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Novel Therapeutic Targets for Ph+ Chromosome Leukemia and Its Leukemia Stem Cells: A Dissertation

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NOVEL THERAPEUTIC TARGETS FOR Ph+ CHROMOSOME LEUKEMIA AND ITS LEUKEMIA STEM CELLS

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CONG PENG

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NOVEL THERAPEUTIC TARGETS FOR Ph+ CHROMOSOME LEUKEMIA AND ITS LEUKEMIA STEM CELLS

A Dissertation Presented By

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Abstract

The human Philadelphia chromosome (Ph) arises from a translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)]. The resulting chimeric *BCR-ABL* oncogene encodes a constitutively activated, oncogenic tyrosine kinase that induces chronic myeloid leukemia (CML) and B-cell acute lymphoblastic leukemia (B-ALL). The BCR-ABL tyrosine kinase inhibitor (TKI), imatinib mesylate, induces a complete hematologic and cytogenetic response in the majority of CML patients, but is unable to completely eradicate BCR-ABL–expressing leukemic cells, suggesting that leukemia stem cells are not eliminated. Over time, patients frequently become drug resistant and develop progressive disease despite continued treatment. Two major reasons cause the imatinib resistance. The first one is the BCR-ABL kinase domain mutations which inhibit the interaction of BCR-ABL kinase domain with imatinib; the second one is the residual leukemia stem cells (LSCs) in the patients who are administrated with imatinib. To overcome these two major obstacles in CML treatment, new strategies need further investigation.

As detailed in Chapter II, we evaluated the therapeutic effect of Hsp90 inhibition by using a novel water-soluble Hsp90 inhibitor, IPI-504, in our BCR-ABL retroviral transplantation mouse model. We found that BCR-ABL mutants relied more on the HSP90 function than WT BCR-ABL in CML. More interestingly, inhibition of HSP90 in CML leukemia stem cells with IPI-504 significantly decreases the survival and proliferation of CML leukemia stem cells in vitro and in vivo. Consistent with these findings, IPI-504 treatment achieved significant prolonged survival of CML and B-ALL

mice. IPI-504 represents a novel therapeutic approach whereby inhibition of Hsp90 in CML patients and Ph+ ALL may significantly advance efforts to develop a cure for these diseases. The rationale underlying the use of IPI-504 for kinase inhibitor–resistant CML has implications for other cancers that display oncogene addiction to kinases that are Hsp90 client proteins.

Although we proved that inhibition of Hsp90 could restrain LSCs *in vitro* and *in vivo*, it is still unclear how to define specific targets in LSCs and eradicate LSCs. In Chapter III, we took advantage of our CML mouse model and compared the global gene expression signature between normal HSCs and LSCs to identify the downregulation of *Pten* in CML LSCs. CML develops faster when *Pten* is deleted in $Pten^{fl/fl}$ mice. On the other hand, Pten overexpression significantly delays the CML development and impairs leukemia stem cell function. mTOR is a major downstream of Pten-Akt pathway and it is always activated or overepxressed when Pten is mutated or deleted in human cancers. In our study, we found that inhibition of mTOR by rapamycin inhibited proliferation and induced apoptosis of LSCs. Notably, our study also confirmed a recent clinical report that Pten has been downregulated in human CML patient LSCs. In summary, our results proved the tumor suppressor role of Pten in CML mouse model. Although the mechanisms of Pten in leukemia stem cells still need further study, Pten and its downstream, such as Akt and mTOR, should be more attractive in LSCs study.

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List of Abbreviations

5-FU	5-fluorouracil
17-AAG	17-(Allylamino)-17-demethoxygeldanamycin
AML	Acute Myeloid Leukemia
AP	Accelerate phase
B-ALL	B cell-acute lymphoid leukemia
BC	Blast crisis
BM	Bone marrow
BMT	Bone marrow transplantation
CML	Chronic myeloid leukemia
CRKL	v-crk sarcoma virus CT10 oncogene homolog (avian)-like
CRK	v-crk sarcoma virus CT10 oncogene homolog (avian)
CP	Chronic phase
CBL	Casitas B-lineage lymphoma
EphB4	Eph receptor B4
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
GRB2	growth factor receptor bound protein 2
GAB2	growth factor receptor bound protein 2-associated protein 2
HSCs	Hematopoietic stem cells
IL3	interleukin 3
GA	geldanamycin

IL6	interleukin 6
LSCs	Leukemia stem cells
LT-HSC	Long term- hematopoietic stem cell
MPD	Myeloproliferative Disorders
PI3K	phosphatidylinositol 3-kinase
PDGFR	platelet derived growth factor receptor
SHC	src homology 2 domain-containing transforming protein
STAT5	signal transducer and activator of transcription 5
SHP2	SH2 domain-containing protein tyrosine phosphatase-2
ST-HSC	short term- hematopoietic stem cell
SCF	Stem cell factor
TCF4	transcription factor 4

CHAPTER I

INTRODUCTION

1.Philadelphia chromosome and chronic myeloid leukemia (CML)

In 1960, Nowell and Hungerford's landmark discovery of the Philadelphia (Ph) chromosome in human CML patients provided a major clue to the cause of CML. This discovery was the first demonstration of a chromosomal rearrangement that is consistently linked to a specific cancer, and had sparked searches for associations of additional chromosomal aberrations with specific forms of cancer. In their study, they examined leukemia cells from patients with chronic phase CML and other leukemia patients. Surprisingly, the leukemia cells from all of seven CML patients showed a consistent tiny chromosome, which then was named as Philadelphia (Ph) chromosome by them [1]. However, none of other types of leukemia patients had been detected to contain this minute chromosome abnormality. Ph chromosome is now known to be present in over 95% of CML cases [2]. In samples from patients with adult B-ALL, the Ph chromosome accounts for 10% to 15% of such cases [2]. CML is characterized by distinct three clinical phases: most patients are in chronic phase (CP), in which patients can still produce mature granulocytes but they have an increased number of myeloid progenitor cells in the peripheral blood. The medium duration of the chronic phase is 3-4 years. The chronic phase is relatively long term and researchers have opportunity to investigate the leukemia cells development. With the disease progression and acquisition of secondary genetic or epigenetic abnormalities, patients enter an accelerated phase (AP) and finally

develop to a blast crisis (BC), in which hematopoietic development has been blocked and immature blasts accumulate in the bone marrow (BM) and migrate into peripheral organs. In BC, about 60% of the patients enter a myeloid blast stage resembling acute myeloid leukemia (AML) and about 30% of the patients develop a pre-B blast stage similar to acute lymphoid leukemia (B-ALL)[3].

The blast crisis only lasts a few months[4] and this aggressive progression of blast crisis is directly caused by different secondary genetic mutations. Over 80% of BC patients have genetic abnormalities in addition of the Ph chromosome. [5] Plenty of oncogenic events have been associated with blast crisis, including loss of *p53*[6, 7], *Myc* amplification[8], deletion of *Rb*[9] and *p16*[10].

2. BCR-ABL oncoprotein and its domain functions

In the effort to identify the origin of the Ph chromosome, the advent of quinacrine fluorescence and Giemsa banding enabled Rowley to show that the Ph chromosome results from a reciprocal translocation between the long arms of chromosomes 9 and 22 t(9;22)(q34;q11) [11]. Nine years later after the identification of the chromosome arms involved in the Ph chromosomal translocation, some key studies from several investigators including Heisterkamp, Groffen, Stephenson, and Canaani led to the definitive characterization of the Ph chromosome structure and its mRNA product. Chromosome mapping studies demonstrated that the human *ABL* gene mapped to chromosome 9 [12]. This gene was shown to be present in the Ph chromosome in CML cells [13-16]. Breakpoints along the Ph chromosome were found to occur within the

breakpoint cluster region (*BCR*) gene, suggesting that *BCR* is also involved in the translocation that creates the Ph chromosome [17]. Hybridization of both *BCR* and *ABL* probes to the unique mRNA product of the Ph chromosome confirmed that Ph chromosome mRNA was composed of *BCR-ABL*.[15, 18, 19] Later, through sequence analysis, the *BCR-ABL* transcript was shown to contain the first 13 to 14 *BCR* exons and exons1a or a2 through a11 of *ABL*. [20] This *BCR-ABL* gene generated a large mRNA product that after splicing, encoded an 8.5-kB *BCR-ABL* chimeric transcript [20]. These studies show that the molecular basis of the Ph chromosome is the *BCR-ABL* oncogene which encodes BCR-ABL protein.

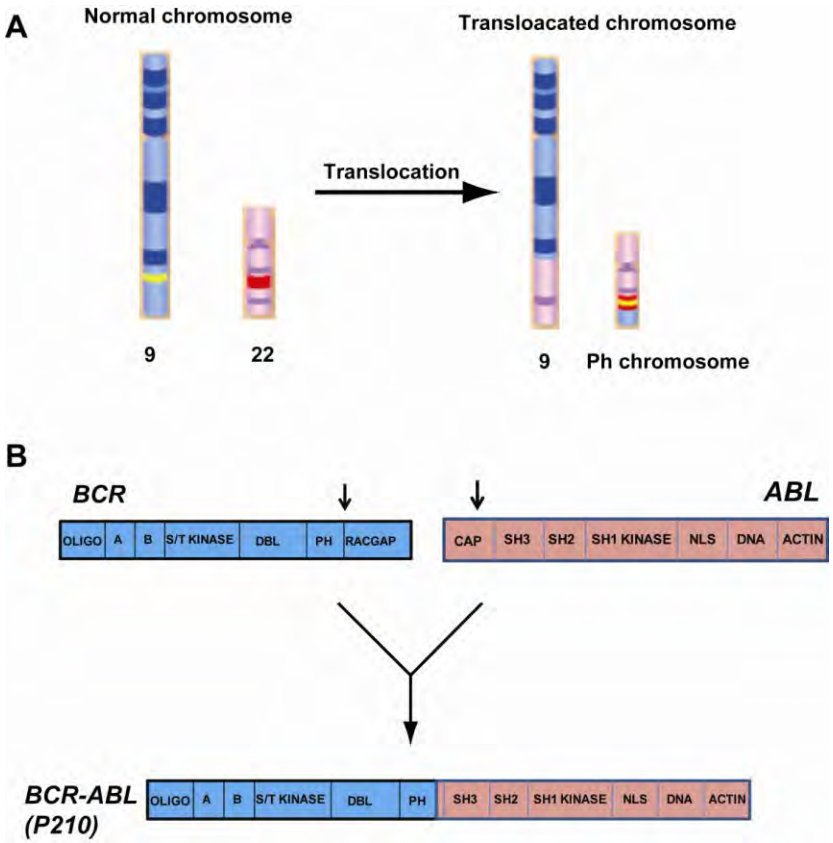

Figure 1. Generation of chimeric *BCR-ABL* oncogenes. (*A*) Schematic diagram of the translocation that creates the Philadelphia chromosome. The *ABL* and *BCR* genes reside on the long arms of chromosomes 9 and 22, respectively. As a result of the (9;22) translocation, a *BCR-ABL* gene is formed on the derivative chromosome 22 (Philadelphia chromosome). (*B*) Locations of breakpoints in *BCR* and *ABL* genes and structure of derived chimeric proteins. The major breakpoint cluster spans 5.8 kb and results in a 8.5 kb mRNA producing *BCR-ABL* (P210). *BCR* sequences are most often fused to *ABL* exon a2 in the hybrid transcript. BCR protein domains include: OLIGO = oligomerization; A and B = A and B boxes; S/TKINASE = serine/threonine kinase; DBL= Dbl homology; PH=Pleckstrin homology; RACGAP = Rac GTPase. ABL protein domains include MYR = myristoylation signal; CAP = CAP hydrophobic residues; SH3 = src homology 3; and remaining ABL domains as described as the SH2 = src homology domain 2, SH1 = src homology tyrosine kinase domain, NLS = nuclear localization domains, DNA = DNA binding sites, and ACTIN = F and G actin binding sites.

c-ABL, a non-receptor tyrosine kinase, is expressed both in the cytoplasm and nucleus and is involved in the cell proliferation, survival and morphogenesis. There is a unique N-terminus followed by a SH3 (Src homology 3) domain, a SH2 domain, and the kinase domain (Figure 1B). There are F-(filamentous) and G-(globular) actin-binding domain, NLS (nuclear localization signal) and DNA-binding sequences in the C-terminus. These NSL and DNA-binding domain are crucial for its nuclear functions.[21] c-ABL shuttles between the cytoplasmic and nuclear compartments. In nuclear, it regulates the cellular response induced by DNA damage, apoptosis and cell growth arrest. [22] Recent study points to a central role of the cytoplasmic c-ABL is in morphogenesis and F-actin dynamics.[23] In addition, c-ABL also plays a critical role in signaling induced by extracellular stimuli. [24]. Plenty of studies indicated the c-ABL function in several cell surface receptors mediated signaling transductions which include T cell receptor[25], angiotensin subtype 1 receptor[26], EphB4 receptor[27] and PDGFR[28]. *Abl*-null mice are

variably affected, and the phenotypes include an increased incidence of perinatal mortality, lymphopenia and osteoporosis. These mice are also smaller, with abnormal head and eye development.[29, 30] BCR is also a signaling protein that contains multiple modular domains (Figure 1B). *Bcr* deficient mice develop normally, but neutrophils of *Bcr-/-* mice showed a pronounced increase in reactive oxygen metabolite production upon activation and were more sensitive to priming stimuli[31]. 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.

The importance of domains of BCR-ABL has been validated *in vivo* using a retroviral transduction/bone marrow transplantation (BMT) mouse model of CML. This BMT-based CML mouse model is an excellent system for the *in vivo* structure-function analysis of BCR-ABL [32], and various mutant forms of BCR-ABL have been expressed in mice using this model. Mice that express 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 cells, indicating that the ABL kinase activity is absolutely essential for BCR-ABL leukemogenesis *in vivo* [33]. It proves that the ABL kinase is an excellent target for treating CML. In addition to the ABL kinase domain, there are other important domains in BCR-ABL, which regulate the kinase activity of ABL or mediate the interaction of BCR-ABL signaling pathways (Fig. 2). The relative importance of various domains of BCR-ABL in neoplastic transformation has been examined *in vitro* and *in*

vivo, including transformation of immortalized fibroblast cell lines, growth-factor-dependent hematopoietic cell lines and primary bone-marrow cells.

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*. However, it only induces lymphoid leukemia with a greatly extended latency in mice.[34] A mutant form of BCR-ABL with a deletion of the SH3 domain does, however, still effectively induce a fatal myeloproliferative disease (MPD).[35] These findings indicate that activation of ABL kinase alone (through the loss of SH3) is not sufficient to cause a CML-like MPD, and that other functional domains/motifs of BCR-ABL are also required for the induction of CML. 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 fibres.[36] A mutant form of BCR-ABL that lacks the BCR-CC domain (ΔCC-BCR-ABL) failed to induce MPD in mice, but, rather, induced a T-cell leukemia/lymphoma only after a long latent period.[37-39] 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.[39] These results demonstrate that the BCR CC 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. Another important motif in the BCR region of BCR-ABL is the 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 [40],

leading to activation of downstream RAS and recruitment of SHP2 and phosphatidylinositol 3-kinase (PI3K).[40, 41] 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.[40, 42] In the BMT CML model, the Y177F mutant form of BCR-ABL has a greatly reduced ability to induce CML in mice, and these mice eventually developed T-ALL or abdominal T-cell lymphomas after a prolonged latent period.[37, 38, 43] These results demonstrate that phosphorylation at Y177 is required for the induction of CML by BCR-ABL.

The SH2 domain of ABL is believed to activate RAS, at least partially, through binding to SHC, which, following tyrosine phosphorylation, can recruit GRB2.[44] Mutations in the SH2 domain of ABL reduced the ability of BCR-ABL to induce a CML-like MPD in mice.[37] The Y1294F point mutation in SH2 domain of BCR-ABL also attenuated leukemogenesis by BCR-ABL.[39] The carboxy-terminal region of ABL is required for the proper function of normal ABL and for the lymphoid leukemogenicity of v-Abl.[45] However, deletion of actin-binding domain of ABL or the entire carboxy-terminal region downstream of the ABL kinase domain did not affect the ability of BCR-ABL to induce CML-like MPD in mice.[46] Therefore, the functions of these domains are dispensable in BCR-ABL-mediated leukemogenesis. It is evident that certain domains/motifs of BCR-ABL bear overlapping functions. Deletions of both SH3 domain and carboxy-terminal proline-rich SH3-binding sites (ABL-PP) of ABL, but not point mutations of each domain, block the ability of BCR-ABL to stimulate spontaneous cell migration on fibronectin-coated surfaces, and greatly reduced BCR-ABL

leukemogenicity in mice.[47] Deletions of both SH3 and SH2 domains of ABL in BCR-ABL also showed more severe defects in CML induction than mutating either domain.[48] 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.

3. BCR-ABL retroviral transduction/transplantation mouse models

CML has been extensively studied and used as a model disease to investigate the molecular basis of leukemia, shedding light on the understanding of other human cancers as well. CML validates the concept that cancer is a genetic disease. CML is derived from the hematopoietic stem cells which harbor the BCR-ABL oncogene and acquire a selective growth advantages over the normal hematopoietic stem cells. These conditions are drawn based on the transplantation experiments in which peripheral blood (PB) cells, total bone marrow (BM) cells, and primitive cells (CD34+) from the CML patients have been transplanted into irradiated nonobese severe combined immunodeficient (NOD/SCID) mice[49]. Recipient mice transplanted with these PB or BM cells showed engraftment of the human leukemia cells in BM for up to 7 months and those transplanted with CD34+ cells showed a greater engraftment of leukemia cells. Although this xenograft model allows evaluating the capability of transplanted human leukemia cells to initiate and maintain CML disease in recipient mice, the mice with engrafted human leukemia cells did not develop lethal leukemia after 7 months. This call for

further improvement of this xenograft model as it is important to establish a faithful CML mouse model for evaluating promising therapeutic compounds and developing new therapeutic strategies.

Much effort has been made to generate mouse models of Ph+ leukemia. *BCR-ABL* transgenic models have been made to express *BCR-ABL* transgene in mice. Different promoters that drive BCR-ABL expression have been tested in these models to express BCR-ABL in different target cells. These promoters include Eμ [50], MPSV-LTR[50], metallothionein[50], BCR [51], and MSCV-LTR[52]. Although all of these models show the expression of BCR-ABL in mice, there are at least two obvious defects in using these models: (1) not all mice harboring BCR-ABL develop myeloproliferative disorder, with some mice only developing lymphoid leukemia; and (2) disease latency is too long, restricting the use of these models in developing therapeutic strategies for CML.
In contrast, the retroviral transduction/transplantation model is a more faithful model of BCR-ABL induced CML. In 1990, Daley et al. co-cultured mouse bone marrow cells with the retroviral producer cells that produced BCR-ABL expressing retrovirus, and transplanted these infected bone marrow cells into lethally irradiated recipient mice.[53] Three different types of diseases were found in the recipients at up to 5 months after bone marrow transplantation. These diseases included CML like myeloproliferative syndrome, acute lymphoblastic leukemia, and a type of tumor involving macrophages. In the meantime, Kelliher et al. also established a retroviral system in which a JW-RX retrovirus expressing BCR-ABL was used to infect 5-FU pretreated donor mice bone marrow cells. After bone marrow transplantation, more than 90% of recipients developed

tumors, with 50% of them developing a myeloproliferative syndrome that shares several features with the chronic phase of chronic myeloid leukemia[54]. Both of these studies proved that BCRABL is the primary cause of myeloproliferative syndromes in mice. However, there was more than one type of disease in the recipients, further as not 100% of mice developed CML with similar disease latency; hence it was still difficult to conduct drug testing experiments using these models. To overcome these deficiencies, improvements on the model system have been made including modified construct, transient retroviral packaging system, and changes of virus infection conditions.

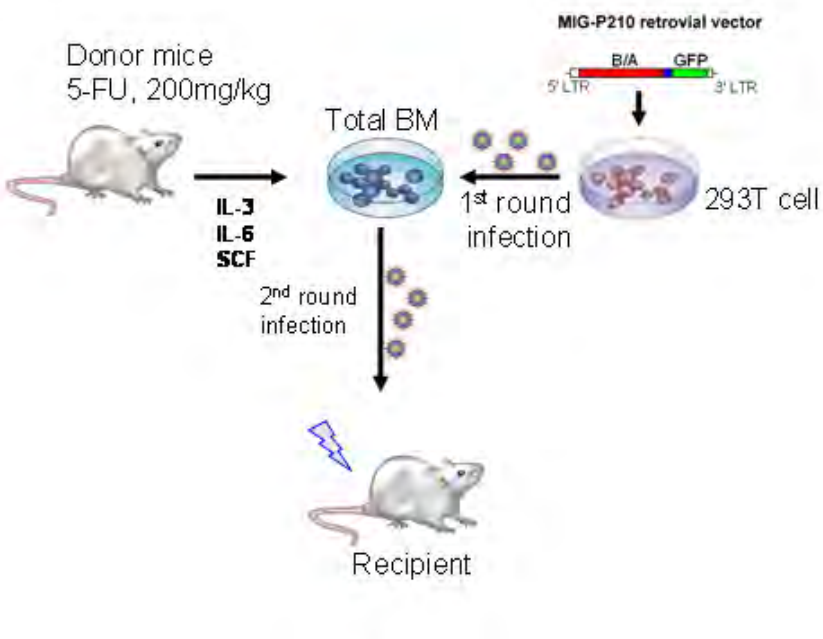

Figure 2. Retroviral transduction/ transplantation model of BCR-ABL induced CML. Donor mice are pretreated with 5-FU (150mg/Kg) for 4 days, and bone marrow cells are stimulated with IL3, IL6 and SCF cytokines in vitro. After infected twice with MSCV-BCR-ABL-GFP retrovirus, donor bone marrow cells are transplanted into lethally irradiated recipients for induction of CML.

In our lab, we use BCR-ABL expressing retrovirus to induce disease mice which mimic human CML or a B cell-acute lymphoid leukemia (B-ALL).[32] To induce CML mice (Figure 2), donor mice are primed by intravenous injection with 5-fluorouracil (5-FU) for 4 days before harvest to remove most of the dividing cells and enrich hematopoietic stem cells and progenitors. Donors are sacrificed and bone marrow is harvested. Cells are cultured in DMEM, with FCS, WEHI-3B conditioned medium, penicillin/ streptomycin, L -glutamine, and several cytokine combinations. During the culture, we transduce the bone marrow cells with high titer BCR-ABL retrovirus for the first round. After first found infection, we change fresh culture medium and continue culture the cells overnight. Second round infection is performed before transplantation of the cells into lethally irradiated recipients. All of these mice will develop CML disease and die of CML within 3-4 weeks. The CML is characterized by massive expansion of maturing granulocytes in peripheral blood, spleen and bone marrow. The peripheral blood leukocyte count at death was about 2–4 $x10^5$/ml, composed predominantly of mature neutrophils. The spleen and liver are greatly enlarged and disrupted by large numbers of myeloid leukemia cells. Notably, all animals have leukemia cell infiltration in the lung and the extensive intraparenchymal hemorrhage, which is the ultimate cause of death of these animals. In most cases, the CML syndrome could be efficiently transferred to secondary recipients by injection of bone marrow.

Because animals with the CML syndrome die within 4 weeks after transplantation, it is impossible to evaluate the transition process which CML will gradually develop to

acute myeloid leukemia (AML) or acute lymphoid leukemia (ALL) which are the common result from clinical observation.[55] As HSCs are enriched and B cell progenitors are eliminated in donor mice after by 5-FU treatment[56], the transduction efficiency of HSCs by BCR-ABL greatly increases and the recipients are prone to develop CML. To mimic the characteristics of acute lymphoid leukemia induced by *BCR/ABL*, we use bone marrow from donors that had not been pretreated with 5-FU. Briefly, we transduce non 5-FU treated donor bone marrow cells with BCR-ABL retrovirus only once then transplant them into lethally irradiated recipients (Figure 3). All recipients will exhibit B cell acute lymphoid leukemia (B-ALL) phenotype within 2 weeks after bone marrow transplantation and die of B-ALL within 8 weeks. All the disease mice have modest splenomegaly (0.2–0.4 g) and lymphadenopathy with infiltration with lymphoblasts, and a bloody pleural effusion, containing high levels of malignant lymphoid cells that appeared to be the cause of death. The leukemia cells highly expressed BCR-ABL and they are negative for myeloid and T lymphoid cell surface markers, but positive for B220, CD19, indicating an immature B cell phenotype. The lymphoid leukemia is efficiently transplanted to secondary recipients, with animals receiving tumor cells from lymph node or pleural effusion succumbing to an identical disease within 4–5 weeks.

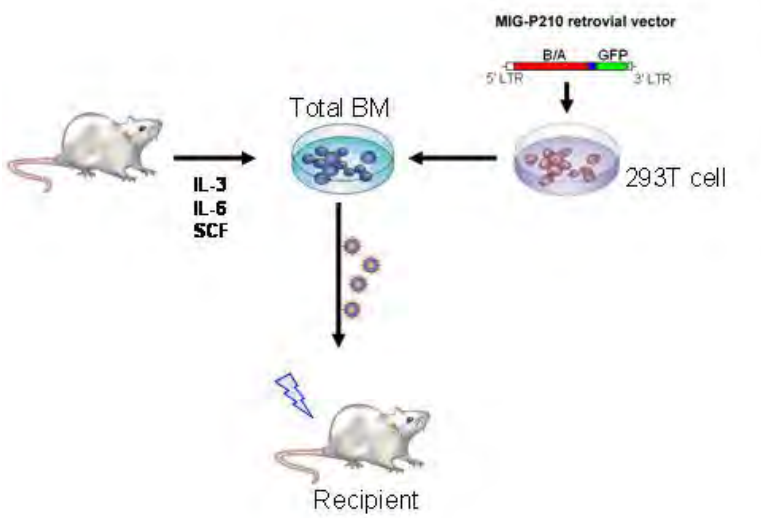

Figure 3. Retroviral transduction/ transplantation model of BCR-ABL induced B-ALL. Donor bone marrow cells from mice without 5-FU treatment are harvested and marrow cells are stimulated with IL3, IL6 and SCF cytokines in vitro. After transduced with MSCV-BCR-ABL-GFP retrovirus only once. Then donor bone marrow cells are transplanted into lethally irradiated recipients for induction of B-ALL.

Based on these two high efficient mouse models, we can induce CML or B-ALL in mice and monitor the disease development carefully. The disease could be induced in most of the inbred mouse strain including C57BL/6, BALB/c, and viable gene knockout mice strains and it facilitates us to evaluate the functions of certain genes in the leukemogenesis. Because all recipients develop CML or B-ALL with a short latency, this provides us an excellent model for evaluating therapeutic agents for CML or B-ALL treatment. As CML is derived from the hematopoietic stem cells which harbor BCRABL oncogene, CML leukemia stem cells can also be studied in our model. In conclusion, these retroviral model systems provide a powerful tool for studying BCR-ABL induced CML and B-ALL disease mechanism and performing translational research.

4. BCR-ABL kinase inhibitors

The essential role of BCR-ABL tyrosine kinase activity for cellular transformation provided the rational for targeting this function therapeutically. Starting in the late 1980s, scientists at Novartis initiated projects on the identification of compounds with inhibitory activity against protein kinases. After several rounds of screen of small che micals, they found one named STI571 (now Imatinib Mesylate) emerged as the most promising compound for clinical development, since it had the highest selectivity for growth inhibition of BCR-ABL–expressing cells. Studies showed that imatinib potently inhibits all of the ABL tyrosine kinases. This includes cellular ABL, viral ABL (*v*-ABL), and BCR-ABL[57, 58]. In contrast, the compound was inactive against serine/threonine kinases, did not inhibit the epidermal growth factor (EGF) receptor intracellular domain, and showed weak or no inhibition of the kinase activity of the receptors for vascular endothelial growth factor (VEGF-R1 and VEGF-R2), fibroblast growth factor receptor 1 (FGF-R1), tyrosine kinase with immunoglobulin and EGF homology-2 (TIE-2 [TEK]), c-MET, and nonreceptor tyrosine kinases of the Src family (Fgr, Lyn, and Lck). The results of the kinase assays were confirmed in cell lines expressing constitutively active forms of ABL such as *v*-ABL, BCR-ABL, where imatinib was found to inhibit ABL kinase activity with 50% inhibitory concentration (IC50) values ranging between 0.1 and 0.35μM[58]. Imatinib potently inhibits the growth of cells expressing BCR-ABL, while up to 10 μM did not inhibit growth of parental or v-SRC–transformed cells[57]. A five-year

follow up study showed that a complete cytogenetic response among 553 patients receiving imatinib was 69% by 12 months and 87% by 60 months. An estimated 7% of patients progressed to accelerated-phase CML or blast crisis, and the estimated overall survival of patients who received imatinib as initial therapy was 89% at 60 months.[59]

To determine how imatinib achieves its high specificity at inhibiting BCR-ABL transformation, the crystal structure of the kinase domain of ABL complexed with imatinib was resolved by Kuriyan's and Zimmermann's groups.[60-62] Both groups showed that imatinib bound to the inactive conformation of ABL in the region where the adenosine base of ATP would bind thereby obstructing ATP binding. So imatinib blocks BCR-ABL kinase activity by blocking the binding between BCR-ABL kinase domain and ATP.

