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Review Article

Novel therapeutic approach to eradicate tyrosine kinase inhibitor resistant chronic myeloid leukemia stem cells

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Although discovery of the tyrosine kinase inhibitor (TKI) imatinib mesylate has significantly improved the prognosis of chronic myeloid leukemia (CML) patients, a rare population of CML stem cells is known to be resistant to TKI therapy, causing recurrence of CML. However, recent progress in CML stem cell biology may present a novel therapeutic avenue for CML patients. In this review, we focus on mechanisms used by CML stem cells to maintain TKI-resistance. Comprehensive approaches including mouse genetics, prospective identification of CML stem cells, and syngenic transplantation techniques have identified several key molecules or signaling pathways, including hedgehog (Hh)/Smo, promyelocytic leukemia (PML), 5-lipoxygenase (5-LO), and fork-head box class O (FOXO), that function in CML stem cell maintenance. Inhibiting some of these factors in combination with TKI administration successfully antagonized resistance of CML stem cells to TKI therapy, resulting in efficient eradication of leukemia cells *in vivo*. Thus, development of methods that sensitize CML stem cells to TKI therapy may lead to novel therapies to treat CML patients. (*Cancer Sci* 2010; 101: 1577–1581)

Chronic Myeloid Leukemia (CML) as a ''Stem Cell Disease''

Hematopoietic stem cells (HSCs), which top the hierarchy of the normal hematopoietic system, are defined by both their ability to reproduce themselves, a property known as self-renewal, and their capacity to give rise to all mature hematopoietic cell lineages throughout an individual's lifetime. Recently, it was revealed that CML contains leukemia stem cells which form a hierarchy similar to that seen in normal hematopoiesis. Human CML is a biphasic myeloproliferative disease (MPD), which initially assumes a chronic phase before progressing to an accelerated phase and then to blast crisis. In CML patients, the initial chronic phase is characterized by massive expansion of myeloid cells. Fialkow *et al.*[1] demonstrated that chronic phase CML cells can produce functionally normal mature blood cells, indicating that CML cells in the chronic phase can differentiate into mature blood cells, like normal HSCs. Acquisition of additional genetic mutations and/or epigenetic alterations results in progression from the chronic phase to an accelerated phase and finally to blast crisis. Indeed, Jamieson *et al.*[2] proposed a model in which abnormal activation of β-catenin in a granulocyte/macrophage progenitor (GMP)-like population in CML patients promotes disease progression from chronic phase to blast crisis. Blast crisis is characterized by accumulation of myeloid or lymphoid blast cells in peripheral blood or bone marrow, indicating that CML cells in blast crisis can proliferate but that their differentiation capacity is blocked. Since CML patients in blast crisis show poor prognosis with short survival, it is critical to treat CML patients during the chronic phase.

Most CML is caused by a translocation between human chromosomes 9 and 22 that generates what is known as the Philadelphia chromosome and resultant breakpoint cluster region-abelson (*BCR–ABL*) fusion gene, which encodes a constitutively active tyrosine kinase.[3] Several lines of evidence indicate that CML cells emerge due to expression of BCR–ABL in normal HSCs (Fig. 1). Transplantation of multipotent murine HSCs expressing BCR–ABL into recipient mice induces CML-like MPD.[4–8] By contrast, CML is not induced in committed murine hematopoietic progenitor cells expressing BCR–ABL.[9] These results demonstrate that chronic phase CML cells originate from multipotent HSCs. In a mouse CML-like MPD model, CML stem cells, which are defined by the ability to induce CML in transplanted mice, can be purified in a rare c-Kit^{+}Lineage^{-} Sca-1^{+} (KLS^{+}) population of mouse CML cells (i.e. in cells that exhibit the marker profile of normal HSCs).[10–14] Interestingly, in human CML patients, CML stem cells are apparently found in a cell fraction expressing cell surface markers characteristic of primitive hematopoietic cells.[15,16] All of these findings support the notion that CML is a ''stem cell disease'' (Fig. 1). In this review, we present evidence from our investigations and those of others supporting a link between molecular mechanisms regulating self-renewal in normal HSCs and CML stem cells, and discuss current efforts aimed at developing novel therapeutics to specifically target tyrosine kinase inhibitor (TKI)-resistant CML stem cells.

