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Critical Molecular Pathways in Cancer Stem Cells of Chronic Myeloid Leukemia: A Dissertation

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CRITICAL MOLECULAR PATHWAYS IN CANCER STEM CELLS OF CHRONIC MYELOID LEUKEMIA

A Dissertation Presented

By

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Abstract

Chronic myeloid leukemia (CML) is a disease characterized by the expansion of granulocytic cells. The BCR-ABL tyrosine kinase inhibitor imatinib, the frontline treatment for Ph+ leukemias, can induce complete hematologic and cytogenetic response in most chronic phase CML patients. Despite the remarkable initial clinic effects, it is now recognized that imatinib will unlikely cure patients because a small cell population containing leukemic stem cells (LSCs) with self-renewal capacity is insensitive to tyrosine kinase inhibitors.

In Chapter I, I briefly review the BCR-ABL kinase and its related signaling pathways. BCR-ABL kinase activates several signaling pathways including MAPK, STAT, and JNK/SAPK. BCR-ABL also mediates kinase-independent pathways through SRC family kinases. I will also discuss pathways involving β-catenin, hedgehog, FoxO and Alox5 are critical to the regulation of self-renewal and differentiation in LSC of CML.

As detailed in Chapter II, I describe our work evaluating the effects of omacetaxine, a novel CML drug inducing cell apoptosis by inhibition of protein synthesis, on self-renewal and differentiation of LSCs and BCR-ABL-induced CML and acute lymphoblastic leukemia (B-ALL) in mice. We found that treatment with omacetaxine decreased the number of LSCs and prolonged the survival of mice with CML or B-ALL.

In chapter III, I describe that *Alox5* is an essential gene in the function of LSCs and CML development. We show evidence that *Alox5* affects differentiation, cell division,

and survival of long-term LSCs. Treatment of CML mice with a 5-LO inhibitor also impaired the function of LSCs similarly and prolonged survival.

In chapter IV, I present evidence of our work showing a further dissection the *Alox5* pathway by comparing the gene expression profiles of wild type and *Alox5*$^{-/-}$ LSCs. We show that *Msr1* deletion causes acceleration of CML development. We also show that *Msr1* affects CML development by regulating the PI3K-AKT pathway and *β-catenin*.

Taken together, these results demonstrate that some pathways including *Alox5* and *Msr1* play an important role in regulating the self-renewal and differentiation of LSC. More efforts should be put into developing the novel strategies that may effectively target LSCs and thus cure CML.

Table of Contents

List of Tables

Chapter I

List of Figures

Chapter I

Chapter II

Chapter III

List of Abbreviations

5-FU: 5-fluorouracil

CML: chronic myeloid leukemia

AML: acute myeloid leukemia

ALL: acute lymphoblastic leukemia

B-ALL: B cell-acute lymphoid leukemia

Ph^+: Philadelphia-positive

BM: bone marrow

BMT: Bone marrow transplantation

CP: chronic phase

AP: accelerate phase

BC: blast crisis

FBS: Fetal bovine serum

FACS: fluorescence-activated cell sorter

HSCs: Hematopoietic stem cells

GMPs: granulocyte macrophage progenitors

CSCs: Cancer stem cells

LSCs: Leukemia stem cells

LT-HSC: Long term-hematopoietic stem cells

ST-HSC: Short term-hematopoietic stem cells

MPP: multiple progenitor cells

MPD: Myeloproliferative disorders

IL3: interleukin 3

IL6: interleukin 6

SCF: stem cell factor

WBC: white blood cell

APC: adenomatous polyposis coli

CKI: choline kinase

GSK3β: glycogen synthase kinase 3β

Hh: Hedgehog

Shh: Sonic hedgehog

Ihh: Indian hedgehog

Dhh: Desert hedgehog

5-LO: 5-lipoxygenase

SHP2: tyrosine phosphatase

PI3K : phosphatidylinositol 3-kinase

Pten: phosphatase and tensin homolog

MAPK: mitogen-activated protein kinase

JNK: c-jun N-terminal kinase

SAPK: stress-activated protein kinase

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

STAT: signal transducers and activators of transcription

PIP3: dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate

FOXO: Forkhead O

G-CSF: granulocyte-colony stimulating factor

PKCβ: protein kinase Cβ

PP2A: Protein phosphatase 2A

LTC-IC: long-term culture-initiating

IFN-α: interferon-α

IFN-γ: interferon-γ

TKIs: tyrosine kinase inhibitors

HE: hematoxylin and eosin

RIPA: radioimmunoprecipitation

Msr1: macrophage scavenger receptor

PMA: Phorbol 12-myristate 13-acetate

DMSO: dimethyl sulfoxide

GSLCs: glioma stem-like cellsl

LTA4: leukotriene A4

LTB4: leukotriene B4

Blt1: B leukotriene receptor 1

GRB2: growth factor receptor-binding protein 2

Preface

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CHAPTER I

Critical molecular pathways in cancer stem cells of chronic myeloid leukemia

The work described in this chapter has been published

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Chen Y, Peng C, Li D, Li S. Molecular and cellular bases of chronic myeloid leukemia. **Protein & Cell**. 2010, 1 (2): 124-132.)

1.1 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 introduction, 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.

1.2 BCR-ABL tyrosine kinase and domain functions

The Ph chromosome is a type of chromosomal rearrangement and produces a new gene:

BCR-ABL. BCR is a signaling protein that contains multiple modular domains. BCR-deficient mice develop normally, and one obvious phenotype is that neutrophils produce excessive levels of oxygen metabolites following their activation [1]. c-ABL, a non-receptor tyrosine kinase, is expressed in most tissues. In a cell, it is distributed in both the nucleus and cytoplasm of cells, and shuttles between the two compartments. It transduces signals from cell-surface receptors for growth factors and adhesion receptors to regulate cytoskeleton structure [2,3]. The fusion of BCR to ABL during the translocation increases the tyrosine-kinase activity of ABL, and brings new regulatory domains/motifs to ABL, such as the growth factor receptor-binding protein 2 (GRB2) and SH2-binding sites [4]. Depending on the precise breakpoints in the translocation and RNA splicing, different forms of BCR-ABL protein with different molecular weights can be generated in patients, such as P190, P210, and P230 [5-7].The importance of some domains/motifs of *BCR-ABL* has been validated *in vivo*. Mice expressing a form of *BCR-ABL* with a point mutation in the ATP-binding site of ABL, which inactivates its kinase activity, do not develop leukemia, even when the fusion protein is expressed in the long-term repopulating hematopoietic stem/progenitor cells, indicating that the ABL kinase activity is absolutely essential for BCR-ABL leukemogenesis *in vivo* [8]. This result is consistent with the finding that the kinase activity of ABL is required for *BCR-ABL*-mediated transformation in cultured cells [9]. It also demonstrated that the ABL kinase is an excellent target for treating CML. In addition to the ABL kinase domain, there are other important domains/motifs in *BCR-ABL*, such as those that regulate the kinase activity of ABL or connect to other downstream signaling pathways (Figure 1). The relative importance of

various domains of *BCR-ABL* in neoplastic transformation has been examined. 1). Deletion of the SH3 domain of ABL results in a mutant form of the protein with increased tyrosine kinase activity, and expression of this truncated protein can transform both fibroblast and haematopoietic cell lines *in vitro*. 2). The amino-terminal coiled-coil (CC) oligomerization domain of BCR is an important activator of ABL kinase activity, and also promotes the association of BCR–ABL with ACTIN fibers [10]. A mutant form of *BCR-ABL* that lacks the BCR-CC domain (ΔCC-BCR-ABL) failed to induce myeloproliferative disorders (MPD) in mice. Reactivation of the kinase activity of ABL by mutating its SH3 domain (through deletion or a P1013L point mutation), rescued the ability of ΔCC-*BCR-ABL* to induce a CML-like MPD in mice [11]. These results demonstrate that the BCR domain is essential for the induction of CML by BCR-ABL in mice, mainly owing to its ability to activate the kinase activity of ABL. 3). GRB2-binding site. GRB2 binds SOS as well as the scaffolding adapter GRB2-associated binding protein 2 (GAB2). Formation of this complex depends on BCR phosphorylation at tyrosine 177 [12], leading to activation of downstream RAS and recruitment of protein tyrosine phosphatase (SHP2) and phosphatidylinositol 3-kinase (PI3K) [12,13]. Mutation of the tyrosine-177 residue of *BCR-ABL* to phenylalanine (Y177F) largely abolished its ability to bind GRB2 without affecting the kinase activity of ABL [12,14]. 4). The SH2 domain of ABL is believed to activate RAS, at least partially, through binding to SHC, which, following tyrosine phosphorylation, can recruit GRB2 [15]. Mutations in the SH2 domain of ABL reduced the ability of *BCR-ABL* to induce a CML-like MPD in mice [16]. The Y1294F point mutation in SH2 domain of *BCR-ABL* also attenuated leukemogenesis

by *BCR-ABL* [11]. Together these structure-function analyses of *BCR-ABL* have shown that the activation of the tyrosine-kinase activity of ABL is necessary but not sufficient to induce CML in mice.

1.3 BCR-ABL kinase-dependent or independent signaling pathways

The potent, constitutively active non-receptor tyrosine-kinase activities of the BCR-ABL oncoproteins are responsible for triggering assorted signaling pathways that promote the growth and survival of hematopoietic cells and the induction of cell transformation. These pathways include those mediated by Ras, mitogen-activated protein kinase (MAPK), c-jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), signal transducers and activators of transcription (STAT), phosphoinositide 3- (PI-3) kinase, and c-Myc, among others(Figure 1). Not all BCR-ABL-expressing cells are killed using kinase-inhibiting and CML stem cells are insensitive to kinase inhibition. Targeted approaches to the kinase-independent signaling pathways of BCR-ABL are also important. Several have been identified to play important roles in CML development and specifically LSC survival, including the Wnt/β-catenin, Hedgehog, Alox5, SRC family kinase and FoxO pathways (Figure 2)

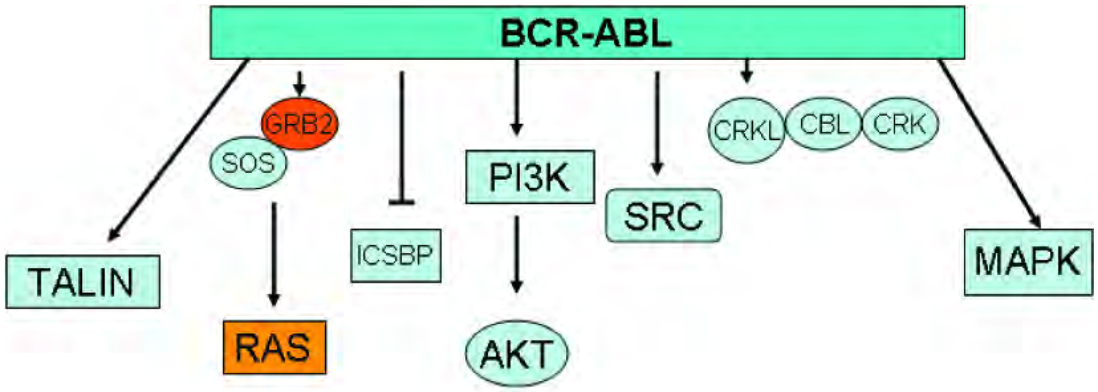


Figure 1. BCR-ABL kinase acitivated signaling pathways.

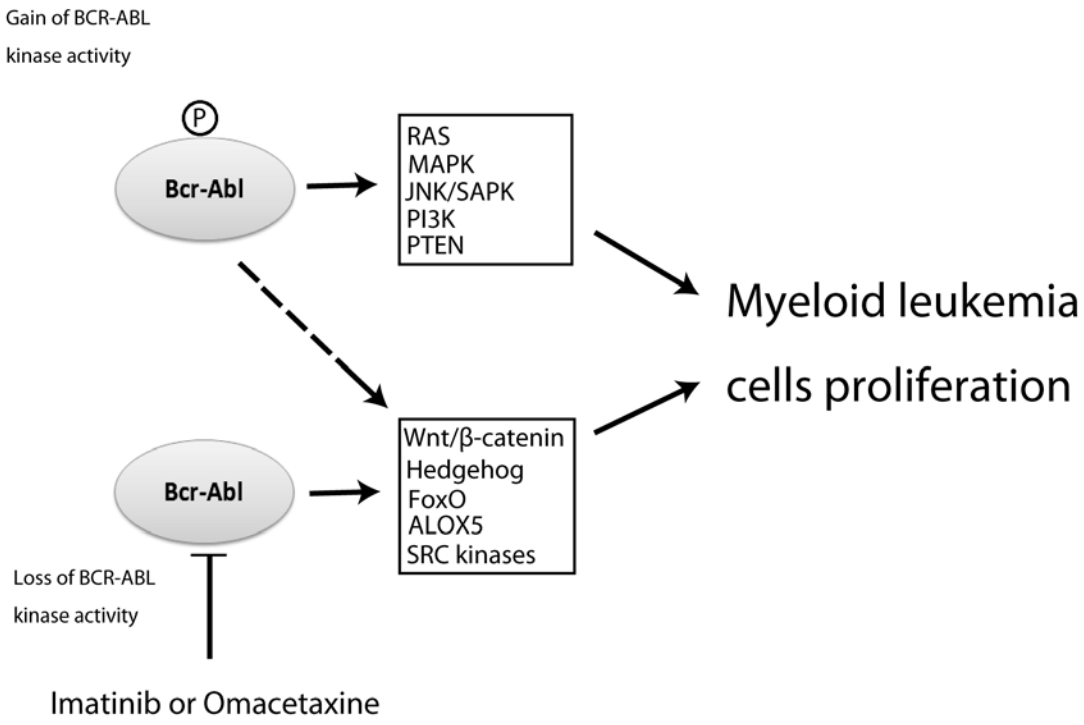


Figure 2. BCR-ABL kinase-dependent or independent signaling pathways.

1.4 Chronic myeloid leukemia

Philadelphia-positive (Ph^+) chronic myeloid leukemia 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 [17]. 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) [18]. 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 [17], suggesting that LSCs are not eliminated. A real cure may require complete eradication of these stem cells. Although BMT is considered 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) [19]. 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 [20]. Although it effectively prolongs the survival of CML patients, imatinib does not show any definite prospect of "cure" [18], as these

imatinib-treated patients in complete cytogenetic remission still contain malignant hematopoietic progenitor cells[21]. The patients are likely required to take the drug for the rest of their lives[22] . 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-γ(IFN-γ) [22], raising a question as to whether IFN-γ played a critical role in the induction of molecular remission by imatinib. Besides these facts, resistance to imatinib develops [20], and one of the major reasons for the resistance is the development of point mutations in the BCR-ABL kinase domain [23,24]. In addition, imatinib does not completely eradicate LSCs [21,25], which does not appear to be related to the BCR-ABL kinase domain mutations [26,27]. 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 introduction, 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[28-30], providing valuable information for identifying critical molecular pathways in LSCs. Recent studies in CML LSCs have been reviewed thoroughly by Goldman and colleagues [31]. Therefore, I 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.

1.5 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 (Figure 3). Of these, the most frequently used CML mouse model is the retroviral bone marrow transduction/transplantation model, which was initially described in 1990s [32-34]. The efficiency of CML induction was later improved to 100%[8,35,36], 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). Then, donor bone marrow cells were transduced with BCR-ABL retrovirus and transplanted into lethally irradiated syngeneic recipient mice [36]. 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[26]. 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[26,27,37].

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 [38]. 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) [39]. Later, BCR-ABL inducible transgenic mice using a tet-off system were developed, with these mice developing rapid ALL[40]. 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[41].

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(SCF), interleukin 3(IL-3), interleukin 6(IL-6) and granulocyte-colony stimulating factor [42,43]. These CD34+ leukemic cells had an *in vitro* progenitor activity and were capable of engrafting immunodeficient NOD/SCID mice [43].

This system had been used to test the inhibitory effects of potential drugs on human CML cells [44].

Figure 3. 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.

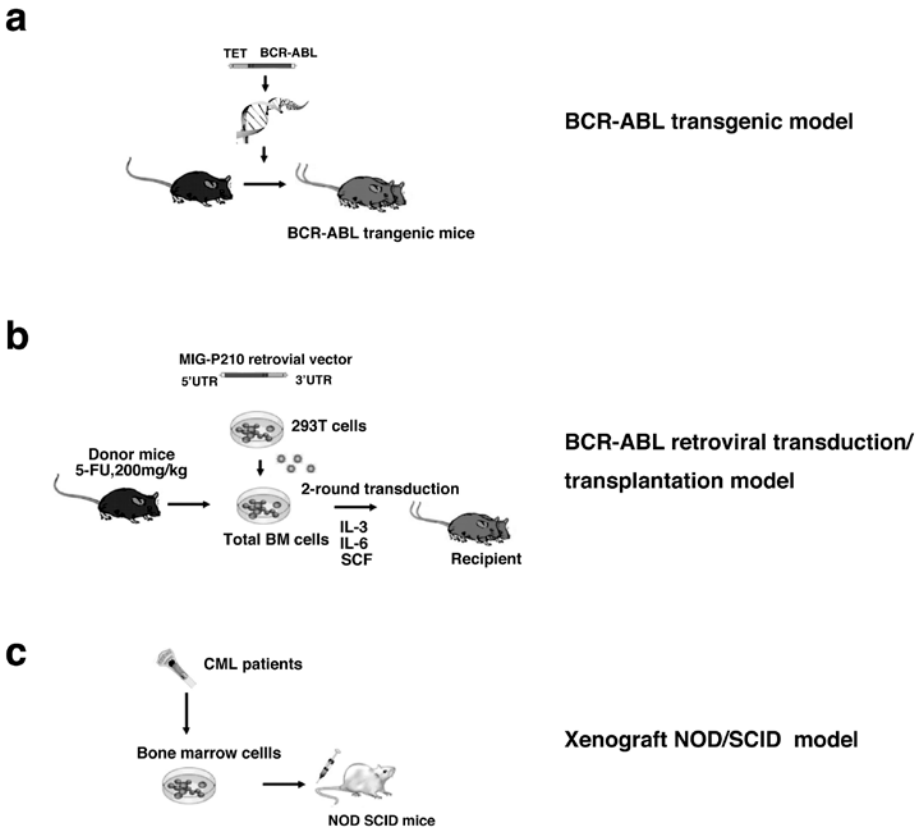


Figure 3. Mouse model for studying BCR-ABL leukemia.

1.6 Cancer stem cells

Cancer stem cells (CSCs) constitute a subpopulation of malignant cells capable of self-renewal and differentiation[45-51]. 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[52,53]. 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 [52,53]. 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 [54].

1.7 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 [55]. Furthermore, Dick, Eaves and colleague reported that enriched $CD34^+$ cells from patients with CML could be transplanted into NOD/SCID mice [42], 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 [56]. 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.

1.7.1 *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 [26], 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 [26].

1.7.2 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 [57], 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 [58]. 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[57] [58]. These results demonstrate that GMPs function as LSCs in CML blast crisis, and these stem cells are similar to LSCs in AML [51].

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.

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 GMP
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

1.7.3 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 [19]. 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 [25]. The fact that imatinib could not target the quiescent BCR-ABL-expressing LSCs made it apparent that imatinib treatment alone could not cure CML [21,25]. 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 [21,25]. The minimal effect of BCR-ABL kinase inhibitor on LSCs was also observed in the CML mouse model [26].

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 [26]. 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 [59]. 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 [26]. 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 [26].

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 [27]. 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[27], 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 [26]. 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 [26]. 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* [60]. These results suggest that inhibition of BCR-ABL kinase activity alone is insufficient to eradicate LSCs [27].

1.8 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. 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[26], 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 (Figure 4).

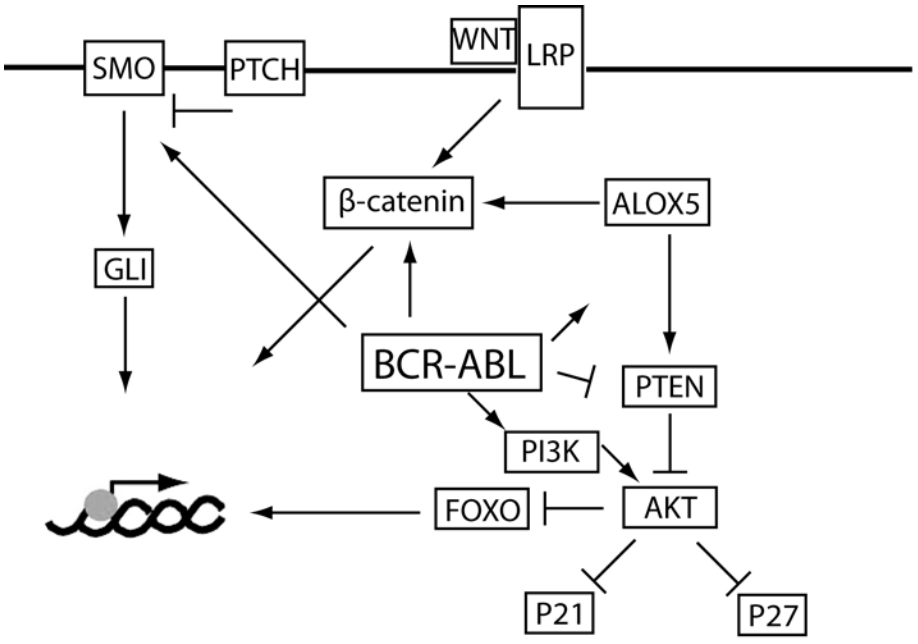


Figure 4. Critical molecular pathways in LSCs

1.8.1 *Wnt/β-catenin pathway*

Wnt/β-catenin signalling plays an important role in HSC development [61]. *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*[62]. The phosphorylated β-catenin recruits an E3 ubiquitin ligase, which targets *β-catenin* for proteasomal degradation [63]. In blast crisis CML patient, *β-catenin* is activated in myeloid progenitors and the activated *β-catenin* translocates to the nucleus [57]. In a CML mouse model, loss of *β-catenin* delays the development of CML [27,64]. 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* [64]. 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 [65].

1.8.2 *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 [66]. Three homologs of Hh genes (Sonic hedgehog (Shh), Indian

hedgehog (Ihh) and Desert hedgehog (Dhh)) have been identified in mammalian system [67-69]. 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* [70-72].

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[73]. 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 [74]. 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 [75,76]. 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 [77-79]. 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 [80]. 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 [80,81]. 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 [80,81]. 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* [81].

1.8.3 *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 [82-89]. 5-LO is responsible for producing leukotrienes, a group of inflammatory substances that cause human asthma [90]. Altered arachidonate metabolism by leukocytes and platelets was reported in association with MPD almost 30 years ago [91]. Several selective 5-LO inhibitors were found to reduce proliferation of and induce apoptosis of CML cells *in vitro* [92,93], 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 [28,29]. 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 [37], providing a possible explanation why LSCs are not

sensitive to inhibition by BCR-ABL kinase inhibitors [26]. Furthermore, recipients of BCR-ABL transduced bone marrow cells from *Alox5*$^{-/-}$ donor mice were resistant to the induction of CML by BCR-ABL [37], 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 [37]. 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).

1.8.4 *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 [30]. *Pten* is often deleted or inactivated in many tumor types, including glioblastoma, endometrial carcinoma, and lymphoid malignancies [94-96]. PTEN is a

phosphatase that dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate(PIP3) in PI3K signaling pathway [97]. PI3K is a key signaling pathway for cell growth, anti-apoptosis, cell division and glucose uptake[98-100]. PI3K is a family of proteins that catalyze transfer of the phosphate of ATP to the D3 position of phosphoinositides. Activated PI3K translocates to the plasma membrane and associates with a receptor tyrosine kinase or its substrates. The activated PI3K produces phosphoinositide-3-phosphate and/or phosphoinositide-3,4,5-triphosphate and those PI3K's products activates downstream signaling pathways, including PDK, AKT and PKC[101-103]. Phosphatases like PTEN can degrade those phosphoinositide-3-phosphate products and further inhibit the cell growth. 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 [104]. A study shows that PTEN is oxidized and inactivated in pancreatic cell lines, and that treatment with a 5-LO inhibitor prevented PTEN degradation [105], 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) [106]. 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 $GFP^{+}Gr\text{-}1^{+}$ myeloid leukemia cells and an increased number of leukemia cells in peripheral blood of the mice [106]. 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 [106], 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*.

1.8.5 *FoxO pathway*

The FOXO (Forkhead O) subfamily of transcription factors plays critical roles in cell cycle arrest, stress resistance and apoptosis [107]. There are four members (FOXO1, FOXO3, FOXO4 and FOXO6), and they are functionally downstream of the PI3K-AKT pathway[108,109]. AKT directly phosphorylates FOXOs members, resulting in the exclusion of FOXOs from the nucleus and degradation in cytoplasma [110]. FOXOs localize to nucleus and regulate apoptosis, cell cycle progression and oxidative stress responses [110-113]. 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[108,109], 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 [108]. 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 [114]. In addition, serial CML transplantation showed that the FoxO3a deficiency severely impairs the ability of LSCs to induce CML [114]. 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 [114].

1.8.6 *Msr1 pathway*

MSR1 is a member of a family termed scavenger receptors and is mostly expressed in macrophages and dendritic cells and bind to a broad range of ligands including Ac-LDL, bacterial surface components and apoptotic cells[115]. MSR1 could mediate receptor internalization and cell adhesion and play important roles in host cell interactions, macrophage adhesion and phagocytosis of apoptotic cells[115]. The binding MSR1 will activate a series of signal pathways, including Src kinase pathway and PI3K kinase-Akt pathway, to promote the cell growth, metabolism, survival and glucose homeostasis[116]. The Src kinase Lyn is associated with the cytoplamic domain of MSR1, and MSR1 will rapidly activate Lyn[117]. Another important signal pathway is PI3K-AKT pathway. PI3K-AKT-GSK3β pathway was activated rapidly during the MSR1 mediated cell adhesion[116].

Recently, MSR1 was reported to be associated with prostate cancer in men of both African American and European genetically although the mechanism of MSR1 regulating cancer development is still unclear[118]. In CML development, *Msr1* is down-regulated by *BCR-ABL* and this down-regulation is partially restored by *Alox5* deletion, and that *Msr1* deletion causes acceleration of CML development. Moreover, *Msr1* deletion markedly increases LSC function through its effects on cell cycle progression and apoptosis. *Msr1* affects CML development through the PI3K-AKT pathway and β-Catenin. The enhancement of *Msr1* function may be of significance in the development of novel therapeutic strategies targeting CML.

1.9 Therapeutic strategies for eradicating LSCs in CML

1.9.1 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 [119], treatment with G-CSF, in combination with imatinib, provides a novel method for treating CML, although current efforts did not result in expected outcome [120]. Another attractive approach is to inhibit autophagy of LSCs using inhibitory compounds such as chloroquine [121], because inhibition of autophagy may promote cell death induced by imatinib [121]. 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 [122].

1.9.2 Targeting critical signaling pathways in LSCs

1.9.2.1 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 [123]. 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 [37]. 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 leukemia 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/ multiple progenitor cells (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.

1.9.2.2 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 [124]. 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 [125]. 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 [125]. 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 2 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.

1.9.2.3 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 [126]. Recently, omacetaxine has been shown to have the capability to kill LSCs effectively *in vitro* and in CML mice [127]. 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 [128]. 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[127]. The clinical efficacy of

omacetaxine on CML could be related to the inhibitory activity of omacetaxine on LSCs [127].

1.9.2.4 Cyclopamine. Cyclopamine belongs to the jervine family of alkaloids derived from the plants of genus *Veratrum*, and is a specific Hedgehog inhibitor [129]. Cyclopamine inhibits the Hh pathway by binding directly to Smo and affecting its protein conformation [130]. 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 [130]. 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 [81].

1.9.2.5 BMS-214662. BMS-214662 is a cytotoxic farnesyltransferase inhibitor capable of targeting and killing non-proliferating tumor cells [131]. 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 [44]. 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. Co-treatment 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 [44,132].

1.9.2.6 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 [133]. FTY720 is a water-soluble, nontoxic PP2A activator with a high oral bioavailability, and has been used as an immunomodulator in Phase III trials for patients with multiple sclerosis [134,135]. 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 [136]. In addition, FTY720 treatment triggers apoptosis of LSCs, showing a great potential for targeting LSCs through activating PP2A.

1.9.2.7 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 [137,138]. Bortezomib is a reversible and specific inhibitor of CT-L activity, and has been used for treating mantle cell lymphoma and multiple myeloma [139]. Proteasomal activity was shown to be increased in CML cells [138], and loss of proteasomal activity showed an inhibitory effect on growth of BCR-ABL$^+$ cell lines sensitive or resistant to imatinib [140]. The effect of bortezomib was also tested in CD34$^+$ cells from CML patients. *In vitro*, bortezomib selectively inhibited colony formation by CD34$^+$ BCR-ABL$^+$ progenitor cells [141]. Bortezomib was also shown to induce apoptosis and inhibit proliferation of CD34+38-, long-term culture-initiating (LTC-IC) cells [142]. Bortezomib also impaired the function of CML LSCs by reducing the engraftment of patient-derived CD34+ CML cells[142]. Importantly, different BCR-ABL mutants, including T315I, H396P and M351T, were sensitive to bortezomib [38], although it also inhibited proliferation and induced apoptosis of normal CD34+38- cells [142].

1.9.2.8 Interferon-α (IFN-α). Interferon-α, an immunomodulatory cytokine, has been used to treat CML [143]. 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 [22]. 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.

CHAPTER II

Inhibitory effects of omacetaxine on leukemic stem cells and BCR-ABL-induced chronic myeloid leukemia and acute lymphoblastic leukemia in mice.

The work described in this chapter has been published (**Chen Y**, Hu Y, Michaels S, Segal D, Brown D, Li S. Inhibitory effects of omacetaxine on leukemic stem cells and BCR-ABL-induced chronic myeloid leukemia and acute lymphoblastic leukemia in mice. **Leukemia**. 2009, 23(8):1446-54)

2.1 Abstract

Omacetaxine mepesuccinate (formerly homoharringtonine) is a molecule with a mechanism of action that is different from tyrosine kinase inhibitors, and its activity in chronic myeloid leukemia (CML) seems to be independent of the BCR-ABL mutation status. Using BCR-ABL-expressing myelogenous and lymphoid cell lines and mouse models of CML and B-cell acute lymphoblastic leukemia (B-ALL) induced by wild-type BCR-ABL or T315I mutant-BCR-ABL, we evaluated the inhibitory effects of omacetaxine on CML and B-ALL. We showed that more than 90% of the leukemic stem cells were killed after treatment with omacetaxine *in vitro*. In contrast, less than 9 or 25% of the leukemic stem cells were killed after treating with imatinib or dasatinib, respectively. After 4 days of treatment of CML mice with omacetaxine, Gr-1^+myeloid leukemia cells decreased in the peripheral blood of the treated CML mice. In the omacetaxine-treated B-ALL mice, only 0.8% of the $B220^+$leukemia cells were found in

peripheral blood, compared with 34% of the $B220^+$leukemia cells in the placebo group. Treatment with omacetaxine decreased the number of leukemia stem cells and prolonged the survival of mice with BCR-ABL-induced CML or B-ALL.