Although imatinib has become a powerful drug clinically, it is much less effective in treating CML patients containing mutations of the BCR-ABL kinase domain. Mutations of kinase domain are found in 50% to 90% of patients with secondary resistance[63-65]. Mutations are detected in many different amino acids, but there are 4 distinguishable clusters: the ATP binding loop (P-loop), T315, M351, and the A-loop. Recently, although several new derivatives of imatinib have been developed and they can overcome most of these mutations, all of these drug are still ineffective against T315I mutated BCR-ABL. Thus, new approaches are needed to treat CML induced by this BCR-ABL mutant.

Another obstacle in CML therapy comes from leukemia stem cell (LSC). LSCs in many types of hematologic malignancies are believed to be a cell population required for initiation and sustaining growth of the leukemia.[66-72] LSCs may arise from normal hematopoietic stem cells or normal progenitor populations.[73] In BCR-ABL induced CML, LSCs phenotypically appear to be similar to the normal hematopoietic stem cells (HSC). Fialkow and colleagues examined different cell types in chronic phase CML patients for the presence of the Ph chromosome.[74, 75] Surprisingly, both granulocytes and erythroid cells from chronic phase CML patients contained the Ph chromosome, even though only myeloid cells are expanded during chronic phase CML. The presence of the Ph chromosome in granulocyte and erythroid lineages suggests that the Ph chromosome is either generated in multiple cell types or originates in a HSC, from which it is passed down to other more differentiated cell lineages. Subsequent purification of HSCs from CML patients by cell surface markers has confirmed the presence of the Ph chromosome in HSCs.[76] The discovery of a clonal LSCs origin of CML suggests that elimination of Ph^+ LSCs and replacement of these cells with normal HSCs should be an effective therapeutic strategy.

To further identify CML stem cells in mice, we have assessed whether BCR-ABL-expressing HSCs function as CML stem cells in our CML mouse model. C57BL/6 (B6) mouse bone marrow (BM) cells transduced with BCR-ABL retrovirus were first sorted into two separate populations of Sca-1^- and Sca-1^+ cells. These two populations of cells were then transferred, respectively, into B6 recipient mice. Only the mice receiving BCR-ABL-transduced Sca-1^+ cells developed and died of CML, diagnosed by detecting

GFP^+ myeloid cells ($Gr\text{-}1^+$) in the peripheral blood of the mice, suggesting that early BM progenitors contain CML stem cells. To narrow down the specific cell lineages that function as CML stem cells, HSCs ($Lin^-c\text{-}Kit^+Sca\text{-}1^+$) were sorted out from BM cells transduced with BCR-ABL retrovirus, followed by transfer into recipient mice. The mice developed and died of CML. To confirm that BCR-ABL-expressing HSCs contain CML stem cells, BM cells from primary CML mice were harvested and sorted for the BCR-ABL-expressing HSCs ($GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$) by FACS. The sorted cells were transferred into recipient mice, and the mice developed and died of CML, indicating that BCR-ABL expressing HSCs function as CML stem cells.[77] When CML mice were treated with imatnib or dasatinib, a more potent BCR-ABL kinase inhibitor, both mice achieved significant longer survival curve and mice received dasatinib treatment died latest. However, all of the mice of imatinib or dasatinib treatment group eventually died as CML, indicating sole inhibition of BCR-ABL kinase is not enough to eliminate LSCs and targeting at least one additional component of BCR-ABL-expressing HSCs is required for curing the disease.[77]

In CML patients, bone marrow $CD34^+Lin^-$ cells, in which normal hematopoietic stem cells (HSCs) reside, are thought to contain CML stem cells and be responsible for disease initiation, progression and resistance to imatinib[38, 78]. Several clinical reports have confirmed withdraw imatinib treatment in those patients who had achieved complete molecular response after imtainib treatment, BCR-ABL expressing leukemia cells relapsed shortly after discontinuation of the therapy.[79, 80] Although restart of the imatinib treatment induced new molecular response and most of the patients could not be detected

BCR-ABL, imatinib still did not eventually cure the disease. These results proved imatinib could not eradicate LSCs in CML patients and LSCs will reinitiate the disease when the inhibition of BCR-ABL kinase activity is removed. Except the clinical observations, human CML LSCs have also been treated by imatinib in vitro.[38] When CFSE labeled LSCs (Lin^-CD34^+) from CML patients were treated with imatinib, almost all dividing cells were killed by imatinib. However, a significant $CD34^+$ population cells were recovered in the undivided peak in all patient cells.[38] This study also indicated the heterogeneity of LSCs in human CML LSCs and the undividing population is the most robust to be eliminated only based on the BCR-ABL kinase inhibitor. To exclude the lower inhibitory efficiency of imatinib on BCR-ABL kinase activity, both of BCR-ABL kinase second generation inhibitors, dasatinib and nilotinib are also assessed in LSCs in vitro treatment. Dasatinib is a novel dual Src and BCR-ABL kinase inhibitor and exhibits greater poterncy than imatinib.[81] Although dasatinib led to significant inhibition of BCR-ABL and its downstream activity, the most primitive quiescent CML LSCs ($CD34^+Cd38^-$) were still resistant to dasatinib treatment.[82] Similarly, nilotinib, a 20-fold-higher potency than imatinib, exerts quipotent antiproliferative effects to $CD34^+$ LSCs and it could not induce apoptosis in LSCs.[83] Taken together, both of CML LSCs in either mouse model or patients are resistant to the BCR-ABL kianse inhibitors, indicating LSCs maintain themselves not only by depending BCR-ABL kinase activity but also by counting on other crucial pathways. Targeting these unknown pathways should provide rational strategies to eventually inhibit or eradicate LSCs.

5. Novel targets for eliminating LSCs in CML

LSCs in CML are BCR-ABL transformed HSCs and they not only self-renew to maintain the LSCs pool but also differentiate to mature leukemia cells. However, as BCR-ABL cannot initiate self-renew in progenitor cells to induce CML, LSCs have to addict to these critical self-renew pathways which are also crucial to normal HSCs to maintain LSCs population. β-catenin, hedgehog and Foxo are all important components in HSCs self-renew program and they are great target in LSCs research. Except these pathways which involve in HSCs self-renew, those HSCs surface markers which promote or maintain HSCs homing capability are also considerated in LSCs therapy because inhibition of LSCs homing or promotion of LSCs migration is also possible strategy to block leukemia development. Finally, investigation of novel LSCs specific pathways is much tough but is also most promising because inhibition of these specific pathways should only affect LSCs but not HSCs.

β-catenin

LSCs are capable of limitless self-renewal and are responsible for the maintenance of leukemia. Because selective eradication of CML LSCs could offer promising therapeutic benefit, there is interest to identify the signaling pathway that controls the LSCs self-renewal. [84] Wnt factors are cysteine-rich lipid-modified proteins that bind to several Frizzled (FZD) receptors. Under physiological conditions, Wnt proteins accumulate β-catenin by inhibiting its glycogen synthase kinase 3 (GSK3)-dependent serine/threonine (S/T) phosphorylation on specific N-terminal residues. The

Wnt/ β -catenin cascade has also pivotal roles in the selfrenewal of hematopoietic stem cells (HSC), as a forced expression of a nondegradable β -catenin (S33-mutant) is sufficient to perpetuate themselves in vitro and sustain bone marrow reconstitution in vivo.[85]

Several groups are investigating the role of β-catenin in the LSCs and try to target β-catenin in the CML therapy. It has been reported that BCR-ABL levels control the degree of β-catenin protein stabilization by affecting its Y/S/T-phospho content in CML cells.[86] BCR-ABL directly interacts with β-catenin, and the Tyr86 (Y86) and Tyr654 (Y654) residues of β-catenin are phosphrylated by BCR-ABL kinase activity. This Y-phosphorylated β-catenin is in a stable conformation and it binds to the TCF4 transcription factor, thus representing a transcriptionally active pool.[86] At same time, another group reported loss of β-catenin could impair normal HSCs and CML LSCs self-renewal.[87] In this study, a conditional β-catenin knockout strain was established. Although normal HSCs were established in the β-catenin deletion condition, they are impaired in long-term growth and maintenance following transplantation. Interestingly, using a BCR-ABL retrovirus mouse model, the authors also demonstrated that loss of β-catenin block the CML development in recipients which are transplated with BCR-ABL transduced bone marrow cells from β-catenin null mice, due in part to the decreased self-renewal of CML LSCs.[87] This result was also confirmed in our study. Our lab recently reported the upregulation of β-catenin by BCR-ABL in our LSCs microarray. To further test the β-catenin function in LSCs in vivo, we used wild type and β-catenin conditional knockout mice as donor and transduced the marrow from these mice with BCR-ABL-

iCre coexpression retrovirus. After 14 days bone marrow transplantation, equal numbers of bone marrow cells from both groups of primary mice were transferred into secondary recipient mice. In the presence of β-catenin, all of mice developed and died of CML, However, all mice were free of CML in absence of β-catenin.[88] These results confirmed the critical role of β-catenin to support LSCs self-renewal and β-catenin also becomes a potential therapeutic target in CML LSCs therapy. A current exciting study proved β-catenin also maintain the self-renewal of AML LSCs which include oncogene transduced HSCs or more differentiated granulocyte-macrophage progenitor (GMP). [89] Inhibition of β-catenin by a reversible COX inhibitor, indomethacin, can reduce the LSCs number in vitro and decrease LSCs frequency in vivo. [89] These exciting results suggest indomethacin treatment might be a promising therapy strategy in CML.

Hedgehog

As early as 1980, the hedgehog (Hh) has been indentified in development of *Drosophila* and mutants of Hh genes alter the segmental pattern of the larva and cause embryonic lethal.[90] Later on, three homologs of Hh genes (Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh)) have been demonstrated in mammalians.[91-93] These Hh proteins are secreted proteins and they can induce signaling transduction in nearby and distant tissues. Generally, in the presence of active Hh protein, they will bind to its specific receptor, Patched (PTC). PTC is a transmemberane protein and negatively regulates another seven transmemberane protein, smoothened (SMO). When SMO is released from the inhibition of PTC, SMO will eventually activate its downstream Gli

transcription factor and Gli will regulate its target gene expression[94], including *Gli1*[95], *Ptch1*[96], *Cyclin D*[97], *Cyclin E*[97], and *Bcl2*[98].

Hh signaling has also been indicated a role in the primitive hematopoiesis based on mouse embryonic stem cell studies. Ihh is a primitive endoderm-secreted signal and it is sufficient to activate embryonic hematopoiesis and vasculogenesis in pre- or early-gastrulation-stage epiblasts.[99] Moreover, study from zebrafish showed the mutations of Hh pathway members or inhibition of Hh pathway with Hh inhibitor, cyclopamine, will result in adult HSC development defect. [100] However, as the functional redundancy of those three Hh members, the individual knock mice does not provide convincing evidence for the role of Hh in adult hematopoiesis. [101-103]

Activation of Hh pathway has been demonstrated in different human cancers. Activating point mutations of *Smo* or inactivating point of mutations of *Ptch* has been shown in medulloblastoma,[104] rhabdomyosarcoma,[105] and sporadic basal cell carcinoma.[106] Recently, Christine Dierks et al showed the hedgehog signaling pathway is active in BCR-ABL expressing leukemia stem cells (LSCs).[107] They first detected the there are about 4 fold induction of *Gli1*and *Ptch1* in CML patients compared with healthy donors. To confirm their clinical findings, they monitored the *Gli, Ptch1and Smo* mRNA level in BCR-ABL expressing LSCs in a retrovirus mouse model. Similarly, *Gli1, Ptch1 and Smo* display enhanced expression in LSCs. When the authors subsequently treated the CML mice with KAAD-cyclopamine, a Smo inhibitor, the LSCs have been significantly inhibited in vivo. Consistent with the treatment, the BCR-ABL transduced Smo deficiency mice fetal liver cells also failed to expand and induce the CML in mice. This

study demonstrated solid evidence to support the role of Hh pathway in leukemogenesis and also confirmed the new strategy in CML treatment that targeting LSCs could significantly prolong the CML latency.

Foxo

The Foxo (Forkhead O) subfamily of transcription factors plays critical roles in cell cycle arrest, stress resistance and apoptosis.[108] There are four members (Foxo1, Foxo3, Foxo4 and Foxo6) in Foxo group and they are all important downstream of the PI3K-Akt pathway which transduces cell survival and proliferation signals from cell surface receptors. Generally, growth factors bind their cell surface receptors and they subsequently active PI3K-Akt. Akt directly phosphorylates Foxos members, resulting in nuclear Foxos exclusion and degradation in cytoplasma.[109] Foxos locate in nucleus and activatly regulate their downstream, resulting in proapoptosis,[109, 110] cell cycle arrest[111] and resistant to oxidative stress response.[112] Although Foxos are vital player in cell cycle regulation, apoptosis and oxidation, individual knockout mice of Foxo1 and Foxo4 did not demonstrate overt hematopoietic phenotype. As these Foxo members could be functionally redundant, a Foxo1/3/4 triple conditional knockout mouse has been established recently.[113] Interestingly, a marked decrease of HSCs was observed when Foxos had been deleted for 4 weeks and the Foxo deficient HSCs are defective in long-term repopulation in vivo. Consistent with above results, a novel Foxo3a knock mice study showed the long-term reconstitution ability of HSCs was significantly inpaired

compared with the wild type mice.[114] Both studies proved a crucial role of Foxos to maintain HSCs long term reconstitution ability. As LSCs are always highly activated in reconstitution ability, Foxos pathway initiate more attention in LSCs research and targeting Foxos pathways might be a rational strategy in CML therapy.

As it is widely believed that BCR-ABL actives Akt and inhibits Foxo to promote leukemia cells proliferation and suppress apoptosis. A recently study provided solid evidence to prove Foxo3a has an essential role in maintaince of CML LSC.[84] In this study, nuclear localization of Foxo3q has been detected in LSCs similar as observed in normal HSCs[114] and the LSCs exhibited lower level of p-Akt, suggesting the Foxo3a remained active in LSCs owing to the decreased Akt phosphorylation. When the

Foxo3a+/+ and *Foxo3a*-/- mice are used as donor mice to induce CML by using a BCR-ABL retrovirus model, there are no difference in CML development in the primary and secondary recipients. However, the absolute numbers of *Foxo3a*-/- LSCs were much lower than in third round recipients that received *Foxo3a*+/+ LSCs. Thus, *Foxo3a*-/- LSCs lost their long-term reconstitution ability and exhaust in the third round transplantation. To further explain the mechanism, apoptosis rate of *Foxo3a*-/- LSCs has been detected and this indicated that Foxo3a is required for LSCs survival because it mediates suppression of apoptosis. Later on, TGF-β pathway has been activated in nuclei of LSCs which will control the Foxo3a nuclear localization and prevent LSCs apoptosis. To test whether inhibition of TGF-β pathway could mess up the Foxo3a localization and induce LSCs apoptosis, CML mice and human CML LSCs have been treated with TGF-β

inhibitor, Ly364947. Significantly, TGF-β inhibitor suppresses LSCs colony-forming ability and prolongs the CML mice disease latency. This inhibitory effect also synergize with imatinib therapy. This story evokes a new ankle to eradicate LSCs by inducing LSCs apoptosis. At the same time, current or more potent TGF-β inhibitors are promising drugs which need to be tested in mouse model and clinical trials.

CD44

Adhesion molecule CD44 is a ubiquitously expressed transmembrane glycoprotein that is extensively spliced and produces many variant isoforms.[115] CD44 mediates cell-cell and cell-extracellular matrix interactions through binding with its major ligand, hyaluronan, a glycosaminoglycan highly expressed in the endosteal region.[116] Also CD44 can bind to other ligands, including osteopontin, fibronectin, and seletin, and mediates cell trafficking, migrating and homing. Beyond its cell adhesion functions, CD44 also transduces several intracellular signals into cell when it binds to its specific ligands.

Interestingly, enhanced expression of CD44 on acute myeloid leukemia (AML) cells has been reported and the expression of CD44 also relates with poor prognosis of AML.[117, 118] Notably, inhibition of C44 by injection of its specific antibody blocked the human AML cells reconstitution in NOD-SCID mice.[119] This CD44 antibody binds to CD44 and selectively eliminates AML leukemia stem cells in vivo by blocking LSCs interaction with its niche.[119] Similar as AML, high expression of CD44 has also been detected in CML LSCs, suggesting CD44 might increases LSCs homing and maintaining

in bone marrow microenvironment to support their normal functions.[120] To prove the CD44 function in LSCs homing, *CD44-/-* mice bone marrow are transduced with BCR-ABL and transplanted into wild type recipients. The disease latency in recipients received BCR-ABL expressing *CD44-/-* cell was much slower than those received wild type cells. To further confirm the role of CD44 in regulating LSCs homing, same number of BCR-ABL expressing wild type or *CD44-/-* cells was directly injected into the femoral bone marrow cavity of the recipients. Strikingly, a similar frequency of CML disease was induced in both groups. These result identified that CD44 is a crucial LSCs surface molecule which can medicate LSCs homing and migration. Subsequently, blocking BCR-ABL expressing bone marrow cells with CD44 specific antibody before the bone marrow transplantation will significantly delay disease development in mice. In summary, the adhesion molecule CD44 mediates LSCs of CML and AML to home and migrate in the mice. Blocking this potent LSCs homing pathway could profoundly inhibit LSCs interact with their microenvironment and suppress LSCs function, eventually prohibiting leukemia development in mice.

6. Novel strategy for identifying therapeutic targets in LSCs

Above studies provide solid data to support the idea that inhibition and eradication of LSCs could suppress the CML development. However, current targets in LSCs are also critical molecules in regulating normal HSCs self-renewal or maintenance. It is still not clear how to find out the LSCs specific signaling pathways and regulators which LSCs addict to. In our lab, we take advantage of our CML model and compare the global genes expression difference in normal HSCs and LSCs by microarray analysis

(Figure. 3). Isolation of LSCs and normal HSCs is a key initial step to harvest 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 are up or downregulated by BCR-ABL in LSCs, and the key is to select candidate genes for further functional tests. Prior to the functional tests, we also confirm expression change of the candidate genes by real-time PCR. The function of a candidate gene can be primarily determined based on the literature or database search. Later on, we will analyze the candidate gene function in its knockout mice or transgenic mice. Spontaneously, we will assess the function of candidate gene in human LSCs and try to transit our bench work to bedside. Finally, we will utilize the specific inhibitors or stimulators in the mouse model or clinical trials to transit our basic research to patients.

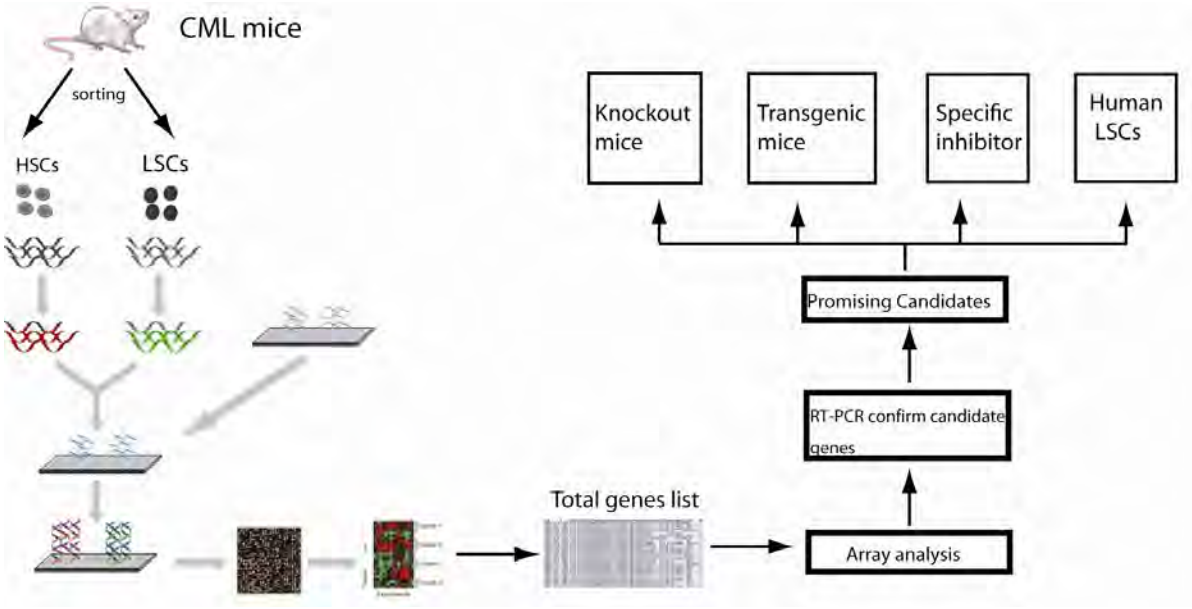

Figure 4. Strategy for identification of genes that play key roles in regulating the functions of LSCs. Normal HSCs and LSCs are sorted from BCR-ABL induced CML mice. Total RNAs are isolated from HSCs and LSCs and microarray is carried out using the RNAs. Total expression changed genes are analyzed as bioinformatics. Expression changes of interested candidate genes are confirmed by RT-PCR. Later, functional tests of candidate genes will be assessed in knockout mice, transgenic mice. Moreover, the expression of candidate genes are also tested in human CML LSCs. Lastly, the possible gene inhibitors will be applied in mouse model or human leukemia cells.

Following this strategy, our lab recent study identified the arachidonate 5-lipoxygenase (5-LO) gene (*Alox5*) is a novel LSCs specific regulator in CML development. *Alox5* has been shown to regulate numerous physiological and pathological progresses, including inflammation and cancer [121-123]. *Alox5* is differentially expressed in $CD34^+$ human CML cells and an *in vitro* study showed that an *Alox5* inhibitor suppressed proliferation and induced apoptosis of K562 cells (a human CML cell line)[124-126], although an off-target effect of the *Alox5* inhibitor needs to be ruled out. These data suggest a possibility that *Alox5* is involved in CML development.

To investigate the role of *Alox5* in BCR-ABL induced CML and –B-ALL, our lab first detected the *Alox5* mRNA level in CML LSCs.[127] We isolated total RNA from these BCR-ABL–expressing LSCs or from the GFP+Lin–c-Kit+Sca-1+ cells that expressed only GFP, and carried out DNA microarray analysis 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 upregulated and this upregulation was not abolished by imatinib treatment. Later on, the upregulation of Alxo5 by BCR-ABL in LSCs was also confirmed by RT-PCR. Next, to study the role of *Alox5* in regulation of

LSC function, wild-type or *Alox5-/-* donor bone marrow cells in B6 background were used to induce CML. 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 BCRABL–transduced bone marrow cells from *Alox5-/-* donor mice were resistant to the induction of CML. The eventual disappearance of myeloid leukemia cells in CML mice in the absence of *Alox5* prompted us to examine whether *Alox5* is required for self-renewal of LSCs. 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-/-* cells could not induce CML at day 20, the percentage or total number of bone marrow LT-LSCs ($GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+CD34^-$) was about half that of ST-LSCs/ MPP cells ($GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+CD34^+$). However, at day 90, the percentage or total number of LT-LSCs was about eightfold higher than that of ST-LSCs/MPP cells. 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, demonstrating that *Alox5* deficiency did not similarly affect differentiation of normal LT-HSCs. pressing *Alox5-/-* bone marrow cells failed to induce CML in secondary recipient mice. This result suggests that *Alox5* deficiency causes the impairment of the function of LSCs. Alox5 deficiency caused the impairment of the function of LSCs, and these cells were $GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$ phenotypically, containing 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.

Zileuton, a drug that has been currently used to treat human asthma, specifically inhibits the enzymatic activity of 5-LO, the product of the *Alox5* gene[128]. To test its therapeutic effect on CML, BCR-ABL transduced BM cells were transplanted into recipient mice to induce CML, and then the CML mice were treated with a placebo, Zileuton or imatinib alone, or two agents in combination. All placebo-treated mice developed and died of CML within 4 weeks after the induction of CML by BCR-ABL, and Zileuton alone was even more effective than imatinib in prolonging survival of CML mice. About 7 weeks after the treatment with Zileuton, GFP^+ $Gr\text{-}1^+$ leukemia cells in peripheral blood of the mice gradually declined and dropped from over 50% to less than 2%, indicating that myeloid leukemia is eventually eliminated. 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. At the early stage of CML development, Zileuton treatment only caused a less marked reduction of white blood cell counts than did imatinib treatment. This therapeutic effect of Zileuton on CML is caused by inhibiting LSCs. Long-term (LT)-LSCs were found to accumulate in BM of the treated mice; however, short-term (ST)-LSCs and multipotent progenitor cells (MPPs were gradually depleted, suggesting that inhibition of 5-LO by Zileuton causes the blockade of differentiation of LT-LSCs. The inhibitory effect of Zileuton are consistent with those from above-described genetic studies using *Alox5*$^{-/-}$ mice, demonstrating that

targeting of the Alox5 pathway is potentially curative for CML, and this idea needs to tested in human CML patients.

7. Hsp90 in CML

Hsp90 is a molecular chaperone which is responsible for managing newborn protein folding and quality control in the cell.[129] Besides involving in the regulation of misfolded proteins, Hsp90 is also a critical regulator maintains the stability and activation of a wide range of client proteins. [130] More important, most of its client proteins are mutated or hyperactivated oncoproteins which are involved in the proliferation, survival and anti-apoptosis in cancer development.[131]

Hsp90 has low ATP-dependent catalytic activity and it could be inhibited by its ATP competitive inhibitors, such as geldanamycin (GA) and its derivative 17-allylamino-17-emethoxygeldanamycin (17-AAG).[132] Hsp90 always function as a heterodimer or homodimer. When Hsp90 is in its ATP-binding conformation, the two N-domains tightly interact and form an activated conformation. Together with the constitutively dimerizated C-domain, the activated Hsp90 could hydrolyze ATP and stabilize its client proteins which are located in its molecular clamp. [133] Prevention of this conformational change by its ATP competitive inhibitors blocks activation and stability of Hsp90's client protein and suppresses the tumor cell proliferation.

HSP90 is constitutively expressed at 2- to 10-fold higher levels in tumor cells compared to their normal counterparts, suggesting that it is critically important for tumor cell growth and survival[134]. As early as the 1980s, Oppermann found HSP90 could

interact with $p60^{v\text{-}Src}$, stabilizing this client oncoprotein but they did not know what the mechanism for stabilizing was[135]. In 1994, Whitesell et al. first identified GA, which works as inhibitor of HSP90. They reported that GA could bind to Hsp90, followed by destabilization of v-src protein. They also observed a rapid, apparent dissociation of p60v-src from Hsp90 in drug-treated cells leading to p60v-src instability. This is the first work which sparked the idea that HSP90 can be designed as a target in tumor therapies. The list of HSP90 client proteins has grown strikingly over the last several years, including Her-2, Akt, Npm, b-Raf, Cdk4,[136] and its inhibitors have achieved several exciting preclinical trials.[134]

In 2000, Won et al. reported that BCR-ABL is a client protein of HSP90 and they observed the degradation of BCR-ABL protein after they treated human CML leukemia cell: K562, which harbors BCR-ABL, with GA[137]. This work indicated Hsp90 should be designed as a target in CML therapies (Figure 4). Later on, Bhalla and colleagues also reported that high (5 μM) concentrations of either GA or 17-AAG induced apoptosis in CML-derived cell lines, as well as in cells transfected with the BCR-ABL gene. [138] Notably, imatinib resistant BCR-ABL mutant cells isolated from patients are also sensitive to 17-AAG treatment.[139] All above studies demonstrate Hsp90 inhibitors could be promising drugs in the CML treatment.