Common Signaling Pathways Underlie Stem Cell Fate in Both Normal Hematopoiesis and CML

Observation that HSCs and CML stem cells share common properties suggests that signaling pathways determining normal HSC fate also govern maintenance of CML stem cell function. Several factors regulating stem cell fate in both normal hematopoiesis and CML leukemogenesis have been identified (Fig. 1). Promyelocytic leukemia (PML) protein functions in diverse cellular activities, including the DNA damage response, apoptosis, cellular senescence, and neoangiogenesis.[17] Ito *et al.*[12] found that PML is highly expressed in normal HSCs and that *Pml* deficiency impairs HSC self-renewal

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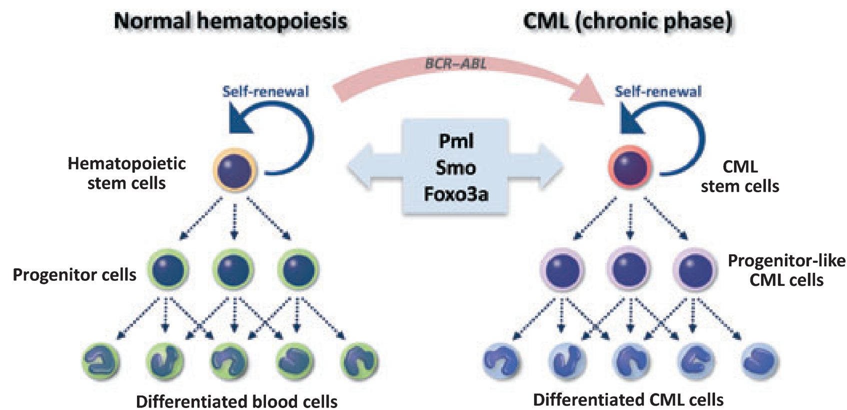

Fig. 1. A link between molecular mechanisms regulating self-renewal activity in normal hematopoietic stem cells (HSCs) and in chronic myeloid leukemia (CML) stem cells. HSCs, which top the hierarchy of the normal hematopoietic system, are defined by both their ability to self-renew and to give rise to all lineages of mature hematopoietic cells throughout an individual's lifetime. A rare population of CML stem cells also can self-renew and generate progenitor-like and differentiated CML cells, indicating that CML cells in the chronic phase form a hierarchy similar to that of normal HSCs. Several lines of evidence demonstrate that the emergence of CML cells requires BCR–ABL expression in normal HSCs. Recently molecules regulating normal HSC fate, including promyelocytic leukemia (Pml), Smo, and forkhead box class O (Foxo)-3a, have also been shown to govern CML stem cell function. These observations support the idea that CML is ''stem cell disease''.

Table 1. The biology and TKI-resistance in CML stem cells based on mouse genetic studies

Disrupted molecule	CML stem cells	Inhibitor	Possible combined therapy	Reference
Pml	Defective ability of CML stem cells to develop CML at third transplantation	Arsenic trioxide	Arsenic trioxide and Ara-C improve survival of CML-affected mice	12
β-Catenin	Defective ability of CML stem cells to develop CML at second transplantation	–	–	11
Alox5	Defective ability of CML stem cells to develop CML at second transplantation	Zileuton	Zileuton and imatinib improve survival of CML-affected mice	45
Smo	Impairment of development of CML and depletion of CML stem cells	Cyclopamin	Cyclopamin and nilotinib prolong survival of CML-affected mice	13,21
Foxo3a	Defective ability of CML stem cells to develop CML at third transplantation	TGF-β inhibitor	TGF-β inhibitor and imatinib improve survival of CML-affected mice, and suppress infiltration of CML cells in lung	14

Arsenic trioxide can reduce PML expression. Zileuton is an inhibitor of 5-lipoxygenase. Cyclopamin stabilizes Smo in an inactive form. TGF-β inhibitor suppresses TGF-β–Foxo signaling. Alox5, arachidonate 5-lipoxygenase; CML, chronic myeloid leukemia; Foxo3a, forkhead box class O 3a; PML, promyelocytic leukemia; TGF-β, transforming growth factor-β.

capacity. Importantly, whereas *Pml*$^{+/+}$ CML stem cells maintain the ability to induce CML at least at the 4th-serial transplantation, *Pml*$^{-/-}$ CML stem cells fail to generate disease at the 3rd-transplantation, demonstrating that PML is essential for CML stem cell maintenance (Table 1). Since *in vivo* treatment with arsenic trioxide (As_2O_3), which can reduce PML expression, eradicates CML stem cells in combination with the anti-leukemia drug Ara-C, PML down-regulation is an important candidate for CML stem cell-targeting therapy.