2.2 Introduction

The Abl tyrosine kinase inhibitors (TKIs) imatinib mesylate and dasatinib, have revolutionized the treatment of Philadelphia-positive (Ph^+) leukemia in both chronic myeloid leukemia (CML) and B-cell acute lymphoblastic leukemia (B-ALL) by targeting and disabling the proliferative signal coming from BCR-ABL. These TKIs elicit high rates of durable complete cytogenetic responses, particularly in CML, however, only a small proportion of treated individuals remain disease free if therapy is discontinued[22,144]. This lack of curative effect of TKIs in Ph^+ leukemias is largely due to insensitivity of primitive leukemic stem cells to these TKIs and the selection of cells expressing TKI resistant BCR-ABL mutants[145]. At present, an anti-stem cell strategy has not been developed for treating these leukemia patients; however a number of agents such as farnesyl transferase inhibitors have shown activity toward leukemic stem cell that is superior to TKIs[44,146]. These findings suggest that non-TKI based therapies may prove useful in targeting leukemic stem cells in Ph^+ leukemias and raise the prospect of improved rates of durable complete molecular remission.

Omacetaxine is a cephalotaxine ester derived from the evergreen tree, *Cephalotaxus harringtonia*, native to China. The chemical structure of omacetaxine is shown in Supplementary Figure 1. Omacetaxine has shown clinical activity alone and

in combination with imatinib in CML patients resistant to imatinib or other TKIs[126,147,148]. However, little is known about whether omacetaxine has an inhibitory effect on leukemic stem cells. In this study, we utilized mouse model of BCR-ABL induced leukemia to investigate the efficacy of omacetaxine toward leukemic stem cells. We show that omacetaxine inhibited the proliferation of CML and B-ALL stem cells and provided a significant survival benefit to mice with CML and B-ALL.

2.3 Results

2.3.1 Omacetaxine suppresses myeloid leukemic cells and improves survival of mice with BCR-ABL induced CML

To investigate the therapeutic effect of omacetaxine (Supplementary Figure 1) on CML, we used a bone marrow transplantation (BMT) mouse model of CML in which bone marrow cells from BALB/c donor mice pretreated with 5-fluorouracil (5-FU), were transduced with BCR-ABL and injected into BALB/c recipient mice to induce CML[36]. Mice transplanted with BCR-ABL-transduced bone marrow were treated with a placebo or omacetaxine. Omacetaxine treatment of CML mice decreased BCR-ABL–expressing (GFP^+) leukemia cells during therapy (Figure 1a and 1b, $P < 0.001$). In addition, splenomegaly in omacetaxine-treated mice (a consistent physical sign in CML) decreased by 88%, compared to placebo (Figure 1b). All placebo-treated mice developed CML and died within 3 weeks after BMT. In contrast, all omacetaxine treated CML mice survived (Figure 1c). Lung hemorrhage caused by infiltration of mature myeloid leukemia cells is a major cause of death of CML mice[36]. We further

evaluated the therapeutic effect of omacetaxine on CML by examining the severity of lung hemorrhages at day 14 after BMT. Compared with placebo-treated mice, much less severe hemorrhages were observed in the lungs of omacetaxine–treated CML mice (Figure 1d). We compared the effect of omacetaxine on BCR-ABL-expressing and non-BCR-ABL-expressing 32D cells, and found that omacetaxine inhibited BCR-ABL-expressing cells more strongly than non-BCR-ABL-expressing cells (Figure 1e).

Figure 1: Omacetaxine reduces circulating GFP^+ cells, reduces spleen weight and improves survival in mice with BCR-ABL-WT-induced CML.

a. Flow cytometric evaluation of the leukemic process in omacetaxine and placebo treated CML mice. Percentage of $Gr\text{-}1^+GFP^+$ cells in peripheral blood of placebo or omacetaxine-treated CML mice were analyzed at day 14 after BMT. **b.** The number of circulating leukemic cells (calculated as percentage of $Gr\text{-}1^+GFP^+$cells×white blood cell count) and spleen weight in mice with BCR-ABL induced CML treated with placebo or omacetaxine for 4 days was determined on day 14 after 0.5 x 10^6 cells transplantation. **c.**Treatment with omacetaxine prolonged the survival of CML mice. Mice with BCR-ABL-induced CML were treated with placebo (n=15) or omacetaxine (0.5 mg/kg for 4 days) (n=15). **d.** Treatment with omacetaxine reduced lung hemorrhage and splenomegaly of mice with BCR-ABL induced CML after treatment with omacetaxine for 4 days. **e.** The inhibitory effect of Omacetaxine on 32D and 32D P210 cells. 32D and BCR-ABL-transduced 32D cells were cultured at 5×10^5 cells per well in 24-well plates, and omacetaxine (20nM) was added to the culture for 48h or 72h. The number of viable cells at the indicated drug concentrations was determined by trypan blue.

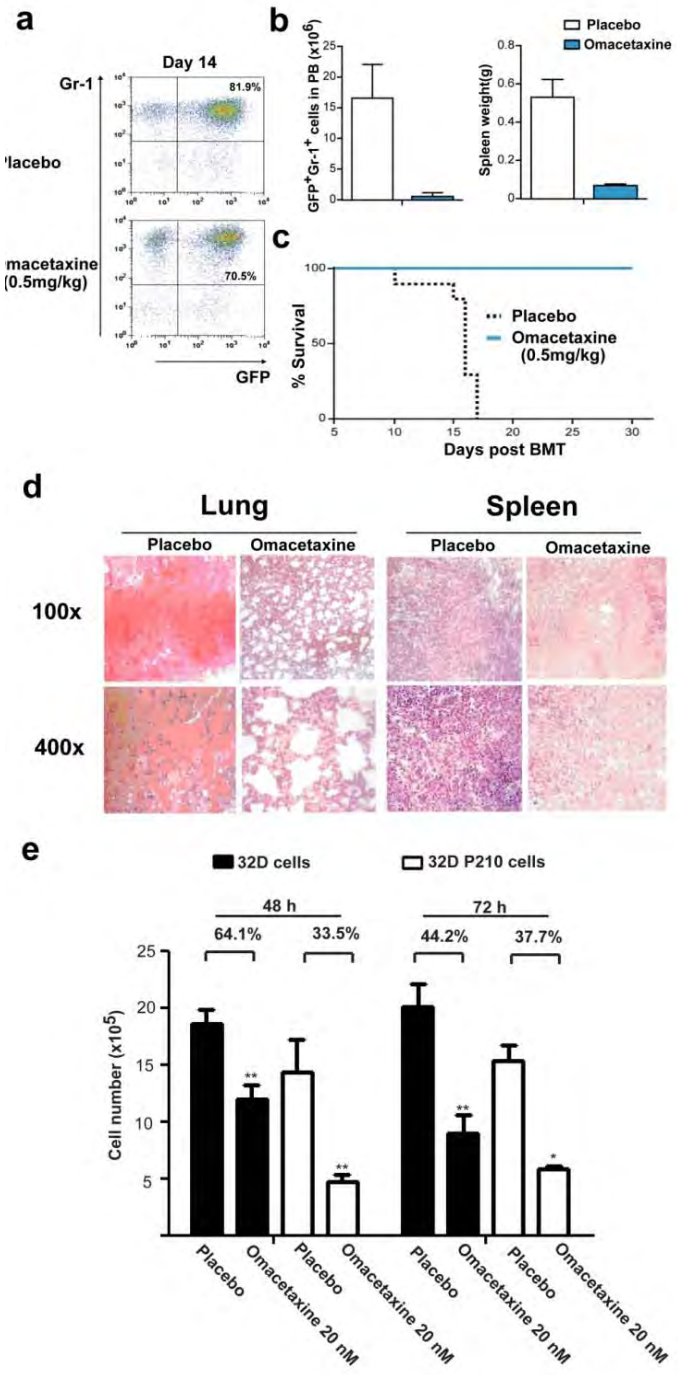


Figure 1: Omacetaxine reduces circulating GFP$^+$ cells, reduces spleen weight and improves survival in mice with BCR-ABL-WT-induced CML.

2.3.2 Omacetaxine suppresses chronic myeloid leukemia stem cells *in vitro* and *in vivo.*

Although imatinib, the BCR-ABL inhibitor, has been shown to prolong survival of mice with BCR-ABL–induced CML, it does not completely stop the progression of CML in our BMT mouse model, due to accumulation of surviving leukemia stem cells[26,149]. The Lin^{-} $cKit^{+}$ $Sca\text{-}1^{+}$ population has been identified as CML stem cells, as these cells confer leukemia in recipient mice in the CML model [26]. To investigate whether omacetaxine has an inhibitory effect on leukemia stem cells, bone marrow cells from CML mice on day 13 after BMT were isolated and treated with omacetaxine at various doses in conditions that support survival and growth of hematopoietic stem cells(HSCs) (Figure 2a) [150,151]. After 6 days in culture, survival of $GFP^{+}Lin^{-}$ $cKit^{+}$ $Sca\text{-}1^{+}$ cells representing leukemia stem cells, and GFP^{+} cells indicating BCR-ABL-expressing cells, was determined. FACS analysis showed that compared with the placebo group, omacetaxine treatment inhibited survival of BCR-ABL expressing leukemia stem cells and total leukemia cells in a dose dependent manner (Figure 2a, $P < 0.001$).

The effect of omacetaxine on leukemia stem cells in CML mice was also examined. Mice with BCR-ABL-induced CML were treated with a placebo, omacetaxine, imatinib or both drugs in combination for 4 days from Day 10 after BMT, and the number of $GFP^{+}Lin^{-}$ $cKit^{+}Sca\text{-}1^{+}$ and total GFP^{+} bone marrow cells was determined by flow cytometry. Consistent with our previous findings[125], imatinib treatment did not lower the percentage

and number of leukemia stem cells and total leukemia cells in bone marrow, compared with the placebo group. Omacetaxine treatment greatly reduced the numbers of both leukemia stem cells and total leukemia cells, while treatment of CML mice with both drugs did not show further effects on reducing the number of leukemia stem cells or total leukemia cells, compared with the mice treated with omacetaxine alone (Figure 2b). The effect of omacetaxine on leukemia stem cells in CML mice was also compared. With the effect of omacetaxine on BCR-ABL-negative stem cells in the same animals. We found that a higher percentage of leukemia stem cells were inhibited by omacetaxine as compared with the percent reduction of BCR-ABL-negative stem cells (Figure 2c).

Figure 2: Omacetaxine inhibits survival of leukemic stem cells *in vitro* and *in vivo*.

a. Bone marrow cells isolated from C57BL/6 (B6) mice with BCR-ABL-induced CML on day 13 afternoon transplant were cultured in vitro (5×10^6 cells/6 cm tissue culture plate) under stem cell conditions ("Materials and Methods") in the presence or absence of omacetaxine (12.5 nM, 25 nM, 50 nM)) for 6 days (changing the stem cell medium containing placebo or omacetaxine at day 3) followed by FACS analysis of leukemia stem cells ($GFP^+Lin\text{-}cKit^+Sca\text{-}1^+$). **b.** Mice with BCR-ABL–induced CML were treated with placebo (n=5) or omacetaxine (0.5 mg/kg, 4 days) (n=5), respectively, for 4 days beginning at day 10 after transplantation. Bone marrow cells were isolated from the treated CML mice, and leukemia stem cells were analyzed by FACS. The numbers of cells represents the average number of leukemia stem cells from the femur and tibia of each treated CML mouse. **c.** Mice transplanted with *MSCV-GFP* induced bone marrow cells were treated with placebo (n=3) or omacetaxine (0.5 mg/kg) (n=3), respectively, for 4 days beginning at day 10 after transplantation. Bone marrow cells were isolated from the treated mice, and hematopoietic stem cells were analyzed by FACS. The numbers of cells represents the average number of hematopoietic stem cells from the femur and tibia of each mouse.

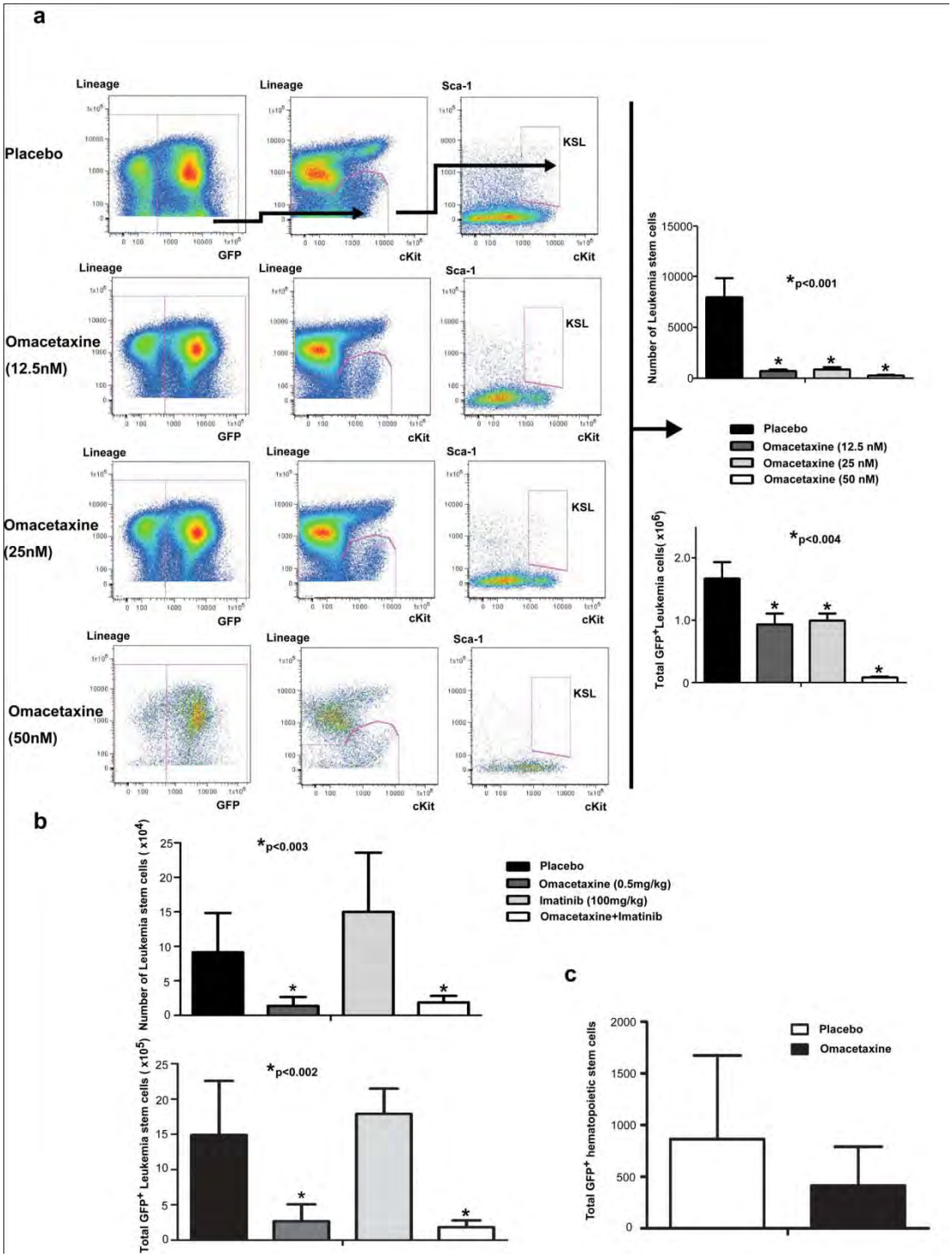


Figure 2: Omacetaxine inhibits survival of leukemic stem cells *in vitro* and *in vivo*.

2.3.3 Omacetaxine improves survival of mice with BCR-ABL-T315I induced CML and suppresses BCR-ABL-T315I leukemia stem cells.

Imatinib does not suppress leukemia cells expressing BCR-ABL-T315I and does not prolong the survival of mice with BCR-ABL-T315I induced CML[19,125]. To investigate whether omacetaxine also inhibits BCR-ABL-T315I induced CML and leukemia stem cells, we transduced bone marrow cells with BCR-ABL-T315I and implanted the transduced cells into BALB/c recipient mice to induce CML. Similar to wild type BCR-ABL induced CML, omacetaxine caused a decrease in peripheral blood BCR-ABL-T315I–expressing (GFP^+) leukemia cells (Figure 3a) and reduced splenomegaly (data not shown). BCR-ABL-T315I-expressing myeloid leukemic cells in peripheral blood of the treated CML mice were reduced greater than 49.5 fold compared to untreated CML mice (Figure 3a), whereas wild type BCR-ABL-expressing myeloid leukemic cells in peripheral blood of the treated CML mice were reduced only 30.2 fold (Figure 1b). These results suggest that omacetaxine was more efficient in inhibiting BCR-ABL-T315I compared to wild type BCR-ABL, which is supported by a previous report[128]. Omacetaxine treatment prolonged the survival of mice transplanted with BCR-ABL-T315I expressing bone marrow cells (Figure 3b). We next examined whether omacetaxine could inhibit the BCR-ABL-T315I induced leukemia stem cells *in vivo*. Mice with BCR-ABL T315I- induced CML were treated with a placebo or omacetaxine for 4 days from Day 10 after BMT. Bone marrow and spleen cells were analyzed by FACS for $GFP^+Lin^-cKit^+CD34^-$ cells. The number of BCR-ABL-T315I

expressing leukemia stem cells was significantly decreased by omacetaxine treatment (Figure 3c).

Figure 3: Omacetaxine improves survival of mice with BCR-ABL-T315I-induced CML.

a. The number of circulating leukemia cells (calculated as percentage of Gr-1^{+}GFP^{+}cells×white blood cell count) in mice with BCR-ABL-T315I–induced CML treated with placebo or omacetaxine was determined on day 14 after transplantation. **b.** Treatment with the omacetaxine prolonged survival of CML mice. Mice with BCR-ABL-T315I induced CML were treated with placebo (n = 15) or omacetaxine (0.5 mg/kg for 4 days) (n=15). **c.** Mice with BCR-ABL-T315I induced CML were treated with a placebo (n=5) or omacetaxine (0.5 mg/kg, 4 days) (n=5), respectively, for 4 days beginning at day 10 after transplantation. Bone marrow cells were isolated from the treated CML mice, and leukemia stem cells were analyzed by FACS. The numbers of cells represents the average number of leukemia stem cells from the femur and tibia of each treated CML mouse.

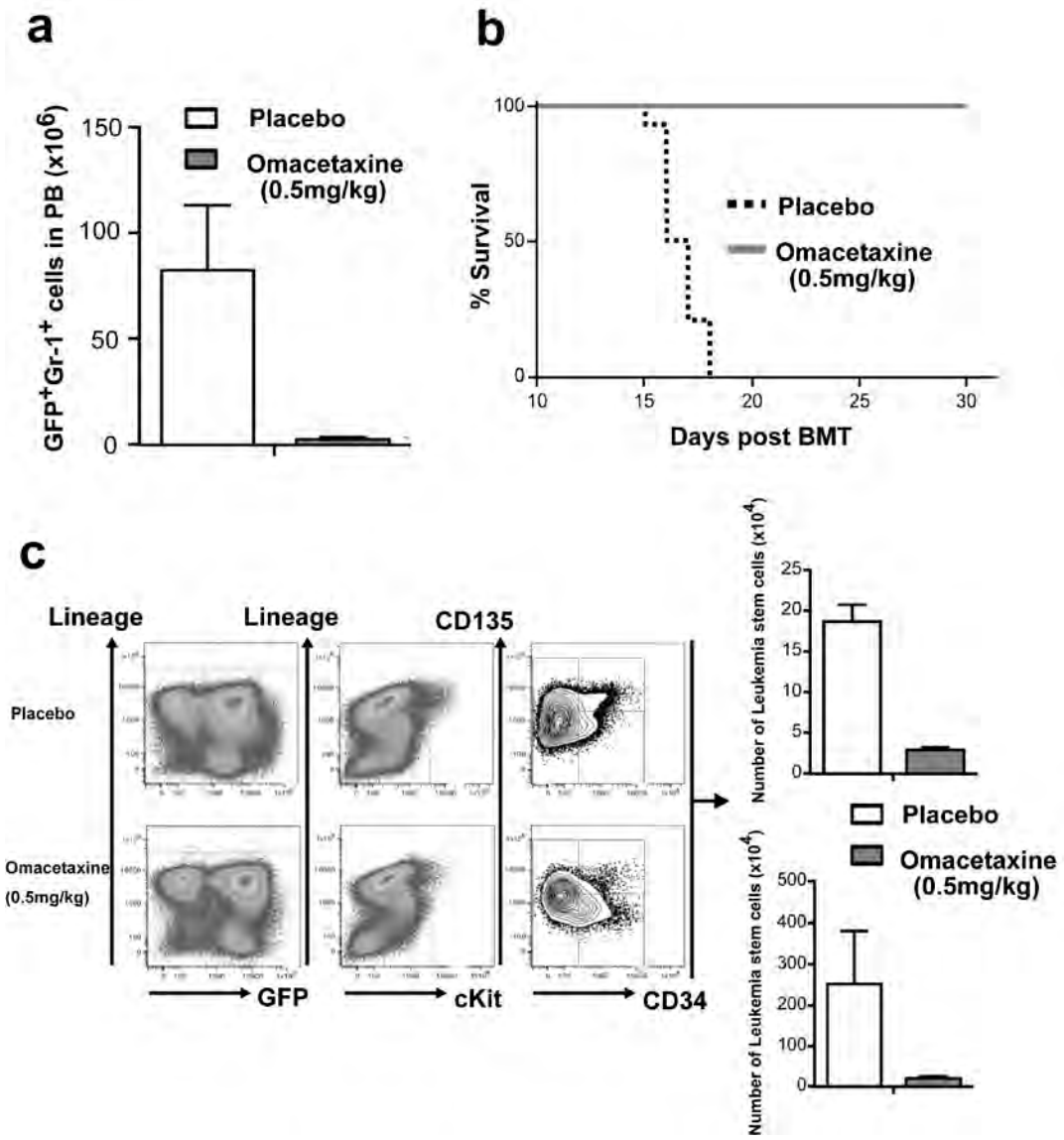


Figure 3: Omacetaxine improves survival of mice with BCR-ABL-T315I-induced CML.

2.3.4 Omacetaxine caused degradation of BCR-ABL protein through inhibiting Hsp90 and reduced Mcl-1 protein level in myeloid leukemic cells.

To understand the possible mechanisms by which omacetaxine inhibits myeloid leukemia cells, we examined the effect of omacetaxine on human myeloid leukemia cells (K562). In a dose-dependent manner, treatment with omacetaxine significantly suppressed K562 cell growth particularly at concentrations of 200 nM or more ($P<0.001$) (Figure 4a). We then compared protein levels in K562 cells after treatment with either 50 nM or 150 nM omacetaxine. After treatment, levels of BCR-ABL protein were decreased in K562 cells in a dose dependent manner (Figure 4b). As BCR-ABL protein is associated with Hsp90 protein[125], we next measured the Hsp90 protein levels and found that the Hsp90 protein levels were also decreased after omacetaxine treatment (Figure 4b). However, Hsp70 which plays a positive role in BCR-ABL–mediated resistance to apoptosis was not changed after omacetaxine treatment (Figure 4b). Down regulation of Mcl-1, a member of the Bcl-2 family, is associated with a substantial decrease in viability of K562 cells, and reduced survival of imatinib-resistant K562 cells[152]. The level of Mcl-1 protein was also greatly reduced following treatment with 150 nM omacetaxine treatment (Figure 4b).

Figure 4: Omacetaxine degrades BCR-ABL by inhibiting HSP90 and suppresses MCL-1 in myeloid leukemia cells.

a. Omacetaxine inhibited K562 cells in a dose dependent manner. The number of viable cells at the indicated drug concentrations was determined by trypan blue. **b.** Omacetaxine inhibited the expression of Hsp90, Abl and Mcl-1 in K562 cells. K562 cells were treated with omacetaxine (50 nM, 150 nM) for 48 hours. Protein lysates were analyzed by Western blotting using antibodies indicated.

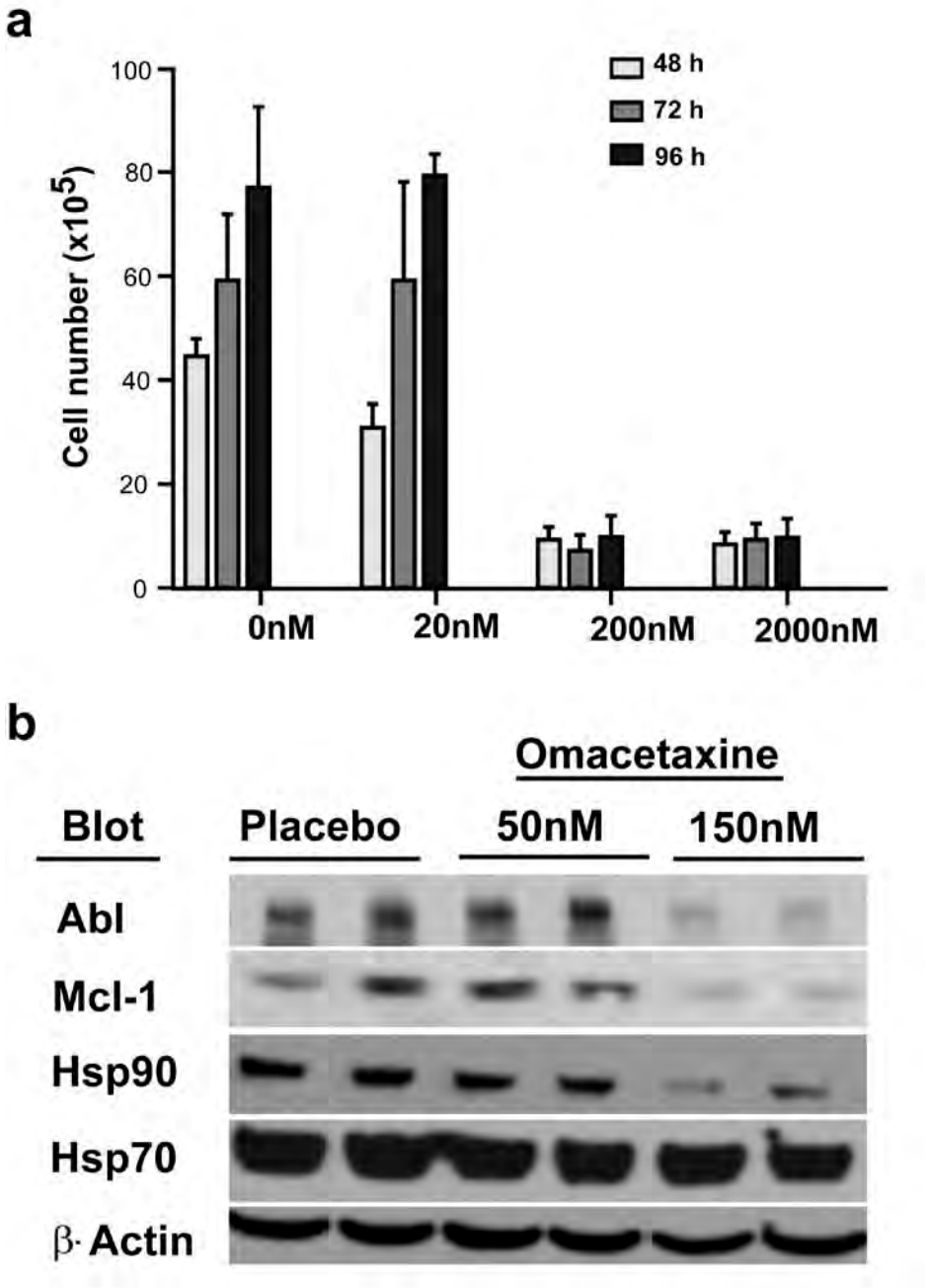


Figure 4: Omacetaxine degrades BCR-ABL by inhibiting HSP90 and suppresses MCL-1 in myeloid leukemia cells.

2.3.5 Omacetaxine improves survival of mice with BCR-ABL induced B-ALL

In contrast to CML(Figure 1), the development of B-ALL induced by BCR-ABL is not significantly affected by TKIs such as imatinib and dasatinib[152,153]. To induce B-ALL in mice, BCR-ABL–transduced bone marrow cells from donor mice that were not pretreated with 5-FU were transplanted into BALB/c mice[36,149]. To determine whether omacetaxine was effective in treating B-ALL, we used the B-ALL model, where pre-B cells express the B220 and CD19 cell surface antigens, and phenotypically resemble de novo Ph^+ B-ALL and lymphoid blast crisis of CML[36,154]. These mice were treated with a placebo, 0.5mg/kg or 1mg/kg omacetaxine daily starting at day 10. After 4 days of treatment, only 2% of cells in peripheral blood were GFP^+ B- leukemia cells in the 1mg/kg group, compared with 20% GFP^+ B-leukemia cells in 0.5mg/kg group or 50% GFP^+ B-leukemia cells in placebo group ($P < 0.001$, Figure 5a and 5b). After 10 days of treatment, less than 2% GFP^+ B-leukemia cells were detected in both drug treatment groups (Figure 5a and 5b) ($P <0.001$). All placebo-treated recipients of BCR-ABL transduced bone marrow developed and died of B-ALL within 4 weeks after BMT (Figure 5c), and all B-ALL mice treated with omacetaxine survived.

Figure 5: Omacetaxine improves survival of mice with BCR-ABL-induced B-ALL

a and **b** Flow cytometric evaluation of the B-lymphoid leukemic process. The B-lymphoid leukemic cells (GFP^+B220^+) were measured after placebo or omacetaxine (0.5 mg/kg or 1 mg/kg, n=4) treatment of B-ALL mice 4 days or 10 days after treatment. **c.** Treatment with the omacetaxine prolonged survival of BCR-ABL induced B-ALL mice. Mice with BCR-ABL induced B-ALL were treated with placebo (n = 15) or omacetaxine (1 mg/kg/day) (n=15).