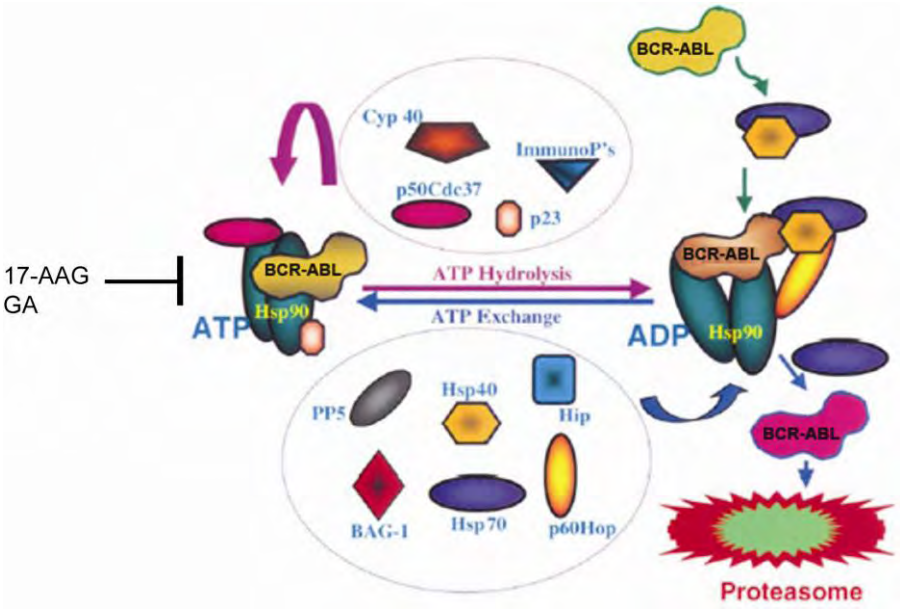

Figure 5. Mechanism of Hsp90-BCR-ABL complex and its inhibition effect. Nucleotide-dependent cycling of the Hsp90-based super-chaperone machine Hsp90 forms the basis of a super-chaperone machine that promotes the proper folding of client proteins so that they can respond to a stimulus or bind ligand. However, the machine is in constant flux and cycles between two Hsp90 conformations, determined by nucleotide binding, which in turn specify which set of cochaperones associate with the chaperone complex. Cochaperones that can associate with one conformation or the other include p23, p50Cdc37, p60Hop, immunophilins, cyclophilins, Hsp70, Hip, phosphatase PP5, Hsp40, and BAG-1. Cycling of this machine is driven by ATP hydrolysis. Although Hsp90 is a weak ATPase, its activity is regulated by cochaperones and dramatically enhanced by client protein binding. A client protein's half-life may be stochastically determined by the length of time it resides in association with the Hsp90-Hsp70 form of the chaperone machine, because at this time, the client protein is susceptible to ubiquitination and delivery to the proteasome. (Modified from Isaacs et al., *Cancer Cell*, 2003, vol. 3, 213-217)

Although GA and 17-AAG can be used in vitro, it is still difficult to use them in mice and clinical trials, as they are highly toxic and lowly soluble. Recently, IPI-504, a novel, water soluble HSP90 inhibitor, was developed by Infinity Pharmaceutical Co[140]. IPI-504 exists as a hydrochloride salt soluble in water in excess of 200mg/mL and is

4000- fold more soluble than 17-AAG. It has been previously been shown that IPI-504 inter-converts with 17-AAG and exists in a pH and enzyme-mediated dynamic redox equilibrium, which has been observed in human clinical trials[140]. These data show that IPI-504 is a promising HSP90 inhibitor which can be used widely in CML mouse model and patients.

8. PTEN and leukemias

The gene *Phosphatase and tensin homologue* (*PTEN*) is deleted or inactivated in many human tumor types, including glioblastoma[141, 142], endometrial carcinoma[143], prostate cancer[144], melanoma[145] and certain lymphoid malignancies.[146] The *PTEN* gene encodes a 403 amino acid protein that shares homology to dual-specificity phosphatases.[141, 142] PTEN demonstrates phosphatase activity against the phospholipid product of PI3K kinase activity, phosphatidylinositol(3,4,5)-trisphosphate (PIP3).[147-149] PIP3 plays a critical role in the regulation of cell survival and growth signaling through the activation of the Ser/Thr protein kinase PDK1 and its downstream target, Akt.[150, 151] Activated Akt is a key signal regulator of PI3K signaling pathway which mainly promotes the cell growth, metabolism, survival, and glucose homeostasis[152]. Pten negatively regulates the PI3K-Akt pathway by blocking the Akt phosphorylation. In mice, a complete null mutation of *Pten* results in early embryonic lethality at E9.5 with abnormally patterned enlarged brains and defective placentas.[153-155] In addition, *Pten* heterozygotes develop a broad range of tumors, including mammary, thyroid,

endometrial and prostate cancers[153-155], as well as autoimmune disease[156] and most *Pten* heterozygotes die within 1 year of birth. To investigate the physiological functions of Pten in adult tissues and organs, several groups already generated various tissue specific *Pten* conditional knockout strains using the *Cre-Loxp* system[157, 158]. Adult mice with a *Pten* conditional deletion in their hematopoietic system develop acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL), and subsequently die in one month[159, 160]. AKT1, the major PTEN downstream target, remains activated upon PTEN inactivation in human cancers[161]. Recent studies showed the deficiency of Akt1 is sufficient to inhibit several different kinds of tumors in *Pten*+/- heterozygous mice, including prostate cancer, endometrial carcinoma, thyroid neoplasia, intestinal polyps, and lymphoid hyperplasia[162]. All of these results confirmed the crucial role of the PTEN and AKT1 pathway in human cancers, including leukemia.

Compared with mutation or deletion of *Pten* reported in lymphoid malignancie[163] and induction of AML in *Pten* conditional deletion mouse models[159, 160], there are few studies to investigate Pten functions in CML. Recently, downregualtion of *PTEN* has been found in $CD34^+$ LSCs in human CML patients by comparing the CML LSCs and normal HSCs gene expression with a microarray analysis, indicating PTEN could be a tumor suppressor in CML. Furthermore, using gene expression profiling, another study showed that the expression of 105-probe sets in mononuclear cells collected from CML patients with raised leukocyte counts who subsequently achieved complete cytogenetic response after 12 months on imatinib, differed substantially from that of patients who failed to achieve any degree of cytogenetic response. In the non-responder cohorts,

downregulation of *PTEN* was also observed. Although how BCR-ABL downregulates PTEN is still unknown, both clinical results confirmed the Pten expression level changes in CML patients. How Pten function as tumor suppressor in CML and whether Pten restrain LSCs in CML development are fundamental questions which need to be detailed investigated.

Chapter II

Inhibition of heat shock protein 90 prolongs survival of mice with BCR-ABL-T315I–induced leukemia and suppresses leukemic stem cells

The work described in this chapter has been published (Peng et al.,2007).

Abstract

Development of kinase domain mutations is a major drug-resistance mechanism for tyrosine kinase inhibitors (TKIs) in cancer therapy. A particularly challenging example is found in Philadelphia chromosome–positive chronic myelogenous leukemia (CML) where all available kinase inhibitors in clinic are ineffective against the BCR-ABL mutant, T315I. As an alternative approach to kinase inhibition, an orally administered heat shock protein 90 (Hsp90) inhibitor, IPI-504, was evaluated in a murine model of CML. Treatment with IPI-504 resulted in BCR-ABL protein degradation, decreased numbers of leukemia stem cells, and prolonged survival of leukemic mice bearing the T315I mutation. Hsp90 inhibition more potently suppressed T315I-expressing leukemia clones relative to the wild-type (WT) clones in mice. Combination treatment with IPI-504 and imatinib was more effective than either treatment alone in prolonging survival of mice simultaneously bearing both WT and T315I leukemic cells. These results provide a rationale for use of an Hsp90 inhibitor as a first-line treatment in CML by inhibiting leukemia stem cells and preventing the emergence of imatinib-resistant

clones in patients. Rather than inhibiting kinase activity, elimination of mutant kinases provides a new therapeutic strategy for treating BCR-ABL-induced leukemia as well as other cancers resistant to treatment with tyrosine kinase inhibitors.

Introduction

The human Philadelphia chromosome (Ph) arises from a translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)].[14] The resulting chimeric *BCR-ABL* oncogene encodes a constitutively activated, oncogenic tyrosine kinase that induces chronic myeloid leukemia (CML) and B-cell acute lymphoblastic leukemia (B-ALL). The BCR-ABL TKI, imatinib mesylate, induces a complete hematologic and cytogenetic response in the majority of chronicphase CML patients,[164] but is unable to completely eradicate BCR-ABL–expressing leukemic cells,[38, 165] suggesting that leukemia stem cells are not eliminated. Over time, patients frequently become drug resistant and develop progressive disease despite continued treatment.[63, 65, 166-169] Resistance is predominantly due to emergence of kinase domain mutations. Three newly developed BCRABL kinase inhibitors—dasatinib,[81]AP23464,[170] and AMN107[171]—inhibit most of imatinib-resistant BCR-ABL mutants at biochemical and cellular levels, but are ineffective against the BCR-ABL-T315I mutant.[172, 173] New approaches are needed to treat drug-resistant forms of CML as well as BCR-ABL–induced B-ALL, a leukemia that does not respond well to available TKIs.[172, 173] Heat shock protein 90 (Hsp90) is a highly conserved, constitutively expressed molecular chaperone that facilitates folding of client proteins such as BCR-ABL, and affects the stability of these proteins.[131, 134, 137, 139, 174] When BCR-ABL

contains resistance-conferring mutations, it becomes even more dependent on Hsp90 in vitro.[139] We therefore evaluated the therapeutic effect of Hsp90 inhibition by using a novel water-soluble inhibitor, IPI-504,[140] in drug-resistant animal models of leukemia induced by BCR-ABL-WT and T315I.

Results

Inhibition of Hsp90 by IPI-504 causes BCR-ABL protein degradation

IPI-504 is the hydroquinone hydrochloride derivative of the well-described Hsp90 inhibitor, 17-AAG; the chemical structure of IPI-504 is shown in Figure 1A. To examine the effects of IPI-504 on stability of BCR-ABL protein and to test whether the degradation of BCR-ABL protein is initiated through IPI-504–induced disassociation of BCR-ABL from Hsp90, T315I-32D myeloid cells were treated with IPI-504 for 30 minutes and 4 hours, respectively. Hsp90 protein was immunoprecipitated and Hsp90-associated BCR-ABL protein was assessed. IPI-504 induced complete disassociation of BCR-ABL and Hsp90 within 30 minutes, followed by loss of BCR-ABL protein at 4 hours (Figure 1B). These results demonstrate that BCR-ABL protein is degraded after inhibition of Hsp90 by IPI-504 and this degradation occurs after disassociation of BCR-ABL from Hsp90. To further demonstrate that IPI-504 mediates the degradation of BCR-ABL through the proteasome, T315I-32D myeloid cells were treated with IPI-504 alone for up to 8 hours or with both IPI-504 and a proteasome inhibitor PS-341[175] that should

inhibit BCR-ABL degradation caused by IPI-504. PS-341 restored IPI-504–mediated depletion of BCR-ABL protein (Figure 1C).

Figure 1. Inhibition of Hsp90 by IPI-504 causes BCR-ABL protein degradation. (A) Structure of IPI-504. (B) IPI-504–induced disassociation of BCR-ABL and Hsp90, and subsequent degradation of BCR-ABL protein. BCR-ABL-T315I–expressing 32D cells were treated with IPI-504 (2 μM) for 30 minutes and 4 hours, respectively. Protein lysates were analyzed by Western blotting using antibodies indicated. WCL indicates whole cell lysate; IP, immunoprecipitation; and IB, immunoblotting. (C) The proteasome inhibitor PS-341 restored IPI-504–mediated depletion of BCR-ABL protein. BCR-ABL-T315I–expressing 32D cells were treated with IPI-504 (2 μM) alone or IPI-504 plus PS-341 (100 nM) for 4 or 8 hours, respectively. Protein lysates were analyzed by Western blotting using antibodies indicated. The well-described Hsp90 client, Akt, was evaluated as a positive control. Note that the cells were pretreated with PS-341 for 30 minutes prior to the cotreatment with IPI-504 and PS-341. The black lines indicate that the lanes that were not adjacent on the same original Western blotting gel were brought together to generate this figure.

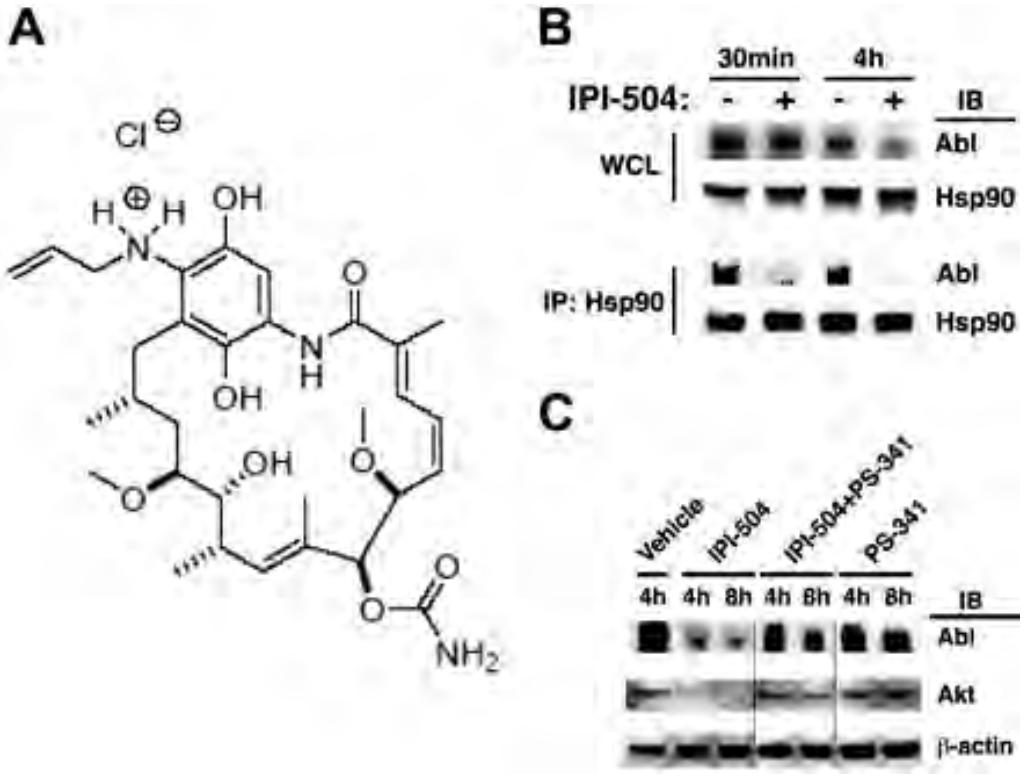

Figure 1. Inhibition of Hsp90 by IPI-504 causes BCR-ABL protein degradation.

Hsp90 is a therapeutic target for BCR-ABL–induced CML

An investigation of whether Hsp90 is an effective target for the treatment of CML in vivo was conducted in the bone marrow transplantation (BMT) mouse model of CML, in which bone marrow cells from BALB/c donor mice pretreated with 5-fluorouracil (5-FU) and transduced with *BCR-ABL* results in development of CML in BALB/c recipient mice.[32] Mice with WT or T315Itransduced bone marrow from 5-FU–treated WT BALB/c donor mice were treated with a placebo, the Hsp90 inhibitor IPI-504, or imatinib alone, or the 2 agents in combination. All placebo-treated mice developed and died of CML within 3 weeks after BMT (Figure 2A). As expected, imatinib treatment was effective to treat WT-induced CML but not CML induced by T315I (Figure 2A). In a dose-dependent manner, treatment with IPI-504 alone significantly prolonged survival of mice with WT CML, but even more markedly prolonged survival of mice with T315I-induced CML (Figure 2A, $P < 0.001$). Inhibition of Hsp90 by IPI-504 appears to be more effective in treating CML induced by T315I than by WT BCR-ABL, consistent with results in Figure 1A and in line with previously reported results with the Hsp90 inhibitor, 17-AAG.20 In both cases, inhibition of Hsp90 results in degradation of mutant BCR-ABL more readily than WT. Treatment of mice with WT CML with both IPI-504 and imatinib was slightly more effective (but statistically insignificant) than with imatinib alone in prolonging survival of the mice (Figure 2A), while treatment of mice with BCR-ABL-T315I–induced CML with these 2 drugs did not further prolong survival of the mice compared

with the mice treated with IPI-504 alone (Figure 2A). Prolonged survival of IPI-504–treated CML mice correlated with decreased peripheral blood BCR-ABL–expressing (GFP-positive) leukemia cells during therapy (Figure 2B, P <0 .001) and less splenomegaly at necropsy (Figure 2C). As lung hemorrhage caused by infiltration of mature myeloid leukemia cells is a major cause of death of CML mice,[32] we further evaluated the therapeutic effect of IPI-504 on CML by examining the severity of lung hemorrhages at day 15 after BMT. Compared with placebo-treated mice, fewer hemorrhages were observed in the lungs of IPI-504–treated mice with BCR-ABLT315I–induced CML (Figure 2D).Western blot analysis of spleen cell lysates from the treated CML mice showed that IPI-504 reduced the levels of BCR-ABL protein in CML mice (Figure 2E).

Figure 2. Hsp90 is a therapeutic target for CML induced by either BCR-ABL-WT or BCR-ABL-T315I. (A) Treatment with the Hsp90 inhibitor IPI-504 prolonged survival of CML mice. Mice with BCR-ABL-WT (left panel) or BCR-ABL-T315I (right panel) induced CML were treated with placebo (n=15 for BCR-ABL-WT; n=13 for BCR-ABL-T315I), imatinib (100 mg/kg, twice a day by gavage) (n =8 for both BCR-ABL-WT and -T315I), IPI-504 (50 mg/kg, once every 2 days by gavage) (n=20 for both BCR-ABL-WT and BCR-ABL-T315I), IPI-504 (100 mg/kg, once every 2 days by gavage) (n=8 for both BCR-ABL-WT; n=7 for BCR-ABL-T315I), and imatinib+IPI-504 (n=12 for both BCR-ABL-WT and -T315I), respectively, beginning at day 8 after transplantation. The IPI-504–treated mice with BCR-ABL-T315I–induced CML lived longer than those with BCR-ABL-WT–induced CML (comparing between left and right panels). (B) Flow cytometry evaluation of the leukemic process in IPI-504 or imatinib treated CML mice. The number of circulating leukemic cells (calculated as percentage of $Gr\text{-}1^{+}GFP^{+}$ cells X white blood cell count) in mice with BCR-ABL-WT (left panel) or BCR-ABL-T315I (right panel) induced CML treated with placebo, imatinib, IPI-504, or the combination of imatinib and IPI-504 was determined on day 14 after transplantation. (C) Spleen weights of CML mice treated with placebo, imatinib, IPI-504, and combination of imatinib and IPI-504. (Left panel) BCR-ABL-WT. (Right panel) BCR-ABL-T315I. (D) Photomicrographs of hematoxylin and eosin–stained lung sections from drug-treated mice at day 14 after transplantation. (E) Western blot analysis of spleen-cell lysates for degradation of BCR-ABL in IPI-504–treated CML mice. IB indicates immunoblot.

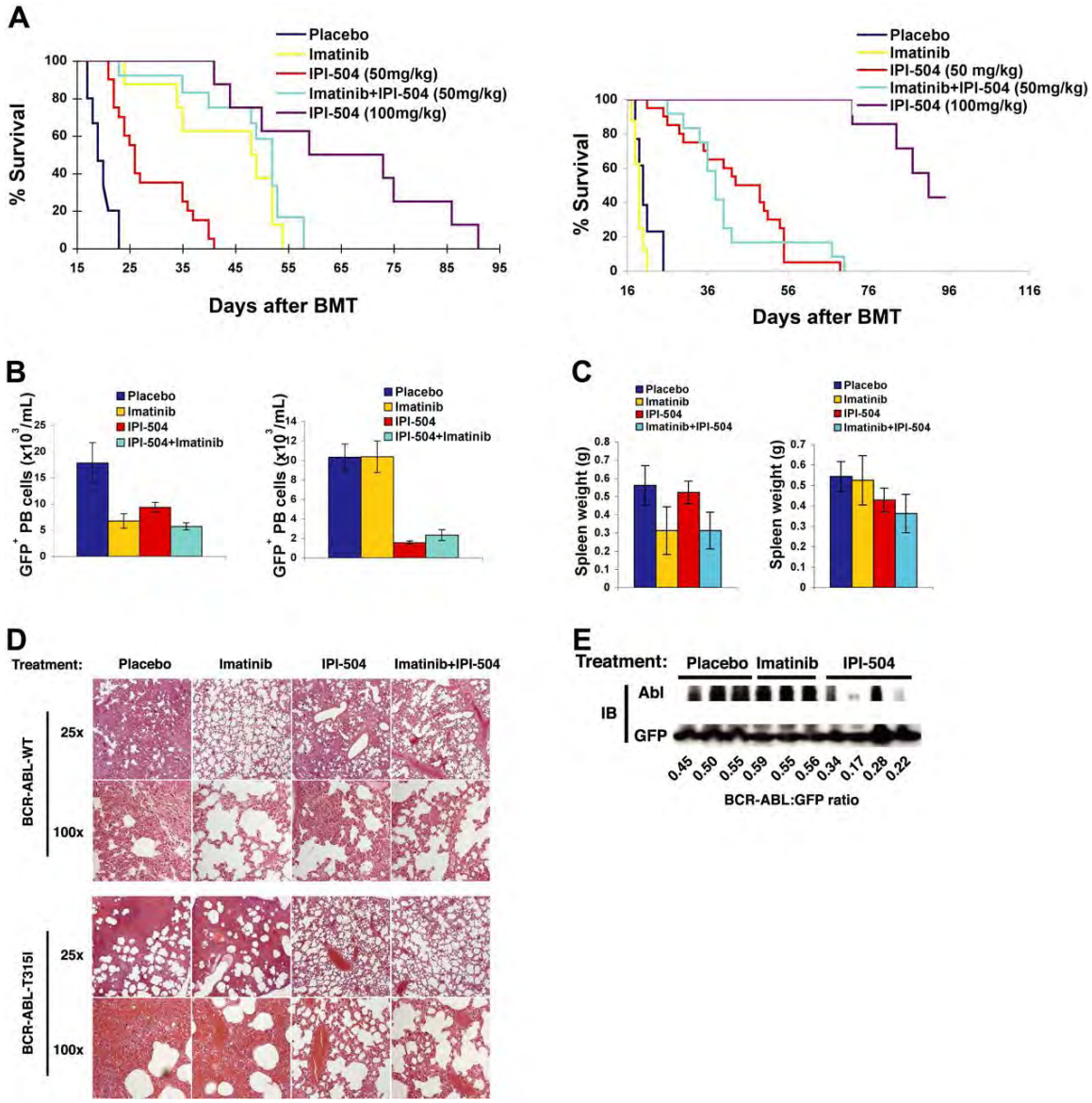

Figure 2. Hsp90 is a therapeutic target for CML induced by either BCR-ABL-WT or BCR-ABL-T315I.

Hsp90 is also a therapeutic target for B-ALL induced by BCR-ABL-T315I

CML often initiates in a chronic phase and eventually progresses to a terminal blastic phase, in which either acute myeloid or acute B-lymphoid leukemia develops.[176] Some Ph+ leukemia patients have B-ALL as their first clinical appearance. B-ALL is similar pathologically to acute B-lymphoid leukemia in the blastic phase of CML. Notably, both forms of acute leukemia do not respond well to available BCR-ABL kinase inhibitors.[172, 173] To model B-ALL in mice, BCR-ABL–transduced bone marrow cells from donor mice that are not pretreated with 5-FU are transplanted into BALB/c mice.[32, 177] In this model, the malignant pre-B cells express the cell surface markers B220 and CD19, and phenotypically resemble de novo Ph_ B-ALL and lymphoid blast crisis of CML.[32, 178] To determine whether inhibition of Hsp90 is effective in treating WT or T315I-induced B-ALL, these mice were treated with a placebo, IPI-504 alone, imatinib alone, or the 2 agents in combination (Figure 3). All placebo-treated recipients of WT or T315Itransduced bone marrow developed and died of B-ALL within 5 to 6 weeks after BMT (Figure 3A). IPI-504 treatment did not prolong survival of mice with BCR-ABL-WT–induced B-ALL (Figure 3A), in contrast to its therapeutic effect on CML induced by BCR-ABL-WT (Figure 2A). Given the dose response seen in the CML study and significant improvement in survival when the dose of IPI-504 is increased from 50 to 100 mg, a similar increase in dose may be needed in B-ALL. However, similar to the effect seen in CML (Figure 2A), IPI-504 treatment significantly prolonged survival of mice with T315I- B-ALL (Figure 3A, P <0.001). Prolonged survival of IPI-504–treated B-ALL mice correlated with decreased numbers of peripheral blood BCR-ABL–expressing

leukemia cells and spleen weights during therapy (Figure 3B-C, $P < 0.001$). Once again, inhibition of Hsp90 by IPI-504 is more effective against tumor cells bearing T315 than BCR-ABL-WT.

Figure 3. Hsp90 is a therapeutic target for B-ALL induced by BCR-ABL-T315I. (A) Treatment with the Hsp90 inhibitor IPI-504 prolonged survival of mice with B-ALL induced by BCR-ABL-T315I (right panel) but not by BCR-ABL-WT (left panel). B-ALL mice treated with a placebo (n=9 for BCR-ABL-WT; n=8 for BCR-ABL-T315I), imatinib (n=8 for BCR-ABL-WT; n=10 for BCR-ABL-T315I), IPI-504 (n=13 for BCR-ABL-WT; n=8 for BCR-ABL-T315I), and combination of imatinib and IPI-504 (n=10 for BCR-ABL-WT; n=8 for BCR-ABL-T315I). (B) Flow cytometric evaluation of the leukemic process in IPI-504– or imatinibtreated mice with B-ALL induced by BCR-ABL-WT (left panel) or BCR-ABLT315I (right panel). The number of circulating leukemic cells (calculated as percentage of $B220^{+}GFP^{+}$cells X white blood cell count) in B-ALL mice treated with placebo, imatinib, IPI-504, or the combination of imatinib and IPI-504 was determined on days 11, 14, and 17 after transplantation. (C)Spleen weights of B-ALL mice treated with placebo, imatinib, IPI-504, and combination of imatinib and IPI-504. (Left panel) BCR-ABL-WT. (Right panel) BCR-ABL-T315I.

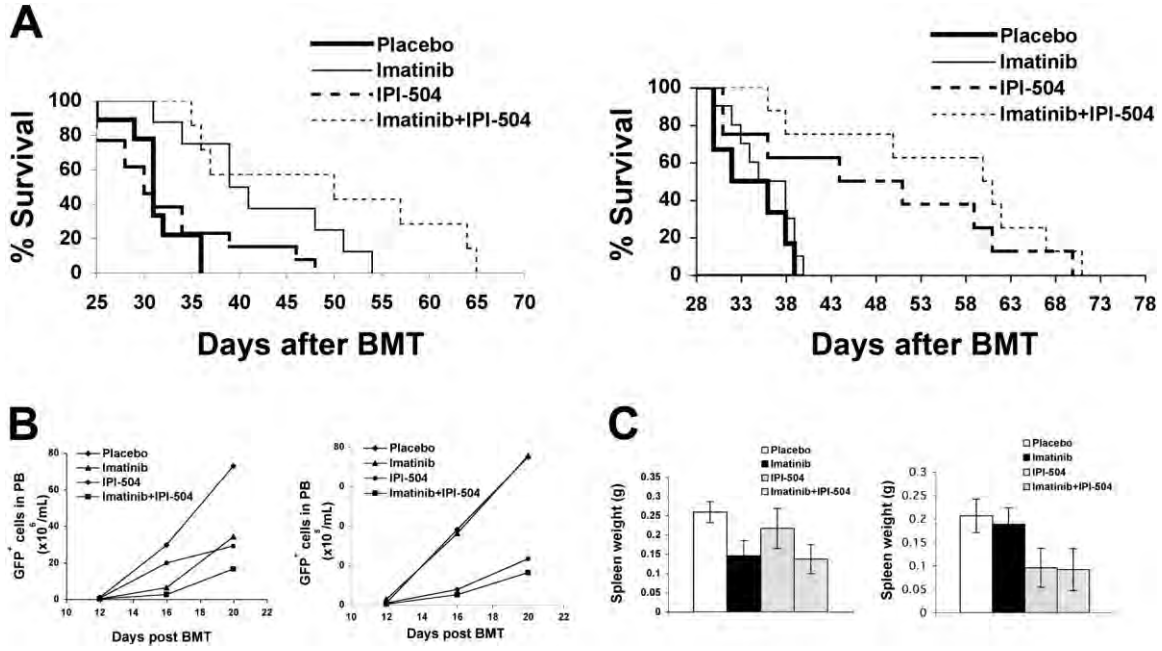

Figure 3. Hsp90 is a therapeutic target for B-ALL induced by BCR-ABL-T315I.