Wnt/β-catenin signaling is implicated in both normal HSC homeostasis and CML leukemogenesis. β-Catenin reportedly functions to regulate normal mouse HSC self-renewal.[11,18,19] Interestingly, Zhao *et al.*,[11] reported that conditional deletion of β-catenin impairs long-term maintenance of CML stem cells in the chronic phase, whereas loss of β-catenin allows acute lymphoblastic leukemia (ALL) to proceed unimpaired (Table 1). Loss of β-catenin can suppress infiltration of CML cells into the lung and liver in mice injected with CML stem cells. Thus, Wnt/β-catenin signaling likely plays a role in maintaining CML stem cells.

Hedgehog (Hh) signaling reportedly regulates self-renewal of normal HSCs and CML stem cells. In the absence of Hh ligands, the 12-pass transmembrane domain receptor Patched (Ptch) inhibits Smo, a seven-pass transmembrane domain receptor.[20] Binding of Hh ligands, including Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh), to Ptch activates Smo, which in turn activates signaling via Gli transcriptional effectors. To understand the role of Hh signaling in normal HSCs and CML leukemogenesis, Dierks *et al.* and Zhao *et al.*[13,21] in parallel studies analyzed the effects of conditional *Smo* deletion in the hematopoietic system. Although the frequency of normal HSCs was unchanged in *Smo*-deficient mice,[13,21] Smo was required for long-term maintenance of HSCs *in vivo*.[13] Interestingly, Smo loss causes CML stem cell depletion and impairs CML development,[13,21] whereas expression of constitutively active Smo increases the frequency of CML stem cells and accelerates CML development.[13] These findings demonstrate that Hh signaling plays an essential role to maintain CML stem cell function.

Forkhead box class O (FOXO) transcription factors also play an important role in the maintenance of normal HSCs and CML stem cells. The FOXO factors include FOXO1, FOXO3a, FOXO4, and FOXO6, all of which are downstream targets of Phosphatidylinositol 3 kinase (PI3K)–AKT signaling.[22] In the

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absence of stimulation by growth factors or insulin, FOXOs are present in the nucleus and activate their transcriptional targets. When a growth factor or insulin binds to the appropriate cell surface receptor, AKT is activated and directly phosphorylates FOXOs, resulting in their nuclear exclusion and degradation in the cytoplasm. Tothova *et al.*[23] reported that in mice with triple conditional deletion of *Foxo1*, *Foxo3a*, and *Foxo4*, the HSC population markedly increases. We and others have reported that Foxo3a alone is essential to maintain the HSC pool,[24,25] indicating that FOXOs are essential to maintain normal HSC self-renewal. Recently, we demonstrated an essential role for Foxo3a in the maintenance of CML stem cells.[14] Previous studies using CML cell lines indicate that BCR–ABL likely activates PI3K–AKT signaling, leading to FOXO nuclear export and suppression of FOXO transcriptional activity.[26–28] However, using a mouse CML-like MPD model we found that whereas non-CML stem cells showed high levels of Akt phosphorylation and cytoplasmic localization of Foxo3a, cells with decreased Akt phosphorylation and nuclear localization of Foxo3a were enriched in the CML stem cell population, despite expression of BCR–ABL. We also showed that the ability of CML stem cells to promote disease at the 3rd-transplantation is significantly decreased by Foxo3a deficiency *in vivo*. In particular, cells deficient in Foxo3a lose the potential to generate malignancies in multiple lineages.[14] Thus, Foxo3a is essential for long-term maintenance of leukemia-initiating potential in CML stem cells.

Resistance of Quiescent CML Stem Cells to TKI Therapy

The development of imatinib mesylate, an inhibitor of ABL kinase, has represented a breakthrough in CML treatment.[29] However, most CML patients require additional therapy due to resistance or intolerance.[30–33] Recent studies report that TKIs including imatinib, nilotinib, and dasatinib are potent TKI inhibitors in differentiated CML cells but are not as effective in quiescent primitive CML stem cells (Fig. 2).[34–39] In addition, mathematical modeling has shown that successful imatinib therapy is characterized by a bi-phasic, exponential decline in the number of CML cells, but that imatinib likely does not deplete CML stem cells in patients in the chronic phase.[40,41] Since residual CML stem cells are the source of disease recurrence, drugs capable of eradicating CML stem cells would provide markedly improved therapeutic benefits to CML patients.[42]

Molecular Mechanisms of TKI-Resistance of CML Stem Cells

Several potential mechanisms underlying TKI-resistance of CML stem cells have been proposed. It has been shown that CML stem cells are quiescent and undifferentiated, resulting in resistance to chemotherapy. CML stem cell properties are possibly regulated by association with a niche. Several lines of evidence indicate that inactivation of key molecules for CML stem cell maintenance possibly contributes to development of CML therapy. For example, pharmacological blockade of Hh signaling by cyclopamine, which stabilizes Smo in an inactive form, impairs CML development by CML stem cells (Table 1).[13,21] Furthermore, combining a TKI (nilotinib) with cyclopamine increased the amount of time to disease recurrence more effectively than did treatment with a TKI alone.[21]