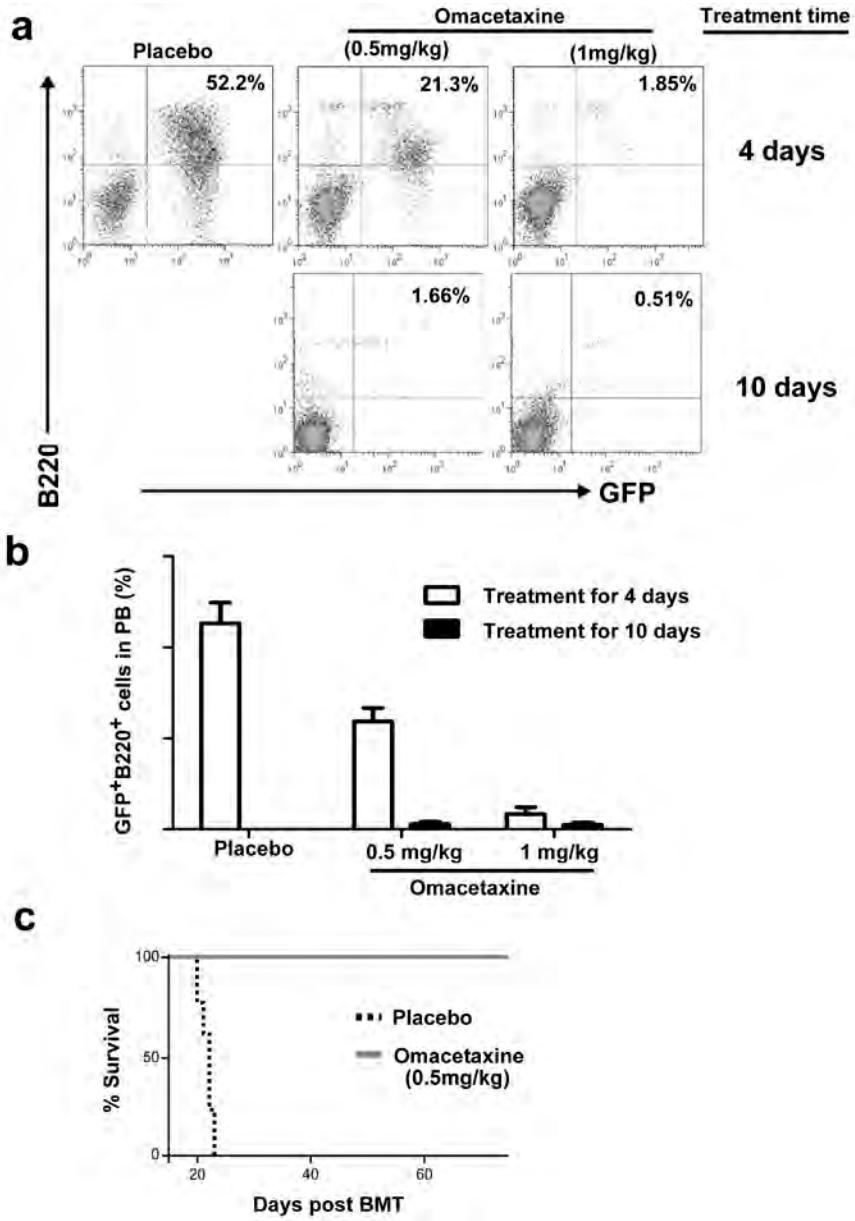


Figure 5: Omacetaxine improves survival of mice with BCR-ABL-induced B-ALL

2.3.6 Omacetaxine inhibits BCR-ABL expression without affecting Hsp90 in B-lymphoid cells expressing BCR-ABL.

We further investigated whether omacetaxine also affects the expression levels of BCR-ABL and Hsp90 proteins in lymphoid cells expressing BCR-ABL. Pre-B cells expressing BCR-ABL or BCR-ABL-T315I from omacetaxine-treated B-ALL mice showed a concentration dependent inhibition of cell proliferation in response to omacetaxine (Figure 6a). We then compared BCR-ABL protein levels between placebo and omacetaxine (50 nM or 150 nM) treatment groups. After the treatment, the level of wild type BCR-ABL protein was slightly lower in omacetaxine treated B-leukemia cells, but the level of BCR-ABL T315I protein was markedly decreased. However, unlike myeloid leukemia cells (Figure 4b), the level of Hsp90 protein did not change after omacetaxine treatment (Figure 6b).

Figure 6: Omacetaxine inhibits B-ALL cells by suppressing BCR-ABL without affecting HSP90.

a. Omacetaxine inhibited pre-B cells expressing BCR-ABL or BCR-ABL-T315I associated with drug concentration. The number of viable cells at the indicated drug concentrations was determined by trypan blue. **b.** Omacetaxine inhibited the expression of Abl in pre-B cells expressing BCR-ABL or BCR-ABL-T315I. These pre-B cells were treated with omacetaxine (50 nM, 150 nM) for 48 hours. Protein lysates were analyzed by Western blotting using antibodies indicated.

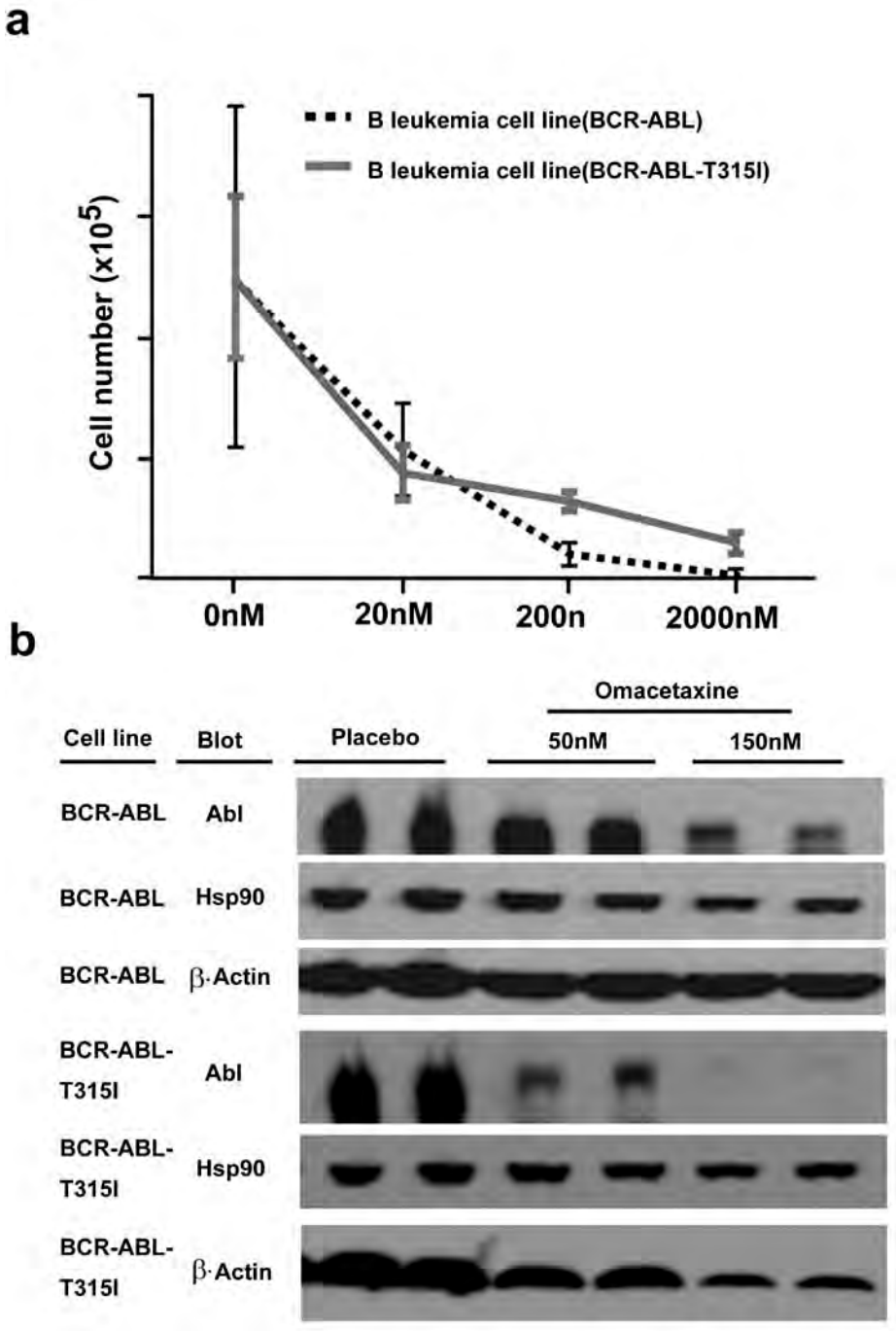


Figure 6: Omacetaxine inhibits B-ALL cells by suppressing BCR-ABL without affecting HSP90.

2.4 Discussion

The BCR-ABL positive leukemia stem cell is a major target for curative therapy of Ph+ leukemias[145]. Although current TKI based therapies have significant clinical efficacy in Ph+ leukemias, these agents do not effectively kill BCR-ABL positive leukemia stem cells and only rarely elicit long lasting complete cytogenetic remission after cessation of therapy [22,144]. This finding has led to investigation of therapeutics that selectively kill BCR-ABL positive leukemia stem cells as potential novel treatments of Ph+ leukemias [44,146]. Here we have shown in animal models of Ph+ CML and B-ALL that omacetaxine effectively targets BCR-ABL positive leukemia stem cells in vivo and confers a significant survival benefit for leukemic mice. In contrast, previous work has shown that the TKI imatinib did not affect the numbers of stem cells found in the circulation or the bone marrow of mice with CML[26]. These findings raise the possibility that clinical efficacy of omacetaxine in CML (where it is currently in phase 2/3 trials) may be due, at least in part, to its inhibiting activity on leukemic stem cells.

The appearance of mutations in the BCR-ABL oncoprotein is a major cause of failure with imatinib therapy of BCR-ABL positive leukemias [155]. Whilst the second generation TKIs dasatinib and nolotinib, inhibit many imatinib resistant mutant BCR-ABL proteins, cells expressing T315I mutant BCR-ABL are resistant to all 3 TKIs currently used to treat CML. Ongoing clinical trials have found that omacetaxine has clinical efficacy in CML patients with T315I mutant BCR-ABL[126]. Here we have

shown that omacectaxine significantly prolonged survival in these mice and dramatically reduced the number of T315I BCR-ABL positive leukemic cells both *in vivo* and *in vitro*. These observations support the clinical use of omacetaxine in CML patients with TKI resistant disease mediated by the T315I mutant BCR-ABL.

The mechanisms for omacetaxine inhibition of Ph^+ leukemic stem cells are unclear. As omacetaxine is an inhibitor of protein synthesis, we hypothesized that the anti-leukemic activity of omacetaxine may be due to the loss of anti-apoptotic proteins. We postulated that omacetaxine may induce apoptosis in BCR-ABL positive CML cells via three potential pathways: (a) by directly reducing the expression of BCR-ABL, (b) by reducing the expression levels of BCR-ABL stabilizing proteins (such as Hsp90), which will lead to the degradation of BCR-ABL, and (c) by reducing the expression of the short lived anti-apoptotic Bcl-2 family protein Mcl-1. We investigated the effect of omacetaxine on the expression of BCR-ABL, Hsp90 and Mcl-1 proteins in CML derived K562 cells and found that omacetaxine treatment induces the loss of BCR-ABL, Hsp90 and Mcl-1. These observations are consistent with the hypothesis that omacetaxine acts by directly or indirectly inhibiting the BCR-ABL, Mcl-1 and Hsp90 pathways in BCR-ABL positive CML cells and raise the possibility that omacetaxine acts on CML leukemic stem cells via reduced levels of these proteins.

Acute lymphocytic leukemia in adults is commonly caused by the expression of BCR-ABL. In general, B-ALL tends to show different symptoms, compared with CML. Omacetaxine removed most GFP^+ $B220^+$ cells from the peripheral blood with less than

1% GFP^+ $B220^+$ cells still detected by FACS and prolonged the survival of B-ALL mice. However, there was no change in Hsp90 protein levels, although BCR-ABL protein level was greatly reduced by omacetaxine. In CML, treatment with omacetaxine reduced the level of both Hsp90 and BCR-ABL protein demonstrating a different role for omacetaxine in treating CML and B-ALL. These results are consistent with our previous observation that BCR-ABL utilizes different signaling pathway to induce CML and B-ALL [149]. Omacetaxine also has potential for treating CML and Ph^+ B-ALL resistant to TKIs [128].

2.5 Materials and methods

Cell lines

Human K562 myeloid leukemia cell line was grown in RPMI 1640 medium containing 10% FCS. To generate the BCR-ABL–expressing pre-B cell lines, bone marrow cells were transduced with the BCR-ABL-WT- or BCR-ABL-T315I-IRES-GFP-MSCV retrovirus, followed by transplantation into recipient mice. The BCR-ABL-expressing cells were isolated from the spleen of a mouse with BCR-ABL induced B-ALL, and pre-B leukemic cells were selected through GFP sorting by fluorescence-activated cell sorter (FACS). To generate the BCR-ABL–expressing 32D line, the cells were transduced with the BCR-ABL-WT- or BCR-ABL-T315I-IRES-GFP-MSCV retrovirus, and the BCR-ABL–expressing cells were selected by GFP sorting by FACS.

Histology

The lungs from the placebo- or drug-treated mice were fixed in Bouin fixative (Fisher Scientific, Pittsburgh, PA) for 24 hours at room temperature, followed by an overnight rinse in water. Ten-µm sections were stained with hematoxylin and eosin (H&E) and observed by a model DMRE compound microscope (Leica, Heidelberg, Germany). All sections were imaged with a 2.5 x PH1 objective (NPLan, NA 0.25) and 10 x PH1 objective (NPLan, NA 0.40). All images were imported into MetaMorph software (Molecular Devices, Downingtown, PA) as a series of tagged image files. All images were then constructed in Adobe Photoshop 7.0 (Adobe, San Jose, CA).

Antibodies and Western blot analysis

Antibodies against c-ABL, Hsp90, Hsp70, Mcl-1 and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protein lysates were prepared by lysing cells in radioimmunoprecipitation (RIPA) buffer, and immunoprecipitation and Western blotting were carried out as described previously.

Bone marrow transduction/transplantation

The retroviral vector *MSCV-IRES-EGFP* carrying the *p210 BCR-ABL* cDNA was used to make high-titer, helper-free, replication-defective ecotropic virus stock by transient transfection of 293T cells using the kat system as previously described. 6- to 10-week-old wild-type BABL/c or C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine, and U.S.A) were used for leukemogenesis experiments. Induction of CML and B-ALL was as previously described. Briefly, to model CML, bone marrow from 5-fluorouracil

(5-FU)-treated (200 mg/kg) donor mice was transduced twice with *BCR-ABL* retrovirus by cosedementation in the presence of IL-3, IL-6, and stem cell factor(SCF). To model B-ALL, bone marrow from non-5-FU-treated donors was transduced without cytokines. Wild-type recipient mice were prepared by 900 cGy (for BABL/c) or 1100 cGy (for C57BL/6) gamma irradiation and a dose of 0.5×10^6 (CML) or 1.0×10^6 (B-ALL) cells transplanted via tail vein injection. Diseased mice were analyzed by histopathological and biochemical analyses as described previously.

Flow cytometry

Hematopoietic cells were collected from peripheral blood and bone marrow of diseased mice, and red blood cells were lysed with NH_4Cl red blood cell lysis buffer (pH 7.4). The cells were washed with PBS, and stained with B220-PE for B cells, Gr-1-APC for neutrophils, and Sca1-APC/c-kit-PE for hematopoietic stem cells. After staining, the cells were washed once with PBS and subjected to FACS analysis.

Culture of leukemia stem cells

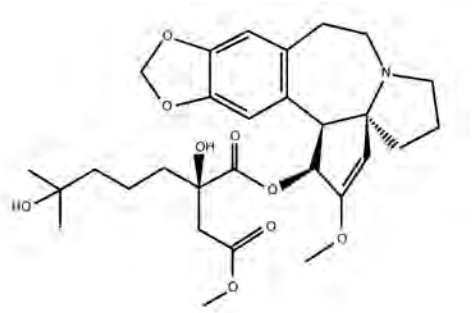
Bone marrow cells isolated from CML mice were cultured *in vitro* in the presence of stemspan SFEM, SCF, IGF-2, TPO, heparin, and FGF as reported previously for culture of hematopoietic stem cells.

Drug treatment

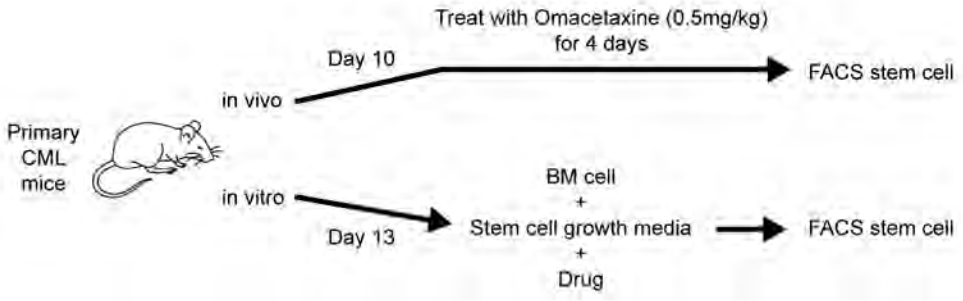
Omacetaxine (ChemGenex Pharmaceuticals, Inc, Menlo Park, CA) was dissolved in 0.9% NaCl to a stock concentration of 1 mg/ml. Further dilutions were made to working concentrations using media or water. Imatinib was dissolved in water directly at a concentration of 10 mg/ml. The drugs were given by either oral gavage for the CML model or by I.P. route for the B-ALL model in a volume of <0.5 ml, once a day, at 0.5 mg or 1.0 mg per kilogram of body weight for omacetaxine and 100 mg per kilogram of body weight per dose of imatinib, beginning at 10 days after bone marrow transplantation.

Statistical analysis

Results are reported as mean ± SD. Differences were evaluated by t test or analysis of variance, and accepted as significance when P value is less than 0.05.



Supplementary Figure1: The structure of omacetaxine mepesuccinate.



Supplementary Figure2: Analysis of leukemic stem cells in vitro and in vivo.

Chapter III

Loss of the Alox5 gene impairs leukemia stem cells and prevents chronic myeloid leukemia

The work described in this chapter has been published (**Chen Y**, Hu Y, Zhang H, Peng C, Li S. Loss of the Alox5 gene impairs leukemia stem cells and prevents chronic myeloid leukemia. **Nature Genetics**. 2009, 41(7):783-92)

3.1 Abstract

Targeting of cancer stem cells is believed to be essential for curative therapy of cancers, but supporting evidence is limited. Few selective target genes in cancer stem cells have been identified. Here we identify the arachidonate 5-lipoxygenase (5-LO) gene (*Alox5*) as a critical regulator for leukemia stem cells (LSCs) in BCR-ABL-induced chronic myeloid leukemia (CML). In the absence of *Alox5*, BCR-ABL failed to induce CML in mice. This *Alox5* deficiency caused impairment of the function of LSCs but not normal hematopoietic stem cells (HSCs) through affecting differentiation, cell division, and survival of long-term LSCs (LT-LSCs), consequently causing a depletion of LSCs and a failure of CML development. Treatment of CML mice with a 5-LO inhibitor also impaired the function of LSCs similarly by affecting LT-LSCs, and prolonged survival. These [4]inhibition can completely inhibit the function of these stem cells.

3.2 Introduction

Cancer stem cells in many types of hematologic malignancies and solid tumors are believed to be a cell population that is required for cancer initiation and must be targeted for effective treatment of the diseases[45-50], however, the direct supporting evidence is still lacking. The challenge includes identification of differences between cancer stem cells and their normal stem cell counterparts, and demonstration of complete control of cancer by targeting these stem cells. Success of an anti-stem cell strategy relies on complete inhibition of function of a gene required for maintenance of cancer stem cells but not normal stem cells. A number of genes have been found to promote or inhibit cancer cell proliferation, but they also play similar roles in regulating normal stem cells. Examples include pathways involved in signaling through Wnt/β-catenin, Hedgehog and Notch[47,48,61,64,156,157], and Bim-1[158,159], p53[156], p16 ^{INK4a}[160], etc. The role of *Pten* in inhibition of cancer stem cells of acute myeloid leukemia (AML) and in the maintenance of normal HSCs is unique, and provides an example that distinguishes AML stem cells from normal HSCs, although *Pten* has an effect on normal HSCs [161]. Because cancer stem cells express markers similar to those on normal stem cells[52,53], the major difference between them should be related to the cancer-initiating genetic changes such as acquiring an oncogene or accumulating a DNA mutation. It is reasonable to hypothesize that these genetic changes cause aberrant expression of genes, consequently turning a normal stem cell into a cancer stem cell. It is essential to identify genes that are functionally required by cancer stem cells but not by normal stem cell counterparts. Some clues for identifying genes that play roles in cancer stem cells could come from studies of normal stem cell

counterparts and different lineages of cancer cells[80,162-164]. It is reasonable to think that biological features of cancer stem cells are reflected by the deference in gene expression between cancer and normal stem cells. The list of aberrantly expressed genes could be huge, and it is critical to identify key genes or pathways that are required for initiating and maintaining cancer stem cells and can be used as targets for inhibiting these cells.

We have tested our hypothesis using BCR-ABL-induced CML as a disease model system, as CML is a stem cell disease and we have previously identified LSCs for CML in mice[26]. In addition, CML stem cells are insensitive to BCR-ABL kinase inhibitors[25,165]. Here we identify *Alox5* as a key gene that regulates the function of LSCs but not normal HSCs in mice. Alox5 has been shown to be involved in numerous physiological and pathological processes, including oxidative stress response, inflammation, and cancer [82-89]. Here we also show that *Alox5* deficiency or inhibition of function of this gene completely prevent the initiation of BCR-ABL-induced CML.

3.3 Results

3.3.1 *Alox5* is essential for CML induction by BCR-ABL

LSCs in CML are insensitive to BCR-ABL kinase inhibitors[25]. To identify genes that are regulated by BCR-ABL in LSCs, we used the bone marrow transplantation (BMT) mouse model of CML as an assay system, in which bone marrow cells from donor mice pre-treated with 5-fluorouracil (5-FU) and transduced with BCR-ABL result in development of CML in recipient mice[36]. We transduced bone marrow cells from C57BL/6 (B6) mice with retrovirus containing BCR-ABL/GFP or GFP alone under conditions for induction

of CML, followed by transplantation of the transduced cells into B6 recipient mice. Some mice were treated with the BCR-ABL kinase inhibitor imatinib to allow identification of genes that were altered by BCR-ABL in LSCs, but this alteration was not restored by inhibition of BCR-ABL kinase activity with imatinib. This approach to identifying pathways that are activated by BCR-ABL but are insensitive to inhibition by imatinib is based on our previous observation that BCR-ABL kinase activity does not relate to all signaling pathways activated by BCR-ABL[26]. 14 days after BMT, bone marrow cells were isolated and subsequently sorted by FACS for LSCs ($GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$) [26]. Total RNA was isolated from these BCR-ABL-expressing LSCs or from the $GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells that only expressed GFP, and DNA microarray analysis was carried out to compare gene expression between BCR-ABL-expressing and non-BCR-ABL-expressing $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells. The *Alox5* gene was up-regulated and this up-regulation was not abolished by imatinib treatment (Figure 1a). There were several genes that were also up-regulated by BCR-ABL and not changed in expression following imatinib treatment (Supplementary Figure 1). The up-regulation of *Alox5* by BCR-ABL in LSCs was confirmed by RT-PCR (Figure 1b). To further support the regulation of *Alox5* by BCR-ABL, we tested whether BCR-ABL up-regulates Alox5 function by measuring levels of leukotriene B4 (LTB4), which is synthesized and metabolized through the 5-LO pathway, in peripheral blood of the transplanted mice. Plasma level of LTB4 was increased in CML mice (Figure 1c)

Next, we studied to study the role of *Alox5* in regulation of LSC function using *Alox5* homozygous knockout (*Alox5*$^{-/-}$) mice. Wild type or *Alox5*$^{-/-}$ donor bone marrow

cells in B6 background were used to induce CML. We first investigated whether Alox5 is required for CML induction by BCR-ABL. Recipients of *BCR-ABL*-transduced bone marrow cells from 5-FU-treated wild type donor mice developed and died of CML within 4 weeks, whereas recipients of *BCR-ABL*-transduced bone marrow cells from *Alox5*$^{-/-}$ donor mice were resistant to induction of CML (Figure 1d). This defective disease phenotype correlated with much less severe infiltration of myeloid leukemia cells in the lung and spleen (Figures. 1e, f). In addition, FACS analysis of CML cells in peripheral blood and bone marrow showed that Gr-1^{+} myeloid leukemia cells grew initially, reached a peak after 2 weeks, then started to decline, and eventually disappeared after 7 weeks in peripheral blood and bone marrow of recipients receiving BCR-ABL-transduced *Alox5*$^{-/-}$ donor bone marrow cells (Figures. 1 g, h). Alox5 deficiency mainly affected growth of BCR-ABL-expressing (GFP^{+}) but not non-BCR-ABL-expressing (GFP^{-}) donor bone marrow cells (Figure 1h), suggesting that Alox5 signaling is much more critical for function of LSCs but not normal HSCs. Together, these results demonstrate that *Alox5* is essential for induction of CML by BCR-ABL.

Figure 1. *Alox5* is essential for the induction of CML induced by BCR-ABL.

a. Bone marrow cells from C57BL/6 mice (B6) were transduced with retrovirus containing BCR-ABL/GFP or GFP alone (BCR-ABL-IRES-GFP-pMSCV or IRES-GFP-pMSCV), and then transferred into B6 recipient mice to induce CML. One group of CML mice was treated with imatinib (150 mg/kg body weight/ per dose, once every 4 hours) for 5 doses beginning at day 13 post bone marrow transplantation (BMT). Bone marrow cells were isolated from CML mice, and were sorted by FACS for $GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells (normal or CML stem cells). Total RNA was isolated from these sorted cells for DNA micorarray analysis. Expression of the *Alox5* gene was up-regulated by BCR-ABL in CML stem cells as compared to the sorted $GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells that did not express BCR-ABL, and this up-regulation was not prevented by imatinib treatment. **b.** Bone marrow cells from C57BL/6 mice (B6) were transduced with retrovirus containing BCR-ABL/GFP or GFP alone to induce CML as described in **a**. Bone marrow cells were isolated from the mice were sorted by FACS for $GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells, and then total RNA was isolated from these sorted cells for comparing *Alox5* expression between GFP vector-transduced normal stem cells and BCR-ABL-transduced LSCs by RT-PCR. Expression of the *Alox5* gene was significantly up-regulated by BCR-ABL in LSCs as compared to the sorted GFP vector-transduced normal stem cells ($p< 0.001$). **c.** The plasma level of LTB4 in recipients of *BCR-ABL*-transduced bone marrow cells was significantly higher than that in recipients of bone marrow cells transduced with GFP-containing retrovirus ($p< 0.05$), and this increased level of LTB4 was not observed in recipients of $Alox5^{-/-}$ bone marrow cells transduced by *BCR-ABL*. These results indicated that BCR-ABL up-regulates LTB4 through Alox5. **d.** Kaplan-Meier survival curves for recipients of *BCR-ABL*-transduced bone marrow cells from wild type or $Alox5^{-/-}$ donor mice (10 mice per group). All recipients of *BCR-ABL*-transduced bone marrow cells from wild type donor mice developed CML and died within 4 weeks after bone marrow

transplantation (days post BMT), whereas recipients of *BCR-ABL*-transduced bone marrow cells from *Alox5*$^{-/-}$ donor mice survived. **e.** Gross appearance of the lungs and spleens showed severe lung hemorrhages and splenomegaly of recipients of BCR-ABL-transduced bone marrow cells from wild type but not *Alox5*$^{-/-}$ donor mice. **f.** Photomicrographs of haematoxylin and eosin-stained lung and spleen sections from recipients of *BCR-ABL*-transduced bone marrow cells from wild type or *Alox5*$^{-/-}$ donor mice. **g.** FACS analysis showed gradual disappearance of GFP^{+}Gr-1^{+} cells in peripheral blood (PB) and bone marrow (BM) of recipients of *BCR-ABL*-transduced bone marrow cells from *Alox5*$^{-/-}$ but not wild type donor mice. **h.** In recipients of BCR-ABL-transduced bone marrow cells from *Alox5*$^{-/-}$ donor mice, GFP^{+}Gr-1^{+} cells in PB gradually decreased with time, whereas the GFP^{-}Gr-1^{+} cells that did not express BCR-ABL gradually increased, showing that *Alox5* deficiency significantly inhibited engraftment of BCR-ABL-expressing but not normal BM cells in the same animals. Mean percentage for each group (n=5) was shown.

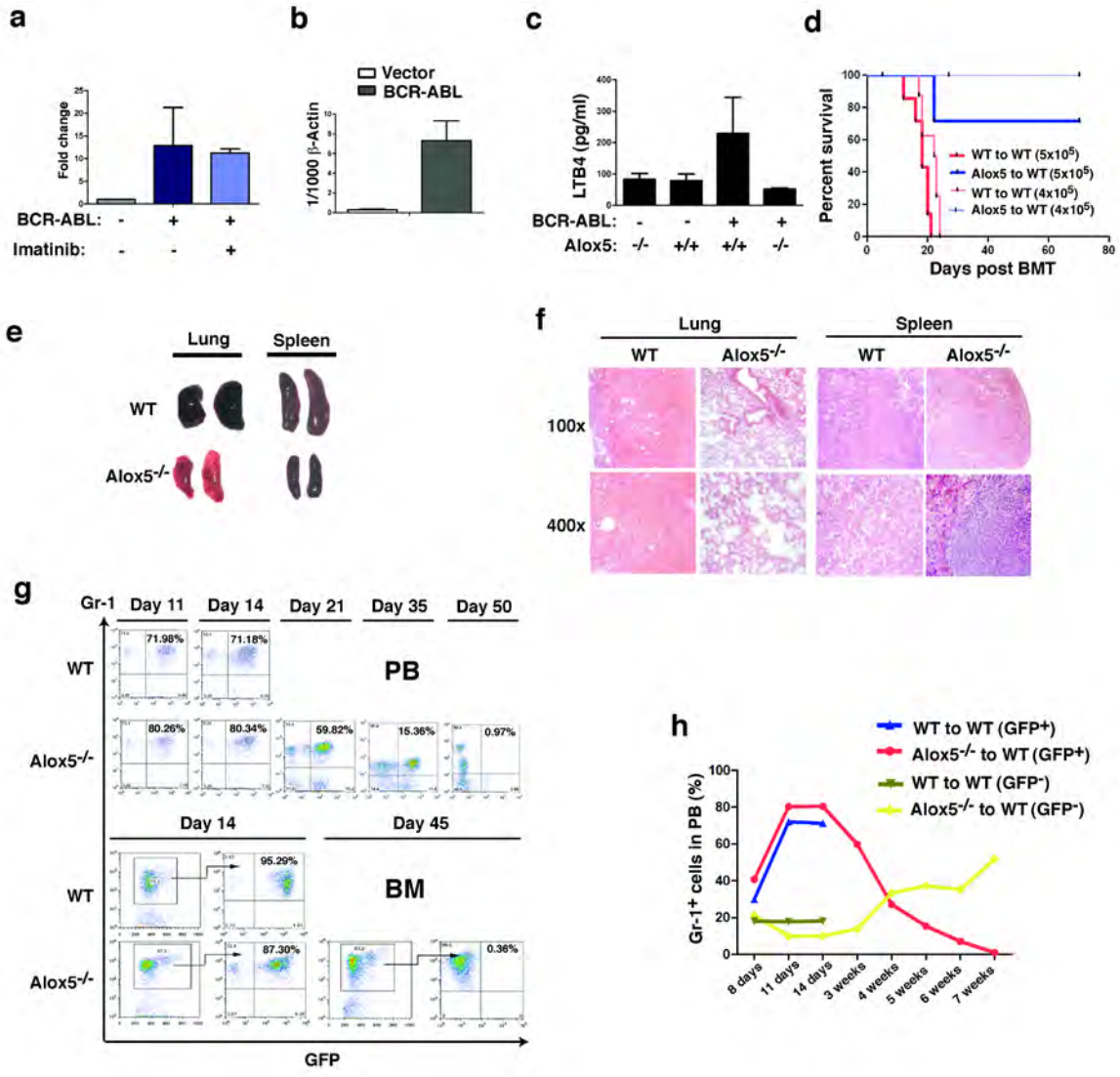


Figure 1. *Alox5* is essential for the induction of CML induced by BCR-ABL.