Hsp90 inhibition has differential effects on BCR-ABL degradation and Hsp70 induction in myeloid and lymphoid cells in vitro.

To investigate why inhibition of Hsp90 is more effective in treating CML than B-ALL (Figures 2-3), we compared the effects of treatment with IPI-504 on BCR-ABL-WT or BCR-ABL-T315I at protein level in a mouse myeloid cell line (32D) and a mouse lymphoid cell line (BaF/3) (Figure 4). BCR-ABL–expressing 32D and BaF/3 cells were treated with different concentrations of IPI-504. After treatment, levels of BCR-ABL-WT protein were dramatically decreased in 32D cells (Figure 4A), but only slightly in BaF/3 cells (Figure 4B). Compared with BCR-ABL-WT, BCR-ABL-T315I was more sensitive to IPI-504–induced degradation in both 32D and BaF/3 cells, but levels of BCR-ABL protein were decreased much more markedly in 32D cells than in BaF/3 cells (Figure 4A-B). These results indicate that inhibition of Hsp90 by IPI-504 affects BCR-ABL stability more strongly in myeloid cells than in lymphoid cells. It has been shown that the Hsp90 antagonists geldanamycin and 17-AAG alter chaperone association of Hsp90 with BCR-ABL and facilitate binding of BCR-ABL to heat shock protein 70 (hsp70), resulting in degradation of BCR-ABL by the proteasome.[137, 138, 179, 180] Recent studies have shown that Hsp70 plays a positive role in BCR-ABL–mediated resistance to apoptosis.[181, 182] If Hsp70 plays a role in decreased sensitivity of B-ALL than CML to IPI-504 treatment, we expect that after IPI-504 treatment, Hsp70 would be induced to a much higher level in BCR-ABL–expressing lymphoid cells than in myeloid cells. However, an increase in intracellular Hsp70 levels was observed in IPI-504–treated BCR-ABL–expressing 32D but not Ba/F3 cells (Figure 4A-B). This observation is consistent with our in vivo

observation in cells from CML and B-ALL mice, which showed that the level of Hsp70 in leukemic cells from IPI-504-treated CML mice is higher than that in leukemic cells from B-ALL mice (Figure 4C-D). Thus, Hsp70 is only a partial explanation for the decreased sensitivity of B-ALL compared with CML upon IPI-504 treatment (Figures 2-3).

Figure 4. Hsp90 inhibition has differential effects on BCR-ABL degradation and Hsp70 induction in myeloid and lymphoid cells in vitro and in vivo. (A) In 32D cells, IPI-504–induced degradation of BCR-ABL-T315I was greater than that of BCR-ABL-WT. BCR-ABL-WT– or BCR-ABL-T315I–expressing 32D cells were treated with different concentrations of IPI-504 for 12 hours. Protein lysates were analyzed by Western blotting using antibodies indicated. (B) In Ba/F3 cells, IPI-504 induced significant degradation of BCR-ABL-T315I but not BCR-ABL-WT. BCR-ABL-WT– or BCR-ABL-T315I–expressing 32D cells were treated with different concentrations of IPI-504 for 12 hours. Protein lysates were analyzed by Western blotting using antibodies indicated. (C) Mice with BCR-ABL-T315I–induced CML were treated with placebo, imatinib (100 mg/kg, twice a day by gavage), and IPI-504 (50 mg/kg, once every 2 days by gavage), respectively, for 8 days, beginning at day 8 after transplantation. At 6 hours after the last dose, protein lysates of leukemic cells from the spleen of the treated CML mice were analyzed by Western blotting using antibodies indicated. The black line indicates that the lanes that were not adjacent on the same original Western blotting gel were brought together to generate this figure. (D) Mice with BCR-ABL-T315I–induced B-ALL were treated with placebo, imatinib, and IPI-504, respectively, for 8 days, beginning at day 8 after transplantation. At 6 hours after the last dose, protein lysates of leukemic cells from the spleen of the treated mice were analyzed byWestern blotting using antibodies indicated. The black line indicates that the lanes that were not adjacent on the same original Western blotting gel were brought together to generate this figure.

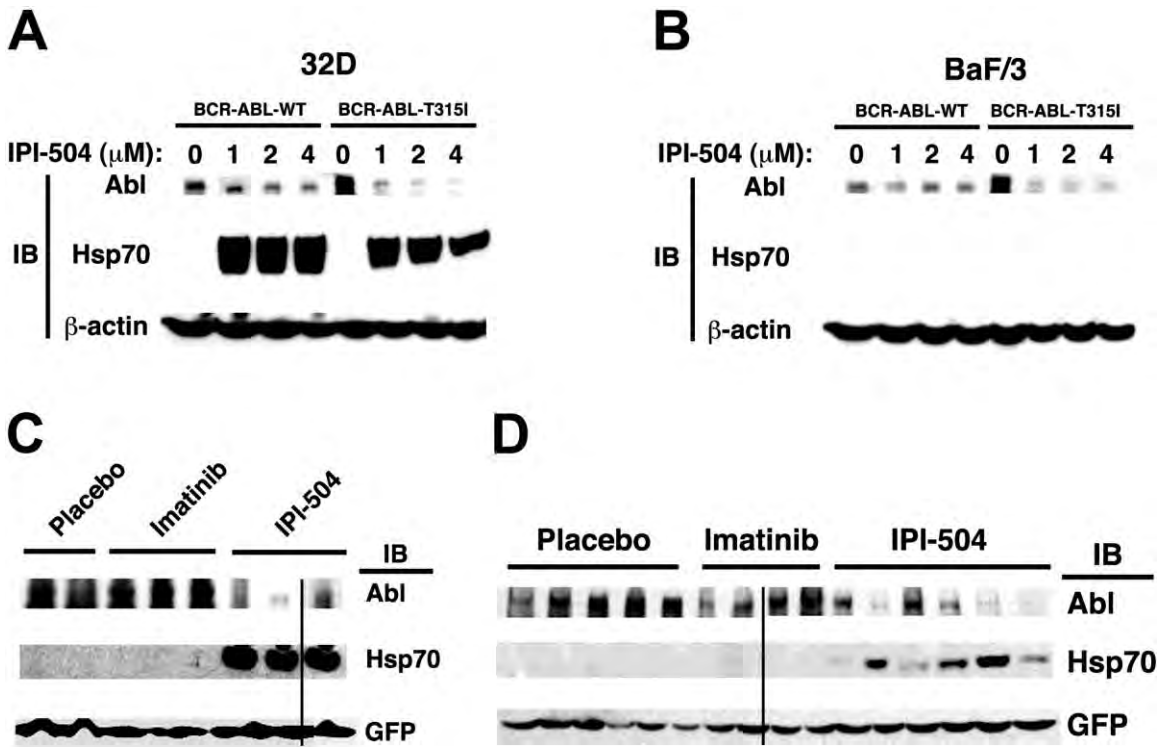

Figure 4. Hsp90 inhibition has differential effects on BCR-ABL degradation and Hsp70 induction in myeloid and lymphoid cells in vitro and in vivo.

Inhibition of Hsp90 suppresses CML stem cells

In the BMT CML model, imatinib prolongs survival of mice with BCR-ABL–induced CML,[177, 183] but does not stop progression of the disease,[177] partially due to the inability of imatinib to completely eradicate leukemia stem cells.[77] Hematopoietic stem cells (HSCs) have been identified in the CML model by showing that the $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ population is sufficient to confer leukemia in recipient mice. To investigate whether inhibition of Hsp90 has an inhibitory effect on leukemia stem cells in CML, we first isolated bone marrow cells from mice with T315I-induced CML and cultured the cells in conditions that support survival and growth of HSCs.[184, 185] During culture, the cells were treated with IPI-504 or imatinib (Figure 5). Six days after the treatment, we analyzed survival of $GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells, representing leukemia stem cells remaining in the culture. FACS analysis showed that compared with the untreated group, imatinib treatment did not lower the percentage and the number of leukemia stem cells, whereas IPI-504 treatment had a dramatic inhibitory effect on the stem cells (Figure 5A, $P < 0.001$). We next tested whether IPI-504 inhibited leukemia stem cells in CML mice. BCR-ABLT315I–induced CML were treated with a placebo, imatinib, or IPI-504 for 6 days, and bone marrow cells were analyzed by FACS for $GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells. Consistent with the in vitro results, imatinib treatment did not lower the percentage and number of leukemia stem cells, compared with the untreated group, whereas IPI-504 treatment had a dramatic inhibitory effect on the stem cells (Figure 5B). To determine whether IPI-504 had an effect on normal HSCs in mice, WT mice were treated with IPI-504 or placebo for 2 weeks. Analysis of bone marrow from these mice showed that there was no change in levels of $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells from any treatment group (Figure 5C), indicating that IPI-504 treatment did not inhibit survival of normal HSCs.

Figure 5. Targeting Hsp90 by IPI-504 inhibits survival of leukemia stem cells. (A) Bone marrow cells isolated from C57BL/6 (B6) mice with BCR-ABL-T315I–induced CML were cultured in vitro (5 X 10^6 cells/6 cm tissue culture plate) under the stem cell condition (~~–M~~aterials and methods") in the presence or absence of IPI-504 (0.1 μM) or imatinib (2 μM) for 6 days (changing the stem cell medium containing placebo or IPI-504 at day 3) followed by FACS analysis of leukemia stem cells ($GFP^+Lin^-c\text{-}Kit^+Sca\text{-}1^+$). (B) Mice with BCR-ABL-T315I–induced CML were treated with a placebo (n=5), imatinib (100 mg/kg, twice a day by gavage) (n=5), and IPI-504 (50 mg/kg, once every 2 days by gavage) (n=5), respectively, for 6 days beginning at day 8 after transplantation. Bone marrow cells were isolated from the treated CML mice, and leukemia stem cells were analyzed by FACS. The numbers of cells represent total leukemia stem cells in average from femur and tibia of each treated CML mouse. (C) IPI-504 had no inhibitory effect on survival of normal HSCs in mice. B6 mice were treated with a placebo (n=5), imatinib (100 mg/kg, twice a day by gavage) (n=5), and IPI-504 (50 mg/kg, once every 2 days by gavage) (n=5), respectively, for 2 weeks. Bone marrow cells were isolated from the treated mice, and were analyzed by FACS.

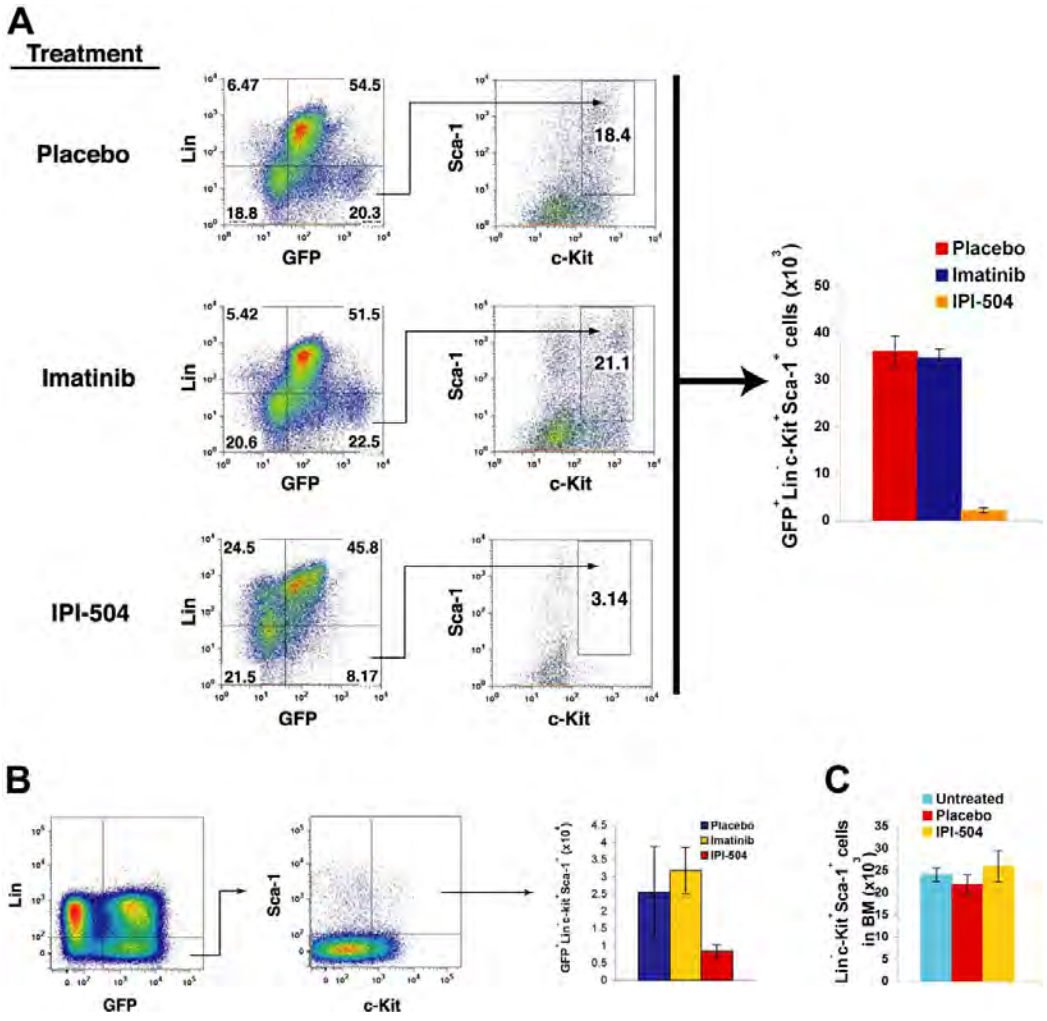

Figure 5. Targeting Hsp90 by IPI-504 inhibits survival of leukemia stem cells.

Inhibition of Hsp90 prevents emergence of the T315I-expressing clones over the WT clones.

The effectiveness of IPI-504 in prolonging survival of mice with CML and B-ALL induced by the T315I mutant (Figures 2-3) suggests that inhibition of Hsp90 would preferentially prevent emergence of the T315I-expressing clones over the WT clones. To test this hypothesis, studies were performed in mice bearing both populations of leukemic cells. In the first study, bone marrow cells (BMCs) from Ly 5.1 and Ly5.2 C57BL/6 mice were transduced with BCR-ABL-T315I and BCR-ABL-WT, respectively. Equal numbers of donor BMCs were mixed and transplanted into recipient mice. Mice were treated with a placebo, imatinib, or IPI-504. During the treatment, FACS analysis was performed to assess the percentages of $GFP^{+}Gr\text{-}1^{+}Ly5.1^{+}$ (representing T315Iexpressing cells) and $GFP^{+}Gr\text{-}1^{+}Ly5.1^{-}$ (representing WT expressing cells) cells in peripheral blood of the CML mice (Figure 6A). In placebo-treated mice, the ratio between T315I- and WT-expressing cells remained unchanged, and in imatinib-treated mice, T315I-expressing cells became dominant. In contrast, with continuous treatment of IPI-504, T315I-expressing cells gradually decreased to a low level (Figure 6A). Mice treated with IPI-504 lived significantly longer than those treated with imatinib (Figure 6A). Consistent with previous data, these results indicate that inhibition of Hsp90 preferentially suppresses T315I-expressing leukemic clones over the WT-expressing clones. In the second study, BMCs from BALB/c mice were transduced with BCR-ABLT315I and BCR-ABL-WT, respectively, and equal numbers of the transduced cells were mixed and transplanted into recipient mice. Mice were treated with a placebo, imatinib, IPI-504, or

both agents (Figure 6B). Mice treated with the combination of IPI-504 and imatinib survived significantly longer than those treated with IPI-504 or imatinib alone. Results from these studies suggest that the combined use of IPI-504 and imatinib would be a viable strategy for preventing emergence of imatinib-resistant clones in the clinic.

Other imatinib-resistant BCR-ABL mutants are also sensitive to Hsp90 inhibition

Other resistance-conferring BCR-ABL kinase domain mutations have been observed in imatinib refractory CML patients, including E225K, M351T, and Y253F.[65, 166] Consistent with the increased dependency of BCR-ABL-T315I on Hsp90, IPI-504 also prolonged survival of mice with CML induced by these mutants (Figure 7).

Figure 6. Inhibition of Hsp90 by IPI-504 preferentially reduces growth of myeloid leukemic cells harboring the BCR-ABL-T315I mutant. (A) Bone marrow cells from C57BL/6-Ly5.2 mice were transduced by BCR-ABL-WT, and bone marrow cells from C57BL/6-Ly5.1 mice were transduced by BCR-ABL-T315I. The transduced cells were 1:1 mixed, and 0.5 X 10^6 mixed cells were injected into each recipient mouse (C57BL/6-Ly5.2). The mice were treated with a placebo (n=10), imatinib (100 mg/kg, twice a day) (n=10), and IPI-504 (50 mg/kg, once every 2 days) (n=10), respectively, beginning at 8 days after BMT. At days 12 and 15 after BMT, GFP_ cells viable cells in peripheral blood of the mice were analyzed for Gr-1^+Ly5.1^+cells that represented BCR-ABL-T315I–expressing myeloid cells. Gr-1^+Ly5.1^- cells represented BCR-ABL-WT–expressing myeloid cells. Percentages of BCR-ABLT315I–expressing myeloid cells in peripheral blood of IPI-504–treated CML mice were further analyzed at days 21 and 28 after BMT. The FACS results for one representative mouse from each treatment group were shown. IPI-504 but not imatinib significantly prolonged survival of the CML mice. (B) Simultaneous inhibition of Hsp90 and BCR-ABL kinase activity with IPI-504 and imatinib significantly prolongs survival of CML mice carrying both T315-expressing and WT-BCR-ABL leukemia cells. BALB/c mice were used to induce CML, and each treatment group had 10 mice.

Figure 7. Other imatinib-resistant BCR-ABL mutants are also sensitive to Hsp90 inhibition. IPI-504 treatment prolonged survival of mice with CML induced by imatinib-resistant BCR-ABL-E225K (n=10), -M351T (n=10), or -Y253F (n=10).

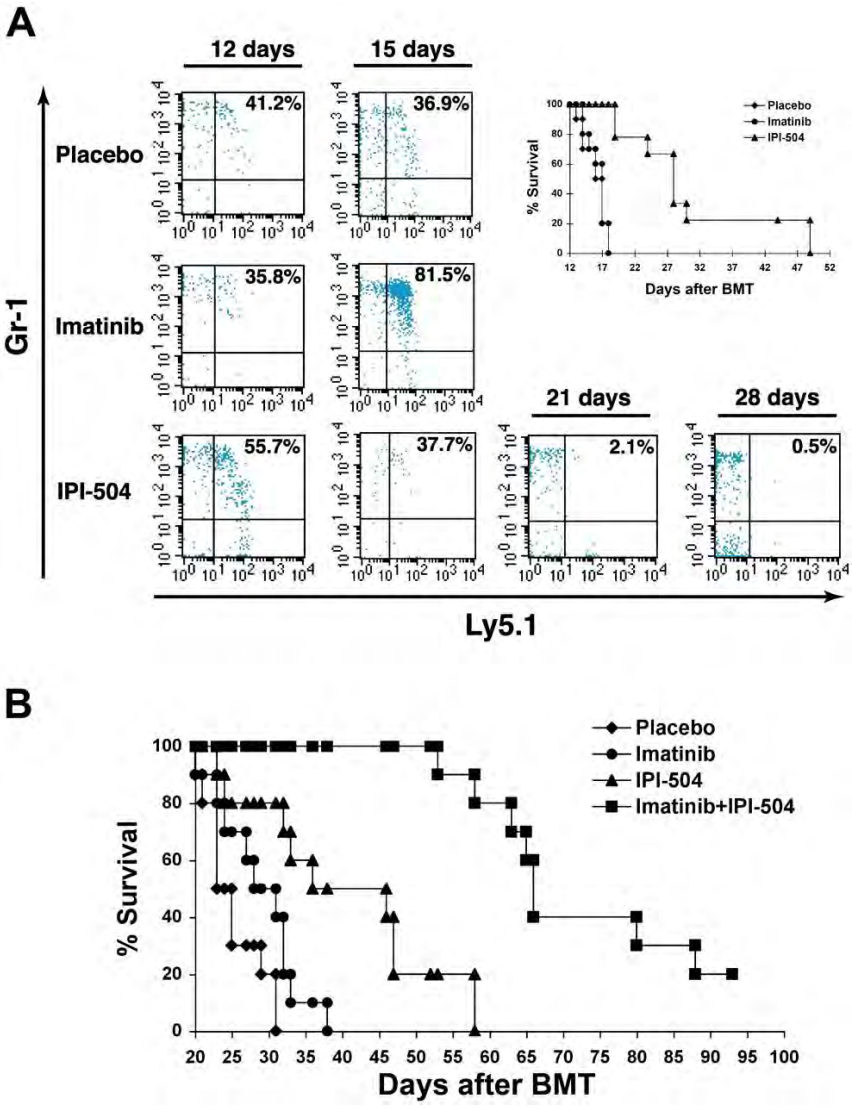

Figure 6. Inhibition of Hsp90 by IPI-504 preferentially reduces growth of myeloid leukemic cells harboring the BCR-ABL-T315I mutant.

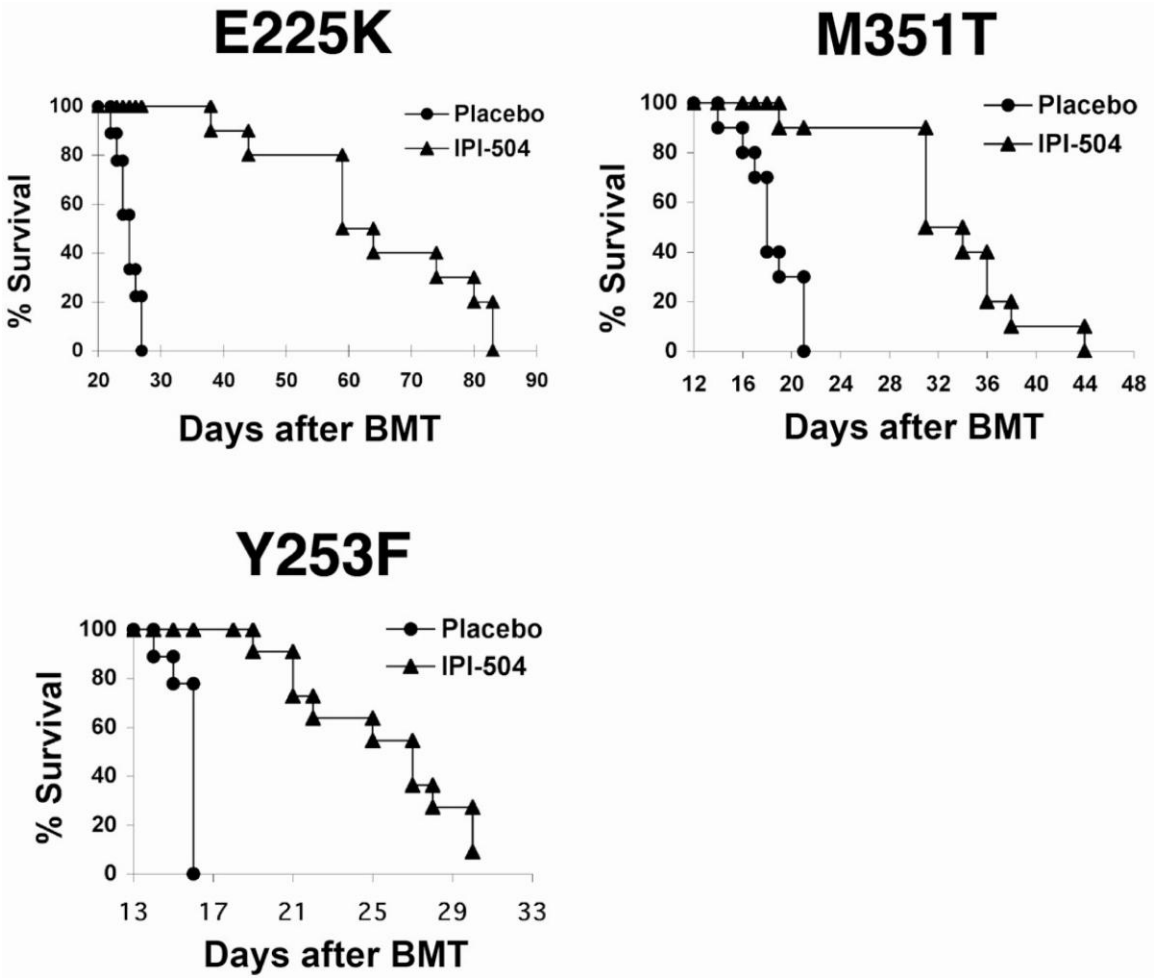

Figure 7. Other imatinib-resistant BCR-ABL mutants are also sensitive to Hsp90 inhibition.

Discussion

While the mechanism of primary resistance to imatinib and dasatinib therapy in CML patients is poorly understood, the mechanisms of secondary resistance have been very well characterized. Kinase domain mutations represent the predominant form of secondary resistance accounting for up to 90% of cases. Currently, no drugs have been effective in treating patients with CML and B-ALL harboring the BCR-ABL-T315I mutation. Recent clinical trials with dasatinib revealed that patients known to have the BCR-ABL-T315I mutation prior to therapy had no objective response to treatment.[173] Thus, as newer tyrosine kinase inhibitors (TKIs) that effectively block other resistant mutations become clinically available, the T315I mutation may become the predominant acquired resistance mutation. The challenge for development of an effective Ph+ leukemia therapy is therefore to develop an alternative treatment strategy that does not rely solely on kinase domain inhibition but rather results in degradation of the offending BCR-ABL protein regardless of its mutational status. Herein, we demonstrate that direct inhibition of Hsp90 function with IPI-504 represents an alternative treatment strategy that results in degradation of the offending BCR-ABL protein regardless of its mutational status. Our findings suggest that inhibition of other targets that impact Hsp90 function might also be effective in murine models of Ph+ leukemia. For example, histone deacetylatase (HDAC) inhibitors that induce acetylation and inhibition of Hsp90 might also be active.[186-190]

While imatinib induces complete hematologic and cytogenetic remission in the majority of newly diagnosed chronic-phase CML patients,[191] molecular remission is

difficult to achieve in these patients. One study designed to look at newly diagnosed chronic phase patients using the standard-dose imatinib (400 mg daily), with a 18-month follow-up showed that only 39% of patients obtained a major molecular response (greater than or equal to 3-log reduction of BCR-ABL mRNA), whereas even fewer patients, 4%, obtained a complete molecular response rate (negativity by reverse transcription–polymerase chain reaction [RT-PCR]).[190] One prediction as to why the patients do not obtain a complete molecular response is perhaps due to imatinib not completely eradicating the BCR-ABL-positive stem cells. It is likely that a small number of leukemic cells remain in imatinib-treated CML patients, and these cells may function as leukemia stem cells responsible for disease relapse. The inhibitory effects of IPI-504 on leukemia stem cells, while appearing to spare the normal hematopoietic stem cells, merit further investigation. Sole inhibition of BCR-ABL by imatinib has limited inhibitory effects on leukemic stem cells in mice. Thus, a pathway distinct from BCR-ABL is likely involved in suppression of survival of leukemic stem cells by IPI-504. A plausible explanation is that BCR-ABL cooperates with a non–BCR-ABL signaling pathway that is driven by an unknown Hsp90 client protein to maintain survival of leukemic stem cells. IPI-504 is able to inhibit both pathways, as would be necessary to suppress leukemic stem cells. The putative pathway that is Hsp90 dependent might be less critical for normal hematopoietic stem cells. The putative non–BCR-ABL pathway in leukemic stem cells requires further study. The inhibitory effects of IPI-504 on BCR-ABL-T315I–expressing cells indicate that Hsp90 may serve as an effective target for treating imatinib- and dasatinib-resistant CML patients, as well as patients with blast crisis or with Ph+ ALL. The simultaneous

use of IPI-504 and imatinib in chronic-phase CML patients might prevent the development of imatinib-resistant clones and inhibit growth of highly proliferative leukemic cells through

inhibition of BCR-ABL kinase activity, thereby providing a rationale for combination strategy(Figure 8). Likewise, early use of IPI-504 to suppress initial B-ALL clones may help prevent the transition of CML to advanced B-ALL caused by the BCR-ABL-T315I mutation.