BCR–ABL drives robust AKT activation, which represses FOXO function. TKIs block this activity and promote FOXO nuclear localization and activation, which induces apoptosis or cell cycle arrest. However, as noted above we observed unexpected nuclear localization of FOXO in CML stem cells in mouse models.[14] Since CML stem cells resist TKI therapy, it is suggested that this ''stem cell paradox'' regarding FOXO activation may reflect stem cell status. Komatsu *et al.* previously reported that introduction of an activated form of FOXO into the CML line KCL22 led to cell cycle arrest, concluding that FOXO3a is a downstream effector of TKI-induced cell cycle arrest in CML cells. This study also reported the interesting finding that FOXO inactivation sensitizes cells to TKI treatment *in vitro*, suggesting that FOXO contributes to resistance to TKI therapy. To address whether FOXO functions as a TKI effector

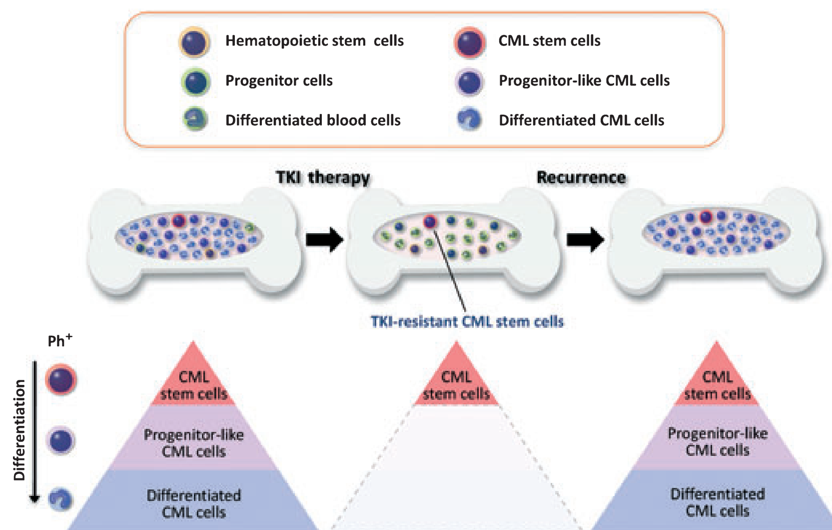

Fig. 2. Residual CML stem cells are responsible for TKI resistance in CML patients. CML arises in HSCs due to activity of the oncogenic BCR–ABL fusion protein. The TKI imatinib, which inhibits ABL tyrosine kinase activity, is now standard therapy for CML. Imatinib can eradicate numerous CML cells, such as progenitor-like CML cells and differentiated CML cells, and significantly improves the prognosis of CML patients in the chronic phase. However, it does not deplete CML stem cells, which top the CML hierarchy and are responsible for disease recurrence. Thus, effective human CML therapy requires measures to eradicate TKI-resistant CML stem cells. Ph^+, Philadelphia chromosome-positive CML cells.

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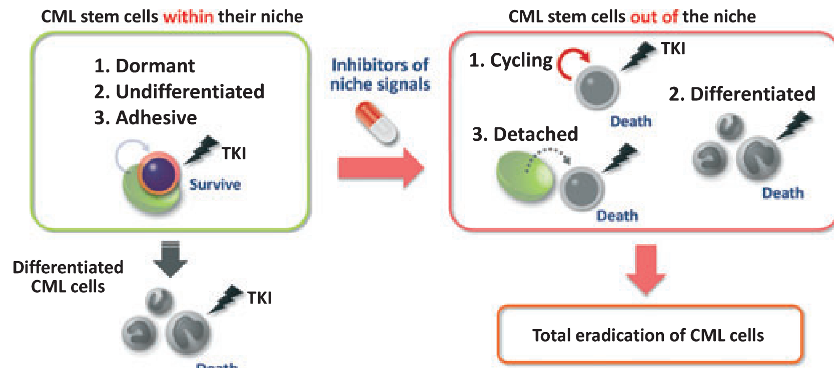

Fig. 3. Possible strategy to eradicate chronic CML cells by inhibiting association of CML stem cells with their niche. Although imatinib can eradicate numerous TKI-sensitive differentiated CML cells, it does not deplete CML stem cells responsible for disease recurrence. Interaction of TKI-resistant CML stem cells with a niche may keep stem cells dormant and undifferentiated. Thus, inhibitors of the niche signal could activate cell cycling or induce differentiation, promoting TKI-sensitivity. Together, factors capable of inhibiting CML stem cell/niche interaction are important candidates for CML therapies.

or an inhibitor of TKI in CML stem cells *in vivo*, we investigated roles of Foxo3a in response to TKI therapy using a CML mouse model.[14] We found that Foxo3a deficiency enhanced sensitivity of CML stem cells to TKI therapy and proposed that Foxo3a plays distinct roles in CML stem cells *versus* non-CML stem cells.[14] In our model, FOXO activation protects CML stem cells against various stresses, including TKI therapy, whereas it induces apoptosis or cell cycle arrest in non-CML stem cells in response to TKI therapy.