3.3.2 *Alox5* transgene rescues defective CML

To further confirm the role of *Alox5* in CML development, we co-expressed *BCR-ABL* and *Alox5* in *Alox5*$^{-/-}$ and wild type bone marrow cells, respectively, by retroviral transduction, followed by transplantation of the transduced cells into recipient mice. The *BCR-ABL*-IRES-*Alox5*-pMSCV construct expressed BCR-ABL and 5-LO in 293T cells (Figure 2a), and induced LTB4 production in mice (Figure 2b). In contrast to no CML induction by BCR-ABL in the absence of *Alox5* (Figure 1), ectopically expressed *Alox5* in *Alox5*$^{-/-}$ bone marrow cells rescued defective CML phenotype, and all mice receiving the *BCR-ABL*-IRES-*Alox5*-pMSCV transduced *Alox5*$^{-/-}$ bone marrow cells died (Figure 2c). In this experiment, we included the control mice that received BCR-ABL transduced *Alox5*$^{-/-}$ bone marrow cells as shown in Figure1, and all these mice survived (data not shown). FACS analysis (Figure 2d) and peripheral blood smears (Figure2e) showed the development of typical CML after the expression of the rescue gene *Alox5*, consistent with the severe infiltration of myeloid leukemia cells in the lung and spleen (Figure 2f). The mice receiving the *BCR-ABL*-IRES-*Alox5*-pMSCV transduced wild type bone marrow cells died faster than those receiving the *BCR-ABL*-IRES-*Alox5*-pMSCV transduced *Alox5*$^{-/-}$ bone marrow cells (Figure 2c), correlating with more myeloid cells in peripheral blood (Figure 2d, e) and more severe infiltration of myeloid leukemia cells in the lung and spleen (Figure 2f). The results from this rescue experiment definitively confirmed that *Alox5* plays a critical role in CML development.

Figure 2. *Alox5* transgene rescues defective CML phenotype. a. The rescue construct **(**BCR-ABL-IRES-Alox5-pMSCV) was used to transfect 293T cells to test for expression of 5-LO with mock- or BCR-ABL-IRES-GFP-pMSCV-transfected cells as controls. BCR-ABL and 5-LO were detected by Western blotting using antibodies against Abl and 5-LO. 5-LO protein was detected in cells transfected with BCR-ABL-IRES-Alox5-pMSCV. **b**. Bone marrow cells from B6 mice were transduced with retrovirus containing IRES-GFP-pMSCV (empty vector) or BCR-ABL-IRES-Alox5-pMSCV, and then transferred into B6 recipient mice to induce CML. The plasma level of LTB4 in recipients of BCR-ABL-IRES-Alox5-pMSCV-transduced bone marrow cells was significantly higher than that in recipients of bone marrow cells transduced with empty vector-containing retrovirus ($p< 0.01$), confirming that the BCR-ABL-IRES-Alox5-pMSCV construct induced LTB4 production in mice. **c.** Kaplan-Meier survival curves for recipients of BCR-ABL-IRES-Alox5-pMSCV-transduced bone marrow cells from wild type (n=10) or *Alox5*$^{-/-}$ (n=9) donor mice. All recipient mice died. **d.** FACS analysis showed appearance of GFP^{+}Gr-1^{+} cells in peripheral blood of recipients of BCR-ABL-IRES-Alox5-pMSCV-transduced bone marrow cells from both wild type and *Alox5*$^{-/-}$ donor mice. **e.** Peripheral blood smears showed accumulation of neutrophils, indicating high white blood cell counts in these CML mice. **f.** Photomicrographs of haematoxylin and eosin-stained lung and spleen sections from recipients of BCR-ABL-IRES-Alox5-pMSCV-transduced bone marrow cells from wild type or *Alox5*$^{-/-}$ donor mice.

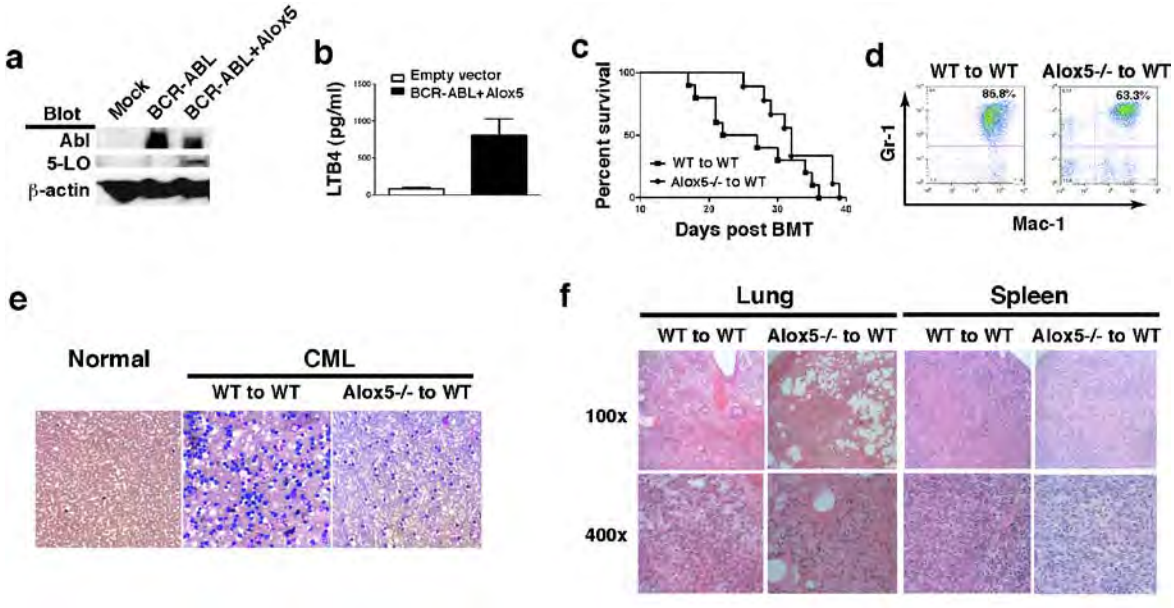


Figure 2. *Alox5* transgene rescues defective CML phenotype.

3.3.3 *Alox5* deficiency impairs the function of LSCs

The eventual disappearance of myeloid leukemia cells in CML mice in the absence of *Alox5* (Figure 1h) prompted us to examine whether *Alox5* is required for self-renewal of LSCs. A biological assay for LSCs is to examine their ability to transfer disease to secondary recipient mice[52,53]. We transferred bone marrow cells from primary recipients of *BCR-ABL*-transduced wild type or *Alox5*$^{-/-}$ donor bone marrow cells to secondary recipient mice. *BCR-ABL*-expressing wild type bone marrow cells transferred lethal CML, whereas *BCR-ABL*-expressing *Alox5*$^{-/-}$ bone marrow cells failed to induce CML in secondary recipient mice (Supplementary Figure 2). This result suggests that *Alox5* deficiency causes the impairment of the function of LSCs.

The impaired CML development from LSCs in the absence of *Alox5* could be caused by decreased number of LSCs. To test this hypothesis, we quantified LSCs ($GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$) and normal hematopoietic stem cells (HSCs) ($GFP^-Lin^-c\text{-}Kit^+Sca\text{-}1^+$) in bone marrow and the spleens of CML mice at day 14 and day 18 after induction of CML. At day 14, *Alox5* deficiency did not cause a reduction of LSCs in bone marrow and the spleens, but did so in bone marrow but not in the spleens at day 18, as compared to LSCs in wild type CML mice (Figure 3a); in the spleen, the number of *Alox5*$^{-/-}$ LSCs was similar to that of wild type LSCs (Figure 3a), indicating that the reduction of LSCs in bone marrow was not due to the migration of bone marrow LSCs to the spleens but due to an intrinsic defect caused by *Alox5* deficiency in LSCs. *Alox5* deficiency did not cause a significant reduction of normal HSCs (GFP^-) in bone marrow

and the spleens of the same animals (Figure 3a), suggesting that *Alox5* is functionally required by LSCs but not by normal HSCs. In addition, we did not observe a homing defect of HSCs lacking *Alox5* (Supplementary Figure 3), which could cause the impaired CML development when the cells were transduced by BCR-ABL (Figure 1). Furthermore, *Alox5* deficiency did not appear to cause a homing defect on LSCs, as the total number of *Alox5*$^{-/-}$ LSCs was not lower than that of wild type LSCs at day 14 after induction of CML (Figure 3a).

Engraftment of donor bone marrow cells in lethally irradiated recipient mice reflects stem cell function. *Alox5* deficiency could cause a decreased engraftment of LSCs, leading to a decrease in LSCs. To test this idea, LSCs were sorted by FACS from bone marrow of CML induced by transplanting BCR-ABL-transduced wild type (CD45.1) or *Alox5*$^{-/-}$ (CD45.2) donor bone marrow cells. Wild type and *Alox5*$^{-/-}$ LSCs were 1:1 mixed, followed by transplantation into lethally irradiated recipient mice. At day 14 or 25 after transplantation, more than 70% or 90% of GFP^{+}Gr-1^{+} cells in peripheral blood of the mice were wild type (CD45.1^{+}) leukemia cells, and all these mice developed CML and died (Figure 3b). Total wild type and *Alox5*$^{-/-}$ bone marrow cells were also 1:1 mixed, followed by transplantation into lethally irradiated recipient mice. At 40 day after BMT, more than 80% of GFP^{+}Gr-1^{+} cells in peripheral blood were wild type (CD45.1^{+}) leukemia cells, and all these mice died of CML (Supplementary Figure4). To further compare biological function between wild type and *Alox5*$^{-/-}$ LSCs, *BCR-ABL*-transduced wild type or *Alox5*$^{-/-}$ bone marrow cells were sorted by FACS for Lin^{-}c-Kit^{+}Sca-1^{+} cells, and equal number of the sorted wild type or *Alox5*$^{-/-}$ cells was transplanted into each

lethally irradiated recipient mouse. At day 14 after transplantation, $GFP^+Gr\text{-}1^+$ cells were detected in peripheral blood of recipient mice receiving *BCR-ABL*-transduced wild type but not *Alox5*$^{-/-}$ bone marrow donor cells (Figure 3c). By day 50 after transplantation, all mice receiving *BCR-ABL*-transduced sorted wild type bone marrow cells died of CML, whereas all mice receiving sorted *Alox5*$^{-/-}$ bone marrow cells survived (Figure 3c). These results indicated that *Alox5* deficiency caused impairment of the function of LSCs, consequently leading to reduced production of leukemia progenitor cells. This conclusion is consistent with our finding that at day 20 after induction of CML, similar numbers of GFP^+ CMP (common myeloid progenitor), GMP (granulocyte-macrophage progenitor), and MEP (megakaryocyte-erythroid progenitor) cells were detected in bone marrow of mice receiving *BCR-ABL*-transduced *Alox5*$^{-/-}$ donor bone marrow cells (Figure 3d); however, at day 45 these cells were much less in bone marrow of mice receiving BCR-ABL transduced *Alox5*$^{-/-}$ donor marrow cells (Figure 3d). By contrast, *Alox5*$^{-/-}$ deficiency did not cause reduction of non-BCR-ABL-expressing myeloid progenitor cells in the same animals (Figure 3d), providing an indirect evidence that *Alox5*$^{-/-}$ deficiency only causes a functional defect in LSCs but not normal HSCs. This conclusion was further confirmed by comparing the engraftment of wild type (CD45.1) and *Alox5*$^{-/-}$ (CD45.2) bone marrow cells by injecting the same number of each type of bone marrow cells into lethally irradiated recipient mice. At 30 day after BMT, the percentage of wild type or *Alox5*$^{-/-}$ bone marrow cells was similar (Supplementary Figure 5). A control experiment showed that after lethal irradiation, 100 % of cells in peripheral blood of recipient mice were donor-derived when assayed at day 30 after BMT (data not shown), allowing the

direct analysis of CD45.1 or CD45.2 cells to reflect the donor-derived wild type (CD45.1) and *Alox5*$^{-/-}$ (CD45.2) bone marrow cells in the same animal.

Figure 3. Loss of *Alox5* impairs the function of CML stem cell. a. BCR-ABL-expressing (GFP^+) and non-BCR-ABL-expressing (GFP^-) $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells (CML stem cells) in BM and the spleens (SPL) were analyzed by FACS in recipients of *BCR-ABL*-transduced BM cells from wild type or *Alox5*$^{-/-}$ donor mice (n=4 for each group at two time points after BMT). Total number of $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells for each mouse was calculated as percentage of $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells x total cell count for the cells from femurs and tabias. Loss of Alox5 caused significant reduction of CML stem cells in BM ($p< 0.05$). **b.** Equal numbers of the sorted $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells from recipients of *BCR-ABL*-transduced bone marrow cells from wild type (CD45.1) or *Alox5*$^{-/-}$(CD45.2) donor mice were mixed, followed by transplantation into lethally irradiated wild type mice. At days 14 and 25 after BMT, FACS analysis showed that the percentages of $CD45.1^+$ cells were much higher than those of $CD45.2^+$ cells. All these mice died of CML, presumably due to the development of CML from $CD45.1^+$ cells. **c.** $Lin^-c\text{-}kit^+Sca\text{-}1^+$ cells sorted by FACS from *BCR-ABL*-transduced BM cells from wild type or *Alox5*$^{-/-}$ mice were injected into lethally irradiated wild type recipient mice (15000 $Lin^-c\text{-}kit^+Sca\text{-}1^+$ cells per recipient mouse). At day 14 after BMT, GFP^+ $Gr\text{-}1^+$ cells in peripheral blood of the mice were analyzed by FACS. *Alox5*-deficient $Lin^-c\text{-}kit^+Sca\text{-}1^+$ poorly engrafted. The mice receiving the *Alox5*-deficient $Lin^-c\text{-}kit^+Sca\text{-}1^+$ cells survived (n=4), whereas the mice receiving the wild type $Lin^-c\text{-}kit^+Sca\text{-}1^+$ cells died of CML (n=2). **d.** FACS analysis indicated the percentages of BCR-ABL-expressing (GFP^+) and non-BCR-ABL-expressing (GFP^-) CMP, GMP, and MEP cells in BM of recipients of BCR-ABL-transduced wild type or *Alox5*$^{-/-}$ donor BM cells (n=4). The results showed that loss of Alox5 caused depletion of

BCR-ABL-expressing but not non-BCR-ABL-expressing CMP, GMP, and MEP cells in BM of the mice.

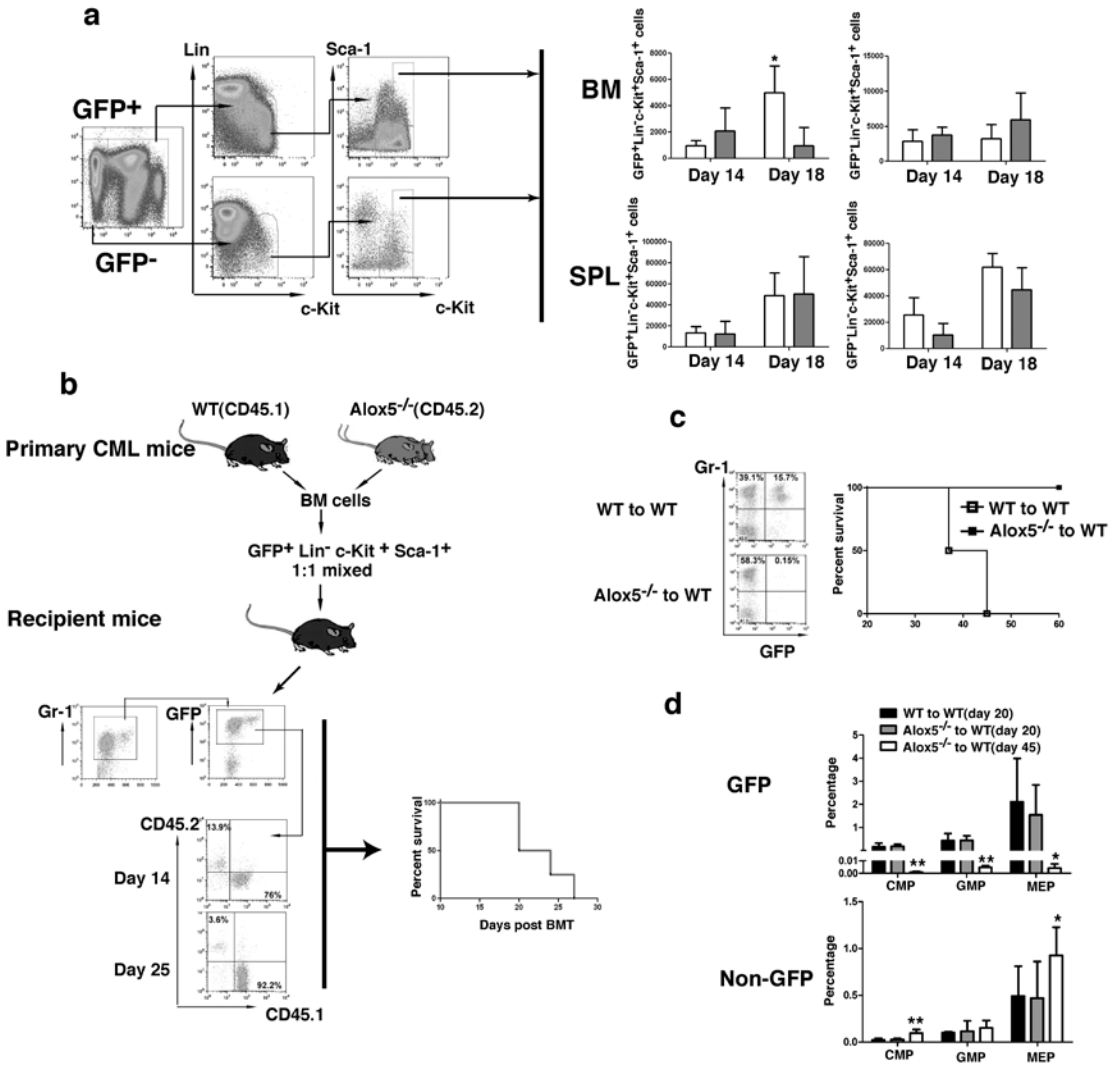


Figure 3. Loss of *Alox5* impairs the function of CML stem cell.

3.3.4 *Alox5* deficiency affects differentiation, cell division and survival of LT-LSCs

Alox5 deficiency caused the impairment of function of LSCs (Figure 3), and these cells were phenotypically $Lin^-c\text{-}Kit^+Sca\text{-}1^+$, including long-term (LT) HSCs, short-term (ST) HSCs, and multipotent progenitor (MPP) cells. We further investigated which of these cell populations is affected by *Alox5* deficiency. *BCR-ABL*-transduced *Alox5*$^{-/-}$ bone marrow cells were transplanted into recipient mice to induce CML, and bone marrow cells from these mice were analyzed by FACS for percentages of total HSCs ($Lin^-c\text{-}Kit^+Sca\text{-}1^+$), LT-HSCs ($Lin^-c\text{-}Kit^+Sca\text{-}1^+CD34^-$), and ST-HSCs /MPP cells ($Lin^-c\text{-}Kit^+Sca\text{-}1^+CD34^+$). At day 20 after induction of CML, the percentage or total number of bone marrow LT-LSCs ($GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+CD34^-$) was about 1/2 of those of ST-LSCs/MPP cells ($GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+CD34^+$) (Figure 4a). However, at day 90, the percentage or total number of LT-LSCs was about 8 fold higher than that of ST-LSCs/MPP cells (Figure 4a). These results suggest that *Alox5* deficiency blocks differentiation of LT-LSCs, preventing these cells from developing CML. In these mice, the percentage of GFP^- LT-HSCs was much lower than that of GFP^- ST-HSCs/MPP cells (Supplementary Figure 6), demonstrating that *Alox5* deficiency does not similarly affect differentiation of normal LT-HSCs. Although loss of *Alox5* caused a relative high percentage of LT-LSCs than that of ST-LSCs/MPP cells, the total number of LT-LSCs declined with time (Figure 4a), suggesting that *Alox5* deficiency caused a gradual depletion of LSCs.

The effect of *Alox5* deficiency on LSCs was further demonstrated in experiments showing that *Alox5* function was inhibited by a selective 5-LO inhibitor Zileuton[166].

BCR-ABL-transduced wild type bone marrow cells were transplanted into recipient mice to induce CML, and LSCs in bone marrow cells of these mice treated with Zileuton (300 mg/kg of body weight, twice a day) were analyzed by FACS at days 20 and 90 after induction of CML. As the treatment went on, the ratio between the percentage of LT-LSCs and that of ST-LSCs/MPP cells became increasing (Figure 4b), suggesting a blockade of differentiation of LT-LSCs. Zileuton treatment did not similarly affect differentiation of GFP^- LT-HSCs in the same animals (Figure 4b), suggesting that inhibition of 5-LO does not suppress normal HSCs. These findings were consistent with those from the studies using *Alox5*$^{-/-}$ mice (Figure 4a). No suppression of normal HSCs was further demonstrated in placebo- or Zileuton-treated mice receiving wild type donor bone marrow cells transduced with MSCV-IRES-GFP retrovirus. At day 14 after BMT, the numbers of $GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells in bone marrow of placebo- and Zileuton-treated mice were compared. Zileuton treatment did not result in a reduction of $GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells in bone marrow and the spleens of the mice (Figure 4c).

The blockade of LT-LSC differentiation was correlated with biological and molecular changes in LSCs. Cell cycle analysis of LSCs in bone marrow of CML mice showed that there was higher percentage of LT-LSCs in the S+G2M phase of the cell cycle in mice receiving BCR-ABL-transduced *Alox5*$^{-/-}$ bone marrow cells than in mice receiving BCR-ABL-transduced wild type bone marrow cells (Figure 4d); however, the percentages of LT-LSCs and ST-LSCs/MPPs in the S+G2M phase of the cell cycle were similar (Figure 4d), suggesting that this differentiation blockade caused a compensatory response of *Alox5* deficient LSCs to the shortage of downstream cell lineages. The higher

percentage of LT-LSCs in the S+G2M phase of the cell cycle in the absence of *Alox5* may also be explained by asymmetric cell division of LT-LSCs. *Alox5* deficiency also caused slightly increased apoptosis of LSCs ($p > 0.05$) (Figure 4e), but the apoptotic ratio between *Alox5*$^{-/-}$ ST-LSCs/MPPs and LT-LSCs was similar to the ratio between *Alox5*$^{+/+}$ ST-LSCs/MPPs and LT-LSCs (Supplementary Figure 7). To study the underlying mechanism for the defective function of LSCs at molecular level, we examined the expressive levels of three regulatory genes of hematopoiesis, β-catenin, GATA-1 and FOG-1[61,167,168]. We found that the defective function of LSCs in the absence of *Alox5* was correlated with the reduction of β-catenin and GATA-1 but not FOG-1 (Figure 4f).

Figure 4. Loss of Alox5 function blocks differentiation of LT-LSCs.

a. Bone marrow cells were isolated from recipients of *BCR-ABL*-transduced bone marrow cells from wild type or *Alox5*$^{-/-}$donor mice, and FACS analysis showed that the percentages and total numbers of LT-LSCs (GFP^{+}Lin^{-}c-Kit^{+}Sca-1^{+}CD34^{-}) and ST-LSCs /BCR-ABL-expressing MPP cells (Lin^{-}c-Kit^{+}Sca-1^{+}CD34^{+}) in recipients of BCR-ABL-transduced *Alox5*$^{-/-}$ (middle and right panels) donor BM cells were much lower than those in recipients of BCR-ABL-transduced wild type (left panel) donor BM cells (n=4). In addition, LT-LSCs in recipients of BCR-ABL-transduced *Alox5*$^{-/-}$ donor BM cells relatively accumulated due to the blockade of differentiation, resulting in depletion of ST-LSCs /BCR-ABL-expressing MPP cells as assayed at day 90 post BMT. **b.** Recipients of *BCR-ABL*-transduced bone marrow cells from wild type donor mice were treated with Zileuton (300 mg/kg, twice a day) beginning at 8 days after BMT. At days 20 and 90 post BMT, FACS analysis showed the depletion of ST-LSCs /BCR-ABL-expressing MPP cells in Zileuton-treated CML mice, indicating a blockade of differentiation of LT-LSCs. In contrast, in the same animals the percentages and total numbers of ST-HSCs /MPP cells were higher than those of LT-LSCs, indicating that Zileuton treatment did not lead to a blockade of differentiation of normal LT-HSCs in Zileuton-treated CML mice. **c.** Mice receiving wild type donor BM cells transduced with MSCV-IRES-GFP retrovirus were treated with a placebo or Zileuton as described in **b**. At day 14 after BMT, the numbers of GFP^{+}Lin^{-}c-Kit^{+}Sca-1^{+} cells in BM and the spleens (SPL) of placebo- and Zileuton-treated mice were compared. Zileuton treatment did not result in a reduction of normal HSCs in the mice. **d.** At day 14 after BMT, bone marrow cells were isolated from

recipients of *BCR-ABL*-transduced bone marrow cells from wild type or *Alox5*$^{-/-}$donor mice. The cells were stained with Hoechst Blue, and DNA contents, represented by the percentages of three LSC populations (total LSCs, LT-LSCs, and ST-LSCs+MPPs) in the S+G2M phase of the cell cycle, was examined by FACS. Mean percentage for each cell population (n=5) was shown. **e.** At day 14 after BMT, bone marrow cells were isolated from recipients of *BCR-ABL*-transduced bone marrow cells from wild type or *Alox5*$^{-/-}$ donor mice. The cells were stained with PI and Hoechst Blue, and the percentages of LSCs positive for PI and Hoechst Blue, representing apoptotic cells, were determined by F ACS. **f**. Bone marrow GFP^{+}Lin^{-}c-Kit^{+}Sca-1^{+} cells were sorted by FACS from recipients of GFP vector- or *BCR-ABL*-transduced bone marrow cells from wild type or *Alox5*$^{-/-}$ donor mice for isolation of total RNA, and expression of β-catenin, GATA-1 and FOG were detected by RT-PCR. Each bar represents the mean value of three samples. BMCs: bone marrow cells.

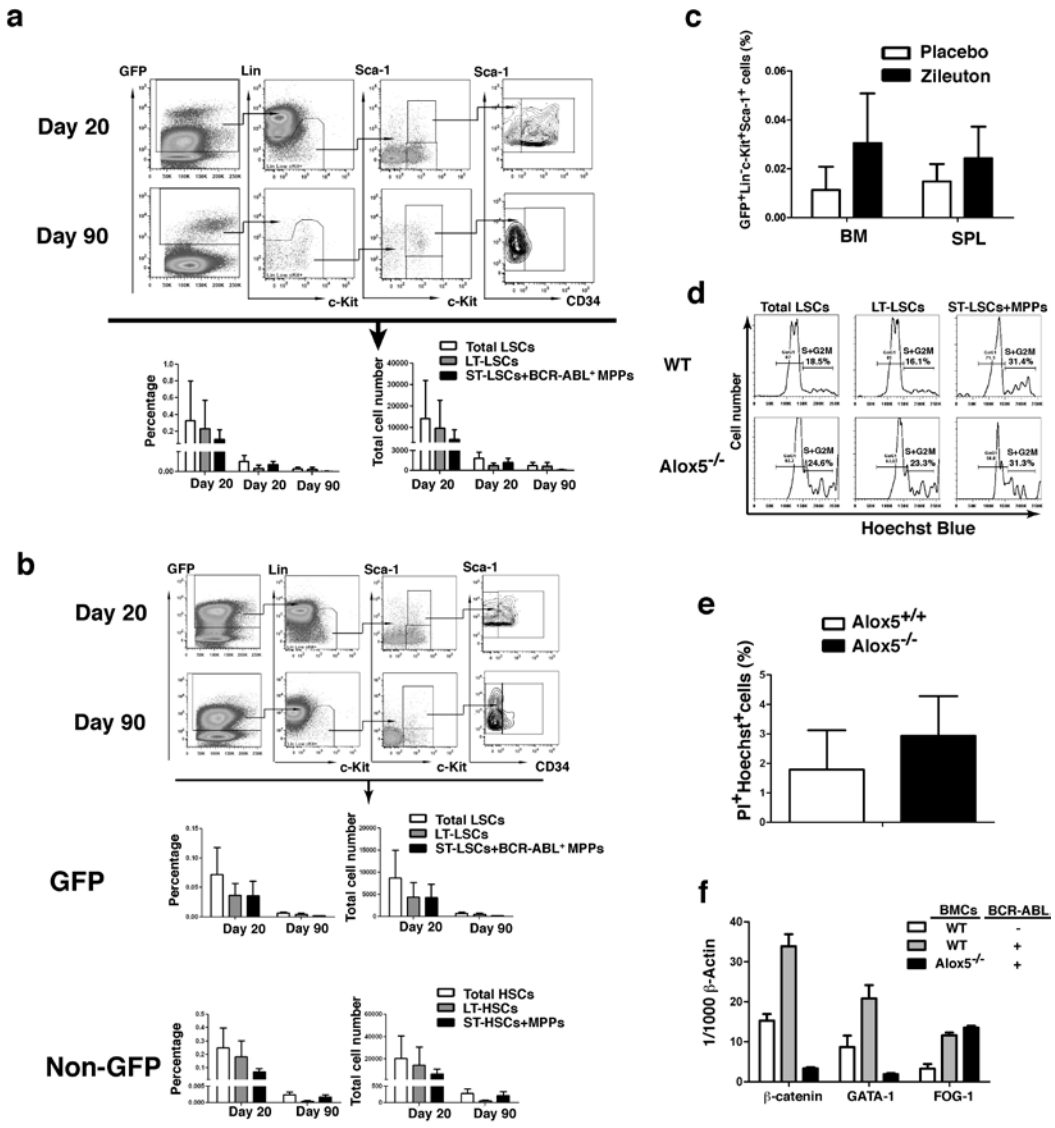


Figure 4. Loss of Alox5 function blocks differentiation of LT-LSCs.

3.3.5 Inhibition of 5-LO prolongs survival of CML mice

Zileuton treatment suppressed LSCs in CML mice (Figure 4). We examined whether 5-LO serves as a potential target in LSCs for treating CML. Mice with BCR-ABL-induced CML were treated with a placebo, the 5-LO inhibitor Zileuton, imatinib alone, or two agents in combination. All placebo-treated mice developed and died of CML within 4 weeks after induction of CML. Zileuton inhibited Alox5 function in CML mice, as plasma level of LTB4 was decreased compared to placebo-treated CML mice (Figure 5a). As expected, imatinib treatment was effective in treating CML, but Zileuton treatment was even more effective (Figure 5b). Treatment of CML mice with both Zileuton and imatinib had a better therapeutic effect than with either Zileuton or imatinib alone in prolonging survival of the mice (Figure 5b). Prolonged survival of Zileuton-treated CML mice correlated with less severe leukemia cell infiltration to the lungs and the spleens (Figure 5c). In peripheral blood of CML mice treated with Zileuton and imatinib, $GFP^+Gr\text{-}1^+$ leukemia cells gradually decreased with treatment, and dropped from over 50% to less than 2%, as analyzed at day 60 after induction of CML (Figure 5d). Although these mice eventually died (Figure 5d), FACS analysis barely detected any $GFP^+Gr\text{-}1^+$ myeloid leukemic cells in peripheral blood (data not shown), indicating that myeloid leukemia was eliminated. Instead, these mice developed ALL, as shown by the presence of GFP^+B220^+ leukemic cells in peripheral blood (data not shown). Zileuton treatment did not have an inhibitory effect on normal myeloid cells ($GFP^-Gr\text{-}1^+$) in peripheral blood of the same animals, as the number of these non-leukemia cells increased during the treatment (Figure 5e). In bone marrow of Zileuton-treated CML mice, $GFP^+Gr\text{-}1^+$

myeloid leukemia cells also dropped to low levels during the treatment (Figure 5f). Prolonged survival of CML mice by Zileuton treatment is consistent with the inhibitory effect of Zileuton on LSCs (Figure 4b). Zileuton treatment caused a reduction of white blood cell counts less dramatically than did imatinib treatment (Supplementary Figure 8), presumably because Zileuton targeted LSCs and imatinib inhibited more differentiated leukemic cells. Zileuton treatment also prolonged survival of mice with CML induced by BCR-ABL-T315I (Supplementary Figure 9).