Although inhibition of Hsp90 significantly prolonged the CML mice survival curve, eventually the mice are still dead as CML after a continuous long-term IPI-504 treatment. There are at least three possible reasons to cause this IPI-504 resistance in CML mice. The first one might be that a Hsp90 mutant has gradually grown up and it facilitates the leukemia cells to achieve the growth advantage over the wild type Hsp90 expressing leukemia cells. Although there are no clinical or mouse models report the Hsp90 mutations occurrence after Hsp90 inhibitors treatment, a recent yeast-based system showed a yeast Hsp90 mutant (A107N), a human Hsp90α mutant (A121N) and a human Hsp90β (A116N) are significantly resistant to Hsp90 inhibitors, including redicicol and 17-AAG.[192] This study reminds us it is necessary to collect the DNA samples from the dying CML mice after IPI-504 long-term treatment and sequence *Hsp90* to check whether there are IPI-504 mutations coming out. The second reason might be LSCs who are resistant to IPI-504 are selected from long-term treatment and these LSCs gradually self-renew and differentiate mature leukemia cells which are also insensitive to Hsp90 inhibition. This possibility has been implicated in our CML mice

and LSCs treatment. When we administrated CML mice with Hsp90, we found a dramatic reduce of LSCs after two weeks IPI-504 treatment. However, IPI-504 did not eliminate all the LSCs in vivo. Later on, these residual LSCs might initiate leukemia development and cause the disease mice death. Similar to in vivo situation, when LSCs were treated with IPI-504 in vitro, there was still about 3% LSCs leftover after 6 days IPI-504 treatment, indicating small percentage of LSCs are still alive and they might responsible to resistance. These results indicate inhibition of sole Hsp90 function is not enough to totally eradicate LSCs and small population of LSCs still can survival, depending on other unknown mechanisms and other chaperon protein might also be involved in this process. More studies are definitely needed to investigate the LSCs functions and how they maintain themselves.

The third possibility might be the inhibitory effect of IPI-504 could not completely block the Hsp90 function. LSCs still can self-renew and initiate the disease based on partial function of Hsp90 after IPI-504 treatment and finally cause the death of mice. To test this possibility, at least two experiments could be carried out. First, a more potent Hsp90 inhibitor could be applied in the same assay. Second, donor bone marrow cells could be collected from Hsp90 deficient mice and CML mice could be induced by these Hsp90 deficient donor cells. Unfortunately, Hsp90β strain is not available because The homozygous mice failed to form a fetal placental labyrinth and died at embryonic day 10.[193] Also the Hsp90α knockout strain is not available. A knockdown strategy by both isoforms shRNA might be substituted in the similar study, providing genetic evidence of Hsp90 in LSCs.

While IPI-504 was active in BCR-ABL–induced B-ALL, the activity was not as pronounced as in CML. Studies to evaluate the mechanism for this difference showed that Hsp70 was more strongly induced in myeloid cells compared with lymphoid cells. Hsp70 is reported to exert antiapoptotic effects in a variety of settings and cell types, including leukemia cells that are exposed to Hsp90 inhibitors.[181, 182] In separate studies, inhibition of Hsp90 was shown to result in increased binding of BCR-ABL to Hsp70, thereby favoring proteasome-mediated degradation of BCRABL.[137,138, 179, 180] Thus, on one hand, Hsp70 induction could counter the effects of Hsp90 inhibition, while other studies suggest that Hsp70 could have a positive influence on the ability of Hsp90 inhibition to result in degradation of BCR-ABL. In addition, imatinib has been shown to decrease the level of Hsp70 in BCR‐ABL‐expressing HL60 cells,[194] supporting the anti‐apoptotic role of Hsp70 in BCR‐ABL‐stimulated cell growth.

We studied further the relationship between Hsp70 inhibition and stability of the BCR-ABL protein. If Hsp70 cooperates with Hsp90 to facilitate degradation of BCR-ABL protein, inhibition of Hsp70 should increase level of BCR-ABL protein in cells. To test this hypothesis, we treated BCR-ABL-expressing 32D myeloid cells with an Hsp70 inhibitor, KNK437.[195] Inhibition of Hsp70 by KNK437 did not prevent BCR‐ABL degradation caused by inhibition of Hsp90 by IPI‐504 (Figure. 9). This result does not support a positive role of Hsp70 in Hsp90‐mediated degradation of BCR‐ABL. However, inhibition of Hsp70 by KNK347 did not synergistically increase IPI‐504‐induced apoptosis of BCR‐ABL‐expressing 32D myeloid cells either (data not shown).

Functional relationship between Hsp70 and Hsp90 needs to be studied further and elucidation of the mechanism of differential sensitivity to Hsp90 inhibition between myeloid and lymphoid leukemia will require more extensive studies, as variation in Hsp70 induction is not likely the cause.

In summary, IPI-504 represents a novel therapeutic approach whereby inhibition of Hsp90 in CML patients and Ph+ ALL may significantly advance efforts to develop a cure for these diseases. The rationale underlying the use of IPI-504 for kinase inhibitor–resistant CML has implications for other cancers that display oncogene addiction to kinases that are Hsp90 client proteins. While resistant conferring kinase-domain mutations were originally described in CML, analogous mutations have been observed in lung cancer, gastrointestinal stromal tumor, and the hypereosinophilic syndrome with resistance to kinase inhibitor therapy.[196] IPI-504 is currently in clinical trials to evaluate its potential for treating cancer that has become resistant to therapy with tyrosine kinase inhibitors such as imatinib.

Figure 8. Combination therapy of CML using Hsp90 and BCR-ABL kinase inhibitors. After imatinib-resistant mutations of BCR-ABL occur in leukemic cells, two types of cells may exist in a patient: cells harboring mutant BCR-ABL (such as BCR-ABL-T315I) and cells harboring wild type BCR-ABL. Treatment with a BCR-ABL kinase inhibitor alone (such as imatinib) would lead toselective growth of leukemic cells harboring mutant BCR-ABL, although leukemic cells harboring wild type BCR-ABL were suppressed. In contrast, treatment with both Hsp90 and BCR-ABL kinase inhibitors (such as IPI-504 and imatinib) would inhibit growth of both types of leukemic cells, with a much stronger inhibition of leukemic cells harboring mutant BCR-ABL. This combination therapy provides a novel therapy for Ph^+ leukemis.

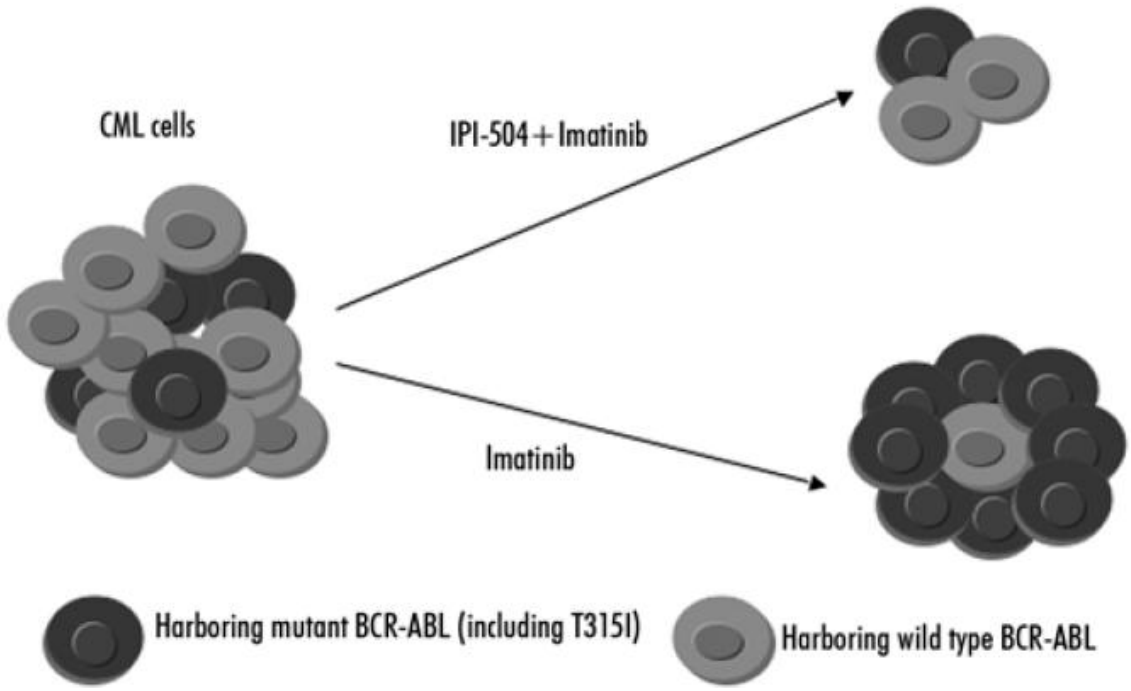

Figure 8. Combination therapy of CML using Hsp90 and BCR-ABL kinase inhibitors.

Figure 9. Inhibition of Hsp70 by KNK437 does not prevent BCR-ABL degradation caused by inhibition of Hsp90 by IPI-504. BCR-ABL-expressing 32D cells were treated with KNK437 (100 or 400 mM) or IPI-504 (2 mM) alone or both for 24 hours. Protein lysates were analyzed by Western blotting using antibodies indicated. Inhibition of Hsp70 by KNK437 did not prevent BCR-ABL degradation caused by inhibition of Hsp90 by IPI-504.

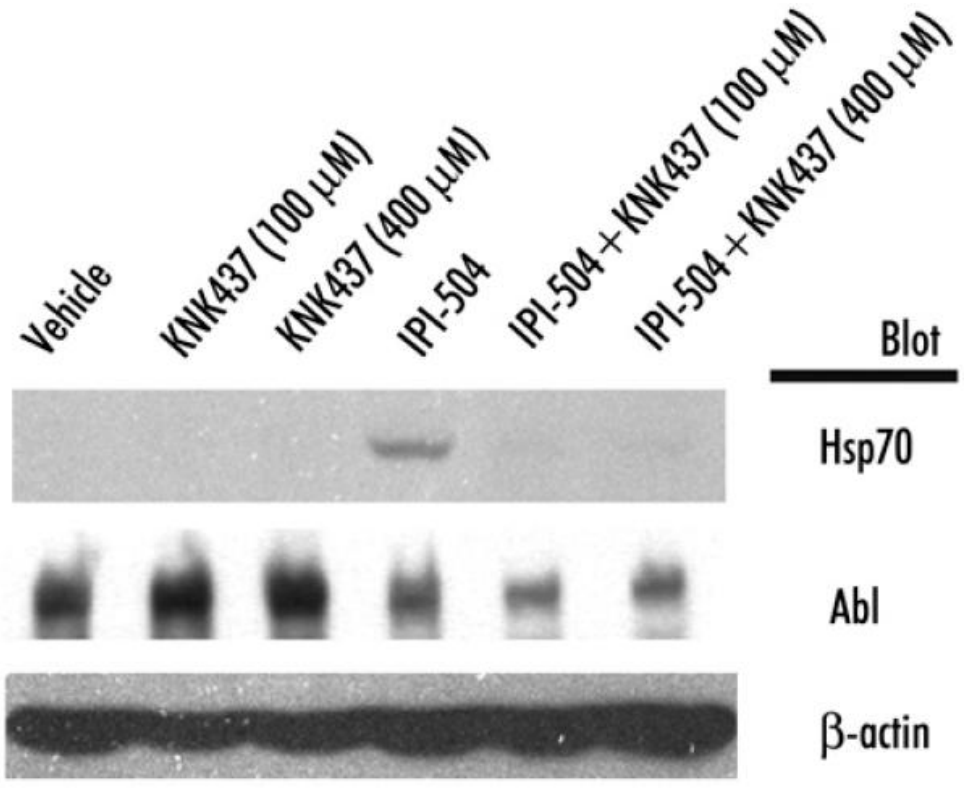

Figure 9. Inhibition of Hsp70 by KNK437 does not prevent BCR-ABL degradation caused by inhibition of Hsp90 by IPI-504

Materials and methods

Cell lines

The 32D myeloid cell line was grown in RPMI 1640 medium containing 10% FCS and 10% WEHI medium. The BaF/3 pre-B-cell line was grown in RPMI 1640 medium containing 10% FCS, 10% WEHI medium, and 50 μM 2-mercaptoethanol. To generate the BCR-ABL–expressing 32D or BaF/3 line, the cells were transduced with the BCR-ABL-WT- or BCR-ABL-T315IIRES-GFP-MSCV retrovirus, and the BCR-ABL–expressing cells were selected by GFP sorting by fluorescence-activated cell sorter (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. 10-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 6.0 (Adobe, San Jose, CA).

Antibodies and Western blot analysis

Antibodies against c-ABL, Hsp90, Hsp70, 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.[197]

Generation of MSCV-BCR-ABLIRES-GFP Virus Supernatant

Culture 293T cells in 15 cm tissue culture dish (there are about 1×10^8 cells in confluent plates). When the 293T cells reach 90% confluence in the 15 cm dish, remove the medium and wash cells once with 1XPBS. Remove PBS, add 3 mL of trypsin–EDTA solution, and stop the reaction by adding 20 mL 293T medium. Collect cells carefully in 50 mL centrifuge tube and spin at 1500 rpm, 10 mins at room temperature. The 293T cells are passaged to 6 cm dish at 4×10^6 cells/dish at the day before transfection. Change 4 mL fresh 293T medium to each dish before transfection. In a 15 mL tube, add 10µg MSCV-BCR-ABL-GFP plasmid, 5 µg Ecopack plasmid, 62 µl 2 M $CaCl_2$ and sterile water to 500 µl total volume. Briefly vortex. Add 500µl 2×HBS to the tube and mix by vortexing for 10s. Gently and quickly drop the DNA/HBS solution onto 293T cells. Rock the dishes forward and backward a few times to achieve even distribution of DNA/Ca3(PO4)2 particles. After 24 h, remove the old medium and add 4 mL fresh 293T medium 9. After 48 h post-transfection of 293T cells, collect the supernatant by 10 mL

BD syringe and filter the supernatant through 0.45 μm syringe filter. Aliquot virus supernatant in 4 mL/tube and store at −80ºC.

Bone marrow transduction/transplantation

The retroviral vector MSCV-IRES-eGFP[198] 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,[199] as previously described.26 Six- to 10-week-old wild-type BABL/c or C57BL/6 mice (The Jackson Laboratory) were used for leukemogenesis experiments. Induction of CML[32] and B-ALL[32, 178] was as described previously. Briefly, to model CML, bone marrow from 5-FU–treated (200 mg/kg) donor mice was transduced twice with *BCR-ABL* retrovirus by cosedentation in the presence of IL-3, IL-6, and 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 1150 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.[32]

Flow cytometry

Hematopoietic cells were collected from peripheral blood and bone marrow

of the diseased mice, and red blood cells were lysed with NH4Cl red blood cell lysis buffer (pH 7.4). The cells were washed with PBS, and stained with B220-PE for B cells, Gr1-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 α-FGF as reported previously for culture of hematopoietic stem cells.[184, 185]

Acknowledgments

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We thank Stephen B. Sampson for critical reading of the paper, Patricia Cherry for secretarial assistance, and Richard A. Van Etten for the BCR-ABL mutant constructs E225K, M351T, and Y253F.

Chapter III

Pten is a tumor suppressor in CML stem cells and BCR-ABL–induced leukemias in mice

The work described in this chapter has been published (Peng et al.,2009).

Abstract

The tumor suppressor gene PTEN is inactivated in many human cancers. However, it is unknown whether PTEN functions as a tumor suppressor in human Philadelphia chromosome positive (Ph^+) leukemia that includes chronic myeloid leukemia (CML) and B-cell acute lymphoblastic leukemia (B-ALL) and is induced by the BCR-ABL oncogene. Using our mouse model of BCR-ABL induced leukemias, we show that Pten is downregulated by BCR-ABL in leukemia stem cells (LSCs) in CML. Furthermore, *Pten* deletion in $Pten^{fl/fl}$ mice causes acceleration of CML development. In addition, overexpression of Pten delays the development of CML and B-ALL, and prolongs survival of leukemia mice. Pten suppresses LSCs and induces cell cycle arrest of leukemia cells. Moreover, Pten suppresses B-ALL development through regulating its downstream gene Akt1. In the end, rapamycin, which specifically inhibit Akt downstream molecule mTOR, could significantly suppress human leukemia cells proliferation and induce apoptosis in these cells. These results demonstrate a critical role of Pten in BCR-ABL induced leukemias and increase Pten expression or inhibition of Akt-mTOR pathway could be a promising strategy to the CML treatment.

Introduction

The human Philadelphia chromosome arises from a reciprocal translocation between chromosome 9 and 22, resulting in the formation of chimeric BCR-ABL oncogene. BCR-ABL encodes a constitutively activated, oncogenic tyrosine kinase[3]. Philadelphia chromosome positive (Ph+) leukemia induced by BCR-ABL includes chronic myeloid leukemia (CML) and B cell acute lymphoid leukemia (B-ALL). The BCR-ABL kinase inhibitor imatinib mesylate induces a complete hematologic and cytogenetic response in the majority of chronic phase CML patients [164], but is unable to completely eradicate BCR-ABL-expressing leukemic cells [38, 165], suggesting that leukemia stem cells are not eliminated. Over time, patients frequently become drug resistant and develop progressive disease despite continued treatment[65, 166, 167]. Moreover, B-ALL is less sensitive to imatinib, suggesting that inhibition of BCR-ABL kinase activity is not enough to suppress B-ALL development. New therapeutic strategies need to be developed for Ph+ leukemia.

Tumors progress to more advanced stages after acquiring additional genetic alterations, and inactivation of tumor suppressor genes are common in human cancers. Phosphatase and tensin homologue (PTEN) [200] is often deleted or inactivated in many tumor types, including glioblastoma [141], endometrial carcinoma [143], and lymphoid malignancies [146]. PTEN is a phosphatase that dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate (PIP3)[147, 148]. PIP3 is a direct product of PI3K activity, and plays a critical role in the regulation of cell survival and growth through activating the Ser/Thr protein kinase PDK1 and its downstream target Akt [150, 151]. Activated Akt mediates

several well-described PI3K responses that include cell survival and growth, cellular metabolism, angiogenesis, and cell migration. Mice with a complete null mutation of *Pten* develop early embryonic lethality at E9.5 [153-155]. *Pten* heterozygous mice die within 1 year after birth and survivors develop a broad range of tumors, including mammary, thyroid, endometrial and prostate cancers [153-155], as well as autoimmunity related to Fas mediated response [156]. Mice with the tissue specific deletion of Pten using the Cre-loxP system have become available for studying physiological functions of PTEN in adult tissues and organs [157, 158]. For example, mice with Pten deletion in adult hematopoietic cells develop and die of acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) [159]. Akt1 is a major downstream signaling molecule of PTEN, and is activated after PTEN is mutated in human cancers. A recent study showed that the deficiency of Akt1 is sufficient to suppress the development of several types of tumors in *Pten* heterozygous mice, including prostate cancer, endometrial carcinoma, thyroid neoplasia, intestinal polyps, and lymphoid hyperplasia.[162] Moreover, the rapamycin, which directly inhibits Akt downstream mTOR, can effectively inhibit the survival and proliferation of AML cells from *Pten$^{fl/fl}$;Mx-1-Cre* AML mice and prolong these disease mice.[160] All of these results demonstrate a crucial role of the PI3K-Pten-Akt pathway in cancer development. In this study, we investigated the role of Pten in the development of BCR-ABL induced CML and B-ALL in mice. We also tested the effect of Pten on LSCs and studied the role of Akt1 as a Pten downstream signaling molecule in B-ALL development. Further, we evaluated the potential role of targeting the Akt1-mTOR

pathway in the treatment of BCR-ABL induced leukemia and the rapamycin inhibitory effect of human CML cells.

Results

PTEN expression is downregulated by BCR-ABL

We performed a global gene expression analysis using DNA microarray to identify genes regulated by BCR-ABL in a BCR-ABL-expressing mouse pre-B cell line (ENU-BCR-ABL cells).[77] Comparing to non-BCR-ABL-expressing parental cells, we observed a 1.43 fold decrease in the level of PTEN mRNA (Figure 1A). The downregulation of PTEN by BCR-ABL was further confirmed by Western blotting in BCR-ABL-expressing Ba/F3 cells (Ba/F3-BCR-ABL) (Figure 1B). We also treated Ba/F3 and Ba/F3-BCR-ABL cells with the BCR-ABL kinase inhibitor imatinib, and found that imatinib did not have an effect on PTEN expression in Ba/F3 cells but caused the restoration of PTEN protein expression back to its endogenous level in Ba/F3-BCR-ABL cells (Figure 1B), indicating that this PTEN downregulation is dependent upon BCR-ABL kinase activity.

There are two p53 binding sites on the human *PTEN* promoter and p53 positively regulate *PTEN* by binding to these two sites [201]. BCR-ABL causes downregulation of p53 in leukemia cells through the upregulation of MDM2 that inhibits p53 transcriptional activation and promotes p53 export and proteasome-dependent degradation in the cytoplasm[202]. In mouse, there is only one p53 binding site on *Pten* promoter and p53 indeed binds to *Pten* promoter in our Ba/F3 murine cell line (Figure1 C). We tested whether Pten downregulation by BCR-ABL correlates with p53 degradation. In BCR-

ABL-expressing Ba/F3 cells, the level of p53 was lower than that in parental Ba/F3 cells, and this reduced P53 level was reversed after imatinib treatment. This result suggests that BCR-ABL might down-regulate Pten through P53 (Figure1 D).

Figure 1. Down-regulation of Pten by BCR-ABL. (A) Total mRNAwas isolated from parental ENU and ENU-BCR-ABL cells for DNA microarray analysis. The level of *Pten* mRNAwas lower in BCR-ABL–expressing ENU cells than in parental ENU cells. (B) *Pten* protein level was also lower in Ba/F3-BCR-ABL cells than in parental Ba/F3 cells. Parental Ba/F3 and Ba/F3-BCR-ABL cells were treated with imatinib (IM; 1μM) for 48 and 72 hours, respectively. Protein lysates were analyzed by Western blot by the use of antibodies indicated. Independent experiments were repeated 3 times. (C) The mouse *Pten* promoter contains only one p53 binding site. (Top) Schematic representation of the mouse *Pten* genomics locus (GenBank accession number NM_008960). The exons are indicated by the black bars 1 to 9. (Middle) Region directly upstream of the *Pten* translation start site. The positions of oligonucleotide probes used for mapping the transcription stat site by PCR are indicated. (Bottom) Nucleotide sequence of the p53 binding site identified based on human p53 binding sequence.27 (D) p53 binds to *Pten* promoter directly. Chip was performed in BaF3 cells to show the binding of p53 to the *Pten* promoter as described in the Methods section.

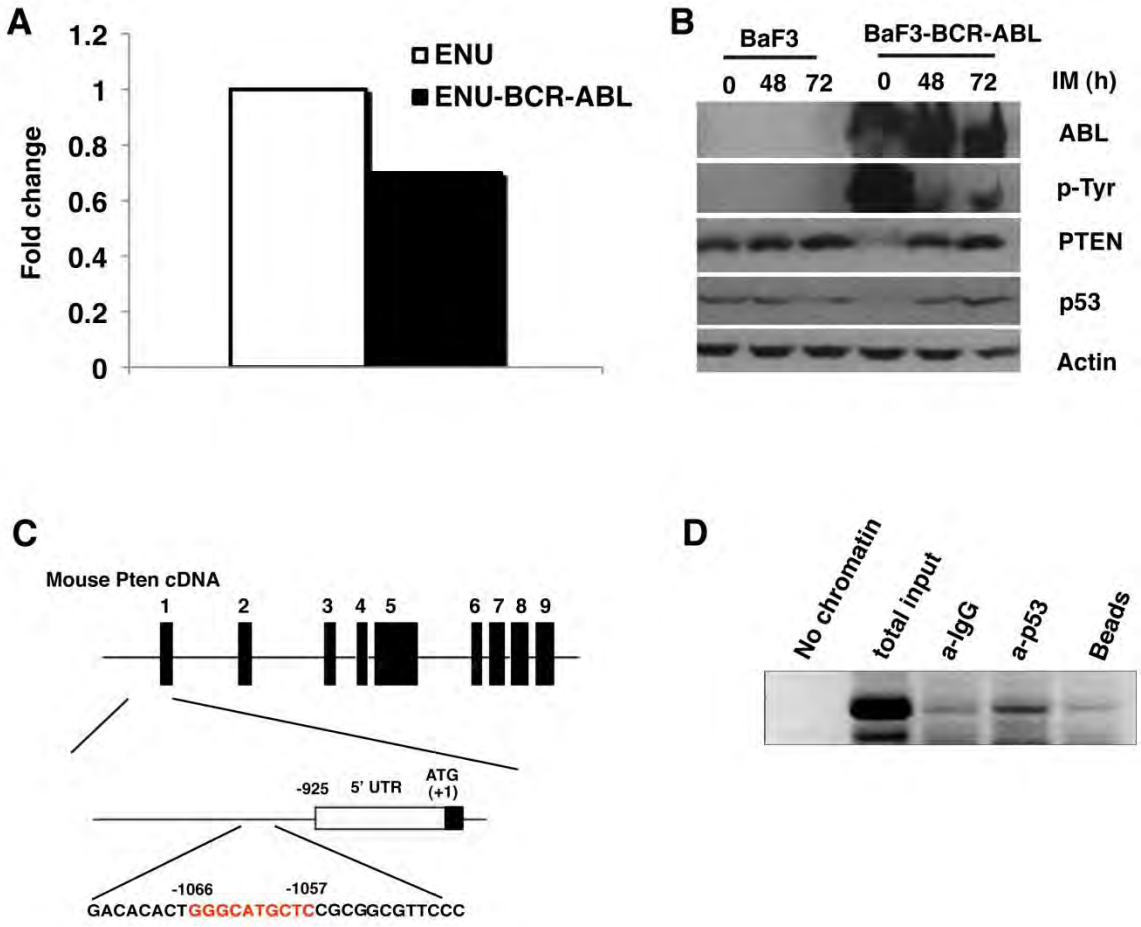


Figure 1. Down-regulation of Pten by BCR-ABL.

Pten deletion causes acceleration of CML development

Because Pten was down-regulated by BCR-ABL (Figure 1), we tested whether Pten functions as a tumor suppressor in CML development by using *Pten* conditional knock mice ($Pten^{fl/fl}$). To delete *Pten* from bone marrow cells of $Pten^{fl/fl}$ mice, we transduced the cells with BCR-ABL-iCre-GFP retrovirus or BCR-ABL-GFP retrovirus as a control (Figure 2A). Western blot analysis showed expression of iCre and a significant decrease of the Pten protein level (Figure 2B), indicating that the *Pten* gene was deleted from the cells. To test whether deletion of Pten affects CML development, we transduced bone marrow cells from $Pten^{fl/fl}$ mice with BCR-ABL-iCre-GFP or BCR-ABL-GFP retrovirus, followed by transplantation of the transduced cells into lethal irradiated recipient mice. Mice receiving donor bone marrow cells transduced with BCR-ABL-iCre-GFP developed CML much faster than those receiving bone marrow cells transduced with BCR-ABL-GFP (Figure 2C; $P < 0.005$). In these CML disease mice, the majority of GFP cells were $Gr1^+$ but not $B220^+$ leukemia cells (supplemental Figure 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). The accelerated death of CML mice in the absence of Pten correlated with a greater percentage of GFP^+Gr1^+ myeloid leukemia cells (Figure 2D) and a greater number of leukemia cells (Figure 2E) in peripheral blood of the mice. Accelerated CML development in the absence of Pten also correlated with more severe infiltration of leukemia cells in the lungs (Figure 2F, H) and splenomegaly (Figure 2G-H). These results demonstrated that Pten is a potent tumor suppressor in BCR-ABL–induced CML.

Pten$^{fl/fl}$;Mx-1-Cre mice develop AML 20 days after PIPC treatment that initiates the deletion of *Pten*.[160] We wondered whether the mice receiving donor bone marrow cells transduced with BCR-ABL-iCre-GFP developed AML, which may contribute to the accelerated death of CML mice in the absence of Pten (Figure 1C). We found that these mice developed typical CML (Figure 2), and we did not observe any signs for AML development (data not shown). To further rule out the possible contribution of AML to the accelerated death of CML mice in the absence of Pten, we transduced normal bone marrow cells from *Pten*$^{fl/fl}$ mice with MSCV-iCre-GFP retrovirus to delete *Pten*, followed by transplantation of the transduced cells into the lethal irradiated recipient mice. Although 20% white blood cells in peripheral blood of the recipient mice were GFP^{+}, indicating that the iCre gene was expressed in the cells, none of these mice developed AML, and all mice survived (supplemental Figure 2). This result suggests that the deletion of *Pten* in non–BCR-ABL–expressing bone marrow cells is insufficient to induce AML in our bone marrow transduction/transplantation model system.

