $CD34^+$ cells compared to $CD34^+$ cells from normal bone marrow donor. The $CD34^+$ cells from CML patients were cultured in the presence of myeloid cytokines. After treatment with FTY720, the PP2A activity was shown to be recovered to the levels in normal $CD34^+$ cells (111). In addition, FTY720 treatment triggers apoptosis of LSCs, showing a great potential for targeting LSCs through activating PP2A.

Bortezomib—The proteasome is an intracellular organelle providing a targeted mechanism for protein degradation via 3 catalytic specificities: chymotrypsin-like (CT-L), trypsin-like (T-L), and post-glutamyl hydrolytic (PG); this process is essential for cell cycle progression, cell proliferation and apoptosis (112, 113). Bortezomib is a reversible and specific inhibitor of CT-L activity, and has been used for treating mantle cell lymphoma and multiple myeloma (114). Proteasomal activity was shown to be increased in CML cells (113), and loss of proteasomal activity showed an inhibitory effect on growth of $BCR\text{-}ABL^+$ cell lines sensitive or resistant to imatinib (115). The effect of bortezomib was also tested in $CD34^+$ cells from CML patients. *In vitro*, bortezomib selectively inhibited colony formation by $CD34^+$ $BCR\text{-}ABL^+$ progenitor cells (116). Bortezomib was also shown to induce apoptosis and inhibit proliferation of CD34+38−, long-term culture-initiating (LTC-IC) cells (117). Bortezomib also impaired the function of CML LSCs by reducing the engraftment of patient-derived CD34+ CML cells (117). Importantly, different BCR-ABL mutants, including T315I, H396P and M351T, were sensitive to bortezomib (23), although it also inhibited proliferation and induced apoptosis of normal CD34+38− cells (117).

Interferon-α (IFN-α)—Interferon-α, an immunomodulatory cytokine, has been used to treat CML (118). Although the underlying mechanism is unclear, IFN-α may target LSCs. In a study involving 12 CML patients who achieved molecular remission on imatinib and

subsequently discontinued the kinase inhibitor therapy, half of them relapsed with detectable BCR-ABL mRNA transcripts, whereas the others remained in molecular remission. All these patients remaining in molecular remission had been previously treated with IFN-α (6). These results are not definitive, but suggest a potential inhibitory effect of IFN-α on LSCs in CML. More work needs to be done to explain how IFN-α inhibits LSCs.

In summary, although current understanding of the biology of LSCs in CML is still preliminary, the identification of several critical target genes such as *Alox5, hedgehog, β-cantenin and FoxO* provides opportunities for developing promising anti-stem cell therapies for curing CML. Future clinical trials for these drugs will determine whether this anti-stem cell strategy is effective in the treatment of CML and perhaps other cancers.