Elevation of LTB4 level was observed in bone marrow cells of CML patients [169]. However, a later report from the same group showed that the level of leukotriene C4 (LTC4) synthase, which is a key enzyme in the biosynthesis of LTC4 and represents a different metabolic pathway from LTB4, was elevated in human CML cells. Therefore, we examined whether BCR-ABL stimulates LTC4 production in CML mice using an ELISA method. We did not observe an increase of plasma LTC4 levels in mice receiving BCR-ABL-expressing wild type bone marrow cells (BCR-ABL+Alox5$^{+/+}$) as compared to mice receiving BCR-ABL-expressing *Alox5* deficient bone marrow cells (BCR-ABL+Alox5$^{-/-}$). The mean level of LTC4 was 128.63 pg/ml for the *BCR-ABL+Alox5*$^{+/+}$ group and 154.84 pg/ml for the *BCR-ABL+Alox5*$^{-/-}$ group, respectively.

Figure 5. Inhibition of Alox5 prolongs survival of CML mice.

a. Zileuton treatment resulted in a reduction of the plasma LTB4 level in CML mice. **b.** Kaplan-Meier survival curves for CML mice treated with a placebo, Zileuton alone, imatinib alone, or both Zileuton and imatinib in combination. Zileuton-treated CML mice were much healthier than placebo-treated CML mice, and inhibition of Alox5 by Zileuton significantly prolonged survival of CML mice. **c.** Photomicrographs of haematoxylin and eosin-stained lung and spleen sections from CML mice treated with a placebo or Zileuton. **d.** FACS analysis showed gradual disappearance of $GFP^{+}Gr\text{-}1^{+}$ cells in PB of CML mice treated with Zileuton. **e.** In CML mice treated with Zileuton, $GFP^{+}Gr\text{-}1^{+}$ cells in PB gradually decreased with time, whereas the $GFP^{-}Gr\text{-}1^{+}$ cells that did not express BCR-ABL gradually increased, showing that inhibition of Alox5 with Zileuton significantly inhibited engraftment of BCR-ABL-expressing but not normal BM cells in the same animals. **f.** FACS analysis showed gradual disappearance of $GFP^{+}Gr\text{-}1^{+}$ cells in BM of CML mice treated with Zileuton.

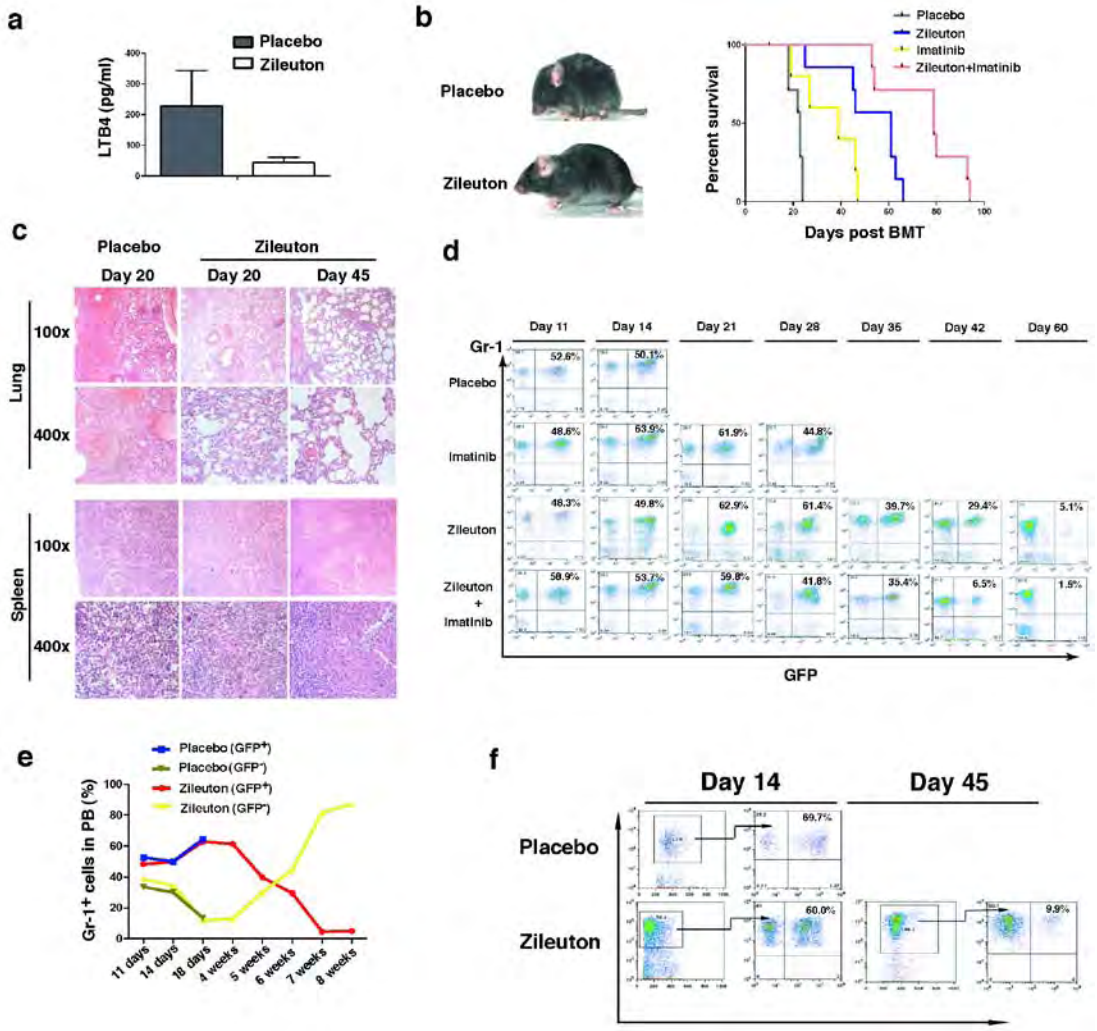


Figure 5. Inhibition of Alox5 prolongs survival of CML mice.

3.3.6 Alox5 deficiency does not significantly affect normal HSCs

Alox5 deficiency or inhibition of 5-LO by Zileuton specifically impaired the function of *Alox5*$^{-/-}$ LSCs but not non-BCR-ABL-expressing *Alox5*$^{-/-}$ HSCs in CML mice (Figures. 4, 5), showing that these *Alox5*$^{-/-}$ HSCs had stronger stem cell ability to engraft recipient mice than did *Alox5*$^{-/-}$ LSCs in the same animals. However, these results did not clearly show whether *Alox5* deficiency affected the function of normal HSCs. This question is important, because it is critical to know whether *Alox5* deficiency or inhibition of 5-LO lowers the function of HSCs below their normal level, especially if *Alox5* were considered as a therapeutic target. We characterized hematopoietic cell lineages in bone marrow and peripheral blood of *Alox5*$^{-/-}$ mice in comparison with those of wild type B6 mice. The percentages of HSCs (Lin$^-$c-Kit$^+$Sca-1$^+$), LT-HSCs (Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^-$), ST-HSCs (Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^+$Flt3$^-$) and MPPs (Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^+$Flt3$^+$) in bone marrow of *Alox5*$^{-/-}$ mice were slightly lower than those of wild type mice (Figure 6a), and the percentages of Gr-1$^+$, Mac-1$^+$, B220$^+$, or CD3E$^+$ cell populations in bone marrow of these mice were similar to those of wild type mice (Figure 6b). In peripheral blood, Gr-1$^+$ and Mac-1$^+$ cells in *Alox5*$^{-/-}$ mice were even higher than those in wild type mice (Figure 6b). These results demonstrated that *Alox5* deficiency did not lead to significant decrease in the numbers of hematopoietic cell lineages.

To compare the function of HSCs between *Alox5*$^{-/-}$ and wild type mice, we first performed engraftment assay, in which several doses of *Alox5*$^{-/-}$ or wild type bone marrow cells were transplanted into lethally irradiated wild type B6 mice. The engraftment ability of *Alox5*$^{-/-}$ bone marrow cells was slightly lower than that of wild type

bone marrow cells, as $5x10^4$ wild type bone marrow cells completely protected death of lethally irradiated recipient mice, whereas the same number of *Alox5*$^{-/-}$ bone marrow cells partially rescued the irradiated mice (Figure 6c). The minor engraftment defect of *Alox5*$^{-/-}$ bone marrow cells was further examined in a competitive reconstitution analysis, in which *Alox5*$^{-/-}$ (CD45.2) and wild type (CD45.1) bone marrow cells were 1:1 mixed and then transferred into recipient mice. 12 weeks after transplantation, the percentages of CD45.2 Gr-1$^+$ and Mac-1$^+$ cells were lower than those of CD45.1 Gr-1$^+$ and Mac-1$^+$ cells in peripheral blood (Figure 6d), indicating that *Alox5*$^{-/-}$ HSCs had slightly lower stem cell function as compared to wild type HSCs. However, the effect of *Alox5* deficiency on normal HSCs is much less than that on LSCs, as the competitive engraftment capability of the sorted *Alox5*$^{-/-}$ LSCs was about thirty fold lower than that of wild type LSCs as assayed at day 25 after CML induction (Figure 3c), comparing to the one fold difference between *Alox5*$^{-/-}$ and normal HSCs (Figure 6d). This was further supported by the observation that *Alox5*$^{-/-}$ LSCs failed to induce CML (Figure 3c). We also compared the numbers of bone marrow CMP, GMP, MEP, and CLP between wild type and *Alox5*$^{-/-}$ mice, and no significant differences were found (Figure 6e), suggesting that there were no significant functional defects in these progenitor cells. The minor functional defect of *Alox5*$^{-/-}$ HSCs promoted us to examine whether there were any cellular changes in cell cycle and gene expression of β-catenin, GATA-1 and FOG-1 in these cells. We found that there were slightly higher percentages of both LT-HSCs and ST-HSCs/MPPs in the S+G2M phase of the cell cycle in *Alox5*$^{-/-}$ bone marrow cells than in wild type bone marrow cells (Figure 6f), in contrast to the only increase in LT-LSCs in S+G2M phase of

the cell cycle in BCR-ABL-expressing *Alox5*$^{-/-}$ bone marrow cells (Figure 4d). This further indicates that there is no differentiation blockade in *Alox5*$^{-/-}$ HSCs and that *Alox5* deficiency has distinct biological effect on normal HSCs and LSCs. To support this idea, we compared the expressive levels of β-catenin, GATA-1 and FOG-1 in HSCs of wild type and *Alox5*$^{-/-}$ mice. We found that the minor functional defect of *Alox5*$^{-/-}$ HSCs was correlated with a decreased expression of GATA-1 and FOG-1, and that β-catenin expression was increased in *Alox5*$^{-/-}$ HSCs compared to that in wild type HSCs (Figure 6g). Different expression patterns of β-catenin, GATA-1 and FOG-1 in *Alox5*$^{-/-}$ HSCs (Figure 6g) and LSCs (Figure 4e) indicate that these three genes function differently in these two types of stem cells.

Figure 6. *Alox5* deficiency does not significantly affect normal HSCs.

a. Bone marrow cells from wild type and *Alox5*$^{-/-}$mice were analyzed by FACS for the percentages of total HSCs (Lin$^-$c-Kit$^+$Sca-1$^+$), LT-HSCs (Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^-$), ST-HSCs (Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^+$Flt3$^-$), and MPPs (Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^+$Flt3$^+$). **b.** Cells from bone marrow and peripheral blood of wild type and *Alox5*$^{-/-}$mice were analyzed by FACS for the percentages of Gr-1$^+$, Mac-1$^+$, B220$^+$, and CD3E$^+$ cells. **c.** Three doses ($1x10^5$, $5x10^4$, and $2.5x10^4$) of wild type or *Alox5*$^{-/-}$BM cells were injected into lethally irradiated recipient mice. Survival curves showed that there was only a minor engraftment defect of BM in *Alox5*$^{-/-}$mice. **d.** *Alox5*$^{-/-}$ (CD45.2) and wild type (CD45.1) BM cells were 1:1 mixed and then transferred into recipient mice (n=20). 12 weeks after BMT, FACS analysis was carried out to compare the percentages of wild type and *Alox5*$^{-/-}$Gr-1$^+$, Mac-1$^+$, B220$^+$, and CD3E$^+$ cells in PB of recipient mice. **e.** BM cells from BM of wild type and *Alox5*$^{-/-}$mice were analyzed by FACS for the numbers of CMPs, GMPs, MEP, and CLPs. **f.** Bone marrow cells were isolated from wild type or *Alox5*$^{-/-}$ mice, and stained with Hoechst Blue. DNA contents in total HSCs, LT-HSCs, and ST-HSCs+MPPs in the S+G2M phase of the cell cycle were examined by FACS. **g**. Bone marrow HSCs were sorted by FACS from wild type or *Alox5*$^{-/-}$ mice for isolation of total RNA, and expression of β-catenin, GATA-1 and FOG were detected by RT-PCR. Each bar represents the mean value of three samples.

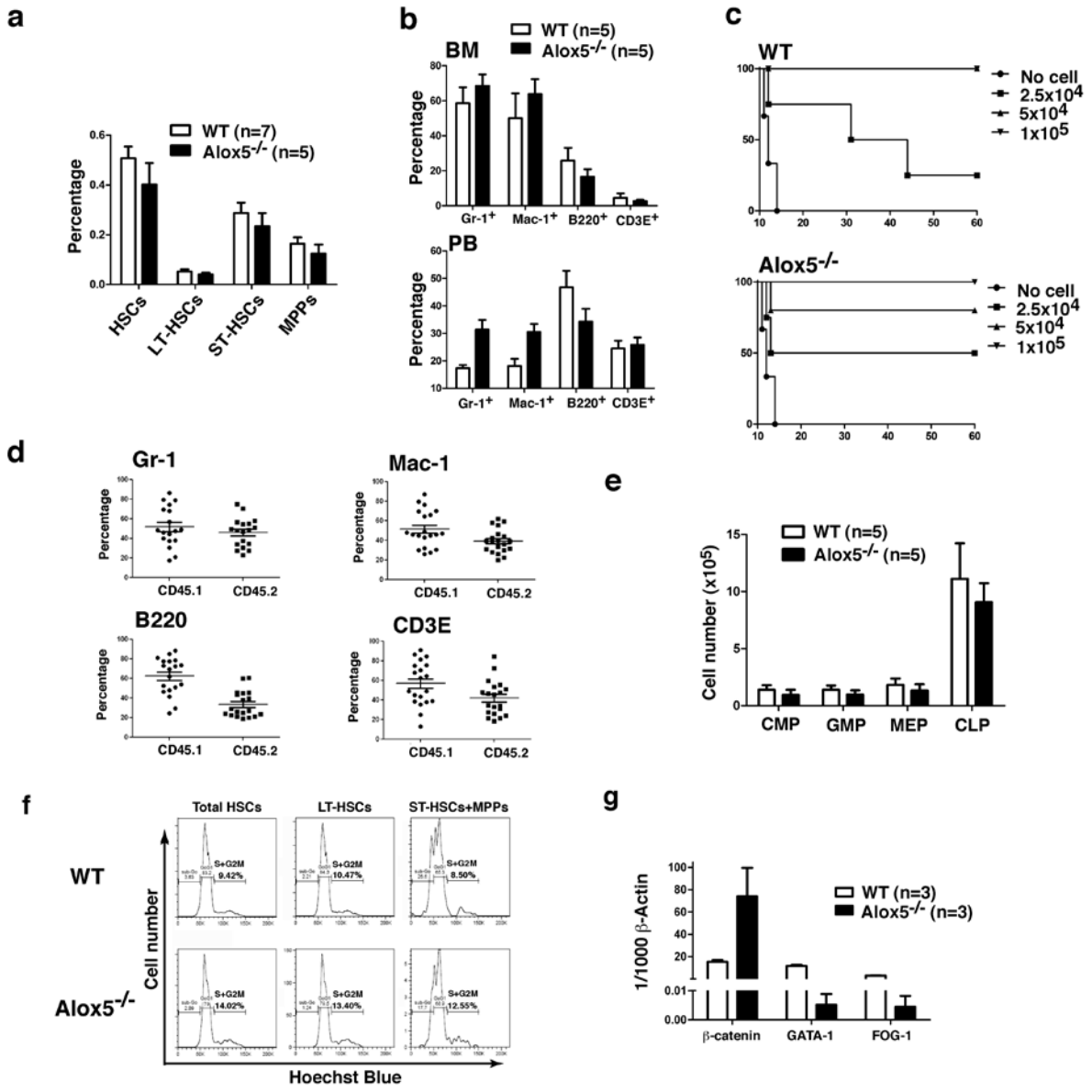


Figure 6. *Alox5* deficiency does not significantly affect normal HSCs.

3.3.7 Alox5 is not required for the induction of lymphoid leukemia by BCR-ABL

We tested whether *Alox5* plays a role in BCR-ABL-induced acute lymphoblastic leukemia (ALL), which originates from committed lymphoid progenitors [36]. We have previously shown that BCR-ABL-expressing pro-B cells serve as LSCs for this disease [26]. Wild type or *Alox5*$^{-/-}$ donor bone marrow cells in B6 background were transduced by BCR-ABL retrovirus followed by transplantation of the transduced cells into lethally irradiated wild type B6 recipient mice. Both groups of mice developed and died of ALL with similar disease latency and survival time (Figure 7a). FACS analysis of lymphoid leukemia cells in peripheral blood of these mice showed that both group of ALL mice had similar percentages (Figure 7b) and numbers (Figure 7c) of B220^{+} leukemia cells in peripheral blood. Together these results showed that *Alox5* did not contribute to the development of ALL induced by BCR-ABL, and suggest that *Alox5* is not required by ALL stem cells, but is specifically required by CML stem cells. Consistent with these findings, BCR-ABL did not up-regulate *Alox5* function, as the plasma level of LTB4 in recipients of *BCR-ABL*-transduced wild type bone marrow cells was not significantly higher compared to recipients of *BCR-ABL*-transduced *Alox5*$^{-/-}$ bone marrow cells (Figure 7d).

Figure 7. *Alox5* is not required for the induction of lymphoid leukemia by *BCR-ABL*.

a. Kaplan-Meier survival curves for recipients of *BCR-ABL*-transduced bone marrow cells from wild type (n=6) or *Alox5*$^{-/-}$ (n=8) donor mice. Both groups of mice developed and died of ALL. **b.** At day 14 post BMT, FACS analysis showed no difference in the percentages of GFP^{+}B220^{+} cells in PB of recipients of *BCR-ABL*-transduced bone marrow cells from wild type or *Alox5*$^{-/-}$ donor mice. **c.** FACS analysis showed similar numbers of GFP^{+}B220^{+} cells in PB of recipients of *BCR-ABL*-transduced bone marrow cells from wild type or *Alox5*$^{-/-}$ donor mice. **d.** The plasma levels of LTB4 in recipients of *BCR-ABL*-transduced bone marrow cells from wild type or *Alox5*$^{-/-}$ donor mice were compared. Loss of *Alox5* did not result in a reduction of the plasma LTB4 level in ALL mice.

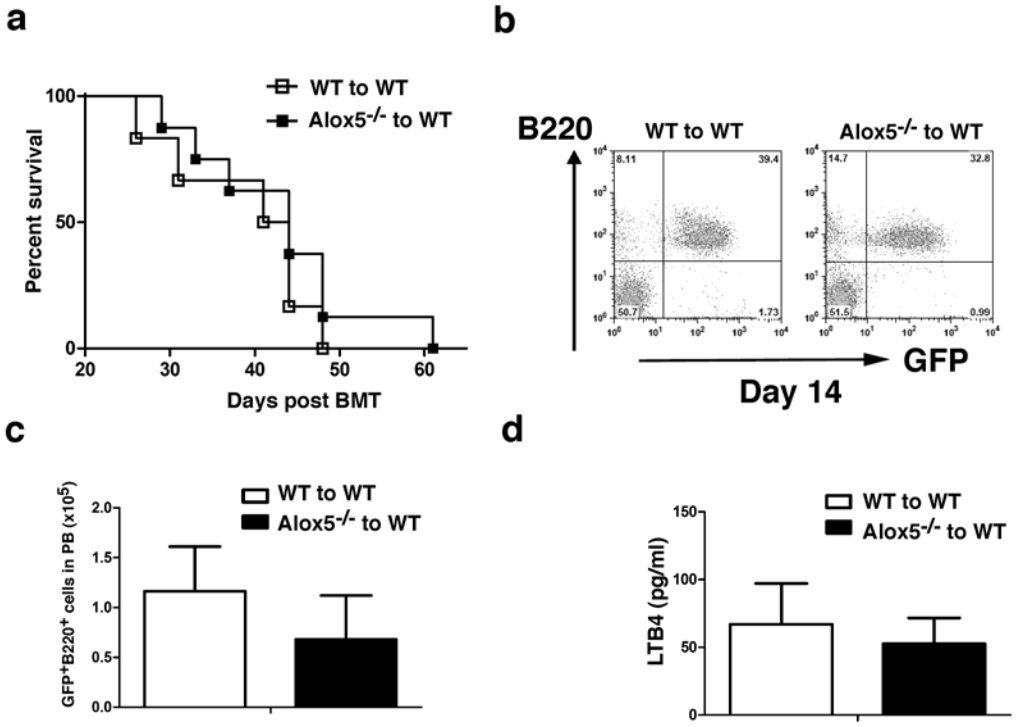


Figure 7. *Alox5* is not required for the induction of lymphoid leukemia by *BCR-ABL*.

3.4 Discussion

The specific role of the *Alox5* gene in regulating function of LSCs but not normal HSCs proposes a new mechanism for how cancer and normal stem cells distinctly self-renew and differentiate, and provides a potential novel strategy for curative therapy of CML induced by BCR-ABL. Although *Alox5* and its related pathways were identified in LSCs of CML, they may play a critical role in regulating LSCs of myeloid leukemias induced by other types of oncogenes. This idea is supported by our preliminary finding that the development of Tel-PDGFR-beta induced myeloid leukemia was also largely prevented in the absence of the *Alox5* gene (data not shown). In addition, similar phenomenon that a gene specifically regulates cancer stem cells but not normal stem cells may likely be found in other types of cancers.

The mechanism for the specific role of the *Alox5* gene in regulating function of LSCs but not normal HSCs remains to be further elucidated. We showed the differential regulation of the beta-catenin gene in wild type and *Alox5* deficient LSCs, partially explaining the specific inhibitory effect of *Alox5* deficiency on LSCs but not on normal HSCs. It is reasonable to think that these two types of stem cells utilize different pathways for self-renewal and differentiation, and *Alox5* represents a critical difference in these stem cells. It is likely that BCR-ABL stimulates transcription of *Alox5* to jointly form a unique pathway critical for LSC function. Because the transcriptional activation of *Alox5* by BCR-ABL is not inhibited by the BCR-ABL kinase inhibitor imatinib, this at least partially explains why imatinib does not inhibit LSCs in CML mice [26]. It is hopeful

that targeting *Alox5* in combination with a BCR-ABL inhibitor that eliminates BCR-ABL protein, such as a heat shock protein 90 inhibitor [125], would lead to a better control of CML. 5-LO inhibitor and imatinib could also be a good combination, with one inhibiting LSCs and another eliminating differentiated leukemia cells.

No studies have shown a role of *Alox5* in regulation of LSCs. Our findings in this study first demonstrate that *Alox5* is a critical regulator of LSCs in CML, providing an *in vivo* system for investigating underlying molecular mechanisms. Human CML microarray studies have shown that *Alox5* is differentially expressed in $CD34^+$ CML cells [28,29], suggesting a role of *Alox5* in human CML stem cells. An *in vitro* study also supports the role of *Alox5* in CML, and in this study the treatment of CML blast cells in culture with 5-LO inhibitors reduced cell proliferation [93], although genetic approach is required to rule out any off-target effects. Our study shows complete eradication of myeloid leukemia in mice by removing and inhibiting 5-LO, prompting us to test this novel therapeutic strategy in human CML patients. *Alox5* function has been linked to many important signaling pathways such as p53 [82], NF-kB [87], and PI3K [87] that is regulated by Pten, which has been shown to play a critical role in AML stem cells in mice [161]. Thus, inhibition of *Alox5* function may hold a promise for treating other types of malignant diseases. In addition, complete blockade of LSCs in CML development by inhibiting Alox5 function also suggests that *Alox5* may activate unknown pathways, and investigation of this *Alox5* network is critical to understand the mechanisms by which LSCs survive, self-renew, and differentiate. By DNA microarray analysis, we showed the differential expression of 50 genes regulated by *Alox5*, providing clues for further

elucidating the mechanisms by which *Alox5* regulated LSC function. Besides LSCs in CML, specific signaling networks are likely found in other cancer stem cells, relative to their normal stem cell counterparts.

3.5 Materials and Methods

Mice. C57BL/6J-CD45.1, C57BL/6J-CD45.2, and homozygous *Alox5* knockout (*Alox5*$^{-/-}$) mice in C57BL/6 background were obtained from The Jackson Laboratory. Mice were maintained in a temperature- and humidity-controlled environment and given unrestricted access to 6% chow diet and acidified water. *Alox5*$^{-/-}$ mice have normal hematopoietic cell counts in bone marrow and peripheral blood[83].

Flow cytometry and identification of leukemia and normal hematopoietic cell lineages. Hematopoietic cells were collected from bone marrow and peripheral blood of the normal and diseased mice, and red blood cells were lysed with NH_4Cl red blood cell lysis buffer (pH 7.4). The cells were washed with PBS, and stained with B220-PE for B cells, Gr-1-APC for neutrophils, Mac-1-PE for macrophage, CD3E-APC for T cells, and Sca-1-PE-cy7/c-Kit-APC-cy7/CD34-APC/CD135 (Flt3)-PE for hematopoietic stem cells. After staining, the cells were washed once with PBS and subjected to FACS analysis.

Bone marrow transduction/transplantation. The retroviral vector MSCV-IRES-eGFP carrying the p210 BCR-ABL cDNA and retroviral transduction and transplantation of mouse bone marrow cells for induction of CML and ALL by BCR-ABL had been described previously[125,149].

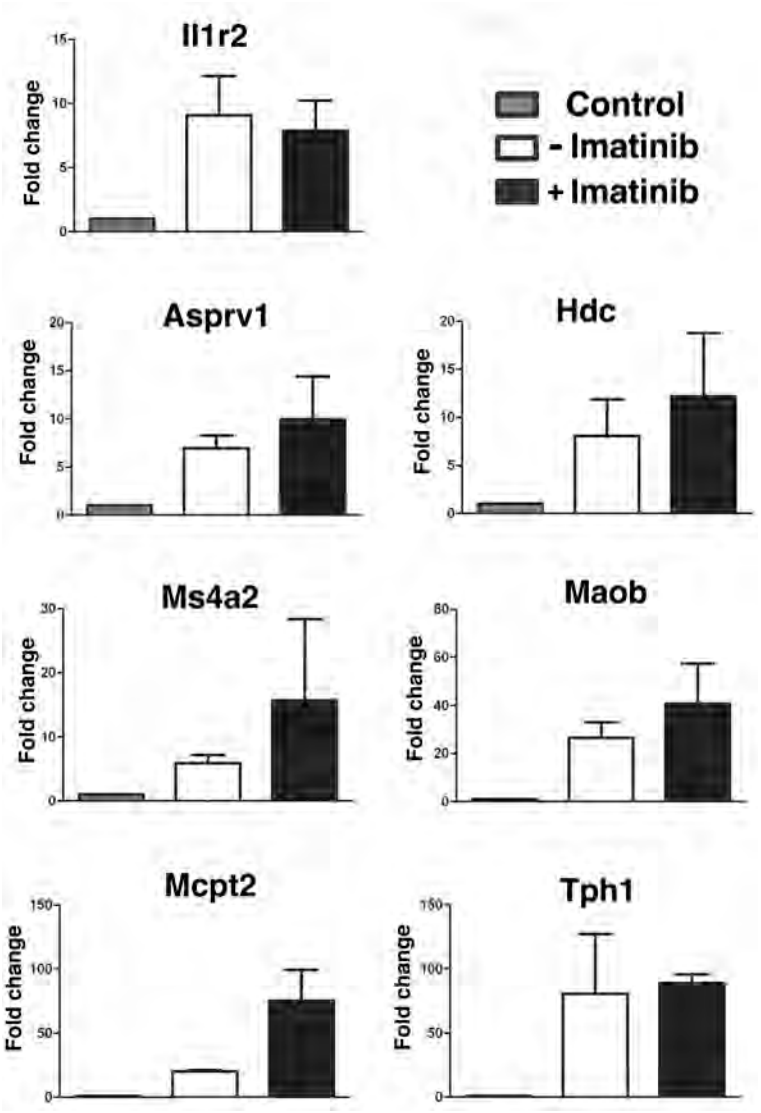
Measurement of plasma LTB4. Plasma of BCR-ABL induced CML mice were collected on day 20 post induction of the disease, and stored at the -80 ^{o}C before use. An enzyme immunoassay was carried out to measure the LTB4 levels in plasma using an ELISA kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer's instruction. Briefly, 50 ul of each standard or plasma sample was added to each well of 96-well plates, followed by adding 50 ul of LTB4 AchE trancer to each well. Then, 50 ul of LTB4 EIA antiserum was added to each well, and incubated the plates at 4 ^{o}C overnight. Next day, the plates were read and the results were analyzed.

Western blot analysis and antibodies: Antibodies against c-ABL, β-actin, and 5-LO□ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protein lysates were prepared by lysing cells in RIPA buffer, and Western blotting was carried out as described previously [4].

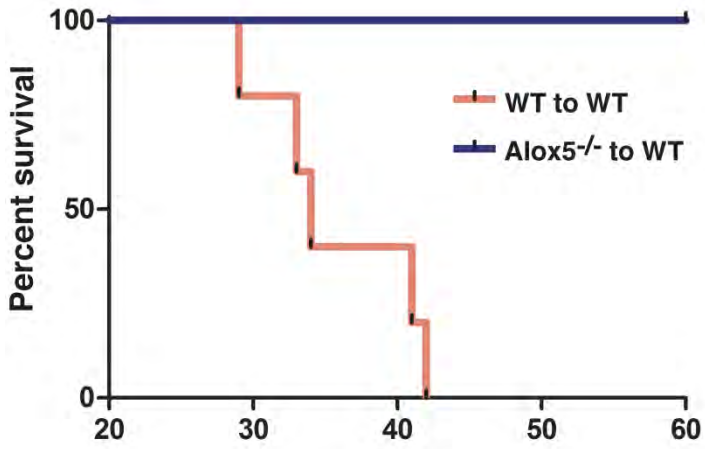
Real-time PCR. Total mRNA was isolated from HSCs or LSCs isolated by the FACS sorting. cDNA was synthesized using the Ovation-Pico cDNA synthesis method. Real-time PCR (RT-PCR) was performed to detect expression of β-catenin, GATA-1 and FOG-1 with the following primers: β-catenin forward primer 5'AACAGGGTGCTATTCCACGACTA-3' and reverse primer 5'-TGTGAACGTCCCGAGCAA-3'; GATA-1 forward primer 5'-ACTGTGGAGCAACGGCTACT-3' and reverse primer 5'-TCCGCCAGAGTGTTGTAGTG-3'; and FOG-1 forward primer 5'-CATAGAGGAGCCCCCAAGTC-3' and reverse primer 5'-

GGCTGCCTCTTCTTCCTTTT-3'. The Power SYBR Green PCR master mix was from Applied Biosystems. The numbers of copies of β-catenin, GATA-1 and FOG-1 mRNA relative to 1,000 copies of β-actin mRNA reflect the abundance of mRNA copies detected.