Figure 2. *Pten* deletion accelerates CML development.
(A) Structure of BCR-ABL-iCre-GFP retroviral construct. (B) BCR-ABL-GFP and BCR-ABL-iCre-GFP retrovirus transduced bone marrow cells from *Pten*$^{fl/fl}$ mice were cultured under the Whitlock-Witte conditions for 1 week. Protein lysates were analyzed by Western blotting with the antibodies indicated. iCre-induced deletion of the *Pten* gene resulted in the removal of Pten protein. (C) Kaplan-Meier-style survival curves for recipients of BCR-ABL-iCre-GFP–transduced bone marrow cells from wild-type (WT; n =6) or PTEN*fl*/fl (PTEN; n=9) mice (P <0.005). (D) The percentage of leukemia cells (GFP^+Gr^+) in recipients of BCR-ABL-iCre-GFP–transduced bone marrow cells from Ptenfl/fl mice was greater than that in recipients of BCR-ABL-iCre-GFP–transduced bone marrow cells from wild-type mice. (E) The total number of leukemia cells (total white blood cell count X percentage of GFP^+Gr1^+ cells) in the peripheral blood of recipients of BCR-ABL-iCre-GFP–transduced bone marrow cells from *Pten*$^{fl/fl}$ mice (PTEN) was greater than that in recipients of BCR-ABL-iCre-GFP–transduced bone marrow cells from wild-type mice (WT). (F) Photomicrographs of hematoxylin and eosin-stained lung sections from recipients of bone marrow cells from PTEN-deficient CML mice (Pten-/-) showed more severe infiltration of the lungs with myeloid leukemia cells than recipients of bone marrow cells from wild-type mice (WT) at day 14 after BMT. (G) Spleen weight of recipients of wild-type (WT) or *Pten*$^{fl/fl}$ (Pten-/-) bone marrow cells transduced with BCR-ABL-iCre-GFP retrovirus at day 14 after BMT (P =0 .028). (H) Gross appearance of the lungs and spleens showed severe lung hemorrhages and splenomegaly in recipients of BCR-ABL-iCre-GFP transduced bone marrow cells from *Pten*$^{fl/fl}$ CML mice (Pten-/-) than in recipients of the transduced wild-type (WT) bone marrow cells.

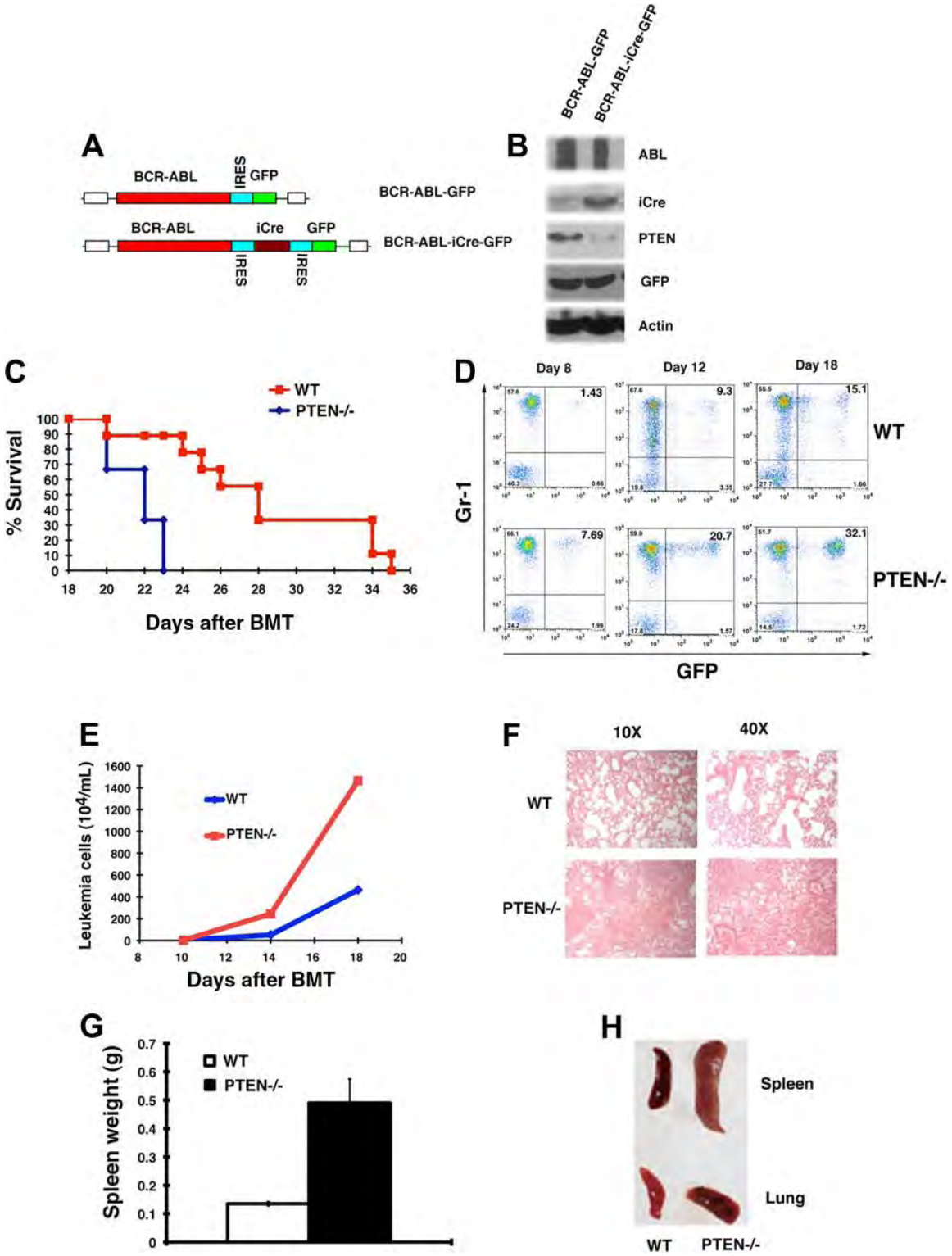


Figure 2. Pten deletion accelerates CML development.

PTEN overexpression delays CML development

CML developed faster in the absence of Pten (Figure 2), indicating that Pten is a tumor suppressor in BCR-ABL–induced leukemia. To further test this idea, we examined whether overexpression of Pten delays CML development.We cloned the *Pten* gene into the BCR-ABL-GFP construct for simultaneous expression of the three genes, BCR-ABL, *Pten*, and GFP (Figure 3A). Western blot analysis showed that this triple-gene retroviral construct allowed overexpression of Pten in cells (Figure 3B). We next transduced donor bone marrow cells from wild-type mice with BCR-ABL-PTEN-GFP or BCR-ABL-GFP retrovirus, followed by transplantation of the transduced cells into recipient mice. CML development was significantly slower in mice receiving bone marrow cells transduced with BCR-ABL-PTEN-GFP than in those receiving bone marrow cells transduced with BCR-ABL-GFP (Figure 3C,P <0.001), indicating that Pten overexpression caused a delay of CML development. The delayed CML development correlated with a less percentage and number of leukemia cells in peripheral blood (Figures 3D,E), and also with less severe splenomegaly (Figure 3F) and infiltration of leukemia cells in the lungs (Figure 3G). These results further support the role of Pten as a tumor suppressor in CML development.

To evaluate whether Pten overexpression in BCR-ABL–expressing cells synergizes with the therapeutic effect of imatinib on CML, we treated mice receiving bone marrow cells transduced with BCR-ABL-PTEN-GFP or BCR-ABL-GFP retrovirus with imatinib. As expected, imatinib treatment prolonged survival of CML mice receiving bone marrow cells transduced with BCR-ABLGFP (Figure 3C; P<0.001).

However, imatinib-treated CML mice receiving bone marrow cells transduced with BCR-ABL-PTENGFP lived significantly longer than those not treated with imatinib (Figure 3C; $P<0.001$). The synergistic effect of Pten overexpression with imatinib treatment correlated with less leukemia cells in peripheral blood of the mice (Figure 3E). To explain how Pten reduced proliferation of leukemia cells, we performed the DNA content analysis to examine the effect of Pten overexpression on cell-cycle progression of these cells. We showed that the percentage of leukemia cells in the S _ G2M phase was much lower in leukemia cells with Pten overexpression than in those without Pten overexpression (Figure 3H; $P<0.01$), indicating that Pten inhibits the proliferation of leukemia cells by inducing a cell-cycle arrest. Furthermore, we examined whether Pten induces apoptosis of leukemia cells by staining the cells with PI and annexin V. Corresponding to the result in the cell-cycle analysis, apoptosis in leukemia cells with Pten overexpression was more severe than in those without Pten overexpression (Figure 3I; $P<0$ .05).

Figure 3. Overexpression of Pten delays CML development. (A) Structure of BCR-ABL-PTEN-GFP retroviral construct. (B) Western blot analysis shows expression of BCR-ABL, PTEN, and GFP from BCR-ABL-PTEN-GFP retrovirus. NIH3T3 cells were transduced with BCR-ABL-GFP or BCR-ABL-PTEN-GFP retrovirus for 3 hours. Then, 2 days later, protein lysates were analyzed be Western blotting by the use of the antibodies indicated. (C) Overexpression of Pten alone or in combination imatinib treatment prolongs survival of CML mice. Mice with CML induced with BCR-ABL-GFP (n=20) or BCR-ABL-PTEN-GFP (n=20) were treated with a placebo (n=7) or imatinib (n=7, 100 mg/kg, twice a day by gavage), beginning at day 8 after transplantation. (D) Flow cytometry analysis showed a slower accumulation of GFP^+Gr1^+ leukemia cells in peripheral blood of recipients of BCR-ABL-PTEN-GFP–transduced bone marrow cells than that in recipients of BCR-ABL-GFP–transduced bone marrow cells. (E) CML was induced with BCR-ABL-GFP or BCR-ABL-PTEN-GFP, and the difference in peripheral blood leukemia cell counts (white blood cell count X the percentage of GFP^+Gr1^+ cells) in CML mice treated with a placebo or imatinib was determined at day 20 after BMT. (F) Spleen weight of CML mice induced with BCR-ABL-GFP or BCR-ABL-PTEN-GFP. (G) Photomicrographs of hematoxylin and eosin–stained lung sections from mice with CML induced with BCR-ABL-GFP or BCR-ABL-PTEN-GFP at day 20 after transplantation. (H) At day 20 after BMT, peripheral blood cells were stained with Gr1 and Hoechst blue. The S + G2Mphase of leukemia cells (GFP^+Gr1^+) was represented by the percentage of Hoechst blue–positive cells. Mean percentage for each cell population (n=3) was shown. (I) At day 20 after BMT, peripheral blood cells were stained with Gr1, Annexin V, and propidium iodide (PI). Apoptotic leukemia cells were represented by the $GFP^+Gr1^+AnnexinV^+PI^+$population. Mean percentage for each cell population (n=3) was shown.

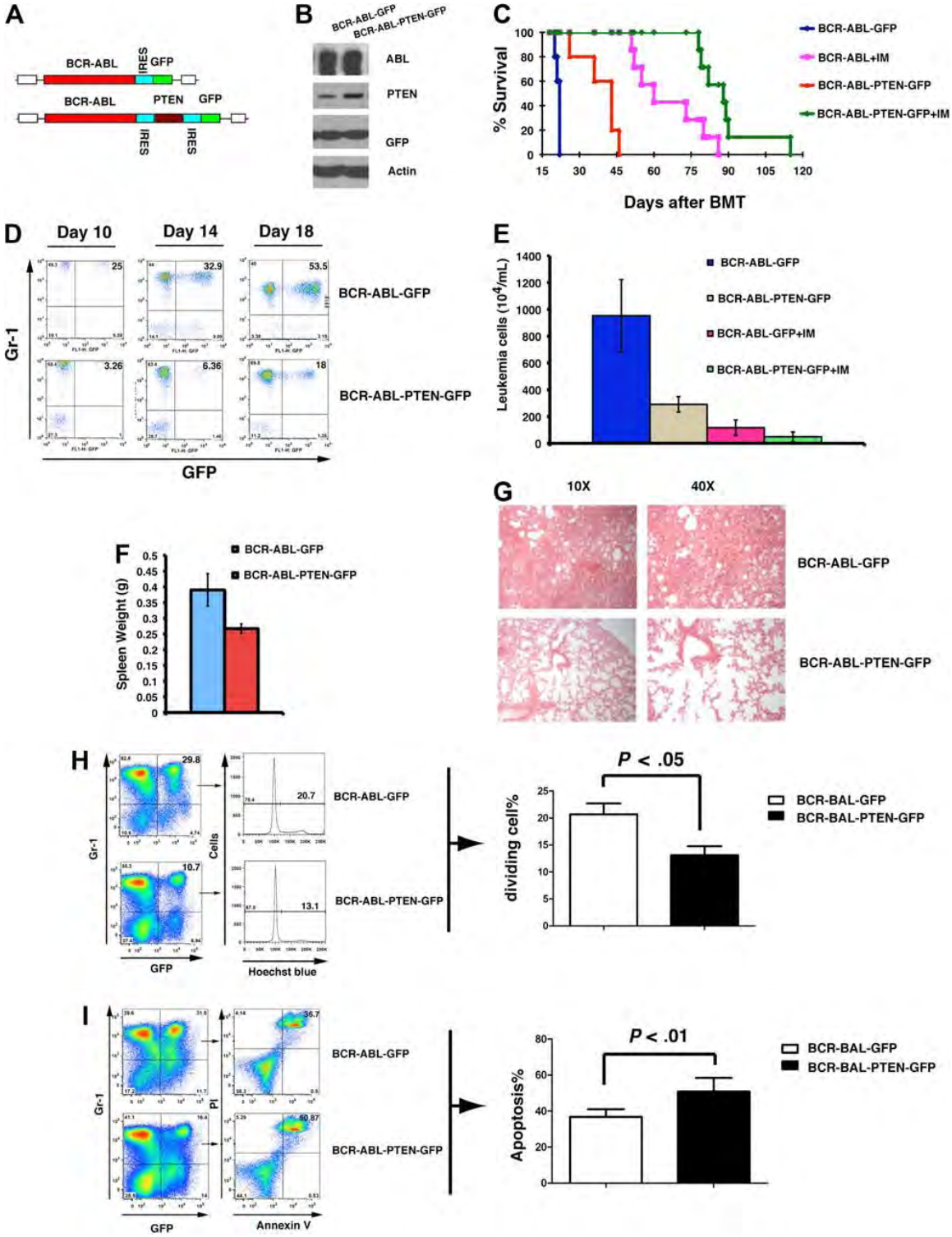


Figure 3. Overexpression of Pten delays CML development.

Pten suppresses CML stem cells

CML is derived from hematopoietic stem cells harboring the *BCR-ABL* oncogene.[203] It is possible that Pten suppresses CMLstem cells, resulting in acceleration of CML when deleted (Figure 2) and delay of CML when overexpressed (Figure 3).We have previously identified BCR-ABL–expressing $Lin^{-}c\text{-}kit^{+}Sca1^{+}$ cells as LSCs in CML induced by BCR-ABL in mice. To test whether Pten expression is affected by BCR-ABL in LSCs, GFP^{+} $Lin^{-}c\text{-}kit^{+}Sca1^{+}$cells were sorted by FACS from CML mice treated with a placebo or imatinib, and total RNA was isolated for DNA microarray analysis. The microarray study showed that *Pten* mRNA was significantly down-regulated approximately 3.59-fold by BCR-ABL, and this down-regulation was restored upon imatinib treatment (Figure 4A; P <0 .001). Correlating with *Pten* down-regulation in LSCs, *p53* was also down-regulated approximately 2.9-fold by BCR-ABL in LSCs (Figure 4B). These results further support our observations in BaF3-BCR-ABL cells (Figure 1).

To test whether Pten functions as a tumor suppressor in LSCs, we transduced bone marrow cells with BCR-ABL-PTEN-GFP or BCR-ABL-GFP retrovirus, followed by transplantation of the transduced cells into recipient mice. At 14 days after the transplantation, bone marrow cells were isolated from CML mice, and LSCs ($GFP^{+}Lin^{-}c\text{-}kit^{+}Sca1^{+}$) were analyzed by flow cytometry. The percentage of LSCs in mice with CML induced by BCR-ABLPTEN-GFP was significantly lower than that in mice with CML induced by BCR-ABL-GFP (Figure 4C), indicating that Pten uppresses LSCs.

To determine whether Pten affects the function of LSCs, we compared the ability to induce CML between LSCs that expressed BCR-ABL-PTEN-GFP and those that expressed BCR-ABL-GFP. At 14 days after BMT, the same number (3×10^4) of GFP^+ $Lin^-c\text{-}kit^+Sca1^+$cells sorted from CML mice receiving BCR-ABL-GFP or BCR-ABL-PTEN-GFP transduced bone marrow cells were transferred into recipient mice. The percentages and numbers of leukemia cells in peripheral blood were monitored at day 25 after BMT. The total number of leukemia cells in CMLmice receiving BCR-ABL-PTEN-GFP transduced bone marrow cells was 4-fold lower than that in CMLmice receiving BCR-ABL-GFP–transduced bone marrow cells (Figure 4E), correlating with a lower percentage of leukemia cells in peripheral blood (Figure 4D). Consistent with less-severe CML induced by BCR-ABL-PTEN-GFP, the survival of mice receiving LSCs transduced with BCRABL-PTEN-GFP was significantly longer than that of mice receiving LSCs transduced with BCR-ABL-GFP (Figure 4F; P<0 .001). These results indicate that Pten suppresses the function of LSCs.

Because rapamycin suppresses AML cells in vitro and prolongs the survival of *Pten*$^{fl/fl}$;Mx-1-Cre AML mice, likely through inhibiting inhibiting AML stem cells, we tested whether rapamycin also inhibits leukemia stem cells in CML. We isolated bone marrow cells from CML mice and cultured the cells under the conditions that support survival and growth of leukemia stem cells from CML mice.[204] During the culture, the cells were treated with rapamycin. At 3 days after the treatment, we calculated the numbers of $GFP^+Lin^-c\text{-}kit^+Sca1^+$cells that remained in the culture based on FACS

analysis and total cell counts (Figure 4G). We showed that inhibition of mTOR by rapamycin also significantly inhibited CML stem cells in vitro.

Figure 4. Pten suppresses leukemia stem cells. (A) BCR-ABL down-regulates *Pten* expression, and this down-regulation is abolished upon imatinib treatment. Bone marrow cells were transduced with GFP or BCR-ABL-GFP retrovirus, followed by transplantation into recipient mice. Some recipients of BCR-ABL-GFP–transduced bone marrow cells were treated with imatinib (100 mg/kg, twice a day by gavage), beginning at day 8 after BMT. At 24 hours later, $GFP^+Lin^-c\text{-}kit^+Sca1^+$cells in bone marrow were sorted from these mice by FACS, and total RNA was isolated for DNA microarray assay. (B) DNA microarray assay shows that the mRNA level of *p53* was down-regulated by BCR-ABL in LSCs. (C) Bone marrow cells were isolated from mice with CML induced with BCR-ABL-GFP or BCR-ABL-PTEN-GFP. The percentage of $GFP^+Lin^-c\text{-}kit^+Sca1^+$ cells in bone marrow was analyzed by FACS. (D) At day 20 after BMT, the percentages of GFP^+Gr1^+ leukemia cells in peripheral blood of recipients of bone marrow cells transduced with BCR-ABL-GFP or BCR-ABL-PTEN-GFP were analyzed by FACS. (E) Total numbers of leukemia cells in peripheral blood of recipients of bone marrow cells transduced with BCR-ABL-GFP or BCR-ABL-PTEN-GFP were analyzed by FACS. (F) Pten overexpression reduces the ability of leukemia stem cells to induce CML. Bone marrow cells from mice with CML induced with BCR-ABL-GFP or BCR-ABL-PTEN-GFP were sorted by lineage-depletion MACS columns (Miltenyi Biotec), followed by FACS analysis for the percentages of $c\text{-}Kit^+Sca1^+$ cells. After normalization, the same number (3 X 104) of $GFP^+Lin^-c\text{-}kit^+Sca1^+$cells from each group was transferred into recipient mice (BCR-ABL-GFP, n=7; BCR-ABL-PTEN-GFP, n=5) to induce CML. (G) Rapamycin inhibits leukemia stem cells from CML mice in vitro. Bone marrow cells isolated from mice with CML induced by BCR-ABL-GFP were cultured (2 X 106 cells/6-cm plate) under the stem cell conditions (see –Methods") in the presence of DMSO or rapamycin (10μM) for 3 days, followed by FACS analysis of leukemia stem cells ($GFP^+Lin^-c\text{-}kit^+Sca1^+$).

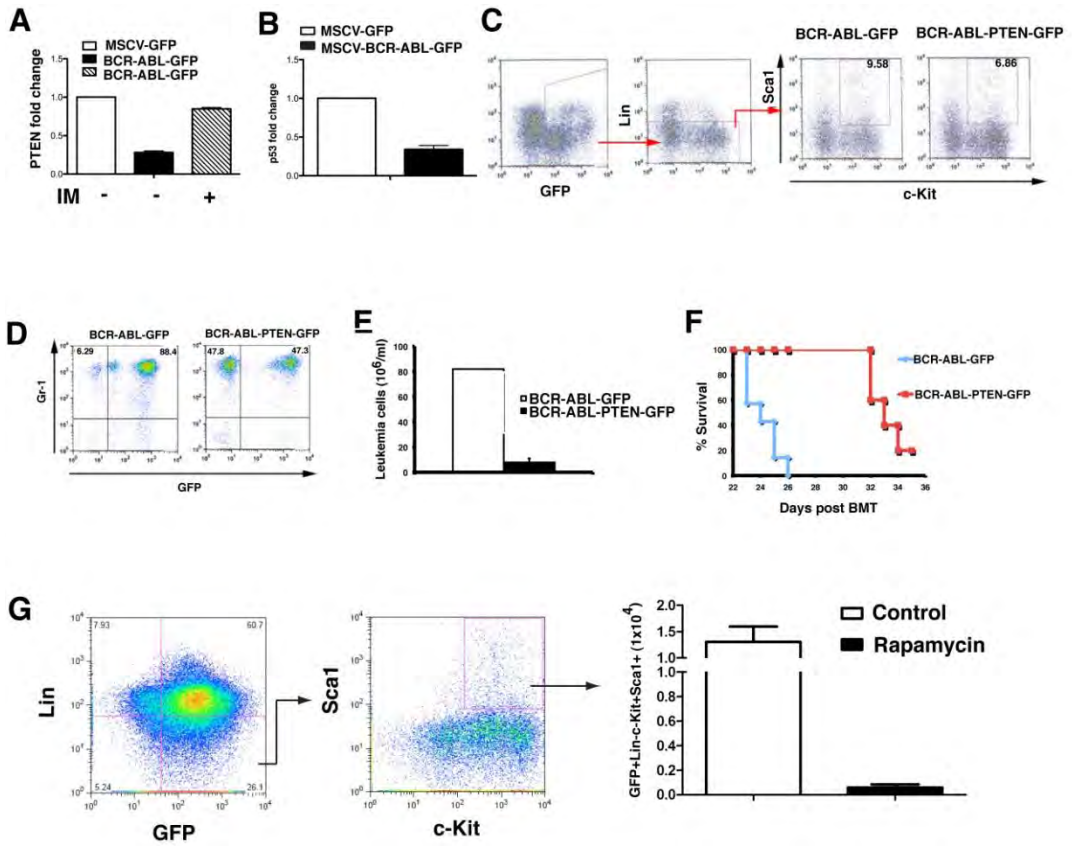


Figure 4. Pten suppresses leukemia stem cells.

Pten overexpression delays B-ALL development

We showed previously in this report that Pten functions as a tumor suppressor in CML development. We determined to examine whether Pten also plays a suppressive role in the development of B-ALL induced by BCR-ABL. To induce B-ALL in mice, donor bone marrow cells were transduced with BCR-ABL-PTEN-GFP or BCR-ABL-GFP retrovirus, followed by transplantation of the transduced cells into lethally irradiated recipient mice, as described previously.[32] All mice receiving bone marrow cells transduced with BCR-ABL-GFP developed and died of B-ALL within 4 to 5 weeks after transplantation (Figure 5A), whereas mice receiving bone marrow cells transduced with BCR-ABL-PTENGFP developed B-ALL with much longer disease latency (Figure 5A, $P<0.004$). The delayed B-ALL development correlated with a lower percentage and number of BCR-ABL–expressing B-lymphoid cells (GFP^+B220^+) in peripheral blood of the mice (Figures 5B,C), in which GFP^+Gr1^+ cells were almost undetectable (supplemental Figure 3).

To evaluate whether PTEN overexpression synergizes with imatinib in treating B-ALL mice, we treated mice receiving bone marrow cells transduced with BCR-ABL-PTEN-GFP or BCR-ABL-GFP retrovirus with imatinib. As expected, imatinib treatment prolonged the survival of B-ALL mice receiving bone marrow cells transduced with BCR-ABL-GFP (Figure 5A). However, imatinibtreated B-ALL mice receiving bone

marrow cells transduced with BCR-ABL-PTEN-GFP lived significantly longer than those not treated with imatinib (Figure 5A; $P<0.001$). The synergistic effect of Pten overexpression and imatinib treatment correlated with fewer leukemia cells in peripheral blood of the mice (Figure 5D). To examine whether Pten is overexpressed in vivo, leading to the inhibition of Akt phosphorylation, Western blot analysis of spleen cell lysates from mice with B-ALL induced by BCR-ABL-GFP or BCR-ABL-PTEN-GFP was performed. We found that Pten was undetectable and that the levels of Akt phosphorylation were high in the majority of mice with B-ALL induced by BCR-ABL-GFP. In contrast, *Pten* was detected and the levels of Akt phosphorylation were low in mice with B-ALL induced by BCR-ABL-PTEN-GFP (Figure 5E).

Figure 5. Pten overexpression delays B-ALL development. (A) Overexpression of Pten alone or in combination with imatinib treatment prolonged survival of B-ALL mice. Mice with B-ALL induced with BCR-ABL-GFP (n=10) or BCR-ABL-PTEN-GFP (n=10) were treated with a placebo (n=5) or imatinib (n=5, 100 mg/kg, twice a day by gavage), beginning at day 8 after BMT. (B) FACS analysis showed a slower accumulation of GFP^+B220^+ leukemia cells in peripheral blood of recipients of BCR-ABL-PTEN-GFP transduced bone marrow cells than that in recipients of BCR-ABL-GFP–transduced bone marrow cells. (C) The difference in peripheral blood leukemia cell counts (white blood cell count X percentage of GFP^+B220^+ cells) in B-ALL mice induced with BCR-ABL-GFP or BCR-ABL-PTEN-GFP was determined at day 12 or 20 after BMT. (D) Mice with B-ALL induced with BCR-ABL-PTEN-GFP were treated with a placebo or imatinib (IM). Peripheral blood leukemia cells were analyzed by FACS at day 35 after BMT. (E) Western blot analysis of spleen cell lysates for Pten overexpression and Akt phosphorylation in mice with B-ALL induced by BCR-ABL-PTEN-GFP or by BCR-ABL-GFP mice. The protein lysates were isolated from the mice at day 20 after BMT. The black line indicates that the lanes not adjacent on the same original sodium dodecyl sulfate–polyacrylamide gel electrophoresis were brought together to generate this figure.