We have also searched for chemical compounds that alter Akt–Foxo status in CML stem cells and found that treatment of CML stem cells with Ly364947, an inhibitor of transforming growth factor (TGF)-β signaling, efficiently activates Akt and suppresses Foxo. Although administration of Ly364947 alone did not extend the survival of CML-affected mice, Ly364947 combined with imatinib significantly reduced recipient lethality and decreased CML stem cell frequency (Table 1).[14] These *in vivo* data demonstrate a critical role for the TGF-β/FOXO axis in maintenance of imatinib-resistant CML stem cells. Interestingly, Yamazaki *et al.*[43] reported that TGF-β is a candidate niche factor to maintain HSC dormancy. Thus the TGF-β pathway may be common to both normal HSCs and CML stem cells. However, we found that inhibition of TGF-β had a more potent effect on CML stem cells than on normal stem/progenitor cells.[14] Supporting this finding, it has been reported that TGF-β signaling is enhanced by the presence of BCR–ABL.[44] These data indicate that inhibition of TGF-β signaling may lead to efficient eradication of residual CML stem cells.

Chen *et al.*[45] propose an alternate mechanism underlying TKI resistance of CML stem cells. The Arachidonate 5-lipoxygenase (*5-LO*) gene (*Alox5*) encodes a lipoxygenase, which plays a role in the synthesis of leukotrienes from arachidonic acid and functions in numerous physiological and pathological processes, including oxidative stress, inflammation, and cancer.[46,47] Gene expression profiling revealed that *Alox5* expression is specifically up-regulated by BCR–ABL.[45] Whereas recipient mice transplanted with *Alox5*$^{+/+}$ CML stem cells died of CML, mice transplanted with *Alox5*$^{-/-}$ CML stem cells survived, indicating that *Alox5* deficiency underlies impaired CML stem cell function. Combined administration of the 5-LO inhibitor Zileuton with imatinib prolonged survival of CML-affected mice (Table 1).[45] Since loss of *Alox5* does not affect normal HSC self-renewal, combined therapy of TKIs and 5-LO inhibitors may bring therapeutic benefits to CML patients in the chronic phase. Interestingly, *Alox5* up-regulation was not inhibited by TKI treatment, which may explain why imatinib does not inhibit CML stem cells. Further investigation regarding BCR–ABL functions not affected by TKI treatment are required in order to understand mechanisms of TKI-resistance in CML stem cells.

Although interferon (IFN)-α was a first line treatment for CML, imatinib has replaced IFN-α therapy due to the higher response rate. However, it was reported that patients previously exposed to IFN-α showed a high rate of long-term complete remission.[48] Interestingly, it has been recently demonstrated that type I IFNs act directly on HSCs to induce cell cycle progression,[49,50] suggesting the possibility that IFN-α pretreatment sensitized the CML stem cells to TKI therapy. Investigation of the IFN signaling on CML stem cells may influence the future treatment of CML.

Future Perspectives

In this review, we have focused on recent advances in our understanding of CML stem cell maintenance. Notably, factors regulating normal HSCs also govern TKI-resistance in CML stem cells, indicating that identifying molecular mechanisms governing normal HSC fate could shed light on activities of CML stem cells. For instance, inhibiting the association between CML stem cells and their microenvironment, or niche, could constitute one therapeutic strategy whereby CML patients could be treated by specific suppression of TKI-resistant CML stem cells (Fig. 3). Since normal HSCs are supported within a niche, TKI-resistant CML stem cells may also be protected and maintained in a dormant, undifferentiated state by interaction with their niche. Thus, inhibitors of niche signals could promote TKI-sensitive phenotypes in CML stem cells by activating cell cycling or inducing differentiation, leading to eradication of CML cells. Continued investigations of differences between normal HSCs and CML stem cells are also critical for development of CML therapy that does not promote severe side effects due to damage to normal HSCs. Further progress in stem cell biology offers promising directions for development of successful CML therapies.

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