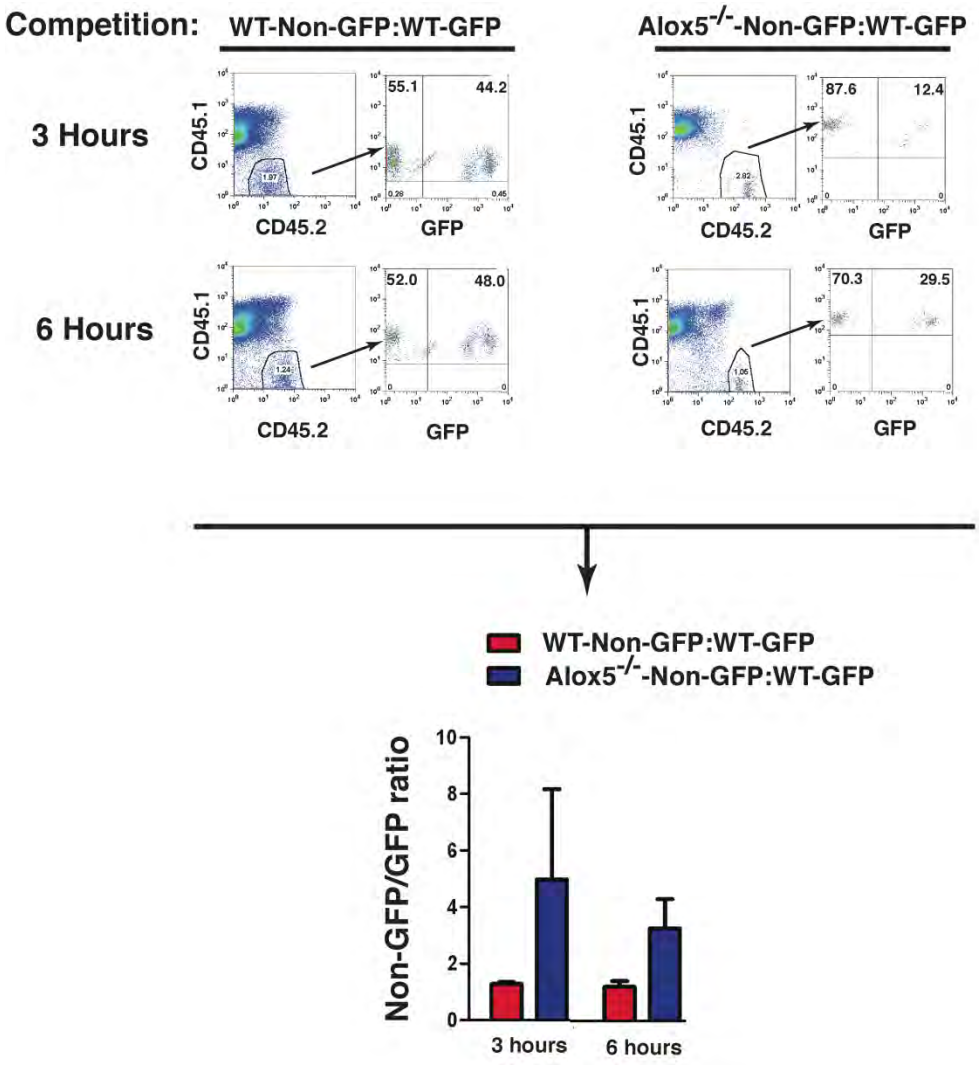
Drug treatment. Zileuton (Critical Therapeutics. Inc) and Imatinib (Novartis) were dissolved in water. The drugs were given orally in a volume of less than 0.3 ml by gavage (300 mg/kg, twice a day for Zileuton, and 100 mg/kg, twice a day for Imatinib) beginning at 8 days after bone marrow transplantation, and continuing until the morbidity or death of the leukemia mice. Water was used as placebo.



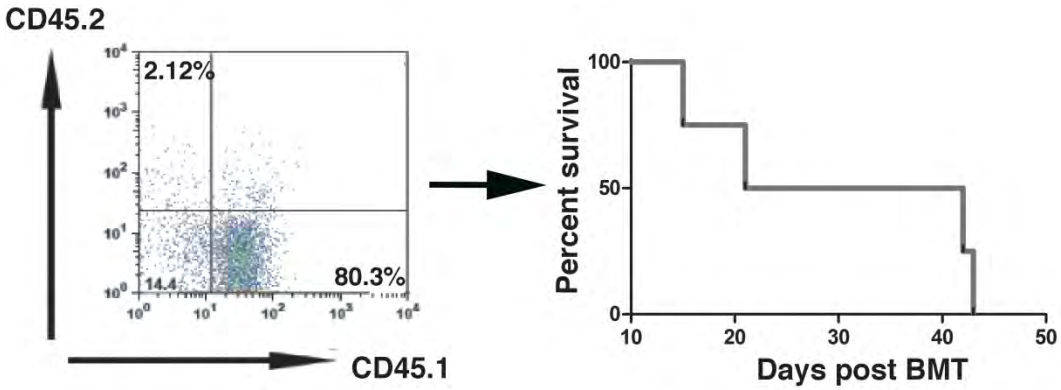
Supplementary Figure 1. Genes that are up-regulated by BCR-ABL in LSCs and not changed in expression following imatinib treatment. Bone marrow cells from C57BL/6 mice (B6) were transduced with retrovirus containing BCR-ABL/GFP or GFP alone to induce CML as described in **Figure 1a**. A group of these mice were treated with imatinib as described in **Figure 1a**. Bone marrow cells were isolated from the mice, and were sorted by FACS for $GFP^{+}Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{+}$ cells. Total RNA was isolated from these sorted cells for comparing *Alox5* expression between GFP vector-transduced normal stem cells and BCR-ABL-transduced LSCs by DNA microarray.



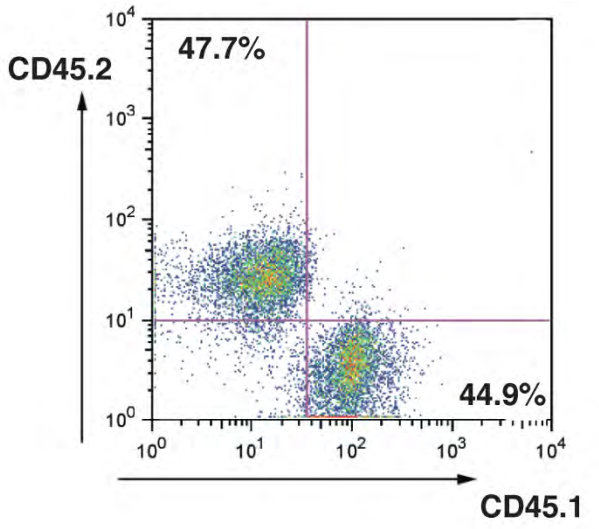
Supplementary Figure 2. Loss of *Alox5* causes failure of BCR-ABL-expressing BM cells to induce CML in secondary recipient mice. Kaplan-Meier survival curves for secondary recipients of $2x10^6$ bone marrow cells from mice receiving *BCR-ABL*-transduced wild type (n=5) or *Alox5*$^{-/-}$ (n=6) donor BM cells.



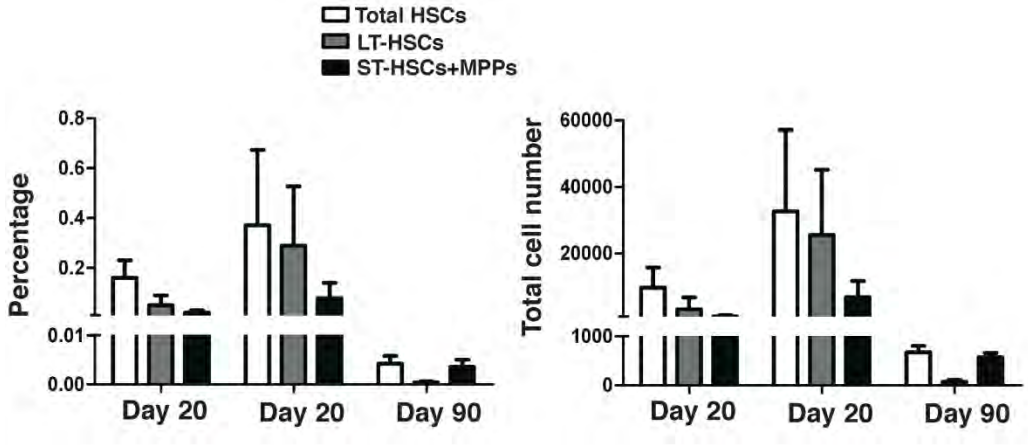
Supplementary Figure 3. *Alox5*$^{-/-}$ bone marrow cells do not have a homing defect. Bone marrow cells ($6x10^6$) from GFP mice (CD45.2) were 1:1 mixed with either bone marrow cells from wild type B6 mice (CD45.2) or those from *Alox5*$^{-/-}$ mice (CD45.2), and then transferred by tail vein injection into each wild type recipient mouse (CD45.1). 3 or 6 hours after the transplantation, By FACS analysis, CD45.2$^+$ bone marrow cells, representing the donor cells, were first identified and then analyzed for the percentages of GFP$^+$ and GFP$^-$ populations. The ration of non-GFP and GFP populations were shown.



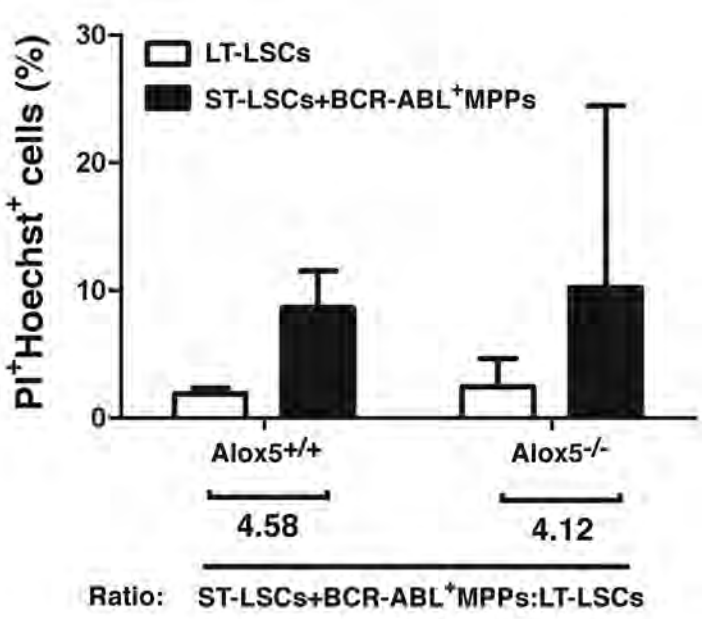
Supplementary Figure 4. Loss of *Alox5* impairs the function of LSCs. BCR-ABL-expressing wild type ($CD45.1^+$) and *Alox5*$^{-/-}$ ($CD45.2^+$) BM cells were 1:1 mixed ($5x10^5$ each), followed by transplantation into lethally irradiated recipient mice. At 40 day after BMT, more than 80% of cells in PB were wild type ($CD45.1^+$) leukaemia cells, and all these mice died of CML.



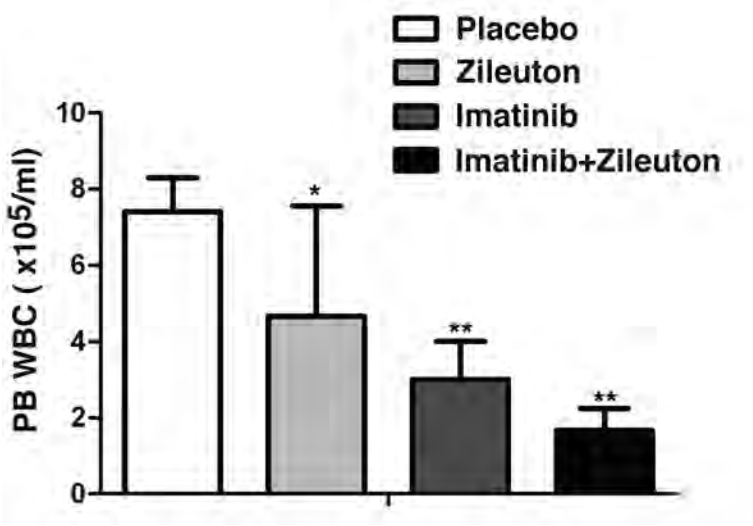
Supplementary Figure 5. Loss of Alox5 does not impair the function of normal stem cells. Alox5$^{-/-}$ (CD45.2) and wild type (CD45.1) BM cells were 1:1 mixed and then transferred into lethal recipient mice. 4 weeks after BMT, FACS analysis was carried out to compare the percentages of wild type and Alox5$^{-/-}$ cells in BM of the recipient mice.



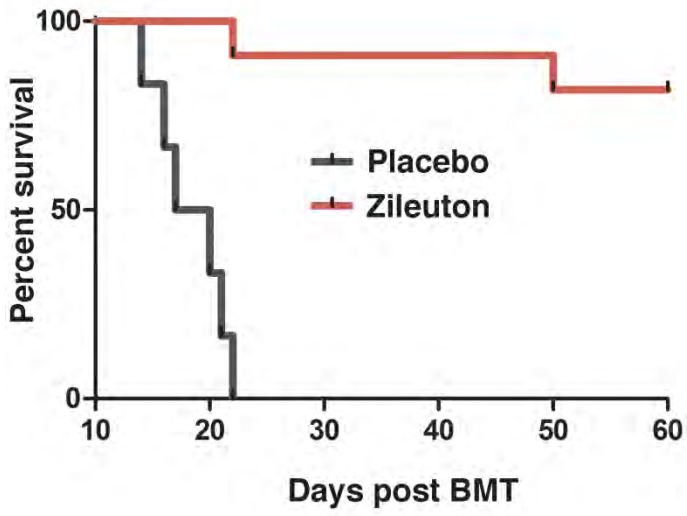
Supplementary Figure 6. *Alox5* deficiency does not lead to blockade of differentiation of normal LT-HSCs. Bone marrow cells were isolated from recipients of *BCR-ABL*-transduced bone marrow cells from wild type or *Alox5*$^{-/-}$ donor mice, and GFP$^-$ cell population (representing normal hematopoietic cells in CML mice) were analyzed by FACS analysis. At 90 days after induction of CML, the percentages and total numbers of LT-HSCs (GFP$^-$Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^-$) were much lower than those of ST-HSCs /MPP cells (GFP$^-$Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^+$) in a similar degree in mice receiving either BCR-ABL-transduced wild type or *Alox5*$^{-/-}$ donor bone marrow cells. These results indicate that *Alox5* deficiency does not lead to blockade of differentiation of normal LT-HSCs.



Supplementary Figure 7. Analysis of apoptosis of LSCs in wild type and *Alox5*$^{-/-}$ mice. At day 14 after BMT, bone marrow cells were isolated from recipients of *BCR-ABL*-transduced bone marrow cells from wild type or *Alox5*$^{-/-}$donor mice. The cells were stained with PI and Hoechst Blue, and the percentages of LT-LSCs ($GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+CD34^-$) and ST-LSCs /BCR-ABL-expressing MPP cells ($Lin^-c\text{-}Kit^+Sca\text{-}1^+CD34^+$) that were positive for PI and Hoechst Blue were determined by FACS. The ratios between ST-LSCs /BCR-ABL-expressing MPP cells and LT-LSCs in the presence and absence of *Alox5* were compared.



Supplementary Figure 8. Comparison of white blood cell (WBC) counts. CML mice were treated with a placebo, Zileuton alone, imatinib alone, or both Zileuton and imatinib in combination as described in Figure 4b. WBCs were collected from the treated mice 14 days after induction of CML.



Supplementary Figure 9. Inhibition of Alox5 prolongs survival of mice with CML induced with BCR-ABL-T315I. Kaplan-Meier survival curves for CML mice treated with a placebo (n=6), or Zileuton (n=11).

Gene	GeneBank accession	Forward primer	Reverse primer
β-catenin	NM_007614	5'-AACAGGGTGCTATTCCACGACTA-3'	5'-TGTGAACGTCCCGAGCAA-3'
GATA-1	NM_008089	5'-ACTGTGGAGCAACGGCTACT-3'	5'-TCCGCCAGAGTGTTGTAGTG-3'
FOG-1	NM_009569	5'-CATAGAGGAGCCCCCAAGTC-3'	5'-GGCTGCCTCTTCTTCCTTTT-3'

Supplementary Table. 1. Sequence of the primers used in real-time quantitative PCR assays.

Chapter IV

A tumor suppressor role for the *Msr1* gene in leukemia stem cells of chronic myeloid leukemia

4.1 Abstract

We have shown that *Alox5* is a critical regulator of leukemia stem cells (LSCs) in a *BCR-ABL*-induced chronic myeloid leukemia (CML) mouse model, and we hypothesize that the *Alox5* pathway represents a major molecular network that regulates LSC function. Therefore, we sought to further dissect this pathway by comparing the gene expression profiles of wild type and *Alox5*$^{-/-}$ LSCs derived from our mouse model for BCR-ABL-induced CML. DNA microarray analysis revealed a small group of candidate genes that exhibited changes in the levels of transcription in the absence of *Alox5* expression. In particular, we noted that the expression of the *Msr1* gene was up-regulated in *Alox5*$^{-/-}$ LSCs, suggesting that *Msr1* plays a tumor suppressor role in LSCs. Using our CML mouse model, we show that *Msr1* is down-regulated by *BCR-ABL* and this down-regulation is partially restored by *Alox5* deletion, and that *Msr1* deletion causes acceleration of CML development. Moreover, *Msr1* deletion markedly increases LSC function through its effects on cell cycle progression and apoptosis. We also show that *Msr1* affects CML development by regulating the PI3K-AKT pathway and *β-catenin*. Together, these results demonstrate that *Msr1* plays a tumor suppressor role in LSCs. The enhancement of *Msr1* function may be of significance in the development of novel therapeutic strategies targeting CML.

4.2 Introduction

Cancer stem cells (CSCs) are believed to be promising targets with the potential to offer curative therapies for some types of cancer, especially leukemias. A number of genes and their pathways, including *Wnt/β-catenin*[27,64], *Hedgehog*[81], *Notch*[156], *Bim-1*[158], *p53*[156], *p16*[160] and *p19*[170], *Pten* [161], *PML* [122], *PP2A* [133], *Alox5* [37], *TGF-beta/FOXO* [114] and *Musashi* [171], have been found to promote or inhibit CSC proliferation. Some of these genes and pathways also play similar roles in regulating the function of normal stem cells[64,108,160]. There is a need to identify CSC-specific genes for developing anti-stem cell strategies. Chronic myeloid leukemia (CML) is a stem cell-derived hematologic malignancy and serves as a good disease for the study of CSC behavior and function. We have previously identified *BCR-ABL*-expressing $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ cells as leukemia stem cells (LSCs) in CML mice [26]. We have demonstrated the critical role of the *Alox5* gene in functional regulation of LSCs [37]. We believe that the *Alox5* pathway represents a major pathway that regulates the function of LSCs, and it will be important to identify other genes that interact with *Alox5*.

In theory, genes regulating LSC function can be grouped in two categories: those that enhance or promote LSC function or those that suppress and/or negatively regulate LSC function. Genetic deletions and/or mutations in the latter group are likely to favor leukemogenesis and a few genes in this category have been identified in LSCs. For example, *Pten* is down-regulated by *BCR-ABL* in LSCs. *Pten* deletion accelerates CML development and delays the disease upon over-expression [106]. *p53* is also down-regulated

by *BCR-ABL* and loss of *p53* accelerates CML development [48,172]. In this study, we identified that macrophage scavenger receptor (*Msr1*) functions as a tumor suppressor in *BCR-ABL*-expressing LSCs and CML development. *Msr1* is a member of scavenger receptor family and is mostly expressed in hematopoietic cells [173]. *Msr1* is important for mediating host-cell interactions, macrophage adhesion and phagocytosis of apoptotic cells [115]. Using *Msr1*$^{-/-}$ mice, we show that *Msr1* delays CML development and regulates the self-renewal and differentiation capacity of LSCs through PI3K-AKT-GSK-3β and *β-catenin* pathways.

4.3 Results

4.3.1 Loss of *Msr1* accelerates CML development

We previously demonstrated that *Alox5* deficiency impairs the function of LSCs and prevents the initiation of *BCR-ABL*-induced CML. To identify the pathways in which *Alox5* gene regulates function of LSCs, we performed a comparative DNA microarray analysis using total RNA isolated from non-*BCR-ABL*-expressing Lin$^-$Sca-1$^+$c-Kit$^+$, *BCR-ABL*-expressing wild type LSCs and *BCR-ABL*-expressing *Alox5*$^{-/-}$ LSCs. Our data identified *Msr1* as a candidate gene important for LSC function, as it was down-regulated by *BCR-ABL* and further restored by loss of *Alox5* in LSCs (Figure 1a). This result was validated by quantitative real-time PCR analysis of non-*BCR-ABL*-expressing Lin$^-$Sca-1$^+$c-Kit$^+$, *BCR-ABL*-expressing wild type LSCs and *BCR-ABL*-expressing *Alox5*$^{-/-}$ LSCs (Figure 1b). To further demonstrate the regulation of *Msr1* by *BCR-ABL*, we compared the cell surface expression levels of MSR1 between LSCs and HSCs by FACS

and found that MSR1 was down-regulated by *BCR-ABL* (Figure 1c). This down-regulation was rescued by the loss of *Alox5* (Figure 1d). These results suggest that *Msr1* plays a tumor suppressor role in LSCs and CML development. To test whether *Msr1* affects CML development, we transduced bone marrow cells from 5-FU-treated wild type or *Msr1*$^{-/-}$ mice with *BCR-ABL-GFP* retrovirus and transplanted these cells into lethally-irradiated recipient mice. Recipients of *BCR-ABL*-transduced bone marrow cells from *Msr1*$^{-/-}$ donor mice developed CML significantly faster than those receiving *BCR-ABL*-transduced bone marrow cells from wild type donor mice (Figure 1e). The accelerated death of CML mice in the absence of *Msr1* also correlated with an elevated percentage of $GFP^{+}Gr1^{+}$ myeloid leukemia cells and an elevated number of total leukemia cells in the bone marrow and spleens of recipient mice (Figure 1f). Accelerated CML development in the absence of *Msr1* correlated with more severe infiltration of leukemia cells in the lung and spleen (Figure 1g and h). These results show a potent tumor suppressor role for *Msr1* in *BCR-ABL*-induced CML.

Figure 1. *Msr1* deletion accelerates CML development

a. Expression of the *Msr1* gene was up-regulated by *Alox5* deletion in LSCs as compared to the sorted wild type $GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells through DNA microarray. **b.** Expression of the *Msr1* gene was significantly up-regulated by *Alox5* deletion in LSCs when compared to wild type LSCs by RT-PCR. Mean value (±s.d.) for each group is shown (**: $p<0.01$). **c.** FACS analysis showed that MSR1 protein was down-regulated by *BCR-ABL* in LSCs when compared to $GFP^-Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells that did not express *BCR-ABL*. **d.** FACS analysis showed that MSR1 protein was up-regulated by *Alox5* deletion in CML stem cells when compared to wild type LSCs. **e.** Kaplan-Meier survival curves for recipients of *BCR-ABL*-transduced bone marrow cells from wild type or $Msr1^{-/-}$ donor mice. All recipients of *BCR-ABL*-transduced bone marrow cells from $Msr1^{-/-}$ donor mice developed CML and died within 3 weeks of bone marrow transplantation (days post BMT), whereas recipients of *BCR-ABL*-transduced bone marrow cells from wild type donor mice survived longer. **f.** FACS analysis showed a higher percentage of $GFP^+Gr\text{-}1^+$ cells in the bone marrow and spleens of recipients of *BCR-ABL*-transduced bone marrow cells from $Msr1^{-/-}$ than wild type donor mice (**: $p<0.01$).Gross pathology of the lungs and spleens showed severe lung hemorrhages and splenomegaly in recipients of *BCR-ABL*-transduced bone marrow cells from $Msr1^{-/-}$ donor mice. **g.** Gross pathology and **h.** hematoxylin/eosin staining of the lungs and spleens showed severe lung hemorrhages and splenomegaly in recipients of *BCR-ABL*-transduced bone marrow cells from $Msr1^{-/-}$ donor mice. Recipient receiving *pMSCV-GFP*-transduced bone marrow cells was shown as a control.

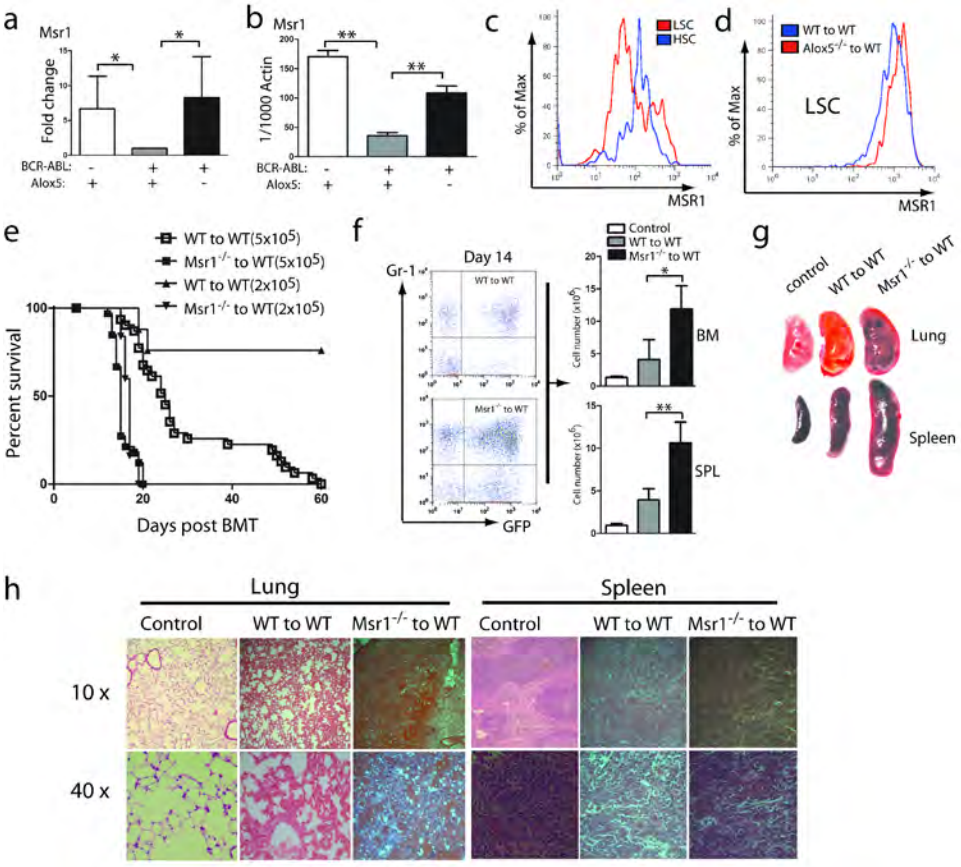


Figure 1. *Msr1* deletion accelerates CML development

4.3.2 *Msr1* over-expression delays CML development

To further test the tumor suppressor role of *Msr1* in CML development, we co-expressed *BCR-ABL* and *Msr1* in wild type bone marrow cells by retroviral transduction and transplanted the transduced cells into recipient mice to induce CML. The *BCR-ABL-GFP* expressing retrovirus was used as a control. The *BCR-ABL-IRES-Msr1-pMSCV* construct expressed *BCR-ABL* and MSR1 in 293T cells (Figure 2a). We next transduced wild type donor bone marrow cells with *BCR-ABL-Msr1* or *BCR-ABL-GFP* retrovirus and transplanted these cells into recipient mice. CML development was significantly slower in mice receiving bone marrow cells transduced with *BCR-ABL-Msr1-GFP* than in those receiving bone marrow cells transduced with *BCR-ABL-GFP*. Some recipient mice did not even develop the CML (Figure 2b). The delayed CML development correlated with fewer leukemia cells in peripheral blood (Figure 2c). In addition, FACS analysis of peripheral blood of recipients receiving *BCR-ABL-Msr1* transduced bone marrow cells showed that the percentage of Gr-1^+ myeloid leukemia cells declined with time (Figure 2d). These results further support the role of *Msr1* as a tumor suppressor in CML development.

Figure 2. *Msr1* over-expression causes a delay of CML development

a. *BCR-ABL* and MSR1 were detected by protein blotting using antibodies against ABL and MSR1. MSR1 protein was detected in cells transfected with *BCR-ABL-IRES-Msr1-pMSCV*. **b.** Kaplan-Meier survival curves for recipients of *BCR-ABL-IRES-pMSCV*-transduced bone marrow cells (n=7) or *BCR-ABL-IRES-Msr1-pMSCV*-transduced bone marrow cells (n=7) donor mice. FACS analysis showed that the total number **c**. and percentage **d.** of Gr-1^+ cells in peripheral blood of recipients of *BCR-ABL-IRES-pMSCV*-transduced bone marrow cells or *BCR-ABL-IRES-Msr1-pMSCV*-transduced bone marrow cells (*: $p<0.05$, **: $p<0.01$).