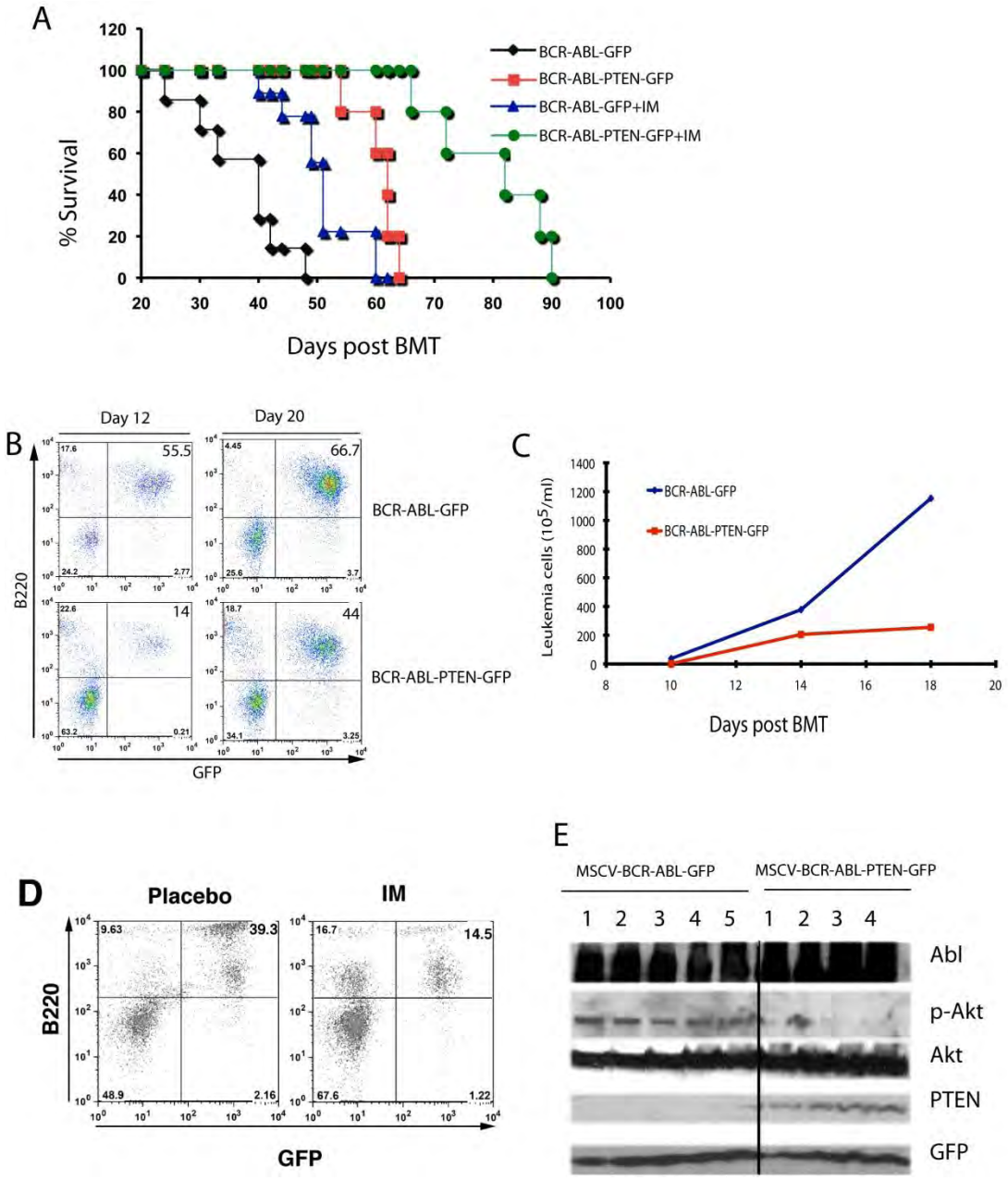


Figure 5. Pten overexpression delays B-ALL development.

Pten delays B-ALL development through its downstream gene Akt1

The Akt pathway is downstream of Pten because Pten inactivation often results in Akt activation in human cancers.[205, 206] There are 3 mammalian Akt genes that share greater than 85% sequence similarity and encode the Akt isoforms 1 to 3.[162] It is still unclear whether the 3 Akt isoforms possess different functional specificities in vivo. A recent study has shown that the deletion of the Akt1 gene has a dramatic inhibitory effect on the development of endometrium carcinoma, prostate cancer, thyroid tumor, and adrenal medulla tumors.[162]*Akt1* deficiency also inhibits the proliferation of lymphoid hyperplasia and expansion of both B- and T-cell populations in *Pten*+/- mice.[162] In addition, the first transforming point mutation in Akt1 (E17K) has been discovered in human breast, colorectal, and ovarian cancers.[200]

Furthermore, fetal liver cells from Eu-Myc transgenic mouse were transduced with this Akt1 (E17K), followed by transplantation into recipient mice. After 16 weeks, 6 of 10 recipients developed pre–pro-B-cell leukemia.8 Here we determined to test whether Akt1 is functionally involved in the development of B-ALL induced by BCR-ABL by using Akt1-/- mice as donors in our B-ALL mouse model. The majority of recipients of BCR-ABL–transduced wild-type bone marrow cells developed and died of B-ALL within 5 to 7 weeks (Figure 6A), whereas recipients of BCR-ABL–transduced Akt1-/- bone marrow cells developed and died of B-ALL with a significantly longer disease delay (Figure 6A; $P<0.005$). This delayed B-ALL development caused by the Akt1 deficiency correlated with a lesser percentage and number of B-leukemia cells ($B220^{+}GFP^{+}$) in the

peripheral blood of the mice (Figure 6B,C). We examined whether *Akt1-/-* have a defect in B-cell development because a reduction of bone marrow pro-B cells, the target cells for BCR-ABL to induce B-ALL, could lead to a delayed disease development. To rule out this possibility, we analyzed bone marrow cells of *Akt1-/-* mice by FACS and found that *Akt1-/-* mice have a normal percentage of pro-B cells ($CD43^{+}B220^{+}$) in bone marrow compared with wild-type mice (supplemental Figure 4).

Because Pten overexpression synergizes with imatinib in treating B-ALL mice (Figure 5A), we examined whether the *Akt1* deficiency also synergizes with imatinib in treating B-ALL. We treated mice receiving wild-type or *Akt1-/-* bone marrow cells transduced with BCR-ABL-GFP retrovirus with imatinib. As expected, imatinib treatment prolonged survival of B-ALL mice receiving BCR-ABL–transduced wild-type bone marrow cells, whereas imatinib treatment more significantly improved survival of B-ALL mice receiving BCR-ABL-GFP–transduced *Akt1-/-* bone marrow cells (Figure 6A; $P<0.001$).

Figure 6. Loss of *Akt1* delays B-ALL development. (A) Gross appearance and Kaplan-Meier–style survival curves for recipients of BCR-ABL–transduced bone marrow cells from wild-type (WT) or *Akt1-/-* mice. B-ALL mice transduced from wild-type mice are treated with placebo (n=20) or imatinib (IM; n=10, 100 mg/kg, twice a day) and B-ALL mice transduced from Akt1_/_ mice are also treated with placebo (n=25) or imatinib (n=10, 100 mg/kg, twice a day). (B) Bone marrow cells were harvested from recipients of BCR-ABL transduced wild-type or *Akt1-/-* bone marrow cells and were stained with antibodies against CD43 and B220 (representing pro-B cells) for FACS analysis. (C) FACS analysis showed the numbers of peripheral blood leukemia cells (GFP^+B220^+) in recipients of BCR-ABL-GFP–transduced wild-type or *Akt1-/-* bone marrow cells.

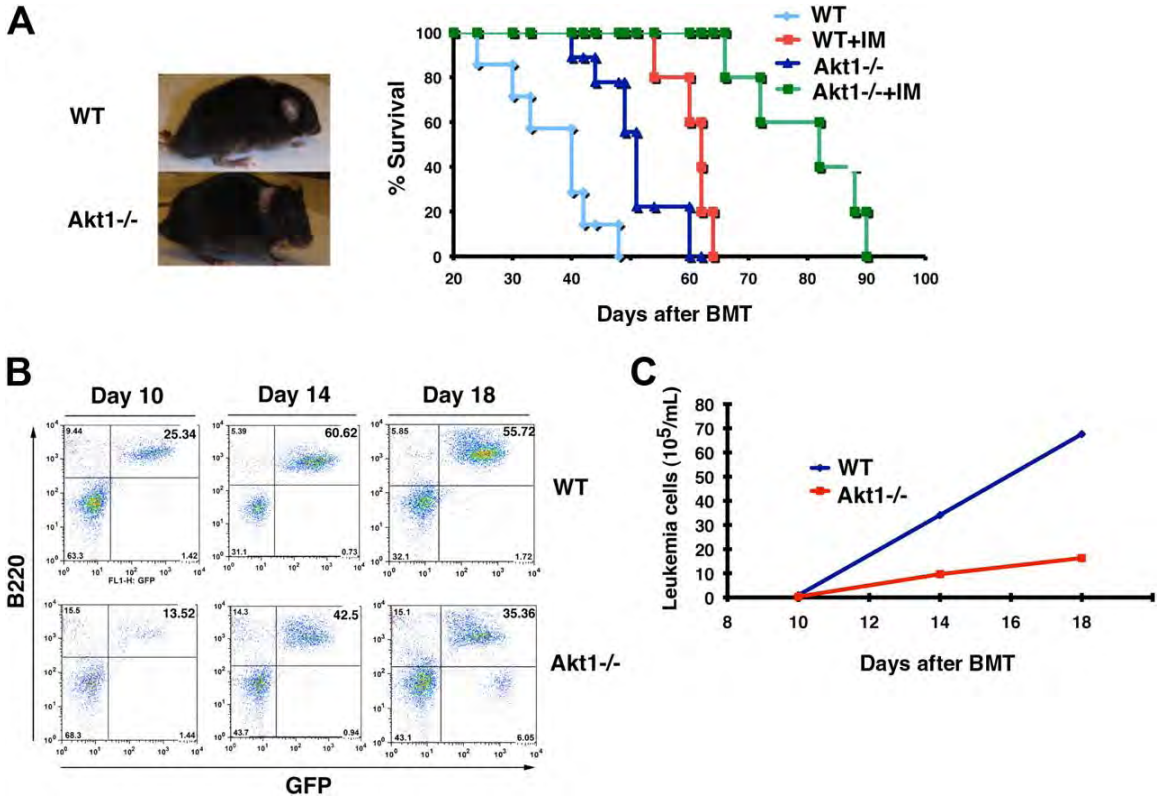


Figure 6. Loss of *Akt1* delays B-ALL development.

Rapamycin inhibits proliferation and induces apoptosis of human CML cells

Recently, downrregulation of *PTEN* mRNA in HSC in CML patients has been reported[207]. To confirm the PTEN expression in human CML cells, PTEN level has been detected in K562 cells. Western blot results showed the lower level of PTEN in k562 cells compared with K562 cells treated with imatinib treatment for 24 hours (Figure 7A). To investigate whether rapamycin can inhibit the K562 proliferation, we treated K562 cells with rapamycin in vitro for 3 days. The significant inhibitory effect has been shown after 48h treatment and further effect was shown after 72h treatment (Figure 7B). At the meantime, the rapamycin dramatically induced the apoptosis rate in K562 cells after 48h treatment (Figure 7C). These data confirmed the PTEN downregulation in human CML cells and indicated the rapamycin could be a promising therapeutic target in the CML.

Figure 7. PTEN down-regulation in K562 cells and inhibition of K562 cells by rapamycin. (A) PTEN protein level was elevated by K562 cells treated with imatinib (IM). K562 cells were treated with imatinib (1μM) for 24 hours, and protein lysates were analyzed by the use of Western blotting with the antibodies indicated. (B) Rapamycin inhibits proliferation of K562 cells. K562 cells were treated with DMSO or rapamycin (10μM) for 48 and 72 hours, and live cells were counted. (C) Rapamycin induces apoptosis of K562 cells. K562 cells were treated with DMSO or rapamycin (10 μM) for 48 hours. Apoptotic cells (Annexin $V^+/7AAD^+$) cells were analyzed by FACS.

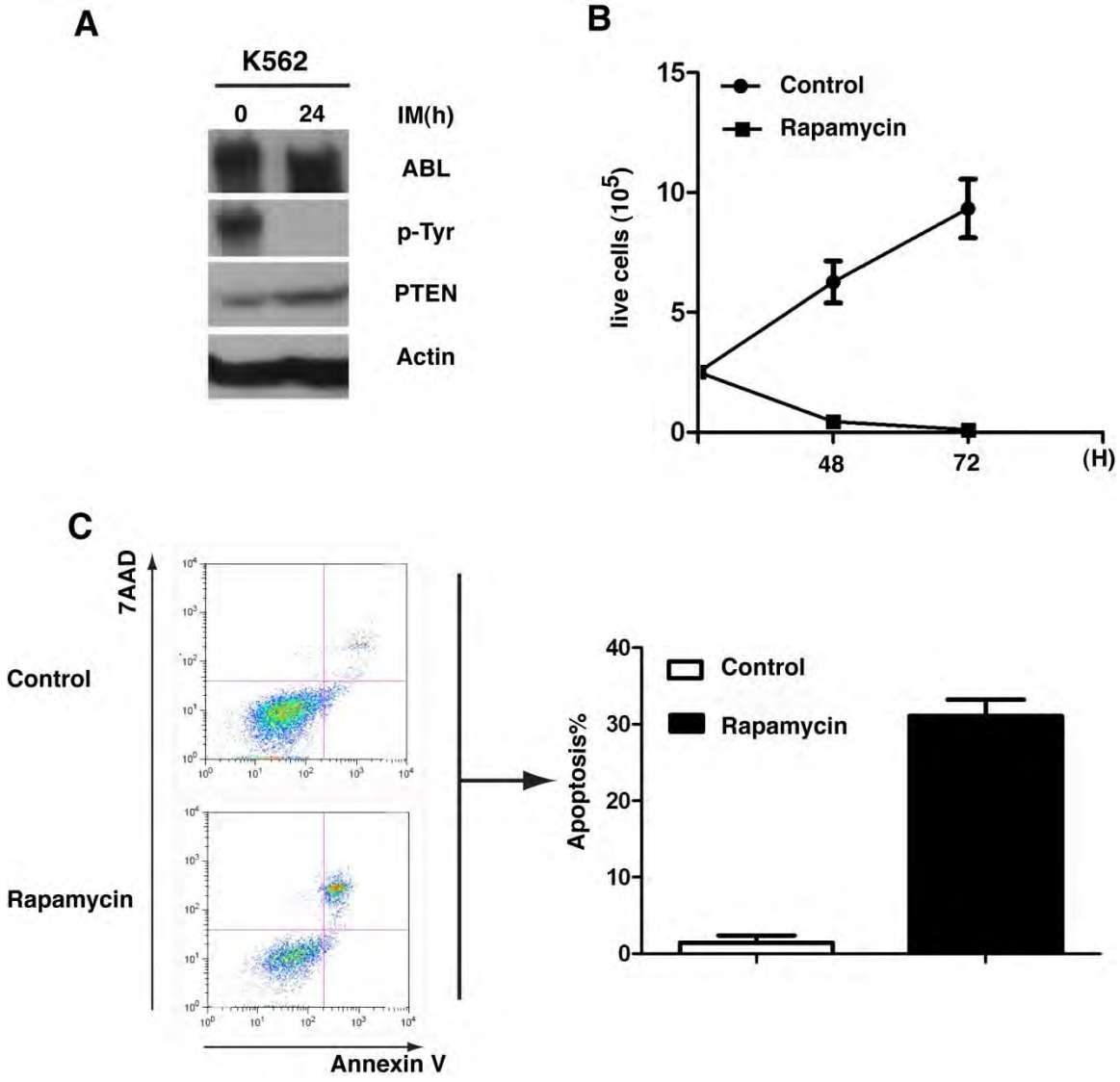


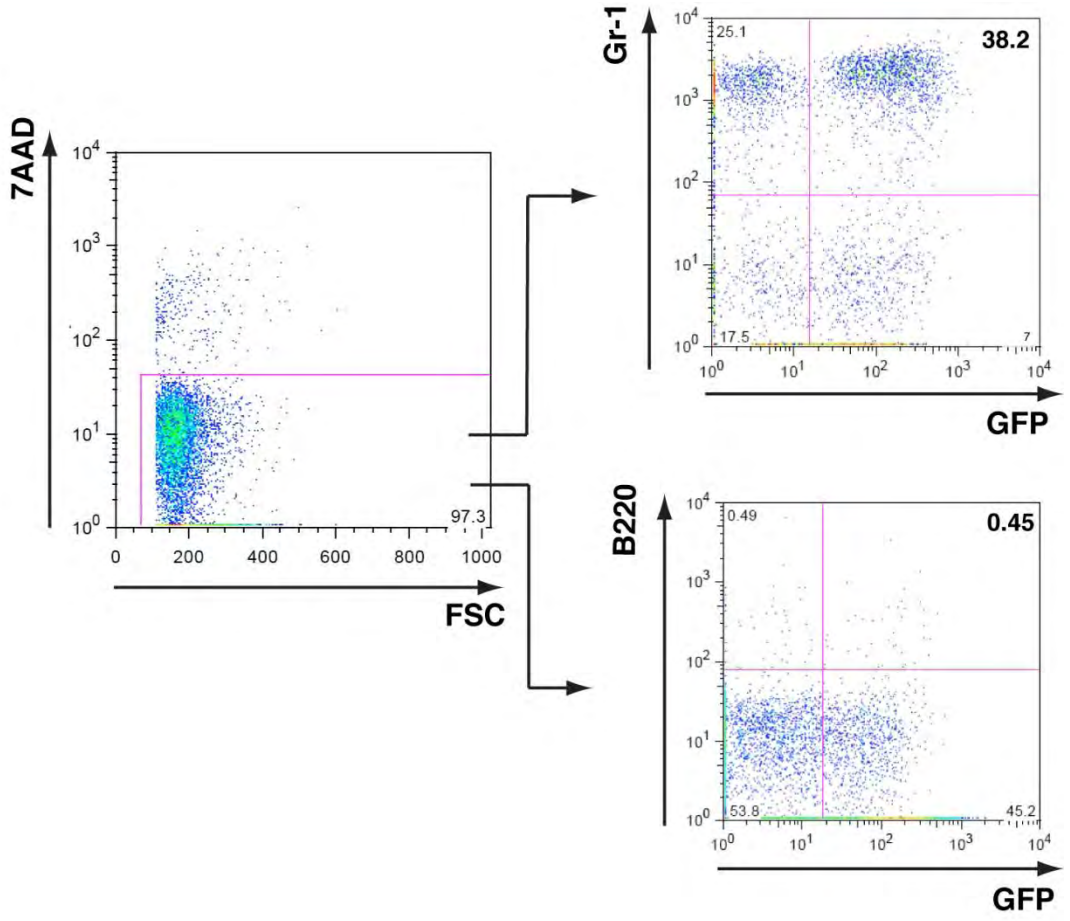
Figure 7. PTEN down-regulation in K562 cells and inhibition of K562 cells by rapamycin.

Discussion

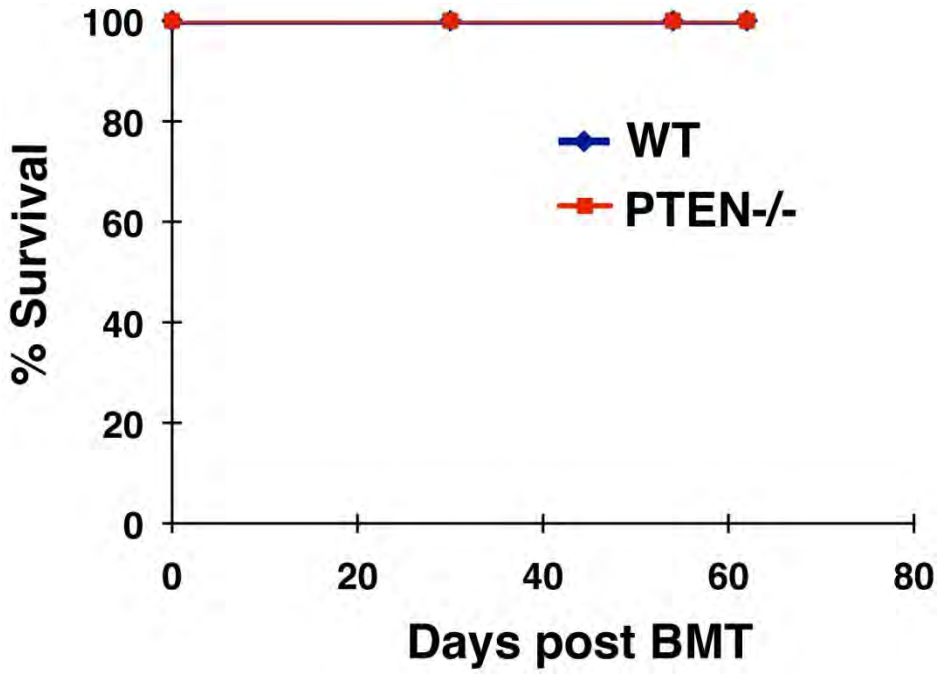
Some tumor suppressor genes have been shown to be inactivated or down-regulated by BCR-ABL in Ph_ leukemia, including PP2A,[208] p53[209] RB,[209] and interferon consensus sequence-binding protein.[210] In this study, we show that the tumor suppressor Pten is also down-regulated by BCR-ABL and that overexpression of Pten delays the development of CML and B-ALL induced by BCRABL. Our DNAmicroarray study shows that *Pten* mRNAlevel was decreased in BCR-ABL–expressing LSCs, indicating that BCRABL regulates Pten at a transcriptional level. Our finding that both *Pten* and p53 are simultaneously down-regulated in BCR-ABL–expressing cells suggests that the Pten down-regulation by BCRABL may be mediated by P53, as PTEN transcription is regulated by p53.[163, 211] p53 has been shown to up-regulate *Pten* by binding to its promoter[201]; in *p53-/-* MEF cells, the level of *Pten* is only 30% of that in the wild-type cells.[212] Besides p53, other mechanisms might also be involved in the down-regulation of Pten by BCR-ABL. An analysis of the *Pten* promoter sequence shows potential binding sites for early growthregulated transcriptional factor 1 (EGR1), and Pten is up-regulated by EGR1 in response to radiation treatment.[213] EGR1 also upregulates Pten, which likely mediates the apoptotic effect of the phosphatase inhibitor calyculin A. There are also pathways that negatively regulate Pten expression. For example, mitogenactivated protein kinase kinase 4 inhibits Pten transcription by activating nuclear factorB that binds to the Pten promoter.[214] In pancreatic cancer cells[215] or mesangial cells,[216] Pten is downregulated by transforming growth factor-. Pten is also regulated at a posttranscriptional level. Phosphorylation of Pten at specific residues in its

C-terminal tail is associated with an increase in its stability,[217-219] whereas phosphorylation at other sites decreases the protein stability.[220] Ubiquitin-dependent degradation of PTEN occurs when human bronchial cells were exposed to zinc ions,[221] and the finding of 2 major conserved ubiquitination sites on PTEN supports this regulation.[222] BCR-ABL may regulate these pathways to down-regulate Pten expression, and these potential mechanisms need to be explored further. PTEN maintains normal hematopoietic stem cells in lineage choice and prevents the leukemia development from leukemia stem cells. Our microarray data show that *Pten* is down-regulated in BCR-ABL–expressing LSCs, suggesting that BCR-ABL regulates the functions of LSCs through regulating Pten expression. This idea is supported by our finding that LSCs in CML mice grew significantly slower when Pten was overexpressed. The role of Pten in LSCs provides a potential strategy for targeting the Pten and its related PI3K/AKT pathways in eradication of LSCs. In this study, we also show that overexpression of Pten delays B-ALL development and that Akt1 is a major downstream signaling molecule of Pten. Moreover, the inhibition of mTOR by rapamycin significantly inhibits proliferation of human CML leukemia cells K562 and leukemia stem cells from CML mice. These findings support the use of the PTEN-PI3K-AKT-mTOR pathway as a target in treating

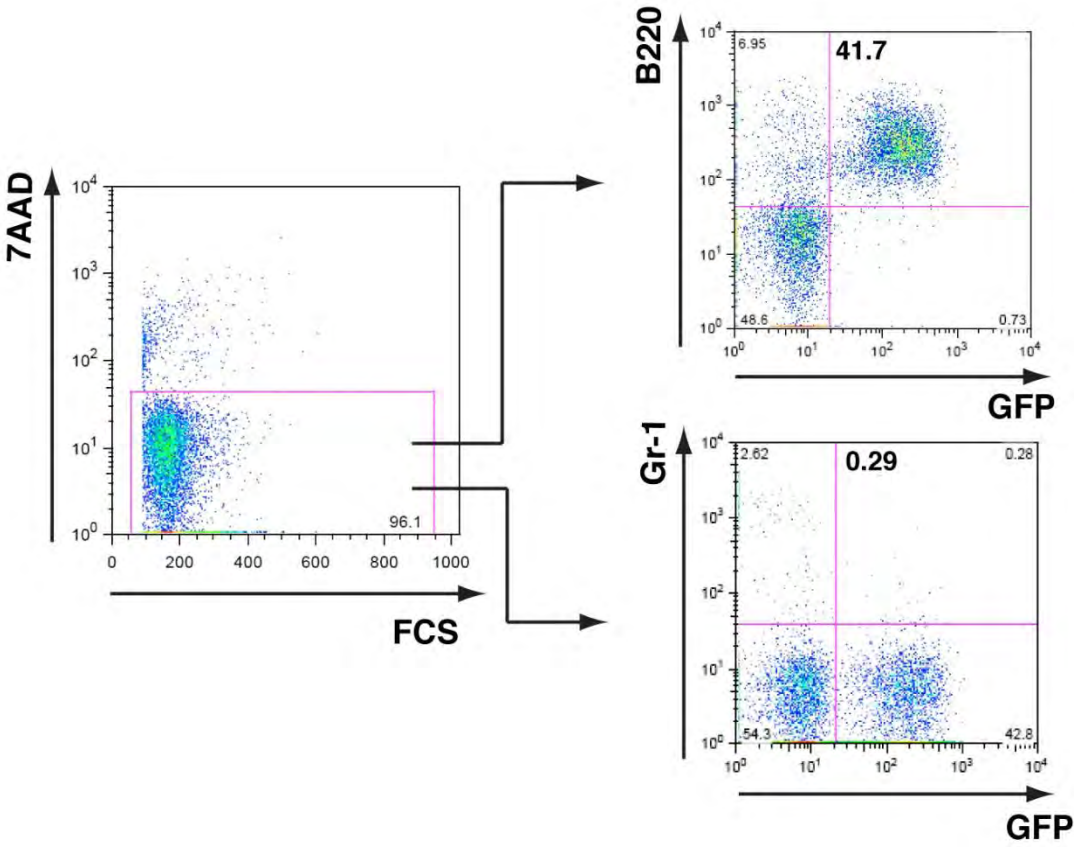
B-ALL, which is not sensitive to imatinib therapy.



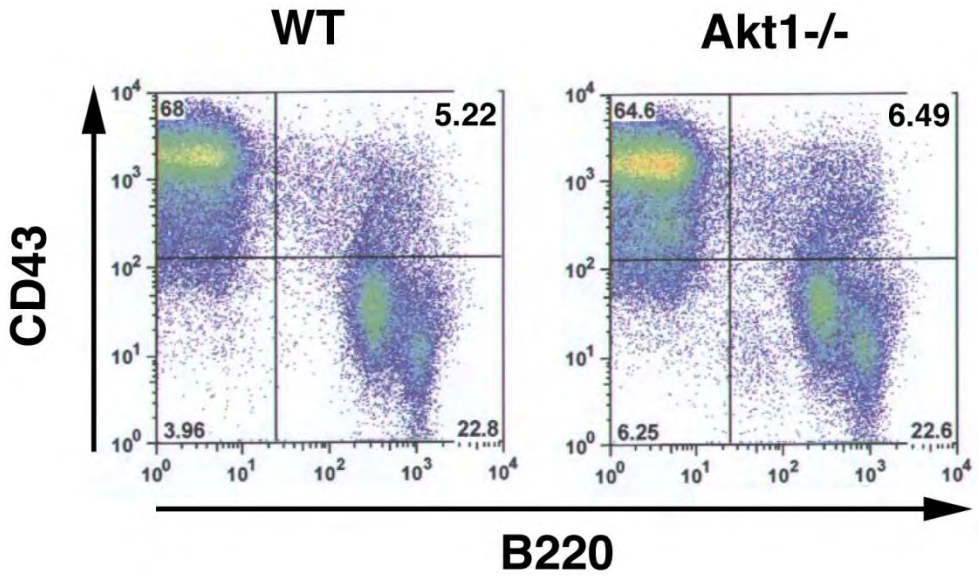
Supplemental Figure 1. FACS analysis of peripheral blood in CML mice. PB cells were collected from CML mice and stained with Gr-1 and B220 and analyzed by FACS. Most of the GFP+ cells are also Gr-1+, indicating they are myeloid leukemia cells.



Supplemental Figure 2. No AML development in CML mice receiving BCR-ABL transduced bone marrow cells from *Pten*$^{fl/fl}$ mice. Bone marrow cells from wild type or *Pten*$^{fl/fl}$ mice were transduced with MSCV-iCre-GFP retrovirus, followed by transplantation of the transdcued cells into the lethal irradiated recipient mice. Survival of the mice was compared between two groups.



Supplemental Figure 3. FACS analysis of peripheral blood in B-ALL mice. PB cells were collected from B-ALL mice and stained with Gr-1 and B220 and analyzed by FACS. Most of the GFP+ cells are also B220+, indicating they are lymphoid leukemia cells.