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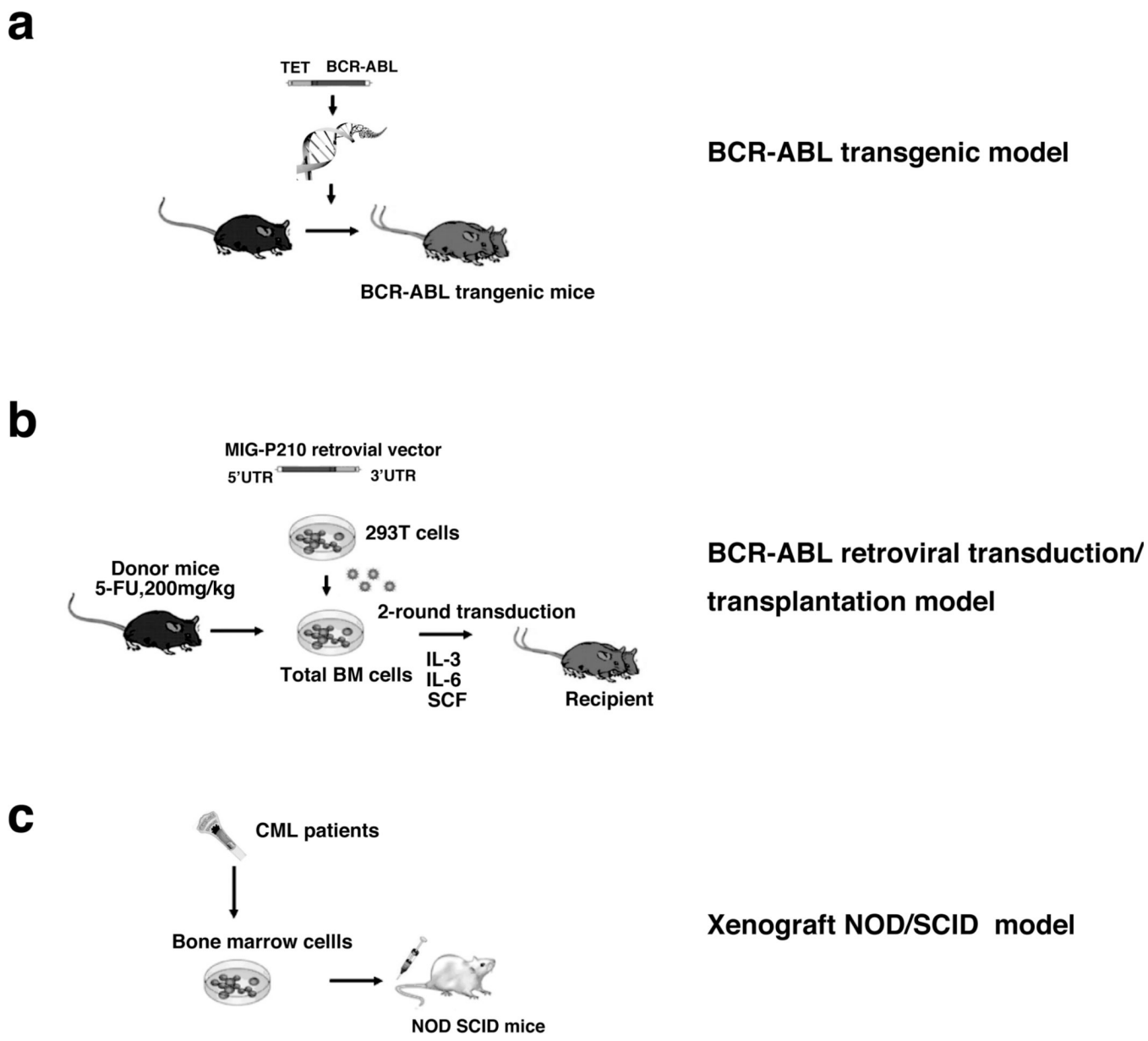

Figure 1. Mouse model for studying BCR-ABL leukemia

There are three major types of mouse models of CML. **a**. BCR-ABL transgenic model. Inducible expression of BCR-ABL controlled by tTA tet-off system in BCR-ABL transgenic mouse. **b**. Retroviral bone marrow transduction/transplantation model. Bone marrow cells from 5-FU treated donor mice are transduced with BCR-ABL retrovirus, cultured in the presence of cytokines, and then transplanted into lethally irradiated syngeneic recipient mice. **c**. Human xenograft NOD/SCID mouse model. Bone marrow or peripheral blood cells from CML patients are purified for CD34+ cells, and then the cells are transplanted into NOD/SCID mice.

Table 1

Comparison of the different properties of LSCs between chronic-phase and blast-crisis CML.

	Chronic-phase CML	**Blast-crisis CML**
LSCs	BCR-ABL transformed HSCs	BCR-ABL transformed GMPs
Human LSC markers	CD34+CD38−CD90+Lin−	CD34+CD38+Lin−
Mouse LSC markers	Lin−c-Kit+Sca-1+	Lin−c-Kit+Sca-1−FcγR+
Activation of Wnt/ β-catenin	Yes	Yes
Other oncogenic mutations	Unclear	Yes
Treatment	Imatinib	Chemotherapy or BMT