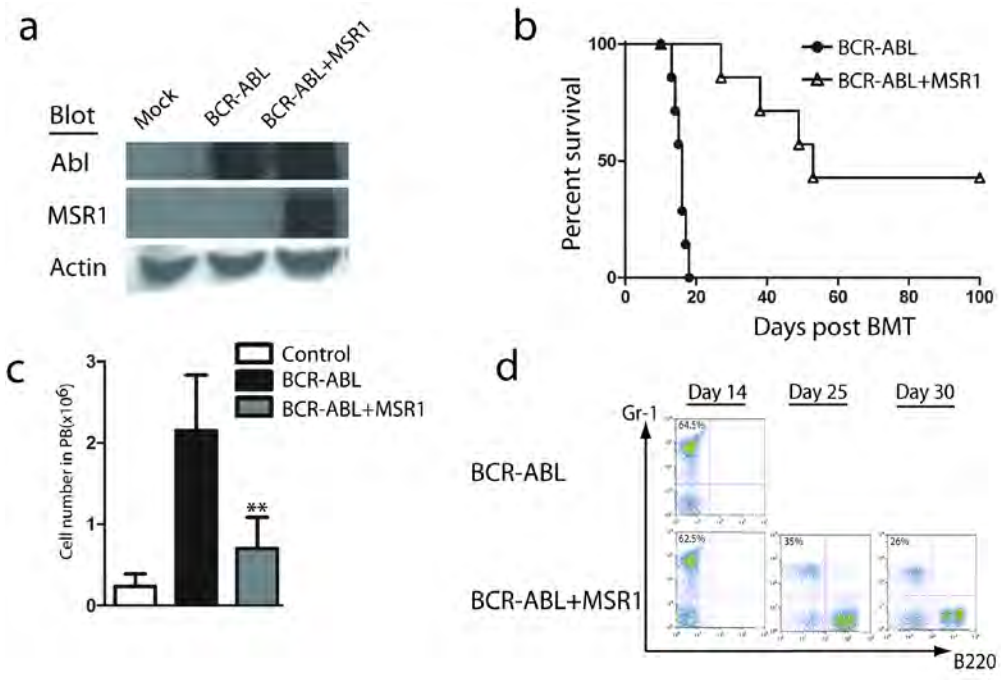


Figure 2. *Msr1* over-expression causes a delay of CML development

4.3.3 *Msr1* deletion causes an increase in LSC function

CML is a stem cell-derived disease and the accelerated development of CML in absence of *Msr1* (Figure 1) prompted us to examine whether *Msr1* regulates the function of LSCs. The accelerated CML development in the absence of *Msr1* could be caused by an increase in the number of LSCs. To test this hypothesis, we quantified LSCs in the bone marrow and spleens of CML mice at 8, 10 and 14 days after CML induction. At day 8, *Msr1* deficiency did not cause an increase of LSCs in either bone marrow or spleen as compared to CML mice receiving BCR-ABL tranduced wild type bone marrow cells. At day 14, a significant increase in *Msr1*-deficient bone marrow LSCs was observed (Figure 3a), but *Msr1* deficiency did not change the number of *Msr1*$^{-/-}$LSCs in the spleen of CML mice (Figure 3a).

The ability to transfer leukemia to next generation recipient mice can be used to examine the biological function of LSCs. We transduced wild type or *Msr1*$^{-/-}$ donor bone marrow cells with a lower titer *BCR-ABL* retrovirus and transferred the transduced cells to recipient mice to induce primary CML. At day 13 after CML induction, bone marrow cells from primary CML mice were transplanted into secondary recipient mice. Subsequently, leukemic bone marrow cells were serially transferred to recipient mice for two more generations. We found that *BCR-ABL*-expressing *Msr1*$^{-/-}$ bone marrow cells could transfer CML up to the fourth generation of recipient mice, while *BCR-ABL*-expressing wild type bone marrow cells only transferred CML once in recipient mice (Figure 3b). These results suggest that *Msr1* deficiency causes an increase in LSC

function. To further evaluate the effect of *Msr1* on LSC function, we sorted wild type (CD45.1) and *Msr1*$^{-/-}$ (CD45.2) LSCs from bone marrow of CML mice by FACS. The sorted wild type and *Msr1*$^{-/-}$ LSCs were mixed in a 1:1 ratio (5000 LSCs for each group), and were transplanted into recipient mice. We observed that at day 10, the percentages of GFP^{+}Gr-1^{+} wild type (CD45.1) and *Msr1*$^{-/-}$ (CD45.2) cells in peripheral blood of the mice were similar, and then gradually increased and eventually became a dominant leukemic cell population (Figure 3c). We also observed that *Msr1* deficiency did not cause an increase in the homing ability of bone marrow cells (Supplementary Figure 1), which could result in accelerated CML development. To explain how the loss of *Msr1* caused an increase in LSC function, we performed a DNA content analysis to examine the effect of *Msr1* deficiency on cell cycle progression of LSCs. We found that the percentage of LSCs in the S+G2M phase was significantly higher in the *Msr1*$^{-/-}$ group than in the wild type group (Figure 3d), indicating that the loss of *Msr1* causes more LSCs to enter the cell cycle, thereby enhancing the proliferation of LSCs. Furthermore, we examined whether *Msr1* deletion reduces apoptosis of LSCs by staining the cells with 7AAD and Annexin V. We observed that apoptosis of *Msr1*$^{-/-}$ LSCs was reduced when compared with wild type LSCs (Figure 3e). Thus, *Msr1* regulates cell cycle progression and apoptosis of LSCs.

Figure 3. *Msr1* deletion affects the function of LSCs

a. Loss of *Msr1* caused a significant increase in LSCs in bone marrow (*: $p<0.05$). Mean value (±s.d.) for each group is shown (*: $p<0.05$). **b.** Kaplan-Meier survival curves for a serial transplantation of recipients of $1x10^6$ bone marrow cells from mice receiving *BCR-ABL*-transduced wild type (n=5) or *Msr1*$^{-/-}$ (n=5) donor bone marrow cells. **c.** Bone marrow cells derived from CML mice induced by transplanting *BCR-ABL*-transduced wild type (CD45.1) or *Msr1*$^{-/-}$ (CD45.2) donor bone marrow cells were isolated. 5×10^3 wild type and *Msr1*$^{-/-}$ LSCs were sorted by FACS, mixed by 1:1 ratio, and transplanted into lethally irradiated secondary recipient mice. At days 10, 20, 25 and 30 after BMT, FACS analysis showed that the percentages of CD45.2$^+$ cells were much higher than those of CD45.1$^+$ cells. All these mice died of CML, presumably due to the development of CML from CD45.1$^+$ cells. **d.** At 14 days post transplantation, bone marrow cells were isolated from recipients of *BCR-ABL*-transduced bone marrow cells from wild type or *Msr1*$^{-/-}$ donor mice. The cells were stained with Hoechst Blue, and the DNA content, represented by the percentages of LSC populations in the S+G2M phase of the cell cycle, was examined by FACS. Mean percentage for each cell population (n=4) is shown (*: $p<0.05$). **e.** At 14 days post transplantation, bone marrow cells were isolated from recipients of *BCR-ABL*-transduced bone marrow cells from wild type or *Msr1*$^{-/-}$ donor mice. The cells were stained with 7AAD and Annexin V, and the percentages of LSCs positive for 7AAD and Annexin V, representing apoptotic cells, were determined by FACS.

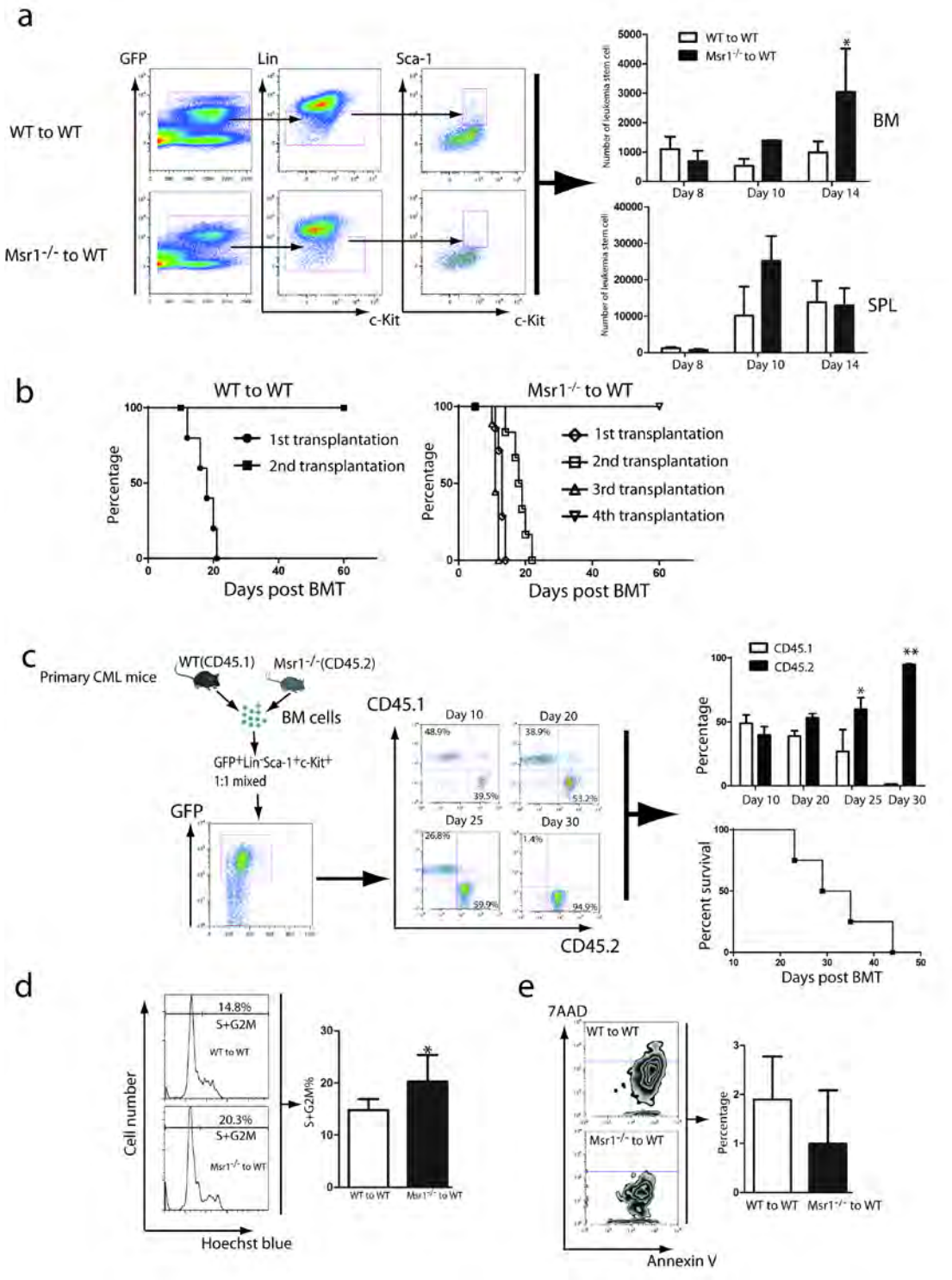


Figure 3. *Msr1* deletion affects the function of LSCs

4.3.4 Induction of *Msr1* expression by PMA causes inhibition of human CML cells and LSCs

Next, we tested whether *Msr1* inhibits human CML cells. Phorbol 12-myristate 13-acetate (PMA) induces differentiation of THP-1 cells, leading to a marked increase in the expression of MSR1 on the cell surface [174]. We found that the level of MSR1 was significantly increased in K562 cells treated with PMA for 48 hours (Figure 4a). We also noted that PMA significantly inhibited the proliferation of K562 cells (Figure 4b). The decreased proliferation of K562 cells was consistent with an increase in apoptosis (Figure 4c). We also tested whether PMA inhibits LSCs from CML *in vitro* under stem cell culture conditions. We cultured bone marrow cells from CML mice in the presence of imatinib (1 μM) or PMA (80 nM) for 3 days, and calculated the total number and percentage of LSCs that remained at the end of the culture based on FACS analysis and total cell counts (Figure 4d). We showed that PMA significantly inhibited LSCs *in vitro*. To further confirm that PMA treatment promotes LSC killing through induction of *Msr1*, we treated bone marrow cells from recipients receiving *BCR-ABL*-transduced bone marrow cells from *Msr1*$^{-/-}$ donor mice with PMA (80 nM) for 3 days and calculated the total number and percentage of LSCs that remained at the end of the culture based on FACS analysis and total cell counts. The percentage of *Msr1*$^{-/-}$LSCs wasn't reduced by PMA treatment although the total number of *Msr1*$^{-/-}$LSCs was reduced about 35% by drug treatment, which is still much lower than WT LSCs treated by PMA (Figure 4e).

Figure 4. PMA inhibits proliferation and induces apoptosis of human CML cells

a. FACS analysis showed that MSR1 protein was up-regulated in K562 cells treated with PMA for 48 h compared to untreated cells. **b.** PMA inhibits proliferation of K562 cells. K562 cells were treated with DMSO or PMA (80 nM) for 48 and 96 hours, and live cells were counted (**: $p<0.01$). **c.** PMA induces apoptosis of K562 cells. K562 cells were treated with DMSO or PMA (80 nM) for 48 hours. Apoptotic cells (Annexin $V^+/7AAD^+$) cells were analyzed by FACS (**: $p<0.01$). **d.** PMA inhibits LSCs from CML mice *in vitro*. Bone marrow cells isolated from mice with CML induced by *BCR-ABL-GFP* were cultured (2×10^6 cells/6cm plate) under stem cell conditions (see Methods) in the presence of DMSO, imatinib (1 μM)or PMA(80 nM) for 3 days, followed by FACS analysis of LSCs (*: $p<0.05$, **: $p<0.01$). **e.** *Msr1*$^{-/-}$ LSCs were treated by PMA in a stem cell culture condition. Bone marrow cells from recipients receiving *BCR-ABL*-transduced bone marrow cells from *Msr1*$^{-/-}$ donor mice were treated with PMA (80nM) for 3 days. The total number and percentage of LSCs that remained at the end of the culture were calculated by FACS analysis and total cell counts. The percentage of *Msr1*$^{-/-}$LSCs wasn't reduced by PMA treatment although the total number of *Msr1*$^{-/-}$LSCs was reduced about 35% by drug treatment, which is still much lower than WT LSCs treated by PMA.

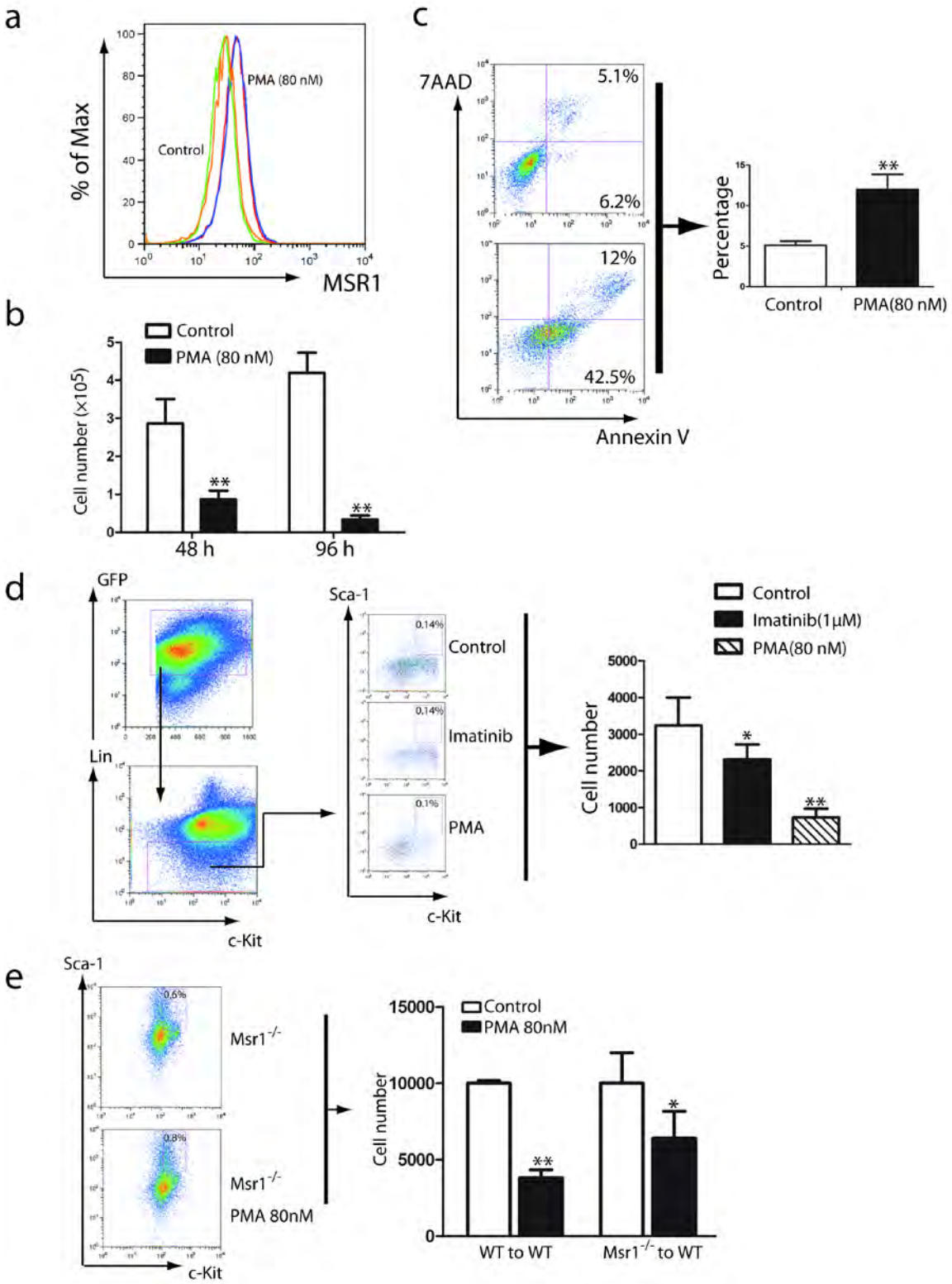


Figure 4. PMA inhibits proliferation and induces apoptosis of human CML

cells4.3.5 *Msr1* deletion does not affect the function of normal HSCs

We further tested whether the loss of *Msr1* affects the function of normal HSCs. We first compared hematopoietic cell lineages in bone marrow and peripheral blood of *Msr1*$^{-/-}$ mice with those of wild type mice. The percentages of HSCs (Lin$^-$c-Kit$^+$Sca-1$^+$), LT-HSCs (Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^-$), ST-HSCs (Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^+$Flt3$^-$) and MPPs (Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^+$Flt3$^+$) in bone marrow of *Msr1*$^{-/-}$mice were only slightly higher than those of wild type mice (Figure 5a), and the percentages of Gr-1$^+$, Mac-1$^+$, B220$^+$ or CD3E$^+$ cell populations in bone marrow were similar between wild type and *Msr1*$^{-/-}$ mice (Figure 5b). To compare the function of HSCs between *Msr1*$^{-/-}$ and wild type mice, we conducted an irradiation rescue assay, in which several doses of *Msr1*$^{-/-}$ or wild type bone marrow cells were transplanted into lethally-irradiated wild type mice. Survival of mice receiving wild type or *Msr1*$^{-/-}$ bone marrow cells was similar (Figure 5c). To more stringently compare the stem cell function, we carried out a competitive reconstitution analysis, in which wild type (CD45.1) and *Msr1*$^{-/-}$ (CD45.2) bone marrow cells were mixed in a 1:1 ratio and then transferred into recipient mice by tail vein or bone injection. Twelve weeks after the transplantation, the percentages of wild type (CD45.1) and *Msr1*$^{-/-}$ (CD45.2) were similar in peripheral blood of the mice (Figure 5d), indicating that *Msr1* did not suppress the function of normal HSCs.

Figure 5. *Msr1* deletion does not affect the function of normal HSCs

a. Bone marrow cells from wild type and *Msr1*$^{-/-}$ mice were analyzed by FACS for the percentages of total HSCs (Lin$^-$c-Kit$^+$Sca-1$^+$), LT-HSCs (Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^-$), ST-HSCs (Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^+$Flt3$^-$) and MPPs (Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^+$Flt3$^+$). **b.** Cells from bone marrow and peripheral blood of wild type and *Msr1*$^{-/-}$ mice were analyzed by FACS for the percentages of Gr-1$^+$, B220$^+$ and CD3E$^+$cells. **c.** Three doses (1×10^5, 5×10^4 and 2.5×10^4) of wild type or *Msr1*$^{-/-}$ bone marrow cells were injected into lethally-irradiated recipient mice. Survival curves showed that there was only a minor engraftment defect of bone marrow cells in *Msr1*$^{-/-}$ mice. **d.** *Msr1*$^{-/-}$ (CD45.2) and wild type (CD45.1) bone marrow cells were 1:1 mixed and then transferred into recipient mice (n=5). 12 weeks after transplantation, FACS analysis showed the percentages of wild type and *Msr1*$^{-/-}$ cells in peripheral blood of recipient mice.

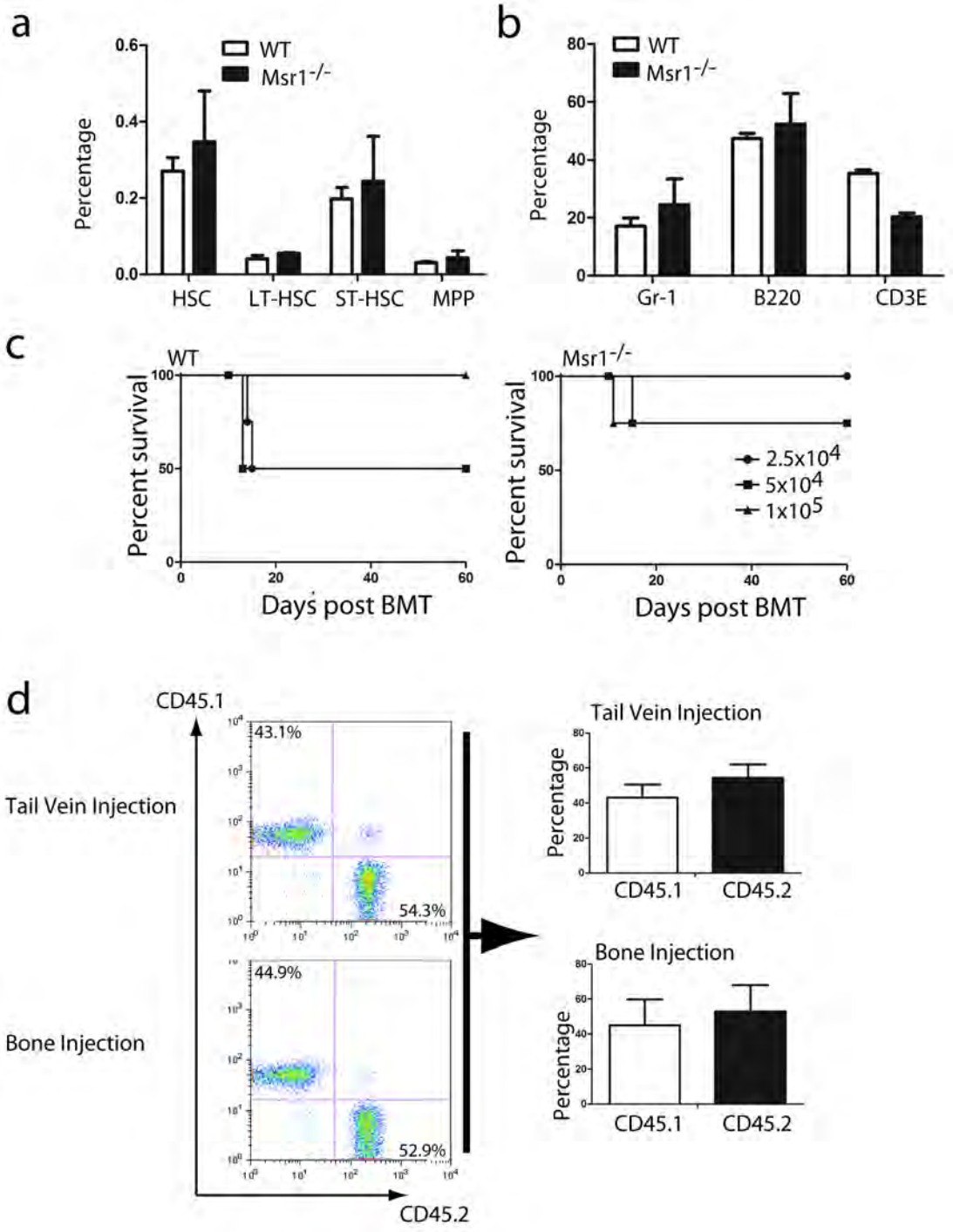


Figure 5. *Msr1* deletion does not affect the function of normal HSCs

4.3.6 *Msr1* affects CML development by regulating the PI3K-AKT-GSK-3β and β-catenin pathways

To determine the signaling pathways involved in *Msr1* function, we analyzed protein lysates isolated from the spleens of CML mice receiving *BCR-ABL*-transduced wild type or *Msr1*$^{-/-}$ donor bone marrow cells. Loss of *Msr1* resulted in a marked activation of the PI3K-AKT-GSK3β pathway and increased expression of β-Catenin (Figure 6a). Conversely, we tested whether the up-regulation of *Msr1* expression inhibits these signaling molecules by treating K562 cells with PMA. PMA markedly inhibited the activation of the PI3K-AKT and reduced expression of β-Catenin (Figure 6b). To confirm whether over-expression of *Msr1* inhibits those signal pathways in *BCR-ABL* expressing leukemic cells, we over-expressed *Msr1* gene in human leukemic cells. We made the *pMSCV-Msr1-GFP* construct and *pMSCV-GFP* retrovirus was used as a control (Supplementary Figure 2a). The *pMSCV-Msr1-GFP* construct expressed MSR1 and GFP while the *pMSCV-GFP* construct only expressed GFP in 293T cells (Supplementary Figure 2b). We next transduced human leukemia cells with *pMSCV-Msr1-GFP* or *pMSCV-GFP* retrovirus, sorted GFP^{+}K562 cells out and further confirmed over-expression of MSR1 in K562 cells by FACS and Western blot (Supplementary Figure 2c and Figure 6d). The over-expression of *Msr1* inhibited the activation of the PI3K-AKT, reduced expression of β-Catenin (Figure 6d) and further suppressed proliferation of leukemia cells (Figure 6e). Together, these results suggest that *Msr1* is a negative regulator between BCR-ABL-activated *Alox5* and the PI3K-AKT-GSK-3β and β-Catenin pathways (Figure 6f).

Figure 6. *Msr1* affects CML development by regulating PI3K-AKT-GSK-3β pathway and β-Catenin

a. *Msr1* regulates signaling pathways involved in the regulation of LSC functions. Spleens of recipients of *BCR-ABL*-transduced wild type or *Msr1*$^{-/-}$ donor bone marrow cells (n=4) at 14 days after transplantation were collected and protein lysates were prepared for expression of a group of signaling molecules. Loss of *Msr1* markedly activated the PI3K-AKT pathway and increased expression of β-Catenin. **b.** Quantification of relative protein expression levels in spleens of recipients of *BCR-ABL*-transduced wild type or *Msr1*$^{-/-}$ donor bone marrow cells. Mean value (±s.d.) for each group (n=4) is shown (*: $p<0.05$, **: $p<0.01$). **c.** PMA inhibits PI3K-AKT signaling pathways and β-Catenin expression in human CML cells. K562 cells were treated with PMA, and protein lysates were collected for expression of a group of signaling molecules. PMA markedly inhibits activity of PI3K-AKTand reduced expression of β-Catenin. **d.** Over-expression of *Msr1* gene inhibits PI3K-AKT signaling pathways and slightly reduces β-Catenin expression in human CML cells. K562 transduced with *pMSCV-Msr1-GFP* or *pMSCV-GFP* retrovirus were cultured and protein lysates were collected for expression of a group of signaling molecules. Over-expression of *Msr1* gene markedly inhibits activity of PI3K-AKTand reduced expression of β-Catenin. **e.** *Msr1* suppresses the proliferation of human leukemic cells. The same number of K562 cells transduced with *pMSCV-GFP* or *pMSCV-Msr1-GFP* was cultured in 24 wells plate. The live cells were counted at 24h, 48h and 96h (*: $p<0.05$, **: $p<0.01$). **f.** A summary of *Msr1* pathway in LSCs of CML.

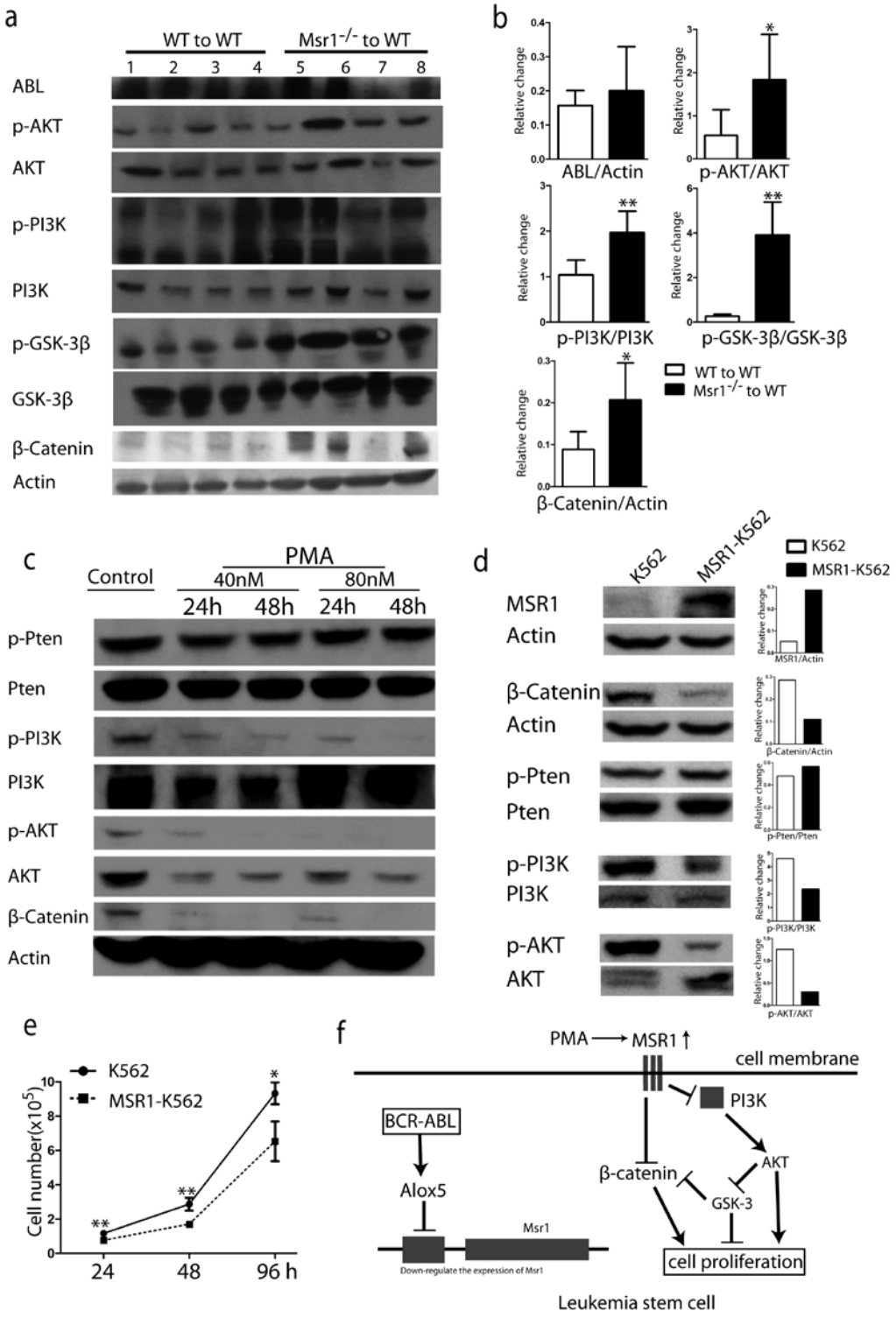


Figure 6. ***Msr1*** **affects CML development by regulating PI3K-AKT-GSK-3β pathway and** ***β-catenin***

4.3.7 *Msr1* does not affect *BCR-ABL*-induced lymphoid leukemia

We also tested whether *Msr1* affects *BCR-ABL*-induced acute lymphoblastic leukemia (ALL), which originates from committed lymphoid progenitors. Wild type or *Msr1*$^{-/-}$ donor bone marrow cells were transduced by *BCR-ABL-GFP* retrovirus, followed by transplantation cells into lethally irradiated wild type recipient mice. Both groups of mice developed and died of ALL with similar disease latency and survival time (Figure 7a). FACS analysis of lymphoid leukemia cells showed that both groups of ALL mice had similar percentages of B220^{+} leukemia cells in peripheral blood of these mice (Figure 7b and c) although the mass of spleen in *Msr1*$^{-/-}$ donor group was slightly more elevated than wild type group (Figure 7d). Together, these results showed that *Msr1* did not affect the development of *BCR-ABL* induced ALL, and suggest that *Msr1* is not required by ALL stem cells, but is required by CML stem cells.