Supplemental Figure 4. *Akt1-/-* mice do not have a defect in B progenitor cells. Bone marrow cells were isolated from wild type or *Akt-/-* mice, and stained with antibodies against CD43 and B220. The percentages of pro-B cells in wild type and *Akt-/-* mice were compared.

Material and Methods

Cell lines

Ba/F3 pre-B-cells were grown in RPMI 1640 medium containing 10% FCS, 10% WEHI medium, and 50 μM 2-mercaptoethanol. Ba/F3-BCR-ABL, parental ENU and ENU-BCR-ABL cells were grown in RPMI 1640 medium containing 10% FCS, and 50μM 2-mercaptoethanol.

Mice

C57BL/6J, B6.129S4-*Pten*^{tm1Hwu}/J (*Pten*$^{fl/fl}$) and B6.129P2-*Akt1*^{tm1Mbb}/J (*Akt1-/-*) mice 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.

Antibodies and Western blot analysis

Antibodies against c-Abl (sc-131), p-Tyr (sc-508), PTEN (sc-7974), p53 (sc-6243) and Actin (sc-1616-R) were purchased from Santa Cruz Biotechnology. Cre (Cat# 69050) antibody was ordered from Novagen. Protein lysates were prepared by lysing cells in radioimmunoprecipitation (RIPA) buffer, and immunoprecipitation and Western blotting were carried out as described previousl[204].

Construction of triple gene coexpression plasmids

The original MSCV-IRES-GFP vector was first modified to add new cloning sites for the restriction enzymes MfeI, NotI and MluI. To do so, the IRES sequence was first amplified by the MSCV primer (CGTCTCTCCCCCTTGAACCTCCTCG) and the IRES-MfeI primer (CATG*CCATGGCAATTG*A*GCGGCCGC*TTGTGGCCATATTATCATC) which contains the new MfeI, NotI and the existing NcoI sites (underlighted). This allowed us to synthesize a new IRES fragment containing the MfeI, NotI and NcoI sites. To replace the original IRES sequence in the original MSCV-IRES-GFP vector with the newly-synthesized IRES, this vector was cut with EcoRI and NcoI, and then the new IRES fragment was cloned into the MSCV-IRES-GFP cut with EcoRI and NcoI, forming a new MSCV-IRES-GFP vector that contains two additional sites, MfeI andf NotI. To add the MluI site to the new MSCV-IRES-GFP vector, an IRES-GFP fragment was amplified from this vector by the MSCV primer and the GFP-MluI primer (CC*ATCGATACGCGT*AAGCTTGGCTGCAGGTCGA) which contains the existing ClaI and the new MluI sites (underlighted). The synthesized IRES-GFP fragment was digested with EcoRI and ClaI, and then cloned into new MSCV-IRES-GFP vector between the EcoRI and ClaI sites to generate the final MSCV-IRES-GFP vector. Compared to the original MSCV-IRES-GFP vector, this final MSCV-IRES-GFP vector contains additional sites MfeI, NotI (before the GFP sequence) and MluI (after the GFP sequence). To clone the BCR-ABL cDNA into this final MSCV-IRES-GFP vector, BCR-ABL was cloned into it at the EcoRI site.

To make the MSCV-BCR-ABL-PTEN-GFP construct, total RNA was isolated from C57BL/6 mice liver tissue for synthesizing the *Pten* cDNA by RT-PCR. The *Pten* cDNA was amplified by PTEN-NotI

(5' A*GCGGCCGC*ATGACAGCCATCATCAAAGAG 3') and PTEN-MluI (5' CG*ACGCGT*TCAGACTTTTGTAATTTGTG 3') primers. The cDNA was sequenced from both ends to confirm the sequence. The *Pten* cDNA was cloned into the MSCV-BCR-ABL-GFP vector between NotI and MluI sites. The IRES-GFP fragment was amplified by MSCV-MluI (cg*acgcgt*AATTCCGCCCCTCTCCCTC) and GFP-MluI (cc*acgcgt*TAAGCTTGGCTGCAGGTCGA) primers using MSCV-GFP as a template, and the IRES-GFP fragment was inserted after the PTEN sequence at the MluI site.

To make MSCV-BCR-ABL-iCre-GFP construct, the iCre (improved Cre) ORF was amplified by iCre-MfeI (CG*CAATTG*ATGGTGCCCAAGAAGAAGAGG) and iCre-ClaI (CC*ATCGAT*TCAGTCCCCATCCTCGAGCAG) using the pBOB-CAG-iCre-SD (addgene, cat#12336) was used as a template. The iCre ORF was cloned into the MSCV-BCR-ABL vector between NotI and MluI sites and the IRES-GFP fragment was cloned at the MluI site after the iCre ORF.

Whitlock-Witte culture

Bone marrow cells were tranduced with BCR-ABL retrovirus and cultured in a 6-well plate in RPMI 1640 medium containing 10% FCS, and 50μM 2-mercaptoethanol for 1 week. Protein lysates were collected and analyzed by Western blotting.

Bone marrow transduction/transplantation

The retroviral constructs MSCV-GFP[24], BCR-ABL-PTEN-GFP or BCR-ABL-iCre-GFP carrying the BCR-ABL cDNA were used to make high-titer, helper-free, replication-defective ecotropic viral stocks by transient transfection of 293T cells using the kat system as previously described [156]. Six- to ten-week-old wild-type C57BL/6 and *Pten*$^{fl/fl}$ (The Jackson Laboratory) mice were used for leukemogenesis experiments. Induction of CML and B-ALL [156] was described previously. Briefly, to induce CML, bone marrow cells from 5-FU–treated (200 mg/kg) donor mice were transduced twice with BCR-ABL retrovirus by cosedentation in the presence of IL-3, IL-6, and SCF. To induce B-ALL, bone marrow cells from non–5-FU–treated donors were transduced with BCR-ABL in the absence of any cytokines. Wild-type recipient mice were prepared by 1150 cGy gamma irradiation. A dose of 0.5 x10^6 (CML) or 1.0x10^6 (B-ALL) cells was transplanted via tail vein injection. Diseased mice were analyzed by histopathological and biochemical analyses as described previously[204] .

Flow cytometry

Hematopoietic cells were collected from peripheral blood and bone marrow of the disease mice, and red blood cells were lysed with NH_4Cl red blood cell lysis buffer (pH 7.4). The cells are washed with PBS, and stained with B220-PE for B cells and Gr1-APC for neutrophils, Sca1-APC/c-Kit-PE for hematopoietic stem cells and Hoechst blue for DNA. After staining, the cells were washed once with PBS and subjected to FACS analysis.

Chromatin immunoprecipitation (Chip)

Chip assay was performed according to the protocol of Chip-TI Express kit (Active Motif, Carlsbad, CA). In brief, BaF3 cells were lyzed and chromatin was broken into smaller fregments from 200bps to 500 bps by sonication. Genomic DNA was then incubated with anti-rabbit immune IgG or anti-p53 antibody, and pre-incubated protein G sepharose beads and precipitated by centrifugation. Recovered protein-bond DNA was applied for PCR reaction with primers: 5'-CAAAGCCGGCGTAGCTC-3' and

5'-ACAAAGAGTCCCGCCACAT-3'.

Culture of leukemia stem cell

Bone marrow cells isolated from CML mice were culture in vitro in the presence of stemspan SFEM, SCF, IGF-2, TPO, heparin and α-FGF as described previously[204].

Drug treatment

Imatinib was dissolved in water directly at a concentration of 10mg/ml, and administered orally by gavage in a volume less than 0.5 ml twice a day at 100mg/kg body weight, beginning 8 days after bone marrow transplantation and continuing until the morbidity or death of leukemic mice. Rapamycin (Calbiochem, Cat# 553210) was dissolved in DMSO and made stock concentration as 1mM.

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Chapter V Discussion and future directions

Although CML has been identified as a blood cancer derived from BCR-ABL transformed HSCs, there is still no specific therapy for it until the emerging of imatinib which blocks BCR-ABL kinase activity and inhibits its functions. Now imatinib has become the first line clinical drug to treat CML patients and achieves a complete hematologic response in major CML patients. A five-year follow up study showed that a complete cytogenetic response among 553 patients receiving imatinib was 69% by 12 months and 87% by 60 months, and the estimated overall survival of patients who received imatinib as initial therapy was 89% at 60 months.[59] However, imatinib still cannot remove all of the BCR-ABL expressing leukemia cells, indicating more mechanisms of CML leukemogenesis and therapies need to be investigated. There are two major reasons to cause this imatinib resistance. The first one is the BCR-ABL kinase mutations, especially the T315I mutant, which can change the BCR-ABL kinase domain conformation and release the inhibition of imatinib on the kinase activity, and it is even still highly resistant to dasatinib and nilotinib, which are more potent to inhibit WT-BCR-ABL kinase activity than imatinib. The second reason is the residual of leukemia stem cells in CML patients who receive the imatinib treatment and they can re-initiate the leukemia cells proliferation and relapse when imatinib is discontinued or the concentration is invalidated.

Hsp90 and T315I-BCR-ABL

To overcome these two major obstacles in CML therapy, novel strategies need to be developed. In my thesis work, we focused on two ways to solve the obstacles mentioned above. First, we not only inhibited the BCR-ABL kinase activity by imatinib, but also affected BCR-ABL oncoprotein stability to induce BCR-ABL oncoprotein degradation during the treatment. Although it has been reported that BCR-ABL is a client protein of chaperon protein, Hsp90, and inhibition of Hsp90 function with its inhibitors, such as GA or 17-AAG, can induce BCR-ABL degradation *in vitro*[137, 138, 179], the therapeutic effects of Hsp90 inhibitors have not been proved in CML mouse models or patients. On the other hand, the high toxicity and low solubility of current Hsp90 inhibitors also prevent their applications in clinical trials. In this part of my work, I evaluated a novel Hsp90 inhibitor, IPI504, which has less toxicity and 4000 folds higher solubility compared with current Hsp90 inhibitors in our CML and B-ALL mouse models. The *in vivo* treatment showed a significant prolonged survival of CML mice after IPI504 administration, consisting with the BCR-ABL *in vivo* degradation and less leukemia cells infiltration in the lung which is the major cause of death in our CML mice. Notably, the T315I-BCR-ABL induced CML mice were more sensitive to IPI504 treatment compared with WT-BCR-ABL induced CML. We supposed the mutant BCR-ABL might rely more on Hsp90 function to stabilize its stability and facilitate its activation of downstream. Our later immunoprecipitation results also confirmed this idea because more T315I- BCR-ABL protein has been immunoprecipitated by same dose of Hsp90 antibody compared with WT BCR-ABL (data not shown). So it is reasonable to observe faster degradation of

T315I-BCR-ABL after IPI504 treatment than WT-BCR-ABL does. Both of these results well explain why mutants of BCR-ABL are more sensitive to Hsp90 inhibition and this could also be a promising strategy to treatment CML patients who harbor the mutant BCR-ABL oncoproteins.

Hsp90 and LSCs

Another exciting finding in this part of work is inhibition of Hsp90 could impair LSCs *in vitro* and *in vivo*. As CML is a disease derived from cancer stem cells, it has become a paradigm model in cancer stem cell biology to investigate the critical pathways and therapeutic targets in cancer stem cells. As LSCs in CML are BCR-ABL transformed HSCs, BCR-ABL is the major player in LSCs to initiate leukemia development. Given the BCR-ABL degradation *in vitro* and *in vivo*, and the significantly prolonged survival of CML mice after IPI504 treatment, inhibition of Hsp90 not only caused BCR-ABL degradation but also might impair LSCs in CML. To confirm this LSCs inhibition, we subsequently treated CML mice total BM which contains LSCs with IPI504 in stem cell enrich condition for 6 days, and found a dramatic inhibition of LSCs percentage and total number. Similar inhibition effect was also observed in CML mice treated with IPI504. This inhibition of LSCs was specifically caused by suppression of Hsp90 function in LSCs because the potential IPI504 side-effect or toxicity has been excluded by following IPI504 treatment in normal WT B6 mice. This promising result indicates LSCs are more sensitive to Hsp90 inhibition than normal HSCs and this result also makes it more confident to apply IPI504 into clinical trial. Communication with Infinity Co. confirmed

the success of IPI504 in the clinical trial I and we also hope it will achieve success in further clinical trials to benefit more CML patients.

Although all CML mice achieved significant longer survival and LSCs were impaired after IPI504 treatment, they still died of CML in the end. There are at least three possibilities to explain the failure of IPI504. The first one could be the drug concentration is not high enough *in vivo*, especially in the long term treatment. As we showed higher concentration of IPI504 achieved better therapeutic effect, we believe increase of IPI504 dose will improve the treatment. Recently, a new version of IPI504, called IPI493 which has higher solubility and lower toxicity than IPI504, has been developed by Infinity Co. We are also evaluating and comparing its therapeutic effect with imatinib and IPI504 in our models. We also hope this new version of Hsp90 inhibitor could achieve better therapeutic effect. On the other hand, a genetic knockout mouse strain could solve this drug concentration problem, as we do not need to worry about the inhibitory efficiency of Hsp90 in knockout condition. However, it is unfortunate that the *Hsp90β* conventional knockout mice are embryonic lethal and the *Hsp90α* conventional knockout mice are also not available now, so it is hard to evaluate their contribution in our BCR-ABL retroviral mouse models. To overcome this obstacle and also to be a future plan, we are planning to establish both *Hsp90α* and *Hsp90β* conditional knockout mice strains. Specific deletion both of these Hsp90 isoforms in mouse hematologic cells will definitely help us to confirm their contribution in CML development.

The second possibility might be the emerging of Hsp90 mutation during the treatment. In normal condition, GA, 17-AAG and IPI504 replace nucleotide in the Hsp90

binding pocket with an affinity much greater than either ATP or ADP, thus effectively short-circuiting the chaperone cycle.[134] If any mutant occurs in this nucleotide binding pocket, it will definitely change the conformation which facilitate Hsp90 binding to IPI504 and cause the insensitiveness to treatment. To find out these potential Hsp90 mutants, we can collect the DNA samples from the dead mice after IPI504 treatment and check the Hsp90 sequence by screening its genomic DNA. If any mutation has been found in the Hsp90 DNA, we can clone this mutant cDNA out and transfect it into 32D-BCR-ABL cell line which is originally sensitive to IPI504 to test whether ectopic expression of this mutant Hsp90 will switch the cells to be insensitive to IPI504 treatment.

The last one might be the residual LSCs could re-initiate the leukemia proliferation to cause the death of mice. Although a dramatic decrease of LSCs has been observed *in vitro* and *in vivo* treatment, a small percentage of LSCs were still residual. They might finally take over the disease initiation and promote leukemia cells proliferation and cause the death of mice. This small residual LSCs population also indicates Hsp90 inhibition alone is not enough to remove all LSCs and part of them can addict to other pathways to bypass the Hsp90 inhibition and remain their self-renewal and differentiation. Further investigations of LSCs critical pathway are still needed to be performed.

Role of Pten in LSCs

To identify critical pathways in LSCs, we compared the global gene expression signature between normal HSCs and LSCs by conducting a DNA microarray analysis.[223] During this whole analysis, we already proved that *Alox5* is a CML LSCs specific regulator and inhibition of *Alox5* by either genetic knockout or inhibition with its specific inhibitor, zileuton, dramatically blocks the CML development.[127] Interestingly, Pten, a most mutated and downregulated tumor suppressor in human cancers, is also listed in the candidate genes.

In the second part of this thesis, we focused on the Pten function in LSCs and CML development. Two of reasons stimulate us to investigate Pten function in LSCs in CML. The first one is although *PTEN* is often deleted or inactivated in many human cancers, including glioblastoma[141], endometrial carcinoma [143], and lymphoid malignancies[146], these are few studies reporting the downregulation or mutation of *Pten* in human CML patients. As our LSCs microarray results suggest Pten plays as a tumor suppressor in LSCs, it is very attractive for us to study Pten function in our CML mouse model. The second reason is two independent groups reported Pten distinguished normal HSCs from leukemia initiating cells and prevented the acute myeloid leukemia (AML) development in mice. These exciting studies revealed the crucial role of Pten in the self-renewal and differentiation of LSCs in AML and invoked us to extend Pten study from AML to CML.

Our results in this part proved Pten functions as a tumor suppressor in LSCs and suppresses CML leukemogenesis. As Pten conventional knockout mice are embryonic lethal and specific deletion of Pten in mouse hematologic cells will induce lethal AML in

mice, we creatively make a triple-gene expression retroviral construct which can simultaneous expresses BCR-ABL, iCre (or Pten) and GFP in a same cell. The advantage of this construct is iCre can delete Pten in every BCR-ABL expressing leukemia cell and we can monitor these leukemia cells proliferation, cell cycle and apoptosis, following the GFP expression. In our study, we did find the acceleration of CML development when Pten was deleted with the MSCV-BCR-ABL-iCre-GFP retrovirus in *Pten*$^{fl/fl}$ mice or a prolonged survival of CML mice induced by MSCV-BCR-ABL-Pten-GFP retrovirus compared with control group. However, we only used MSCV-BCR-ABL-GFP retrovirus induced CML mice as controls. The different sizes of dual-gene and triple-gene constructs might affect the titer of retrovirus. In the future experiments, to make more accurate control, a loss-of-function form of iCre could be cloned to make a MSCV-BCR-ABL-iCre(mutant)-GFP construct, which will be a real control to MSCV-BCR-ABL-iCre-GFP construct and they can achieve more reliable results by excluding the titer issue.

Potential mechanisms of Pten in LSCs

Although Pten is intensively studied in solid tumors and T cell-acute lymphoid leukemia (T-ALL)[224-227], little is known about Pten in CML until we show that Pten inhibits LSCs and CML development.[204] This result is supported by a clinical study which compared global gene expression between normal CD34+ HSCs and CD34+ subsets from six patients with chronic phase CML. Besides the changes of gene expression for several adhesion molecules, transcription factors, cell cycle and stem cell

fate regulators, *PTEN* was also downregulated.[207] Another study showed that the gene expression profiles of mononuclear cells from CML patients who are non-responder for imatinib treatment also indicated the Pten downregulation.[228] However, the mechanisms of Pten regulation of LSCs in CML still need to be investigated. We noticed that the level of phosphorylated-Akt (p-Akt) was significantly lower in leukemia cells in CML mice when Pten was overexpressed [204], suggesting that p-Akt is a critical in Pten pathway and inhibition of Akt could be a rational therapeutic strategy in treatment. This idea is supported by our finding that induction of B-cell acute lymphoblastic leukemia (B-ALL) in mice was largely compromised when Akt1 was absent, as shown by the prolonged survival of recipients of BCR-ABL transduced *Akt1* deficient bone marrow cells mice.[204] The involvement of Akt1 in cancer has been shown in endometrial tumor, prostate cancer, thyroid tumor, adrenal medulla tumors and intestinal polyps in *Pten*+/- mice.[162] However, the roles of the Akt family members (Akt1, Akt2 and Akt3) in CML are still needed to be studied in the future. We have shown that expression of the *Alox5* gene is upregulated by BCR-ABL in CML LSCs,[127] and it has been reported that Alox5 activates Akt through oxidation and inhibition of Pten.[229] This potential pathway between Alox5, Pten and Akt are great targets in LSCs therapies.

When *Pten* is specifically deleted in mouse hematopoietic cells, the mice develop acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL), and all mice died within 4 weeks.[159, 160] LSCs in these mice are highly enriched in Lin-Sca1+cKit+Flk2-CD48- population.[160] A blockade of differentiation from LT-HSC (Lin-Sca1+cKit+Flk2-) to ST-HSC (Lin-Sca1+cKit+Flk2+) was also found in *Pten* deficient mice, causing an

eventual exhaustion of LT-HSC.[159] Increased percentage of S+G2M dividing HSCs was observed in *Pten* deficient mice, indicating that Pten functions as a molecular switch governing the G0-G1 transition between the quiescent and activated states of LT-HSCs to maintain normal HSCs pool.[159] The role of Pten in cell cycle control is consistent with our result that Pten expression induces cell cycle arrest in BCR-ABL expressing leukemia cells.[204] In addition, cyclin D1 is a well known target of the PI3K-Akt pathway,[230] maintaining cells at G1 stage in preparation for G1/S phase transition. In *Pten* deficient AML mice, high number of cyclin D1-expressing cells were detected in bone marrow, suggesting that cyclin D1 is downstream of Pten in cell cycle regulation of HSCs. Thus, the role of cyclin D1 in cell cycle regulation of LSCs in CML requires further study. Furthermore, after rapamycin administration, LSCs were depleted and normal HSCs restored in *Pten*-deficient AML mice[159], indicating that Pten maintains normal HSCs pool and suppresses LSCs through inhibition of mTOR. It has been reported that PML (promyelocytic leukemia protein) plays a role in normal HSCs and BCR-ABL transduced quiescent LSCs, facilitating leukemia initiation and maintenance.[231] *Pml* deficiency promoted transition of LSCs from quiescent to activated stage and *Pml-/-* LSCs finally failed to initiate CML disease contrary to wild type LSCs after serial transplantation. As PML is a repressor of mTOR, inhibition of mTOR with rapamycin restored *Pml-/-* HSCs and the long-term reconstitution functions of LSCs. This study is controversy with the role of Pten in AML LSCs and our finding in CML, indicating the function of mTOR complex still needs more studies. Furthermore, rapamycin is also could be tested in

different CML cell lines to confirm its inhibition of leukemia cell proliferation and induction of apoptosis and it is should be a promising reagent in future CML clinical trial.

Another *Pten* conditional deletion mouse model demonstrated that *Pten* partial deletion in mouse fetal liver HSCs and their differentiated progeny led to a myeloproliferative disorder, followed by acute T-lymphoblastic leukemia (T-ALL).[232] In this study, the *Pten* deficient $Lin^{-}c\text{-}Kit^{mid}CD3^{+}$ population was shown to be the T-ALL LSCs through a serial transplantation assay. Interestingly, ablation of one allele of *β-catenin* significantly delayed the occurrence of acute leukemia. We and others have also shown that β-catenin plays a key role in maintaining LSCs in CML[87, 88] and AML[89].

All of above results allow us to draw a potential picture connecting Pten with other key pathways involved in survival and proliferation of LSCs, including β-catenin, p53, Alox5, PI3K/Akt/mTOR pathways (Figure 1). These pathways are disturbed in CML, AML and other malignancies, and targeting of the pathways may be beneficial to patients.

Taken together, the two parts of my thesis work broaden the existing BCR-ABL signaling pathway. The chaperon protein, Hsp90, can stabilize BCR-ABL and facilitate it to continually active its downstream, including PI3K, Akt, Grb2, MAPK et, al. When Hsp90 is inhibited by its specific inhibitor, such as IPI504, BCR-ABL will be released from Hsp90 complex and degraded by proteasome, finally shutting down its signaling transduction and suppressing the leukemogenesis. On the other hand, BCR-ABL can downregulate Pten expression and release its inhibition of p-Akt which is critical to promote leukemia cells proliferation. Notably, BCR-ABL also might inactive Pten function through upregulate Alox5 expression to oxidize Pten and facilitate

leukemogenesis (Figure 2). In this network, several therapeutic targets, such as Hsp90, Alox5, Akt, mTOR, are so promising in CML treatment and their novel inhibitors might achieve significant benefit for CML patients.

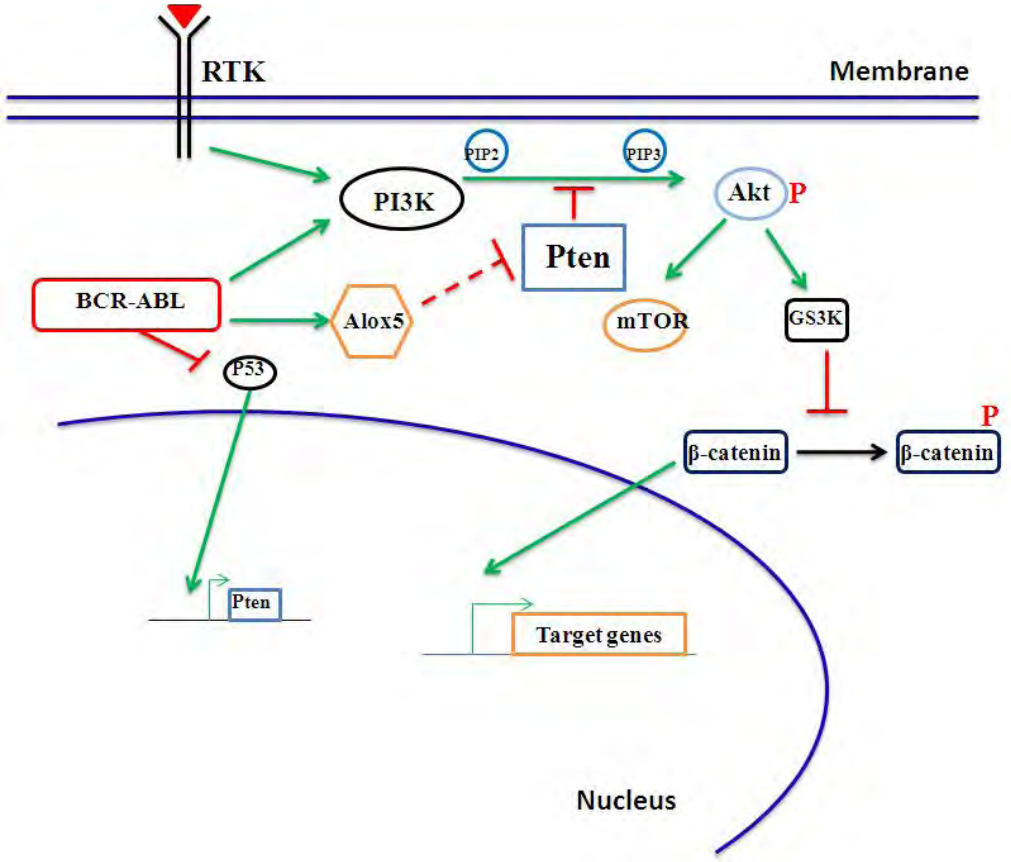

Figure 1. Potential Alox5/Pten/Akt/GSk3-β/β -catenin network in CML LSCs. BCR-ABL actives PI3K which switches PIP2 to PIP3 and activates Akt, followed by the activation of mTOR and β-catenin nuclear translocation to maintain LSCs self-renew and leukemia initiating. BCR-ABL also downregulates Pten by suppressing p53 which binds to Pten promoter and upregulates Pten expression. Moreover, BCR-ABL upregulates Alox5 expression which potentially inhibits Pten function and promotes Akt phosphorylation by oxidation of Pten.

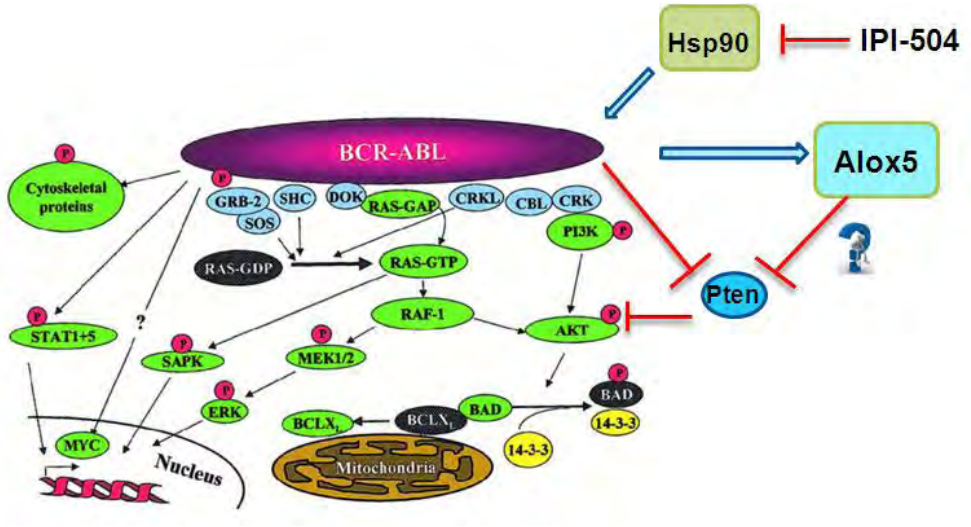

Figure 2. Network of BCR-ABL signaling transduction pathway. Novel components are added in the existing BCR-ABL signaling transduction network. Hsp90 stabilizes BCR-ABL and its inhibition by IPI504 induces BCR-ABL degradation. BCR-ABL downregulates Pten to release its inhibitory effect on Akt phosphorylation and upreuglates Alxo5 expression which might inactive Pten function and active Akt phosphorylation in leukemia cells. (Modified from Brain Druker, *Blood*, 2009, vol 112, 4808-4817)

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