Figure 7. *Msr1* does not affect BCR-ABL-induced lymphoid leukemia

a. Kaplan-Meier survival curves for recipients of *BCR-ABL*-transduced bone marrow cells from wild type (*n*=10) or *Msr1*$^{-/-}$ (*n*=8) donor mice. Both groups of mice developed and died of ALL. **b** and **c** FACS analysis showed similar numbers of GFP^{+}B220^{+} cells in peripheral blood of recipients of *BCR-ABL*-transduced bone marrow cells from wild type (*n*=5) or *Msr1*$^{-/-}$ (*n*=5) donor mice. **d.** Spleen masses of recipients of *BCR-ABL*-transduced bone marrow cells from *Msr1*$^{-/-}$*(n*=9*)* donor mice is similar to wild type *(n*=4*)* donor mice.

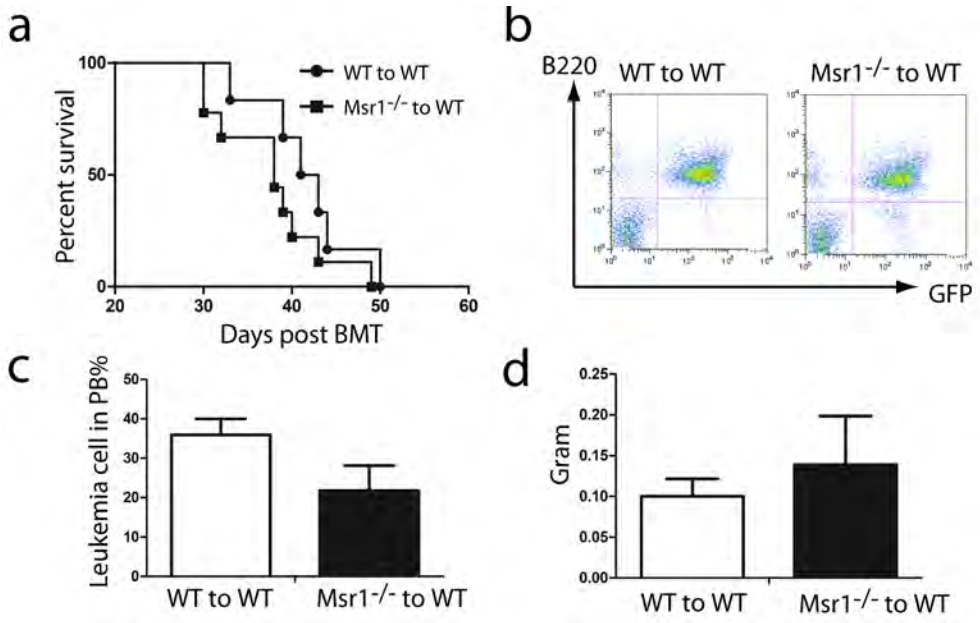


Figure 7. *Msr1* does not affect BCR-ABL-induced lymphoid leukemia

4.4 Discussion

In this study, we show that the reduction of LSCs in the absence of *Alox5* is partially due to an up-regulated expression of *Msr1* since *Msr1* inhibits the proliferation of LSCs. Our finding suggest that both *Msr1* and *Alox5* are in the same pathway and that *Msr1* functions downstream of *Alox5* as a tumor suppressor gene in *BCR-ABL*-induced CML. Compared to well studied tumor suppressor genes like *Pten* and *p53*, *Msr1* may represent a new group of genes with tumor suppressive activities, because deletion of this gene in mice does not result in tumor development (data not shown). Genetic changes to *Msr1* have been shown to be related to prostate cancer risk, although the underlying mechanism is unclear[118] . Our data demonstrate that the expression of *Msr1* is down-regulated by *BCR-ABL*. Loss of *Msr1* function leads to accelerated development of CML in mice, and this disease phenotype can be rescued by ectopic *Msr1* expression. Together, the results in this study reveal the functional relationship between *Msr1* and *Alox5*, i.e. BCR-ABL activates *Alox5* in LSCs, leading to reduced expression of *Msr1*. Thus, restoration of *Msr1* expression provides a strategy for shutting down the *Alox5* pathway required for LSC function [37]. Thus, the impetus for the identification of genes critical to LSC function in CML is the opportunity to find pathways that may be targeted for eradicating CML LSCs. Remarkably; enhanced *Msr1* expression may provide a means for eliminating LSCs and preventing CML. Our findings that the pharmacological induction of *Msr1* impairs *BCR-ABL* expressing LSCs, as well as human CML cells, lend credence to the notion that *Msr1* agonists may be used as a novel therapeutic strategy for eradicating LSCs in CML. Targeting *Alox5* and *Msr1* in combination with the *BCR-ABL*

kinase inhibitor imatinib or a BCR-ABL protein inhibitor, such as an HSP90 inhibitor, may provide a novel approach that leads to better clinical control of CML and the depletion of LSCs.

Our results also provide insight into the cellular mechanism for CML development in the absence of *Msr1*. The CML serial transplantation assay and LSC competition assay suggest that the accelerated CML development is caused by the significantly enhanced ability of LSCs to self-renew and induce leukemia in the absence of *Msr1*. The enhanced function of LSCs in the absence of *Msr1* is due to an increase in the number of LSCs in S+G2M phase and a decrease in LSC apoptosis. However, *Msr1* deficiency does not appear to significantly affect normal HSCs, which is consistent with our observation that *Alox5* deletion only affects LSCs instead of normal HSCs[83 37].

The molecular mechanism for the functional regulation of LSCs by *Msr1* remains largely unknown. We show that the loss of *Msr1* function leads to activation of the PI3K-AKT pathway and up-regulated expression of *β-catenin*. By contrast, up-regulation of *Msr1* by PMA inhibits the activity of the PI3K-AKT pathway and reduces the expression of *β-catenin*. We and others have recently shown that *β-catenin* plays a critical role in CML LSCs [27,64], and that *Akt* affects the progression of *BCR-ABL*-induced leukemia in a mouse model [106]. Thus, the regulation of these LSC-related genes by *Msr1* indicates its potential role in the development of *BCR-ABL*-induced CML. Because *Alox5* regulates expression of *β-catenin* in LSCs[37]and loss of *Alox5* up-regulatesthe expression

of *Msr1*, we propose that *Alox5* and *Msr1* are functionally linked and critical for LSC function. More studies are needed to explain how *Msr1* suppresses LSCs in CML.

4.5 Materials and methods

Mice

C57BL/6, B6.SJL-Ptprca Pepcb/BoyJ, homozygous *Alox5* knockout (*Alox5*$^{-/-}$) mice and homozygous *Msr1* knockout (*Msr1*$^{-/-}$) mice in a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, Maine). Mice were maintained in a temperature- and humidity-controlled environment and given unrestricted access to 6% chow diet and acidified water.

Microarray

To perform the microarray to compare the gene profiles, we used the bone marrow transplantation mouse model of CML as an assay system, in which bone marrow cells from donor mice pre-treated with 5-fluorouracil (5-FU) and transduced with BCR-ABL result in development of CML in recipient mice. We transduced bone marrow cells from C57BL/6 (B6) mice or *Alox5*$^{-/-}$ mice with retrovirus containing *BCR-ABL*/GFP or GFP alone under conditions for induction of CML, followed by transplantation of the transduced cells into B6 recipient mice (30 recipient mice for each group). 14 days after BMT, bone marrow cells were isolated and subsequently sorted by FACS for LSCs ($GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$). Total RNA was isolated from these BCR-ABL-expressing LSCs or from the $GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells that only expressed GFP, and DNA microarray

analysis was carried out to compare gene expression among the different groups by using Affymetrix Chips.

Construct

Human *Msr1* cDNA are kindly provided by Dr.Sulahian (Harvard School of Public Health). To make the *MSCV-BCR-ABL-Msr1* construct, *Msr1* cDNA was amplified by *Msr1*-EcorI 5'GAATTCATGGAGCAGTGGGATCACTTTC3' and *Msr1*-ClaI 5'ATCGATTTATAAAGTGCAAGTGAC3'. The cDNA was sequenced from both ends to confirm the sequence. The *Msr1* cDNA was cloned into the *pMSCV-BCR-ABL-GFP* vector between MfeI and ClaI sites.

To make the *pMSCV-Msr1-GFP* construct, *Msr1* cDNA was amplified by *Msr1*-EcorI 5'GAATTCATGGAGCAGTGGGATCACTTTC3' and *Msr1*-ClaI 5'ATCGATTTATAAAGTGCAAGTGAC3'. The cDNA was sequenced from both ends to confirm the sequence. The *Msr1* cDNA was cloned into the *pMSCV -GFP* vector at the EcorI site.

Cell lines

Human K562 myeloid leukemia cell line was grown in RPMI 1640 medium containing 10% FCS. To generate the *Msr1*-expressing K562 cell lines, K562 cells were transduced with the *pMSCV-Msr1-GFP* retrovirus, followed the *Msr1*-expressing cells were selected by GFP sorting by fluorescence-activated cell sorter (FACS). K562 cells transduced with the *pMSCV-GFP* retrovirus were used as a control. The livable cells were counted by Trypan Blue staining.

Flow cytometry and identification of leukemia and normal hematopoietic cell lineages.

Hematopoietic cells were collected from the bone marrow and peripheral blood of the normal and diseased mice. Erythrocytes were lysed in NH_4Cl red blood cell lysis buffer (pH 7.4). The cells were washed with PBS and stained with B220-PE for B cells, Gr-1-APC for neutrophils, Mac-1-PE for macrophages, CD3E-APC for T cells and Sca-1/c-Kit/CD34/CD135 (Flt3)/CD204 for HSCs. All of these antibodies were purchased from Ebioscience Inc and Serotec Inc. After staining, the cells were washed once with PBS and subjected to FACS analysis.

Bone marrow transduction/transplantation

The retroviral vector *MSCV-IRES-GFP* carrying the *p210 BCR-ABL* cDNA and retroviral transduction/transplantation of mouse bone marrow cells for induction of CML and ALL by *BCR-ABL* have been described previously[36,149].

Western blot analysis and antibodies

Antibodies against c-ABL, β-actin, β-catenin and MSR1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and antibodies against p-Pten, Pten, p-PI3K, PI3K, p-AKT, AKT, p-GSK-3β and GSK-3β from Cell Signal Technology (Danvers, MA). Protein lysates were prepared by lysing cells in RIPA buffer and western blotting was carried out as described previously [4].

In vitro culture of LSCs and drug treatment

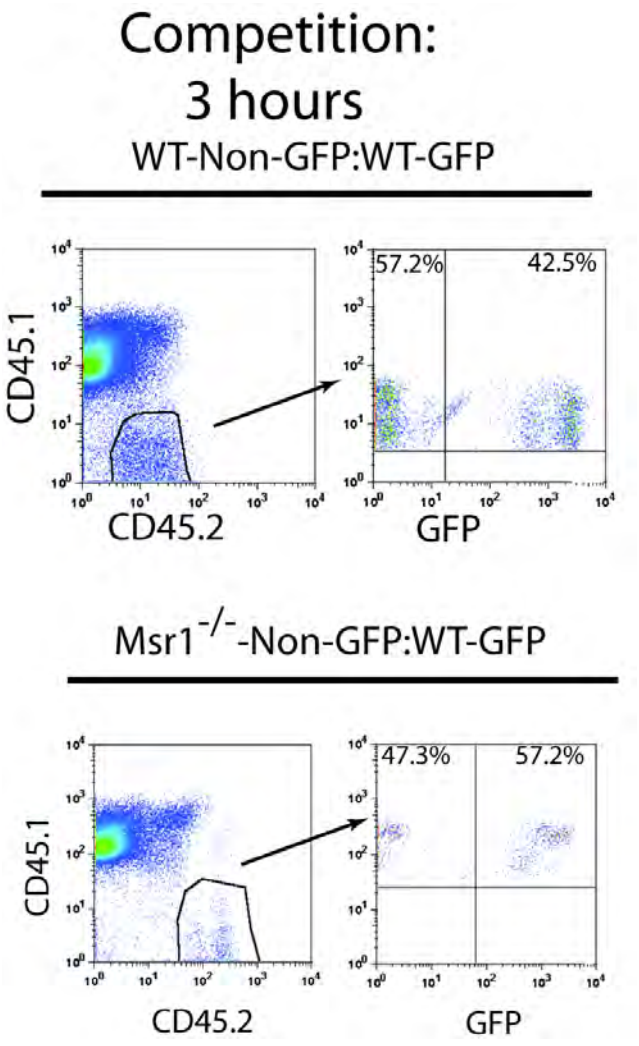
Bone marrow cells isolated from CML mice were cultured *in vitro* in the presence of Stemspan SFEM (Stemcell Technologies, Vancouver, CA), stem cell factor, insulin-like growth factor-2, thrombopoietin, heparin, and α-fibroblast growth factor as described previously [125]. PMA (Sigma) were dissolved in dimethyl sulfoxide (DMSO) to make stock solution at 10mM, and were then diluted in culture media to different concentrations for use.

Histology

The lungs from the placebo- or drug-treated mice were fixed in Bouin fixative (Fisher Scientific, Pittsburgh, PA) for 24 hours at room temperature, followed by an overnight rinse in water. Ten-μm sections were stained with hematoxylin and eosin (H&E) and observed by a model DMRE compound microscope (Leica, Heidelberg, Germany). All sections were imaged with a 2.5 x PH1 objective (NPLan, NA 0.25) and 10 x PH1 objective (NPLan, NA 0.40). All images were imported into MetaMorph software (Molecular Devices, Downingtown, PA) as a series of tagged image files. All images were then constructed in Adobe Photoshop 7.0 (Adobe, San Jose, CA).

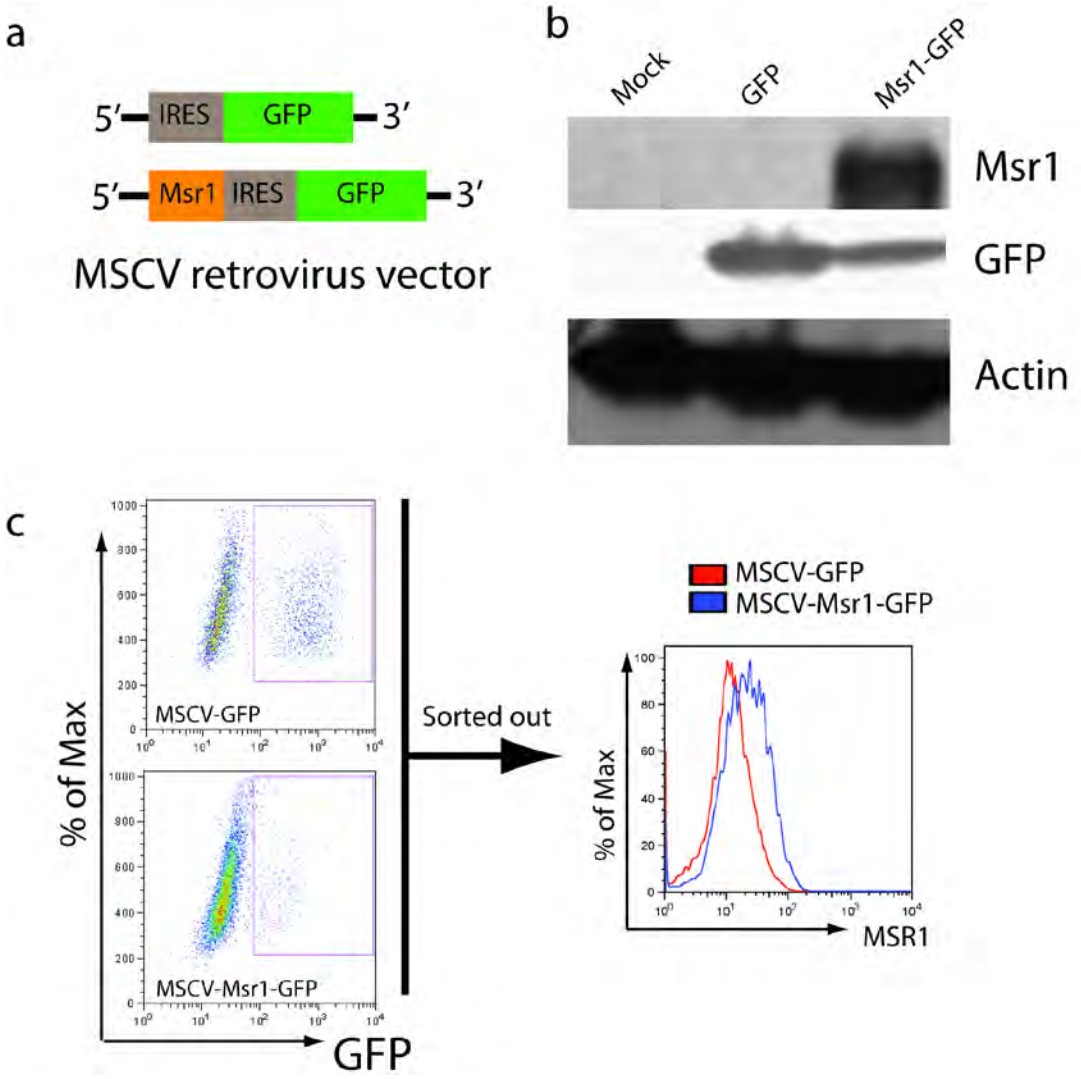
Statistics

Statistical analyses were performed by using Student *t* Test (*: $p<0.05$, **: $p<0.01$) (GraphPad Prism v5.01 software for Windows, GraphPad Software, San Diego, CA USA).



Supplementary Figure 1. *Msr1*$^{-/-}$ bone marrow cells do not exhibit a homing defect

Bone marrow cells ($6x10^6$) from GFP mice (CD45.2) were mixed 1:1 with either bone marrow cells from wild type mice (CD45.2) or those from *Msr1*$^{-/-}$ mice (CD45.2) and then transplanted by tail vein injection into wild type recipient mice (CD45.1). Three hours after the transplantation, by FACS analysis, CD45.2$^+$ bone marrow cells, representing the donor cells, were first identified and then analyzed for the percentages of GFP$^+$ and GFP$^-$ populations. The ratio of non-GFP and GFP populations were shown.



Supplementary Figure 2. *Msr1* is over-expressed in human leukemic cells. **a.** *pMSCV-Msr1-GFP* construct was made and *pMSCV-GFP* retrovirus was used as a control. **b.** *pMSCV-Msr1-GFP* construct expressed MSR1 and GFP together while the *pMSCV-GFP* construct only expressed GFP in 293T cells. **c.** Human leukemic cells were tranduced with *pMSCV-Msr1-GFP* or *pMSCV-GFP* retrovirus. GFP^{+}K562 cells were sorted out and the expression of MSR1 was measured by FACS.

Final Summary and Discussion

The work described in this chapter has been published (**Chen Y**, Li D, Li S. The Alox5 gene is a novel therapeutic target in cancer stem cells of chronic myeloid leukemia. **Cell Cycle.** 2009, 8(21): 3488-3492.)

In my Ph.D thesis, I focus on looking for the new target on leukemia stem cells of CML. First, we evaluated the effects of Omacetaxine on *BCR-ABL*-induced chronic myeloid leukemia and acute lymphoblastic leukemia in mice. Second, we used our CML mouse model and compared the global gene profile between normal HSCs and LSCs to identify the up-regulation of *Alox5* in CML LSCs. Finally, we sought to further dissect this pathway by comparing the gene expression profiles of wild type and *Alox5*$^{-/-}$ LSCs derived from our mouse model for *BCR-ABL*-induced CML. In particular, we identified that *Msr1* plays a tumor suppressor role in LSCs.

Our strategy for targeting LSCs

A straightforward strategy for targeting LSCs is through inhibiting development-related genes that play roles in regulation of both normal HSCs and LSCs, as many of these genes have been identified and initially found to play critical roles in the development of normal HSCs. However, a potential problem of this strategy is that normal stem cells could be simultaneously inhibited during a long-term cancer treatment, leading to severe side effects potentially. One strategy for inhibiting LSCs is to target genes that play crucial roles in functional regulation of LSCs but not normal HSCs, and obviously, the challenge for taking this approach is to first identify these key stem cell-

specific genes, as expression of many genes can be altered by an oncogene including *BCR-ABL* in CML and not all these genes are essential for survival and self-renewal of LSCs. The *Alox5* gene is probably the first LSC-specific gene identified and shown to play a critical role in LSCs but not normal HSCs, and our identification of this gene in LSCs shows a good example for how we design a strategy for targeting LSCs. In our study, the *Alox5* gene was picked for further functional study based on our comparison of gene expression profiles between normal HSCs and LSCs by DNA microarray analysis, in which *Alox5* is shown to be up-regulated by *BCR-ABL*, which was confirmed by real-time PCR analysis. *Alox5* has been shown to be associated with many important signaling pathways including *P53* and *PI3K*, and is thought to be involved in many different types of diseases[87,161]. We believe that the *Alox5* pathway represents a major molecular network that regulates the function of LSCs, and much more work needs to be done to fully dissect this pathway.

Recently, *Alox5* was identified as a novel target in glioma stem-like cells (GSLCs) and its inhibition with Nordy exhibits therapeutic implications through inducing GSLC differentiation. *Alox5* gene was shown to have a heterogeneous expression pattern in human glioblastoma cells and its preferential expression in a GSLC population. The synthetic Alox5 inhibitor (Nordy) was reported to inhibit self-renewal and induced differentiation of GSLCs in vitro and in vivo. The Alox5 inhibitor was found to attenuate the growth of GSLCs in vitro and reduced the GSLC pool through a decrease in the CD133 (+) population and abrogated clonogenicity. The Alox5 inhibitor appeared to exert its effect via astrocytic differentiation by up-regulation of GFAP and down-

regulation of stemness related genes, rather than by inducing apoptosis of GSLCs. The growth inhibition of xenografted glioma by Nordy was more long-lasting compared with that of the akylating agent BCNU, which exhibited significant relapse on drug discontinuation resulting from an enrichment of GSLCs. And transient exposure to Nordy reduced tumorigenecity of GSLCs and induced differentiation of the xenografts[175].

Targeting of cancer stem cells (CSCs) have become an important issue in developing new therapies of cancer. As described above, we believe that the key to eradicating CSCs is to identify critical pathways responsible for regulating self-renewal and differentiation of CSCs but not normal stem cells. The *Alox5* study in leukemia stem cells and glioma stem-like cells has shown that this strategy is feasible. Using CML induced by *BCR-ABL* as a model disease, here we propose a strategy for identifying new therapeutic targets in CSCs (Figure 1). Isolation of LSCs and normal HSCs is a key initial step to obtaining high-quality RNA for microarray analysis. In our study, *BCR-ABL*-expressing HSCs ($GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$) represent LSCs in CML mice. It is important to realize that the microarray analysis will show many genes that were up- and down-regulated by *BCR-ABL* in LSCs, and the key is to select candidate genes for further functional tests. Prior to the functional tests, we always confirm expression change of a gene of interest by real-time PCR. The function of a candidate gene can be primarily determined based on the literature or database search. The last step is to functionally test a candidate gene using knockout or transgenic strains.

Future directions

Imatinib effectively inhibits BCR-ABL kinase activity, but does not remove BCR-ABL protein, which may partially explain why imatinib does not eradicate LSCs in CML. In fact, we have shown that total number and percentage of LSCs in bone marrow of CML mice gradually increase with time during imatinib treatment, although the BCR-ABL kinase activity is greatly inhibited by imatinib. These results indicate that inhibition of BCR-ABL kinase activity alone is insufficient to completely shut down BCR-ABL, and that other strategies need to be developed. Our demonstration of the role of Alox5 in regulating the function of LSCs but not normal HSCs identifies the first LSC-specific gene in CML, and provides us with an exciting opportunity to explore the regulatory molecular pathways in LSCs. In our *Alox5* study, we have primarily linked the *Alox5* pathway to activation of β-catenin and a cell surface receptor Msr1, as we showed that loss of *Alox5* caused down-regulation of β-catenin expression and up-regulation of Msr1 in LSCs, but not in normal HSCs. Several important questions remain to be answered:

1) How does *Alox5* regulate the development of cancer stem cells? Recently, the Alox5 gene was reported to be essential for self-renewal and differentiation of glioma stem cells[175]. It has also been shown that the *Alox5* pathway is utilized early during the differentiation of embryonic stem cells into cells of a myeloid lineage[176]. These findings suggest a unique role for the *Alox5* gene in the development of both normal stem cells and cancer stem cells. Our BCR-ABL mouse model is a good tool to study the mechanism of *Alox5* in cancer stem cells. To investigate how *Alox5* gene is activated in LSCs, *Alox5* promoter studies will be initiated to analyze its transcriptional regulation. The key genes regulating self-renewal and differentiation of stem cells like OCT4, SOX-

2 and CD133 will also be tested in LSCs with or without *Alox5* gene. The mechanism by which Alox5 regulates these stem cell -specific genes will also be investigated. This study will help us to establish the potential link among *BCR-ABL*, *Alox5* gene and cancer stem cells and identify new targets for inhibiting the LSCs of CML.

2) What is the function of other genes of the *Alox5* pathway in LSCs? Our results indicate that *Alox5*, a gene critical to the production of leukotrienes in the arachidonic acid metabolism pathway, is required for CML development, likely through its effect on CML stem cell maintenance. Next, it is important to understand the function of other genes of arachidonic acid metabolism like *Alox12/15* and *PEG2* in LSCs and CML development. It is also necessary to understand the function of leukotrienes and other leukotriene related genes in LSCs. For example, leukotriene B4 (LTB4) is produced by 5-LO and exported from the cell by specific transporter proteins. LTB4 acts by binding to the B leukotriene receptor 1 (*Blt1*), which is located on the outer plasma membrane of structural and inflammatory cells. To characterize the function of LTB4 in LSCs, LSCs will be treated with LTB4 and the proliferation of LSCs will be measured. The function of LTB4 receptor Blt1 in LSCs will also be examined using *Blt1* knockout mouse. LTA4 hydrolase (*Lta4h*) is another key gene in leukotriene metabolism, which converting leukotriene A4 (LTA4) the product of Alox5 to leukotriene B4 (LTB4). The function of LTA4 and *Lta4h* in LSCs is not clear. The function of *Lta4h* in LSCs may be examined using *Lta4h* knockout mice.

3) How does *BCR-ABL* inhibit Msr1 expression and how does Msr1 regulate PI3K-AKT pathway? Msr1 is a cell surface receptor expressing on HSCs and is down-regulated by *BCR-ABL*. Msr1 suppresses the CML development and enhances the function of LSCs. It is still not clear that how *BCR-ABL* inhibits Msr1 expression, what signaling pathway or transcription factors link the *BCR-ABL* and Msr1, and how those signaling pathways are changed by the loss of *Alox5* gene. Over-expression of Msr1 inhibits PI3K and AKT pathway in human leukemia cells. The mechanism of Msr1 inhibiting PI3K and AKT kinases activity is still unknown. For example, will Msr1 activate or inactivate any potential phosphorylation, ubiquitination, acetylation, methylation and other post-translational modification sites of PI3K and AKT? Will Msr1 also activate or inactivate other signaling pathways? Mass spectrometry analysis of PI3K and AKT post-translational modification from cells with or without over-expressed of Msr1 will help to identify any potential phosphorylation, ubiquitination, acetylation, methylation and other post-translational modification sites of PI3K and AKT. Microarray or RNA-Seq analysis will be help to find out whether Msr1 activates or in activates other signaling pathways.

We believe that all these studies will help us to understand the mechanism of self-renewal or differentiation of cancer stem cells and find a new strategy to effectively target cancer stem cells for developing curative therapies.

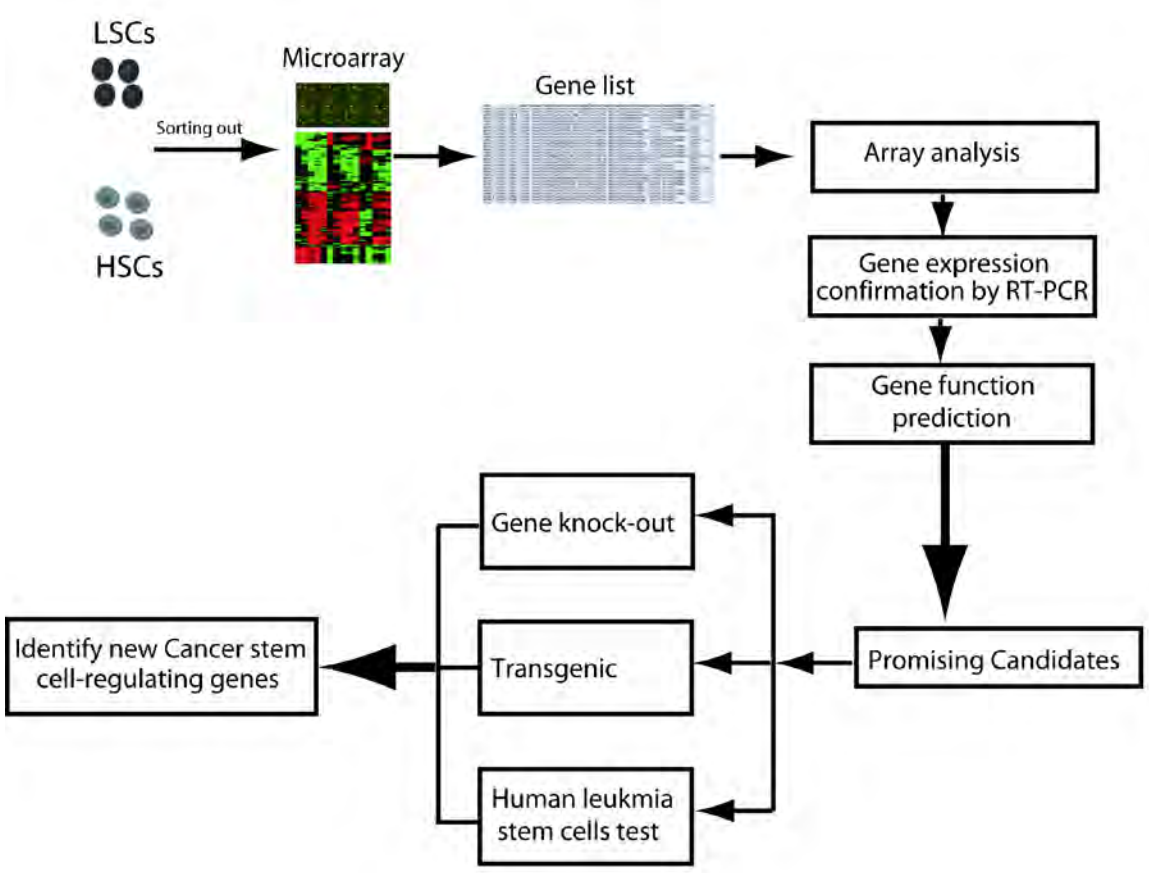


Figure 1. Our strategy for identification of genes that play key roles in regulating the functions of LSCs. One of the most critical steps in this approach is to analyze DNA microarray data to provide a short list of candidate genes.

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